

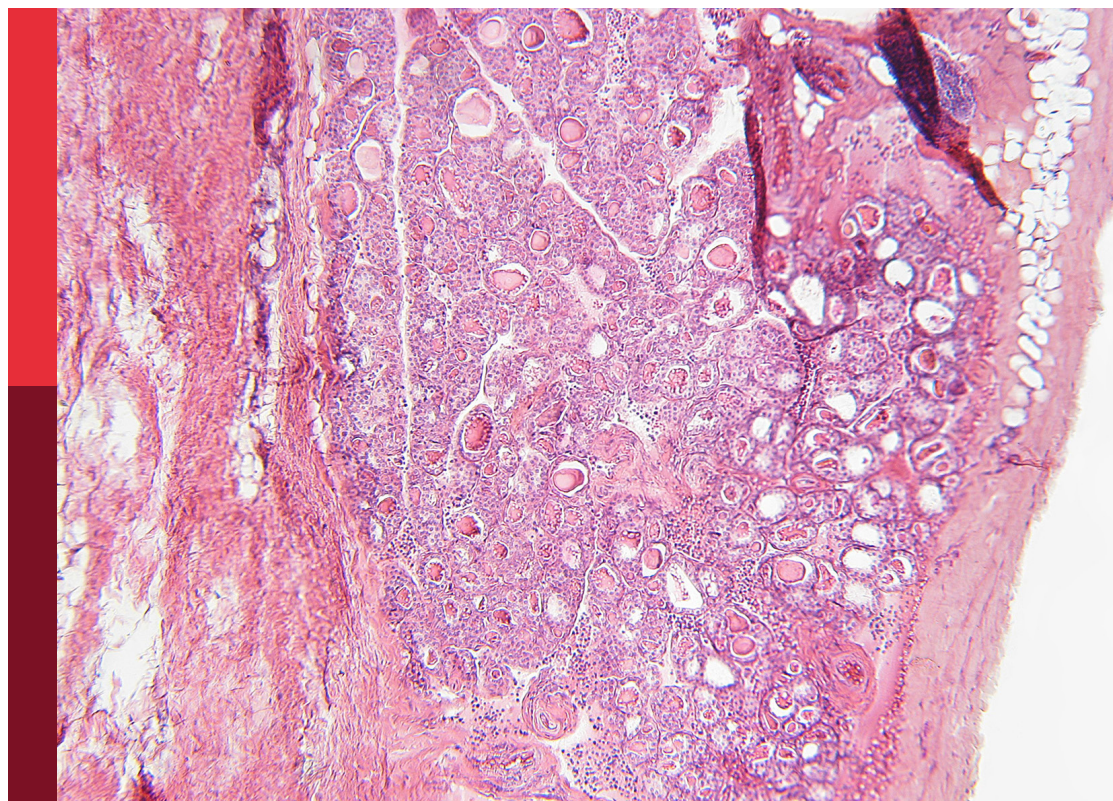
Maternal-fetal interface: new insight in placenta research, volume II

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Maternal-fetal interface: new insight in placenta research, volume II

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Table of contents

- 05 **Editorial: Maternal-fetal interface: new insight in placenta research, volume II**
Cilia Abad, Mariana Farina, Alicia E. Damiano and Reinaldo Marín
- 08 **Phenotype of early-onset fetal growth restriction in sheep**
Amy E. Sutherland, Tegan A. White, Charmaine R. Rock, Beth R. Piscopo, Ingrid Dudink, Ishmael M. Inocencio, Zahrah Azman, Yen Pham, Ilias Nitsos, Atul Malhotra, Tamara Yawno, Graeme R. Polglase, Graham Jenkin, Emily J. Camm, Beth J. Allison and Suzanne L. Miller
- 20 **The interplay of inflammation and placenta in maternal diabetes: insights into Hofbauer cell expression patterns**
Zdenek Tauber, Adela Burianova, Katerina Koubova, Max Mrstik, Marie Jirkovska and Katerina Cizkova
- 32 **The effects of leptin on human cytotrophoblast invasion are gestational age and dose-dependent**
Kristen K. Rumer, Shilpi Sehgal, Anita Kramer, Kevin P. Bogart and Virginia D. Winn
- 45 **Analysis of the evolution of placental oxidative stress research from a bibliometric perspective**
Ailing Chen, Mengyuan Tian, Zouqing Luo, Xiaohui Cao and Yanfang Gu
- 74 **Comparative study on pregnancy complications: PGT-A vs. IVF-ET with gender-specific outcomes**
Ling Guo, Xiao Li, Anliang Guo, Yufeng Wang, Yue Liang, Yan Li, Xinbo Xu and Hong Lv
- 84 **Polystyrene microplastics exposition on human placental explants induces time-dependent cytotoxicity, oxidative stress and metabolic alterations**
Ashelley Kettylem Alves de Sousa, Keyla Silva Nobre Pires, Isadora Hart Cavalcante, Ismin Cristina Lira Cavalcante, Julia Domingues Santos, Maiara Ingrid Cavalcante Queiroz, Ana Catarina Rezende Leite, Alexandre Carmo Crispim, Edmilson Rodrigues da Rocha Junior, Thiago Mendonça Aquino, Rodrigo Barbano Weingrill, Johann Urschitz, Stephanie Ospina-Prieto and Alexandre Urban Borbely
- 99 **Trained innate immunity as a potential link between preeclampsia and future cardiovascular disease**
Ivo Carrasco-Wong, Javiera M. Sanchez, Jaime A. Gutierrez and Delia I. Chiarello
- 110 **A stress paradox: the dual role of the unfolded protein response in the placenta**
Diba Chowdhury, Chloe E. Jang, Patrick Lajoie and Stephen J. Renaud

- 124 **Umbilical vein remodeling is associated with pregestational maternal overweight**
Kamilla Batista da Silva Souza, Luana Caroline Hochberger, Felipe Egon Castrignano Camargo, Gabriely Santos Silva, Giovanna Castrignano Camargo, João Pedro Lourenço Mello, Fernanda Cristina Alcantara Dos Santos, Fernanda Regina Giachini, Núbia de Souza Lobato and Paula Cristina de Souza Souto
- 135 **Epigenetics of maternal-fetal interface immune microenvironment and placental related pregnancy complications**
Xueqin Ma, Xin Chen, Xuefeng Mu, Min Cao and Yan Zhang
- 152 **Happy feet: the key roles of podosomes and invadopodia in trophoblast invasion at the maternal-fetal interface**
Padma Murthi, Emily Overton, Shaun P. Brennecke and Rosemary J. Keogh



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Editorial: Maternal-fetal interface: new insight in placenta research, volume II

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KEYWORDS

placenta, maternal-fetal interface, immune tolerance, cellular invasion, metabolism

Editorial on the Research Topic

Maternal-fetal interface: new insight in placenta research, volume II

The maternal-fetal interface is a dynamic and complex microenvironment where the delicate balance between immune tolerance, cellular invasion, and metabolic regulation determines pregnancy success and the health of both mother and child (1). Recent research continues to unravel the intricate mechanisms at play, revealing new insights into how cellular structures, immune responses, metabolic stress, and environmental exposures converge to shape pregnancy outcomes.

The objective of the Research Topic “*Maternal-Fetal Interface: New Insights in Placenta Research Volume II*” was to bring together original research articles and reviews highlighting recent advances in understanding placental functions and its impact on the health of the mother and her future child. This Research Topic encompasses a diverse range of contributions, including six original articles, a mini-review, three reviews, and a systematic review.

A striking theme emerging from current research is the central role of specialized cellular projections—podosomes and invadopodia—in trophoblast invasion at the maternal-fetal interface (2). These “invasive feet” are crucial for normal placental development, yet their classification remains debated due to the discovery of hybrid structures that blur conventional definitions (2). This conceptual ambiguity highlights the need to re-evaluate how these invasive mechanisms are defined and studied, particularly in the context of pregnancy complications such as placenta accreta and preeclampsia, where trophoblast invasion is either excessive or insufficient.

Epigenetic regulation has become a central focus in understanding the immune microenvironment at the maternal-fetal interface. Mechanisms such as DNA methylation, histone modifications, and non-coding RNA activity are now recognized as crucial for orchestrating the immune balance required for successful implantation and

placental development (3). Disruptions in these epigenetic processes have been associated with a range of placenta-related complications, highlighting the potential of targeted interventions that modulate gene expression without altering the underlying DNA sequence (4).

The placenta's capacity to regulate protein folding and respond to cellular stress is a critical factor in ensuring a healthy pregnancy (Chowdhury et al.). The unfolded protein response (UPR), activated by endoplasmic reticulum (ER) stress, plays a dual role: while a basal level of UPR activity is essential for normal placental development, prolonged or excessive ER stress can lead to cellular dysfunction and adverse pregnancy outcomes (5, 6). This “stress paradox” illustrates the delicate balance between physiological adaptation and pathological disruption, emphasizing the importance of ER homeostasis for both maternal and fetal well-being.

Preeclampsia (PE) remains one of the most formidable challenges in obstetric medicine, due not only to its acute clinical risks but also to its long-term health implications. Recent work suggests that trained innate immunity (TRIM)—a process by which exposure to danger signals during PE epigenetically reprograms maternal immune cells—may provide a mechanistic link between pregnancy complications and future cardiovascular disease (7). This persistent pro-inflammatory state, driven by epigenetic changes, may help explain the elevated risk of hypertension, heart disease, and stroke observed in women with a history of PE. Advancing our understanding of TRIM could open novel avenues for prevention and therapy, both during pregnancy and beyond.

Maternal metabolic status, particularly overweight and obesity, is increasingly recognized as a key modifier of placental and vascular structure (8). Research has demonstrated that maternal overweight is associated with significant remodeling of the umbilical vein, including increased wall thickness and collagen deposition. These structural alterations may compromise the efficiency of nutrient and oxygen delivery to the fetus, potentially influencing long-term health outcomes. Such findings reinforce the importance of optimizing maternal health before and during pregnancy, not only for favorable perinatal outcomes but also for mitigating intergenerational health risks.

Environmental factors are increasingly recognized as significant threats to placental function. Microplastics, once considered as pollutants affecting marine ecosystems, have now been detected in human placental tissue (9). Experimental studies demonstrate that exposure to polystyrene microplastics induces cytotoxicity, oxidative stress, and metabolic disturbances in placental explants, raising serious concerns about their potential to impair fetal development and contribute to pregnancy complications. Given the pervasive presence of microplastics in the environment, further research into their impact on human health is both urgent and essential.

Research into placental oxidative stress has expanded significantly, with growing emphasis on the mechanisms governing antioxidant defenses, gene expression regulation, and the pathophysiology of pregnancy-related diseases (10, 11). Bibliometric analyses reveal a shift toward investigating emerging

risk factors, environmental exposures, and the complex interplay between inflammation, stem cells, and immune regulation. These trends reflect a broader understanding that placental health is influenced by an intricate network of genetic, epigenetic, metabolic, and environmental factors.

Advances in reproductive technology, such as preimplantation genetic testing for aneuploidy (PGT-A), are also reshaping our understanding of pregnancy complications (12). Emerging evidence suggests that PGT-A may reduce the risk of preeclampsia in pregnancies involving male fetuses, although this protective effect does not appear to extend to female fetuses. This sex-specific difference underscores the complexity of fetal-maternal interactions and highlights the importance of personalized approaches in fertility treatment and pregnancy management.

Finally, the interaction between inflammation and placental function in the context of maternal diabetes provides valuable insights into the roles of Hofbauer cells, unique and specialized fetal immune cells within the placenta (13). Distinct inflammatory activation profiles and enzyme expression patterns observed in type 1 and gestational diabetes suggest that while immune tolerance is largely preserved, structural and vascular alterations in the placenta may still compromise fetal health. These findings underscore the multifaceted nature of placental adaptation to maternal metabolic and inflammatory states.

Collectively, these studies portray the placenta as both a sentinel and a mediator of maternal and fetal health, finely attuned to a wide array of internal and external signals. The key challenge is to translate these growing molecular and cellular insights into effective interventions capable of preventing or alleviating pregnancy complications, safeguarding maternal health, and ensuring the healthiest possible start for future generations.

Author contributions

CA: Conceptualization, Writing – original draft, Writing – review & editing. MF: Conceptualization, Writing – original draft, Writing – review & editing. AD: Conceptualization, Writing – original draft, Writing – review & editing. RM: Conceptualization, Writing – original draft, Writing – review & editing.

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Phenotype of early-onset fetal growth restriction in sheep

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Introduction: Fetal growth restriction (FGR) is a common pregnancy complication, caused by placental insufficiency, with serious adverse consequences for development *in utero* and postnatal wellbeing. There are no antenatal treatments to improve growth or organ development in FGR, and animal models are essential to mimic the physiological adaptations in FGR and to assess potential interventions. This study aimed to identify the temporal nature of reduced developmental trajectory in fetuses with FGR, and to examine the effects of common factors that may mediate differential growth such as glucocorticoid treatment. We hypothesised that the trajectory of growth would be adversely impacted by FGR.

Methods: FGR was induced via surgical placental insufficiency in fetal sheep (89 days gestation/0.6 gestation; n=135) and compared to age-matched controls over the last third of gestation and into neonatal life (n=153).

Results: Body weight of FGR fetuses/lambs was significantly reduced compared to controls ($p<0.0001$) from 127 days of gestation (term is 148 days), with increased brain:body weight ratio ($p<0.0001$) indicative of brain sparing. All biometric measures of body size were reduced in the FGR group with the exception of biparietal (head) diameter. The trajectory of body growth in the last trimester of sheep pregnancy was significantly reduced in the FGR group compared to controls, and stillbirth rate increased with longer gestation.

Discussion: This work provides a well characterised FGR animal model that mimics the known physiological adaptations in human pregnancy and can be used to determine the efficacy of potential interventions.

KEYWORDS

FGR, IUGR, brain sparing, neurodevelopment, brain injury, asymmetric growth, preterm, postnatal

Introduction

Fetal growth restriction (FGR) is a complex obstetric condition in which fetal growth is pathologically reduced, most often because the placenta fails to deliver an adequate supply of oxygen and nutrients to support normal fetal development (1, 2). FGR is common, affecting 6–9% of pregnancies in high-resource countries (3, 4). *Placental insufficiency* is the umbrella term used to describe abnormal development and function of the placenta (1), characterised by reduced uteroplacental blood flow, reduced gas and substrate transfer across the placenta, and reduced fetal growth. In response to placental insufficiency, the growth restricted fetus experiences hypoxia (5), initiating a haemodynamic response to preferentially supply essential organs (brain and heart) by redistributing cardiac output (6). In FGR, this adaptive response can be prolonged, which results in cerebrovascular compensation (brain sparing) and asymmetric fetal growth with relatively spared head size but a thinner and/or shorter body (7). A decade ago, the terms FGR or IUGR (intrauterine growth restriction) were often used interchangeably with small for gestational age (SGA). The 2016 consensus definition for FGR has provided an essential framework to delineate infants with pathological FGR, and greater susceptibility for mortality and morbidity, from SGA infants who are constitutionally small but otherwise healthy. SGA is now used to describe any infant <10th percentile for estimated fetal weight or birth weight relative to gestational age and sex, while true FGR is defined as an estimated fetal weight <10th percentile together with antenatal Doppler indices of uteroplacental dysfunction, or estimated fetal weight <3rd percentile as a sole factor (2).

Infants with FGR are often delivered preterm, particularly when the FGR is early-onset (diagnosed at less than 32 weeks' gestation) (8), and FGR is the strongest risk factor for perinatal death/stillbirth (8, 9). Infants born preterm are likely to be exposed to antenatal glucocorticoids to induce lung maturation, but these glucocorticoids may have differential effects on organ development in FGR and appropriately grown fetuses (10, 11). After birth, FGR is associated with neonatal cardiovascular, respiratory and neurological morbidities at significantly elevated rates compared to appropriate for gestational age infants (7). For example, heart shape and cardiovascular function are altered (12–14) and infants born with FGR spend more time on ventilation and in neonatal intensive care than age-matched appropriately grown infants (15). Despite the presence of brain sparing *in utero*, infants born growth restricted have an increased likelihood of neurodevelopmental deficits in childhood that include poor cognitive function and reduced intelligent quotient (IQ) scores (3, 16, 17) as well as a 10–30-fold increased risk of developing the motor deficit cerebral palsy (18). Determining organ-specific structural and functional changes associated with placental insufficiency and FGR requires appropriate animal models in which major organ development and physiological adaptations replicate the known deficits in human FGR.

There are multiple animal models of placental insufficiency, chronic fetal hypoxia and/or FGR, utilising small and large animal experimental designs (19, 20). A recent systematic review reports

that mice and rats were the most used animals for intervention studies in FGR (79%) followed by sheep (16%) (21). The clinical complexity and heterogeneous nature of human FGR means that no single animal model will perfectly mimic the human condition. However, animal models can provide a standardised set of parameters and outcomes allowing the assessment of outcomes of interest. For example, human early-onset FGR is often complicated by very preterm birth which makes it difficult to distinguish the effects of FGR alone on organ development. Animal models can be used to perform separate studies of FGR, very preterm birth, glucocorticoid exposure, and combinations of these factors, such as FGR and very preterm birth (20, 22). Large animal studies of FGR, often in fetal sheep, permit long-term and *in utero* monitoring of the fetus with the addition of vascular catheters and other monitoring techniques (10, 19, 23–27). Subsequently, studies in large animals allow the examination of the fetal physiological response to placental insufficiency and chronic hypoxia, together with the progression of indices of specific organ structural and functional derangement. In turn, therapeutic interventions can be assessed.

The use of a well characterised and clinically useful animal model of FGR is essential for translational studies to improve outcomes for infants born growth restricted. We have been studying FGR in sheep for more than 15 years (28). Herein, we present combined cohort data to describe the phenotype of our model. We utilise a technique called single umbilical artery ligation (SUAL), undertaken during surgery, which causes placental insufficiency via infarction of approximately half of the ovine placental cotyledons as first described in the late 1960's (29). The SUAL procedure can be carried out at different timepoints in sheep pregnancy, and we have done this to investigate and compare the effects of early-onset FGR (0.6 gestation) versus late-onset FGR (0.72 gestation) on brain development (30). In the work presented here, we include all animals from our early-onset FGR cohort, including singleton pregnancies that we predominantly use to examine the effects of FGR versus control on lambs after birth, and twin pregnancies in which we induce FGR in one fetus, while the second fetus acts as an internal control, used predominantly for fetal studies. We have examined fetal and neonatal lamb outcomes at various ages, allowing us to describe the progression of FGR on organ development. The aim of this study was to characterise the impact of early-onset placental insufficiency on the temporal nature of reduced developmental trajectory in fetuses with FGR, and to examine whether common factors that mediate altered growth (antenatal glucocorticoids, singleton vs twin, fetal sex) differentially affected FGR animals across biometric measures of growth and organ development, compared to gestation-matched control animals.

Materials and methods

All animal experiments were approved by the Hudson Institute Animal Ethics Committee (approval numbers MMCA 2014/04, MMCA, 2016/19, MMCA 2016/24, MMCA 2016/62, MMCA 2017/38, MMCA 2019/02, MMCA 2019/04, MMCA 2020/06, MMCA

2022/08) and were performed between 2014 and 2023. Animal experiments were conducted as per the National Health and Medical Research Council of Australia Code of Practice for the Care and Use of Animals for Scientific Purposes (Eighth Edition) and comply with the ARRIVE guidelines for reporting animal research (31). Animals had free access to food and water, with food only removed 18 hours prior to surgery.

Animal preparation

Singleton ($n=58$) or twin-bearing ($n=149$) Border-Leicester crossbred time-mated pregnant ewes were obtained from Monash Animal Research Platform Gippsland Field Station. On arrival into the Monash Health Translation Precinct Animal Facility, ewes were weighed and this was recorded. At fetal gestational age 88–90 days gestation (0.6 gestation; term is 148 days), surgery was undertaken on the pregnant ewe for induction of placental insufficiency and FGR (30, 32). Briefly, ewes were anaesthetised by injection of sodium thiopentone (Pentothal, 1g in 20mL; Jurox, NSW, Australia) followed by intubation and maintenance of anaesthesia with 1.5–2.5% inhaled isoflurane in O₂ (Isoflo; Abbott Australasia, Botany, NSW, Australia). An incision was made in the uterus, overlying the fetus. The back legs and torso of the fetus were removed up to the level of the abdomen, and within the umbilical cord the two arteries and veins were identified. A small incision was made in the umbilical cord sheath, approximately 3cm from the fetal abdomen, and one umbilical artery was isolated. Single umbilical artery ligation (SUAL) was undertaken by placing two silk sutures around this artery and tying these tightly (Figure 1). In control fetuses, sham-SUAL was performed by isolating and handling the umbilical artery, but with no ligation. In the case of twin-bearing ewes, one fetus was randomly chosen to undergo the SUAL procedure and the other was a sham-SUAL control. A subset of fetuses from twin pregnancies (FGR and control), were instrumented with a femoral artery catheter (inner diameter 0.5mm, outer diameter 1.0mm; Critchley Electrical, NSW, Australia) for blood sampling. Each fetus was returned to the

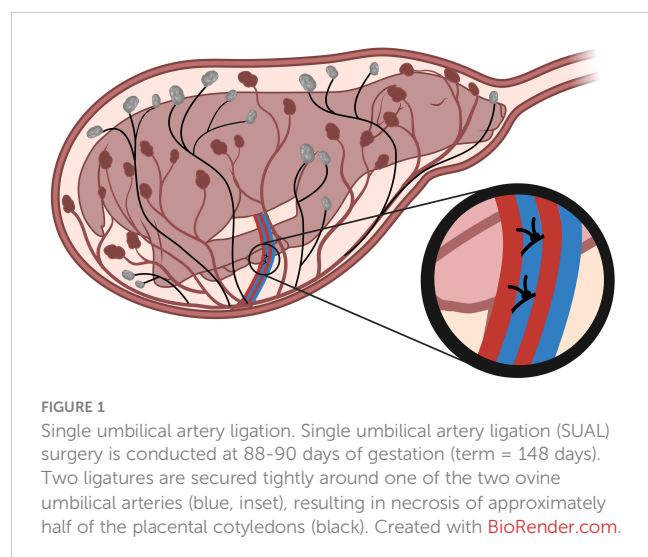
uterus and the uterine incision was closed. A jugular vein catheter was placed in the ewe to allow intravenous antibiotic administration (1g Ampicillin, Aspen Pharmacare Australia, Australia; 500mg Engemycin, Coopers Animal Health, Australia) once daily for the first three days after surgery. The ewes and fetuses were monitored daily to confirm wellbeing. In animals with femoral artery catheters, a fetal blood sample (~200μL) was collected once each day (at approximately 9am) for immediate assessment of blood pH, oxygen saturation (SaO₂%), partial pressure of oxygen (PaO₂), partial pressure of carbon dioxide (PaCO₂), lactate and glucose using an ABL 700 blood gas analyser (Radiometer, Copenhagen, Denmark). Fetal catheters were also flushed again in the afternoon with heparinised saline in an effort to maintain patency of the small fetal femoral artery catheters. In the fetal sheep with post-mortem scheduled for 136–138 days gestation, cord blood was collected from the umbilical vein (~5mL) at the time of post-mortem for assessment of cortisol concentration as per manufacturer's instructions (ABIN1052524, Blue Gene, Shanghai).

In a subset of ewes, betamethasone was administered as part of their experimental protocol. The ewes received an intramuscular betamethasone injection (11.4mg; Celestone Chronodose, Schering Plough, Australia) 48 hours and 24 hours prior to post-mortem at 127 days gestation. In lambing cohorts, ewes received betamethasone as above, as well as intramuscular mifepristone (50mg; Mifepristone Linepharma, MSHealth, Australia) 48 hours prior to delivery at gestational age 136 days; these lambs were used in the 24 hour and 4 week postnatal cohorts. To ensure the ewe and lamb had an opportunity to bond we did not intervene during delivery unless necessary for animal welfare. Birth weights and measures of lamb well-being were recorded once the ewe had interacted with the lamb and were completed as quickly as possible to minimise the amount of time the lamb was separated from the ewe.

At scheduled post-mortem, ewes and their fetuses or lambs were euthanised with intravenous phenobarbitone (100mg/kg IV, Lethobarb, Virbac Pty Ltd, Peakhurst, Australia) at ~110 days gestation, ~127 days gestation, ~136–138 days gestation, or at 24 hours postnatal age or 4 weeks postnatal age. All fetuses and lambs had biometry measurements, body weights and organ weights recorded at post-mortem.

Statistical analysis

Data are presented as the mean $\pm$ the standard error of the mean (SEM), with the exception of the body weight for the normative growth curve, as undertaken in human presentation of similar data. Blood gas parameters were analysed using a mixed-effects model and postnatal weight gain was analysed using two-way repeated measures ANOVA with time and fetal growth status (FGR or control) as variables, and *post-hoc* Šidák test where appropriate. Body weight and brain/body weight at post-mortem, as well as the effects of fetal sex, betamethasone, and singleton vs twins were analysed using a two-way ANOVA, and *post-hoc* Šidák test where required. Organ weight and biometry comparisons between cohorts (control and FGR) within a gestational age were assessed using t-



test. To determine growth trajectory, body weights from separate cohorts collected across gestational ages in control and FGR animals were used to calculate Pearson’s correlation coefficient, and we then compared between groups by analysing whether the coefficient of determination (slope of the lines) between FGR and control groups were different. Statistical comparisons were carried out using GraphPad Prism (GraphPad Software, San Diego, CA, USA, version 9.5). Significance was accepted when $p<0.05$.

Results

Cohort information and fetal arterial blood gas parameters

Data for the number of animals in each group, and survival rates to post-mortem, are presented in Table 1. In the 110 days gestation cohort, we include data from $n=5$ FGR fetuses and 10 control fetuses, with a survival rate for FGR of 63% (5 twins, 3 triplets). In the 127 days gestation cohort we include data from $n=98$ FGR fetuses and $n=105$ control fetuses, with a survival rate for FGR of 78% (6 singletons, 121 twins). The 136-138 days gestation cohort includes data from $n=4$ FGR fetuses and $n=9$ control fetuses (8 twins). The 24 hours postnatal age cohort includes $n=19$ FGR lambs and $n=17$ control lambs (21 singletons, 11 twins), and the 4 week postnatal age cohort includes $n=9$ FGR lambs and $n=12$ control lambs (31 singletons, 1 twin). As only 1 death occurred postnatally (1 FGR lamb in 4 weeks postnatal age cohort due to accidental death by mother) we have combined FGR survival rate from the 136-138 days gestation, 24 hours postnatal age and 4 weeks postnatal age cohorts to result in a survival rate for FGR of 62%.

TABLE 1 Success rates for each cohort.

110 days gestation cohort*	Surgery	Survive to post-mortem	Survival rate
Control	N=11	N=10	91%
FGR	N=8	N=5	63%
*Includes 3 surprise triplets where the 3 rd fetus was added to the control group			
127 days gestation cohort	Surgery	Survive to post-mortem	Survival rate
Control	N=125	N=105	84%
FGR	N=125	N=98	78%
136-138 days gestation cohort*	Surgery	Survive to post-mortem	Survival rate
Control	N=40	N=38	95%
FGR	N=52	N=32	62%
*Includes 24 hour and 4 week postnatal groups as only 1 FGR postnatal death			

Of the lambs in the postnatal age groups, $n=2$ control and $n=3$ FGR lambs in the 24 hours postnatal group required respiratory support in the form of supplemental oxygen immediately after birth (via face mask). All lambs were kept with their mother for the duration of the experimental period however $n=4$ control and $n=9$ FGR lambs in the 24 hours postnatal age cohort and $n=4$ FGR lambs in the 4 weeks postnatal age cohort required bottle feeding in the days following birth. Lambs were given supplemental bottle feeding with milk expressed from the ewe if not consistently gaining weight (measured 4 hourly) within the first 48 hours of life. Bottle feeds were supplemental only, no lamb was exclusively bottle fed. In ewes carrying a single fetus, maternal weight prior to surgery who were assigned to the control or FGR groups was not different (58.1 ± 5.9 kg control fetus or 60.7 ± 1.3 kg FGR fetus; $p=0.41$). Our overall survival rate for SUAL-induced FGR at 0.6 gestation in singleton and twin pregnancies combined was 73%. We do however point out that the survival rate of FGR in the older gestation cohort (136-138 days gestation plus lambs) at post-mortem was reduced to 62%, indicative of an increasing risk of stillbirth as gestation progressed in FGR. In total, we present data for $n=153$ control fetuses/lambs and $n=135$ FGR fetuses/lambs following early-onset placental insufficiency.

In fetuses that were instrumented with an arterial catheter ($n=36$ FGR and $n=36$ control), blood samples were taken daily between day 1 to 10 after surgery and arterial blood gas parameters assessed (Figure 2). Two-way repeated measures ANOVA showed that FGR was associated with reduced circulating partial pressure of oxygen (PaO_2 ; $p=0.002$; Figure 2A), reduced oxygen saturation (SaO_2 ; $p<0.0001$; Figure 2B), increased partial pressure of carbon dioxide (PaCO_2 ; $p<0.0001$; Figure 2C), reduced glucose concentration ($p=0.006$; Figure 2E), and elevated lactate ($p=0.033$; Figure 2F). There was no effect of FGR on pH ($p=0.16$; Figure 2D). We also observed a gestational age-related increase in PaCO_2 ($p<0.0001$) and decrease in pH ($p=0.005$) over the course of the study, reflecting an overall demise in placental function as gestation progressed. Fetal cortisol concentrations were also analysed in a small subset of umbilical cord blood collected from control ($n=4$) and FGR ($n=4$) fetuses from the 136-138 days gestation cohort. Compared to control fetuses, circulating levels of cortisol tended to be elevated in FGR fetuses (88.3 ± 17.3 ng/mL vs 53.4 ± 8.3 ng/mL) but this was not statistically significant ($p=0.12$; intra assay coefficient of variance 5.3%).

Body weights

All body weights of control and FGR cohorts were recorded at post-mortem. Two-way ANOVA showed that body weight increased significantly across the ages assessed ($p<0.0001$) but was significantly reduced in FGR animals compared to controls ($p<0.0001$; Figure 3A). Within age groups, FGR animals weighed less at 127 days gestation ($p<0.001$), 136-138 days gestation ($p<0.001$), 24 hours postnatal age ($p<0.001$) and at 4 weeks postnatal age ($p<0.001$). Brain weight corrected for body weight was significantly reduced over time ($p<0.0001$; Figure 3B) but was significantly increased in FGR animals compared to control groups

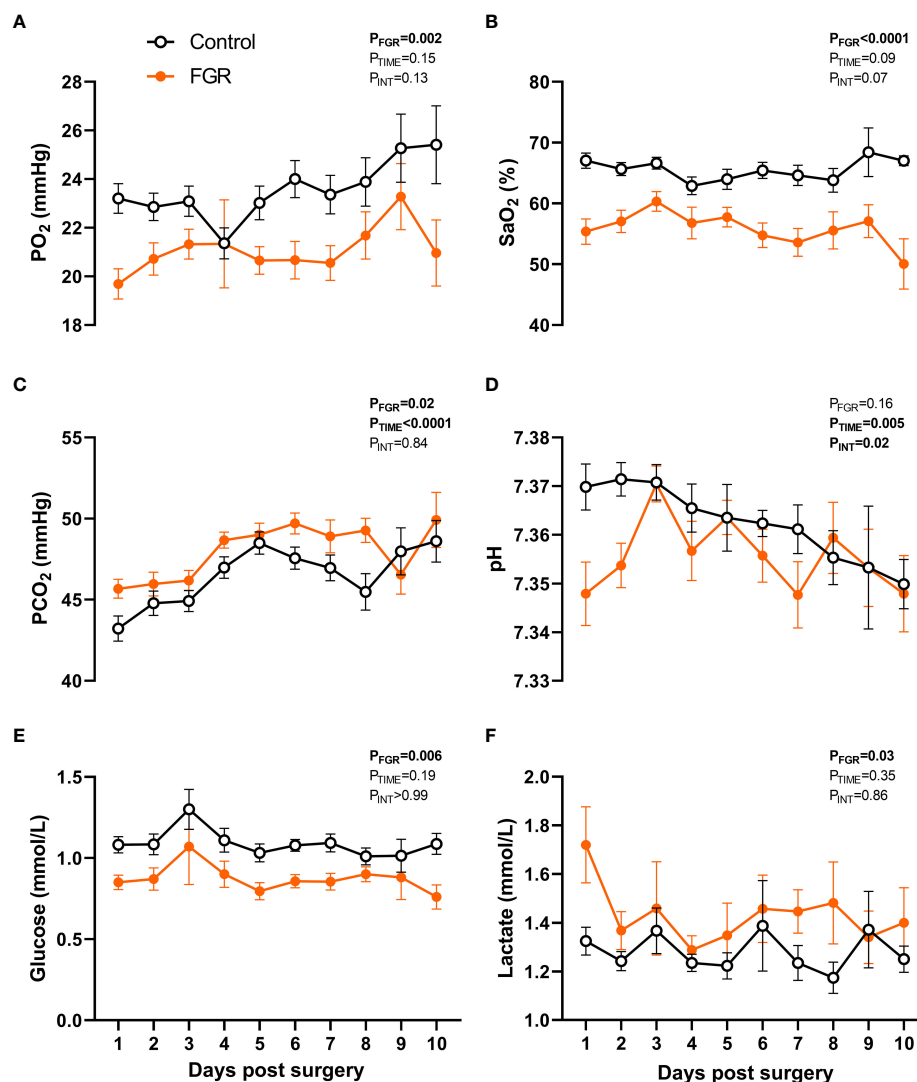


FIGURE 2

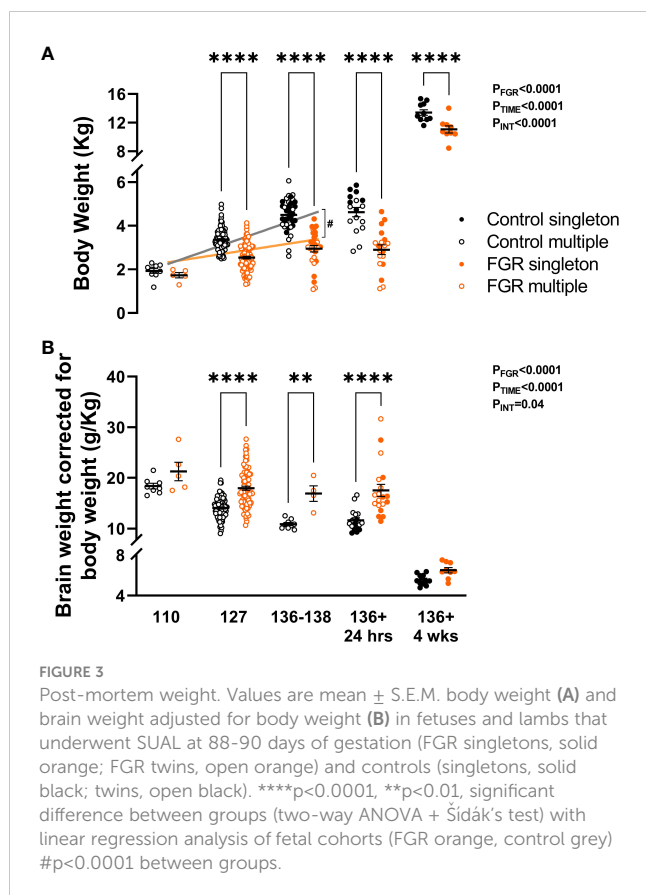
Fetal arterial blood values. Values are mean $\pm$ S.E.M. partial pressure of oxygen [PO_2 ; (A)], oxygen saturation [SaO_2 ; (B)], partial pressure of carbon dioxide [PCO_2 ; (C)], pH (D), glucose concentration (E) and lactate concentration (F) in arterial blood samples from fetuses that underwent SUAL at 88-90 days of gestation (FGR, orange) and controls (black) (mixed effects model). Group sample size ranged between $n=7-33$ for controls and $n=5-35$ for FGR fetuses.

($p<0.0001$). Within age groups, FGR animals had significantly increased brain to body weight ratio at 127 days gestation ($p<0.001$), 136 days gestation ($p=0.005$) and 24 hours postnatal age ($p<0.001$). Within the control group only, multiple regression analysis showed that body weight was significantly increased from 110 to 127 days gestation ($p<0.0001$) and between 127 to 136-138 days gestation ($p<0.0001$). Conversely, within the FGR group body weight did not increase between 110 and 127 days gestation ($p=0.44$), nor did it increase between 127 and 136-138 days gestation ($p=0.22$). Linear regression showed a significant upward trajectory of body weight in the control fetal groups ($r^2 = 0.56$, $p<0.0001$) and in the FGR group ($r^2 = 0.13$, $p<0.0001$). The slope of the lines was significantly different ($p<0.0001$) when comparing the control and FGR group, indicating that overall growth trajectory was adversely impacted in the FGR cohort.

The effects of fetal sex, betamethasone and singleton vs twins

The effects of male vs female sex and maternal betamethasone administration was investigated in fetuses at 127 days gestation. Using a two-way ANOVA, we did not observe an effect of fetal sex on body weight ($p=0.72$; Figure 4A) or brain weight corrected for body weight ($p=0.11$; Figure 4B). Betamethasone administration significantly reduced body weight ($p=0.019$; Figure 4C) but did not significantly alter brain weight corrected for body weight ($p=0.067$; Figure 4D).

In the 136-138 days gestation and 24 hours postnatal age cohort, we investigated the effect of singleton versus twin pregnancy. Two-way ANOVA revealed that twin pregnancies were associated with a significant reduction in fetal body weight



in both control and FGR fetuses compared to singleton pregnancies ($p=0.01$; **Figure 4E**). Twin pregnancy did not alter brain weight corrected for body weight ($p=0.10$; **Figure 4F**).

From **Figure 3**, it is evident that the largest dataset available is from the animals with post-mortem at 127 days gestation ($n=105$ control and $n=98$ FGR fetuses). Accordingly, we used this data to create our normative distribution of body weight in our control fetal sheep (**Figure 5**), with a mean body weight of 3.35 ± 0.50 kg, and for which we marked the 10th (2.71 kg) and 90th (3.99 kg) percentiles for normal body weight distribution. The mean body weight of FGR fetuses at this gestational age was 2.54 ± 0.06 kg, which sits between the 5th–6th percentile on the normative growth chart.

In lambs followed up to 4 weeks of age (**Figure 6**), body weight was recorded at the time of birth and then weekly until post-mortem. Two-way ANOVA revealed a significant increase in body weight over time ($p<0.0001$) in both groups, but the weight of the FGR lambs was significantly lower compared to control lambs ($p=0.0002$). *Post-hoc* analysis shows that compared to control lambs, body weight of FGR lambs was significantly lower at birth ($p=0.002$), week 1 ($p=0.0001$), week 2 ($p=0.0004$), week 3 ($p=0.02$), week 4 ($p=0.02$) and at post-mortem (PM; $p=0.01$). Linear regression analysis showed that there was no difference in the trajectory of postnatal growth between the control and FGR cohorts ($p=0.06$).

Biometry and organ weights

In the 127 days gestation group, we examined the effects of placental insufficiency and FGR on key organ weights in growth restricted and control cohorts (**Table 2**) recorded at post-mortem. After adjustment for body weight at 127 days (3.36 ± 0.05 kg control vs 2.54 ± 0.06 kg FGR; $p<0.0001$), brain weight, adrenal weight, and kidney weight were all significantly increased in the FGR offspring (all $p<0.0001$). The ratio of heart, lung, liver and spleen weight to body weight were not different in control and FGR groups ($p>0.05$). Similarly, in the 127 days gestation group we recorded measures of fetal biometry (**Table 2**) – crown rump length, tibia length, femur length, lower limb length, abdominal circumference and biparietal diameter. Results showed that crown-rump length, abdominal circumference, femur length, tibia length and lower leg length were significantly reduced in the FGR animals compared to the control group ($p<0.01$). In this same cohort, we made qualitative observations on the gross morphology of placental cotyledons from control and FGR offspring. The placental cotyledons from control fetuses were almost always healthy in appearance, with a predominance of type B placentomes (33), while more than 50% of placentomes associated with FGR offspring were necrotic and remaining placentomes showed a predominance of type C flattened shape and to a lesser extent type D morphology.

Discussion

Experimental models of placental insufficiency and FGR are critical tools to advance our understanding of the physiology of altered growth in FGR fetuses, and for the translation of new therapies to improve outcomes. Animal models of FGR allow characterisation of the fetal response to placental insufficiency, the ontogeny of growth deficits, and cellular mechanisms of organ-specific impairments that are associated with FGR. In the current study, we present novel results to show the temporal progression of reduced body growth in FGR during late gestation in response to early-onset placental insufficiency induced by single umbilical artery ligation (SUAL). In human pregnancies, placental insufficiency may be caused by maldevelopment of the placenta or secondary damage to a normal placenta, but with the common features of reduced oxygen and nutrient transfer (1). In the current sheep study we replicated fetal hypoxaemia and hypoglycaemia relative to control fetuses, and redistribution of fetal combined cardiac output to favour the brain. In human infants, an estimated fetal weight (EFW) less than the 3rd percentile relative to growth charts is described as 'severe' FGR (2, 34). The large number of samples available in the current ovine study permitted us to plot a normal ovine birthweight chart, and to determine that the mean body weight for our cohort of FGR sheep fetuses is between the 5th–6th percentile on the normal growth chart, thus representing a moderate to severe degree of fetal growth restriction. We assessed multiple time points for separate cohorts of animals for data

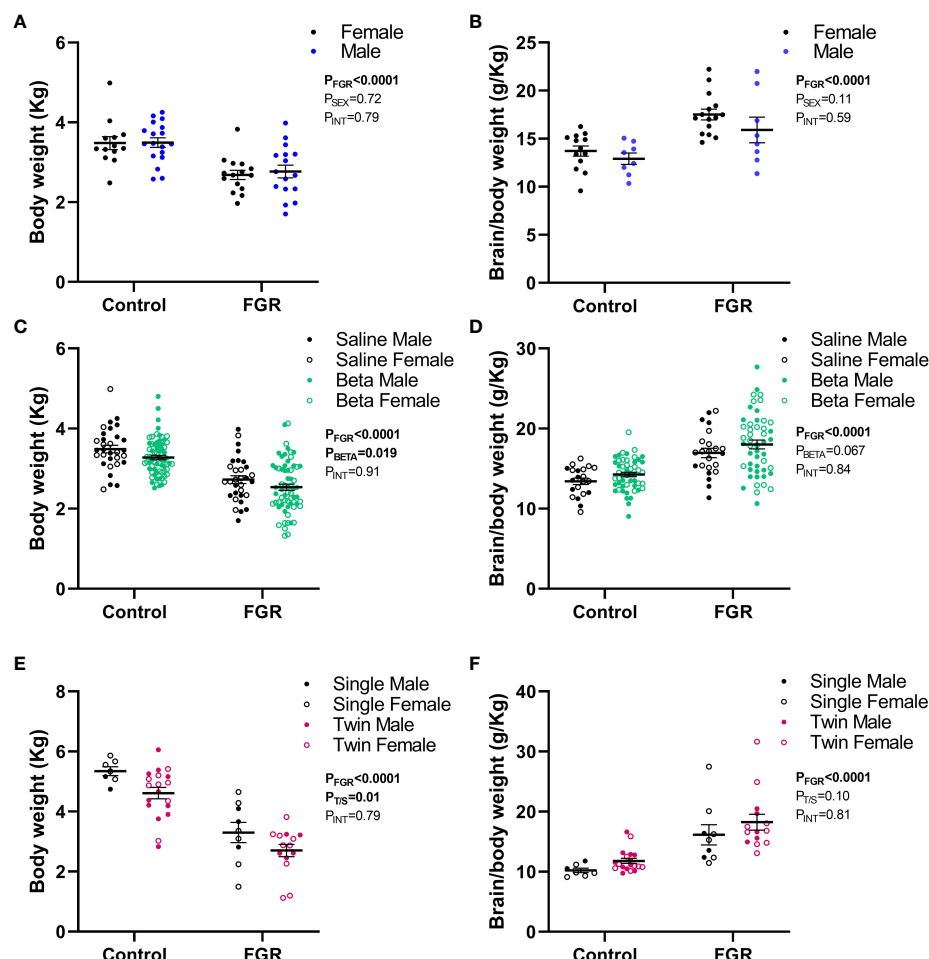


FIGURE 4

Effect of sex, betamethasone and number of fetuses on post-mortem weight. Values are mean $\pm$ S.E.M. body weight (A) and brain weight corrected for body weight (B) from female (black) and male (blue) fetuses at 127 days of gestation, body weight (C) and brain weight corrected for body weight (D) in fetuses that received saline (black, male closed circles, female open circles) or betamethasone (green, male closed circles, female open circles) at 127 days of gestation and body weight (E) and brain weight corrected for body weight (F) in singleton (black, male closed circles, female open circles) and twin (pink, male closed circles, female open circles) fetuses at 136–138 days of gestation and lambs at 24 hours postnatal age that underwent SUAL at 88–90 days of gestation (FGR) and controls (two-way ANOVA).

collection over the final third of gestation and then into the neonatal period, and we show that fetal growth trajectory is significantly reduced in response to placental insufficiency and FGR.

Placental insufficiency is the most well recognised cause of FGR (1) and uteroplacental dysfunction is now incorporated into the diagnostic criteria for clinical FGR through altered Doppler flow patterns (2). We adopted the model of SUAL because it induces placental insufficiency, causing gross and microscopic evidence of placental infarction in about half of the ovine placental cotyledons (29). The surgery to undertake SUAL can be conducted across a range of gestational ages, and previously we have compared the effects of early-onset and late-onset placental insufficiency and FGR on fetal brain development (30). All animals in the data presented in this study were from the early-onset cohort. Over the duration of fetal blood sample collection, arterial oxygenation was reduced by 15% compared to control, and glucose was 20% lower. This is in good agreement with mild chronic hypoxaemia observed in human infants when cord blood was sampled at birth (35). It is well

described that *in utero* fetal hypoxaemia induces an adaptive cardiovascular response to redistribute cardiac output to essential organs, notably the brain, and hence this response is often called brain sparing (6). In the current study, fetal hypoxaemia was present within the first week after the onset of placental insufficiency relative to the controls, and while fetal body weight was not significantly reduced at 110 days gestation (>20 days after onset of placental insufficiency), body weight was reduced at 127 days gestation. Notably, in the control group there was a significant increase in weight gain between 110 to 127 days gestation, and again between 127 to 136–138 days, indicative of the positive trajectory of normal fetal growth during late gestation. In the FGR cohort, there was no significant increase in fetal weight between either 110 to 127 days, or 127 to 136–138 days gestation, confirming that the growth trajectory of FGR fetuses was severely impacted. We also examined the effects of perinatal factors on gross body weight in control and FGR offspring and found that fetal sex was not associated with altered growth, however both antenatal glucocorticoid

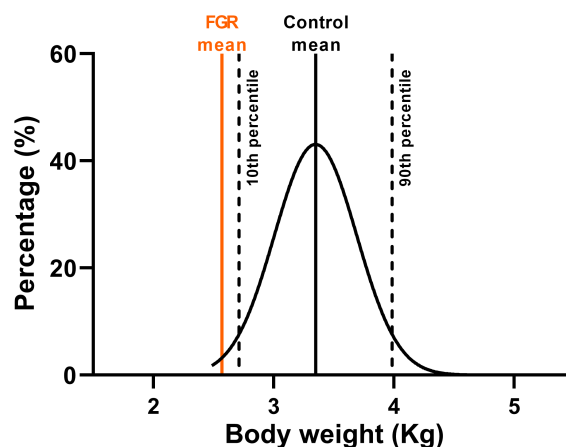


FIGURE 5

Ovine growth normative curve. Normal distribution of body weight from control fetuses (black) at 127 days of gestation from twin pregnancies with mean FGR body weight at 127 days of gestation (orange).

(betamethasone) administration and being a twin were associated with reduced growth in both control and FGR fetuses. Previous studies in fetal sheep have shown growth restriction with antenatal glucocorticoid exposure (36), which is an effect also seen in human infants with a single course of antenatal glucocorticoids who then deliver at term (37). In the current study the degree of growth restriction induced by betamethasone was similar in control and FGR offspring, with no interaction between factors.

Prolonged redistribution of cardiac output in response to chronic fetal hypoxia produces asymmetric fetal growth, via the brain sparing response. This adaptive haemodynamic response is caused by local vasodilatation of the fetal cerebral vascular bed and a sustained increase in fetal peripheral vascular resistance and bradycardia that are mediated by carotid chemoreflexes (6). In the clinical situation, this response to fetal hypoxia is detected as a change in cerebral perfusion using Doppler ultrasound, predominantly via a vasodilatation of the middle cerebral artery

(MCA) with evidence of reduced pulsatility index (PI) (38). A decreased MCA PI is correlated with worsening fetal hypoxaemia (39) and increasing incidence of brain injury (40–42). Ultrasound measures of fetal biometry are also used to diagnose FGR, most commonly head circumference, abdominal circumference, biparietal diameter and femur length (43). In this sheep study, we were able to replicate multiple biometric measures to demonstrate reduced fetal growth, including significantly reduced birth weight, reduced abdominal circumference and reduced femur length. Brain to body weight ratio was significantly increased, demonstrating asymmetric growth restriction and brain sparing. Lung, liver and spleen weight tended to be lower with FGR, these were not significant to control values, and we also observed that adrenal and kidney weight to body weight ratios were significantly increased, indicating preferential sparing of these organs in the FGR cohort. While brain sparing was apparent in this cohort of FGR offspring, our studies to date demonstrate that the brain is not

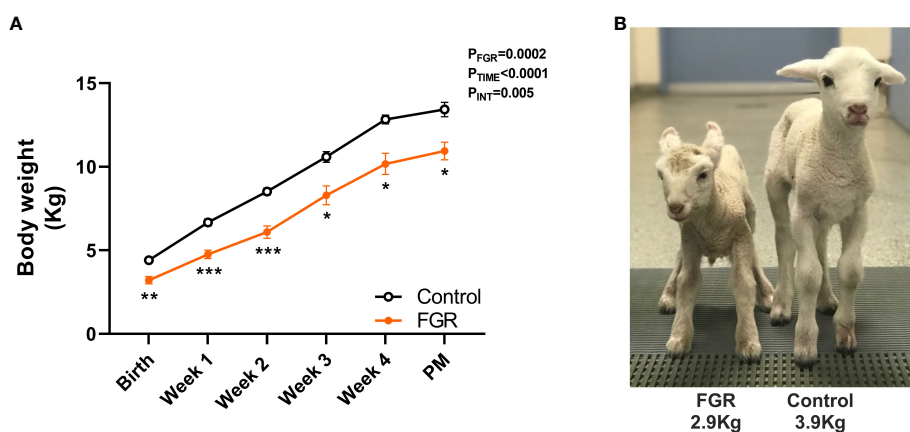


FIGURE 6

Postnatal growth. Values are mean $\pm$ S.E.M. body weight (A) in lambs that underwent SUAL at 88–90 days gestation and were born at 136 days of gestation with post-mortem (PM) at 1 month postnatal age (FGR, orange; $n=9$) and controls (black; $n=10$). *** $p<0.001$, ** $p<0.01$, * $p<0.05$ significant difference between groups (two-way repeated measures ANOVA + Šidák's test). Image of FGR and control lamb at 24 hours postnatal age (B). Linear regression analysis revealed the slope of the lines were not different between control and FGR lambs ($p=0.056$).

TABLE 2 Body and organ weights, and biometric measures of growth from 127 day cohort.

	Control N=69-89	FGR N=69-86
Body weight (Kg)	3.36 ± 0.05	2.54 ± 0.06****
Brain: body weight (g/Kg)	14.07 ± 0.22	17.97 ± 0.40****
Heart: body weight (g/Kg)	8.32 ± 0.18	8.17 ± 0.21
Adrenal: body weight (g/Kg)	0.12 ± 0.00	0.15 ± 0.00****
Lung: body weight (g/Kg)	30.07 ± 0.63	29.08 ± 0.90
Kidney: body weight (g/Kg)	7.01 ± 0.16	7.81 ± 0.16***
Liver: body weight (g/Kg)	31.82 ± 0.65	30.42 ± 0.70
Spleen: body weight (g/Kg)	1.46 ± 0.05	1.36 ± 0.05
Crown rump length (cm)	43.97 ± 0.43	40.05 ± 0.43****
Biparietal diameter (cm)	8.90 ± 0.26	8.48 ± 0.27
Abdominal circumference (cm)	32.45 ± 0.30	29.34 ± 0.41****
Femur length (cm)	11.88 ± 0.20	10.63 ± 0.20****
Tibia length (cm)	14.60 ± 0.23	13.29 ± 0.21****
Lower leg length (cm)	13.95 ± 0.28	12.91 ± 0.27**

****p<0.0001, ***p<0.001, **p<0.01 compared to control.

spared from injury (22, 32, 44–47). We are however mindful that the peak in perinatal brain growth occurs earlier in sheep than in humans (48), in part we accounted for this by inducing early lamb delivery, but future studies should match brain function and structure outcomes in lambs (49, 50) to more comprehensively determine the consequences of early-onset FGR on longer term neurodevelopmental outcomes.

Across the gestational ages studied in this ovine model of FGR, we report the survival rate for the FGR procedure. Overall, this figure is 73% survival of FGR fetuses/lambs to scheduled post-mortem. When examined more closely, we noted a 62% survival rate in the late gestation cohort. This is not surprising, given that it is well described clinically that FGR is the strongest risk factor for stillbirth/perinatal death (8, 9). Two clinical trials have evaluated the effects of the timing of delivery in infants with early-onset FGR (the GRIT and TRUFFLE trials) (51). Both studies were designed to compare immediate delivery versus delayed delivery guided by fetal cardiovascular monitoring, and while they were not powered to assess the effects of gestational age at delivery, results show an increased likelihood of fetal or neonatal death in the delayed delivery cohort (51). This is supported by population data to show that FGR is a significant risk factor for late preterm stillbirth, with undetected FGR linked to a 5-fold increased risk of stillbirth (9). Future studies could utilise this ovine model of FGR to elucidate whether there are physiological signs or biomarkers in the growth restricted fetus prior to fetal demise.

Early-onset FGR, diagnosed prior to 32 weeks fetal gestational age, is the more severe form of fetal growth restriction, associated with preterm birth, significant fetal hypoxia and cardiovascular adaptation (5). Early-onset FGR occurs less commonly than late-onset FGR (early-onset comprises 20-30% of FGR cases), but is

linked to high rates of perinatal mortality, and high likelihood of both neonatal and long-term morbidities of respiratory control, the cardiovascular system, and neurological function (7). Clinical trials to reduce mortality and morbidity in this cohort are extremely limited, but one trial was the international STRIDER trial – Sildenafil therapy in dismal prognosis early-onset fetal growth restriction – which hypothesised that maternal sildenafil citrate treatment in early-onset FGR would improve perinatal wellbeing via increased uteroplacental perfusion with sildenafil administration (52). Unfortunately, the STRIDER trial was halted prematurely due to an indication in the Dutch STRIDER study that sildenafil may have been associated with an increased rate of neonatal death (53). This finding supports the strong need to firstly assess the effects of treatments in well characterised animal models of FGR (23, 54, 55).

The first documented studies of SUAL were undertaken by Emmanouilides in 1968 (29), subsequent studies have added information on the late-onset model (56, 57), reporting mixed success, particularly when the SUAL procedure occurs later. Over time in our studies, we have found that early-onset SUAL in pregnant sheep (before 100 days gestation) is much more reliable at producing FGR than late onset (after 105 days gestation), with a better survival rate of >70%. Over time, we have also noted differential effects of analgesia in this animal model; when we commenced this work our analgesic of choice for sheep studies was fentanyl, however fentanyl itself has effects on fetal haemodynamics, including tachycardia and cerebral vasodilation (58) and we noted high rates of FGR fetal death that we surmised were due to fentanyl interfering with the brain sparing cardiovascular response that follows placental insufficiency. All ewes are now given oral or rectal paracetamol for pain relief. A limitation of the current study was that we did not collect and record weights or perform morphometric analysis on the placental cotyledons. Anecdotally, we observed that >50% of placental cotyledons from FGR offspring were necrotic and remaining healthy placentomes were more likely to be a type C with a flattened morphology that is associated with intrauterine hypoxia (33). This study provides a large overall sample size of n=135 FGR offspring and n=153 control offspring, however we acknowledge that these animals are derived from multiple studies and therefore some outcome measures were not available for all animals; for example there is a relatively small cohort of animals with fetal blood gas parameters and these were difficult to maintain during chronic recordings. Thus, a novelty of this work is that we compared control and FGR animals at various fetal and neonatal timepoints, but the experimental design means that individual fetuses were not tracked over time, rather the results of multiple animals were grouped.

In summary, we induced early-onset FGR at 88-90 days (0.6) of sheep gestation and measured physical characteristics and organ development compared with control offspring over the last trimester of ovine pregnancy and into neonatal life. Our results clearly define the phenotype of FGR in this model, with chronic fetal hypoxaemia apparent *in utero*, and no difference in fetal weight between the FGR and control groups at 110 days gestation but then significant growth restriction thereafter as gestation progresses. We observed a significant upward trajectory of growth in the control

fetuses during the last trimester of pregnancy, but the trajectory of fetal development was significantly reduced in the FGR cohort. Finally, this ovine model of FGR is associated with stillbirth as gestation progresses, which may provide a novel animal model to assess the fetal physiological responses associated with fetal deterioration. The use of preclinical animal models of pregnancy and birth compromise allows specific characterisation of how structural deficits underpin functional deficits of multiple organ systems including the brain, heart and lungs (27, 32, 50, 59–61). In animal experiments, the wellbeing of the fetus can be monitored *in utero*, postnatal clinically applicable assessments such as MRI and haemodynamic monitoring can be performed (62–64), and these can all be correlated with cellular level assessment of structural alterations in organs collected at post-mortem (64, 65). Such studies to reveal fundamental relationships between organ structure and function are essential to better our understanding of how fetal compromise alters the trajectory of development, and these studies cannot be undertaken in humans. Well characterised animal models of placental insufficiency and FGR are also critical for examination of therapeutics to target improved growth, organ development and function.

Data availability statement

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

Ethics statement

The animal study was approved by Hudson Institute of Medical Research Animal Ethics Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AS: Data curation, Formal analysis, Investigation, Project administration, Visualization, Writing – original draft, Writing – review & editing. TW: Investigation, Writing – original draft, Writing – review & editing. CR: Investigation, Writing – original draft, Writing – review & editing. BP: Investigation, Writing – original draft, Writing – review & editing. ID: Investigation, Writing – original draft, Writing – review & editing. II: Investigation, Writing – original draft, Writing – review & editing. ZA: Investigation, Writing – original draft, Writing – review & editing. YP: Investigation, Writing – original draft, Writing – review & editing. IN: Investigation, Writing – original

draft, Writing – review & editing. AM: Investigation, Writing – original draft, Writing – review & editing. TY: Investigation, Writing – original draft, Writing – review & editing. GP: Investigation, Funding acquisition, Writing – original draft, Writing – review & editing. GJ: Investigation, Writing – original draft, Writing – review & editing. EC: Investigation, Writing – original draft, Writing – review & editing. BA: Conceptualization, Formal analysis, Investigation, Funding acquisition, Project administration, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing. SM: Conceptualization, Formal analysis, Investigation, Funding acquisition, Project administration, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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The interplay of inflammation and placenta in maternal diabetes: insights into Hofbauer cell expression patterns

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Introduction: Inflammation of the placenta is harmful to both the fetus and the mother. Inflammation is strongly associated with diabetes, a common complication of pregnancy. Hofbauer cells (HBCs), unique immune system cells of fetal origin in the placenta, play complex roles, including growth of placental villi and their branching, stromal remodelling, and angiogenesis.

Methods: Our study investigated the expression of IL-1 β , IL-10, CYP2C8, CYP2C9, CYP2J2 and sEH in HBCs from patients with type 1 diabetes mellitus (T1DM) and gestational diabetes mellitus (GDM) compared to healthy controls using immunohistochemistry. We also assessed the structure of the villus stroma using Masson's trichrome.

Results: In T1DM, HBCs showed inflammatory activation characterised by increased IL-1 β and decreased CYP epoxygenase expression compared to normal placentas. Conversely, significant inflammation in HBCs appeared less likely in GDM, as levels of IL-1 β and CYP epoxygenases remained stable compared to normal placentas. However, GDM showed a significant increase in sEH expression. Both types of diabetes showed delayed placental villous maturation and hypovascularisation, with GDM showing a more pronounced effect.

Conclusion: The expression profiles of IL-1 β , CYP epoxygenases and sEH significantly differ between controls and diabetic placentas and between T1DM and GDM. These facts suggest an association of the CYP epoxygenase-EETs-sEH axis with IL-1 β expression as well as villous stromal hypovascularisation. Given the stable high expression of IL-10 in both controls and both types of diabetes, it appears that immune tolerance is maintained in HBCs.

KEYWORDS

hofbauer cells, placental inflammation, type 1 diabetes mellitus, gestational diabetes mellitus, CYP epoxygenases, soluble epoxide hydrolase

Introduction

The placenta is an important organ that provides the link between the mother and the developing fetus during pregnancy. It is not only important for the nutrition of the fetus, but also for the establishment of immunological tolerance, a prerequisite for a successful pregnancy. The unique composition and interplay of humoral and cellular factors at the feto-maternal interface contribute significantly to this. Macrophages are the second most abundant immune cells in the human placenta. The current view is that they can be classified into the following groups according to their origin, location and functional characteristics: placenta-associated maternal macrophages (PAMMs) and Hofbauer cells (1).

Hofbauer cells (HBCs) are the only known elements of the placental immune system that are of fetal origin. They are most commonly found in the stroma of placental villi, usually close to blood vessels or the superficial trophoblast (2). They are thought to originate from mesenchymal progenitor cells of the chorionic villi and yolk sac at early stages of embryonic development and from fetal haematopoietic monocytes at later stages of development (2–9). HBCs have been identified in the placenta as early as day 18 of intrauterine development and persist until the time of delivery, but their number has been shown to gradually decrease from 4–5 months of development (7). They are relatively large (10–30 μm) cells with an abundant, highly vacuolated and granular cytoplasm (10). Initially described as oval cell elements, HBCs of star and spindle shape have subsequently been identified (7, 10–12).

HBCs are considered to be alternatively activated M2 macrophages (13–17). This corresponds to the expression of CD163, CD209, CD206 antigens and the production of IL-10, TGF β , VEGF-A, FGF-2, osteopontin, MMP-9 and TIMP-1 (1, 18). HBCs fulfil a variety of functions during pregnancy. They participate in villous growth and branching, in remodelling of extracellular matrix and they also promote angiogenesis and vasculogenesis. They are involved in the maintenance of immunological tolerance at the feto-maternal interface, in elimination of apoptotic cells and they also exhibit antimicrobial activity (1, 2, 19). Interestingly, HBCs, similar to classically activated M1 macrophages, are able to produce IL-1 β , IL-8, CCL-2,3 and 4, factors typically associated with inflammation (1, 20).

Hyperglycaemia in pregnancy is a common complication with an incidence of 16.7% of live births according to the International Diabetes Federation. It can manifest itself as diabetes mellitus in pregnancy, also known as type 1 (T1DM) or type 2 (T2DM) pregestational diabetes. Most cases of hyperglycaemia in pregnancy (84%) are gestational diabetes mellitus (GDM) (21). In normal pregnancy, a physiological state of insulin resistance directs maternal nutrients preferentially to the feto-placental unit, allowing for adequate fetal growth. In GDM, insulin resistance is more severe and disrupts the intrauterine environment, resulting in accelerated fetal development with an increased risk of macrosomia (22, 23). In contrast, T1DM is a deficiency in insulin production by the islets of Langerhans as a consequence of their autoimmune damage (24).

Metabolic disorders such as diabetes and obesity are closely associated with systemic inflammation, including placental one

(25, 26). The factors that lead to the initiation of the inflammatory response in patients with diabetes are not yet fully understood. However, it is clear from *in vitro* studies that hyperglycaemia stimulates TLR-dependent inflammatory pathways, including TLR2, TLR4 and their downstream molecules, leading to overproduction of proinflammatory cytokines by macrophages (27, 28). This condition contributes to the development of insulin resistance and impaired glucose tolerance, as shown by studies conducted mainly on adipose tissue macrophages and adipocytes (29, 30). In addition to the above, it is also known that hyperglycaemia in pregnancy is associated with delayed maturation of placental villi (31, 32).

The phenotype of macrophages and their cytokine production is also related to the level of epoxyeicosatrienoic acids (EETs) (29, 33, 34). EETs play a variety of biological roles in the body and are known for their anti-inflammatory features. EETs are derived from arachidonic acid by CYP epoxygenases. Arachidonic acid (AA) is a ω -6 unsaturated fatty acid which is a part of normal diet. Its concentration in fetal plasma is higher than in maternal plasma and it is preferentially transported across the placenta over non-essential fatty acids and its precursor linoleic acid (35, 36). Arachidonic acid is also a component of the phospholipids of cytoplasmic membranes from which it is released by the activity of phospholipase A2 (37). EETs are known to be synthesised in humans mainly by the CYP2C and CYP2J subfamilies. The CYP2C subfamily consists of four enzymes, namely CYP2C8, CYP2C9, CYP2C18 and CYP2C19. CYP2J2 is the only member of the CYP2J subfamily in humans. The activity of CYP2C and CYP2J2 results in a total of four regioisomers of EETs, namely 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET, the latter two being the most abundant. Individual CYP epoxygenases produce EET regioisomers in different ratios (38, 39). EETs are highly active substances with short biological half-lives. They are either incorporated back into biomembranes or hydrolysed by soluble epoxide hydrolase (sEH) to biologically less active dihydroxyeicosatrienoic acids (DHETs) (40). In addition to CYP epoxygenases, AA can also be metabolised in cells by three different enzymatic pathways, namely cyclooxygenase, lipoxygenase and CYP ω -hydroxylases.

The effect of hyperglycaemia on the biological behaviour of HBCs has been studied to a limited extent. The results are highly contradictory and the possible further consequences for the placenta as a whole remain unknown (28, 41). Because EETs seem to play a role in the pathophysiology of diabetes, the aim of this study was to describe the expression profiles of EET-generating (CYP2C8, CYP2C9, CYP2J2) and EET-degrading (sEH) enzymes in HBCs from patients with T1DM and GDM compared to healthy controls. Moreover, it is known that EETs are involved in immunomodulation and inflammation, thus we also detected IL-10 and IL-1 β . In addition, HBCs are known to play a role in extracellular matrix remodelling and angiogenesis, and simultaneously, EETs are pro-angiogenic molecules. It is also known that diabetes is associated with delayed maturation of placental villi and changes in vasculature. We therefore investigated whether the IHC profiles obtained correspond to structural changes in the placental villous stroma.

Material and methods

Tissue samples

A total of 54 formalin-fixed, paraffin-embedded samples of term placentas from healthy controls ($n = 18$) and patients with type 1 diabetes mellitus (T1DM, $n = 22$) and gestational diabetes mellitus (GDM, $n = 14$) were used. The samples were collected in 1999–2011 at the Department of Gynaecology, Obstetrics and Neonatology, First Faculty of Medicine and General University Hospital in Prague. It is a retrospective study using archival material. Routine hematoxylin-eosin staining was performed on all samples used to assess tissue integrity. All available characteristics of the samples are summarised in [Table 1](#). The use of the samples was approved by the Ethics Committee of the University Hospital and the Faculty of Medicine and Dentistry, Palacký University, Olomouc (ref. no. 151/23).

Confirmation of Hofbauer cells in tissue samples

HBCs are the only elements of immune system found within the villous stroma. The presence of HBCs was confirmed in routine staining by their typical morphology and localization. Importantly, there is no marker specific for HBC. HBCs are considered as M2 macrophages. Thus, M2 markers, such as CD206, CD163, or CD209 are suitable for their detection ([1](#), [18](#)). Based on these, we used CD206 for their detection in our study.

Immunohistochemistry

All proteins of interest were detected in 4 μm thick paraffin sections by two-step indirect immunohistochemistry. Briefly, slides were deparaffinised, hydrated and antigens were unmasked by heat antigen retrieval in citrate buffer pH 6. For CD86 immunostaining, Tris/EDTA buffer pH 9 was used. Non-specific background staining was blocked with Protein Block (Dako) for 30 minutes at room temperature (RT). The samples were then incubated with primary antibodies for 1 hour at RT. The following primary antibodies were used: rabbit polyclonal antibodies against CD206 (Abcam; ab64693) at a dilution of 1:1000, IL-10 (Abcam; ab34843) at a dilution of 1:400, IL-1 β (Novus Biologicals, NBP1-19775) at a dilution of 1:100, CYP2C8 (ProteinTech, 16546-1-AP) at a dilution of 1: 50 and CYP2J2 (Abcam, ab151996) at 1:100 dilution and mouse monoclonal antibody against CD86 (Abcam; ab220188) at 1:50 dilution, CYP2C9 (Novus Biologicals, NBP2-01381) at 1:400 dilution and sEH (Santa Cruz, sc166916) at 1:200 dilution. Appropriate dilutions of primary antibodies were determined by staining positive control samples as recommended by the manufacturer. Antibodies were diluted in Dako REALTM Antibody Diluent (Dako). Visualisation was performed using the Mouse/Rabbit PolyDetector DAB HRP Brown Detection System (Bio SB). Tissue sections were counterstained with haematoxylin. Tris buffer (pH 7.6) was used for washing between steps. The

samples were then dehydrated and coverslipped. Positive and negative controls were included in the immunostaining of samples to verify the staining process. As a negative control, the primary antibody was replaced with Tris buffer followed by incubation with the detection system.

The semi-quantitative evaluation of the staining intensity under the microscope according to the signal intensity was graded as follows: 0 for negative tissue, 1 for weak signal, 2 for moderate signal and 3 for strong signal. The intensity was evaluated in 100 HBCs in each sample and median of these values represents the final staining intensity of the sample. The samples were evaluated twice at separate times by an experienced histologist. The samples were blinded before evaluation. The results were summarized in graphs. The distribution of staining intensities is shown in the graphs as points (one point represents one sample) and the columns show the medians.

Masson's trichrome staining

The collagen and vascular fractions in the placental villi were evaluated after Masson's trichrome staining. For image analysis, RGB images of 10 different fields of vision with a resolution of 1920x1200 pixels, saved as.tiff, were obtained using an Olympus BX40 light microscope equipped with an Olympus DP71 camera at 200x magnification for each sample. The tissue was carefully evaluated by experienced histologist using ImageJ software. The villous area, collagen area and vascular area of 15 terminal villi from each patient were detected. The binary mask for these structures were saved for measurement. AutoThreshold combined with Analyze Particles function were used to detect villi area. Vascular area was annotated manually. The collagen fraction was detected using Trainable Weka Segmentation plugin, which combines image segmentation and machine learning algorithm ([42](#)). The areas of villus, collagen and blood vessels were measured (in pixel unit). The calculation was as follows:

$$\text{collagen fraction} = \frac{\text{collagen area}}{(\text{villi area} - \text{vascular area})}$$

$$\text{vascular fraction} = \frac{\text{vascular area}}{\text{villi area}}$$

The results are summarised in graphs showing the distribution of values in each field of vision (individual points) and the mean $\pm$ SD (red and black lines).

Statistical evaluation

The obtained immunostaining intensities, as well as for trichrome staining, were evaluated by Kruskal-Wallis test to find differences between the studied groups, followed by Dunn's multiple comparison test at the level of significance $p < 0.05$ for all test performed. Statistically significant results are marked with an asterisk (*) in the graphs and figures. We used: * for $p < 0.05$, ** for

TABLE 1 Characteristics of patient samples.

type 1 diabetes mellitus (T1DM)							
No.	mother´s age (years)	duration of diabetes (years)	GlyHb %	gestation week	delivery	placenta (g)	newborn (gender/g/cm)
1	34	20	7.7	38	C-section	655	F/3630/49
2	35	6	3.8	38	C-section	645	M/3680/52
3	33	15	3.7	40	C-section	620	M/3790/51
4	21	7	5.8	37	spontaneous	550	M/2920/47
5	36	17	4.0	39	C-section	770	F/4070/53
6	23	1	3.1	39	spontaneous	490	F/3170/49
7	36	15	3.3	38	C-section	475	F/2860/46
8	31	13	4.5	37	C-section	560	M/3570/51
9	28	14	4.8	39	C-section	500	M/3390/48
10	24	11	4.7	37	C-section	400	M/2400/42
11	38	27	5.0	38	C-section	680	F/4290/52
12	42	14	4.4	36	C-section	695	M/4200/55
13	31	7	3.8	40	C-section	615	F/2880/47
14	33	10	4.8	39	spontaneous	610	M/3430/49
15	38	4	5.0	35	C-section	310	F/1845/41
16	30	10	4.3	40	spontaneous	560	F/3900/51
17	29	6	7.2	38	C-section	620	F/4480/53
18	36	23	4.1	34	C-section	370	F/2230/46
19	34	15	3.4	39	spontaneous	525	M/3210/49
20	34	5	9.4	32	C-section	754	M/2900/45
21	30	21	5.2	32	C-section	845	M/3260/50
22	22	20	7.7	38	C-section	760	M/3950/49
gestational diabetes (GDM)							
No.	mother´s age (years)	treatment	gestation week		delivery	placenta (g)	newborn (gender/g/cm)
23	27	diet	39		C-section	450	M/3010/50
24	28	diet	38		spontaneous	570	M/3140/43
25	28	–	39		C-section	1000	M/3760/51
26	27	insulin	40		spontaneous	520	M/3250/50
27	29	insulin	39		spontaneous	560	M/3080/50
28	32	diet	41		spontaneous	585	F/3940/54
29	31	diet	39		spontaneous	725	M/3555/53
30	30	insulin	37		spontaneous	500	M/3020/50
31	31	diet	40		spontaneous	550	F/3840/50
32	34	insulin	40		spontaneous	590	F/3280/50
33	37	–	39		spontaneous	570	F/3470/51
34	29	–	38		spontaneous	405	F/2910/47

(Continued)

TABLE 1 Continued

type 1 diabetes mellitus (T1DM)							
No.	mother´s age (years)	duration of diabetes (years)	GlyHb %	gestation week	delivery	placenta (g)	newborn (gender/g/cm)
No.	mother´s age (years)	treatment		gestation week	delivery	placenta (g)	newborn (gender/g/cm)
35	30	insulin		37	spontaneous	476	F/2570/46
36	33	diet		39	spontaneous	340	F/3330/51
controls							
No.	mother´s age (years)		gestation week		delivery	placenta (g)	newborn (gender/g/cm)
37	30		39		C-section	900	M/3700/
38	27		40		C-section	555	F/3030/
39	30		39		C-section	635	M/3870/52
40	24		40		C-section	620	M/3730/53
41	26		38		C-section	435	F/2780/45
42	33		38		C-section	700	M/3470/50
43	37		39		C-section	740	M/4110/53
44	24		38		spontaneous	390	F/2890/50
45	36		38		C-section	770	F/4200/51
46	30		39		spontaneous	600	F/3380/49
47	38		39		spontaneous	535	M/3490/50
48	31		39		C-section	605	M/3560/50
49	23		39		spontaneous	580	F/3520/52
50	31		38		C-section	590	F/3175/50
51	31		39		C-section	603	F/3880/50
52	30		41		C-section	575	M/3180/49
53	30		41		C-section	640	M/3820/52
54	30		39		C-section	460	F/3250/49

p< 0.01, *** for p< 0.001 and **** for p< 0.0001. All calculations were performed using GraphPad Prism 8 software.

The contribution of samples characteristics such as mother age, foetal sex, gestation age, delivery, and diagnosis to obtained IHC results were evaluated using ordinal logistic regression. These tests were perfomed by TIBCO Statistica software at the level of significance p< 0.05.

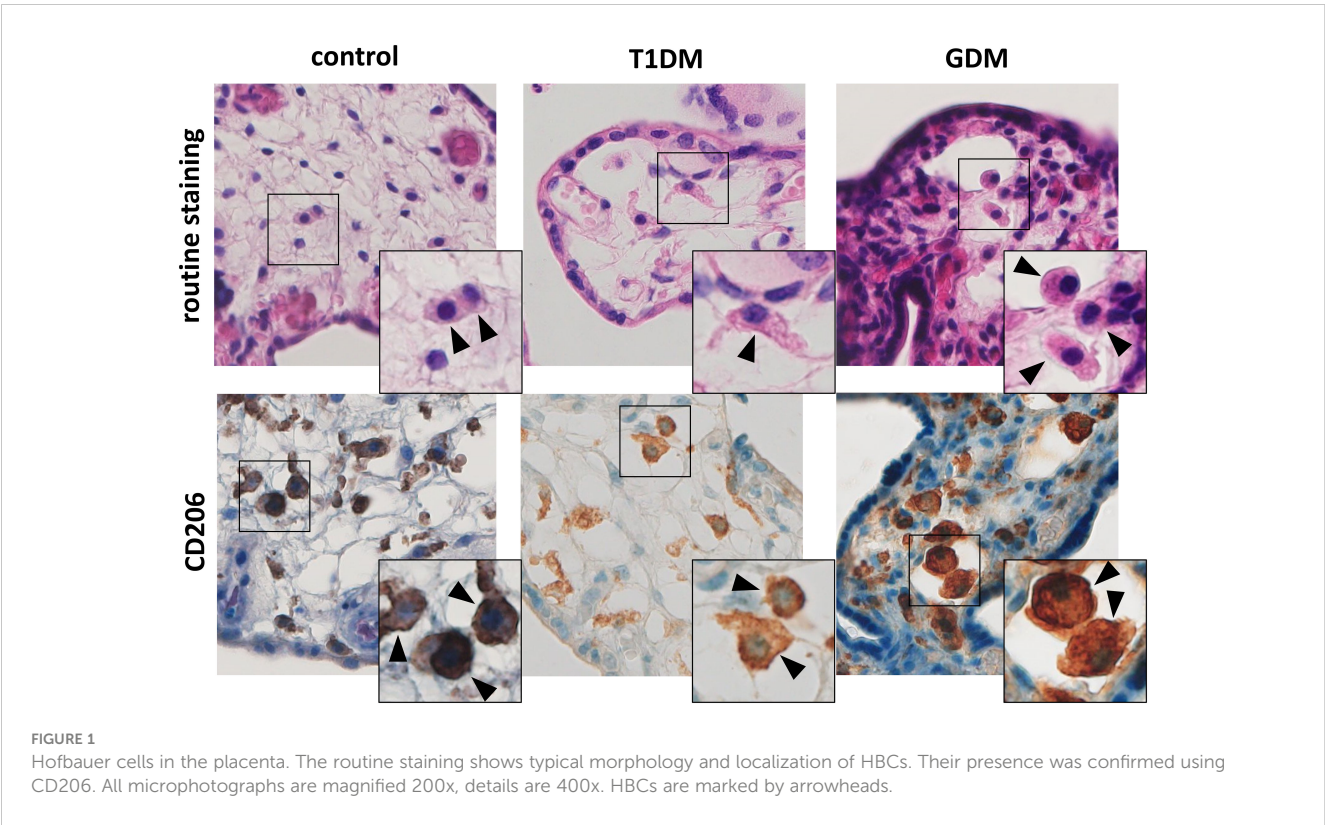
Results

Confirmation of HBCs and immunostaining profiles of IL-1β and IL-10

We detected CD206+ cell in villous stroma in all samples tested. These cells showed typical morphology and localization

of HBCs (see Figure 1). Moreover, all the samples were also negative for CD86, a marker for M1 macrophages (data not shown).

We confirmed the expression of IL-1β and IL-10 in HBCs in normal term, T1DM and GDM placentas. Both interleukins showed cytoplasmic staining, consistent with their expected intracellular localization. The expression levels of the interleukins studied in normal, T1DM and GDM placentas are summarised in Figure 2 together with representative micrographs. IL-1β showed significant differences between the groups evaluated. Its expression is significantly higher in placenta with T1DM compared to control (p = 0.002) as well as in placenta with GDM (p< 0.0001). There was no difference between control and GDM placenta (p = 0.9688). Surprisingly, the staining intensity of IL-10 showed a very uniform and high expression in all three groups (p = 0.6903, Kruskal-Wallis test).

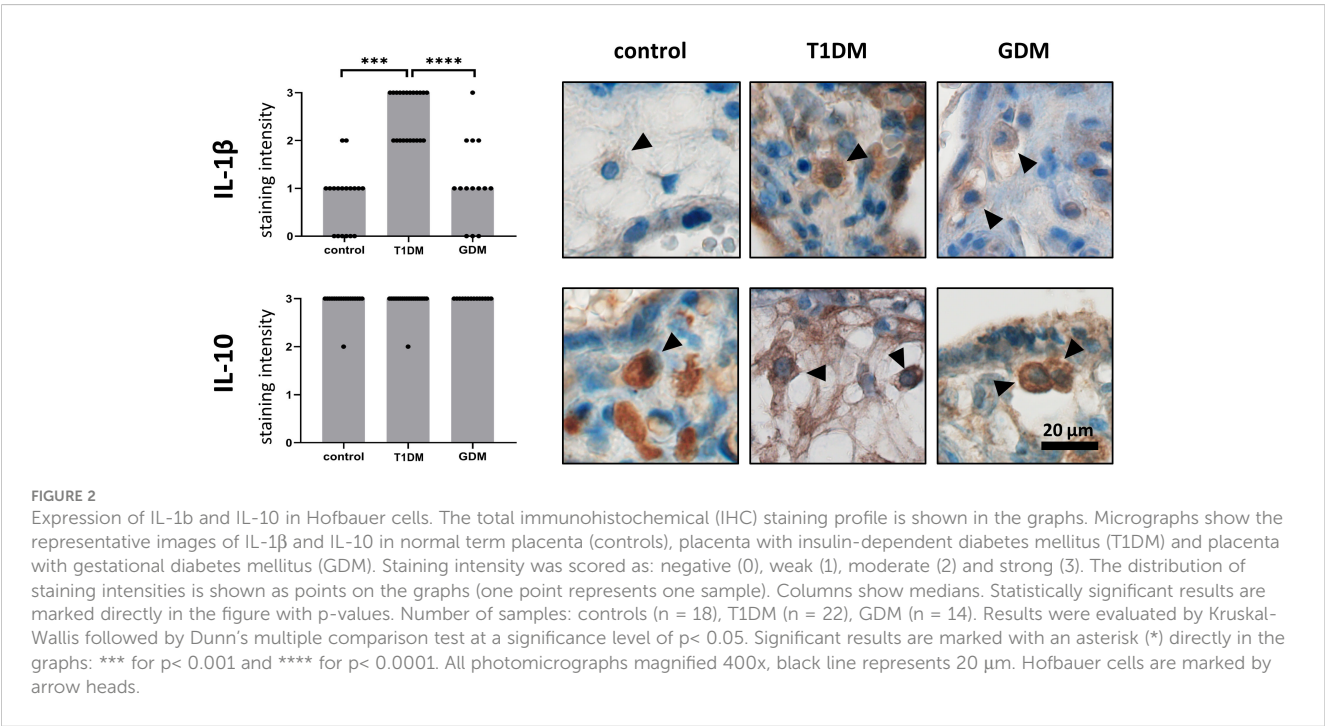


Immunostaining profiles of CYP epoxygenases and sEH

Expression of CYP2C8, CYP2C9, CYP2J2 and sEH was confirmed in HBCs in normal term placenta, T1DM and GDM placenta. All studied antigens showed cytoplasmic staining in

accordance with their expected intracellular localisation. The expression levels of the tested CYP epoxygenases and sEH in normal, T1DM and GDM placenta are summarised in [Figure 3](#) together with representative micrographs.

The highest staining intensity of CYP2C8 was found in controls (median 3) and the lowest in T1DM (median 1). These two groups



were statistically different ($p = 0.0003$). The expression of CYP2C8 in GDM was in the middle of the previous groups. However, there was no significant difference compared to controls ($p = 0.4415$) or T1DM ($p = 0.1086$). In addition, CYP2C8 showed the highest staining intensity among the epoxigenases tested in normal term placentas. CYP2C9 also showed the lowest level in T1DM, but the highest median staining intensity was detected in GDM. CYP2C9 staining intensities differed significantly only between T1DM and GDM ($p = 0.0030$). The difference between controls and T1DM did not reach significance ($p = 0.0790$). There was no difference between controls and GDM ($p = 0.7234$). The median CYP2J2 staining intensity was highest in controls (median 1.5) and showed comparable levels in both types of diabetes. CYP2J2 expression was significantly different only between controls and T1DM ($p = 0.0006$). Comparisons between controls and GDM

($p = 0.3502$) and between T1DM and GDM ($p = 0.2020$) were not significant. Expression of sEH was barely detectable in controls and T1DM, but clearly distinguishable in GDM (median 2 vs 0 in controls and T1DM). The expression of sEH differed significantly between GDM and controls ($p = 0.0009$) and between both types of diabetes ($p < 0.0001$).

Influence of sample characteristics on immunohistochemical profiles of studied antigens

In general, the ordinal logistic regression reveals that mother age, gestational age, foetal sex, delivery does not significantly contribute to IHC results. Only the diagnosis significantly

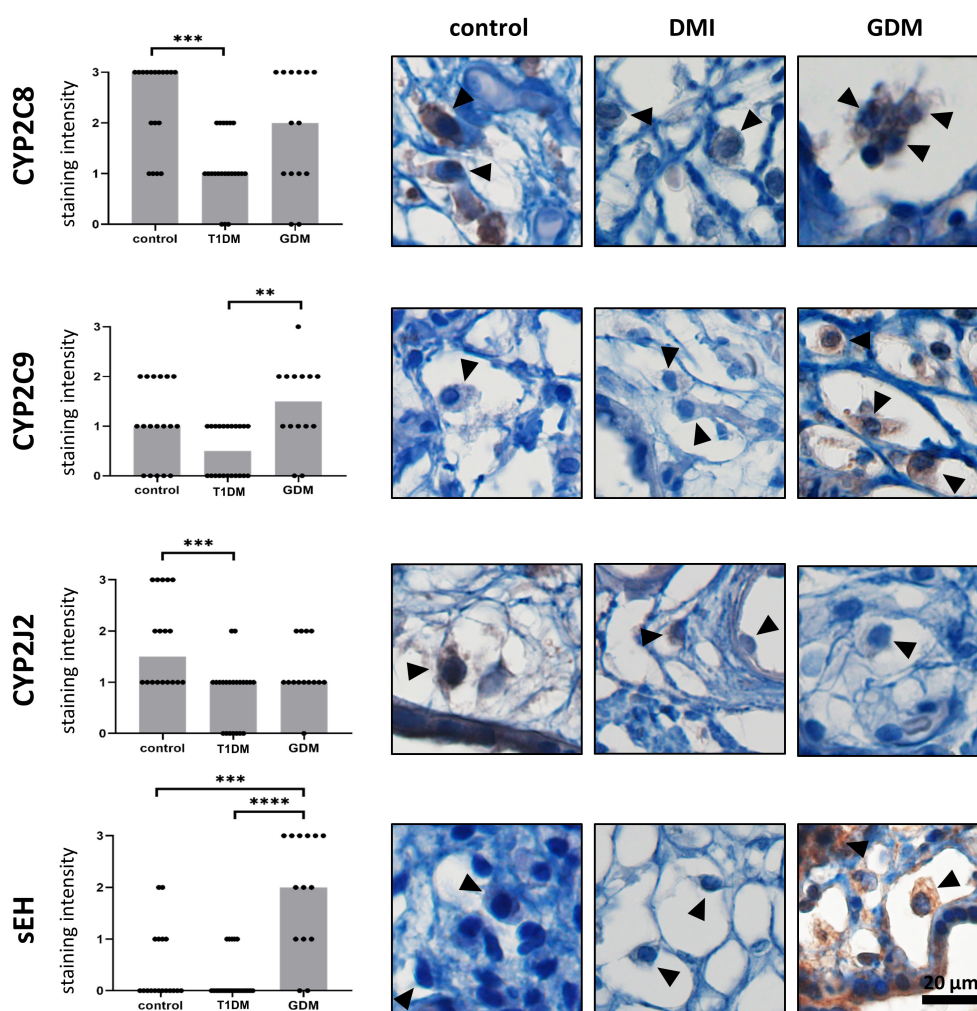


FIGURE 3

Expression of CYP2C8, CYP2C9, CYP2J2 and sEH in Hofbauer cells. The total immunohistochemical (IHC) staining profile is shown in the graphs. Micrographs show the representative images of the protein of interest in normal term placenta (controls), placenta with insulin dependent diabetes mellitus (T1DM) and placenta with gestational diabetes mellitus (GDM). Staining intensity was scored as: negative (0), weak (1), moderate (2) and strong (3). The distribution of staining intensities is shown as points on the graphs (one point represents one sample). Columns show medians. Statistically significant results are marked directly in the figure with p-values. Number of samples: controls ($n = 18$), T1DM ($n = 22$), GDM ($n = 14$). Results were evaluated by Kruskal-Wallis followed by Dunn's multiple comparison test at a significance level of $p < 0.05$. Significant results are marked with an asterisk (*) directly in the graphs: ** for $p < 0.01$, *** for $p < 0.001$ and **** for $p < 0.0001$. All photomicrographs magnified 400x, black line represents 20 μm . Hofbauer cells are marked by arrow heads.

contributed to obtained results (see Table 2), with only one exeption, namely mother age and CYP2J2 expression.

Characterization of villous stroma

Masson’s trichrome staining was used to assess the maturity of the placental terminal villi. The collagen fraction was highest in controls and significantly different from both T1DM ($p < 0.0001$) and GDM ($p < 0.0001$). There was no difference between T1DM and GDM ($p = 0.5852$). Means $\pm$ SD for controls, T1DM and GDM were plotted: 0.4140 ± 0.1789 , 0.3388 ± 0.1868 and 0.3176 ± 0.2202 , respectively. The vascular fraction was also highest in controls and significantly different from both T1DM ($p = 0.0002$) and GDM ($p < 0.0001$). Moreover, there was significant difference between T1DM and GDM ($p = 0.0235$), GDM showed the lowest values with a mean of 0.1415 ± 0.0962 (T1DM: 0.1766 ± 0.1300 , controls: 0.2162 ± 0.1373). For results see Figure 4.

TABLE 2 Factors affecting immunohistochemical profiles.

		Wald Statistics	p-value
IL-1beta	diagnosis	20.6322	< 0.0001
	mother’s age	1.8870	0.1695
	gestation week	0.0435	0.8348
	delivery	1.2590	0.2618
	foetal sex	0.6440	0.4223
CYP2C8	diagnosis	9.9729	0.0068
	mother’s age	3.6581	0.0558
	gestation week	0.7867	0.3751
	delivery	0.3010	0.5833
	foetal sex	1.1284	0.2881
CYP2C9	diagnosis	8.5217	0.0141
	mother’s age	3.1056	0.0780
	gestation week	0.0036	0.9523
	delivery	0.2078	0.6485
	foetal sex	0.4085	0.5228
CYP2J2	diagnosis	14.3997	0.0007
	mother’s age	8.2808	0.0040
	gestation week	3.5801	0.0585
	delivery	0.5688	0.4507
	foetal sex	0.7210	0.3958
sEH	diagnosis	17.0155	0.0002
	mother’s age	1.8727	0.1712
	gestation week	1.1486	0.2838
	delivery	1.8359	0.1754
	foetal sex	0.0653	0.7984

Results of ordinal logistic regresion, level of significance: $p < 0.05$. Significant factors are bold.

Discussion

We investigated the phenotype and expression profiles of IL-1 β , IL-10, CYP2C8, CYP2C9, CYP2J2 and sEH in HBCs from patients with T1DM and GDM compared to healthy controls. To our knowledge, this is the first study that directly compares these parameters in two types of diabetes and healthy controls. We used immunohistochemistry to detect antigens of interest in HBCs in situ. This approach eliminates contamination by macrophages of maternal origin.

Diabetes is known to be closely associated with inflammation (43). Our work confirmed that HBCs maintain the M2 phenotype in both T1DM and GDM by expressing CD206. This is consistent with previous studies describing that HBCs maintain the M2 phenotype in GDM (44, 45) as well as in uncontrolled T2DM (46). Conversely, other authors have described a decrease in the M2 phenotype with an increase in the M1 phenotype in both GDM and T1DM (28, 43). However, it is not clear whether Pan et al. only determined HBCs or also included decidual marcophages. Sisino et al. assessed the M1 phenotype based on CD68 positivity, although according to current opinion, CD68 antigen is considered as pan-macrophage marker and not specific for the M1 phenotype.

Although HBCs maintained the M2 phenotype, they expressed IL-1 β . We found that the two types of diabetes differed significantly in IL-1 β expression. In HBCs, IL-1 β was significantly upregulated only in T1DM and remained unchanged in GDM. This is consistent with previous findings (28, 41). Sisino et al. demonstrated a significant increase in the expression of proinflammatory cytokines, including IL-1 β , in T1DM (28). Schliefssteiner et al. showed that the cytokines IL-1 β , TNF α , IL-6 and IL-10 were not significantly altered in GDM compared to controls (41). Although we found a difference in the expression of IL-1 β between T1DM and GDM, the level of the anti-inflammatory cytokine IL-10 in HBCs was comparable in all groups tested. This may suggest that immunological tolerance at the feto-maternal interface is not affected. IL-10 plays an essential role in the establishment and maintenance of pregnancy and prevents preterm labour, which is often associated with inflammation. In addition, IL-10 regulates immune cell phenotypes and functions by modulating oxidative phosphorylation and glycolysis (47, 48).

According to some authors, there is a strong possibility that in diabetes, HBCs may undergo an inter-subgroup conversion or potentially adopt a novel, distinct phenotype, rather than simply maintaining M2 polarisation or transitioning to the pro-inflammatory M1 phenotype (49). This hypothesis is also supported by the fact that the production of proinflammatory cytokines is not solely associated with the M1 phenotype. This is supported by the IL-1 β and IL-10 expression profiles detected in the current study as well as by our previous results clearly demonstrating the colocalization of CD206 and IL-1 β expression in HBCs (50). In addition to M1 macrophages, the M2b subtype is also capable of producing proinflammatory cytokines (41). The basic classification into M1 and M2 macrophages seems to be oversimplified. The placenta is an organ where a new classification of macrophages has been proposed, abandoning the

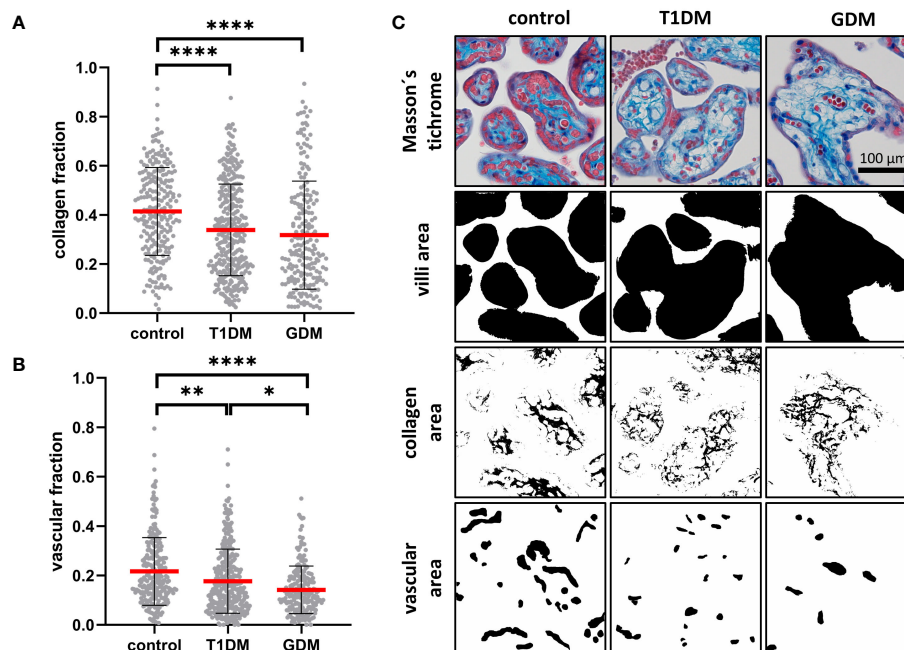


FIGURE 4

Characterisation of placental villi. (A, B) Collagen and vascular fractions of placental villi obtained from normal term placenta (controls), placenta with insulin dependent diabetes mellitus (T1DM) and placenta with gestational diabetes mellitus (GDM). Red line represents mean, error bars represent SD, each point represents one terminal villus. Number of terminal villi: controls (n = 270), T1DM (n = 330), GDM (n = 210). Results were evaluated by Kruskal-Wallis followed by Dunn's multiple comparison test at a significance level of $p < 0.05$. Significant results are marked with an asterisk (*) directly in the graphs: * for $p < 0.05$, ** for $p < 0.01$, and **** for $p < 0.0001$. (C) Representative micrographs showing Masson's trichrome staining (collagen is blue) and binary representation of total villus area, collagen area, and vascular area (created with ImageJ software). These areas were measured and used to calculate the collagen and vascular fractions. All micrographs are magnified 200x, black line represents 100 μm.

M1/M2 division and taking into account more developmental and functional aspects (1).

Among other functions, EETs also regulate the immune response. They are generally regarded as anti-inflammatory molecules. It is known that the level of EETs in tissues is determined by the ratio of the expression of EET-generating enzymes (CYP epoxygenases) and EET-degrading enzymes (sEH) (51). To the best of our knowledge, direct measurement of EETs and DHETs levels from paraffin-embedded tissue samples is not possible. In normal placenta, we found a high expression of CYP epoxygenases accompanied by a low expression of sEH, suggesting higher concentrations of EETs in a given cell type. In T1DM, there is a decrease in the expression of CYP epoxygenases, while the level of sEH does not change. In contrast, in GDM, CYP epoxygenase expression is maintained or decreases slightly, but sEH expression increases significantly. This relationship suggests different levels of EETs in T1DM and GDM.

There is a lack of information in the current literature on the interplay between CYP epoxygenases and interleukins in HBCs. The only paper suggesting a relationship between IL-1 β , IL-10 and CYP epoxygenases is our previous work (50) in which we studied normal embryonic, early fetal and term placentas. We identified CYP2C8 as a key epoxygenase in HBCs. We demonstrated a strong positive correlation of CYP2C8 expression with IL-10 and a strong negative correlation of CYP2C8 expression with IL-1 β during the study period. In current study, we found low expression of CYP epoxygenases and

barely detectable sEH in HBCs together with inflammatory activation in T1DM. In GDM, the expression levels of CYP epoxygenases were not significantly affected compared to control, suggesting preserved EET synthesis. However, sEH expression was significantly increased, suggesting the occurrence of DHET metabolites.

In addition to HBCs, the relationship between cytokines and EETs has been established in other tissue macrophages. Recently, both EETs and sEH inhibition have been shown to reduce the expression of NLRP3 inflammasome-related molecules, suppress calcium overload and reactive oxygen species (ROS) production in macrophages (52). Interestingly, Bystrom et al. conclude that epoxygenases are immunomodulators that regulate macrophages depending on the underlying activation state (33). In the case of classical macrophage activation (M1), inhibition of epoxygenases led to an increase in TNF α and COX-2 expression, which was reversed by the addition of exogenous 11,12-EETs. Surprisingly, in the case of alternative activation (M2), inhibition of epoxygenases led to a decrease in TNF α . In mouse models, EETs attenuated the M1 phenotype and the production of pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF α , while preserving the M2 phenotype (29, 34).

However, the role of DHETs in the regulation of inflammation is not clear as they have been studied less than EETs. It has been shown that 5,6-EET and, surprisingly, 5,6-DHET downregulate IL-6 production in macrophages (53). In addition, Diclofenac increased 5,6-DHET levels in obesity-related inflammatory states, suggesting its anti-inflammatory role (54). A recent study by

Bergmann et al. investigated the role of 14,15-DHET in the inflammatory response in burn injured mice. They found that 14,15-DHET functionally impairs neutrophil activation, ROS production, acidification and the expression of CXCR1 and CXCR2, the latter potentially inhibiting migration. This impairs the function of neutrophils as key elements of innate immunity. They also showed that DHET administration did not lead to a change in systematic IL-6 levels. This suggests that the increase in EETs and not the decrease in DHETs may be responsible for the decrease in IL-6 (55). Information mentioned above could explain the differences we detected in the inflammatory activation of HBCs in T1DM and GDM. As the exact role of DHETs in inflammation is not fully understood, further research is needed in this area, particularly in the tissue context.

One of the major roles of HBCs during normal pregnancy is their involvement in the process of placentation, specifically promoting angiogenesis, extracellular matrix remodelling, growth, and villous branching. This is suggested by the demonstration of VEGF, MMP-9 and TIMP-1 expression in HBCs (1). Diabetes manifests itself by delayed maturation of placental villi and their pathological vascularisation (31, 32). We confirmed it by finding a lower collagen fraction and hypovascularisation in placentas with T1DM and GDM compared to controls. Although collagen content was comparable in both types of diabetes, interesting results were obtained for vascularisation. We detected more pronounced hypovascularization in GDM than in T1DM. Given the described IHC profiles of CYP epoxygenases and sEH in HBCs in T1DM and GDM, the causes of hypovascularization may be different. EETs are known to be proangiogenic molecules. The low expression of CYP epoxygenases in HBCs in T1DM suggests that their concentrations are low, which could lead to a decrease in vascularisation. On the other hand, the expression of CYP epoxygenases is preserved in GDM. However, the high expression of sEH in HBCs in GDM could lead to EET hydrolysis and the presence of DHET metabolites. The exact role of DHET metabolites in angiogenesis is not well understood. 56 suggested that DHETs are inactive in terms of angiogenesis (56). More recently, Kumar et al. suggested that high levels of DHETs may reduce Notch signalling in angiogenesis (57). However, more research confirming potential anti-angiogenic effect of DHETs is needed since sEH could be pharmacologically targeted.

Generally, it seems that a high level of EETs is desirable in HBCs. EETs promote the M2 phenotype of macrophages, limit inflammation, help overcome insulin resistance and improve glucose tolerance, and influence angiogenesis (58, 59). Our findings may have potential implications for the pharmacological management of different types of diabetes during pregnancy. Currently available analogues of EETs (60) and in particular sEH inhibitors are in clinical trials (61, 62). The use of EET analogues may have a beneficial effect, particularly in T1DM. On the other hand, pharmacological inhibition of sEH seems to be a suitable approach in GDM. Further research in this area is therefore highly desirable.

Taken together, T1DM, we demonstrated inflammatory activation of HBCs based on high IL-1 β expression accompanied by a marked decrease in CYP epoxygenase expression compared to normal term placenta. In contrast, inflammatory activation of

HBCs is unlikely to occur in GDM, as the expression of both IL-1 β and CYP epoxygenases does not change significantly compared to normal placenta. However, in contrast to T1DM and normal placenta, there is a significant increase in sEH expression. This suggests a possible link between the CYP epoxygenase-EETs-sEH axis and IL-1 β expression, confirming its anti-inflammatory effect. In both types of diabetes, we observed a delayed maturation of placental villi and their hypovascularization, which was more pronounced in GDM than in T1DM. Based on the different expression profiles of HBCs between T1DM and GDM, a different pharmacological approach to influence placental structure and function seems to be desirable. Given the stable high expression of IL-10 in both controls and both types of diabetes, it appears that immune tolerance is maintained in HBCs.

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 humans were approved by Ethics Committee of the University Hospital and the Faculty of Medicine and Dentistry, Palacký University, Olomouc. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

ZT: Investigation, Writing – original draft. AB: Data curation, Investigation, Writing – review & editing. KK: Formal analysis, Investigation, Validation, Writing – review & editing. MM: Data curation, Investigation, Writing – review & editing. MJ: Formal analysis, Writing – review & editing. KC: Conceptualization, Data curation, Methodology, Supervision, Visualization, Writing – review & editing.

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Conflict of interest

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

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The effects of leptin on human cytotrophoblast invasion are gestational age and dose-dependent

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Introduction: Leptin and its receptors are expressed by the human placenta throughout gestation, yet the role of leptin in early human placental development is not well characterized. Leptin is overexpressed in the placentas from preeclamptic (PE) pregnancies. PE can result from the impaired invasion of fetal placental cells, cytotrophoblasts (CTBs), into the maternal decidua. We hypothesized that elevated leptin levels would impair human CTB invasion.

Methods: The effects of leptin on the invasion of human CTBs were evaluated in three cell models, HTR-8/SVneo cells, primary CTBs, and placental villous explants using invasion assays. Further, leptin receptor expression was characterized in all three cell models using RT-PCR. Further phosphokinase assays were performed in HTR-8/SVneo cells to determine signaling pathways involved in CTB invasion in response to differential leptin doses.

Results: We found that, prior to 8 weeks gestation, leptin promoted CTB invasion in the explant model. After 11 weeks gestation in explants, primary CTBs and in HTR-8/SVneo cells, leptin promoted invasion at moderate but not at high concentrations. Further, leptin receptor characterization revealed that leptin receptor expression did not vary over gestation, however, STAT, PI3K and MAPK pathways showed different signaling in response to varied leptin doses.

Discussion: These data suggest that the excess placental leptin observed in PE may cause impaired CTB invasion as a second-trimester defect. Leptin's differential effect on trophoblast invasion may explain the role of hyperleptinemia in preeclampsia pathogenesis.

KEYWORDS

invasion, leptin, leptin receptor, placenta, preeclampsia, trophoblast

1 Introduction

Leptin, a 16kDa multifunctional peptide hormone secreted primarily by adipocytes, is also secreted by the placenta (1, 2). Leptin's role has been implicated in energy homeostasis, immune function, and reproduction (3–5). Throughout human gestation, placental trophoblasts produce leptin and express leptin receptors (LepR) suggesting leptin likely plays a role in placental development (6, 7). Leptin levels in pregnancy depend both on maternal body mass index (BMI) and gestational age (8, 9). Over the course of gestation, as the placenta enlarges, circulating leptin levels increase, peak in the second trimester, and fall back to normal levels postpartum (10).

There are several lines of evidence to indicate that leptin is critical in promoting the invasion of placental cytotrophoblasts (CTB). In the homozygous female ob/ob (leptin-deficient) mouse, early placental invasion can only occur if the mice are administered with exogenous recombinant human ob protein, leptin (11, 12). Further, leptin promotes invasion of primary mouse CTB in a matrix metalloproteinase (MMP) dependent manner. The promotion of murine CTB invasion is seen only in early gestation and is lost in later gestation (13). Leptin has been demonstrated to exert a dose-dependent effect on MMPs and fetal fibronectin in human cytotrophoblast cells implicating its role in CTB invasion (14). However, the dose-dependent effect of leptin on human pCTB invasion has not been directly determined.

Several key features of a human pregnancy suggest that leptin may have different effects on CTB invasion compared to what has been demonstrated in mice. Mammalian placental structure and development are not conserved. Humans have one of the most invasive placentas; human CTBs invade through the decidua into the myometrium to establish the basal plate. By contrast, murine CTBs invade only to a shallow depth in the decidua (15). Further, primarily only human pregnancies are affected by preeclampsia (PE), a disorder characterized by maternal hypertension and proteinuria or other end organ damage after 20 weeks of gestation, which can arise from inadequate CTB invasion earlier in pregnancy (16). PE has not been observed to occur spontaneously in other species except higher apes (17). Increased leptin at the protein and/or transcript level has been reported in the maternal serum and placentas of preeclamptic women as compared to controls even after accounting for maternal BMI (18–30). As early as 13 weeks of pregnancy, a woman's risk of PE increases with increasing serum leptin levels. In fact, for every 10ng/ml increase in serum leptin, the risk of preeclampsia increases by 30% (31, 32). Interestingly, one of the risk factors for PE is obesity which has the characteristic of hyperleptinemia (33). These associations between leptin and PE raise the question of whether leptin impairs human CTB invasion. We hypothesize that high levels of leptin inhibit human CTB invasion. To test this hypothesis, the effects of varying concentrations of leptin on human trophoblast invasion of early and mid-gestation human CTBs were evaluated using HTR-8/SVneo cells, primary CTBs, and placental villous explants. Further, the expression of LepR was characterized and the leptin impact on downstream signaling pathways was explored. Understanding the effects of leptin on human CTB invasion is important for a better understanding of both normal placentation and PE pathogenesis.

2 Methods

2.1 Tissue collection

Biopsy specimens of tissue from elective pregnancy terminations were collected using protocols approved by the University of Colorado Institutional Review Board. Exclusion criteria included evidence of infection, hydropic changes, and known genetic or fetal anomalies. Gestational age was determined using standard dating criteria (34, 35). Tissue was washed extensively in cold phosphate-buffered saline (PBS, RICCA Chemical Company) and processed within one hour of the procedure. Tissue was immediately processed for isolation of primary CTBs (pCTB), villous explants, formalin-fixed for histology, or snap frozen and stored at -80°C for later RNA or protein isolation.

2.2 HTR-8/SVneo cell culture

HTR-8/SVneo immortalized first-trimester human trophoblast cells (derived by Charles Graham (36) were cultured in RPMI 1640 medium (Hyclone) supplemented with 5% fetal bovine serum (Hyclone), 100 U/ml penicillin and 100 g/ml streptomycin (Invitrogen). Cells were maintained in monolayer cultures on plastic at 37°C in 5% CO₂, 95% air, and 95% humidity.

2.3 Primary cytotrophoblast and villous explant isolation

To confirm findings obtained with the HTR-8/SVneo cells with more physiologic systems we used both primary CTB and villous explants. Primary CTB isolations were done as previously described (37). Briefly, villi were dissected away from the chorionic and basal plates to isolate the pure population of primary CTBs. A series of enzymatic digestions were used to isolate CTBs which were further purified over a Percoll gradient. The purity of each CTB preparation was determined by spinning aliquots of each preparation onto slides (Shandon Cytospin 4, Thermo Scientific) for immunofluorescent staining. Cells were fixed with 4% paraformaldehyde for one hour, permeabilized with 10% cold methanol for 10 minutes, and blocked by incubating with 3% BSA (wt/vol), 0.1% Triton X-100 (vol/vol), and 0.5% Tween 20 (vol/vol) in PBS for one hour at room temperature. The cells were incubated overnight at 4°C with anti-cytokeratin-7 antibody (1:100, Dako, clone OV-TL 12/30) to identify trophoblasts, anti-vimentin antibody (1:200, Abcam, clone SP20) to identify contaminating stroma or decidual cells or anti-CD45 antibody (1:50, Dako, clone 2B11+PD7/26) to identify contaminating immune cells. Species-appropriate Alexa Fluor conjugated antibodies (1:1000, Invitrogen) were used as secondary antibodies. Nuclei were stained with DAPI and the cells were mounted in Vectashield containing 4',6-diamidino-2-phenylindole (Vector Laboratories). Imaging was done using a Leica DM 5000B fluorescence microscope equipped with a Leica DFC 350FX digital camera (Leica Microsystems, Bannockburn, IL; and Leica-Camera, Solms, Germany). Cytotrophoblast purity

ranged from 80-95+% and the degree of purity did not affect the observed phenotype.

Villous explants were isolated as previously described (38, 39). Villous tissue (~5mm) containing 1-4 cell columns was collected from 5-11 weeks gestation and was plated on a 24-well hanging chambers with 0.4 μ m pores (Millipore) coated with 120 μ l of Matrigel (BD Bioscience) per well. The explants were incubated overnight in 20 μ l explant medium (DMEM: F12K medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin) in the top of the chamber and 500 μ l explant media in the bottom chamber. The following morning, 200 μ l of explant medium was added to each chamber. Explants were cultured for an additional 24-48 hours until cell column development was evident; villi lacking cell column development were discarded. Conditioned medium from the top of the wells was then removed and combined. Unused conditioned medium was stored at -20°C for use on subsequent experimental days.

2.4 Cell invasion assay

HTR-8/SVneo or pCTBs were seeded in invasion chambers with 8 μ m pores size membrane, (Costar) coated with 10 μ l of Matrigel Basement Membrane Matrix. (BD Bioscience) (20,000 HTR-8/SVneo cells or 200,000-250,000 pCTBs per well). The invasion chambers were placed in a 24-well cell culture plate. Invasion medium [HTR-8/SVneo: RPMI 1640. pCTBs: DMEM high glucose (Hyclone) supplemented with 2% Nutridoma-SP (Roche) and 50 μ g/ml gentamycin (Gibco)] containing 0, 10, 80, 160, or 320 ng/ml human recombinant leptin (Sigma) was added in both the upper chamber and the bottom well. The medium was replaced every 24 hours and the invasion cultures were harvested at 48 hours (HTR-8/SVneo) or 72 hours (pCTBs). Chambers were washed three times in PBS, fixed for 20 minutes in 3% paraformaldehyde at 4°C and permeabilized in ice-cold methanol for 5 minutes at 4°C. Invasion chambers were stored at -20°C until immunofluorescent staining. HTR-8/SVneo chambers were rehydrated in PBS and then mounted in a DAPI-containing mounting medium (Vector Labs) to stain the nuclei. The pCTB chambers were rehydrated in PBS and then incubated overnight at 4°C in anti-cytokeratin-7 antibody followed by anti-mouse Alexa Fluor 488 antibody for one hour. Invasion was quantified by counting invasive nuclei (HTR-8/SVneo) or cytokeratin-7 positive cellular projections (pCTBs) on the lower surface of the filter membrane. Counts were averaged from 2-5 membranes per leptin concentration per experiment. HTR-8/SVneo experiments were performed three times. Primary CTB isolations from 6 placentas (13-24-week gestations) were evaluated independently. The average invasion at each leptin concentration was normalized to the average invasion at 0 ng/ml leptin. The data were then combined after normalization.

Villous explants were grouped by size and number of cell columns and then randomly assigned to treatment condition; the first treatment day was defined as Day 0. Explants were fed with 70% explant medium, 30% conditioned medium (described above), and 0, 10, 80, 160, or 320 ng/ml leptin in the upper chambers and explant medium with corresponding leptin concentration in the

lower chambers. The leptin-containing medium was replenished on Days 1 and 2. Explants were photographed on Days 0, 1, 2, and 3. An Olympus SZX12 dissecting scope equipped with an Olympus DP72 digital camera was used to photograph the explants and the areas of trophoblast outgrowth were manually outlined. The traced area reflects both CTB migration over the surface and invasion into the Matrigel. The area on Day 3 was normalized to the area on Day 0 to account for baseline differences.

2.5 Absolute cell number determination

HTR-8/SVneo cells (20,000 cells per well) were plated on 96 well plates coated with 10 μ l of Matrigel. Cells were incubated in RPMI 1640 medium containing leptin at 0, 10, 80, 160, or 320 ng/ml. The medium was replaced at 24 hours and the cells were retrieved at 48 hours by trypsinization with mechanical disruption. Cell numbers were quantified using a hemocytometer. Two counts per well with three wells per concentration were averaged at each leptin concentration. The absolute cell number was normalized to the average cell number at plating. The experiment was repeated twice.

2.6 Leptin ELISA

Secretion of leptin into the conditioned medium was quantified using an Alpco ELISA for human leptin following manufacturer standard protocol and provided standards. Twenty microliters of conditioned medium samples from invasion assays were analyzed in duplicate. Additional standard curves using the same leptin as used in the invasion assays diluted in appropriate culture medium were also evaluated. The assay had a limit of detection of 0.5 ng/ml, intra-assay variation of approximately 4.6%, and inter-assay variation of 6.1%, as reported by the manufacturer.

2.7 Zymography of MMP-2 and -9 activity

Conditioned medium from the HTR-8/SVneo and pCTB invasion assays were collected at 24, 48, and 72 (pCTBs only) hours, frozen in liquid nitrogen, and stored at -80°C. An equal volume of conditioned medium from each experimental condition was treated with Lammeli sample buffer without boiling or reduction. Samples were frozen/thawed in liquid nitrogen/37°C three times before separation on 8% polyacrylamide gels containing 3 mg/ml porcine gelatin A (Sigma) at 100 V for 90 minutes at 4°C. After electrophoresis, the gels were soaked in 2.5% Triton X-100 twice for 30 minutes at RT and incubated in Zymogen buffer (50 mM Tris-HCl pH 8.0 and 5 mM CaCl₂) at 37°C for 48-72 hours to allow proteinase digestion of its substrate. Gels were stained with Coomassie blue and visualized on a Biorad Chemi Doc XRS. Proteolytic activities appeared as white bands of lysis against a dark background of stained gelatin. Explant cultured medium contains a serum that has inherent MMP activity negating the ability to evaluate MMP activity in the explant model system.

2.8 Detection of LepR by immunohistochemistry

Formalin-fixed, paraffin-embedded biopsy tissues were serially sectioned. Five-micrometer-thick tissue sections were cut on a microtome and mounted on super frost glass slides. The sections were deparaffinized and rehydrated before immunostaining. The goat ABC kit (Vector) was used for LepR staining. ObR N20 antibody (1:50) was used to detect all LepR isoforms, ObR C20 antibody (1:100) was used to detect the long isoform (Santa Cruz). Visualization was done with diaminobenzidine (DAB, Dako) and hematoxylin QS (Vector Laboratories) was used for the nuclear counter stain. Negative control staining was performed without primary antibodies. A Nikon Eclipse 80i microscope equipped with a Q-imaging Retiga 100R digital camera and NIS-Elements Advanced Research version 2.30 software was used for visualization and photography. Each sample was evaluated for intensity (1-3) and percent-positive-trophoblasts (0: <10%, 1: 10-50%, 2: 50-90%, 3 >90%) and the values were multiplied to calculate the immunoreactivity score.

2.9 RT-PCR to detect LepR isoforms

Total RNA was isolated from HTR-8/SVneo cells, snap-frozen explants, or pCTBs using Trizol reagent following the manufacturer's protocols (Invitrogen). RNA was quantified by measuring the absorption at 260 nm and stored at -80°C . cDNA was generated with ImProm-II reagents (Promega) from 1 μg of RNA according to manufacturer's protocols. Templates were amplified with Promega PCR 2X master mix according to standard protocols and primers synthesized by Integrated DNA Technologies (sequences in Table 1). 18S primers were used as an amplification control. Primer specificity was confirmed by demonstrating uniqueness through NCBI BLAST and detecting a single amplicon band at the predicted size on an ethidium bromide embedded 1.5% agarose gel. Briefly, RT-PCR was performed

under the following conditions: denaturation at 95°C for 2 minutes, 35 amplification cycles of: 95°C for 45 seconds, 60°C for 30 seconds, 72°C for 60 seconds followed by a final extension at 72°C for 10 minutes.

2.10 RT-qPCR to detect LepR expression changes in HTR8/SVneo cells treated with increasing leptin dose

1×10^5 HTR8/SVneo cells were seeded in a 12-well plate and treated with 0, 10, 80, 160 and 320 ng/ml of leptin for 24 and 48 hours. Post-treatment the cells were lysed and total RNA was isolated and quantified. cDNA was generated with iScript (Bio-rad) from 1 μg of RNA using the manufacturer's protocol. qPCR was performed with the fluorescent Taqman method using panLepR primers. The thermal cycling conditions were: 50°C for 2 min (AMPerase activation), 95°C for 10 min (Taq activation), 95°C for 15 sec for denaturation, and 60°C for 1 min for annealing and extension. To determine expression levels of LepR isoforms, qPCR based on the Syber Green fluorescence method was implemented using isoform-specific primers (Table 1). The thermal cycling conditions were: 95°C for 2 minutes; 40 cycles of 95°C for 45 seconds and 60°C for 30 seconds; and a melt curve analysis using temperatures from 60°C to 95°C . For quantification of gene expression changes, the $2^{-\Delta\text{ddCt}}$ method was used to calculate the fold change relative to the control (0 ng/ml). The gene expression values for LepR at each dose were normalized to 18S. All measurements were conducted in triplicates.

2.11 Phospho-kinase assay

The human phosphokinase array kit (R and D systems) was used according to the manufacturer's protocol. Briefly, HTR-8/SVneo cells were cultured on Matrigel for an hour and then stimulated with 0, 80 or 320 ng/ml leptin for 30 minutes (24 well plates, 1:1 Matrigel: Serum-free media, 500,000 cells/well). Wells were washed with PBS and lysates were harvested in lysis buffer. The lysate was centrifuged, and the supernatant was incubated with Blots A and B overnight at 4°C (167 μl lysate per part per sample) that had been pre-blocked in array buffer 1. Blots A and B are designed to identify different phosphorylations with minimal cross-reactivity. The blots were washed, incubated in the presence of A or B antibody cocktails, washed, incubated in A and B detection antibody cocktails, and washed again. Streptavidin-HRP detection reagent was then added, and blots were washed one last time. Visualization of the immunoreactive spots was done using chemiluminescence (Western Lightning Plus -ECL, Perkin Elmer) and exposure to XR film (Kodak). Film imaging and spot densitometry were performed using Biorad Chemi Doc XRS and QuantityOne software. Each blot contains duplicate spots for analyses of phosphorylation sites, 46 unique phosphorylation sites between the two blots. The average background signal from negative control spots was subtracted from each individual phospho-spot. The average background-corrected signal of duplicate spots from each treatment condition is reported as a fold change from the average

TABLE 1 pan Lep R and isoform-specific primer sequences.

	Forward Primer Sequence	Reverse Primer Sequence	Amplicon Size (base pairs)
LepR long (b)	5'- ACA GCA TCA GTG ACA TGT GGT CCT -3'	5'- CCA AGG GTT GTC TCT GGC TTT CGT -3'	244
LepR short (a)	5'- GAA GCC CGA AGT TGT GTT TGT GCT -3'	5'- TTT GCA TCA CGT CCC ATT TCC CGT -3'	212
LepR short (c)	5'- GAT GCT TGA AGG CAG CAT GTT CGT -3'	5'- TTC TTG GGT TCT CAC AGA GGG -3'	202
18s	5'- ATC CCT GAA AAG TTC CAG CA - 3'	5'- CCC TCT TGG TGA GGT CCA TG -3'	186

background-corrected signal of the equivalent spots from 0 ng/ml treated lysates.

2.12 Statistics

One-way ANOVA with Tukey post-test for multiple comparisons was carried out to determine statistically significant differences, $p < 0.05$ was considered significant.

3 Results

To determine the effect of leptin on human CTB invasion, we utilized three different model systems from the first and second trimesters of human pregnancy, the timeframe of maximal CTB invasion. The three systems were HTR-8/SVneo immortalized human trophoblast cells, pCTB and placental villous explants. The absolute concentration of leptin in placental tissue is unknown; however maternal serum levels of leptin vary during pregnancy from 40 to over five hundred ng/ml (40) based on many factors including maternal BMI, gestational age, and disease state such as PE. To model this wide range of serum leptin concentrations, we evaluated the effects of 0–320 ng/ml leptin on the invasion of human CTBs.

3.1 Leptin promotes invasion of HTR-8/SVneo cells and primary human CTBs in a dose-dependent manner

We first evaluated the effect of leptin on the invasion of the HTR-8/SVneo cells, a cell line derived from first-trimester placenta commonly used as an *in vitro* model of non-cancerous human cytotrophoblast and invasive trophoblasts. Leptin had a dose-dependent effect on the invasion of HTR-8/SVneo cells. Treatment of HTR-8/SVneo cells with 80 ng/ml leptin promoted

invasion over no leptin treatment. At 160 and 320 ng/ml leptin, the invasion was significantly decreased as compared to invasion at 80 ng/ml but did not differ from invasion in the absence of leptin (Figure 1A). To determine whether changes in the invasion were due to actual changes in invasive phenotype and not due to changes in cell number, we evaluated the effect of leptin on HTR-8/SVneo cell number when cultured on Matrigel. HTR-8/SVneo cell number increased over 48 hours in the absence of leptin, but leptin dose did not differentially affect HTR-8/SVneo cell number (Figure 1B). Therefore, the differences in the invasion were due to a direct effect of leptin on CTB invasion rather than an alteration in cell number. We next evaluated the effect of leptin on the invasion of primary human CTBs to determine if the dose-dependent effect would be recapitulated in a more physiologic system. Leptin impacted the invasion of second-trimester human pCTBs. As with the HTR-8/SVneo cells, 80 ng/ml leptin promoted maximal invasion. Invasion at both 160 and 320 ng/ml leptin was significantly lower than at 10 and 80 ng/ml leptin but did not differ from invasion in the absence of leptin (Figure 1C). This effect is not likely due to alterations in CTB number since pCTBs do not proliferate on Matrigel under these normoxic conditions (41, 42). Of note, no gross differences in apoptotic or mitotic nuclei were observed. As leptin is known to be secreted by the human placenta, we determined if the secretion of endogenous leptin by CTBs in our culture systems could account for the biological differences seen. We measured leptin in the conditioned medium by ELISA. Leptin was not secreted at detectable levels (< 0.5 ng/ml) by HTR-8/SVneo cells or pCTBs during the timeframe of culture on Matrigel. Therefore, endogenous leptin secretion is unlikely to be influencing the observed phenotypes.

3.2 Leptin's impact on the invasion of explant CTBs varied by gestation and dose

The effect of leptin on the invasion of CTBs in human placental villous explants varied with both dose and gestational age. Villous

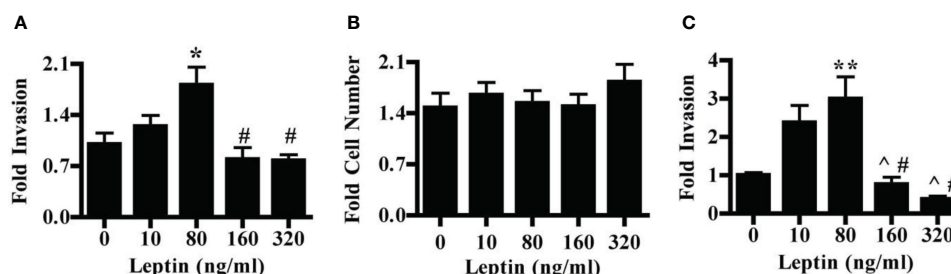


FIGURE 1

Leptin has a differential effect on HTR-8/SVneo and second-trimester human pCTB invasion. (A) Invasion of HTR-8/SVneo cells through Matrigel-coated invasion chambers was evaluated in the presence of 0, 10, 80, 160 or 320 ng/ml leptin. Data normalized to invasion at 0 ng/ml leptin are combined from three independent experiments: $n=12, 8, 14, 13$, and 12 invasion chambers per respective leptin concentration. (B) Absolute cell number of HTR-8/SVneo cells cultured on Matrigel in the presence of 0–320 ng/ml leptin was evaluated. Number of cells after 48 hours was normalized to the number of cells plated at the start of the experiment. Normalized data from three independent experiments were combined, $n=12$ wells per concentration. (C) Invasion of pCTBs plated as described for (A) Primary CTBs isolated from six placentas (13–24 weeks gestation) were evaluated independently and normalized data combined; $n=20, 19, 19, 13, 7$ invasion chambers per respective leptin concentration. Data are reported as mean fold change from 0 ng/ml $\pm$ standard error of mean (SEM). * $p < 0.05$ vs. 0 ng/ml, ** $p < 0.01$ vs. 0 ng/ml, ^ $p < 0.05$ vs. 10 ng/ml, # $p < 0.01$ vs. 80 ng/ml.

explants provide a model system for exploring the effects of leptin on CTB invasion in a context that maintains complex cellular interactions as well as the secretion of stromal and syncytial factors. CTB invasion in villous explants was observed from Day 1 to Day 3 (Figure 2A). In the earliest samples, less than 8 weeks gestation, leptin promoted invasion with increasing dose, reaching significance at 320 ng/ml (Figure 2B). Leptin did not affect the invasion of explant CTBs from 8–10 weeks gestation (Figure 2C). However, leptin had a differentially invasive response in explant CTBs after 11 weeks of gestation, similar to what was observed for HTR-8/SVneo cells and pCTBs (Figure 2D).

3.3 Leptin does not affect MMP-2 and -9 activity in HTR-8/SVneo and pCTB

Leptin has been shown to alter MMP activity in murine as well as in human CTBs and may mediate the effect of leptin on invasion. Therefore, we evaluated the activity of MMP-2 and MMP-9 in the HTR-8/SVneo and pCTBs by gelatin zymography. HTR-8/SVneo cells expressed very low levels of active MMP-9 and differences in activity in response to leptin were not observed. HTR-8/SVneo cells also expressed pro- and active MMP-2, both were unaffected by leptin. Primary CTBs mainly expressed active MMP-9, and had very

low levels of pro-MMP-2 and undetectable active MMP-2. MMP levels and activity were not affected by leptin concentration at any gestational age in pCTBs (Figure 3). The original gel image illustrating the enzymatic activity of MMP2 and MMP9 following leptin treatment in pCTB (17 weeks) and HTR8/SVneo cells is shown in Supplemental Figure I. As the villous explants are cultured in serum, which contains substantial MMP activity, we were not able to evaluate leptin effects on MMP activity in the explant system. Leptin effects on MMP-2 and -9 do not explain the differential effect of leptin on CTB invasion.

3.4 Leptin receptor expression does not change during the first half of pregnancy

The effect of leptin on CTB invasion could be mediated by LepR. Therefore, we characterized leptin receptor expression in our model systems. Immunohistochemistry was used to localize the receptors by cell type and across gestation up to 20 weeks. We analyzed the villous explants used in the invasion assays as well as biopsies from additional placentas. Two different LepR antibodies were used to isolate isoform-specific expression. The first antibody recognizes an N-terminal antigen and detects all LepR isoforms (N-20). This antibody showed immunoreactivity in all trophoblasts at all

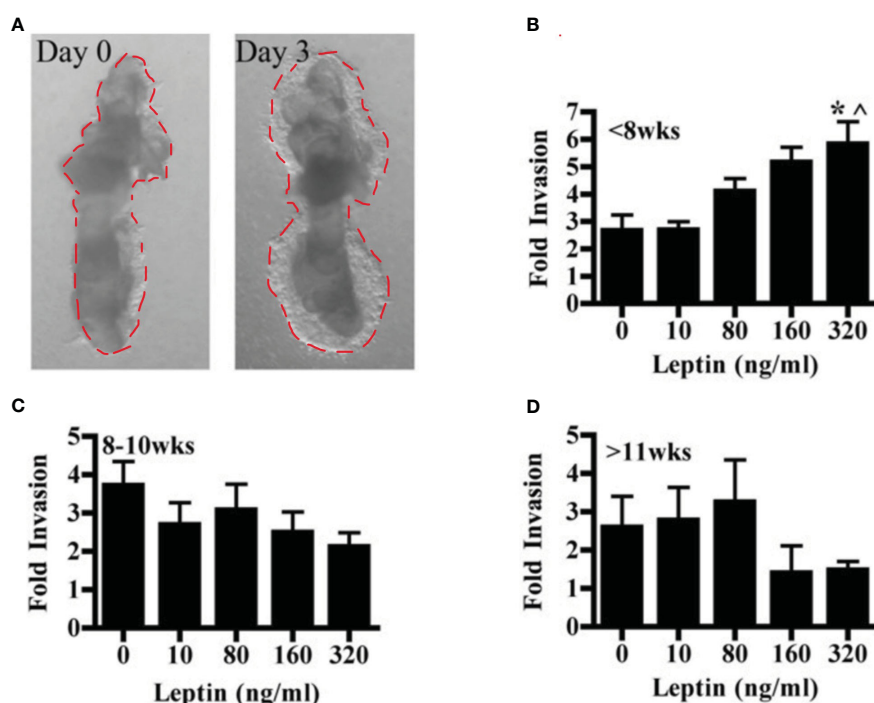


FIGURE 2

Leptin affects human explant CTB invasion in a dose and gestational age-dependent manner. Invasion of placental villous explants plated on Matrigel was evaluated in the presence of 0–320 ng/ml leptin. The area of Matrigel invaded by CTBs after three days of leptin treatment (Day 3) is normalized to the area invaded on Day 0, before the initiation of leptin treatment. Normalized data are combined from independent experiments. (A) A representative image of the CTB invasion on Days 0 and 3. (B) Fold change invasion of <8 weeks gestation explant CTBs increases with increasing leptin dose (5- and 7-week gestation placentas; n=6–8 explants per leptin concentration). (C) Fold change invasion of 8–10-week gestation explant CTBs is not differentially affected by leptin (8, 9, and 10-week gestation placentas; n=8–14 explants per leptin concentration). (D) Fold change invasion of explant CTBs from two independent 11-week gestations had a differentially invasive trend in response to leptin (n=5–6 explants per leptin concentration). Data are reported as mean fold change in the area invaded on Day 3 compared to Day 0 $\pm$ SE. *p<0.05 vs. 0 ng/ml, ^p<0.05 vs. 10 ng/ml.

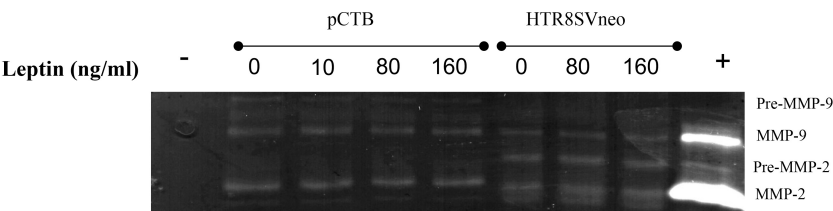


FIGURE 3
Leptin did not change MMP-2 or MMP-9 activity in human CTBs cultured on Matrigel. Culture medium from the top chamber of the invasion assays was collected and analyzed for MMP-2 and -9 activity by gelatin zymography. Representative images are shown from the conditioned medium after 24 hours for 17-week pCTBs and HTR-8/Svneo cells (conditioned medium from n=3 invasion assays). Similar results were seen for pCTB regardless of gestational age (n=4, 17-23 weeks gestation). Serum-free medium served as the negative control (-), and medium containing serum served as the positive control (+).

gestational ages including syncytiotrophoblasts and CTB in the villi, CTBs in the cell column, and extravillous trophoblasts (Figure 4A). The second antibody recognizes a C-terminal antigen unique to the long receptor isoform (C-20). Immunoreactivity with this antibody

was observed in syncytiotrophoblasts and CTB in the villi, CTBs in the cell column, and extravillous trophoblasts (Figure 4B). A comparison of LepR expression by semi-quantitative scoring of immunoreactivity did not reveal differences in expression from 5-

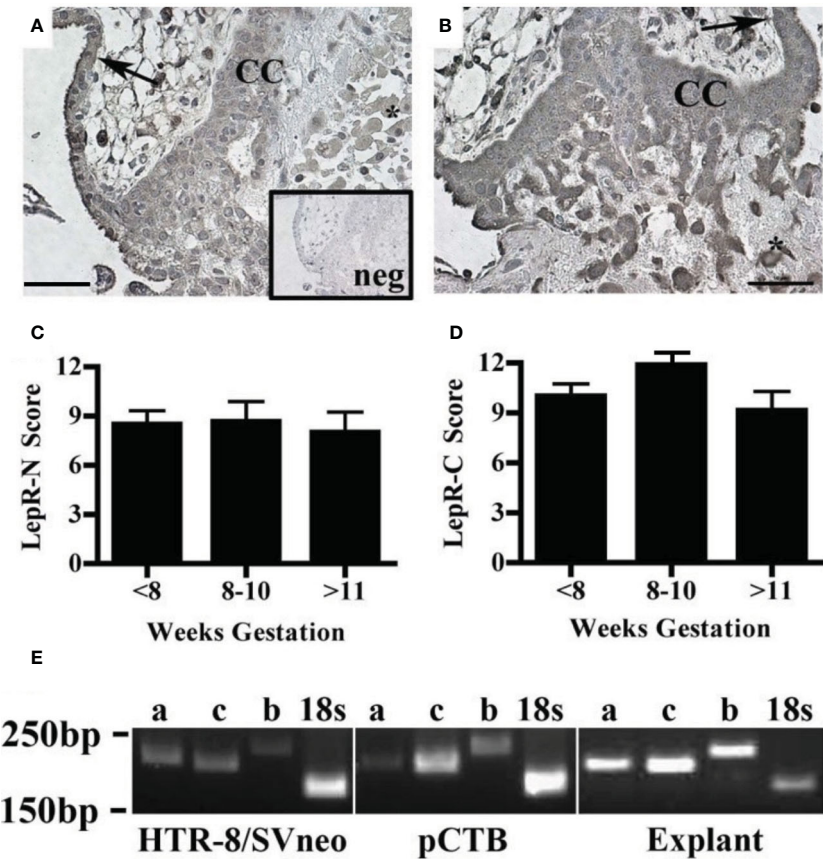


FIGURE 4
Human placental LepR expression did not change across the first half of gestation. Formalin-fixed, paraffin-embedded human placental biopsy tissue sections were evaluated by immunohistochemistry for LepR with antibodies that detect (A) all isoforms (N-20 antibody) or (B) exclusively the long isoform (C-20 antibody). LepR expression was found in all trophoblast populations at all gestational ages (<8 weeks n=8, 8-10 weeks n=6, 11-20 weeks n=7). Representative images containing villous and basal plate trophoblasts are shown; arrow indicates villous CTBS, CC indicates cell column CTBS, *indicates extravillous trophoblasts, and scale bar represents 50µm. The inset in A shows negative control. (C) Immunohistochemistry scores of LepR-N expressions (all isoforms) and (D) LepR-C expression (long isoform only). An intensity score (0-3) was multiplied by the percent-positive trophoblasts score (<10%=1, 10-50%=2, 50-90%=3, >90%=4) for each sample evaluated at 10X magnification. (E) RT-PCR was performed using LepR isoform-specific primers [a=short isoform 2 (212bp), c=short isoform 3 (204bp), b=long isoform 1 (244bp)] on total mRNA from all three model systems; 18S (186bp) was used as an amplification control. For all model systems and all gestational age windows evaluated all three isoforms were expressed. Gel blots are representative of HTR-8/Svneo (n=3), pCTB (n=6; 24 weeks shown), and explants (n=4; 11 weeks shown).

20 weeks (Figures 4C, D). Because the long LepR isoform is expressed in all trophoblast populations at all gestational ages evaluated, we are unable to make conclusions about the short LepR isoforms from immunohistochemistry studies. We, therefore, conducted RT-PCR using isoform-specific primers to determine if the short isoforms (LepRa and LepRc) as well as the long isoform (LepRb) is expressed in our model systems. In HTR-8/SVneo cells, pCTBs, and explant tissue, mRNA for all three isoforms were expressed (Figure 4E).

3.5 Leptin receptor expression does not change with differential leptin treatment in HTR8/SVneo cells

Leptin's impact on the invasion of cytotrophoblasts (CTB) may be attributed to potential variations in LepR expression in response to varying concentrations of leptin. To investigate whether an increasing dose of leptin induces or downregulates leptin receptor expression, HTR8/SVneo cells were treated with 0, 10, 80, 160, and 320 ng/ml of leptin for 24 and 48 hours. Quantitative polymerase chain reaction (qPCR) analyses were performed using both pan LepR and isoform-specific primers to assess the mRNA expression levels of distinct LepR isoforms. The results revealed no statistically significant differences in LepR expression in HTR8/SVneo in response to increasing doses of leptin. Although at 24 hours, panLepR expression shows a bell-shaped response to increasing concentration of leptin, the differences were statistically non-significant (Figure 5A). Next, using HTR8/SVneo cells we explored if any of the Lep R isoform expression is impacted by increasing leptin dose and could be attributed differentially invasive

phenotype. Further, LepR isoform-specific expression also did not show any significant change in response to varied leptin doses (Figure 5B). Similar results were obtained at 48 hours (Figures 5C, D). Of interest, even the fold change ratios of long to short isoforms did not show any significant changes in response to leptin doses (Supplementary Figure II). This indicates a lack of modulation in leptin receptor expression under the experimental conditions. Due to the limited availability of tissue samples and primary CTB cell numbers, we were unable to replicate the same assay in the other two model systems (pCTBs and villous explants). While the ratios of long/short LepR isoforms did not change in response to leptin, however, we cannot rule out the possibility that the relative ratio of short LepR isoforms can change over gestation.

3.6 Leptin has a dose-dependent effect on signaling pathways

Next, we were interested in unraveling the molecular mechanism of leptin's differential effect on human trophoblast invasion. One possibility for the differential invasion in normal trophoblasts in response to different concentrations of leptin is the differential activation of cell signaling pathways. An untargeted approach was implemented to evaluate 46 unique protein phosphorylations in response to 0, 80, and 320 ng/ml of leptin in HTR-8/SVneo cells. The phosphorylation signal on the arrays in response to leptin was normalized to signal in the absence of leptin (represented by the y-axis set to 1). A total of 16 proteins showed greater than a 20% difference in phosphorylation between 0, 80, and 320ng/ml leptin. Three different patterns of phosphorylation

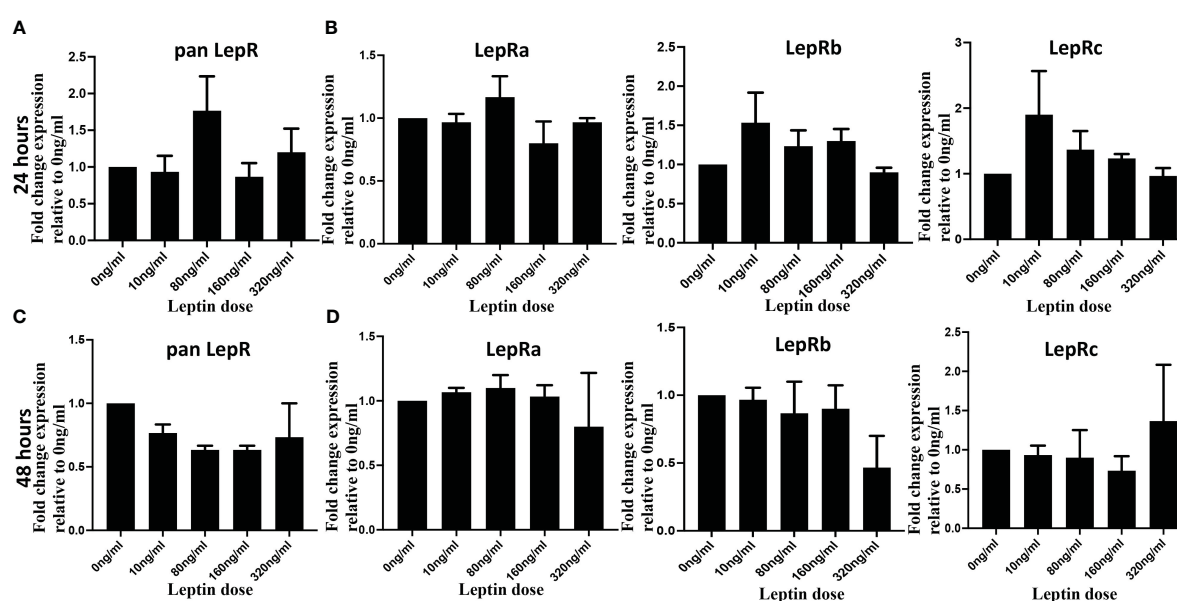


FIGURE 5

Leptin did not change LepR expression in HTR8/SVneo cells. HTR8/SVneo cells were treated with indicated concentration of leptin and total RNA was isolated 24 hours (Upper panel) and 48 hours (Lower panel) hours post-treatment. Total RNA was used to perform qPCR to determine mRNA levels of Leptin receptor using pan Lep R primers (A, B) and isoform specific primers (C, D). Bar graphs represent the mean fold change relative to the control (0 ng/ml). The gene expression values for LepR at each dose were normalized to 18S. Data are depicted as Mean $\pm$ SEM from three independent experiments.

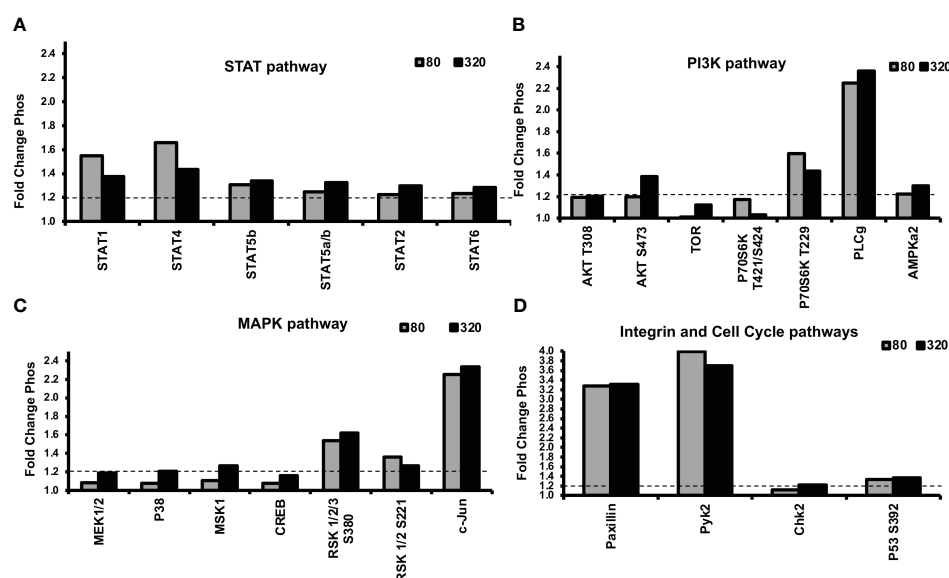


FIGURE 6

Alterations in STAT, PI3K, and MAPK signaling protein phosphorylation were evident in response to leptin, as determined by a phosphokinase array. HTR-8/SVneo cells, cultured on matrigel for an hour were treated with 0, 80, or 320 ng/ml leptin for 30 minutes and were subjected to phosphokinase array analysis. Out of 46 unique phosphorylation sites evaluated, 16 sites showed greater than a 1.2-fold (20%) difference in phosphorylation between 0, 80, and 320 ng/ml leptin. The dotted line represents a threshold of 1.2-fold. Leptin doses 80 and 320 ng/ml are shown as gray and black bars respectively. Changes in phosphorylation in (A) STAT pathway, (B) PI3K pathway (C) MAPK pathway, and (D) Integrin and Cell Cycle Pathways. STAT, PI3K, and MAPK signaling may be involved in the differential invasive response to leptin.

emerged. The proteins showing the same level of phosphorylation at 80 and 320 ng/ml of leptin were categorized as “uninvolved”, and those showing increased phosphorylation at 80 ng/ml of leptin and less phosphorylation at 320 ng/ml of leptin were designated as “Bell-shaped”. The proteins that showed increased phosphorylation at 80 ng/ml of leptin and a further increase in phosphorylation at 320 ng/ml of leptin were categorized as “further augmented”. Each pattern was given a letter designation (U= uninvolved, B= bell-shaped, F= further augmentation). Less phosphorylation or increased phosphorylation at 320 ng/ml of leptin could result in reduced invasion. The proteins were grouped by signal transducer and activator of transcription proteins (STATs), phosphoinositide-3-kinase (PI3K), and Mitogen-activated protein kinase (MAPK) pathways (pathways typically associated with leptin signaling).

Many of the STATs showed increased phosphorylation in response to leptin treatment (Figure 6A). STATs 1 and 4 showed bell-shaped pattern phosphorylation and may be involved in the differential invasion of trophoblasts in response to leptin. The remaining STATs did not show patterns suggestive of involvement in the bell-shaped pattern. STATs 5, 2, and 6 showed pattern U phosphorylation; STAT3 was not found to be induced by 30 minutes of leptin treatment.

The PI3K pathway also showed a phosphorylation response to leptin treatment (Figure 6B). AKT, a member of the PI3K pathway, showed site-specific phosphorylation at different leptin doses. Site T308, which PDK-1 phosphorylates, was equally stimulated by both doses (80 and 320 ng/ml) of leptin, suggesting a U pattern whereas site S473, which is phosphorylated by mTORC2, suggests an F pattern of phosphorylation. mTOR showed increased phosphorylation at 320 ng/ml whereas 80 ng/ml was no different from baseline, suggesting an

F pattern of phosphorylation. P70 S6 kinase, which is downstream of mTOR's isoform, mTORC1, showed no phosphorylation at site T389, on the contrary, its site T229 (phosphorylated by PDK1) and T421/S424 (phosphorylated by MAPK) showed B pattern phosphorylation. These observations suggest that the differential leptin invasive response may be driven through AKT/mTOR/p70 S6.

Many proteins in the MAPK pathway were phosphorylated in response to leptin treatment (Figure 6C). Two transcription factors c-jun and Adenosine 3'5' cyclic monophosphate (cAMP) response element binding protein (CREB) showed F pattern phosphorylation. MKK1/2 (MEK1/2), p38, and mitogen and stress-activated protein kinase (MSK-1) also showed F pattern phosphorylation where only 320 ng/ml leptin resulted in greater than a 20% increase in phosphorylation compared to no leptin. P42/p44 phosphorylation was undetected at 30 mins of leptin stimulation. P90 ribosomal S6 kinase (RSK)-1/2/3, showed U pattern phosphorylation at both S380 and S221 activation sites, which does not support the role of RSK in the differential invasion. The phosphorylation array data suggest that many proteins in the MAPK stress response pathways are stimulated by 320 ng/ml leptin, including transcription factors which may ultimately be inhibiting invasion at 320 ng/ml leptin.

Other proteins that were phosphorylated in response to leptin include the cell cycle pathway proteins (Chk2 and p53) and integrin pathway proteins [Paxillin and non-receptor proline-rich tyrosine kinase (pyk-2)] (Figure 6D). Chk2 and p53 showed U pattern phosphorylation and hence are unlikely to be involved in differential invasive response to leptin. Pyk-2 showed B-pattern phosphorylation however the other members of the integrin

signaling complex such as Paxillin, FAK (Focal adhesion kinase), and Src in contrast showed no change in phosphorylation with leptin treatment (data not shown). However, we speculate that leptin and integrin signaling could be involved in crosstalk as evidenced by differences in Pyk-2 phosphorylation at 80 and 320ng/ml of leptin dose.

4 Discussion

This study shows that the effects of leptin on human CTB invasion are gestational age and dose-dependent. Our hypothesis that high levels of leptin would inhibit trophoblast invasion did not fully capture the complexity of human CTB responses to leptin over gestation. Before 8 weeks gestation, leptin promoted explant CTB invasion in a positive dose-response manner. Between 8–10 weeks gestation, leptin had no significant effects on explant CTB invasion. After 11 weeks gestation, leptin demonstrate a differential invasive response in explant CTBs, HTR8/SVneo cells, and pCTBs model systems revealing a complex relationship between leptin and invasion over gestation. We investigated leptin's impact on MMP activity in our model systems (HTR8/SVneo cells and pCTB) and found that MMP levels and activity were not affected by leptin concentrations. The effect of leptin on CTB invasion could be mediated by LepR and hence we confirmed that all the isoforms of LepR are expressed in our model systems. We also determined that LepR isoform expression does not change in HTR8/Svneo cells when exposed to differential leptin dose. This led us to explore if differential leptin concentrations might activate distinct signaling pathways, influencing CTB invasion. Unbiased exploration of signaling in response to leptin concentrations in HTR-8/SVneo cells suggests roles for several STAT, PI3K, and MAPK signaling pathway molecules in the differential invasive response to leptin.

In early gestation, human CTBs are highly invasive as they establish the maternal/fetal interface. In the second trimester, the invasive capacity decreases and is complete by the end of the second trimester. Thus, it is conceivable that CTB's ability to respond to invasive stimuli (i.e., leptin) would change with gestation. Our results could reflect that leptin in early gestation signals CTBs to invade. In mid-gestation, moderate levels of leptin continue to enhance invasion. Gestational age-dependent responses have also been found for murine CTBs, where leptin promoted the invasion of primary CTBs isolated at day 10 of gestation but had no effect on murine CTBs isolated from day 18 (mice have a 21-day gestation) (13). Although both human CTBs and murine CTBs show changes in responsiveness to leptin over gestation, the character of these responses is different. Our data shows a bell-shaped invasive response to leptin that is distinct from what has been observed in murine models. However, there is a report of leptin dose-response in HTR8/SVneo cells consistent with findings in murine models (43). It is noteworthy, that we were able to demonstrate the bell-shaped invasive response in primary CTBs which corroborates with our findings in HTR8/SVneo cells conferring confidence in the reproducibility and relevance of our data.

The leptin effects on invasion were not explained by MMP-2 or -9 activity. Leptin was previously found to stimulate MMP-2 and/or -9 activity in primary human CTBs isolated from early, but not late, gestation when cultured on glass or plastic; mid-gestation was not evaluated in those studies (14, 44). Differences between our results and previous studies may be due to differences in culture conditions. Culturing primary human CTBs on glass/plastic induces them to differentiate toward a syncytial phenotype, whereas culture on Matrigel induces differentiation toward an invasive phenotype. These different cell types very well may have different responses to leptin. Further, MMP activity may be maximal in CTBs differentiated on Matrigel and therefore not able to be further stimulated by leptin. Our results suggest that some other aspect of invasion rather than MMP-2 or -9 activity is responsible for the differential response to leptin.

The question remains why does the CTB response to leptin change over gestation? The cells may become leptin-resistant with increasing gestational age. However, our data do not support this idea as human CTBs isolated throughout the second trimester retain the ability to increase invasion in response to 80 ng/ml leptin. An alternative possibility is that human CTBs are engaging different signaling machinery, potentially through the expression of different receptors. Regarding LepR, our data indicate that all LepR isoforms are present at all gestational ages and in all our model systems. These findings are consistent with those of other groups (6, 45, 46). Changes in either total LepR or long/short LepR isoform levels over gestation or in response to leptin dose itself do not appear to account for the change in responsiveness to leptin.

A differential response of cells to leptin is not without precedent. Human monocytes increase cholesterol synthesis in response to leptin up to 100 ng/ml but suppress cholesterol synthesis at higher concentrations. At lower concentrations, monocytes engage mitogen-activated protein kinase (MAPK) and phosphatidyl-3-kinase (PI3K) signaling, but actions at higher concentrations involve protein kinase C (PKC) signaling (47). Subsets of rat ventromedial and accurate hypothalamic nuclei neurons have a differential excitation/inhibition response to leptin, also possibly mediated by PI3K and AMP-activated protein kinase (AMPK) (48). The differential leptin action on human CTB may similarly be regulated by engaging different signaling pathways at different concentrations. LepR signaling is also known to be subject to negative feedback by suppressor of cytokine signaling (SOCS)-3. SOCS-3 levels or other signaling pathway molecules may change over gestation or in response to leptin dose. Another possible mechanism is leptin impacting the degree of LepR extracellular cleavage, thereby altering the ability of LepR to signal. Increasing doses of leptin up to 100ng/mL has been shown to decrease LepR cleavage and therefore increase cellular responsiveness to leptin (49). This maybe contributing to the increase in invasion response in our data from 0 to 80ng/mL treatments. Whether higher doses of leptin would further decrease LepR cleavage not known, but if holds then would not explain the decreasing response to leptin at the higher doses. Another contributing factor to the differential response of cells to leptin could be saturation of LepR which has been previously

reported in pathological pregnancies (50). It has been observed in trophoblast explants that increasing dose of leptin can increase signaling through LepR (51) but overstimulation of LepR leads to negative modulation of signaling pathways (52). Alternatively, LepR signaling capacity may change via alterations in membrane localization. In other tissues, high leptin levels induce receptor internalization which (53, 54), if occurring in our systems, may result in a loss of response to leptin at higher concentrations.

To determine which signaling pathways are involved in the leptin-induced differential invasive response of trophoblast cells, we implemented an untargeted approach using a phosphokinase array on the HTR-8/SVneo cell model system. The phosphorylation data suggest the roles of STAT, PI3K, and MAPK signaling in the differential invasive leptin response in HTR8/SVneo cells. STAT1 and STAT4 showed a differential pattern of phosphorylation. STAT1 is known to be phosphorylated by leptin signaling through LepR. Further, STAT1 expression is known to be increased in the processes of CTB differentiation into EVT. The expression of STAT1 in EVT suggests it may be involved in the process of trophoblast invasion. STAT4 has not previously been reported to be phosphorylated by leptin signaling through LepR. STAT4 is generally thought of as being involved in immune responses. However, as with STAT1, STAT4 expression is increased in the process of CTB differentiation to EVT. The promoters of many genes upregulated in EVT have putative STAT4 response elements further adding support to the role of STAT4 in trophoblast invasion (55). The differential phosphorylation pattern of STAT1 and STAT4 responses to leptin as well as their association with trophoblast invasion and differentiation suggest these may be involved in the differential response and warrant follow-up studies.

Many proteins in the MAPK pathway were phosphorylated in response to leptin treatment. Two transcription factors, c-jun and CREB showed F pattern phosphorylation. C-jun is involved in diverse processes in many different tissues. CREB also affects many processes including cell migration and differentiation. MKK1/2 (MEK1/2), p38 and MSK-1 also showed F pattern phosphorylation. p38 can activate MSK-1 which can in turn activate CREB. MAPK pathway data suggested that many proteins in the MAPK stress-responsive pathways are stimulated by 320ng/ml leptin, including transcription factors, which may ultimately contribute reduction in the invasion at 320 ng/ml.

PI3K/AKT signaling pathway may also play a role in leptin-induced CTB invasion, but it is not likely in response to PI3K activation. Rather, leptin may be signaling through the AKT/mTOR/p70 S6 kinase pathway to alter invasion.

Specifically, STAT1/4, the p38 stress pathway, and p70 S6 kinase proteins look promising for involvement in the failure of invasion at high leptin doses. mTOR/p70 S6 kinase inhibition has been shown to inhibit cell proliferation and invasion in trophoblast cell models (56). The phosphorylation data suggest that STAT, PI3K, and MAPK signaling may be involved in the differential invasive response of leptin in HTR-8/SVneo cells and warrants further investigation. Although the invasion pattern of pCTBs and HTR-8/SVneo cells show similar response to leptin, the signaling cascades in HTR-8/SVneo cells maybe different, given it is a

transformed cell line, than those in pCTBs derived from primary tissue. Nonetheless, the HTR-8/SVneo results allude to the pathways that can be involved in the differential invasive response of leptin. Inhibiting and activating these pathways in the various model systems would further clarify how these pathways are involved in the loss of leptin-induced invasion at the higher doses.

When we consider hyperleptinemia observed in preeclampsia, a disorder of insufficient trophoblast invasion, excess leptin may be hindering placental invasion as part of the pathologic process. While all women with obesity do not develop PE, our data provides a potential mechanism explaining the association of obesity with PE. Women who enter pregnancy with higher levels of leptin may have a leptin response in the second trimester that does not optimally promote invasion. There are several possibilities for how excess leptin could prevent maximal CTB invasion in PE. Our data support that exposure of CTBs to higher levels of leptin after 11 weeks could create a situation where the trophoblasts fail to invade to a physiologic depth. Our studies would suggest that the effect of leptin on CTB invasion in PE may be a second-trimester-specific defect. This concept is consistent with the finding that women who have higher leptin levels at 13 weeks gestation are at higher risk of subsequently developing PE (31, 32). Another possibility, which our studies do not address, is that CTBs from placentas of pregnancies complicated by PE may have an inherent defect, which prevents them from responding to the leptin-induced promotion of invasion.

5 Conclusion

This work looks at the direct effect of leptin on first and second-trimester human CTB invasion. Leptin has varying yet reproducible effects dependent on leptin dose and the gestational age of the tissue tested. Leptin differentially modulates human CTB invasion without affecting MMP-2 and MMP-9 activities or LepR isoform expression. While changes in leptin responsiveness are not due to differences in LepR expression our data suggest that they may be due to differential downstream signaling. This work demonstrates that the HTR-8/SVneo cell models in regards to leptin response recapitulates second trimester pCTBS and therefore can be used for further understanding of the differential response of human CTBs to leptin. Further, this work provides fundamental information for considering potential mechanisms of how elevated maternal leptin levels may contribute to the pathogenesis of PE, through alterations in CTB invasion. Clarifying the role of LepR and leptin signaling in the differential invasive response of primary trophoblasts and confirming this response in recently developed trophoblast stem cell models (57) will provide important insights into the molecular mechanisms involved in regulators of CTB invasion and the potential role in the pathogenesis of PE.

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 humans were approved by University of Colorado Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because human samples were obtained from discarded tissue provided by the clinic. Patients signed a surgical consent form that provided an opt-in/opt-out option for procedure tissues to be used for research. There was no direct consent required for this study as approved by the local IRB.

Author contributions

KR: Conceptualization, Writing – review & editing, Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft. SS: Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. KB: Writing – original draft, Methodology. AK: Methodology, Writing – original draft. VW: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fendo.2024.1386309/full#supplementary-material>

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Analysis of the evolution of placental oxidative stress research from a bibliometric perspective

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Background: Research on placental oxidative stress is pivotal for comprehending pregnancy-related physiological changes and disease mechanisms. Despite recent advancements, a comprehensive review of current status, hotspots, and trends remains challenging. This bibliometric study systematically analyzes the evolution of placental oxidative stress research, offering a reference for future studies.

Objective: To conduct a comprehensive bibliometric analysis of the literature on placental oxidative stress to identify research hotspots, trends, and key contributors, thereby providing guidance for future research.

Methods: Relevant data were retrieved from the Web of Science Core Collection database and analyzed using VOSviewer, CiteSpace, and the bibliometrix package. An in-depth analysis of 4,796 publications was conducted, focusing on publication year, country/region, institution, author, journal, references, and keywords. Data collection concluded on 29 April 2024.

Results: A total of 4,796 papers were retrieved from 1,173 journals, authored by 18,835 researchers from 4,257 institutions across 103 countries/regions. From 1991 to 2023, annual publications on placental oxidative stress increased from 7 to 359. The United States (1,222 publications, 64,158 citations), the University of Cambridge (125 publications, 13,562 citations), and Graham J. Burton (73 publications, 11,182 citations) were the most productive country, institution, and author, respectively. The journal *Placenta* had the highest number of publications (329) and citations (17,152), followed by the *International Journal of Molecular Sciences* (122 publications). The most frequent keywords were "oxidative stress," "expression," "pregnancy," "preeclampsia," and "lipid peroxidation." Emerging high-frequency keywords included "gestational diabetes mellitus," "health," "autophagy," "pathophysiology," "infection," "preterm birth," "stem cell," and "inflammation."

Conclusion: Over the past 3 decades, research has concentrated on oxidative stress processes, antioxidant mechanisms, pregnancy-related diseases, and gene expression regulation. Current research frontiers involve exploring pathophysiology and mechanisms, assessing emerging risk factors and environmental impacts, advancing cell biology and stem cell research, and understanding the complex interactions of inflammation and immune

regulation. These studies elucidate the mechanisms of placental oxidative stress, offering essential scientific evidence for future intervention strategies, therapeutic approaches, and public health policies.

KEYWORDS

placenta, oxidative stress, bibliometrics, citespace, VOSviewer, emerging topics, research focus

1 Introduction

Placental oxidative stress has been a focal point in the forefront of biological and medical research. As the key organ facilitating the exchange of substances between the mother and the fetus, the placenta plays a crucial role in maintaining fetal development and maternal health during pregnancy. Research on the placenta encompasses various aspects, including its structure, function, mechanisms of development, and potential complications (Burton and Jauniaux, 2023; Phengpol et al., 2023; Zhang et al., 2023a; Hu et al., 2020). Among these, the study of oxidative stress within the placenta has drawn significant attention (Phengpol et al., 2023; Zhang et al., 2023a; Hu et al., 2020; Myatt and Cui, 2004). Oxidative stress, characterized by an imbalance between reactive oxygen species (ROS) and antioxidant defenses, is a condition resulting from the disruption of the cellular and extracellular environment (Phengpol et al., 2023; Zhang et al., 2023a; Hu et al., 2020; Burton and Jauniaux, 2011). This condition is closely associated with numerous pregnancy-related disorders, such as preeclampsia (Phengpol et al., 2023; Zhang et al., 2023a; Hu et al., 2020; Roberts and Cooper, 2001; Steegers et al., 2010), gestational diabetes (Phengpol et al., 2023; Zhang et al., 2023a; Hu et al., 2020; Fisher et al., 2021), and intrauterine growth restriction (IUGR) (Phengpol et al., 2023; Zhang et al., 2023a; Hu et al., 2020; Burton and Jauniaux, 2018). Understanding the relationship between oxidative stress and placental function is essential for developing therapeutic strategies to mitigate these adverse outcomes.

Over the past decades, the field of oxidative stress research has made advancements. These progressions are reflected not only in the accumulation of scientific knowledge but also in the continual development of methodological approaches and technological innovations. However, given the vast amount of scientific literature, systematically organizing and analyzing the progress, hotspots, and trends in this field has become a pressing challenge. In this context, bibliometrics offers a novel perspective by employing mathematical and statistical methods to quantitatively analyze scientific literature.

Bibliometric analysis, which involves the quantitative evaluation of published literature, provides valuable insights into the development and trends of a specific research area. By analyzing publication patterns, citation networks, and keyword frequencies, researchers can identify key contributors, emerging themes, and potential gaps in the literature. The objective of this bibliometric study is to perform a comprehensive analysis of the literature on “placental oxidative stress,” uncovering its historical evolution and forecasting future research directions.

We specifically utilized bibliometric methods in conjunction with tools such as the bibliometrix package, CiteSpace, and

VOSviewer to analyze publications on “placental oxidative stress” from the Web of Science Core Collection. Our analysis encompasses the distribution of annual publications, countries, institutions, authors, source journals, keyword co-occurrence, and co-citations. The goal of this bibliometric analysis is to gain an in-depth understanding of the current state, hotspots, and future development trends in placental oxidative stress research. This study not only enhances our comprehension of the historical and contemporary landscape of placental oxidative stress research but also provides valuable resources and insights for researchers aiming to navigate and contribute to this dynamic field. Ultimately, our findings aim to guide clinical practice and scientific research in this area.

2 Methods

2.1 Data collection and retrieval strategy

To enhance the representativeness and accessibility of the data, we conducted a literature search in the Web of Science Core Collection on 29 April 2024. Figure 1 illustrates the data collection and retrieval strategy. We specified the search terms using the “Topic” (TS) field, which encompasses the title, abstract, author keywords, and Keywords Plus. The search query was structured as follows: TS = (Placenta* AND (“Oxidative stress” OR “Reactive oxygen species” OR “ROS” OR “Free radicals” OR “Oxidative damage” OR “Oxidative injury” OR “Oxidative imbalance” OR “Antioxidant defense” OR “Redox imbalance” OR “Oxidative markers” OR “Lipid peroxidation” OR “Protein oxidation” OR “Antioxidant enzymes”)). We limited the publication type to articles and reviews, without imposing any time or language restrictions. A total of 4,796 records were retrieved, encompassing publications, authors, countries, institutions, journals, keywords, and citations. These records were exported in the format of complete records.

2.2 Data analysis

Bibliometric data analysis was conducted using VOSviewer (v1.6.19), CiteSpace (v6.1. R6 Basic), and the bibliometrix package (version 4.1.3) within the R statistical environment (version 4.3.1). Preliminary descriptive statistics on the number of publications and citations per year, country, and author were generated using the bibliometrix package. Additionally, this package was employed to analyze the distribution of publications, collaboration patterns between countries/regions, authors’ productivity over time, the top 10 highly cited references and co-

Web of Science Core Collection, TS= (Placenta* and ("Oxidative stress" or "Reactive oxygen species" or "ROS" or "Free radicals" or "Oxidative damage" or "Oxidative injury" or "Oxidative imbalance" or "Antioxidant defense" or "Redox imbalance" or "Oxidative markers" or "Lipid peroxidation" or "Protein oxidation" or "Antioxidant enzymes"))

Performed on April 29, 2024

5007 records identified

Limit the document type to "Article" or "Review Article"

4796 records identified

Bibliometric analysis

FIGURE 1
Flow-chart of the search process.

cited references, trend topic analysis, and word cloud visualization. VOSviewer was utilized for data extraction and visualization of countries, institutions, authors, and keywords. CiteSpace was used to analyze country collaborations, perform cluster analysis of co-cited references, create a dual-map overlay of journals related to “placental oxidative stress,” analyze keyword timeline cluster maps, and detect keyword bursts for the top 25 keywords exhibiting the highest burst strength.

3 Results

3.1 Annual global publication outputs on placental oxidative stress

The earliest publication on “placental oxidative stress” dates back to 1991, with a total of 4,796 articles identified (Figure 2A). Annual publication trends reveal an 8.33% growth rate, indicating a steady increase in interest since 1991 (Figure 2B). Publications increased from 7 to 23 between 1991 and 1995, reflecting early academic interest despite low activity. Between 1996 and 2000, publications increased from 31 to 50, reflecting a surge in research interest. From 2001 to 2010, publication growth accelerated, especially after 2008, marking a phase of high-speed development. During 2011 to 2020, publication numbers remained high with fluctuating increases. In 2021, the number of publications peaked at 412, demonstrating high research activity and academic interest. Despite a slight decline from 2022 to 2023, the count remained substantial, indicating sustained interest. The field has been cited 177,497 times, averaging 37.01 citations per article. The

stable upward trend in citations over the past 30 years underscores the growing research interest in “placental oxidative stress.” These findings underscore the field’s significance and provide a foundation for future research directions and strategies.

3.2 Distribution and co-authorship of countries/regions

Research on “placental oxidative stress” has emerged as a global hotspot, involving researchers from 103 countries/regions across six continents (Figure 3A). The United States, China, and England lead in publication counts, collectively accounting for over 50% of the total research output, reflecting their significant interest. The H-index rankings also emphasize the United States, England, and Canada as top contributors (Table 1). CiteSpace analysis of international collaboration networks identifies the United States, England, Italy, China, Australia, Japan, and France as central nodes (Figure 3B). VOSviewer analysis of co-authorship, with a minimum of five publications, shows the United States, England, and China dominating in Total Link Strength. England, Canada, and the United States lead in average citations, indicating high research quality and strong international collaboration (Table 1).

VOSviewer further classifies countries into nine collaboration clusters based on co-authorship strength (Figure 3C). The United States leads the violet cluster, with strong ties to China, England, Canada, Australia, and the Philippines. China heads the dark brown cluster, collaborating closely with Australia, New Zealand, and Ghana. England’s turquoise cluster includes Canada, Scotland, Israel, Singapore, and Wales. Japan’s orange



FIGURE 2

A bibliometric analysis of research on placental oxidative stress. (A) Bibliometric Data Overview. (B) Distribution of "Placental Oxidative Stress" publications over time.

cluster features collaborations with South Korea, Sweden, Indonesia, and Bangladesh. Italy, Poland, and Switzerland are central to the largest cluster, the dark red cluster, comprising 13 countries. India leads the blue-purple cluster, collaborating with Iran, South Africa, and Nigeria. Brazil forms the green cluster with Spain, Chile, and Mexico. Germany's deep blue cluster includes the Netherlands, Belgium, and Norway, while Turkey's dark yellow-green cluster collaborates with France, Egypt, and Saudi Arabia.

These findings underscore the extensive international collaboration and influential contributions in placental oxidative stress research, providing a foundation for future research directions and collaborations.

3.3 Distribution of research institutions and authors

3.3.1 Distribution of research institutions

Using VOSviewer, the study analyzed institutional co-authorship in research on placental oxidative stress. The analysis applied "Association Strength" with a minimum document threshold of 20, examining 4,257 institutions, of which 77 met the criteria. Table 2 shows that the University of Cambridge led

with 125 papers (2.61%), followed by the University of Melbourne (88 papers, 1.83%), the University of Mississippi (83 papers, 1.73%), Monash University (53 papers, 1.11%), and the University of Pittsburgh (51 papers, 1.06%). Notably, four of the top ten institutions are based in Australia, and three are in the United States. The University of Pittsburgh recorded the highest average citations per paper (140.24), followed by the University of Cambridge (108.50) and Harvard University (91.81). The University of Melbourne exhibited the highest total link strength (107), reflecting its strong collaborative connections within this research field.

Figure 3D categorizes the leading institutions into eight clusters. The green cluster includes the University of Cambridge, the University of Pittsburgh, the University of Manchester, the University of Alberta, and the University of Milan. The dark blue cluster groups Australian institutions such as the University of Melbourne, Monash University, and Griffith University. The dark red cluster features the University of Mississippi, Harvard University, Nanjing Medical University, and the Universidad de Buenos Aires. The blue-purple cluster comprises the University of Queensland, the University of Toronto, the University of São Paulo, and the University of Chile. The blue-green cluster includes Karolinska Institutet, Chongqing Medical University, and the

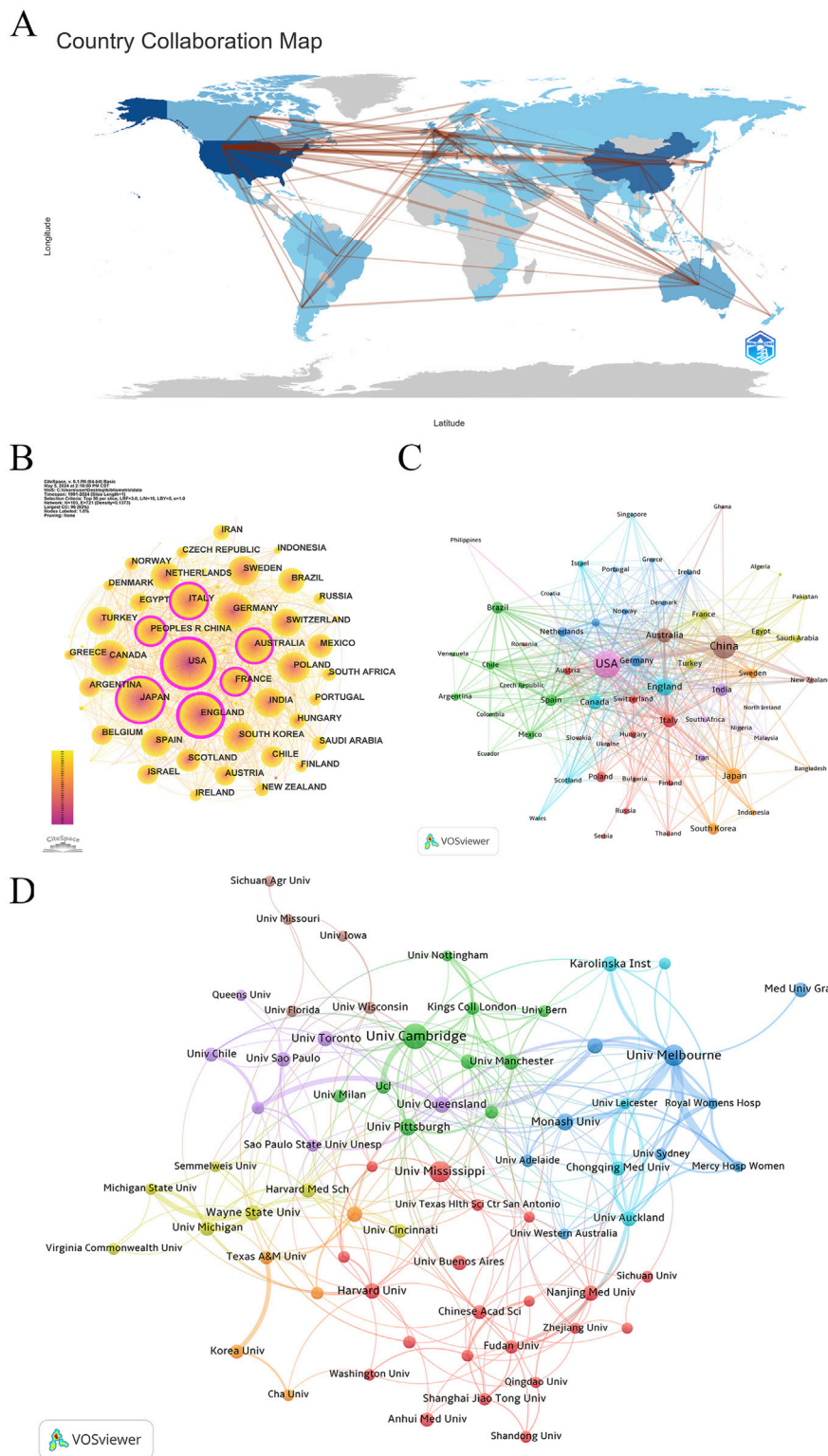


FIGURE 3 Collaboration network of countries/regions/institutions. **(A)** Distribution and collaboration of publications among countries/regions. **(B)** CiteSpace: visualizing clusters of cooperation among countries/regions. **(C)** VOSviewer: visualizing clusters of cooperation among countries/regions. **(D)** Visualization map of institutional collaboration.

TABLE 1 Top 10 most productive countries in Placental Oxidative Stress Research.

Rank	Country	Publications n (%)	Total citations	Average citations	H-index	Total link strength	Centrality
1	United States	1222 (25.53)	64,158	52.5025	119	612	0.28
2	China	810 (16.92)	14,718	18.1704	54	249	0.14
3	England	396 (8.27)	32,904	83.0909	88	374	0.23
4	Japan	305 (6.37)	9185	30.1148	50	107	0.12
5	Australia	280 (5.85)	11,833	42.2607	54	242	0.11
6	Canada	231 (4.83)	16,613	71.9177	60	168	0.02
7	Italy	201 (4.2)	7458	37.1045	46	175	0.19
8	India	177 (3.7)	3403	19.226	32	44	0.01
9	Brazil	167 (3.49)	2873	17.2036	29	97	0.03
10	Spain	166 (3.47)	4728	28.4819	33	188	0.08

TABLE 2 Top 10 most productive institutions in the research on Placental Oxidative Stress.

Rank	Institution	Country	Publications n (%)	Total citations	Average citations	Total link strength
1	University of Cambridge	England	125 (2.61)	13,562	108.50	55
2	University of Melbourne	Australia	88 (1.83)	3504	39.82	107
3	University of Mississippi	United States	83 (1.73)	3821	46.04	8
4	Monash University	Australia	53 (1.11)	1417	26.74	38
5	University of Pittsburgh	United States	51 (1.06)	7152	140.24	21
6	University of Queensland	Australia	48 (1)	1956	40.75	54
7	Karolinska Institutet	Sweden	45 (0.94)	2366	52.58	18
8	University of Toronto	Canada	44 (0.92)	2441	55.48	15
9	Griffith University	Australia	42 (0.88)	1350	32.14	28
10	Harvard University	United States	42 (0.88)	3856	91.81	22

University of Auckland. The dark yellow-green cluster brings together Wayne State University, the University of Michigan, and Harvard Medical School. The orange cluster includes the University of Texas Medical Branch, Korea University, and Southern Medical University. Finally, the dark taupe cluster consists of the University of Wisconsin-Madison, the University of Florida, and the University of Iowa.

These findings highlight the leading institutions and their collaborative networks in placental oxidative stress research, providing valuable insights into the global research landscape and identifying potential opportunities for future collaborations.

3.3.2 Author distribution

Using the bibliometrix package in R, we analyzed author distribution in placental oxidative stress research. Graham J. Burton from the University of Cambridge emerged as the most prolific author with 73 publications, followed by Eric Jauniaux from University College London with 47 publications, and Babbette LaMarca from the University of Mississippi with 45 publications

(Table 3). Graham J. Burton also had the highest average citations and H-index among the top authors.

Analyzing authors’ annual research output revealed that Graham J. Burton, Eric Jauniaux, and Leslie Myatt have consistently contributed to the field over the past 2 decades (Figure 4A). Recent years have seen increased activity from Denise C. Cornelius, Hongbo Qi, and Lorena M. Amaral, indicating significant progress in specific research areas.

Using VOSviewer for co-authorship analysis, we set the parameters to “association strength” with a minimum document threshold of 8. Out of 167 qualifying authors, VOSviewer classified them into clusters based on co-authorship frequency and density (Figure 4B). Graham J. Burton has close collaborative ties with Eric Jauniaux, Tereza Cindrova-Davies, Dino A. Giussani, Leslie Myatt, and D. Stephen Charnock-Jones. Another network includes Babbette LaMarca, Denise C. Cornelius, Lorena M. Amaral, Tarek Ibrahim, and Nathan Campbell. Additionally, Anthony V. Perkins collaborates with James S. M. Cuffe, Bill Kalionis, Tina Bianco-Miotto, Jing Li, and Shaun P. Brennecke.

TABLE 3 Top 10 most productive authors in the Field of Placental Oxidative Stress.

Rank	Author	Institution	Publications n (%)	Total citations	Average citations	H-index
1	Burton, Graham J	University of Cambridge	73 (1.52)	11,182	153.18	51
2	Jauniaux, Eric	University College London	47 (0.98)	6959	148.06	31
3	LaMarca, Babbette	University of Mississippi	45 (0.94)	1985	44.11	24
4	Menon, Ramkumar	University of Texas Medical Branch Galveston	42 (0.88)	1721	40.98	23
5	Perkins, Anthony V	Griffith University	36 (0.75)	1261	35.03	22
6	Myatt, Leslie	Oregon Health and Science University	35 (0.73)	3911	111.74	27
7	Cornelius, Denise C	University of Mississippi Medical Center	31 (0.65)	1163	37.52	16
8	Qi, Hongbo	Chongqing Medical University	29 (0.6)	520	17.93	14
9	Amaral, Lorena M	University of Mississippi	28 (0.58)	1153	41.18	14
10	Mitsumori, Kunitoshi	Tokyo University of Agriculture and Technology	28 (0.58)	500	17.86	14

Co-citation analysis identified Graham J. Burton, Eric Jauniaux, James M. Roberts, Leslie Myatt, and Christopher W. Redman as the five most frequently cited authors, highlighting their central roles and substantial influence in the research network (Figure 4C). Their citation counts significantly surpass those of other authors, underscoring their key positions in the field of placental oxidative stress.

3.4 Subject and journal distribution

3.4.1 Subjects

The analysis of publication volume identified Obstetrics and Gynecology, Reproductive Biology, and Biochemistry and Molecular Biology as the top three subjects in placental oxidative stress research (Table 4). Additional key subjects included Developmental Biology, Cell Biology, and Toxicology. These results demonstrated a strong focus on medical and biological disciplines, highlighting the interdisciplinary nature of placental oxidative stress studies and their broad implications for maternal and fetal health.

3.4.2 Journal distribution

An in-depth analysis of journal distribution was conducted using the bibliometrix package in R, identifying 1,173 journals publishing relevant articles. The top 10 journals with the highest number of publications on “placental oxidative stress” are led by *Placenta*, with 329 articles, accounting for 6.86% of the total publications (Table 5). *Placenta* has accumulated 17,152 citations and an H-index of 67, underscoring its significant impact and authoritative status in obstetrics and gynecology. The International Journal of Molecular Sciences follows with 122 publications. Other notable journals include *PLOS One*, *Reproductive Sciences*, and *Antioxidants*, which also exhibit substantial publication volumes and academic influence. The *American Journal of Obstetrics and Gynecology*, although not at the forefront in publication volume, ranks highly in average citations

and has an impact factor of 8.7, reflecting its prominent position in the field.

A co-citation analysis using VOSviewer was conducted to analyze journal relationships (Figure 5A). In the resulting visualization, journals were represented by nodes, and lines illustrated co-citation relationships. Node size corresponded to the number of publications, while the thickness of connecting lines reflected the strength of associations between journals. Stronger connections, indicated by thicker lines, suggested more frequent co-citations and higher similarities in research topics and methodologies.

Additionally, a dual-map overlay of journals generated in CiteSpace revealed key details about journal relationships and citation patterns. The left side of the overlay represented citing journals, while the right side showed cited journals, with subject areas and citation paths clearly identified. Two prominent citation pathways, distinguished by orange and green hues, highlighted the prevalent citations extending from journals in fields such as molecular/biology/genetics and health/nursing/medicine, to those in domains like molecular biology/immunology and medicine/clinical medicine (Figure 5B). These findings offered valuable insights into the flow of knowledge and academic influence across disciplines, underscoring the interdisciplinary nature and wide-reaching impact of placental oxidative stress research.

3.4.3 Top 10 highly cited and co-cited articles

Using the bibliometrix package in R, a comprehensive analysis of cited and co-cited references was conducted, encompassing 4,176 articles with a total of 177,497 citations and a median citation count of 16. Table 6 and Figure 6A detail the top 10 most cited articles, with the 2010 Lancet article “Pre-eclampsia” leading with 2,422 citations and the highest Normalized Global Citations index of 29.71.

Table 7 lists the top 10 most frequently co-cited references. The article “Oxidative stress in the placenta” is the most co-cited with 330 mentions, followed by “Onset of maternal arterial blood flow

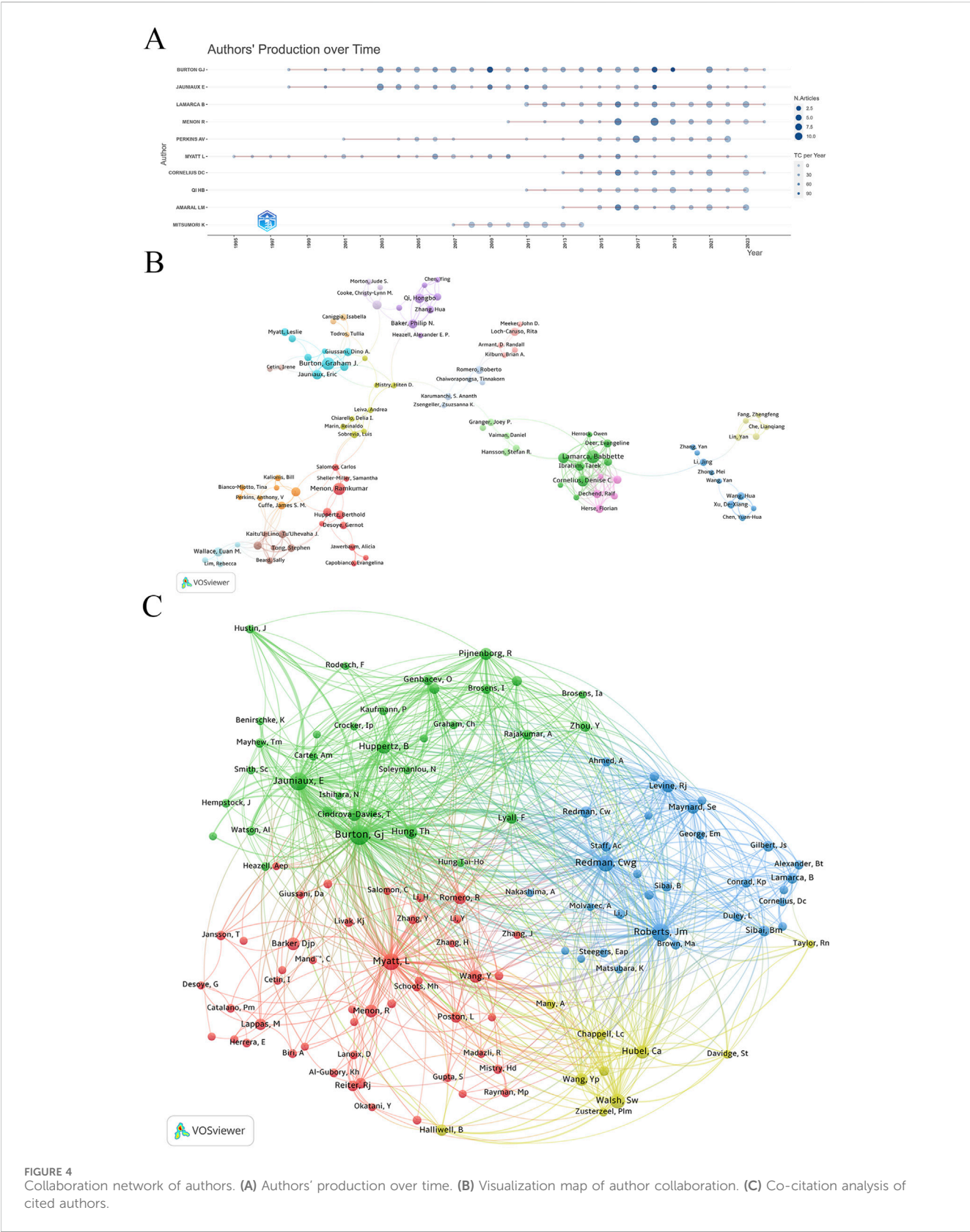


FIGURE 4 Collaboration network of authors. (A) Authors' production over time. (B) Visualization map of author collaboration. (C) Co-citation analysis of cited authors.

and placental oxidative stress” with 320 co-citations, and “Excess placental soluble fms-like tyrosine kinase 1 (sFlt1)” with 299 co-citations. Notably, “Latest advances in understanding preeclampsia”

and “Onset of maternal arterial blood flow and placental oxidative stress” are among both the top cited and co-cited references, indicating their foundational impact in the field.

TABLE 4 Top 10 subject categories in the field of Placental Oxidative Stress.

Rank	Web of science categories	Publications n (%)
1	Obstetrics Gynecology	973 (20.288)
2	Reproductive Biology	805 (16.785)
3	Biochemistry Molecular Biology	708 (14.762)
4	Developmental Biology	469 (9.779)
5	Cell Biology	424 (8.841)
6	Toxicology	398 (8.299)
7	Endocrinology Metabolism	360 (7.506)
8	Pharmacology Pharmacy	321 (6.693)
9	Medicine Research Experimental	288 (6.005)
10	Physiology	281 (5.859)

CiteSpace was employed to analyze co-citation relationships (Figure 6B), revealing a network of 966 nodes and 4,426 links, organized into eight major clusters: mitochondrial dysfunction, angiogenic factors, reduced uterine perfusion pressure, hypertensive pregnancy, recent insights, peroxide-induced vasoconstriction, uterine cells, and human umbilical arterial vasoconstriction. This analysis highlights the diverse research themes within placental oxidative stress and emphasizes key areas of ongoing investigation.

TABLE 5 Top 10 journals in the field of Placental Oxidative Stress.

Journal	Publications n (%)	Total citations	Average citations	H-index	JCR category	Category quartile	Journal impact factor (2023)
Placenta	329 (6.86)	17,152	52.13	67	Obstetrics and Gynecology	Q1	3
International Journal of Molecular Sciences	122 (2.54)	3067	25.14	29	Biochemistry and Molecular Biology	Q1	4.9
Plos One	89 (1.86)	2904	32.63	31	Multidisciplinary Sciences	Q1	2.9
Reproductive Sciences	71 (1.48)	1158	16.31	20	Obstetrics and Gynecology	Q2	2.6
Antioxidants	67 (1.40)	518	7.73	13	Biochemistry and Molecular Biology	Q1	6
Scientific Reports	63 (1.31)	1513	24.02	23	Multidisciplinary Sciences	Q1	3.8
American Journal of Obstetrics and Gynecology	61 (1.27)	4585	75.16	39	Obstetrics and Gynecology	Q1	8.7
Journal of Maternal-Fetal and Neonatal Medicine	59 (1.23)	1517	25.71	20	Obstetrics and Gynecology	Q1	1.7
Biology of Reproduction	52 (1.09)	1854	35.65	27	Reproductive Biology	Q1	3.1
Free Radical Biology and Medicine	47 (0.98)	2344	49.87	24	Biochemistry and Molecular Biology	Q1	7.1

3.5 Keyword analysis

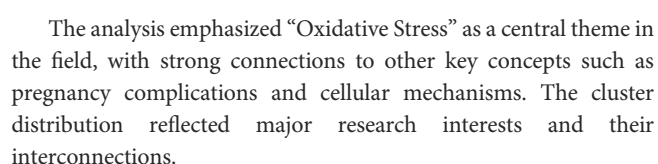
3.5.1 Keyword distribution and co-occurrence analysis

The word cloud analysis using the bibliometrix package (Figure 7A) revealed the distribution of keywords, with frequent terms including “oxidative stress,” “expression,” “pregnancy,” “preeclampsia,” “lipid peroxidation,” and “gene expression,” highlighting central research themes in the field. Further analysis using VOSviewer, applying the association strength method with a minimum keyword occurrence threshold of 50, identified 149 relevant keywords from a dataset of 15,440 (Figure 7B). The co-occurrence network showed “Oxidative Stress” as the most frequently mentioned keyword, followed by “Pregnancy” and “Preeclampsia.”

Four main clusters emerged among the top 50 keywords:

Green Cluster: Led by “Oxidative Stress” and including related terms such as Pregnancy, Placenta, Inflammation, Gene Expression, Risk, Intrauterine Growth Restriction, Exposure, and Metabolism.

Blue Cluster: Focused on terms like Preeclampsia, Hypertension, Nitric Oxide, Nitric Oxide Synthase, Pathogenesis, Endothelial Growth Factor, Normal Pregnancy, and Blood Flow. Red Cluster: Centered on terms such as Expression, Apoptosis, Cells, Trophoblast, *In Vitro*, Hypoxia, Activation, Growth, Mitochondria, Mechanisms, Reactive Oxygen Species, and Stress. Yellow Cluster: Highlighted keywords like Lipid Peroxidation, Women, Human Placenta, Antioxidants, Plasma, Superoxide Dismutase, Vitamin E, and Glutathione.



The burst detection analysis of keywords over the past 20 years revealed significant frequency increases, highlighting emerging trends in the field. **Figure 8A** presents the top 25 keywords with the highest burst strength. “Lipid peroxidation” had the strongest

TABLE 6 Top 10 most cited references in the field of Placental Oxidative Stress.

Title	Journal	Year of publication	Total Citations	Normalized global citations
Pre-eclampsia	Lancet	2010	2422	29.71
Latest advances in understanding preeclampsia	Science	2005	1996	19.38
Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense	Toxicology and Applied Pharmacology	2005	1117	10.84
Pathogenesis and genetics of pre-eclampsia	Lancet	2001	973	9.43
Sampling and Definitions of Placental Lesions Amsterdam Placental Workshop Group Consensus Statement	Archives of Pathology and Laboratory Medicine	2016	954	24.19
Rheological and Physiological Consequences of Conversion of the Maternal Spiral Arteries for Uteroplacental Blood Flow during Human Pregnancy	Placenta	2009	795	10.39
Onset of maternal arterial blood flow and placental oxidative stress - A possible factor in human early pregnancy failure	American Journal of Pathology	2000	782	10.08
Pre-eclampsia part 1: current understanding of its pathophysiology	Nature Reviews Nephrology	2014	688	13.99
Oxidative stress	Best Practice and Research Clinical Obstetrics and Gynaecology	2011	679	12.55
Homologs of gp91phox: cloning and tissue expression of Nox3, Nox4, and Nox5	Gene	2001	672	6.52

burst from 2004 to 2010, followed by key terms such as “vitamin E,” “free radical,” “*in vitro*,” “messenger RNA,” “gestational diabetes mellitus,” “plasma,” “soluble endoglin,” “human placenta,” and “health.” Keywords like “lipid peroxidation,” “vitamin E,” and “free radical” gained prominence over the past decade, while recent surges in “gestational diabetes mellitus,” “autophagy,” and “pathophysiology” indicate emerging research interests.

3.5.3 Trend topics analysis

The trend topics analysis conducted using the bibliometrix package provided insights into the evolution of keyword and subject trends over time. Figure 8B outlined key developments:

1994-1998: Early research focused on “oxygen radicals,” “teratogenicity,” and “*in vitro*,” with less emphasis on “placental form” and “chemical hepatocarcinogenesis.”

1998-2008: Emerging themes included “ascorbate,” “induced lipid-peroxidation,” and “immunohistochemical localization,” alongside persistent topics like “pregnancy-induced hypertension,” “rat liver,” and “prostacyclin.”

2008-Present: Significant growth occurred in keywords such as “lipid peroxidation,” “vitamin E,” and “superoxide,” with “oxidative stress” and “expression” emerging as central themes. Established topics like “pregnancy-induced hypertension,” “carcinogenesis,” and “free radicals” remained relevant.

The evolution of research topics reflected a shift from foundational studies to a more detailed focus on biochemical processes and their implications. The increasing research on “lipid peroxidation,” “vitamin E,” and “superoxide” underscored their current importance.

3.5.4 A timeline visualization in CiteSpace

The CiteSpace timeline view provides an overview of the evolution of research topics in “placental oxidative stress” from

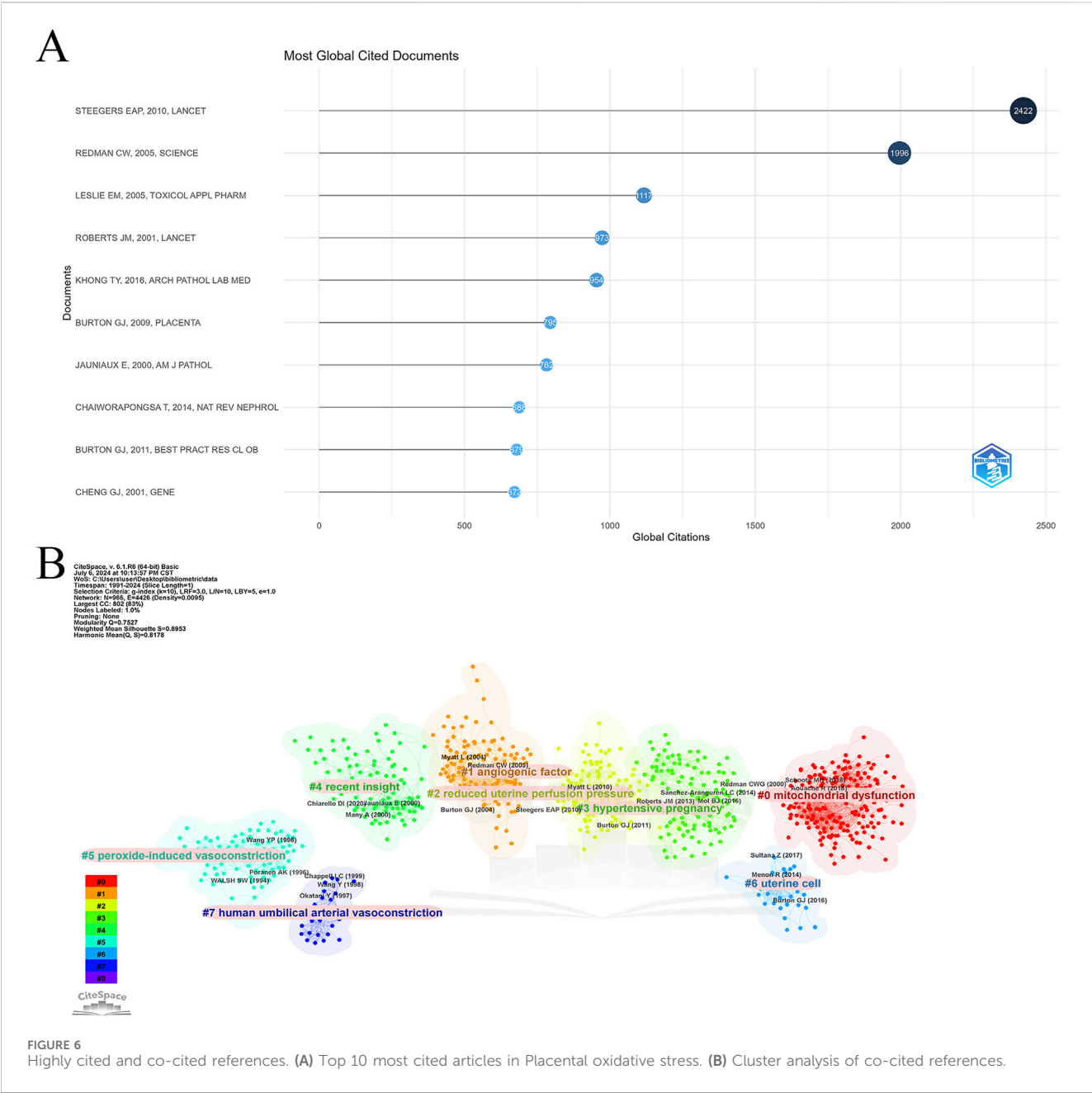
1991 to 2024. Using a 1-year time slice and focusing on the top 50 keywords, the analysis generated a network with 796 nodes and 5,992 links (density = 0.0189) (Figure 8C). The top 10 clusters identified were: Lipid Peroxidation, Vitamin E, Devoid Diet, Tumor Promotion, Cell, Umbilical Artery, Acid, Heat Shock Protein, DNA Damage and Preeclampsia.

The timeline view highlighted key research themes and their progression over time. Early keywords (1991-2000) include “oxidative stress,” “expression,” “pregnancy,” “preeclampsia,” and “lipid peroxidation,” reflecting foundational research topics. Recent keywords (2020-2024) such as “pathophysiology,” “obesity,” “outcome,” and “air pollution” indicate emerging areas of focus and new challenges in the field.

4 Discussion

This bibliometric study employs CiteSpace, VOSviewer, and the Bibliometrix package to perform an in-depth analysis of the literature related to “placental oxidative stress,” providing a comprehensive overview of the field’s research outputs and advancements. We conducted a quantitative analysis of annual publication volumes, country distributions, research institutions, author contributions, interdisciplinary interactions, journal distributions, and keywords.

Among the top ten authors, Professor Graham J. Burton and Eric Jauniaux have forged a close collaborative relationship, offering novel and profound insights into the mechanisms governing placental formation and function during early pregnancy. Their joint research has significantly expanded our understanding of how the placenta supports fetal growth and development (Burton and Jauniaux, 2023; Phengpol et al., 2023; Zhang et al., 2023a; Hu et al., 2020; Fortis et al., 2018; Anto et al., 2023; Amaral et al., 2013).



Specifically, their systematic investigations have revealed the pathophysiological basis of pregnancy complications such as preeclampsia (Phengpol et al., 2023; Zhang et al., 2023a; Hu et al., 2020; Fortis et al., 2018; Anto et al., 2023; Amaral et al., 2013; Burton et al., 2019), gestational diabetes mellitus (GDM) (Phengpol et al., 2023; Zhang et al., 2023a; Hu et al., 2020; Fortis et al., 2018; Anto et al., 2023; Amaral et al., 2013; Ferreira et al., 2023), and fetal growth restriction (Phengpol et al., 2023; Zhang et al., 2023a; Hu et al., 2020; Burton and Jauniaux, 2018; Fortis et al., 2018; Anto et al., 2023; Amaral et al., 2013). These findings address key pathological pathways including oxidative stress (Phengpol et al., 2023; Zhang et al., 2023a; Hu et al., 2020; Fortis et al., 2018; Anto et al., 2023; Amaral et al., 2013; Jauniaux and Burton, 2016; Cindrova-Davies et al., 2018), endoplasmic reticulum stress (Phengpol et al., 2023; Zhang et al., 2023a; Hu et al., 2020; Fortis

et al., 2018; Anto et al., 2023; Amaral et al., 2013; Burton and Yung, 2011), and mitochondrial dysfunction (Phengpol et al., 2023; Zhang et al., 2023a; Hu et al., 2020; Fortis et al., 2018; Anto et al., 2023; Amaral et al., 2013; Yung et al., 2019), opening new avenues for prevention and treatment strategies. Their research also highlights the potential adverse impacts of environmental factors, including air pollution (Phengpol et al., 2023; Zhang et al., 2023a; Hu et al., 2020; Fortis et al., 2018; Anto et al., 2023; Amaral et al., 2013; Fussell et al., 2024; Bearblock et al., 2021), hypoxic conditions, and high-altitude exposure (Phengpol et al., 2023; Zhang et al., 2023a; Hu et al., 2020; Fortis et al., 2018; Anto et al., 2023; Amaral et al., 2013; Kurlak et al., 2016; Tissot et al., 2010) on placental function and pregnancy outcomes, significantly raising awareness about the link between environmental health risks and pregnancy outcomes. Methodologically, Professors Burton and Jauniaux have utilized a

TABLE 7 Top 10 most co-cited references in the field of Placental Oxidative Stress.

Rank	Title	Journal	Year of publication	Number of references co-cited
1	Oxidative stress in the placenta	Histochemistry and Cell Biology	2004	330
2	Onset of maternal arterial blood flow and placental oxidative stress - A possible factor in human early pregnancy failure	The American Journal of Pathology	2000	320
3	Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia	The Journal of Clinical Investigation	2003	299
4	Latest advances in understanding preeclampsia	Science	2005	232
5	Placental oxidative stress: From miscarriage to preeclampsia	Journal of the Society for Gynecologic Investigation	2004	229
6	Circulating angiogenic factors and the risk of preeclampsia	The New England Journal of Medicine	2004	221
7	Oxidative stress in the pathogenesis of preeclampsia	Proceedings of the Society for Experimental Biology and Medicine	1999	203
8	Protein measurement with the Folin phenol reagent	The Journal of Biological Chemistry	1951	192
9	Pre-eclampsia	Lancet	2005	181
10	Trophoblastic oxidative stress in relation to temporal and regional differences in maternal placental blood flow in normal and abnormal early pregnancies	The American Journal of Pathology	2003	176

range of modern biological tools, including RNA-Seq transcriptome sequencing (Phengpol et al., 2023; Zhang et al., 2023a; Hu et al., 2020; Fortis et al., 2018; Anto et al., 2023; Amaral et al., 2013; Prater et al., 2021), digital PCR for high-precision quantification (Hu et al., 2020; Fortis et al., 2018; Anto et al., 2023; Amaral et al., 2013; Allerkamp et al., 2021), and advanced ultrasound imaging techniques (Hu et al., 2020; Fortis et al., 2018; Anto et al., 2023; Amaral et al., 2013; Jauniaux et al., 2005). The integration of these technologies has greatly deepened our understanding of the molecular mechanisms underlying placental physiology and pathology while driving innovations in placental research techniques. Overall, the research by Professor Burton and Jauniaux spans multiple disciplines including biology, medicine, and environmental science, promoting deep communication and interdisciplinary collaboration, and injecting new vitality into the comprehensive development of placental science. Their work has made significant contributions to improving maternal and infant health globally.

Professor Babbette LaMarca’s research provides a profound analysis of gestational hypertension, particularly preeclampsia (Fortis et al., 2018; Anto et al., 2023; Amaral et al., 2013; Deer et al., 2023), emphasizing the central role of immune cell subpopulations such as T cells (Fortis et al., 2018; Anto et al., 2023; Amaral et al., 2013; Hogg et al., 2024; Deer et al., 2021a), B cells (Fortis et al., 2018; Anto et al., 2023; Amaral et al., 2013; Herrock et al., 2023), and natural killer cells (Fortis et al., 2018; Anto et al., 2023; Amaral et al., 2013; Cunningham et al., 2020), as well as their released cytokines [e.g., IL-17 (Fortis et al., 2018; Anto et al., 2023; Fitzgerald et al., 2023), TNF- α (Cunningham et al., 2020), AT1-AA (Herrock et al., 2023)] in the initiation and progression of the disease. Her work further explores the complex associations between key pathophysiological processes such as placental ischemia (Bakrania et al., 2020), endothelial dysfunction (Deer

et al., 2021b), and mitochondrial oxidative stress (Cunningham et al., 2020), with gestational hypertension. Notably, Professor LaMarca focuses on mitochondrial dysfunction (Deer et al., 2021c) and oxidative stress (Vaka et al., 2022) as critical mediators of gestational hypertension and its severe complications (e.g., multi-organ dysfunction), revealing their central role in the disease process. Based on this, she has investigated potential strategies for treating or alleviating gestational hypertension through targeting mitochondrial function (Vaka et al., 2021) and oxidative stress (Deer et al., 2021b). Particularly noteworthy is her extensive discussion of AT1-AA as a core molecule, with its role in mediating gestational hypertension and related pathophysiological changes detailed in multiple papers. This has led to new therapeutic approaches involving the blockade of the AT1-AA pathway to improve disease conditions and related complications (Hogg et al., 2024; Herrock et al., 2023; Cunningham et al., 2018). Additionally, Professor LaMarca’s research utilizes animal models to simulate gestational hypertension and its pathophysiological changes (Cornelius et al., 2015), providing an important platform for understanding disease mechanisms and evaluating treatment interventions. Through various experiments, the study assesses the potential effects of several therapeutic strategies, including vitamin D supplementation (Faulkner et al., 2016), IL-10 supplementation (Harmon et al., 2015), and magnesium sulfate treatment (Johnson et al., 2014), offering valuable insights for clinical practice. In summary, Professor LaMarca’s research not only deepens our understanding of the pathogenesis of gestational hypertension but also opens new scientific pathways and directions for the prevention and treatment of this disease, with significant theoretical and practical implications.

Professor Ramkumar Menon extensively explored the interactions at the maternal-fetal interface under various



understanding of molecular communication mechanisms at the maternal-fetal interface and laid the foundation for developing EV-based diagnostic and therapeutic strategies. Professor Menon also elucidated key mechanisms through which oxidative stress contributes to pregnancy complications, emphasizing its role in driving conditions such as preterm birth and premature rupture of membranes (Arita et al., 2019; Jin et al., 2018). His work provided critical insights into the biological mechanisms underlying these complex pregnancy issues. On a technical level, Professor Menon

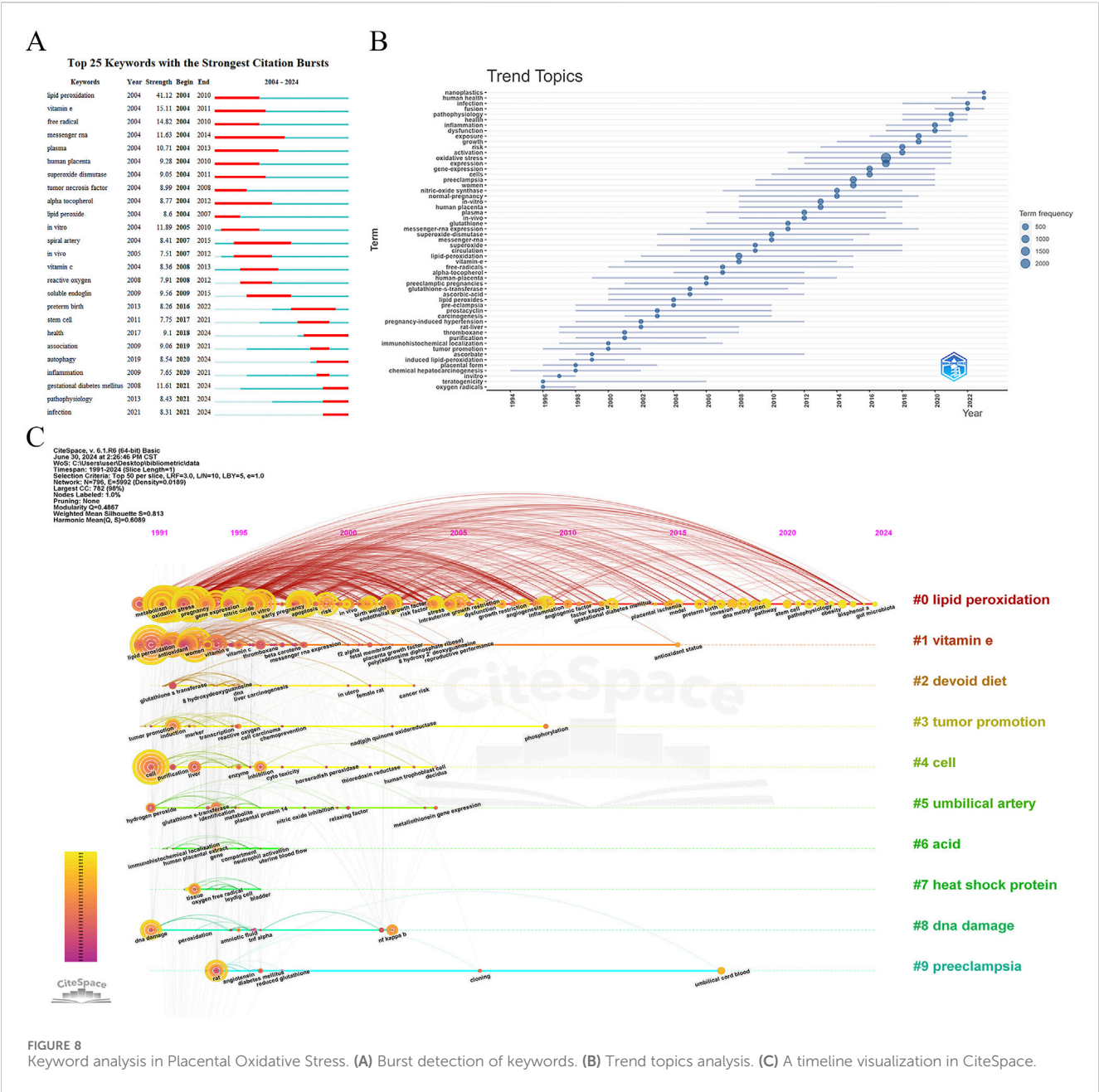


FIGURE 8
Keyword analysis in Placental Oxidative Stress. (A) Burst detection of keywords. (B) Trend topics analysis. (C) A timeline visualization in CiteSpace.

introduced innovative technologies into pregnancy research, including organ-on-chip technology to simulate *in vivo* microenvironments (Vidal et al., 2024), microfluidic systems to optimize drug testing and pharmacokinetic analysis (Kammala et al., 2023), and quantitative proteomics for analyzing changes in protein expression at the maternal-fetal interface (Menon et al., 2019). These advancements enhanced the precision and reproducibility of research, offering powerful tools for investigating the complex physiological and pathological processes in pregnancy, thereby advancing the field of pregnancy science.

Professor Anthony V. Perkins extensively investigated the dynamic changes in placental mitochondria throughout pregnancy (Bartho et al., 2020; Fisher et al., 2020; Holland et al., 2018), including pathological conditions such as gestational diabetes

(Fisher et al., 2021) and preeclampsia (Holland et al., 2018). He systematically evaluated the significant impacts of these changes on pregnancy outcomes, (Cuffe JS. et al., 2017; Perkins, 2011), focusing on how oxidative stress contributes to complications by impairing placental function (Habibi et al., 2021a). In addition to these findings, Professor Perkins concentrated on nutritional interventions, particularly selenium (Hogan and Perkins, 2022) and iodine supplementation (Habibi et al., 2021a), assessing their potential to counteract the harmful effects of oxidative stress on the placenta and fetus. Through a series of well-designed studies, he demonstrated that appropriate trace element supplementation enhances the placenta's antioxidant defenses (Habibi et al., 2021b), supporting normal placental function and fetal development (Hogan and Perkins, 2022). His research emphasized the importance of adequate trace element

supplementation, including selenium, iodine, and iron, in optimizing placental function (Richard et al., 2017), promoting fetal growth, and improving pregnancy outcomes (Hofstee et al., 2019). Furthermore, Professor Perkins explored the application of placental-derived biomarkers for the early prediction and diagnosis of pregnancy complications (Cuffe JS. et al., 2017). By analyzing the patterns of these biomarkers and their associations with pregnancy outcomes, he advanced early detection and intervention strategies (Cuffe J. et al., 2017), contributing significantly to maternal and infant health research.

The top ten highly-cited articles in “placental oxidative stress” showcase advancements and deeper insights in the field. Several articles concentrated on the pathophysiology of preeclampsia (Roberts and Cooper, 2001; Steegers et al., 2010; Redman and Sargent, 2005), examining its mechanisms, genetic underpinnings, and recent developments, underscoring the prominence of this condition in related research. Other key studies explored the role of multidrug resistance proteins in tissue defense (Leslie et al., 2005), the sampling and classification of placental lesions (Khong et al., 2016), and the rheological and physiological effects of maternal spiral artery remodeling on uterine-placental blood flow (Burton et al., 2009). Research into oxidative stress mechanisms (Burton and Jauniaux, 2011; Jauniaux et al., 2000) and the cloning and tissue expression of Nox family homologs (Cheng et al., 2001) also demonstrated the breadth and depth of inquiry in the field. These highly-cited studies not only highlight current research priorities but also provide essential references for future directions.

Frequently cited references among these articles focus on the role of placental oxidative stress in pathological processes such as pregnancy failure (Jauniaux et al., 2000; Burton and Jauniaux, 2004) and preeclampsia (Hubel, 1999; Sibai et al., 2005), marking oxidative stress as a critical research area. In-depth analyses of preeclampsia, including the excessive expression of soluble fms-like tyrosine kinase one in its pathogenesis (Maynard et al., 2003) and the involvement of circulating angiogenic factors (Levine et al., 2004), reveal the complexity and scope of research in the domain. Additionally, references related to protein measurement methods (Lowry et al., 1951) and the connection between spatiotemporal variations in placental blood flow and oxidative stress (Jauniaux et al., 2003) offer vital technical support and theoretical guidance for ongoing research. These widely cited references lay the groundwork for the field and provide valuable insights for future investigations and clinical intervention strategies.

In the VOSviewer keyword co-occurrence network analysis, frequent joint appearances of keywords within clusters indicated their significant role in shaping the content of the field. Four color-coded clusters emerged from the analysis:

The green cluster revealed the broad impact of “Oxidative Stress” on pregnancy, placental function, and fetal development, with a particularly prominent focus (Aouache et al., 2018; Cheong et al., 2016). Keywords such as “Pregnancy” and “Placenta” pointed to the central role of oxidative stress in these physiological processes. Close associations among keywords like “Inflammation,” “Gene-Expression,” and “Risk” provided deeper insights into oxidative stress mechanisms and underscored its pivotal role in various pregnancy complications.

Oxidative stress contributed significantly to the pathogenesis of conditions such as preeclampsia (Tenorio et al., 2019), GDM (Sha

et al., 2019), and fetal growth restriction (Moon et al., 2021) through inflammatory factor activation. These disruptions in placental function involved excessive inflammatory cytokine release (Bernardi et al., 2012), reduced oxidase activity (Mou et al., 2020), and elevated oxidative stress biomarkers (Zhou et al., 2019). Maternal factors such as overweight (Phengpol et al., 2023), obesity (Liang et al., 2018), environmental pollutant exposure (Huang et al., 2017; Riggs et al., 2020), and nutritional deficiencies (Dominguez-Perles et al., 2019) exacerbated oxidative stress and inflammation, further promoting pregnancy complications. These findings emphasized the importance of managing environmental and lifestyle factors during pregnancy. Moreover, certain natural compounds [e.g., astaxanthin (Xuan et al., 2016), pomegranate polyphenols (Chen et al., 2023), and mangiferin (Sha et al., 2019)] and medications [e.g., vitamin D (Nunes et al., 2022), magnesium sulfate (Han et al., 2018), and folic acid (Zhang et al., 2023a)] showed potential in reducing oxidative stress and inflammation, offering promising therapeutic strategies for clinical intervention.

The green cluster also highlighted dynamic changes in gene expression profiles during pregnancy, tightly linked to oxidative stress. Oxidative stress regulated specific genes, such as phospholipase A2 (Brien et al., 2017) and GPX1 (Endler et al., 2016), and indirectly influenced gene activity through epigenetic modifications like DNA methylation (Garcia-Contreras et al., 2019), affecting pregnancy outcomes and offspring health. Environmental factors [e.g., Wi-Fi radiation (Vafaei et al., 2020), maternal obesity (McCoski et al., 2018)], physiological changes (Miyagami et al., 2013), nutritional supplementation (Shorey-Kendrick et al., 2021), and assisted reproductive technologies (Zhang et al., 2010) all impacted gene expression in the placenta and fetus, presenting new perspectives for understanding pregnancy pathophysiology.

Oxidative stress posed a significant risk in assisted reproductive technologies (Mauchart et al., 2023), calling for antioxidant strategies to improve safety. Polymorphisms in DNA repair genes, such as APE1 and XRCC1, were closely linked to heightened risks of preeclampsia (Vural et al., 2009) and preterm birth, underscoring the genetic component in risk assessment. Exposure to pollutants like microplastics (Liu et al., 2023), heavy metals (Shachar et al., 2013), and organophosphate flame retardants (Li et al., 2023) further increased the risk of pregnancy complications and offspring health issues, highlighting the need for environmental protection. Maternal nutritional deficiencies, such as those in vitamin D (Fondjo et al., 2024) and selenium (Lewandowska et al., 2019) along with adverse lifestyle habits like smoking (England and Zhang, 2007) and physical inactivity (Huang et al., 2019), elevated the risks of preeclampsia, preterm birth, and metabolic syndrome. These findings underscored the necessity of multifactorial assessments in predicting and managing pregnancy risks, with future research focused on exploring the mechanisms of these interactions to develop more precise preventive and intervention strategies.

The green cluster included keywords related to fetal growth and development, such as “Intrauterine Growth Restriction,” “Fetal Growth,” “Birth Weight,” and “Preterm Birth.” These terms reflected the significant impact of oxidative stress on intrauterine growth patterns (Yoshida et al., 2018) and fetal birth weight (Hu et al., 2020), highlighting its role as a major risk factor for adverse

pregnancy outcomes, including preterm birth (Menon, 2014; Stefanovic et al., 2019). Oxidative stress affects placental function through various mechanisms, negatively impacting fetal growth and development (Luo et al., 2018; Matsubara and Sato, 2001; Jones et al., 2013). These mechanisms include the regulation of mitochondrial content and cell cycle progression, influencing placental development (Luo et al., 2018), as well as the production of large amounts of ROS by enzymes like NAD(P)H oxidase, which elevate oxidative stress levels in the placenta (Matsubara and Sato, 2001). Additionally, changes in nitric oxide synthase activity further modulate oxidative stress, affecting the development of organs such as the fetal kidneys (Figueroa et al., 2016). Maternal nutrition also plays a critical role in fetal growth. While the intake of ω -3 fatty acids may enhance placental antioxidant capacity, it does not effectively prevent IUGR caused by placental ischemia-reperfusion injury (Jones et al., 2013). In contrast, maternal supplementation with nutrients like folic acid can alleviate IUGR induced by high-fat diets by reducing placental inflammation and oxidative stress (Zhang et al., 2023a). Research on the relationship between IUGR and oxidative stress has revealed a strong correlation through the detection of oxidative stress markers in placental tissue and fetal serum. Elevated levels of lipid peroxidation products, such as malondialdehyde, in IUGR fetal serum, along with decreased activity of antioxidant enzymes like superoxide dismutase, have been observed (Llanos and Ronco, 2009). Furthermore, increased levels of heavy metals in the placenta suggest that environmental pollutants may impair fetal growth by inducing oxidative stress (Llanos and Ronco, 2009). Oxidative stress not only affects fetal development but also has significant implications for maternal health. For instance, IUGR pregnancies are often associated with endothelial dysfunction and elevated systemic oxidative stress, which may contribute to the occurrence of IUGR (Yoshida et al., 2018). Additionally, imbalances in angiogenic factors and oxidative stress biomarkers have been noted in older pregnant women, potentially linking these factors to adverse pregnancy outcomes (Odame et al., 2018). Various antioxidant interventions have been shown to effectively reduce oxidative stress and improve fetal growth in IUGR cases. Antioxidants such as N-acetylcysteine, hydroxychloroquine (Dai et al., 2024), and melatonin (Alers et al., 2013; Miller et al., 2014) have demonstrated efficacy in mitigating oxidative stress and autophagy levels, promoting healthier fetal development. Autophagy, a cellular protective mechanism, has also been found to play a vital role in reducing placental apoptosis and oxidative stress (Zhang et al., 2023b).

Oxidative stress significantly contributes to low birth weight. In malnourished pregnancies, maternal supplementation with melatonin enhances placental efficiency and birth weight by upregulating antioxidant enzyme expression in the placenta, thereby safeguarding placental function and fostering fetal development (Richter et al., 2009). This underscores the potential of antioxidants in ameliorating adverse pregnancy outcomes. Additionally, research indicates that as maternal body mass index increases, nitrate stress levels in the placenta markedly rise, whereas oxidative stress levels may not exhibit a corresponding increase and may even decline in some instances (Roberts et al., 2009). This observation suggests a potential balance between nitrate and oxidative stress in obese pregnancies, potentially

serving as a protective mechanism for the placenta. Additionally, research has focused on placental function in IUGR and low birth weight fetuses, revealing that these placentas are more prone to oxidative damage, mitochondrial dysfunction, and impaired angiogenesis (Hu et al., 2020). These findings further emphasize the crucial role of oxidative stress in the occurrence of IUGR and low birth weight. Notably, exposure to certain environmental pollutants, such as trichloroethylene, has been associated with elevated placental oxidative stress, contributing to fetal growth restriction (Loch-Carusio et al., 2019). These discoveries underscore the importance of regulating oxidative stress to maintain fetal health and highlight the necessity of managing oxidative stress during pregnancy to minimize potential risks (Bedell et al., 2021). Future research should further investigate the interactions between oxidative stress, placental function, and fetal development, along with the specific applications of antioxidants in preventing and treating pregnancy complications such as IUGR.

The significant role of oxidative stress in preterm birth and its associated complications reveals how various endogenous and exogenous factors disrupt the balance of the antioxidant system, thereby increasing the risk of preterm delivery. The antioxidant system plays a critical role in fetal development and the newborn's adaptation to the oxygen-rich extrauterine environment, underscoring the close relationship between preterm birth, immature antioxidant system development, and elevated oxidative stress (Davis and Auten, 2010). Both endogenous and exogenous oxidative stress triggers, such as heavy metal exposure (Ahamed et al., 2009), environmental pollutants, and inflammation (Agarwal et al., 2018), can impair placental function and lead to adverse pregnancy outcomes, including preterm birth (Joo et al., 2021). Research into the specific mechanisms of preterm birth has highlighted the importance of mitochondrial oxidative stress in both preterm delivery and fetal brain injury, while exploring potential therapies aimed at mitigating oxidative stress and inflammation through the induction of the Nrf2 signaling pathway (Chen et al., 2024). Additionally, studies have examined the impact of fetal sex and prenatal glucocorticoid exposure on placental antioxidant balance, indicating that male fetuses may face a higher oxidative stress risk following glucocorticoid exposure (Stark et al., 2011). Multiple studies have established a strong link between preterm birth and oxidative stress by detecting oxidative stress markers in placental tissue and umbilical cord blood. Changes in lipid peroxidation products and antioxidant enzyme activity in preterm placentas (Zadrozna et al., 2009; Ferguson et al., 2015), along with elevated levels of oxidative stress markers like isoprostane in umbilical cord blood (Perrone et al., 2016), suggest a connection to preterm birth. The potential role of antioxidants in preventing preterm birth has been investigated, although certain antioxidants, such as ω -3 polyunsaturated fatty acids, may exhibit pro-oxidant effects under specific conditions, highlighting the need for careful consideration of timing and dosage (Stark et al., 2013; Boulis et al., 2014). On the other hand, some compounds with potential antioxidant and anti-inflammatory properties, such as hydroxylated fullerenes and nicotinamide, have shown promise in preventing preterm birth in animal models (Wakimoto et al., 2015; Lappas and Permezel, 2011). Preterm newborns face unique challenges related to iron metabolism and oxidative stress, given their distinct iron handling and insufficient antioxidant capacity.

These newborns are at risk of both iron deficiency and iron overload, necessitating individualized management strategies that take into account multiple factors (Raffaelli et al., 2020). Comprehensive exploration of oxidative stress mechanisms in preterm birth and its related complications offers new insights into the pathophysiology of preterm birth, while providing valuable guidance for future prevention and treatment strategies. Further research should focus on the development of the antioxidant system, the mechanisms of oxidative stress triggers, and the application of antioxidants in preterm birth prevention, with the aim of creating more effective prevention and treatment approaches.

The clustering of terms like “DNA-Damage,” “Insulin-Resistance,” and “Obesity” pointed to strong connections between oxidative stress and broader pathological processes. These connections extended from molecular mechanisms, such as DNA damage (Fujimaki et al., 2011; Singh et al., 2020), to metabolic disruptions like insulin resistance (Feng et al., 2020; Scioscia et al., 2009), further encompassing the public health challenge of obesity. The analysis underscored the complexity and significance of oxidative stress in pregnancy and related diseases, demonstrating its far-reaching impact. Keywords related to maternal health, such as “Pregnant-Women,” “Gestational Diabetes Mellitus” and “Maternal Obesity” prominently appeared in the green cluster, highlighting the impact of oxidative stress not only on fetal health but also on maternal wellbeing. Oxidative stress was recognized as a teratogenic mechanism during embryonic development, particularly in diabetic embryopathy (Coughlan et al., 2004). Studies identified significant alterations in oxidative stress in the placentas of patients with GDM, with elevated levels of nitrotyrosine serving as direct evidence of oxidative stress (Lyall et al., 1998). Additionally, oxidative stress negatively influenced the expression of inflammatory cytokines and antioxidant enzymes in the placentas of GDM patients, though these effects were less evident in adipose tissue (Lappas et al., 2010). To explore potential treatments, various natural extracts, such as mulberry, acacia, and ginkgo leaf extracts, were evaluated for their effects on maternal and fetal outcomes, oxidative stress levels, and lipid profiles in GDM rat models (Volpato et al., 2011; Volpato et al., 2008; Rudge et al., 2007). The findings suggested the potential of these natural extracts to alleviate oxidative stress and improve pregnancy outcomes. Moreover, oxidative stress was found to enhance the activity of matrix metalloproteinases-2 and -9 in the placenta-fetal unit of diabetic rats, potentially exacerbating placental dysfunction (Pustovrh et al., 2005). Beyond natural extracts, research investigated the influence of both endogenous and exogenous oxidative stress triggers on adverse pregnancy outcomes, including preeclampsia, fetal growth restriction, GDM, and preterm birth (Joo et al., 2021). Studies also examined the relationship between adiponectin and oxidative stress markers in GDM patients and their newborns (Shang et al., 2018), as well as oxidative and antioxidant status in GDM patients diagnosed under International Association of the Diabetes and Pregnancy Study Groups criteria (Shang et al., 2015). A cohort study conducted in the Thai population further compared oxidative stress biomarkers between GDM and non-GDM patients, finding a significant association between GDM and inflammatory processes, reflected in higher oxidative stress and apoptosis markers (Rueangdetnarong et al., 2018).

To identify new therapeutic targets, interactions among oxidative stress, endoplasmic reticulum stress, inflammation, mitochondrial function, and signaling pathways were explored. Apocynin mitigated oxidative stress and inflammation in GDM by inhibiting the TLR4/NF- κ B signaling pathway (Liu et al., 2020). Cryptotanshinone significantly reduced blood glucose levels, oxidative stress, inflammation, and NF- κ B activation in GDM mice, while increasing insulin levels in the placenta and blood (Wang N. et al., 2020). Asperulosidic acid alleviated oxidative stress and inflammation in the GDM placenta by suppressing NF- κ B and MAPK signaling pathways (Wu et al., 2022). Additionally, studies assessed the potential benefits of physical activity (Cid and Gonzalez, 2016), specific nutrients like copper (Ergaz et al., 2014) and lutein (Lorenzoni et al., 2013), and the drug nigericin in reducing oxidative stress in GDM patients. Notably, increased placental and fetal lipoprotein-associated phospholipase A2 in GDM patients might offer protection against oxidative stress (Schliefssteiner et al., 2017). The upregulation of nuclear factor erythroid 2-related factor 2 and antioxidant enzymes in the GDM placenta could represent protective mechanisms against oxidative stress (Manoharan et al., 2019). However, not all studies supported the efficacy of these protective mechanisms. For instance, while vitamins and antioxidants reduced fetal oxidative stress, they failed to restore normal growth (Ornoy et al., 2009).

Research also examined the impact of GDM on neonatal cardiovascular health, particularly the increased intima-media thickness of the aorta and the role of oxidative stress in this process (Triantafyllidou et al., 2023). The roles of oxidative stress, fatty acids, and neurotrophic factors in GDM were explored (Jadhav et al., 2020), along with the association between GDM, autism spectrum traits, and attention deficit hyperactivity disorder symptoms, although placental inflammation and oxidative stress cytokines were not found to mediate these associations (Zhu et al., 2021). In summary, this series of studies provides rich data support for understanding the role of oxidative stress in GDM and its related complications, and offers potential targets for the development of new therapeutic strategies.

GDM (Gauster et al., 2017; Lappas et al., 2011) and obesity (Duan et al., 2018; Hu et al., 2021) both significantly increase oxidative stress in the placenta, posing risks to maternal and fetal health during pregnancy. Research indicates that maternal obesity is not only associated with metabolic abnormalities and reduced antioxidant capacity in pregnant women but may also affect placental function, leading to fetal oxidative stress and metabolic alterations (Malti et al., 2014; Zavalza-Gomez, 2011; Ballesteros-Guzman et al., 2019). Elevated levels of oxidative stress markers, such as malondialdehyde, protein carbonyls, nitric oxide, and superoxide anions, have been observed in the placentas of obese pregnant women, alongside decreased levels of antioxidants like reduced glutathione and superoxide dismutase. This imbalance in redox status could have long-term effects on fetal metabolic and immune programming (Malti et al., 2014; Hernandez-Trejo et al., 2017). Additionally, obesity may contribute to placental mitochondrial dysfunction and impaired angiogenesis, further exacerbating oxidative stress in the placenta (Hu C. et al., 2019; Napso et al., 2022). Notably, alterations in the gut microbiota of obese pregnant women have been linked to increased oxidative

stress in the placenta, suggesting a potential role for gut microbiota in obesity-mediated placental dysfunction (Hu et al., 2021). To explore therapeutic interventions, studies have evaluated the effects of resveratrol supplementation on oxidative stress in obese pregnant women and their fetuses. The results demonstrated that resveratrol improved maternal metabolic status and significantly reduced oxidative stress in the placenta and liver, offering new strategies for managing obesity during pregnancy (Rodriguez-Gonzalez et al., 2022). Research has also focused on the impact of maternal obesity on the renal health of offspring. Offspring of obese pregnant women may experience lipid accumulation, inflammation, oxidative stress, and fibrosis in the kidneys, increasing the risk of developing kidney diseases (Phengpol et al., 2023). An exploratory study evaluated the effects of physical activity and sedentary time during pregnancy on placental oxidative stress markers in obese women. The findings suggest that increased physical activity and reduced sedentary time may correlate with lower expression levels of certain oxidative stress markers in the placenta, underscoring the importance of lifestyle interventions during pregnancy (Zafaranih et al., 2022). Obesity significantly influences oxidative stress in both pregnant women and their fetuses, potentially involving complex physiological and pathological mechanisms. Future research should further investigate the role of obesity-mediated oxidative stress in pregnancy complications and develop effective interventions to improve health outcomes for obese pregnant women and their offspring. These insights highlighted the need for comprehensive strategies to assess and manage oxidative stress during pregnancy (Bedell et al., 2021), aiming for an integrated approach that protects both maternal and fetal health.

The blue cluster primarily focuses on pregnancy-related diseases and their underlying pathophysiological mechanisms. The cluster, with a central focus on “Preeclampsia,” strongly associated with keywords like “Hypertension,” “Nitric-Oxide,” and “Nitric-Oxide Synthase,” highlighting their frequent co-mention in the literature. This co-occurrence indicated the critical roles and interactions of these factors in preeclampsia’s pathophysiology. Research linked preeclampsia to significant oxidative stress, which reduced nitric oxide (NO) bioavailability (Nunes et al., 2021) through the inhibition of endothelial nitric oxide synthase (eNOS) activity (Amaral et al., 2013). Vitamin D deficiency further aggravated the reduction of NO by influencing oxidative stress responses in human umbilical vein endothelial cells (Nunes et al., 2021). L-arginine depletion in preeclampsia contributed to eNOS conversion into an oxidant form, decreasing NO production, while oxidative products such as 4-oxo-2(E)-nonenal exacerbated eNOS dysfunction (Guerby et al., 2019). Inhibition of inducible nitric oxide synthase demonstrated potential for blood pressure reduction in experimental studies (Amaral et al., 2013), and antioxidants like N-acetylcysteine helped restore NO-mediated function in the placenta of preeclampsia patients (Bisseling et al., 2004). Heat shock protein 70 (Barut et al., 2010), homocysteine, and asymmetric dimethylarginine also played key roles in regulating NO metabolism and the development of preeclampsia (Demir et al., 2012). Overall, the imbalance between NO and reactive oxygen species, particularly oxidative stress-induced eNOS inhibition, emerged as a critical aspect of preeclampsia’s pathogenesis. Future studies should

further investigate the molecular mechanisms and potential interventions related to this imbalance.

The frequent appearance of keywords such as “Pathogenesis,” “Endothelial Growth-Factor,” “Blood-Flow,” “Necrosis-Factor-Alpha,” “Blood-Pressure,” and “Endothelial Dysfunction” underscored the central role of vascular endothelial dysfunction (Sanchez-Aranguren et al., 2014; Cindrova-Davies, 2009), inflammatory responses (Tenorio et al., 2019; Harmon et al., 2016; Michalczyk et al., 2020), and blood flow regulation disorders (Parra et al., 2005; Yoshihara et al., 2022) in preeclampsia’s pathogenesis and related hypertensive diseases. These interactions collectively drove the progression of preeclampsia. Keywords like “Placental Oxidative Stress,” “Cytokines,” “Fetal Growth Restriction,” “Trophoblast Invasion,” and “Angiogenic Factors” revealed critical aspects of preeclampsia’s complex pathophysiology, including placental dysfunction (Biron-Shental et al., 2010), oxidative stress responses (Marin et al., 2020), abnormal angiogenesis (Turpin et al., 2015), and fetal growth restriction (Takagi et al., 2004). The role of the placenta in preeclampsia pathology and the impact of oxidative stress (Anto et al., 2023), cytokine network imbalances (Rusterholz et al., 2007; Stark, 1993), trophoblast cell dysfunction (Wang GJ. et al., 2020), and angiogenesis disorders (Anto et al., 2024; Edvinsson et al., 2022) were emphasized.

Finally, the appearance of keywords such as “Tumor-Necrosis-Factor,” “Tyrosine Kinase-1,” and “Endoplasmic-Reticulum Stress” suggested potential roles for immune-inflammatory responses (Kim et al., 2017), signaling pathway abnormalities (Kim et al., 2017; Burke et al., 2016), and cellular stress responses (Redman et al., 2022) in preeclampsia’s pathogenesis, further emphasizing the complexity of this multifactorial disease involving widespread biological processes and molecular network imbalances (Burton et al., 2019).

The keywords in the red cluster form a highly integrated knowledge network closely related to cell biology, oxidative stress, and mechanisms of related diseases. This cluster centers around high-frequency keywords such as ‘Expression,’ ‘Apoptosis,’ ‘Cells,’ and ‘Trophoblast,’ highlighting the central roles of cellular expression regulation, cell apoptosis, and trophoblast cells in these research domains. These keywords not only represent fundamental processes in cell biology but also clearly indicate the critical roles of these processes within complex physiological and pathological environments, particularly in the contexts of pregnancy and placental function.

Additionally, the appearance of keywords such as “In-Vitro,” “Hypoxia,” “Activation,” “Growth,” and “Mitochondria” further underscores the significant impacts of *in vitro* experimental models (Depoix et al., 2017), hypoxic environments (Zhao et al., 2021) on cellular functions, and the crucial roles of mitochondria in cell growth (Ma et al., 2019), energy metabolism (Napso et al., 2022), and stress responses (Burton et al., 2017). The co-occurrence of these keywords reveals multiple important aspects in cell biology research, including the establishment of experimental models, cellular adaptive responses to environmental conditions, and the pivotal role of organelles, especially mitochondria, in maintaining cellular homeostasis.

Notably, the co-occurrence of keywords such as “Reactive Oxygen Species,” “Stress,” “Disease,” “Angiogenesis,” and

“Oxygen” reveals the close relationship between oxidative stress responses, abnormal angiogenesis, and the placenta. Research has shown that ROS not only participates in physiological processes of normal pregnancy, such as placental angiogenesis (Yang et al., 2022) and trophoblast function (Walker et al., 2020), but also plays a key role in various pregnancy complications, including preeclampsia [(Matsubara et al., 2015), (Matsubara et al., 2010)], intrauterine growth restriction (Luo et al., 2018), and fetal loss (Zhang et al., 2019). ROS mediates oxidative stress responses by regulating signaling pathways (Adebambo et al., 2018), affecting gene expression (Wang B. et al., 2020), and impacting cellular functions (Adebambo et al., 2018), thereby influencing pregnancy outcomes. Furthermore, insulin resistance and elevated androgen levels (Hu M. et al., 2019) can increase ROS production, further exacerbating the risk of pregnancy complications. Notably, the application of antioxidants has shown potential in alleviating ROS-mediated damage (Al-Gubory et al., 2010; Khera et al., 2017), providing new insights for clinical intervention. Future research should continue to explore the specific mechanisms of ROS in pregnancy and develop more effective antioxidant therapies to improve pregnancy outcomes and safeguard maternal and fetal health.

The balance between pro-angiogenic and anti-angiogenic factors is crucial for maintaining normal pregnancy. In particular, pathological states such as preeclampsia and intrauterine growth restriction are associated with significant inhibition of angiogenesis (Shi et al., 2021), along with increased oxidative stress (Anto et al., 2024) and changes in the expression of angiogenesis-related genes (Fortis et al., 2018). Additionally, factors such as maternal nutritional status (Anto et al., 2023), environmental factors (Bai et al., 2022), pharmacological interventions (Soobryan et al., 2017), and genetic variations (Fortis et al., 2018) all impact placental angiogenesis. Notably, certain natural compounds (Park et al., 2020) and drugs (Doganlar et al., 2019) have shown potential in promoting angiogenesis, mitigating oxidative stress, and improving pregnancy outcomes. Future research should further explore the specific mechanisms of angiogenesis in pregnancy and develop more effective intervention strategies to improve the prognosis of patients with pregnancy complications by modulating the angiogenesis process.

Furthermore, the clustering of keywords such as “Toxicity,” “Liver,” “Damage,” and “Dysfunction” reveals the broad pathological processes potentially involved in oxidative stress and cellular dysfunction. These processes include the toxic effects and mechanisms of various chemicals (Abdollahzade et al., 2021; Cattani et al., 2014), environmental pollutants (Saben et al., 2014), pharmaceuticals (Lu et al., 2023), and nanomaterials (Wang and Wang, 2020) on the placenta, trophoblast cells, embryo, and fetus. They collectively highlight the central roles of oxidative stress (Huang et al., 2018), apoptosis (Ali et al., 2021), genotoxicity (Kostic et al., 2022), endocrine disruption (Perez-Albaladejo et al., 2017), and mitochondrial dysfunction (Naav et al., 2020) in these toxic effects. Notably, research emphasizes the placenta as a critical target for these toxic effects and its key role in protecting the fetus from external harmful substances. Simultaneously, these studies reveal the potential applications of various antioxidants (Jan et al., 2011), nutrients (Hassoun et al., 1997), and other

bioactive substances (Lee et al., 2023) in mitigating or counteracting these toxic effects. The keyword “Liver” primarily focuses on liver diseases (Pillai and Gupta, 2005), tumors (Puatanchokchai et al., 2006), and their prevention, progression, and treatment (Phannasorn et al., 2022). The terms “Damage” and “Dysfunction” indicate that the impact of oxidative stress is not confined to a specific disease or organ but widely affects multiple systems and organ functions. This co-occurrence of keywords not only expands our understanding of the spectrum of oxidative stress-related diseases but also emphasizes the core role of cellular dysfunction in these diseases, suggesting that future research should focus more on the mechanisms of oxidative stress-induced cellular dysfunction and its specific roles in various diseases.

The co-occurrence of keywords such as “Protein,” “Differentiation,” “Proliferation,” “Inhibition,” “Invasion,” “Receptor,” and “Autophagy” further enriches our understanding of cellular biological processes. These keywords address mechanisms across several layers, including protein functions (Colleoni et al., 2013), cell differentiation and proliferation (Bolnick et al., 2017), signal transduction (Rundlof and Arner, 2004), and cell invasion and migration (Yang and Shang, 2021). The close association of these keywords reveals multiple complex aspects of cell biology research, including the critical roles of proteins in determining cell fate (Rundlof and Arner, 2004), regulatory mechanisms of cell proliferation and differentiation (Dai and Lu, 2022), and the complexity of intercellular interactions and signal transduction (Murphy et al., 2008). Additionally, the appearance of keywords such as “Pregnancies,” “Messenger-RNA,” “Identification,” “ROS,” and “Gene” highlights the significant roles of oxidative stress, gene expression regulation, and reactive oxygen species in cell fate decisions during pregnancy. These keywords reveal the complexity and uniqueness of cellular biological processes under the special physiological state of pregnancy and the critical roles of oxidative stress and gene expression regulation in this process.

The yellow cluster focuses on oxidative stress, antioxidant mechanisms, and related biomarkers, centering around high-frequency keywords like “Lipid-Peroxidation” and “Antioxidant.” This cluster primarily explores the formation and effects of antioxidants and lipid peroxidation in human placental and fetal tissues. Specifically, it covers studies on free radical scavenging enzyme activity and lipid peroxidation in placental tissues of miscarriage patients (Biri et al., 2006), NADPH and iron-dependent lipid peroxidation in human placental microsomes, and lipid peroxidation, antioxidant defenses, and acid-base status in umbilical cord blood at birth (Milczarek et al., 2007). Additionally, the cluster investigates the effects of various substances, such as melatonin (Milczarek et al., 2000), pollutants (Lee et al., 2015), and drugs (Rojas et al., 2022) on lipid peroxidation in the placenta and fetus, as well as changes in lipid peroxidation and antioxidant enzyme activity under different physiological and pathological conditions (Johnston et al., 2016; Loverro et al., 1996). Collectively, these studies reveal the complexity of lipid peroxidation and antioxidant states in placental and fetal tissues and their crucial roles in pregnancy and fetal development.

The emergence of keywords such as “Human Placenta” and “Women” indicates that this research network may particularly focus on oxidative stress and antioxidant mechanisms within the context of female physiology, especially within the placenta. The co-

occurrence of keywords like “Plasma,” “Superoxide-Dismutase,” “Vitamin-E,” “Glutathione,” and “Selenium” further underscores the critical role of antioxidant defense systems in maintaining cellular homeostasis (Wang and Walsh, 2001; Liu et al., 2010) and highlights the potential biomarker value of antioxidants and enzymes present in plasma (Rani et al., 2010). The appearance of the term “Supplementation” suggests the application of antioxidants as an intervention strategy in related research (Poston et al., 2011; Pressman et al., 2003). Additionally, the clustering of keywords such as “Melatonin,” “Free-Radicals,” and “Antioxidant Enzymes” reveals the production of free radicals during oxidative stress (Lista et al., 2010), the occurrence of lipid peroxidation (Gupta et al., 2005), and the pivotal role of antioxidant enzymes in scavenging free radicals (Vanderlelie et al., 2005) and protecting cells from oxidative damage (Okatani et al., 2001). Notably, the appearance of “Messenger-RNA Expression” and “Alpha-Tocopherol” adds a new dimension to this research network, indicating a potential connection between oxidative stress and gene expression regulation (Than et al., 2009), as well as the specific mechanisms through which particular antioxidants contribute to cellular protection (Vieira et al., 2018). Finally, although keywords like “Thromboxane” and “Acid” appear less frequently in this cluster, they represent broader areas of oxidative stress research, such as the relationship between oxidative stress and thrombosis (Vaughan and Walsh, 2005), and acid-base balance in physiological and pathological processes (Kinalski et al., 2001).

Several core themes have emerged as significant research hotspots, providing clear directions for researchers. Oxidative stress and lipid peroxidation processes have been longstanding focal points. The close relationship between “lipid peroxidation” and “oxidative stress” highlights the potential harm lipid peroxidation products inflict on placental function during oxidative stress, offering critical insights for the development of subsequent intervention strategies. Additionally, the role of antioxidants, particularly natural or synthetic agents such as “vitamin E,” has been widely recognized for mitigating oxidative stress and protecting placental health, providing new perspectives on nutritional supplementation and therapeutic approaches during pregnancy. Pregnancy-related disorders have become key research priorities due to their strong association with oxidative stress. Understanding these conditions is essential for elucidating disease mechanisms and optimizing clinical management. The complex roles of gene expression and regulation in placental oxidative stress responses have also gained clarity. The frequent appearance of “gene expression” and related keywords underscores the importance of genetic research in revealing molecular mechanisms underlying oxidative stress, prompting researchers to focus more on genetic factors in this field.

Emerging directions and fields are also worth attention. Pathophysiology and mechanism exploration are gaining prominence, with the rise of keywords such as “pathophysiology” and “mechanism” indicating that deeper investigation into the pathophysiological mechanisms of placental oxidative stress could provide theoretical support for precision therapies. Emerging risk factors and environmental influences have garnered increasing attention, with the appearance of keywords like “obesity,” “bisphenol A,” and “air pollution” revealing potential threats posed by environmental factors to placental health and

pregnancy outcomes, thus offering a scientific basis for public health policies. The growth of cellular biology and stem cell research, especially the increased focus on “cell,” “stem cell,” and “autophagy,” highlights the growing interest in cellular processes related to placental oxidative stress. Stem cell therapy and autophagy regulation could open new avenues for future therapeutic strategies. Furthermore, inflammation and immune regulation remain enduring research frontiers, with the complex interactions between placental oxidative stress and these processes being increasingly revealed. The sustained high frequency and recent rise of keywords such as “inflammatory cytokine” and “inflammation” underscore the critical role inflammation and immune regulation play in maintaining placental homeostasis and preventing related diseases, encouraging further exploration in this area.

Based on the analysis of core themes and research frontiers, this paper provides a scientific basis for policymakers to develop public health policies. Emphasis should be placed on the role of oxidative stress and lipid peroxidation in adverse pregnancy outcomes, encouraging further research to design effective interventions. Additionally, addressing emerging risk factors such as obesity, bisphenol A, and air pollution is crucial. Policymakers should implement targeted public health measures to alleviate their detrimental effects on placental health and pregnancy outcomes. Potential actions include advocating for stricter air pollution regulations and launching public health education campaigns to increase awareness of the risks associated with obesity and environmental pollutants.

Employing bibliometrics to explore the field of “placental oxidative stress” offers a novel perspective and facilitates a deeper understanding of its trends. Unlike traditional literature reviews, the study innovatively employed multiple bibliometric tools such as CiteSpace, VOSviewer, and the R package bibliometrix. The integrated use of these tools enabled a comprehensive and systematic extraction and analysis of data, revealing key research dynamics in the field of placental oxidative stress. By combining systematic searches with quantitative statistical analysis, the approach provided a more data-driven and quantitative view of the research landscape.

However, the study had certain limitations. First, the data were sourced solely from the Web of Science Core Collection, potentially overlooking relevant information from other databases such as Embase and MEDLINE. This choice stemmed from the fact that the Web of Science Core Collection is the most commonly used database for bibliometric analysis, offering timely and comprehensive citation updates. Nonetheless, challenges in integrating data from different sources persist due to limitations in the analytical software. Second, the sensitivity of the analysis algorithms may have missed emerging topics related to “placental oxidative stress.” Despite employing multiple software packages for analysis, the information provided remained constrained by the limitations of bibliometric algorithms. Future advancements in bibliometric methods may help address these challenges.

Additional challenges arose during the bibliometric analysis. First, scientometric research primarily relies on co-citation analysis, which can be influenced by “citation distortion”—the selective use of citations to support unverified scientific claims. This issue, although detectable through close examination of research hotspots, remains

a notable limitation. Second, limiting the search to the Web of Science Core Collection may have restricted the range of publication types retrieved. In many databases such as PubMed and Embase, full-text references and citation lists are unavailable, complicating efforts to merge references from different databases and often requiring significant manual intervention. Future software developments may enable the simultaneous analysis of results from various databases while reliably removing duplicates automatically. Third, the analysis did not cover all publications related to “placental oxidative stress,” as only original articles and reviews were included, and the Web of Science’s stricter inclusion criteria for scientific journals, compared to databases like PubMed, further constrained the scope. Lastly, the inherent lag in publications and subsequent citations made it difficult to capture the latest research trends. However, the impact of this lag was significantly reduced by considering the context in which articles were cited and who conducted the citations, as cited articles might have been recently published.

In summary, despite its limitations, the bibliometric analysis employed in this study provided valuable insights into research trends in the field of placental oxidative stress and suggested new directions for future research. To further improve the analysis in this area, future studies should consider integrating multiple databases and analytical methods for more comprehensive and in-depth insights.

5 Conclusion

This study provides an in-depth bibliometric analysis of the field of “placental oxidative stress” aiming to reveal trends, research hotspots, and future directions. The study identifies key research hotspots focused on oxidative stress and lipid peroxidation processes, with “lipid peroxidation” as a critical event indicating potential risks to placental function. The role of antioxidants, particularly natural or synthetic substances such as vitamin E, in mitigating oxidative stress and protecting placental health is well-recognized, offering new insights for nutritional supplementation and therapeutic strategies during pregnancy. Pregnancy-related diseases, particularly “preeclampsia” and “pregnancy-induced hypertension,” are significant research focuses due to their close association with oxidative stress, which is crucial for understanding disease mechanisms and optimizing clinical management. Additionally, the complex role of gene expression and regulation in the placental oxidative stress response is increasingly evident, highlighting the importance of genetic-level research in elucidating molecular mechanisms. Emerging research directions include

exploring pathophysiological mechanisms, focusing on new risk factors and environmental impacts, advancing cell biology and stem cell research, and continuing to investigate inflammation and immune regulation. These developments are expected to enhance our understanding of placental oxidative stress and advance therapeutic strategies.

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.

Author contributions

AC: Funding acquisition, Writing–original draft. MT: Data curation, Writing–original draft. ZL: Data curation, Writing–original draft. XC: Investigation, Writing–review and editing. YG: Investigation, Writing–review and editing.

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Conflict of interest

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

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Comparative study on pregnancy complications: PGT-A vs. IVF-ET with gender-specific outcomes

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The safety and clinical effectiveness of preimplantation genetic testing for aneuploidy (PGT-A) in improving pregnancy outcomes for sub-fertile patients remains controversial. Potential sex-based differences in the relationship between PGT-A and pregnancy complications have not been investigated, which could guide the appropriate clinical application of PGT-A. In this secondary analysis of data from a multicenter, randomized, controlled, non-inferiority trial (NCT03118141), 940 women who achieved singleton live birth during the trial were included to estimate the between-group differences in pregnancy complications following PGT-A versus conventional *in vitro* fertilization (IVF) vary with fetal sex. Logistic regression analysis was used to adjust for possible confounders, and subgroup analysis was also performed. Among male fetuses, the risk of maternal preeclampsia was significantly lower after PGT-A compared to conventional IVF treatment (3.37% vs. 7.88%; adjusted OR, 0.40; 95% CI, 0.17–0.92; $P = 0.032$). However, this protective effect was not observed in pregnancies with female fetuses (3.63% vs. 3.38%; adjusted OR, 1.04; 95% CI, 0.36–3.00; $P = 0.937$). In addition, no significant sex-dependent differences in the risks of other pregnancy complications or neonatal outcomes were detected between PGT-A and conventional IVF groups ($P > 0.05$). In summary, PGT-A was associated with a decreased risk of maternal preeclampsia in singleton pregnancies with male fetuses, highlighting its potential utility in preeclampsia prevention in addition to spontaneous abortion rate reduction.

KEYWORDS

preimplantation genetic testing for aneuploidy, *in vitro* fertilization and embryo transfer, singleton pregnancy, fetal sex, preeclampsia

Introduction

Preimplantation genetic testing for aneuploidy (PGT-A), a derivative technology of *in vitro* fertilization and embryo transfer (IVF-ET), has become increasingly utilized to improve outcomes in the field of assisted reproduction (1). The goal of PGT-A is to identify euploid embryos that are suitable for transfer, thereby increasing the live birth rates and reducing the risk of adverse pregnancy outcomes, such as spontaneous abortion (2). Some studies have shown that PGT-A can significantly improve pregnancy outcomes compared to conventional IVF, especially in women of advanced maternal age (3, 4). However, our recent randomized controlled study indicates that despite a perceived reduction in the frequency of pregnancy loss among clinical pregnancies for the PGT-A group, it does not positively affect cumulative live birth rates following embryo transfer in patients with a good prognosis, nor does it impact other obstetric pregnancy or neonatal outcomes (5). This provokes debates regarding whether the purported clinical effectiveness and benefits of PGT-A justify the risks intrinsic to the testing process. Despite controversy surrounding the invasiveness of this procedure for transferred embryos, PGT-A is currently utilized in approximately half of all IVF cycles in the USA, with its application steadily increasing worldwide (6). Thus, evidence regarding the effectiveness and safety of PGT-A is urgently needed for guiding the correct indications and optimal utilization.

Preeclampsia, a common pregnancy complication characterized by *de novo* development of high blood pressure ($\geq 140/90$ mm Hg) concurrent with proteinuria (≥ 300 mg/L per 24 hours) after 20 weeks of gestation, remains a leading cause of maternal and perinatal mortality and morbidity worldwide (7) and is associated with a substantial long-term risk for cardiovascular disease (8). One previous report showed a higher risk of preeclampsia associated with frozen single blastocyst transfer compared to fresh single blastocyst transfer (9). It is now broadly accepted that placental dysfunction, originating from the trophectoderm of the embryo, contributes to preeclampsia pathogenesis (7). Given that PGT-A necessitates biopsy of embryonic trophectoderm cells for genetic examination, it could provide valuable insights into preventing diseases related to embryonic trophectoderm abnormalities, such as preeclampsia. However, the accuracy and reliability of PGT-A results should be carefully validated due to potential confounding factors.

Furthermore, emerging evidence indicates that selecting euploid embryos via PGT-A may be misleading, as the genetic features of the trophectoderm do not fully represent the chromosomal status of the inner cell mass, which eventually develops into the fetus. Given the self-correction ability of aneuploid cells in the embryo and the presence of mosaic cells in the placenta of healthy pregnancies, as revealed in recent studies (6, 10, 11), PGT-A could potentially produce high false-positive rates for aneuploidy, resulting in a waste of transferrable embryos. Moreover, an increasing number of studies highlight that placenta formation and function during pregnancy are sex-dependent, and that male fetuses carry a higher risk of pregnancy complications, including gestational diabetes, gestational hypertension, preeclampsia, and eclampsia

(12–14). However, studies investigating the effects of PGT-A on pregnancy complications, particularly those related to placental dysfunction, have generally disregarded differences in fetal sex (15).

Our group conducted a multicenter, randomized, controlled, non-inferiority trial and observed no improvement in the cumulative live birth rate by PGT-A compared to IVF in women with a good prognosis (5). In this study, we further performed a *post hoc* exploratory secondary analysis among women who achieved a singleton live birth during this trial and assessed the differences in pregnancy complications between male and female fetuses in PGT-A versus conventional IVF.

Materials and methods

Design and participants

The original multicenter, randomized, controlled clinical trial (registration number: NCT03118141) was conducted sequentially from July 2017 through June 2018, and the primary outcomes and protocols of the trial are published (5). Briefly, 1212 women who planned to undergo their first IVF cycle with a good prognosis for live birth were enrolled. A good prognosis was defined by patient age of 20 to 37 years and the availability of three or more high-quality blastocysts. All participants were randomized and then assigned to either the PGT-A group or the conventional IVF group (each group included 606 women). In the present analyses, the subjects without a live birth or with a multiple pregnancy were excluded, resulting in a total cohort of 940 women who achieved singleton live birth for further evaluation (Figure 1).

Study procedure

Ovarian stimulation was performed through long or short regimens of gonadotropin-releasing hormone (GnRH) agonist or with a GnRH antagonist. The detailed schedule for controlled ovarian hyperstimulation has been previously described (5, 16). Human chorionic gonadotropin (hCG), GnRH agonist, or both were administered to trigger oocyte maturation when at least two follicles were ≥ 18 mm. All oocytes were inseminated by intracytoplasmic sperm injection (ICSI), and viable embryos were cultured to the blastocyst stage. According to the trial design, three high-quality blastocysts selected by means of morphological criteria (17) in the PGT-A group underwent trophectoderm biopsy. In each cycle, a euploid blastocyst in the PGT-A group or a high-quality blastocyst in the conventional IVF group was chosen for transfer. Endometrial preparation before frozen embryo transfer was performed with either a natural ovulation cycle, an artificial regimen, or an ovulation induction cycle. Luteal-phase support was administered in both groups. These techniques have been previously described in detail (16, 18). In this trial, up to three blastocyst-stage embryos were transferred either in the PGT-A group or conventional IVF group within 1 year after randomization until live birth or pregnancy

termination. All pregnancy and neonatal outcomes were recorded in detail. Only singleton live births were included in the present study.

Study outcomes

The primary outcomes were pregnancy complications, principally including the incidence of preeclampsia in subjects with singleton live births following PGT-A or conventional IVF. Secondary outcomes were neonatal outcomes, mainly including mean birth weight, as well as the incidence of small for gestational age (SGA) and large for gestational age (LGA) of singleton fetuses. The parameters of controlled ovarian hyperstimulation and other pregnancy complications were also analyzed. Preeclampsia was defined as hypertension appearing after 20 weeks of gestation with proteinuria (≥ 0.3 mg urinary protein per 24 h) (19, 20). SGA and LGA were defined as smaller than the 10th percentile or larger than the 90th percentile of birth weight at the corresponding gestational week, respectively, with consideration of fetal sex (21).

Statistical analysis

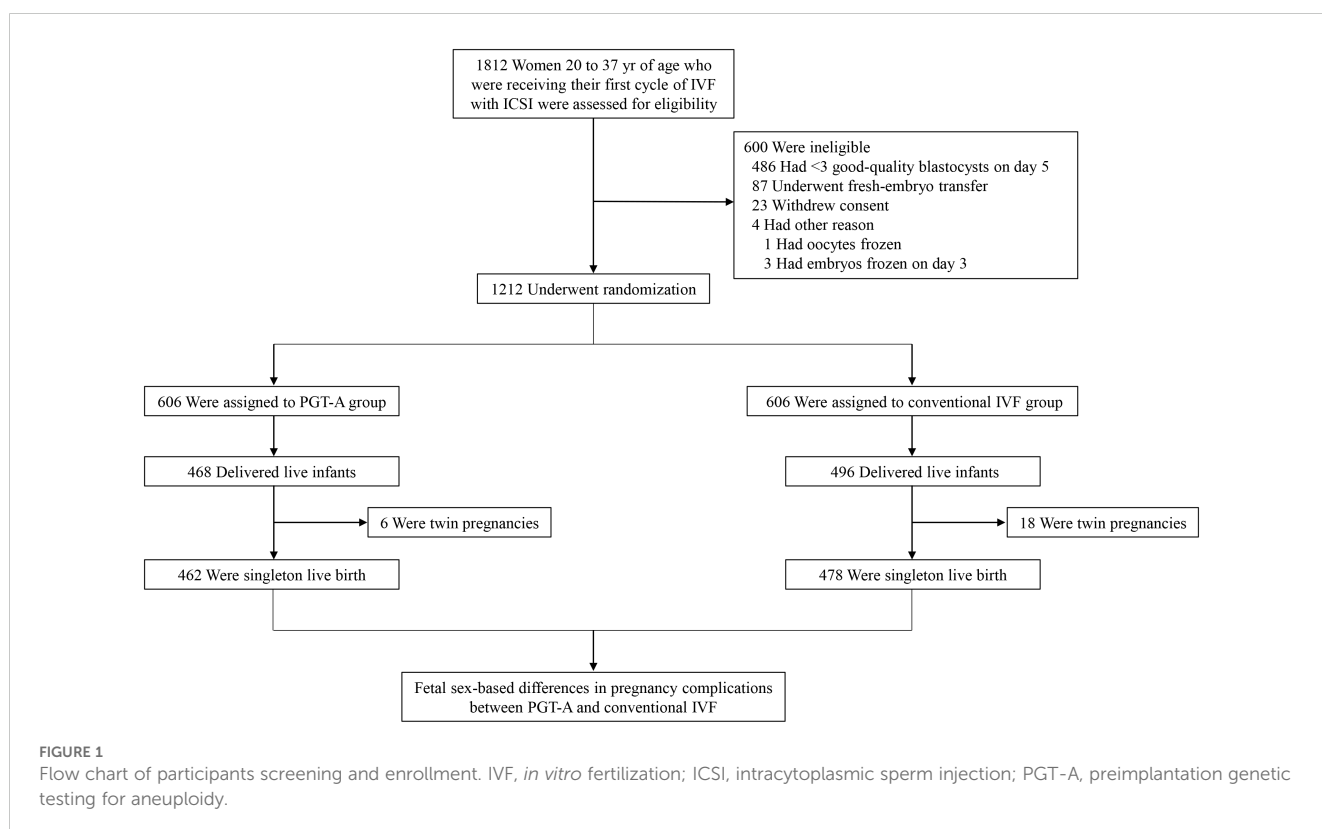
Statistical analyses were performed using SPSS 26.0 for Windows (IBM, Armonk, NY, USA). The Kolmogorov–Smirnov test, in conjunction with histogram and Q-Q plot, was used to test for normality in the distribution of data. Continuous variables with normal distributions are presented as the mean $\pm$ standard deviation (SD) and were analyzed by independent *t* test. The median with interquartile range is used to show data with a non-

normal distribution and compared by the Mann–Whitney U test. Categorical variables are presented as counts (percentages) and were compared using either the chi-square or Fisher's exact test. Multivariable logistic regression analysis was performed to adjust for baseline characteristics. Multivariable linear regression or logistic regression analysis was used to investigate potential associations between PGT-A and pregnancy complications, adjusting for maternal age, body mass index (BMI), serum anti-Müllerian hormone (AMH) level, number of oocytes retrieved, number of high-quality embryos on day 5 or 6, endometrial thickness before embryo transfer, PGT-A vs. conventional IVF, fetal sex, and interaction between PGT-A vs. conventional IVF and fetal sex. Values of $P < 0.05$ were considered statistically significant.

Results

Baseline characteristics

This study included a total of 940 women who achieved singleton live birth after PGT-A or conventional IVF during the trial. Among them, 462 women were randomly assigned to the PGT-A group, and 478 women were assigned to the conventional IVF group. As shown in Table 1, there were no significant differences in baseline characteristics between the two groups ($P > 0.05$), including maternal age, BMI, duration of infertility, type of infertility, indications for IVF, antral follicle count, serum thyroid stimulating hormone (TSH), AMH, follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, progesterone,



testosterone and prolactin levels. Additionally, the results indicated that the proportion of male fetuses in the PGT-A group was slightly higher than in the conventional IVF group. This difference may be due to the limitations of sample size or could suggest that PGT-A may have some influence on sex ratios, warranting further investigation.

Ovarian response and embryo quality

The outcomes of controlled ovarian hyperstimulation in the two groups are compared in [Table 2](#). No significant differences were observed in the duration of ovarian stimulation, gonadotropin dose, serum estradiol, LH and progesterone levels on the hCG trigger day,

endometrial thickness on the hCG trigger day, number of oocytes with diameters ≥ 14 mm and ≥ 18 mm on the hCG trigger day, number of retrieved, metaphase II (MII), and two pronuclei (2PN) oocytes, number of high-quality embryos on day 3 and number of high-quality embryos on day 5 or 6 ($P > 0.05$). This indicates that ovarian response and embryo quality were equivalent between the two groups.

Pregnancy complications

Multivariable regression analyses showed no significant differences ($P > 0.05$) in the interaction between fetal sex and PGT-A vs. conventional IVF regarding risks of pregnancy complications, including gestational age at delivery, incidence of

TABLE 1 Baseline characteristics of the participants who achieved singleton live birth after PGT-A vs. conventional IVF.

Characteristics	PGT-A (N = 462)	Conventional IVF (N = 478)	P values
Age (years)	28.89 $\pm$ 3.53	29.18 $\pm$ 3.49	0.205
BMI (kg/m ²)	23.00 $\pm$ 3.39	22.96 $\pm$ 3.45	0.873
Duration of infertility (years)	3.26 $\pm$ 2.03	3.44 $\pm$ 2.19	0.203
Type of infertility (n, %)			0.106
Primary	303 (65.58)	337 (70.50)	
Secondary	159 (34.42)	141 (29.50)	
Indication for IVF (n, %)			0.121
Ovulatory dysfunction	33 (7.14)	23 (4.81)	
Tubal factor	232 (50.22)	269 (56.28)	
Endometriosis	3 (0.65)	2 (0.42)	
Male factor	69 (14.94)	82 (17.15)	
Combined factors	97 (21.00)	84 (17.57)	
Unexplained	28 (6.06)	18 (3.77)	
Antral follicle count	22.24 $\pm$ 10.62	21.58 $\pm$ 9.71	0.321
TSH (μ IU/mL)	2.24 $\pm$ 1.41	2.18 $\pm$ 1.22	0.542
AMH (ng/ml)	6.40 (4.14-10.18)	6.34 (4.18-9.67)	0.542
FSH (IU/L)	5.82 (4.99-6.84)	5.96 (5.11-6.77)	0.097
LH (IU/L)	5.51 (3.96-8.08)	5.71 (4.05-8.01)	0.670
Estradiol (pg/ml)	35.30 (26.83-46.04)	36.75 (27.85-49.01)	0.157
Progesterone (ng/ml)	0.40 (0.21-0.66)	0.40 (0.22-0.66)	0.972
Testosterone (ng/ml)	0.32 (0.22-0.45)	0.32 (0.24-0.46)	0.642
Prolactin (ng/ml)	15.87 (11.79-20.71)	16.22 (12.17-22.40)	0.319
Sex of fetuses (n, %) †			0.019
Male	267 (58.04)	241 (50.42)	
Female	193 (41.96)	237 (49.58)	

Data are presented as the mean $\pm$ standard deviation, median (interquartile range) or n (%). $P < 0.05$ was considered statistically significant. † Data were missing regarding fetal sex in 2 women in the PGT-A group. Abbreviations: PGT-A, preimplantation genetic testing for aneuploidy; IVF, *in vitro* fertilization; BMI, body mass index; TSH, thyroid stimulating hormone; AMH, anti-Mullerian hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

TABLE 2 Comparison of COH outcomes of singleton pregnancy after PGT-A vs. conventional IVF.

Characteristics	PGT-A (N = 462)	Conventional IVF (N = 478)	P values
Duration of ovarian stimulation (days)	9.80 ± 1.88	9.88 ± 2.13	0.542
Gonadotropin dose (IU)	1493.75 (1171.88-1875.00)	1425.00 (1200.00-1950.00)	0.566
Estradiol level on HCG trigger day (pg/mL)	5667.50 (4108.50-7603.75)	5569.00 (4283.50-7271.50)	0.795
LH level on HCG trigger day (pg/mL)	1.99 (1.26-3.39)	2.05 (1.35-3.23)	0.926
Progesterone level on HCG trigger day (ng/L)	0.87 (0.59-1.23)	0.80 (0.53-1.17)	0.092
Endometrial thickness on HCG trigger day (mm)	10.65 ± 2.24	10.70 ± 2.18	0.696
≥14 mm oocytes on HCG trigger day	17.77 ± 5.40	17.09 ± 5.50	0.053
≥18 mm oocytes on HCG trigger day	7.75 ± 4.20	7.42 ± 4.11	0.218
No. of oocytes retrieved	20.31 ± 7.72	19.56 ± 6.65	0.112
No. of MII oocytes	17.34 ± 5.65	17.06 ± 5.33	0.436
No. of 2PN oocytes	13.60 ± 4.67	13.16 ± 4.40	0.133
No. of high-quality embryos on day 3	9.54 ± 4.32	9.30 ± 4.04	0.388
No. of high-quality embryos on day 5 or 6	7.36 ± 3.14	7.02 ± 2.82	0.086

Data are presented as the mean ± standard deviation or median (interquartile range). *P* < 0.05 was considered statistically significant. Abbreviations: PGT-A, preimplantation genetic testing for aneuploidy; IVF, *in vitro* fertilization; HCG, human chorionic gonadotropin; LH, luteinizing hormone; MII, metaphase II; 2PN, two pronuclei.

TABLE 3 Comparison of pregnancy complications of singleton pregnancy after PGT-A vs. conventional IVF.

Characteristics	PGT-A (N = 462)	Conventional IVF (N = 478)	Adjusted OR or absolute difference (95% CI)	P values for interaction between PGT-A and fetal sex
Gestational age at delivery (weeks)	39.21 ± 1.62	39.23 ± 1.48	0.02 (-0.38-0.42)	0.920
Male fetuses	39.11 ± 1.66	39.09 ± 1.56	-0.01 (-0.29-0.27)	
Female fetuses	39.36 ± 1.57	39.38 ± 1.37	-0.02 (-0.30-0.27)	
Cesarean section (n, %)	288 (64.29)	306 (66.67)	0.89 (0.49-1.58)	0.679
Male fetuses	171 (65.52)	159 (68.24)	0.85 (0.57-1.26)	
Female fetuses	117 (62.57)	147 (65.04)	0.92 (0.60-1.40)	
Preterm delivery (n, %)	27 (5.84)	23 (4.81)	0.51 (0.15-1.71)	0.278
Male fetuses	16 (5.99)	15 (6.22)	0.91 (0.43-1.93)	
Female fetuses	11 (5.70)	8 (3.38)	1.60 (0.62-4.15)	
Gestational diabetes (n, %)	47 (10.17)	54 (11.30)	0.81 (0.34-1.92)	0.630
Male fetuses	30 (11.24)	33 (13.69)	0.84 (0.49-1.43)	
Female fetuses	17 (8.81)	21 (8.86)	0.99 (0.50-1.97)	
Gestational hypertension (n, %)	10 (2.16)	7 (1.46)	3.29 (0.39-27.65)	0.272
Male fetuses	6 (2.25)	2 (0.83)	3.03 (0.59-15.48)	
Female fetuses	4 (2.07)	5 (2.11)	0.89 (0.22-3.57)	
Preeclampsia (n, %)	16 (3.46)	27 (5.65)	0.34 (0.09-1.29)	0.112
Male fetuses	9 (3.37)	19 (7.88)	0.40 (0.17-0.92)	
Female fetuses	7 (3.63)	8 (3.38)	1.04 (0.36-3.00)	

(Continued)

TABLE 3 Continued

Characteristics	PGT-A (N = 462)	Conventional IVF (N = 478)	Adjusted OR or absolute difference (95% CI)	P values for interaction between PGT-A and fetal sex
Placenta previa (n, %)	4 (0.87)	7 (1.46)	5.50 (0.32-94.02)	0.240
Male fetuses	3 (1.12)	2 (0.83)	1.78 (0.27-11.81)	
Female fetuses	1 (0.52)	5 (2.11)	0.28 (0.03-2.50)	
Preterm rupture of membrane (n, %)	33 (7.14)	31 (6.49)	0.54 (0.19-1.56)	0.252
Male fetuses	19 (7.12)	20 (8.30)	0.83 (0.42-1.63)	
Female fetuses	14 (7.25)	11 (4.64)	1.53 (0.66-3.51)	

Data are presented as the mean ± standard deviation, or n/N (%). $P < 0.05$ was considered statistically significant. The covariables in the overall regression models were maternal age, BMI, AMH, number of oocytes retrieved, number of high-quality embryos on day 5 or 6, endometrial thickness before embryo transfer, PGT-A vs. conventional IVF, fetal sex, and interaction between PGT-A vs. conventional IVF and fetal sex. For each fetal sex subgroup, the regression model included covariables of maternal age, BMI, number of oocytes retrieved, number of high-quality embryos on day 5 or 6, endometrial thickness before embryo transfer, and PGT-A vs. conventional IVF. Abbreviations: PGT-A, preimplantation genetic testing for aneuploidy; IVF, *in vitro* fertilization; OR, odds ratio; CI, confidence interval.

cesarean section, pre-term delivery, gestational diabetes, gestational hypertension, preeclampsia, placenta previa, or pre-term rupture of the membrane (Table 3). However, subgroup analysis based on fetal sex demonstrated a significantly lower risk of preeclampsia following PGT-A compared to conventional IVF in male fetuses (3.37% vs. 7.88%; adjusted OR, 0.40; 95% CI, 0.17-0.92; $P = 0.032$) (Figure 2A and Supplementary Tables 1, 2). This protective effect was not observed in pregnancies with female fetuses (3.63% vs. 3.38%; adjusted OR, 1.04; 95% CI, 0.36-3.00; $P = 0.937$) (Figure 2B, Supplementary Table 3), suggesting that the impacts of PGT-A on preeclampsia incidence is dependent on fetal sex.

Neonatal outcomes

As shown in Table 4, no significant differences were found in neonatal outcomes attributable to the interaction between fetal sex

and PGT-A vs. conventional IVF ($P > 0.05$), including mean birth weight, incidence of SGA, LGA, neonatal respiratory distress syndrome (RDS), neonatal jaundice, neonatal infection and neonatal hospitalization of >3 days. For each fetal sex subgroup analysis, no significant difference in neonatal outcomes was detected between the PGT-A group and the conventional IVF group.

Discussion

To our knowledge, no previous studies have examined the potential relationships between PGT-A and fetal sex in relation to the risk of pregnancy complications. In this *post hoc* exploratory secondary analysis of data from a multicenter randomized controlled trial, we provided evidence that PGT-A treatment may decrease the risk of preeclampsia in women carrying singleton male fetuses compared with those pregnant women who underwent

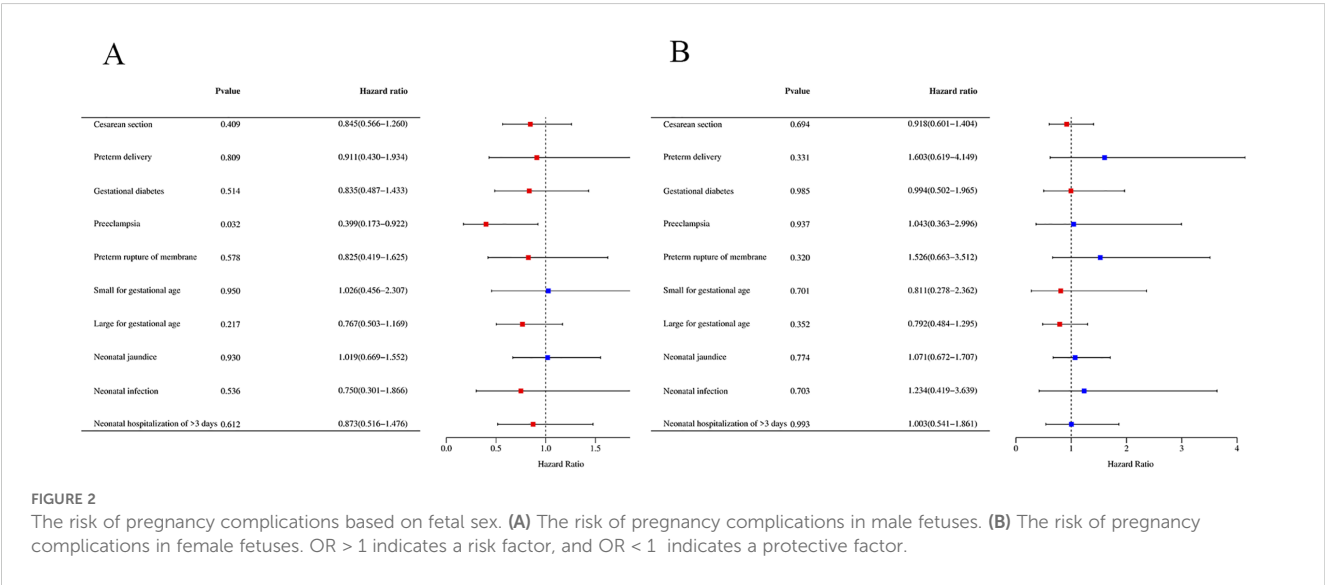


TABLE 4 Comparison of neonatal outcomes and birth weight of singleton pregnancy after PGT-A vs. conventional IVF.

Characteristics	PGT-A (N = 462)	Conventional IVF (N = 478)	Adjusted OR or absolute difference (95% CI)	P values for interaction between PGT-A and fetal sex
Mean birth weight (g)	3417.24 ± 487.71	3449.33 ± 488.19	-58.74 (-185.50-68.03)	0.363
Male fetuses	3440.79 ± 498.67	3506.49 ± 533.68	57.12 (-34.26-148.50)	
Female fetuses	3389.77 ± 471.68	3391.20 ± 430.52	0.27 (-86.32-86.87)	
SGA (n, %)	20 (4.35)	21 (4.39)	1.21 (0.32-4.59)	0.776
Male fetuses	14 (5.24)	12 (4.98)	1.03 (0.46-2.31)	
Female fetuses	6 (3.11)	9 (3.80)	0.81 (0.28-2.36)	
LGA (n, %)	91 (19.78)	114 (23.85)	0.95 (0.50-1.81)	0.876
Male fetuses	55 (20.60)	63 (26.14)	0.77 (0.50-1.17)	
Female fetuses	36 (18.65)	51 (21.52)	0.79 (0.48-1.30)	
Neonatal RDS (n, %)	3 (0.65)	5 (1.05)	0.09 (0.00-2.43)	0.153
Male fetuses	1 (0.37)	4 (1.66)	0.22 (0.02-2.02)	
Female fetuses	2 (1.04)	1 (0.42)	2.48 (0.20-30.06)	
Neonatal jaundice (n, %)	113 (24.46)	110 (23.01)	0.96 (0.51-1.78)	0.886
Male fetuses	65 (24.34)	57 (23.65)	1.02 (0.67-1.55)	
Female fetuses	48 (24.87)	53 (22.36)	1.07 (0.67-1.71)	
Neonatal infection (n, %)	16 (3.46)	18 (3.77)	0.56 (0.14-2.29)	0.423
Male fetuses	9 (3.37)	11 (4.56)	0.75 (0.30-1.87)	
Female fetuses	7 (3.63)	7 (2.95)	1.23 (0.42-3.64)	
Neonatal hospitalization of >3 days (n, %)	55 (11.90)	59 (12.34)	0.90 (0.40-2.01)	0.792
Male fetuses	34 (12.73)	33 (13.69)	0.87 (0.52-1.48)	
Female fetuses	21 (10.88)	26 (10.97)	1.00 (0.54-1.86)	

Data are presented as the mean ± standard deviation, or n/N (%). *P* < 0.05 was considered statistically significant. The covariables in the overall regression models were maternal age, BMI, AMH, number of oocytes retrieved, number of high-quality embryos on day 5 or 6, endometrial thickness before embryo transfer, PGT-A vs. conventional IVF, fetal sex, and interaction between PGT-A vs. conventional IVF and fetal sex. For each fetal sex subgroup, the regression model included covariables of maternal age, BMI, number of oocytes retrieved, number of high-quality embryos on day 5 or 6, endometrial thickness before embryo transfer, and PGT-A vs. conventional IVF. Abbreviations: PGT-A, preimplantation genetic testing for aneuploidy; IVF, *in vitro* fertilization; OR, odds ratio; CI, confidence interval; SGA, small for gestational age; LGA, large for gestational age; RDS, respiratory distress syndrome.

conventional IVF treatment. Notably, this potential benefit of PGT-A treatment was not observed in mothers who delivered female fetuses. In addition, no obvious relationship between PGT-A and fetal sex in other pregnancy complications or neonatal outcomes, such as the mean birth weight, SGA, or LGA, was observed.

Given the unavoidable invasive nature of embryo biopsy during PGT-A, the choice of embryonic stage for biopsy usually with cautious to minimize potential adverse impacts on embryo development. Current methods of PGT-A involve polar body biopsy (22), cleavage-stage biopsy (23), and blastocyst-stage biopsy (24). Among these, blastocyst-stage biopsy is most common due to its improved genetic testing accuracy and lesser impact on the inner cell mass. As the prevalence recognition of mosaic embryos which refer to the embryos possess two or more genetically different sets of cells, it has been realized that the biopsy

and test of 5-10 trophoctoderm cells cannot accurately reflect aneuploidy throughout the embryo and for the inner cell mass, let alone predicting pregnancy outcomes after embryo transfer (11, 25). Several studies have found that PGT-A can improve rates of clinical (3) and ongoing pregnancies (26), as well as effectively reduce the occurrence of adverse pregnancy outcomes such as pre-term birth and low birth weight (27). However, more recent randomized controlled studies have suggested that PGT-A does not improve ongoing pregnancy or live birth rates after frozen embryo transfer compared with conventional IVF treatment (28, 29). Our original randomized controlled trial also found that PGT-A does not improve the cumulative live birth rates after embryo transfer in patients with a good prognosis, except for the rate of first-trimester pregnancy loss, nor does it affect other pregnancy complications or neonatal outcomes (5). These inconsistent

findings might be partially attributed to the self-correction ability of aneuploidy cells and the recognition of mosaic embryos as a normal stage in embryo development (30, 31).

While PGT-A may not accurately depict the chromosomal status of the inner cell mass, it could still theoretically aid in the prevention of diseases related to placental development, considering its ability to identify aneuploidy in the trophoctoderm, which forms the placenta. Studies have demonstrated the presence of aneuploid cells in placentas of otherwise chromosomally normal pregnancies (32). It has been established that the placenta serves as a repository for unrepaired aneuploid cells, which can rectify embryonic endoderm aneuploidy, protect the fetus from aneuploidy, and promote implantation (10, 31). Notably, sex-based differences in disease risk, pathophysiology, clinical manifestations, and responses to clinical interventions have gained significant research attention recently (33). It has been indicated that the formation and function of the placenta are sex-dependent, with male fetuses having smaller placentas than female fetuses, suggesting an increased risk of adverse pregnancy events for male fetuses (12–14, 34). Recent studies suggests that the underlying mechanism of this observed phenomenon may be that the female placenta is more sensitive to abnormal intrauterine stress signals. This sensitivity could confer adaptive benefits through the inactivation of one X chromosome during preimplantation (35).

Abnormalities in the embryonic trophoctoderm are known to be associated with the occurrence of pregnancy complications such as preeclampsia, which arises from impaired placental development (7). Given that the PGT-A during the trophoctoderm development stage could potentially eliminate aneuploid cells that are resistant to self-correction later on, we hypothesize PGT-A could provide protective benefits against placental dysfunction-related pregnancy complications such as preeclampsia. Based on a secondary analysis of a large randomized controlled trial, our study provides evidence supporting the notion that PGT-A reduces the risk of maternal preeclampsia in singleton pregnancies carrying male fetuses compared to conventional IVF. It is important to note the differences in fetal sex between the PGT-A and conventional IVF groups, as these differences could influence the observed outcomes. While our findings suggest a potential protective effect of PGT-A, the mechanisms remain unclear, possibly relating to the timing or self-correction of mosaic cells in male versus female fetuses.

Ethical considerations surrounding sex selection in reproductive technologies, especially PGT-A, must be carefully examined. Choosing embryos based on sex can raise significant concerns about societal preferences for male or female offspring, potentially exacerbating gender imbalances. Additionally, while IVF is associated with an increased risk of preeclampsia, factors such as maternal age, underlying health conditions, and multiple gestations also play crucial roles (36). Currently, the proportion of male births during normal pregnancies in our country is relatively high (37), which may further influence these ethical dynamics, including the complex interplay of societal norms, reproductive choices, and potential long-term effects on gender ratios within the population. Further research is essential to disentangle these variables and comprehend the broader implications of our findings.

This study has several strengths, including the use of data from a prospective multicenter randomized controlled trial, which greatly minimizes potential bias compared to retrospective observational studies. We also have access to comprehensive data regarding baseline characteristics, as well as obstetric and neonatal outcomes. However, the robustness of our analysis was restricted by a relatively small sample size (N=940 singleton births), necessitating further larger studies. Furthermore, given that the original randomized controlled trial only enrolled women predicted to have a good prognosis for a live birth, whether our findings can be extended to patients with a predicted poor prognosis, such as those with chromosomal abnormalities that are indications for PGT-A treatment, needs further evaluation. Additionally, as the original study focused on cumulative live birth rates, the available information on pregnancy complications, particularly preeclampsia, is quite limited. Future research could explore detailed clinical characteristics related to preeclampsia for a more comprehensive understanding.

In conclusion, our study demonstrates that the sex of the fetus significantly influences the maternal risk of preeclampsia in singleton pregnancies following PGT-A treatment as compared to conventional IVF treatment. Specifically, pregnant women carrying male fetuses are likely to experience a decreased risk of preeclampsia after undergoing PGT-A treatment compared to those who received conventional IVF treatment. In this sense, our findings suggest a potential value in considering the sex-dependent influence of PGT-A treatment on the pathogenesis of preeclampsia, beyond its known protective role in reducing the rate of spontaneous abortion. In the future, additional large cohort studies are needed to not only reaffirm our findings but also to uncover the possible underlying mechanisms for these observed correlations.

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 humans were approved by the ethics committees of the Reproductive Medical Center of Shandong University (reference number 2021140). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

LG: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Formal analysis,

Data curation. XL: Writing – original draft, Methodology. AG: Writing – review & editing, Software, Formal analysis. YW: Writing – original draft, Formal analysis. YLiang: Writing – original draft, Formal analysis. YLi: Conceptualization, Writing – review & editing. XX: Writing – review & editing, Conceptualization. HL: Project administration, Funding acquisition, Writing – review & editing, Conceptualization.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fendo.2024.1453083/full#supplementary-material>

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Polystyrene microplastics exposition on human placental explants induces time-dependent cytotoxicity, oxidative stress and metabolic alterations

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Introduction: Microplastics (MPs) are environmental pollutants that pose potential risks to living organisms. MPs have been shown to accumulate in human organs, including the placenta. In this study, we investigated the biochemical impact of 5 µm polystyrene microplastics (PS-MPs) on term placental chorionic villi explants, focusing on cytotoxicity, oxidative stress, metabolic changes, and the potential for MPs to cross the placental barrier.

Methods: Term placental chorionic explants were cultured for 24 hours with varying concentrations of PS-MPs, with MTT assays used to determine the appropriate concentration for further analysis. Cytotoxicity was assessed using the lactate dehydrogenase (LDH) release assay over a period of up to 72 hours. Reactive oxygen species formation and antioxidant activity were evaluated using biochemical assays. Metabolomic profiling was performed using proton nuclear magnetic resonance (1H NMR).

Results: Placental explants exposed to 100 µg/mL of PS-MPs showed a significant increase in cytotoxicity over time ($p < 0.01$). Levels of mitochondrial and total superoxide anion ($p < 0.01$ and $p < 0.05$, respectively) and hydrogen peroxide ($p < 0.001$) were significantly elevated. PS-MP exposure resulted in a reduction in total sulfhydryl content ($p < 0.05$) and the activities of antioxidant enzymes superoxide dismutase ($p < 0.01$) and catalase ($p < 0.05$), while

glutathione peroxidase activity increased ($p < 0.05$), and the oxidized/reduced glutathione ratio decreased ($p < 0.05$). Markers of oxidative damage, such as malondialdehyde and carbonylated proteins, also increased significantly ($p < 0.001$ and $p < 0.01$, respectively), confirming oxidative stress. Metabolomic analysis revealed significant differences between control and PS-MP-exposed groups, with reduced levels of alanine, formate, glutaric acid, and maltotriose after PS-MP exposure.

Discussion: This study demonstrates that high concentrations of PS-MPs induce time-dependent cytotoxicity, oxidative stress, and alterations in the TCA cycle, as well as in folate, amino acid, and energy metabolism. These findings highlight the need for further research to clarify the full impact of MP contamination on pregnancy and its implications for future generations.

KEYWORDS

metabolomics, microplastics, oxidative stress, placenta, polystyrene

1 Introduction

The annual global production of plastics has reached an unprecedented 400.2 million tons in 2022 (1). This surge in plastic production, coupled with improper waste management, is increasingly contributing to plastic pollution, which is released into natural habitats and leads to significant environmental and potential health risks. Notably, plastic waste constitutes over 75.0% of marine debris, primarily due to its rigid and non-biodegradable nature (2).

The environmental contamination results in the formation of smaller particles known as microplastics (MP), defined as “*synthetic solid particles or polymeric matrices, with regular or irregular shapes and sizes ranging from 1 μm to 5 mm, of either primary or secondary manufacturing origin, and insoluble in water*” (3). The MPs are broadly categorized into two types: primary MPs, which are intentionally incorporated into consumer and commercial products such as cosmetics, microfibers, hygiene products, clothing and fishing nets; and secondary MPs, which arise from the degradation of larger polymers due to physical, chemical, or biological processes in terrestrial or marine environments, such as food packages, water bottles, bags, tire debris etc. (4). Furthermore, MPs can be mainly classified into six categories based on their chemical composition: polyethylene (PE), polystyrene (PS), polypropylene (PP), polyurethane (PU), polyvinyl chloride (PVC), and polyethylene terephthalate (PET) (5). Among these, PS, PET, PE and PP are some of the most widely used plastics and the most common polymers studied (6, 7).

The growing concern over MPs pollution highlights the urgent need for comprehensive research and effective management strategies to mitigate its impact on ecosystems and human health. The life cycle of MPs typically begins with the release of primary or secondary microplastics into the terrestrial and aquatic ecosystems,

followed by their transport into water systems (4). Several solutions have been proposed to reduce the amount of such environmental waste. For instance, Osman and colleagues proposed the conversion of PET into solid or liquid fuel, and PET plastic and biomass waste into effective magnetic sorbents for use in waste remediation (7, 8). Such innovations would facilitate the conversion of this abundant waste stream into valuable products, such as energy, while promoting concepts like the circular economy and waste management hierarchy for non-biodegradable waste (7). Another interesting approach is the blending of PS and PU plastic waste into composite resins, which could be reused as binders in the formulation of emulsion paints (9).

Indeed, such innovative ways to deal with MPs waste are much needed, as growing evidence suggest that these MPs can infiltrate the food chain of aquatic organisms and accumulate in their tissues, progressively ascending through the trophic levels (2). This chronic and escalating pollution poses significant threat to all the biodiversity in Earth's ecosystems, as MPs have been shown to not only bioaccumulate, but also impact a wide variety of living organisms, including animals (10, 11). The MPs have been identified in both invertebrates and vertebrates, not only within their immune and digestive systems but also accumulating in their tissues and fluids. In addition to the MPs retention in their tissues, adverse health effects and even mortality have been reported to a variety of animals (12, 13).

In humans, the placenta was the first organ to be reported with MPs presence (14, 15). Afterwards, several other studies have documented the presence of MPs in placentas worldwide (16–23), and in other human organs (24–28). Nevertheless, significant gaps in the data regarding exposure and hazard remain, necessitating further investigation. Consequently, it is currently not possible to perform a human health risk assessment (HRA) for contamination by plastic pollutants (29). Although HRA for the MPs are still

debatable, the toxicity on human health caused by MPs may be due to the nature of plastic; such is the case of styrene-butadiene rubber (30); the other one is the size of the particles (31).

Such growing evidence from diverse research groups worldwide supports the idea that MPs not only accumulate in placentas but also indicates the possibility that they could affect the fetal development. In the absence of large epidemiological studies, both *in vivo* and *in vitro* studies can delineate the molecular initiating event and subsequent key pathways that lead to potential adverse outcomes (32).

Experimental *in vivo* models of MPs exposure during pregnancy have demonstrated increased cytotoxicity, oxidative stress, inflammation, and cognitive and neurological changes in the offspring (33–36), as well as reproductive toxicity (37). *In vitro*, studies using placental cell lines, such as HTR-8/SVneo, Bewo and Swan 71 cells have demonstrated that PS MPs can be internalized and change certain cell functions, which in turn, would alter the placenta physiology (38–40). Advancing research in this area with primary placental cells or tissues is crucial for assessing the potential hazards and risks of MPs exposure to maternal and fetal health (41). Therefore, our study aims to determine whether exposure to MPs on term placental chorionic villi explants can induce biochemical and metabolic alterations in the placenta.

2 Materials and methods

2.1 Sample collection

Term placentas were obtained from a cohort of 40 anonymous healthy pregnant women (average age of 25.9 ± 5.2 years old), carefully matched for gestational delivery week (37.8 ± 2.6 weeks), who provided consent following uncomplicated pregnancies and underwent caesarean section deliveries. Noteworthy, these placentas were not analyzed for MP they eventually could already contain. Ethical approval for research involving human subjects was obtained from the Ethics Committee (CAAE 58129422.3.0000.5013). Sampling took place at Hospital Universitario Professor Alberto Antunes (HUPAA) of the Federal University of Alagoas (UFAL) between February 2023 and September 2023. Exclusion criteria encompassed gestational diabetes, gestational hypertensive diseases, chorioamnionitis, membrane rupture, and any other maternal medical conditions, as well as known fetal aneuploidy, fetal growth restriction, and macrosomia.

2.2 Term chorionic villi explant culture

The placental explants were obtained following a protocol based on the methods initially developed by Trowell (42), with current modifications implemented and extensively published (43). Cotyledon fragments were thoroughly rinsed in glass-filtered phosphate buffer solution (PBS, 1.6 μ m Whatman GF/A, Sigma-Aldrich, St. Louis, MO, USA) to remove maternal blood and to avoid baseline plastic exposure or any contamination by MP. Only the villous tissue was minced with a number 21 scalpel, generating

approximately 5 mg of tissue per well. These placental explants were cultured in individual glass Petri dishes in DMEM/F12 culture medium (Sigma-Aldrich) with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% gentamicin (Sigma-Aldrich), and incubated at 37°C and 5% CO₂. To prevent contamination from laboratory-derived plastics, all laboratory materials, typically constituted by plastic, were substituted for glass materials. Whenever a plastic substitute could not be employed, this limitation was addressed in the text.

2.3 Polystyrene microplastic exposure to placental explants

Polystyrene microplastics (PS-MPs) of 5 μ m functionalized with carboxylic surface groups were used (Magsphere, Los Angeles, CA, USA). Thus, the structure and composition of polystyrene were corroborated using Raman spectroscopy (XploRA Raman spectrometry; spectral range 300–2200 cm⁻¹, 532 and 785 nm laser diodes, 600 lines per mm grating, Horiba, Japan) and analyzed with KnowItAll software (Wiley Science Solutions, Hoboken, NJ, USA), and the free microplastic libraries SLOPP and SLOPPe (Supplementary Figure 1). No pre- or post-treatments were performed in the PS-MPs. Exposure time and concentration were defined in subsequent experiments, also based on the results reported in literature (44). Bisphenol A (BPA) (Sigma-Aldrich) was employed as positive control for oxidative stress experiments at the concentration of 1 μ M according to the literature (45).

2.4 MTT assay

Placental explants were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-ditetrazolium bromide (MTT) assay, following the manufacturer's protocol (INLAB, SP, Brazil). Explants were treated with PS-MP concentrations of 0.1, 1, 10, and 100 μ g/mL (w/v) for 24 h to establish an experimental non-lethal concentration of PS-MP. Briefly, placental explants were transferred to a 96-well plastic plate, and incubated for 4 h at 37°C with 5 mg/mL of MTT in DMEM/F12 (Sigma-Aldrich). Following PS incubation, the absorbance was measured at 540 nm using a Bio-Rad Multiwell Plate reader (Bio-Rad, Hercules, CA, USA). All experiments were performed in triplicates (n=3).

2.5 LDH release assay in placental explants

PS-MP cytotoxicity was measured using the Pierce LDH Cytotoxicity Assay Kit, according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, placental explants were treated or not with PS-MP for 24, 48 and 72 h for a time-dependent cytotoxicity assessment. Following PS-MP exposure, 50 μ L of conditioned medium from each sample were transferred to a 96-well plastic plate, and 50 μ L of Reaction Mixture were added. After 30 minutes, protected from

light, 50 μ L of Stop Solution were added to each well, and the absorbance was measured at 490 and 680 nm on a Bio-Rad Multiwell Plate reader (Bio-Rad). To calculate the percentage of cytotoxicity, the LDH activity of the spontaneous LDH Release Control (water-treated) was subtracted from the PS-treated sample LDH activity and divided by the total LDH activity [(Maximum LDH Release Control activity) – (Spontaneous LDH Release Control activity)], and multiplied by 100, according to the manufacturer. The experiments were made in triplicates, and the cytotoxicity for each n was calculated (n=3). Noteworthy, we have accompanied the control group dynamics of LDH release through time, to make sure that the placental explants were not naturally dying over time, and up to 72 h of culture they were considered viable (Average O.D. of 1.22 at 24 h, 1.3 at 48 h, and 1.529 at 72 h, while the maximum LDH release positive control had an average O.D. of 2.23 at 24 h, 2.3 at 48 h, and 2.46 at 72 h).

2.6 Oxygen superoxide production

The $O_2^{\bullet-}$ production was evaluated by two methods. The first method focused on the overall production of $O_2^{\bullet-}$ by nitroblue tetrazolium (NBT) (Sigma-Aldrich) (46). After 1 h of incubation, a solution of 1 M KOH and DMSO (v/v) was added, and the placental explants were transferred to a 96-well plastic plate. The absorbance was measured at 630 nm using a spectrophotometer (AJX-6100PC, Micronal). The second method was performed to access only the mitochondrial $O_2^{\bullet-}$ production, through mitoSOX staining (47) at 5 μ M at an excitation wavelength (λ_{ex}) of 510 nm, emission wavelength (λ_{em}) of 580 nm, and a 10 nm slit width for both excitation and emission. (Invitrogen, Waltham, MA, USA), and according to the manufacturer's instructions. The fluorescence was measured with two probes (Invitrogen) and a spectrofluorimeter (Shimadzu RF5301, Shimadzu, Columbia, MD, USA). All experiments were conducted in triplicates (n=3).

2.7 Hydrogen peroxide levels

The production of H_2O_2 was monitored using the fluorescent probe Amplex Red (10 μ M, Thermo Fisher Scientific) (48). This experiment was conducted with peroxidase (1 U/mL) at an excitation wavelength (λ_{ex}) of 563 nm, emission wavelength (λ_{em}) of 587 nm, and a 3 nm slit width for both excitation and emission. The H_2O_2 was expressed in μ mol/mg of protein-1 and rendering to a calibration curve. All experiments were conducted in triplicates (n=8).

2.8 Superoxide dismutase activity

The placental explants homogenate (80 μ g protein) were incubated in 50 mM sodium carbonate buffer ($NaCO_3/NaHCO_3$ at pH = 10.2 with 0.1 mM EDTA) at 37°C. The reaction was initiated by adding 20 μ L epinephrine (150 mM) in acetic acid

(0.05% v/v). The absorbance was measured at 480 nm with a dual-beam scanning spectrophotometer (AJX-6100PC, Micronal) for 5 min. One SOD unit was defined as the amount of protein to inhibit the autoxidation of epinephrine (1 μ M) per minute. The results were expressed as U mg-1 protein (49). All experiments were conducted in triplicates (n=5).

2.9 Catalase activity

To determine the decomposition of H_2O_2 in water and oxygen, 40 μ g equivalent of protein (from the placental explants homogenate) was added to 50 mM phosphate buffer ($KH_2PO_4 + Na_2HPO_4$ with pH = 7.0) at 25°C. The reaction was started by adding 10 mM of H_2O_2 . The analytical signal decrease was monitored at 240 nm with a dual-beam scanning spectrophotometer (AJX-6100PC, Micronal) for 2 min. One unit of CAT was defined as the amount of protein required to convert 1 μ M of H_2O_2 to H_2O and O_2 per minute (50). The results were expressed as K mg-1 protein and all experiments were conducted in triplicates (n=5).

2.10 Glutathione peroxidase activity

The GPx activity was monitored by determining the decrease in nicotinamide adenine dinucleotide phosphate (NADPH) analytical signal at 340 nm using a scanning spectrophotometer (AJX-6100PC, Micronal) for 3 min at 20°C (51). The mixture contained 80 μ g protein (from the placental explants homogenate), 0.05 M Na_2HPO_4 (pH = 7.0), 5 mM EDTA, 84 μ M NADPH, 1.1 mM sodium azide, 1.5 mM of GSH, 0.1 U of GR, and 90 μ M of H_2O_2 . One enzyme unit was defined as the amount required for the oxidation of 1 μ M of NADPH min-1 mg-1 of protein. The results were expressed as U mg-1 protein and all experiments were conducted in triplicates (n=5).

2.11 Quantification of the redox state by reduced glutathione and oxidized glutathione relation

The GSH/GSSG ratio was assessed by measuring the levels of GSH and GSSG (52). The homogenates containing 50 μ g and 100 μ g of protein and o-phthalaldehyde (1 mg mL-1) were utilized. For the GSH assay, 0.1 M NaH_2PO_4 (pH 8.0) and 0.005 M EDTA were used as buffer, whereas 0.1 M NaOH was the buffer used for the GSSG reaction. Fluorescence was monitored using a spectrofluorometer (Flex Station 3, Molecular Devices LLC, San Jose, CA, USA) with an excitation wavelength (λ_{ex}) of 350 nm and an emission wavelength (λ_{em}) of 420 nm. An analytical curve based on known concentrations of GSH and GSSG was used to determine the concentrations of these compounds in the samples. All experiments were conducted in triplicates (n=5).

2.12 Sulfhydryl group content

Total protein quantification of the placental explants was conducted using the Bradford method, and a BSA solution (3 mg mL⁻¹) was used as a standard for total protein determination. The total SH content in the samples was determined based on the reaction with 5,5'-Dithiobis (2 Nitrobenzoic Acid) (DTNB, Sigma-Aldrich). The homogenate (100 µg protein) was incubated in the dark with DTNB (500 µM) for 30 min. Afterwards, the final volume was adjusted with extraction buffer (50 mM Tris-HCl with 1.0 mM EDTA, pH 7.4), and the absorbance was measured at 412 nm with a dual-beam scanning spectrophotometer (AJX-6100PC, Micronal, Sao Paulo, Brazil) (53). All experiments were conducted in triplicates (n=5).

2.13 Malondialdehyde content

A total of 450 µg of tissue homogenate protein was combined with 200 µL of 30% (v/v) trichloroacetic acid (TCA). Subsequently, Tris-HCl (10 mM) was added and centrifuged at 1180 g for 10 min at 4°C. Then, the collected supernatant was mixed with 0.73% (m/v) thiobarbituric acid, which reacted with lipid peroxidation products to form a pink-colored compound. Following this, the mixture was incubated for 15 min at 100°C and cooled down. The absorbance was measured using a spectrophotometer (AJX-6100PC, Micronal) at 535 nm. The results were expressed in mmol mg⁻¹ of protein (54). All experiments were conducted in triplicates (n=5).

2.14 Protein carbonylation

The quantification of carbonyl group formation in 100 µL of tissue homogenate was conducted using the reaction with 2,4-dinitrophenylhydrazine (DNPH) (55). The reaction control was treated with 2.5 M HCl, whereas the samples groups were treated with 10 mM DNPH (diluted in 2.5 M HCl). Subsequent to room temperature incubation, 2 M trichloroacetic acid (TCA) was added, followed by centrifugation at 10,000 g for 10 min at 4°C. After sequential steps of centrifugation and washing, an ethanol and ethyl acetate mixture (1:1 v/v) was added. The groups were finally suspended in 6 M guanidine and centrifuged again. The collected supernatant was used for absorbance measurement at 370 nm using a plate reader, normalized to the protein content (Tecan Infinite 200 pro, Männedorf, Switzerland). The results were expressed in pmol mg⁻¹ protein. All experiments were conducted in triplicates (n=5).

2.15 Proton nuclear magnetic resonance-based metabolomics

The supernatants of 72 h explant cultures were prepared by adding 350 µL of 0.1 M phosphate buffer containing 3-[Trimethylsilyl] propionic-2,2,3,3-d₄ acid sodium salt at 0.1 mM in D₂O. The prepared samples were then transferred to 5 mm NMR tubes. All ¹H NMR spectra were acquired on a Bruker 600 MHz

spectrometer Avance III (Bruker BioSpin, Billerica, MA, USA) equipped with a PA BBO 600 S3 BBF-H-D-05 Z SP probe. Before the experiments, the NMR spectrometer was calibrated daily following strict standard operating procedures to ensure the highest spectral quality and reproducibility. A sealed tube containing a 99.8% MeOD standard sample was used to ensure that supernatants were run at 298 K. The 'edte' command controlled the temperature's stability over time. The second calibration with sucrose aimed to optimize water suppression, and spectrometer sensitivity. An automatic tune and matching procedure, deuterium signal lock, and automatic shimming routine were performed before each experiment. The NMR spectra were recorded using the 1D NOESY pulse sequence with water suppression (pulse program: noesygp1d). For all experiments, 128 scans were recorded with 32 k data points, a spectral width of 20.029 ppm, and a relaxation delay of 4 s. The spectral processing was realized automatically using TopSpin 3.5 (Bruker BioSpin, Billerica, MS, United States), performing phase and baseline correction, and calibrating the TSP reference signal at δ0.00 ppm. The peaks were identified using the Human Metabolome Database (HMDB) (The Metabolomics Innovation Centre, Canada) and the ChenomX NMR Suite (Chenomx Inc., Canada). Criteria such as chemical shifts and multiplicity were employed for peak identification. The dataset underwent further processing using the R statistical software, version 4.1, along with the R package PepsNMR (R Core Team, Vienna, Austria). The spectral region spanning from 4.7 to 5.1 ppm, which includes the residual water signal, was selectively suppressed. Biomarker discovery and classification were performed using the online platform MetaboAnalyst version 6 (www.metaboanalyst.ca).

The supervised model Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was executed to evaluate the capacity of metabolomics data in distinguishing PS-MP exposed samples from control samples, considering the complete set of identified metabolites. The defined confidence region was set to 95%, and one hundred permutations were employed for model evaluation. Significant metabolites (biomarkers) were selected by analyzing loadings and variable importance in the projection (VIP) plot from the OPLS-DA model, indicating the variables crucial for group discrimination. Additionally, a heatmap analysis was applied to reveal metabolic patterns between the groups and a t-test with false discovery rate (FDR)-corrected p values was employed to assess the significance of metabolites. The metabolomics biomarkers data were correlated with oxidative stress parameters. Finally, to estimate the association between the two datasets, it was applied the Pearson correlation coefficient and a multiblock Partial Least Squares Discriminant Analysis (PLS-DA) model, also known as Data Integration Analysis for Biomarker Discovery using Latent Components (DIABLO) (56). All experiments were conducted in triplicates (n=16).

2.16 Zeta potential

The 100 µg/mL PS-MP in DMEM/F12 culture media had their zeta potential measured by a nano/zeta sizer ZS90 (Malvern Instruments Ltd) with 250 scans made in five different samples.

2.17 Statistical analysis

With the exception of metabolomics already described, the statistical analyses was performed using the one-way ANOVA followed by Bonferroni multiple comparisons. All the statistical analyses were performed using GraphPad (version 7.0; GraphPadSoftware, Inc.), with $p < 0.05$ considered statistically significant.

3 Results

3.1 Polystyrene exposition is cytotoxic for placental explants

Exposure to different concentrations of PS-MP for 24 h did not affect explant viability as determined by the screening MTT test (Figure 1A). Based on these results of viability, the concentration of PS-MP to be tested in all further experiments were 100 $\mu\text{g/mL}$, as it was not only inducing viability reduction, but the increased tendency observed caught our attention.

To assess if such concentration would present time-dependent cytotoxicity, the LDH release assay was performed after exposure of the placental explants for 24, 48 and 72 h. The cytotoxicity after 24 h was $10.37 \pm 3.79\%$, after 48 h was $23.19 \pm 3.09\%$, and after 72 h was $40.32 \pm 7.02\%$. The 30% increase in cytotoxicity from 24 to 72 h was statistically significant ($p < 0.01$) (Figure 1B).

3.2 Polystyrene exposition leads to oxidative stress on placental explants

As MP-PS exposure showed time-dependent cytotoxicity to placental explants, we evaluated if they would trigger an oxidative stress response. We assessed the production of mitochondrial $\text{O}_2^{\bullet-}$ and overall $\text{O}_2^{\bullet-}$ levels in placental explants using MitoSOX staining and the NBT assay, respectively. Both methods

demonstrated a significant increase in $\text{O}_2^{\bullet-}$ levels, with mitochondrial $\text{O}_2^{\bullet-}$ increasing by 3.5-fold and total $\text{O}_2^{\bullet-}$ showing a similar elevation upon exposure to PS-MP ($p < 0.01$ and $p < 0.05$, respectively) (Figures 2A, B). The similar pattern was observed in the analysis of H_2O_2 generation, which greatly increased from 6.29 ± 0.43 A.U. to 30.10 ± 0.43 A.U. ($p < 0.001$) (Figure 2C). The activity of antioxidant enzymes was also assessed. SOD catalyzes $\text{O}_2^{\bullet-}$ in O_2 and H_2O_2 , helping to regulate mitochondrial function as it scavenges $\text{O}_2^{\bullet-}$, produces H_2O_2 and inhibits ONOO- production by protecting NO, while CAT and GPx are responsible of breaking H_2O_2 in O_2 and H_2O . We observed a great reduction of SOD and CAT activity when explants were exposed to PS, as CAT was reduced from 0.056 ± 0.007 K mg⁻¹ protein in the control group to 0.014 ± 0.008 K mg⁻¹ protein in the PS group ($p < 0.05$) (Figure 2D). Similarly, SOD was reduced from 428.5 ± 34.58 U mg⁻¹ protein in the control group to 150.4 ± 49.84 U mg⁻¹ protein in the PS group ($p < 0.01$) (Figure 2E). In contrast, GPx activity was increased from 0.036 ± 0.002 U mg⁻¹ protein in the control group to 0.061 ± 0.008 U mg⁻¹ protein ($p < 0.05$) (Figure 2F).

Since GPx activity was increased, we evaluated GSH and GSSG levels. While GSH had a tendency of reduction in the PS group (Figure 2G), and GSSG was unchanged (Figure 2H), their ratio was reduced in the PS group, from 3.84 ± 0.43 to 2.91 ± 0.38 ($p < 0.05$) in the control group (Figure 2I). Altogether, these results indicate the presence of oxidative stress due to reduced SOD and CAT capabilities, and a reduced GSH/GSSG ratio coupled with increased GPx activity indicates this antioxidant defense system is actively engaged in counteracting the oxidative stress.

Another well-known marker of oxidative stress is the total SH content, which prevent uncontrolled oxidation of proteins by Cystein aminoacids. We observed a clear reduction of the total SH content, from 93.93 ± 10.84 mmol mg⁻¹ protein in the control group to 56.05 ± 2.96 mmol mg⁻¹ protein in the PS exposed group ($p < 0.05$) (Figure 2J).

Due to the observation that exposure to PS-MP results in an increase in all reactive oxygen species (ROS), it is crucial to

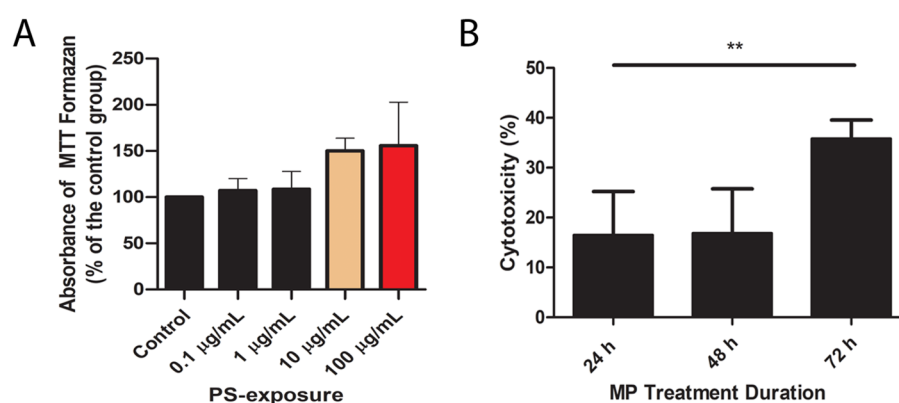


FIGURE 1

Polystyrene exposure induces cytotoxicity in placental explants, as measured by metabolic activity and membrane integrity. (A) MTT assay results show the effects of increasing PS concentrations (0.1, 1, 10, and 100 $\mu\text{g/mL}$) over 24 h, indicating reduced metabolic activity. (B) LDH release assay evaluates membrane damage following exposure to 100 $\mu\text{g/mL}$ PS over 24, 48, and 72 (h) Experiments were conducted in triplicate ($n=3$) with results analyzed via one-way ANOVA and Bonferroni correction. ** $p < 0.01$ compared to control.

comprehend the extensive oxidative stress experienced by placental explants. Therefore, we conducted further analyses on ROS production indicators, including measurements of malondialdehyde (MDA) and carbonylated protein levels. MDA is one of the final products of polyunsaturated fatty acids peroxidation, while carbonylation of proteins is defined as the covalent, nonreversible modification of the side chains of cysteine, histidine and lysine residues by lipid peroxidation end products, resulting in their loss of function. Our results showed that MDA level substantially increased, from $0.66 \pm 0.52 \mu\text{M mg}^{-1}$ protein in the control group to $4.26 \pm 0.60 \mu\text{M mg}^{-1}$ protein in the PS group ($p < 0.001$) (Figure 2K). This elevation was concomitant with a rise in protein carbonylation, escalating from $9.18 \pm 1.20 \text{ nmol mg}^{-1}$ protein in the control group to $29.16 \pm 4.06 \text{ nmol mg}^{-1}$ protein in the PS group ($p < 0.01$) (Figure 2L).

3.3 Placental chorionic villi metabolism is disrupted by PS-MP exposure

All supernatant samples underwent analysis using ^1H NMR. Figures 3A, B depict the metabolite identification and overlapped spectra of PS-exposed and control groups. We employed orthogonal projections to latent structures discriminant analysis (OPLS-DA) for its ability to distinguish predictive from non-predictive (orthogonal) variations. As such, OPLS-DA revealed substantial metabolite differences, separating both clusters (Figure 3C). The model exhibited a high degree of separation between groups and good predictive performance through cross-validation (Q^2 0.747, R^2Y 0.855, and $p < 0.01$) (Figure 3D). The variable importance in the projection (VIP) score highlighted multiple significant alterations, with formate, valine, tyrosine,

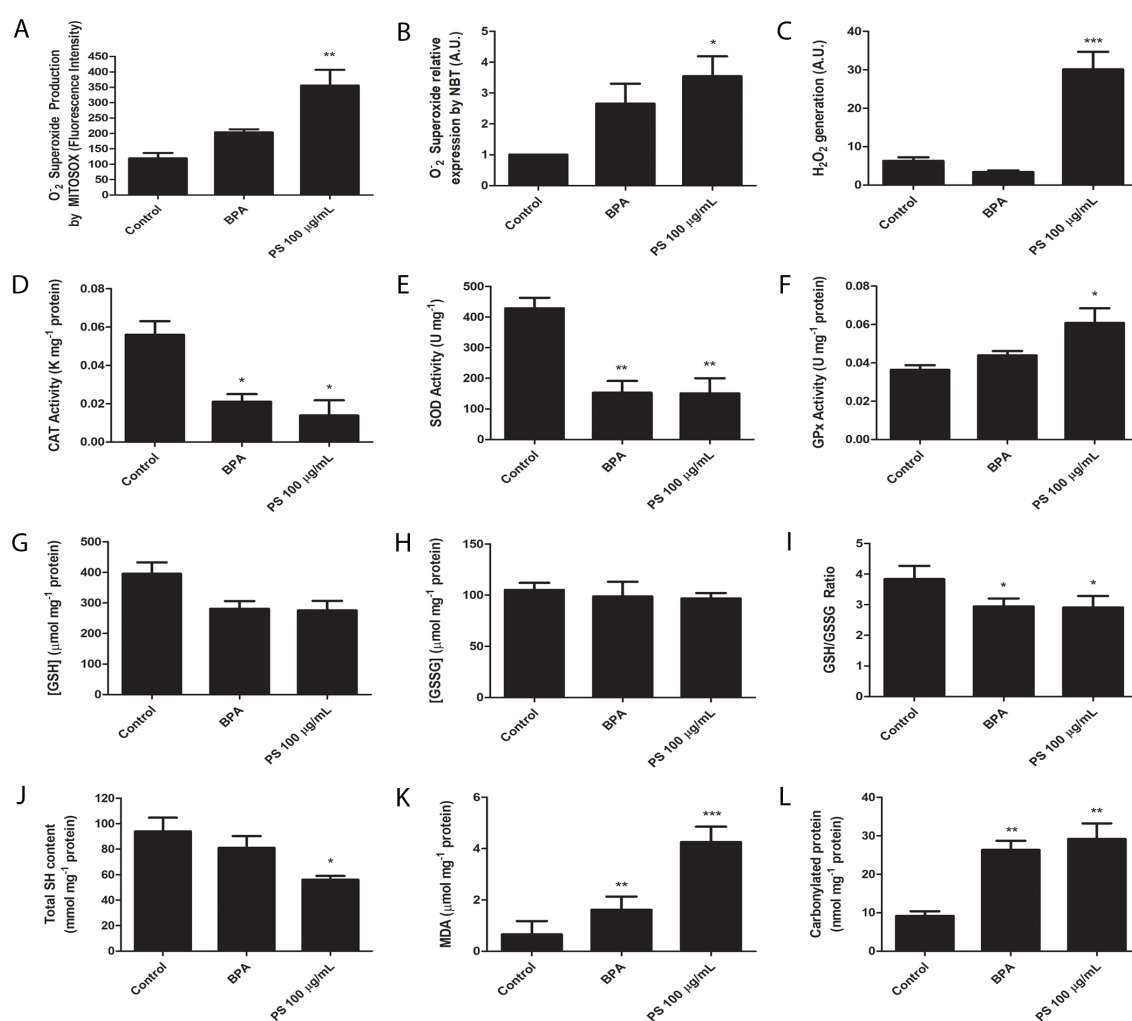


FIGURE 2

Placental explants exposed to 100 $\mu\text{g/mL}$ PS microplastics or 1 μM bisphenol A (BPA, positive control) exhibit changes in oxidative stress markers. (A) Oxygen superoxide ($\text{O}_2^{\bullet-}$) production by mitochondria, (B) $\text{O}_2^{\bullet-}$ total amount, (C) hydrogen peroxide (H_2O_2) production, (D) catalase (CAT) activity, (E) superoxide dismutase (SOD) activity, (F) glutathione peroxidase (GPx) activity, (G) reduced glutathione ([GSH]) levels, (H) oxidized glutathione ([GSSG]) levels, (I) GSH/GSSG ratio, (J) total sulfhydryl (SH) content, (K) malondialdehyde (MDA) levels, and (L) carbonylated protein levels. All experiments were conducted in triplicates, and $n=3$ for MitoSOX analysis, $n=8$ for H_2O_2 analysis, and $n=5$ for the other experiments. One-way ANOVA followed by Bonferroni multiple comparisons was used for all experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control.

isoleucine, isobutyrate, hypoxanthine, and alanine being the most important variables (Figure 3E). Furthermore, the heatmap visualization of both groups illustrated the similarities among samples within the control or PS groups and the main differences between the analyzed groups (Figure 3F), corroborating findings from the correlation plots and OPLS-DA.

The relative concentrations, calculated by normalizing the molar concentration of each metabolite to the total molar concentration of all metabolites for each sample in the two groups, were compared using box-and-whisker plots. The data obtained demonstrated that the PS-MP exposed group exhibited lower levels of alanine ($p = 0.03$, Figure 4), formate ($p < 0.001$, Figure 4), glutaric acid ($p = 0.02$, Figure 4), and maltotriose ($p = 0.03$, Figure 4), whereas showing increase in valine ($p = 0.03$, Figure 4) and tyrosine ($p < 0.001$, Figure 4), confirming the results of previous analyses. These findings suggest potential

impairments in folate, and amino acid metabolisms, and alterations in the TCA cycle (Figure 5).

Additionally, the observed metabolic changes correlated with oxidative stress alterations found in placental explants (Supplementary Figure 2). The heatmaps highlight the Pearson correlation among metabolites and between metabolites and oxidative stress (Supplementary Figures 2A, B). Pairwise variable associations were also extracted from a similarity matrix derived from the DIABLO model (Supplementary Figures 2C, D). This measure, analogous to a correlation coefficient, estimates the variables' association relevant to group discrimination. Consequently, the reduced antioxidant system directly correlated with reduced glucose, maltotriose, carnitine, and glutaric acid metabolites, while CAT-reduced levels correlated negatively with lactate levels. The increased GPx, negatively correlated with almost all metabolites (isoleucine, isobutyrate, tyrosine, phenylalanine,

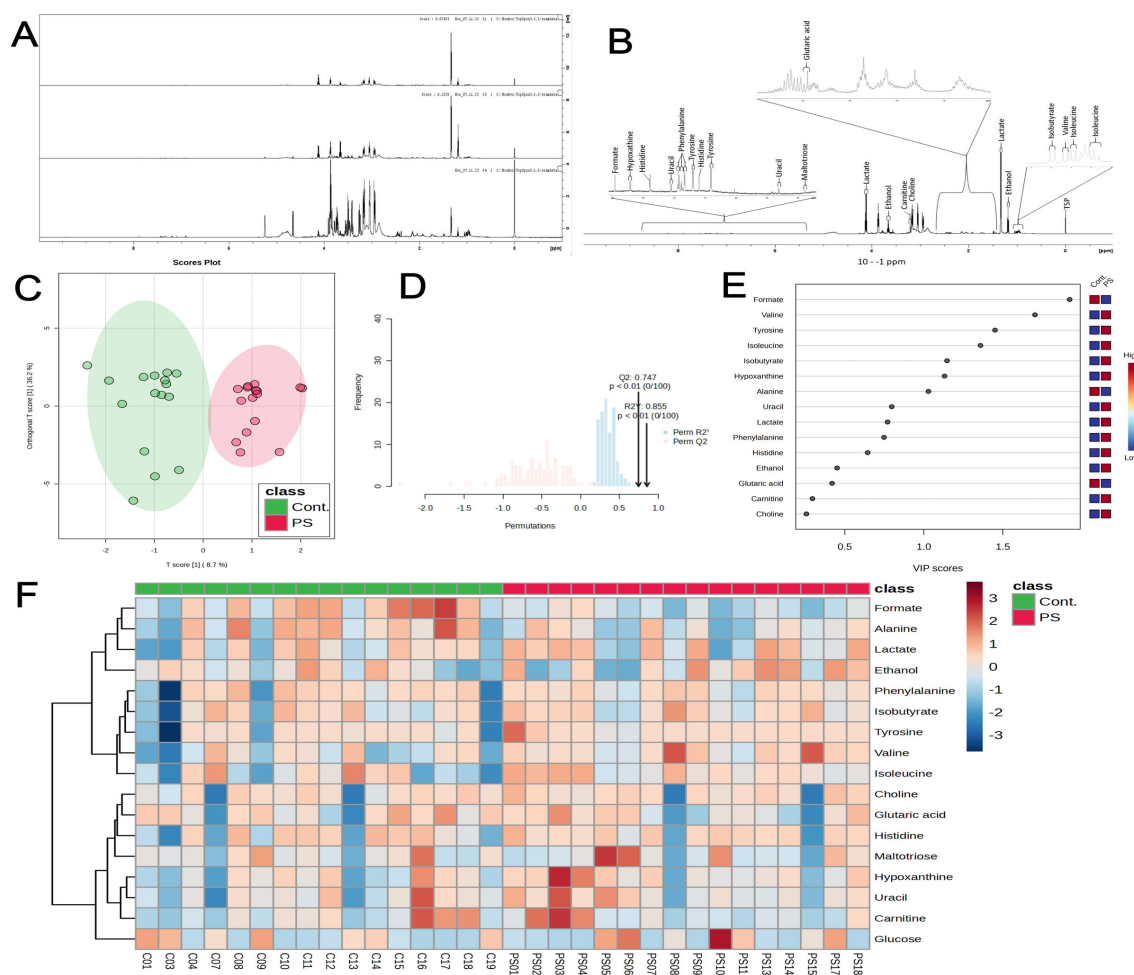


FIGURE 3

¹H NMR spectroscopy reveals metabolic alterations in placental chorionic villi explants following 72 h of PS exposure (100 μ g/mL). (A) Representative ¹H NMR spectra of control, PS-exposed samples, and pure culture medium. (B) List of most prominent metabolites identified in the samples. (C) Score plot of orthogonal projections to latent structures discriminant analysis (OPLS-DA), showing the control group (red) and PS-exposed group (green) ($Q^2 = 0.747$, $R^2Y = 0.855$). (D) Cross-validation and permutation tests (100 permutations) confirm statistical significance ($p < 0.01$). (E) Variable Importance in Projection (VIP) score. Variables with $VIP > 1$ are essential for group discrimination, and higher VIP scores indicate more relevant variables in a classification context. The red-blue tiles on the right side of the VIP plot depict the mean expression of each metabolite for the indicated group. (F) Heatmap illustrates changes in metabolite concentrations between control (green) and PS-exposed (red) groups. Reddish and blueish tiles indicate increased and reduced concentrations, respectively. All metabolomics supernatant experiments were conducted in triplicates pooled together ($n=16$).

lactate, histidine, alanine, carnitine, and formate). Additionally, the reduced GSH/GSSG ratio positively correlated with valine, ethanol, isoleucine, isobutyrate, tyrosine, phenylalanine, lactate, histidine, and alanine reduction. Also noteworthy is the negative correlation between the increase in carbonylated proteins with glutaric acid and hypoxanthine (Supplementary Figure 2).

4 Discussion

Microplastics have been detected in over 1,300 species, ranging from farm animals and aquatic organisms to birds and insects (57). Evidence of MPs ingestion has been observed in numerous ecosystems, and this trend spans various trophic levels (58). The absorption by the body and tissues translocation can lead to toxicity, physical damage, and chemical harm, including the leaching of toxic additives like endocrine disruptors (59, 60). Moreover, understanding the environmental impacts of MPs is a urgent concern, with growing need to quantify effects within risk assessments (61). Moreover, assessing the environmental impacts of MPs remains challenging due to their variability in polymer size,

form, and types, chemical additives, colorants and plasticizers, altogether with different levels of degradation by weathering (57). As such, it is important to highlight the need for detailed MP characterization, relevant controls, and weathering for a correct MP characterization (60). Despite all advancements, several key knowledge gaps remain, with challenges in data comparability, nanoplastics measurements and quantification, and understanding of the mechanisms behind health MP effects (57).

To try to understand MP effects in the human placenta, this study represents a pioneering effort to utilize freshly collected human term placental explants for assessing the impact of MPs on human health, particularly during pregnancy. Our findings reveal that PS-MPs exhibit toxicity that disrupts cellular functions, particularly through oxidative stress. This is consistent with results from other *in vitro* studies involving human cells (38–40), confirming the potential hazards to both maternal and fetal health.

To the best of our knowledge, placental villi explants have not been utilized to investigate the uptake or toxicity of MPs, a fact supported by a review of human *in vitro* and *ex vivo* placental models (41). Despite the debate over the value of term placental

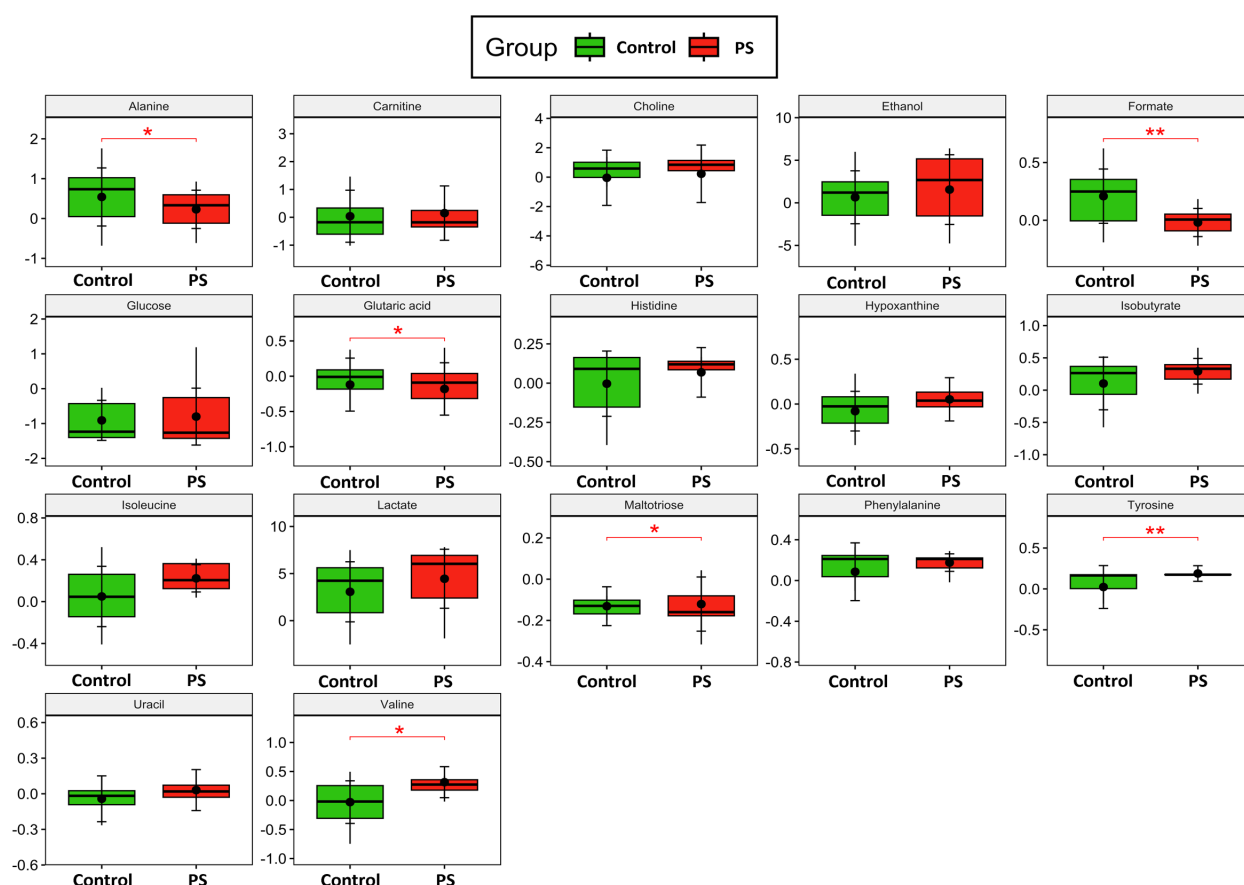


FIGURE 4

Metabolite Analysis. Box-and-whisker plots depict the relative concentrations of critical variables contributing to the separation of the control (green) and polystyrene (PS) microplastics-exposed (red) groups. Y-axes are represented in relative units, and medians are indicated by horizontal lines within each box, the horizontal brackets and asterisks denote the level of statistical significance for each metabolite. The black dots within each boxplot represents the mean value for each group, while the vertical brackets indicate the standard deviation. All metabolomics supernatant experiments were conducted in triplicates and pooled together (n=16). p values in the respective graphs indicate univariate t-test analysis with FDR correction.

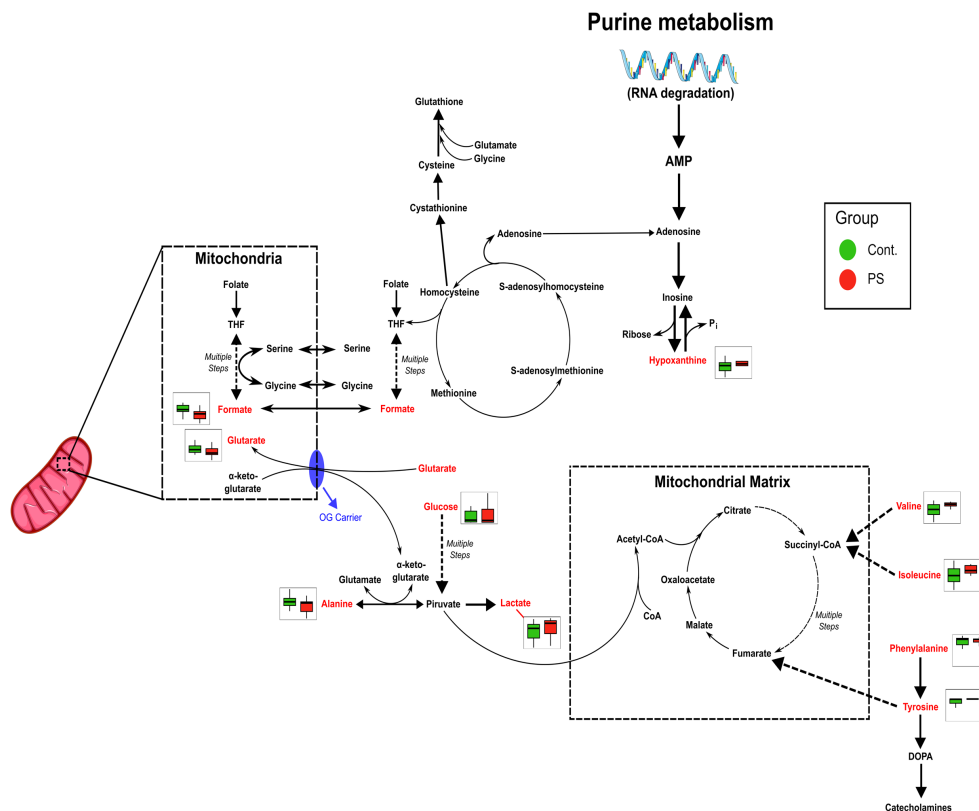


FIGURE 5

Metabolic pathways affected by PS exposure are inferred from heatmap data, showing alterations in folate metabolism, energy production (TCA cycle) and amino acid metabolism. Pathway impairments suggest potential disruptions in mitochondrial function and energy homeostasis.

explants due to high rates of basal cell death reported (35), several manuscripts state otherwise (43, 62, 63), aligning to our results. It is important to highlight that LDH levels need to be monitored closely because term placental villi explants do indeed lose viability fast *in vitro*. As such, we carefully observed the LDH release in our control groups, finding low variations up to 72 h of culture, which worked in our case, but it is a limitation for studies that need a longer analysis. Despite the limitations, placental explants offer an ethically accepted model with greater complexity than cell line cultures, providing human specificity, a complex mixture of cells and extracellular matrix molecules, and still remain one of the closest experimental models to analyze the functional unit of the placenta within all its complexity.

As such, the explants were exposed to the PS-MPs at various concentrations (0.1–100 µg/mL), since PS is one of the most common polymers found in human placentas (18), and it is one of the most common MP polymers used on experimental studies (38, 64). One limitation in MPs experimental studies is the lack of diversity on commercial polymer types, being the most commonly found and used, PS, PE, and PP. All these polymers are commonly listed among the top five environmental contaminants and are known to bioaccumulate in living organisms. A plethora of research has been made assessing differential toxicity of these polymers, and mostly data depict that they are toxic in different experimental models, but no polymer-specific toxicity has been described amid them (65–67). Nonetheless, in mice instilled for

these polymers, only PS was able to induce pulmonary inflammation (68), and in comparison to other polymers such as PVC and PET, PS seems to be less cytotoxic in HepG2 cells (69).

Herein we employed 5 µm commercially purchased PS-MP, and the size was intentionally selected. Our previous study found that term placentas collected in 2021 in Hawai'i, USA, contained MPs with an average size of 5.14 ± 0.75 µm (19), which is very similar to the commercial MP employed in this study. Besides the size, surface charge and concentration can play a decisive role in the uptake mechanisms and toxicity profile of particles (70). The MP-PS particles used in our study are functionalized with carboxyl groups (-COOH), and they are negatively charged, with a zeta potential of -1.67 mV (Supplementary Figure 3). It is known that -COOH groups acquire negative charges in aqueous environments due to weathering, thus influencing the behavior and interaction with other pollutants in the environment (71, 72), which was also one feature strategically chosen to better mimic the chemical surface type of a PS-MP in the environment, slightly reducing the gap between pristine and weathered MPs. Nonetheless, it is important to highlight that the MP used was pristine, completely round, and assumed to not have chemical additives, which are characteristics not similar to weathered MPs.

Regarding the chosen concentration, it is impossible to know for sure which concentration of MP would be the most similar to concentrations found in pregnant women. No large epidemiological studies exist to determine the MPs average concentration in the

blood, even less studies on pregnant women. The closest we have is one study that estimated the total amount of MPs in the blood as 1.6 $\mu\text{g/mL}$, from 22 Dutch volunteers (29), but we still need robust epidemiological studies to access the real concentration of MPs in the worldwide population, and using more than one technique to characterize these MPs. Moreover, we currently lack knowledge regarding the precise daily absorption of microplastics, its bioaccumulation rate in different organs and their resistance to degradation and elimination from the body (40). The concentration of MPs in human placentas is also debatable, with a recent study showing up to $126.8 \pm 147.5 \mu\text{g/g}$ of MPs in 10 placentas from the USA (23), which would even align with the high *in vitro* concentration used herein. Nevertheless, we could have chosen 1 or 10 $\mu\text{g/mL}$ concentrations to this study, but we were initially intrigued by that tendency increase in the 24 h MTT assay, followed by the time-dependent cytotoxicity observed in the LDH release assay after 72 h of exposure. Therefore, we decided to investigate the mechanisms involved using 100 $\mu\text{g/mL}$ of PS-MP. The chosen concentration was also consistent with those reported in previous studies investigating transplacental transport, which used similar concentrations, and even exceeded, using up to 1,000 $\mu\text{g/mL}$ of PS-MP in cell lines (41).

The observed increase in cytotoxicity confirms the findings from several studies demonstrating PS-MNP cytotoxicity in pregnant mice and their offspring (73, 74), placental cell lines (38, 39), and embryo stem cells (44). This result was different from two previous studies that reported that only positively charged PS-MNPs were able to induce cytotoxicity, whereas negatively charged ones not (75, 76). It is worth noting that alterations in the steric shielding of the charge on a surface moiety, resulting from subtle distinctions in the chemical structure of the charge-bearing moiety, might affect the cellular uptake and toxicity of the MNPs (77).

In addition to increased cytotoxicity, PS-MP exposure induced humungous levels of reactive oxygen species (ROS), such as $\text{O}_2^{\cdot-}$ and H_2O_2 . Antioxidant enzyme levels were mostly reduced, with lower SH content, diminished CAT and SOD activity, and a decreased GSH/GSSG ratio. The evident oxidative stress resulted in elevated levels of MDA and carbonyl proteins, suggesting substantial damage to lipids and proteins due to the heightened levels of ROS. Our findings regarding cytotoxicity align with previous reports in the literature, which extensively document the induction of oxidative stress following exposure to PS-MPs (78–80). Additionally, increased production of ROS, oxidative stress, and mitochondrial dysfunction have been consistently reported in various human cell types (81–84). In this context, the results of this study support the idea that the long-term exposure of plastic particles produces higher levels of ROS, which could improve toxicity and cause other adverse pregnancy outcomes (40).

Not only did PS-MP increased cytotoxicity, oxidative stress, protein carbonylation, and lipid peroxidation, but they affected the entire placental metabolism. The disrupted amino acid levels may indicate disturbances in protein metabolism, while the altered levels of hypoxanthine and formate correlate directly with deficiencies in folate and purinergic metabolism. Collectively, these changes are associated with oxidative stress responses and mitochondrial

dysfunction, which contribute to metabolic imbalances. Oxidative stress can impair the enzymes involved in gluconeogenesis, TCA cycle, amino acid catabolism, and energetic metabolism (85), which are known to change the metabolites previously described. Such changes are also present in important gestational diseases, such as preeclampsia and gestational diabetes mellitus (86). The oxidative stress and the metabolic changes can limit the supply of essential nutrients to the fetus, leading to intrauterine growth restriction, stillbirth, preterm birth, neurodevelopmental disorders, and postnatal health issues (87, 88). Similar results in PS-MNP exposure of intestinal cell lines, with oxidative stress, increased glycolysis, disrupted energy, and glutamine metabolism changes have been described (89). Perturbations in biotin metabolism, lysine degradation, and glycolysis/gluconeogenesis pathways in pregnant mice exposed to PS-MNP have also been described (90). In other studies, the researchers found changes in the lipids metabolism in different cell lines (91, 92), in addition to studies that demonstrated disruptions of glucose, energy, and lipid metabolisms in zebrafish and eels (93, 94). Additionally, Li and collaborators (95), reported disruptions of redox homeostasis, carbohydrate, and energy metabolism in barley, indicating solid and preserved alterations of this pathways across different types of experimental models. Therefore, it is essential to highlight that our results indeed demonstrate that PS-MPs are harmful to human placentas, altering key biochemical mechanisms that can reverberate to other molecular effects and pathways, which might be detrimental to fetal development, in complete consonance to what has been observed in wild and experimental animals.

Mechanistically, the oxidative stress and the metabolic changes observed herein seem to be happening mostly due to a mitochondrial dysfunction, although we need further research to prove this hypothesis in our model. The mitochondria, as a central hub of cellular energy metabolism and redox balance, are especially susceptible to environmental stressors. The MPs cause NADPH oxidase 4 (NOX4)-mediated mitochondrial dysfunction, as demonstrated by membrane potential changes, impaired cellular energy metabolism, and repression of mitochondrial respiration in different cell models (96, 97). A review compiled all the mitochondrial alterations caused by MPs exposition: disruption of mitochondrial membrane potential, induction of mitochondrial depolarization, mitochondrial damage, mtDNA damage, and mitochondrial oxidative stress, leading to the conclusion that MPs can indeed be involved in mitochondrial dysfunction (98). Possibly caused by this mitochondrial dysfunction, the remarkable oxidative stress we found is leading to oxidative damage to cellular components, including lipid peroxidation and protein carbonylation. Other interesting evidences of mitochondrial dysfunction are the altered TCA cycle and amino acid metabolism. Studies have shown that MP exposure disrupts these metabolic pathways, leading to a shift toward glycolysis as an alternative energy source, while disruptions in amino acid metabolism, indicate alterations in protein synthesis and degradation (99, 100).

In summary, this study indicates that PS-MP exposure induces time-dependent cytotoxicity, oxidative stress, and metabolic alterations in placental explants, highlighting potential risks for

maternal and fetal health. The results highlight the immediate risks associated with MPs exposure, highlighting an urgent need for targeted resources and strategic initiatives to address plastic pollution at the environmental level, since it is where humans are being exposed. This includes the exploration of new degradation strategies for the effective removal of MP, such as biodegradation, advanced oxidation degradation, and photocatalytic degradation (40). Given the substantial implications of these findings, it is paramount to expand our research efforts, elucidating the ramifications of varied MP exposures on both pregnancy and fetal development, as well as the comprehensive effects of MP contamination on subsequent generations. Particularly, which other effects PS-MP and other polymers can cause in the placenta, and how these changes would affect fetal development, including epigenetic alterations. By demonstrating the impact of PS-MP on placental tissues, this study contributes to the growing body of evidence that underscores the need for comprehensive public health strategies and policies to mitigate the effects of plastic pollution. Moving forward, future research should concentrate on the comparative toxicity of PS-MPs with other prevalent polymers, and chemical mixtures, to better understand the relative risks to human pregnancy.

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 humans were approved by CAAE 58129422.3.0000.5013. Carlos Arthur Cardoso Almeida, Federal University of Alagoas, Brazil - carlos.almeida@icf.ufal.br - Coordinator of the Ethics Committee. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

AdS: Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. KP: Writing – review & editing, Methodology, Investigation. IC: Writing – review & editing, Methodology. IC: Writing – review & editing, Methodology, Investigation. JS: Writing – review & editing, Methodology. MQ: Writing – review & editing, Methodology, Investigation, Formal analysis. AL: Writing – review & editing, Validation, Supervision, Data curation. AC: Writing – review & editing, Software, Methodology, Investigation. Ed: Writing – review & editing, Software, Formal analysis. TA: Writing – review & editing, Validation, Supervision, Data curation. RW: Writing – review & editing, Writing – original draft. JU: Writing – review & editing, Resources, Funding acquisition. SO: Writing – review & editing,

Writing – original draft, Project administration, Methodology, Data curation. AB: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Funding acquisition, Data curation, Conceptualization.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fendo.2024.1481014/full#supplementary-material>

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Trained innate immunity as a potential link between preeclampsia and future cardiovascular disease

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Preeclampsia (PE) is a complex pregnancy syndrome characterized by hypertension with or without proteinuria, affecting 2–6% of pregnancies globally. PE is characterized by excessive release of damage-associated molecular patterns (DAMPs) into the maternal circulation. This DAMP-rich milieu acts on innate immune cells, inducing a proinflammatory state characterized by elevated cytokines such as IL-1 β and IL-18. This proinflammatory state in the mother and placenta results in the endothelial dysfunction strongly associated with cardiovascular disorders. While the immediate maternal and fetal risks of PE are well-documented, accumulating evidence indicates that PE also confers long-term cardiovascular risks to the mother, including hypertension, coronary heart disease, stroke, and heart failure. The underlying mechanisms connecting PE to these chronic cardiovascular conditions remain unclear. This article explores the potential role of trained innate immunity (TRIM) as a mechanistic link between PE and increased long-term cardiovascular risk. We propose that the persistent exposure to DAMPs during PE may epigenetically reprogram maternal innate immune cells and their progenitors, leading to TRIM. This reprogramming enhances the inflammatory response to subsequent stimuli, potentially contributing to endothelial dysfunction and chronic inflammation that predispose women to cardiovascular diseases later in life. Understanding the role of TRIM in PE could provide novel insights into the pathophysiology of PE-related cardiovascular complications and identify potential targets for therapeutic intervention. Further research is warranted to investigate the epigenetic and metabolic alterations in innate immune cells induced by PE and to determine how these changes may influence long-term maternal cardiovascular health.

KEYWORDS

preeclampsia, maternal cardiovascular health, long-lasting effects, trained immunity, DAMPs (damage-associated molecular pattern molecules)

Preeclampsia and maternal long-lasting effects

Preeclampsia (PE) is a serious syndrome of pregnancy, characterized by hypertension with or without proteinuria, which can lead to the severe condition of eclampsia (1). In patients without signs of proteinuria, the diagnosis of the syndrome considers the presence of thrombocytopenia or elevated liver transaminase levels (2). It is estimated that in general population the prevalence of preeclampsia is 2–6% (3). The syndrome is subclassified into either early-onset PE (EOPE) or late-onset PE (LOPE) (1), with the onset of pathological signs falling into <34th and >34th week of gestation, respectively, being EOPE the syndrome which presents the most severe additional symptoms and signs, such as proteinuria, hepatic damage or pulmonary edema, among others (1).

Increasing evidence shows that the syndrome has a long-lasting deleterious effect on their cardiovascular health. Thus, four meta-analysis made on 22 (4), 43 (5), 15 (6) and 21 studies (7) showed that in short-, medium- and long-term (*i.e.* follow-up from 1 month to 34 years (4–7)), women who had preeclampsia have increased risk of I) heart failure (adjusted risk ratio [aRR], 4.19; 95% confidence interval [CI], 2.09–8.38) (4); II) coronary heart disease (aRR, 2.50; 95% CI, 1.43–4.37) (4); III) coronary heart death (aRR, 2.10; 95% CI, 1.25–3.51) (4); IV) CV disease (aRR, 1.85; 95% CI, 0.80–4.29) (4) and odds ratio (OR), 2.28; 95% CI 1.87–2.77) (5); V) CV disease death [aRR, 2.21; 95% CI, 1.83–2.66 (4), OR, 2.89, 95% CI 1.71–4.89 (5) and RR, 2.29; 95% CI, 1.73–3.04) (6)]; VI) stroke (aRR, 1.81; 95% CI, 1.29–2.55) (4); VII) stroke death (aRR, 1.97; 95% CI, 0.80–4.88) (4); VIII) cerebrovascular disease (RR, 2.03; 95% CI, 1.54–2.67) (6); IX) peripheral arterial disease (RR, 1.87; 95% CI, 0.94–3.73) (6); and, X) hypertension [RR, 3.13, 95% CI 2.51–3.89) (5) and OR, 3.19, 95% CI, 1.52–6.70 (7)]. Thus, the effects of preeclampsia not only impact maternal health during the pregnancy but also induce subclinical alteration which can remain silent for years, increasing her cardiovascular risk. In this regard, increased microalbuminuria, was found in association with high hypertension risk in mothers who had PE 7 (8) and 10 (9) years before. Although, the pathophysiological mechanism of the findings is unknown, this slight alteration of the glomerular filtration is independently associated with high stroke (10) and coronary heart disease (11) risks. Nowadays, there is no knowledge about the pathophysiological mechanisms underlying the cardiovascular risk in mothers who had PE.

It has been agreed that the most likely etiology of PE is a poor remodeling of the spiral arteries and veins during early placentation. Meanwhile, LOPE appears to be linked to maternal factors, such as the inability of the cardiovascular system to meet the increasing metabolic needs of the fetoplacental unit, rather than issues with the placentation process (12). The PE condition maintains a pernicious low blood flow in a condition of high pressure (Jet-type), generating vascular mechanical stress, hypoxia (13), and syncytiotrophoblast (STB) dysfunction (14). STB is a multinucleated cell layer of fetal origin that covers the chorionic villi and is in direct contact with maternal blood (15). Thus, it has been

reported that STB stress induced by the Jet-type blood flow can be characterized by: 1) STB damage markers, such as increased mitochondrial dysfunction, apoptotic markers, reticulum stress, oxidative stress, and inflammation; 2) An excessive release of microvesicles, exosomes, and cell fragments (16); and, 3) an increased release of ‘Damage-associated molecular patterns’ (DAMPs) (17). In PE, increased DAMPs activate the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome, which promotes IL-1 β and IL-18 maturation (18).

DAMPs are host to intracellular molecules that are not usually found in cell-free form. They can activate ‘Pattern Recognition Receptors’ (PRRs) mainly in innate immune cells, which are responsible for recognizing pattern molecules of microorganisms (19). PRRs are classified into several classes, including Toll-like receptors (TLR), nucleotide-binding oligomerization domain, Leucine-rich repeats, nucleotide-binding domain leucine-rich repeat containing receptors, retinoic acid-inducible gene 1 (RIG-1) -like receptors, and the C-type lectin receptors (20). PRRs are found in various cell types including monocytes/macrophages (21), neutrophils (22), and endothelial cells (23). The innate immune cells activated by DAMPs via TLR, switch from a tolerogenic, anti-inflammatory phenotype to a cytotoxic, pro-inflammatory phenotype (24). The activation of PRRs favors the proinflammatory status by inducing the secretion of proinflammatory cytokines (25). Under conditions of hypoxia and oxidative (17), reticulum (26), and mitochondrial (27) stress, all of them found in STB stress (16), the STB increases the releasing of several DAMPs, such as HMGB1 or cell-free fetal DNA (17). In this sense, the STB stress contribute to the maternal pro-inflammatory milieu, which includes several increment level of circulating DAMPs and cytokines (28), are part of the pathophysiological signs of the syndrome and could activate and maintain the proinflammatory profile of innate immune cells and endothelial cell dysfunction observed in the syndrome (29). Both early-onset and late-onset preeclampsia appear to share systemic and placental inflammation as a common pathophysiological feature.

Proinflammatory status in preeclampsia

The immunological mechanisms underlying preeclampsia have been extensively explored (24, 30), highlighting the role of adaptive and innate immune pathways in the development of this condition. During the physiological pregnancy, the inflammatory profile of the maternal immune system changes, being proinflammatory during placentation. This is proposed as a requirement for an effective invasion of the placenta and remodeling of the spiral arteries (31). In the 2nd gestation trimester, the profile changes to anti-inflammatory which is the basis of tolerance toward the fetal-placental unit. At the time of delivery, the profile becomes proinflammatory contributing to labor (31). Therefore, a large part of the pregnancy takes place with strong maternal immunomodulation, which is manifested by: I) high levels of anti-inflammatory cytokines (*e.g.* IL-10), immunosuppressants

(e.g. progesterone), suppressive (e.g. HLA-G), and tolerogenic molecules (e.g. TGF- β) (32); and by II) an increase of anti-inflammatory immune cells that including macrophages with the anti-inflammatory phenotype (M2), and a reduction ratio of helper T lymphocytes, Th1:Th2 and Th17:Tregs (33). In general, the placenta favors immunotolerance, either through the expression of human leukocyte antigen G (HLA-G) that reduces the reactivity of natural killer lymphocytes (34), or by releasing extracellular vesicles, which induce the secretion of anti-inflammatory cytokines from the macrophages that engulf them (35). Contrarily, PE is characterized by presenting a proinflammatory state in the mother and placenta (36), which includes an increment of circulating proinflammatory mediators (e.g. TNF- α and IL-6), diminishing of anti-inflammatory cytokines (e.g. IL-10) (36), decreased circulating levels of progesterone (37), decreased HLA-G expression (38), higher ratio lymphocytes Th1:Th2 and Th17:Tregs (36), and increased activation of monocytes, neutrophils, and macrophages (39). In PE, the unbalance toward the proinflammatory status is associated with endothelial activation, leading to endothelial dysfunction and high blood pressure (40).

Cardiovascular disorders and endothelial dysfunction/activation

Preeclampsia has been associated with microvascular dysfunction, which may contribute to the increased risk of obstructive coronary artery stenosis observed in women with a history of this condition, especially when preeclampsia is associated with preterm delivery or stillbirth (41). In physiological status, the endothelium shows a balance between vasodilator and vascular constrictor molecules, such as nitric oxide and Endothelin, respectively, which helps to maintain an anti-inflammatory and antithrombotic function (42). Cardiovascular disorders are strongly associated with endothelial dysfunction (43), characterized by an alteration in endothelium-dependent vascular relaxation, oxidative stress, and the inflammatory activation of endothelial cells (44). During endothelial activation, an overexpression of proinflammatory cytokines, chemokines, and adhesion molecules has been described in endothelial cells (43). Thus, systemic inflammation is associated with vascular diseases (45) and hypertension (46). Increasing evidence indicates that immune cells are directly involved in the onset of hypertension. In IFN- γ KO mice, a murine model of hypertension (DOCA+salt model) did not show the characteristic increment of the blood pressure of the model (47), being observed only when CD8⁺ T lymphocytes from hypertensive WT mice were transferred to knock-out (KO) individuals (47). However, not only are T cells implicated in the onset of hypertension but also innate immune cells, such as dendritic cells, monocytes/macrophages, natural killer cells, and neutrophils (48). Although adaptive immune cells have an important role in the onset of cardiovascular disorders, recently, innate cells have become relevant in the phenomenon due to the persistent activation of PRRs. It is proposed that cardiovascular diseases are related to a maladaptive inflammatory response of innate cells (49). In this sense,

it was reported that circulating monocytes isolated from patients with coronary artery disease have increased NLRP3 and caspase-1 expression, both related to inflammasome formation and the elevated production of the downstream cytokines, such as IL-1 β and IL-18 (50). Classically, it is described that inflammasome formation triggers pyroptosis (51). However, the neutrophils in a DAMP-rich inflammatory milieu are resistant to this type of cell death, becoming a permanent producer of IL-1 β (52).

Monocytes and neutrophils and their association with cardiovascular pathologies

Meta-analysis of 27 studies evidenced that intermediate- and non-classical monocytes are increased in persons with cardiometabolic disorders and cardiovascular disease (53). On the other hand, a high neutrophil-to-lymphocyte ratio is associated with several cardiovascular conditions, as evidenced by a meta-analysis of 38 studies (54), being proposed as a biomarker of cardiovascular health. These innate immune cells are tightly involved in the magnitude of cardiovascular lesions or alterations. In the case of animals subjected to myocardial infarction and reperfusion, the depletion of neutrophils prior to the infarct showed a significant reduction in infarct size (55). During atherogenesis, neutrophils in the intima release reactive oxygen species and proteases, which alter the endothelium integrity, allowing the recruitment and extravasation of monocytes and its further differentiation to macrophages (Reviewed in (56)). The myeloperoxidase released by neutrophils oxidates LDL, enhancing the amount of oxLDL, which together with activated macrophages generates the foam cells (56). However, despite the pro-inflammatory profile of the cardiovascular event, high basal levels of pro-inflammatory cytokines precede the occurrence of cardiovascular pathologies. Thus, a meta-analysis showed that high levels of IL-6, IL-18, and TNF α increase the risk of non-fatal myocardial infarction or coronary heart disease death (57). All those cytokines can induce endothelial dysfunction (58, 59). The activated neutrophils secrete the pro-inflammatory cytokines TNF α , IL-1 β and IL-12 (60), and activated monocytes secrete IL-1 β , IL-6, TNF α (61). The latter suggests that chronic activation of monocytes and neutrophils can impact endothelial function increasing the risk of cardiovascular pathologies.

In the context of preeclampsia, the pro-inflammatory environment includes activated monocytes, showing increased expression of CD11b, ICAM-1, CD14, and TLR4, an overproduction of reactive-oxygen-species (ROS), and altered secretion of cytokines (62). In the same way, neutrophil concentration is increased in maternal blood (63) along with an increment of the neutrophil activation markers CD11b and CD62L (64). Considering that a proinflammatory milieu is shared characteristic between preeclampsia and cardiovascular disorder, then an alteration of the performance of the immune system could be part of the mechanisms that increase the long-lasting cardiovascular risk in women who had preeclampsia. In this sense, "Trained Innate Immunity" (65) could be

the mechanism since it has recently been involved as a player in the onset of cardiometabolic diseases (65, 66).

'Trained Innate Immunity' (TRIM)

TRIM is defined as 'the long-term functional reprogramming of innate immune cells, which is evoked by exogenous or endogenous insults leading to an altered response toward a heterologous second challenge after the return to a non-activated state' (67). TRIM can be triggered by DAMPs, including oxidized low-density lipoprotein (oxLDL) (66), lipoprotein(A) (68), catecholamines (69), aldosterone (70), heme (71), vimentin (72), uric acid (73), S100-alarmin (74) and HMGB1 (75). DAMPs can induce TRIM by signaling through PRRs, including the receptor for advanced glycation end products (RAGE) (76), CD36 (77), and the five types of PRRs (77). Even though there is no report about TRIM in preeclampsia, it is possible to propose that it could occur since several DAMP levels are increased in maternal blood (17, 78).

The effects of TRIM induction mediated by DAMPs trigger metabolic and epigenetic modification that will lead to memory. For instance, cellular metabolic changes are necessary for epigenetic reprogramming (79), including an overexpression of glycolytic enzymes after the first stimulus (80) and an increment of lactate due to higher glucose consumption (81). Fumarate and succinate are produced as intermediates in the tricarboxylic acid cycle and glycolysis, and those intermediate are increased in trained macrophages (82). Additionally, oxidative phosphorylation also in enriched in trained cells (83). The influence of cellular metabolism on epigenetic mechanisms is already known. In TRIM, the accumulation of fumarate inhibits the demethylase activity of KDM5, a lysine demethylase of histones (84).

There is no report about TRIM being induced in preeclampsia. However, in maternal preeclamptic monocytes showed basal intracellular reactive-oxygen-species and increased oxidative burst after stimulation, which is indicative of a potentiated oxidative phosphorylation (85), similar to the observation made in monocytes trained with oxLDL (83). Noteworthy, mothers with PE, exhibited an increased percentage of classical monocytes-2 (CD14⁺⁺, CD16⁻, HLA-DR⁺) and a decreased percentage of non-classical monocytes (CD14⁺, CD16⁺⁺) prior to delivery (86, 87). Since classical monocytes-2 are considered as pro-tolerogenic (88) while non-classical monocytes are associated with pro-inflammatory responses (88), the altered levels observed in PE-pregnancies are proposed to reflect a compensatory mechanism aimed at counterbalancing low-grade chronic inflammation (87). Interestingly, although monocyte-2, considered as monocyte-myeloid derived suppressive cell (89), mainly by its capacity of differentiate naïve CD4⁺ T cells to CD4⁺, CD25⁺, Foxp3⁺ regulatory T-cell (Treg) (90), in Psoriasis, the induced-Treg differentiated by monocytes-2 showed a deficient suppressive activity (91). The latter suggest that in preeclampsia, a similar phenomenon could be occurring, since circulatory Treg in preeclampsia shows reduced function, with reduced expression of FOXP3 and reduced IL-10 and TGF- β secretion (92). However,

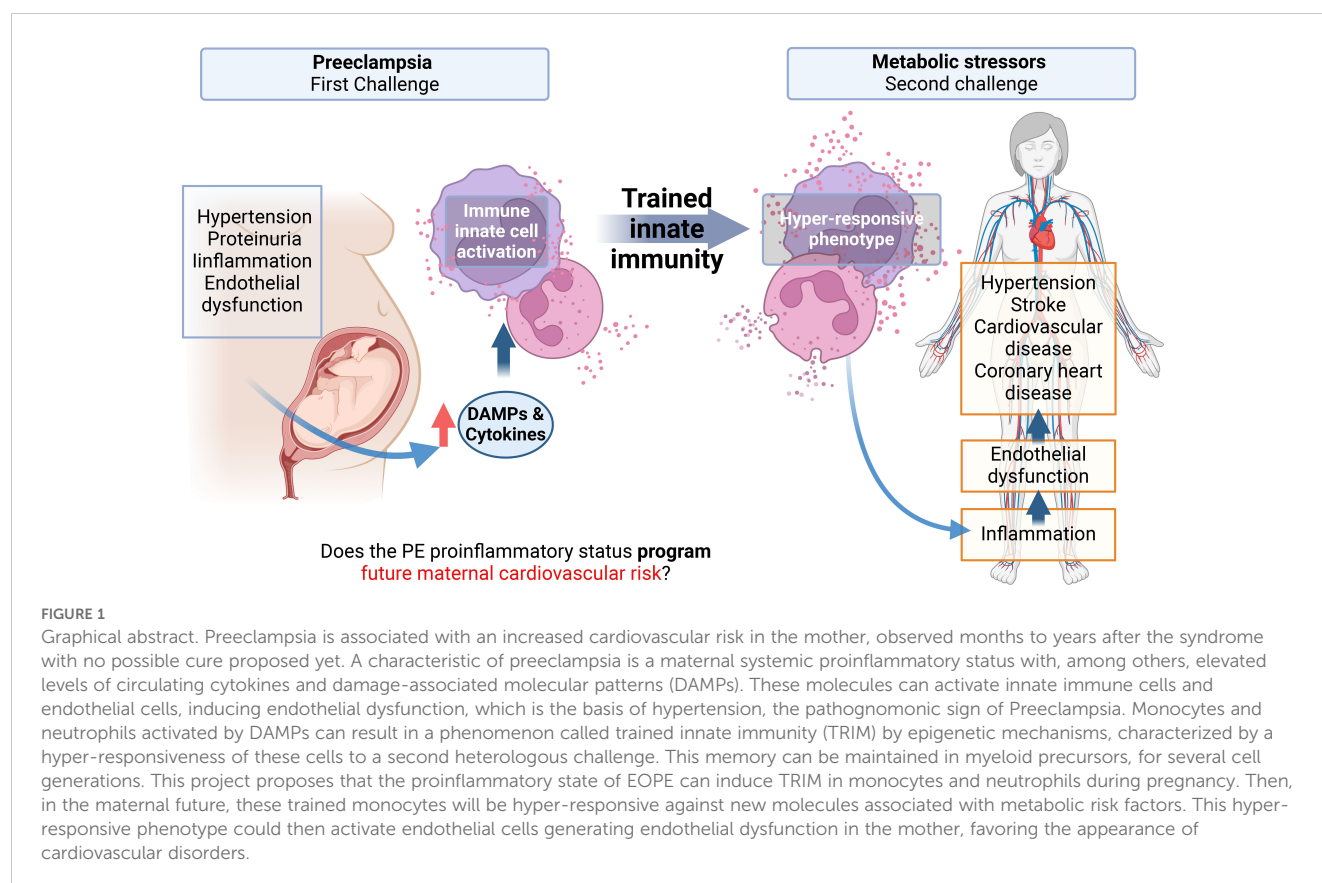
there is no data on the role of monocyte subpopulations in PE or their frequency during the postpartum period. Consequently, the potential permanent programming of monocyte subpopulations remains unknown.

Resident natural killer cells (NK) in decidua (dNK) is an essential cell type during the placentation due to its activity that include the induction of the remodeling of spiral arteries by the disruption of its vascular smooth muscle cells (93) and by the interaction with extravillous trophoblast cells (94) promoting its invasion activity an arterial remodeling through INF- γ and VEGF α secretion (95). Noteworthy, the dNK from multiparous mothers showed a higher response to trophoblast interaction characterized by enhanced INF- γ and VEGF α secretion, in association with an open state of chromatin of their locus, among other loci (95). Thus, it is proposed that the physiological pregnancy can promote epigenetically a tolerance to future pregnancies (95). In preeclampsia, dNK are increased in decidua but showing reduced activity (e.g. reduced INF- γ secretion) (96), which impact in the spiral arteries remodeling. In this scenario, also, it is possible to propose a memory in dNK in PE, since the mothers that have a prior pregnancy with preeclampsia have the greatest relative risk (RR) of PE in a new pregnancy, with a RR of 8.4 (7.1 to 9.9, 95% CI) (97). Also, other conditions increase the risk of PE, such as chronic hypertension with a 5.1 of RR (4.0 to 6.5, 95% CI); pregestational diabetes with a RR of 3.7 (3.2 to 4.3, 95% CI); and, pre-pregnancy BMI>30 with a RR of 2.8 (2.6 to 3.1, 95% CI) (97). In all this pathologies the activity of NK is reduced (98–100). It is proposed that NK exhaustion can be produced by chronic inflammation (101) which is found in chronic hypertension (102), diabetes (103), obesity (104), and preeclampsia (described above). Then, chronic inflammation observed in several pathologies with high risk of PE may generate a pro-exhaustion memory in circulatory and decidual NKs favoring the onset of PE.

TRIM is associated with cardiovascular disorders (105), making it possible that PE-induced long-term TRIM could impact endothelial homeostasis. Then, as shows the Figure 1, we proposed the proinflammatory status of preeclampsia constituted at least by high concentration of pro-inflammatory cytokines and increased levels of several DAMPs (compiled in Table 1) is associated with the activation of innate cells, including monocytes and neutrophils. This context, as was discussed above, could be conducive to TRIM acquisition during the syndrome. Then, in a short-medium or long-term, the maternal trained innate cells could over respond to new challenges and generate a strong and fast proinflammatory status disturbing the cardiovascular physiology of women (see Figure 1). Regarding the moment during the pregnancy at which DAMPs could initiate in PE the challenge in innate immune cells is not clear. However, DAMPs seem to have permanent participation in the pathophysiology of the syndrome.

DAMPs and the pathophysiology of preeclampsia

There is no clue as to whether DAMPs could be involved in the origin of PE, however it is possible to propose that DAMPs



could maintain and even amplify the pro-inflammatory status of the syndrome. Exposure of control placental explants to PE serum increased HMGB1 release (133). Ex vivo induction of oxidative stress and hypoxia to control placenta increased liberation of several DAMPs, including HMGB1, HSP70, S100A8, S100A12 and Cell free-fetal DNA (18). The latter together with the findings that the receptors TLR-2, -3, -4, and -9 are increased in syncytiotrophoblast in PE (134) suggest a self-maintaining of the DAMP-induced proinflammatory status of the placenta.

DAMPs may enter maternal circulation, as several with high maternal plasma levels in PE are listed in Table 1, including cell-free DNA, crystals, and proteins. Although most of them can be released by the placenta it is not possible to determine the original source of them. However, DAMPs could induce alteration in endothelium. Thus, microvascular endothelial cells HMEC-1 treated with recombinant HMGB1 elevated the expression of the adhesion molecule ICAM-1 favoring the arrest of the monocyte cell line U937 on them (133). Cell-free mitochondrial DNA (cfmtDNA) also generated a similar effect, in this case cfmtDNA could increase macrovascular endothelial cell EA.hy926 permeability, together with the rising of ICAM and E-selectin expression which favored the arrest of primary polymorphonuclear leukocyte (135). In the other hand, the cfmtDNA released by hypoxic murine trophoblast reduced the endothelial-dependent vasodilation in abdominal aorta, partially mediated by NLRP3 since the effect on cfmtDNA diminished in NLRP3^{-/-} animals (136). The latter together with the fact that preeclampsia curses along endothelial dysfunction

(137) suggests that elevated circulating DAMPs may be part of the syndrome.

Maternal plasma in PE shows elevated levels of the anti-angiogenic protein soluble fms-like tyrosine kinase 1 (sFLT-1) (138). sFLT-1 is proposed to be one of the responsible for endothelial dysfunction in PE by the sequestration of VEGF resulting in the endothelial expression of the adhesion proteins ICAM and VCAM and the vasoconstrictor peptide endothelin-1 (139). sFLT-1 can be released by THP-1-derived macrophages followed by the activation of inflammasome in a GSDMD dependent manner (140). In this sense the DAMPs hyaluronan and HSP70 (141), and uric Acid (142) generated a strong inflammasome activation in primary monocytes from mothers with PE probably contributing to the high levels of IL-1 β and IL-18 found in PE-maternal plasma (142). The activation of inflammasomes also participates in the releasing of DAMPs since the induction of pyroptosis led to the liberation of HMGB1 (143).

In the most severe cases of preeclampsia, mothers have a higher risk of thrombotic events during pregnancy (144). In fact, the plasma from mothers with PE had fast and strong thrombin generation compared to control pregnancy plasma (122). The same study showed that plasma from preeclampsia patients strongly induced NETosis in neutrophils from healthy donors (122). NETosis is a neutrophil-specific activation characterized by the release of neutrophil extracellular traps (NETs), which consist of chromatin and antimicrobial proteins (145). In PE high levels of NETs were found in maternal circulation (146). The link between

TABLE 1 Maternal circulatory DAMPs with increased levels in Preeclampsia.

DAMP	Level in control and preeclamptic pregnancy	Ref.	Physiological sub-cellular distribution	Physiological function	Receptor that sense DAMP
Cell-free fetal DNA (cffDNA)	Control: 46.9 [20.8-78.2]	(106)	Nucleus	Source of all intrinsic genetic information (107)	TLR-9 (108)
	PE: 594.8 [240-1090.4]				
	GE/mL. Median [IQR]				
Cell-free mitochondrial DNA (cfmtDNA)	Control: 239.3 [197.4-297.1]	(109)	Mitochondrial matrix	Encodes 13 crucial proteins that are part of the oxidative phosphorylation system (110)	TLR-9 (111)
	PE: 271.5 [220.2-335.1]				
	Copy number. Median [IQR]				
Cell-free heme (cfHeme)	Control: 1.63 ± 0.22	(112)	Cytosol	Component of several proteins contributes to vasodilation, cellular signaling, iron balance, and provides antioxidant and cellular protection (113)	TLR-4 (114)
	PE: 3.18 ± 0.35				
	µM. Mean ± SD				
Cyclophilin A (CypA)	Control: 8.71 (5.03–54.12)	(115)	cytosol	Crucial for protein folding, modulates immune response by activating T cells and producing cytokines (116)	CD147 (integrin β2) (117)
	PE: 48.35 (8.12–58.91)				
	ng/mL. Median (min–max)				
Heat Shock Protein 70 (HSP70)	Control: 643.4 [12.7–1084.9]	(78)	Cytosol, nucleus, ER, and mitochondria	Assists in the correct protein folding, prevents aggregation, participates in the degradation of damaged proteins (118)	TLR-2 and TLR-4 (119)
	PE: 901.1 [401.6–1263.8]				
	pg/mL. Median [IQR]				
High Mobility Group Box 1 (HMGB1)	Control: 2.1 [1.5–4.7]	(120)	Nucleus	Organizes DNA and nucleosomes in the nucleus, facilitating gene transcription (121)	RAGE and TLR-4 (76)
	PE: 5.5 [2.3–78.1]				
	ng/mL. Median [IQR]				
Histones (Hs)	Control: 1.31 ± 0.54	(122)	Nucleus	Compact DNA into nucleosomes and regulate gene expression through its post-translational modifications (123)	TLR-4 (124) Clec2d (125)
	PE: 4 ± 0.85				
	Fold change. Mean ± SD				
Hyaluronan (HA)	Control: 58.9 [2.5–180.7]	(78)	Extracellular matrix	Provide structural support, and promoting healing by facilitating cell migration and proliferation (126)	CD44 (127)
	PE: 127.5 [20.7–287.6]				
	ng/mL. Median [IQR]				
Myeloid-related protein 8, MRP8 (S100A8) and MRP14 (S100A9). Heterodimer Calprotectin (S100A8/A9)	Control: 552 (471–651)	(128)	Cytosol	Myelomonocytes metal-chelating antimicrobial protein of the innate immune response (129)	TLR-4 (130)
	PE: 1081 (865–1569)				
	µg/L. Median (95% CI)				
Uric Acid (UA)	Control: 4.2 [2.8–4.8]	(78)	Cytosol	Uric acid is the end product of exogenous and endogenous purine metabolism (131)	P2X7 (132)
	PE: 6.1 [4.5–10.1]				
	ng/dL. Median [IQR]				

NETs thrombosis is based on the capacity of human nuclear-DNA and histones for inducing thrombin generation (147). Interestingly, intact-NETs or assembled histones are unable to induce thrombin generation, indicating that NETs must be disassembled to have coagulatory activity. Based on the latter, DAMPs may favor the elevated risk of thrombotic events in mothers-with-PE due to the high levels of circulatory histones (see Table 1), and to the induction of NETosis by HMGB1 (148).

In the context of PE, as Table 1 and Figure 2 show, several DAMPs have increased maternal circulatory concentration in PE,

DAMPs elevated in maternal circulation in preeclampsia

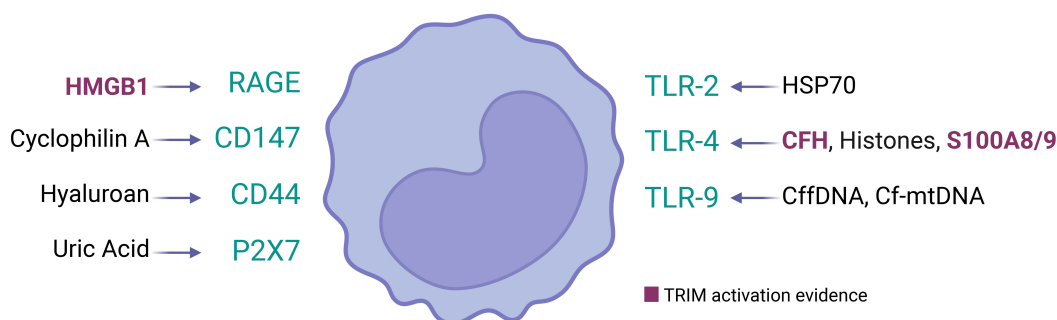


FIGURE 2

DAMPs elevated in maternal circulation in preeclampsia and its receptors in innate immune cells. Several DAMPs have high plasmatic concentration in mothers with preeclampsia, each of them can be sensed by pattern recognition receptors in cell membrane of innate immune cells (represented as monocyte). The DAMPs/Receptor are: High Mobility Group Box 1 (HMGB1) (120)/RAGE and TLR4 (114); Cyclophilin A/CD147 (117); Hyaluronan (78)/CD44 (127); Uric Acid (UA) (78)/P2X7 (132); Heat Shock Protein 70 (HSP70) (78)/TLR2 and TLR4 (119); Cell-free heme (CFH) (112)/TLR-4 (149); Histones (122)/TLR-4 (124) and Clec2d (125); Calprotectin (S100A8/A9) (128)/TLR-4 (123); Cell-free fetal DNA (cffDNA) (106)/TLR-9 (108); Cell-free mitochondrial DNA (cfmtDNA) (109)/TLR-9 (150). Among them, only HMGB1 (75), cell-free heme (151), and S100A8/9 (152) have been showed as TRIM inducers. However, the circulatory DAMPs in PE may act collectively to promote TRIM acquisition in innate immune cells during the syndrome.

suggesting that TRIM could be induced in innate cells during the syndrome. The latter is supported by the evidenced ability of cell-free heme (151) and HMGB1 (75) to induce TRIM. In the case of S100A8/A9, the evidence indicates a possible dual role as pro-inflammatory molecules (153) but also as an immune modulator (152). The latter indicates that it will be necessary to evaluate not only if individual DAMPs with high levels in PE can induce TRIM but also how collectively high levels of different DAMPs affect TRIM acquisition.

TRIM on myeloid progenitors

The fact that mature innate immune cells are short-lived (*i.e.* Half-life 5-7 days (154, 155)), raise the unanswered question about how PE-induced TRIM could last years. In this scenario, it would be necessary that the precursors of innate cells also be involved. In this sense, human hematopoietic stem and progenitors cells (HSPCs) showed permanent alterations after *in vivo* Bacille Calmette-Guérin (BCG) vaccination (150), a classic TRIM inducer (156). The latter included, a permanent (*i.e.* at least 90 days post-vaccination) transcriptional reprogramming in HSPCs, leading to an upregulation of genes associated with myeloid and granulocytic cell lineage priming, generating a myeloid differentiation bias within HSPCs, and enhanced proinflammatory response to various stimuli of mature peripheral blood mononuclear cells (150). Regarding DAMPs, TRIM induced by heme in mice showed an increase in myeloid-biased long-term hematopoietic stem cells and multipotent progenitors with an expansion of myeloid-biased, associated to elevated chromatin accessibility in genes associated with myeloid differentiation of HSPCs, also there

was a significant and permanent increase in mature myeloid cells (*i.e.* neutrophils and monocytes), with an enhanced phagocytic activity (151). HSPCs express TLR-2, -4, and -9, and their activation induces the differentiation and expansion to macrophages (157) suggesting that DAMPs may reach bone marrow and promote TRIM-associated permanent modifications.

Conclusions

Preeclampsia is a severe multisystemic syndrome which manifest with different pathological characteristics (*i.e.* EOPE and LOPE). Additionally, it remains unclear what are the mechanisms that induce the syndrome. However, one fact is clear: mothers who had PE were at higher cardiovascular risk. Thus, mothers not only face a serious pregnancy pathology, but this syndrome will probably also affect their future health. Therefore, an understanding of the mechanisms that underlie higher cardiovascular risk is crucial. Trained innate immunity has recently changed the paradigm that adjudicated the immune memory only to T/B cells from adaptive immunity, and this type of epigenetic memory is a mechanism with a clear potential to impact cardiovascular physiology. This epigenetic memory could explain the increased cardiovascular risk observed in women who have experienced PE, potentially triggered by future health challenges throughout their lives. However, further research is required to explore this hypothesis, as no study has directly examined this possibility to date. If PE-related sterile inflammation can induce TRIM, testing seems to be mandatory since several research groups are focused on TRIM modulation (149). Thus, this offers a certain possibility to improve the future maternal health of women who have preeclampsia.

Author contributions

IC-W: Writing – review & editing, Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft. JS: Writing – review & editing, Conceptualization, Data curation, Investigation. JG: Writing – review & editing. DC: Validation, Writing – review & editing.

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A stress paradox: the dual role of the unfolded protein response in the placenta

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The placenta is a temporary organ that forms during pregnancy and is essential for fetal development and maternal health. As an endocrine organ, proper placental function requires continual production, folding, and transport of proteins and lipids. Central to these processes is the endoplasmic reticulum (ER), a dynamic organelle responsible for maintaining cellular protein and lipid synthesis and processing. ER stress occurs when there is an accumulation of unfolded or misfolded proteins, which triggers the activation of cellular pathways collectively called the unfolded protein response. Unfolded protein response pathways act to alleviate the misfolded protein burden and restore ER homeostasis, or if unresolved, initiate cell death. While prolonged ER stress has been linked to deficient placental function and adverse pregnancy outcomes, basal activation of unfolded protein response pathways is required for placental development and function. This review explores the importance of ER homeostasis in placental development and function, examining how disruptions in ER stress responses may contribute to adverse pregnancy outcomes.

KEYWORDS

ER stress, UPR, placenta, trophoblast, decidua

1 Introduction

The endoplasmic reticulum (ER) is a membrane-bound organelle that plays a central role in the synthesis, folding, and transport of proteins, as well as lipid production and calcium storage. It is a dynamic structure, adapting and remodeling itself in response to cellular demands. This adaptability is critical for maintaining cellular proteostasis, thereby balancing protein synthesis, folding, and degradation. ER stress occurs when the capacity of the ER to properly fold proteins is overwhelmed, leading to an accumulation of misfolded or unfolded proteins in the ER. ER stress triggers the activation of cellular signaling pathways designed to take corrective actions and restore proteostasis, or to initiate cell death pathways if the burden is prolonged or severe. While ER stress and downstream

signaling can occur in any cell, those with a high demand for protein synthesis such as hormone-producing cells are particularly susceptible. The placenta is one such organ tasked with producing numerous hormones and other proteins critical for fetal development and pregnancy success, where disruptions in proteostasis can have severe consequences for maternal and fetal health. In this review, we will present the importance of ER homeostasis for placental development and function, and discuss evidence linking ER stress with deficient placentation and adverse pregnancy outcomes.

2 The placenta

The placenta is a transient yet highly sophisticated organ that intimately connects the gestational parent and fetus. It forms the primary interface separating maternal and fetal tissue and facilitates metabolic exchange, endocrine functions, and fetal protection. The placenta regulates the exchange of nutrients, oxygen, carbon dioxide, and other substances between maternal and fetal circulations, while restricting the transfer of pathogens, xenobiotics, maternal immune cells and many other potentially harmful substances from accessing fetal blood (1, 2). The placenta is also an adaptive organ that can integrate information on maternal nutrient availability and fetal demands, and undergo dynamic morphological and molecular changes to support fetal development (3). As an endocrine organ, the placenta produces a plethora of hormones and other factors that regulate maternal adaptations to pregnancy along with fetal growth and development (4, 5).

3 An overview of placental structure

The human placenta is arranged into highly branched tree-like villi containing an inner core of macrophages, fibroblasts, pericytes, connective tissue, and capillaries that connect to the fetal circulation through the umbilical vessels. The villous core is separated from maternal blood by a trophoblast bilayer: an inner portion of cytotrophoblasts (CTBs) resting on a basement membrane, and an outer syncytiotrophoblast (STB) layer (Figure 1). CTBs are self-renewing progenitor cells that initially form a continuous layer during early pregnancy, and replenish the STB layer by fusing into it. In late pregnancy, the proportion of CTBs dwindles and they form a disjointed layer beneath the STB. The STB layer, on the other hand, is a multinucleated entity with a vast interconnected cytoplasm that lines the placental villi and bathes in maternal blood. It forms the primary boundary between maternal blood and fetal tissue, and is responsible for numerous placental functions including maternal-fetal gas and nutrient exchange (6, 7). Given its proximity to maternal blood, STB is responsible for the production and metabolism of numerous hormones, such as human chorionic gonadotropin (hCG). These factors are deposited into maternal circulation to alter maternal physiology and metabolism for fetal benefit. Furthermore, since the STB does not contain intercellular junctions, it forms a semi-exclusive barrier that restricts the passage of many substances from accessing fetal circulation (8).

At sites where large anchoring villi contact the decidua basalis (the specialized endometrial tissue adjacent to where the placenta forms), CTBs differentiate into a distinct cell-type: extravillous trophoblasts (EVTs). Proximally, EVT's proliferate into stratified cellular columns. At the distal tips of these columns, cells stop replicating and gain invasive properties. Invasive EVT's infiltrate into the decidua basalis and inner third of the myometrium. EVT's are versatile cells that affix the placenta to the decidua basalis, interact with decidual stromal cells and immune cells to support immunological tolerance, and remodel uterine blood vessels and glands to ensure that a consistent supply of nutrients and oxygen are delivered to the placenta to support fetal sustenance (9).

The decidua basalis is a dynamic tissue derived from the endometrium adjacent to where the placenta forms. Transformation of the endometrium into the decidua begins during the secretory phase of the menstrual cycle and accelerates upon fertilization. Decidualization involves the differentiation of fibroblast-like endometrial stromal cells (ESCs) into polygonal decidual stromal cells (DSCs) along with extensive development of uterine glands and blood vessels, processes that are tightly regulated by estrogen and progesterone (10). Transformation of ESCs into DSCs is associated with a substantial increase in the metabolic demand and secretory activity of the cells that serve essential roles in early embryo nutrition and maternal-fetal communication (11).

Placental structure exhibits marked diversity between species. In humans and closely-related primates, the placenta is termed "hemochorial" since maternal blood directly contacts trophoblasts. Other species with hemochorial placentation include common laboratory rodents like mice, rats, and guinea pigs. Although these species exhibit notable differences in placental anatomy and physiology compared to humans, there are also many similarities. For example, placentas of mice, rats, and guinea pigs have a labyrinth zone containing syncytialized trophoblasts that specialize in nutrient and gas exchange (akin to the placental villi in humans), and a junctional zone adjacent to the decidua basalis that anchors the placenta to the decidua and is the site where invasive trophoblasts emanate (analogous to human EVT's). Consequently, rodents are often used as laboratory models to gain deeper mechanistic insight into placental development and maternal-placental-fetal interactions (12). Although this review will focus mostly on placentation in humans, much knowledge has been derived from studies using rodent models and these studies will be mentioned when appropriate.

4 Placenta-associated pregnancy complications

Deficient placental development and function is a major culprit underlying severe pregnancy complications that compromise maternal and fetal well-being and survival. For example, inadequate placental development resulting in placental insufficiency (inadequate maternal blood supply to the placenta) can lead to preeclampsia—a common and dangerous pregnancy disorder characterized by sudden-onset maternal hypertension, endothelial dysfunction, and organ damage. In many cases, in preeclampsia the fetus does not

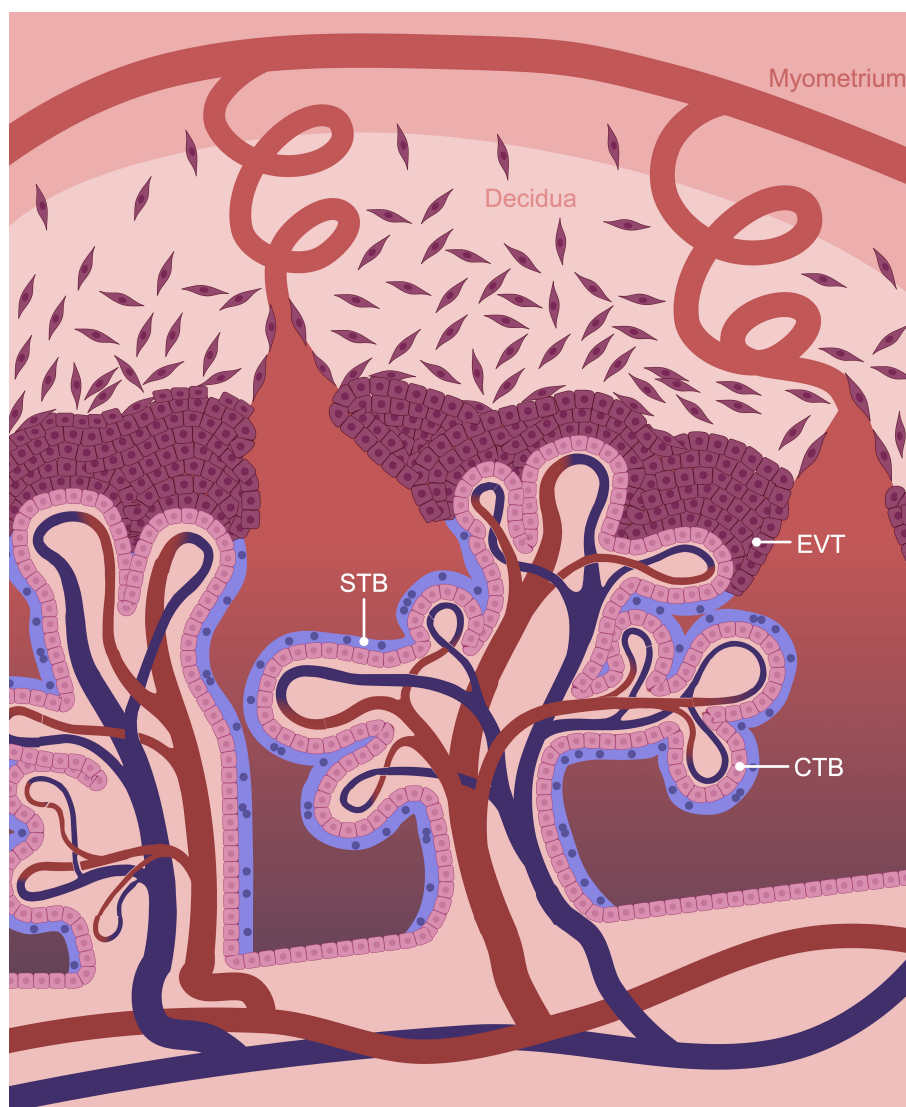


FIGURE 1

Schematic representation of the maternal-fetal interface. The human placenta is composed of chorionic villi which are lined by a trophoblast bilayer containing an inner cytotrophoblast (CTB) and outer syncytiotrophoblast (STB) layer. Extravillous trophoblasts (EVTs) form the cell columns at the tips of anchoring villi adjacent to the decidua and are the source of invasive trophoblasts. Created in BioRender. Chowdhury, D. (2024) <https://BioRender.com/f22l744>.

obtain adequate oxygen and nutrients, resulting in fetal growth restriction (FGR) and the potential for long-term health deficiencies. Placentas from preeclampsia and FGR often exhibit evidence of hypoxia, oxidative stress and inflammation—conditions that can adversely affect ER homeostasis and result in ER stress (13–15). In this review, we will discuss ER homeostatic mechanisms required for placental development and function, and describe how these mechanisms are perturbed following cellular exposure to stress during placenta-associated pregnancy complications.

5 ER stress

Protein turnover in the placenta increases throughout pregnancy, requiring efficient quality control mechanisms to

ensure that the protein folding capacity of the ER meets protein synthesis demands (16, 17). Key components of this quality control machinery include ER-resident chaperones such as binding immunoglobulin protein (BiP; also called glucose regulated protein 78, GRP78), calreticulin, calnexin, and protein disulfide isomerases (PDIs). These chaperones assist proteins in achieving their native conformation (18). However, if chaperones cannot keep pace with protein folding demands, an accumulation of unfolded or misfolded proteins in the ER lumen can result, leading to ER stress and the activation of the unfolded protein response (UPR) (19). The role of ER-associated chaperones for placentation and decidualization are summarized in [Supplementary Table 1](#).

ER stress can occur due to an accumulation of abnormal lipids or misfolded proteins in the ER (20, 21). The accumulation and aggregation of misfolded proteins can occur due to increased rates

of protein synthesis, deficiency in autophagy, nutrient deprivation, and dysregulated calcium levels (22, 23). Many cell types experience a high protein load during various stages of differentiation and maturation, resulting in ER stress. Induction of ER stress can have morphological and biochemical consequences to cells. Morphologically, ER stress can result in dilation of ER cisternae to increase the lumen size and accommodate the sudden increase in protein accumulation (24, 25). Many cells that experience ER stress undergo a degree of epithelial-to-mesenchymal transition, including increased N-cadherin levels and decreased E-cadherin levels (26). Biochemically, ER stress can activate several signaling pathways including the UPR, in an effort to resolve the stress and restore proteostasis (27).

Recent studies have characterized early-onset preeclampsia as a proteinopathy due to presence of misfolded proteins in maternal urine and serum (28–30). Misfolded oligomeric proteins and amyloids, arising from defective chaperone function or impaired autophagy, may either deposit in the placenta from maternal circulation or be produced directly by placental cells (31). Several amyloidogenic proteins, including amyloid- β , transthyretin, and α -1-antitrypsin, have been identified in the preeclampsia-associated misfoldome and proposed as potential diagnostic markers for preeclampsia (31–33). Deposition of protein aggregates in the placenta may induce cellular toxicity, impair nutrient and gas exchange, and lead to placental insufficiency. Exposure of placental explants to serum from preeclamptic pregnancies induces ER stress and the formation of syncytial knots, suggesting that maternal serum in preeclampsia may contain ER stress-inducing factors that promote protein aggregation in placental

cells (34, 35). While the placenta can efflux misfolded proteins into maternal blood, the accumulation of protein aggregates in maternal blood may pose risks for maternal organs (31). Therefore, targeting of misfolded proteins and resolution of ER stress could be promising therapeutic strategies to restore cellular homeostasis and improve pregnancy outcomes (36–38).

6 The UPR in placentation

The UPR is a conserved cellular response to ER stress. In principle, the UPR seeks to correct the accumulation of unfolded and misfolded proteins in the ER, thereby restoring ER homeostasis. Basal levels of UPR activation promote cellular homeostasis, but in cases of prolonged or severe ER stress, unmitigated UPR activation can lead to cell death (39, 40). The UPR pathway consists of three ER-resident sensor proteins: inositol-requiring enzyme 1 (IRE1), protein kinase R-like ER kinase (PERK), and activating transcription factor 6 (ATF6), all of which trigger a transcriptional response to adapt the ER folding environment by upregulating genes involved in protein folding and degradation (Figure 2) (41). The following sections will summarize the role of IRE1, PERK, and ATF6 signaling in the context of placentation and decidualization.

7 IRE1

IRE1 is a transmembrane protein that is evolutionarily conserved from yeast to humans (42, 43). It contains a serine/

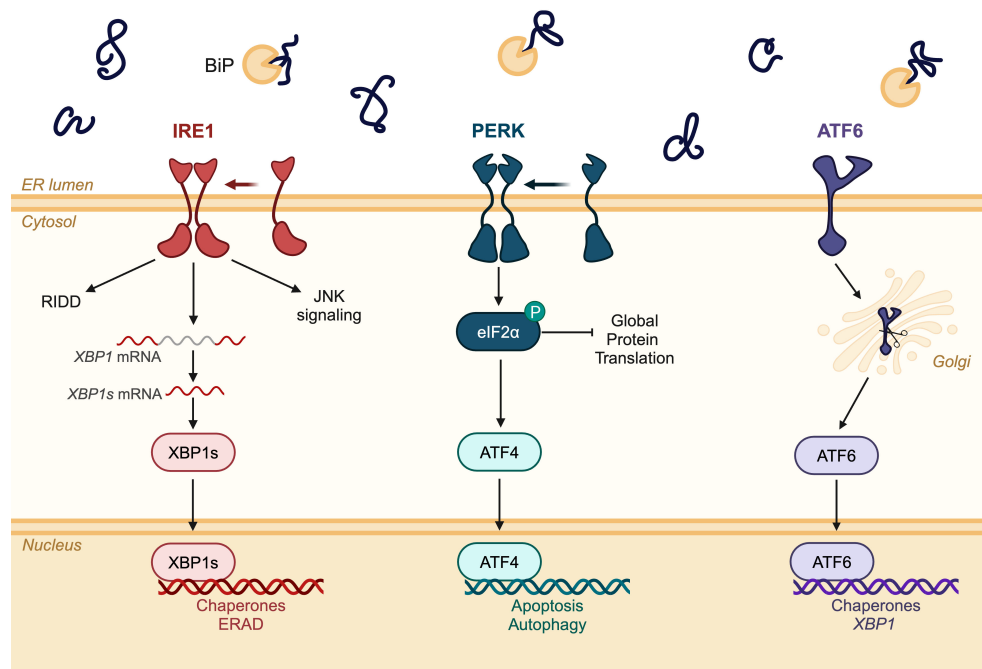


FIGURE 2

Schematic diagram of the three signaling branches of the unfolded protein response (UPR). Inositol-requiring enzyme 1 (IRE1); protein kinase R-like ER kinase (PERK); activating transcription factor 6 (ATF6); regulated IRE1-dependent decay (RIDD); jun N-terminal kinase (JNK). Created in BioRender. Chowdhury, D. (2024) <https://BioRender.com/r03w626>.

threonine kinase domain and a cytosol-facing domain with endoribonuclease activity. There are two isoforms of IRE1 in mammals – IRE1 α and IRE1 β – with IRE1 α being the ubiquitously expressed isoform (44). Upon detection of misfolded or unfolded proteins, IRE1 homodimerizes and activates its kinase function through trans-autophosphorylation of Ser⁷²⁴, Ser⁷²⁶, and Ser⁷²⁹ in the kinase activation loop (45). Once activated, IRE1 splices out a 26-nucleotide intron from the X-box binding protein 1 (*XBP1*) mRNA sequence, encoding for the spliced XBP1 (XBP1s) transcription factor (46). Thus, IRE1 activation is typically measured through the phosphorylation of IRE1, splicing of *XBP1* mRNA, or the accumulation and nuclear localization of the XBP1s transcription factor. XBP1s transcriptionally regulates genes encoding various chaperones, ER-associated degradation (ERAD) components, and proteins involved in lipid biosynthesis to relieve ER stress (47). The endoribonuclease activity of IRE1 can also degrade other mRNA sequences through regulated IRE1-dependent decay to reduce protein synthesis in the ER lumen (48, 49). While the activation of IRE1 is associated with cell survival mechanisms to relieve ER stress, persistent IRE1 activity can induce apoptosis by activating tumor necrosis factor receptor-associated factor 2, apoptosis signal-regulating kinase 1, and mitogen-activated protein kinases (26, 50).

7.1 IRE1 is required for placental development in mice

IRE1 α (herein referred to as IRE1) is essential for placental development in mice. Embryos and placentas with a deletion in *Ern1*, the gene that encodes IRE1, are smaller than wild-type and heterozygous siblings, and die around embryonic day 12.5 (51–53). *Ern1*^{−/−} placentas have reduced blood spaces in the labyrinth zone compared to wild-type mice, accompanied by decreased expression of vascular endothelial growth factor, suggesting that IRE1 contributes to placental vascular development (52–54). There does not appear to be an effect on decidualization in these mice (52). Interestingly, activation of IRE1 signaling, based on detecting IRE1 phosphorylation and *Xbp1* splicing, occurs in the placenta throughout early and midgestation but not in the embryo (52), suggesting that IRE1 signaling is particularly important for placental development. In line with this observation, midgestation lethality is avoided in mice lacking *Ern1* in embryonic tissue but not trophoblasts (using a *Mox2*^{+/Cre} transgenic mouse), demonstrating that the cause of embryonic demise in *Ern1*^{−/−} mice is due to defective placental development (52). Therefore, IRE1 appears to have a critical role for placental development in mice.

In line with an essential role of IRE1 signaling for placentation, high levels of XBP1s are detectable in the mouse placenta, but not in other embryonic organs (52). XBP1s is also spatiotemporally expressed in the mouse uterus where it is mainly detected in epithelial cells during early pregnancy and then subsequently in DSCs (55, 56). To investigate whether canonical IRE1-XBP1s signaling is involved in placental development, *Xbp1*^{−/−} mice have been generated. Interestingly, embryos and placentas with a

deletion in *Xbp1* exhibit embryonic lethality between embryonic day 12.5 and 14.5; however, unlike *Ern1*^{−/−} mice, the cause of lethality is attributed to liver hypoplasia. There does not seem to be a notable difference in placental morphology or vascular endothelial growth factor levels between *Xbp1*^{−/−} mice and wild-type mice (52, 57). Therefore, the essential role of IRE1 in mouse placental development may be through a noncanonical signaling pathway independent of XBP1s.

7.2 IRE1 in human STB formation

There is strong evidence that IRE1 signaling is active and required during human STB formation. Activation of IRE1 signaling may be downstream of cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) signaling, a well-established inducer of trophoblast syncytialization (58, 59). PKA phosphorylates IRE1 on Ser⁷²⁴ (58, 60), and also activates cAMP-response element binding protein, which is a transcriptional activator of *ERN1* (61). STB formation *in vitro* using primary CTBs from term placenta is associated with IRE1 activation. Similar results are observed using BeWo cells, a CTB-like choriocarcinoma line that fuses to form STB-like cells after exposure to forskolin (62). Interestingly, the addition of small molecule inhibitors targeting IRE1, or RNA-interference targeting all three UPR branches, reduces CTB fusion, hCG secretion, and autophagy (62). Therefore, similar to its important role in mouse placentation, IRE1 appears to be required for human STB formation.

Why is IRE1 signaling needed for STB formation? While this answer remains unclear, it may be related to the increased synthesis and secretion of numerous proteins involved in the differentiation of CTBs and their fusion into STBs. Increased demand for protein synthesis during STB formation can exceed the ER's folding capacity, resulting in the activation of the UPR to adapt to the new protein burden. Since IRE1-XBP1s signaling promotes the production of chaperones and ERAD components, its activation during STB formation may promote protein folding and clearance to ensure proper proteostasis in the ER, thereby enabling STB function. IRE1 activation may also promote the expression of genes involved in phospholipid biogenesis to remodel the ER membrane and expand the ER lumen during STB formation (63, 64).

Apart from responding to ER stress signals, XBP1s transcriptionally regulates the expression of differentiation-associated genes in other cells (65, 66). It is therefore possible that XBP1s may exert transcriptional control of genes needed for STB formation and function, such as ER-resident proteins needed for protein processing of STB-related hormones. As one example, the prototypical STB hormone, hCG, is a glycoprotein containing 11 disulfide bonds. These disulfide bonds are essential for hCG to fold into its proper protein configuration (67–69). XBP1s transcriptionally regulates the expression of PDIs, which are needed in the ER for disulfide bond formation and stability of this protein (70). Furthermore, XBP1s also transcriptionally regulates secretory pathway components, including those involved in protein glycosylation and vesicle trafficking needed for

maturation and secretion of hCG. Thus, IRE1-XBP1s signaling may be required for the synthesis, processing, and secretion of hCG, along with other STB-associated proteins (71).

CTB fusion involves dynamic changes in membrane composition and membrane-membrane interactions facilitated by syncytins, resulting in the formation of fusion pores, merging of membranes, and intermixing of cytoplasm between adjacent cells (72). While there is no evidence to-date that IRE1-XBP1s signaling transcriptionally regulates the expression of syncytins or their receptors, a role for IRE1-XBP1s signaling has been identified in other models of cell fusion. For example, deletion of IRE1 in mouse muscle satellite cells inhibits myoblast fusion and impairs skeletal muscle regeneration. IRE1-XBP1s signaling directly controls the expression of several genes involved in myoblast fusion, including *Mymk* (encoding Myomaker) (73). IRE1-XBP1s may similarly regulate the expression of genes involved in STB formation; or at minimum, may be needed to produce chaperones and other proteins that facilitate the synthesis, transport, and membrane presentation of fusogens.

Aside from direct transcriptional control of fusogens, IRE1 may play a role in cell fusion during STB formation by interacting with filamin A, an actin binding protein that regulates cytoskeletal organization. IRE1 acts as a scaffold by recruiting kinases, such as protein kinase C alpha, to promote phosphorylation of filamin A and induce cytoskeletal remodeling, an essential process that occurs during cell fusion (74–76). Furthermore, IRE1 may facilitate cell fusion by initiating contact sites between the ER and plasma membrane through store-operated calcium channels, which regulate intracellular calcium levels and maintain lipid homeostasis. IRE1 interacts with stromal interacting molecule 1 (STIM1), a sensor of calcium levels in the ER lumen (77). When calcium levels in the ER lumen are low, STIM1 undergoes a conformational change to promote calcium entry from the cytosol to the ER lumen (77, 78). Therefore, by interacting with STIM1, IRE1 may regulate cytosolic calcium levels, which along with increased cAMP, is a requirement for STB formation (79).

7.3 IRE1 in human EVT formation and function

IRE1 signaling is enriched in first trimester EVT's compared to CTBs, and gene set enrichment analysis of EVT's derived from human trophoblast stem cells shows enrichment of XBP1s-induced chaperones (80). Furthermore, the same study showed that inhibition of IRE1 using a small molecule inhibitor, 4μ8c, reduces cell surface HLA-G expression, suggesting that IRE1-XBP1s signaling is active during EVT formation and may be required for the synthesis and/or transport of cell surface antigens.

While the specific role of IRE1-mediated signaling in EVT formation and invasiveness is not fully understood, interactions with other factors deemed important for EVT function have been noted. For instance, Krüppel-like factor 6 (KLF6) is a transcription factor highly expressed in the placenta that modulates EVT formation and invasiveness. Using HTR-8/SVneo cells, an

immortalized cell-line derived from first trimester placental outgrowths and often used as a model of invasive EVT's, silencing KLF6 reduces IRE1 and XBP1s levels, suggesting that KLF6 may be upstream of IRE1 signaling. Interestingly, both KLF6 and IRE1 deficient mouse models are embryonic lethal at embryonic day 12.5, suggesting that they may be part of a similar developmental pathway (52, 81). IRE1 may also promote the expression of high-temperature requirement A serine peptidase 1 (HTRA1) following exposure to ER stress conditions, which promotes invasion of HTR-8/SVneo cells (82, 83). Notably, in other cells, IRE1-XBP1s signaling promotes the transcription of genes encoding factors associated with epithelial-to-mesenchymal transition, such as *SNAI1*, *SNAI2*, *ZEB2* and *TCF3* (84). EVT's undergo epithelial-to-mesenchymal transition as they gain invasive properties (85), but further studies are needed to determine whether IRE1-XBP1s signaling may contribute to this aspect of EVT development.

7.4 IRE1 signaling during human decidualization

There is evidence that IRE1-XBP1 signaling is active during decidualization. For example, cytoplasmic and nuclear reactivity for unspliced and spliced variants of XBP1 are detected in both DSCs and glandular epithelium (86). Decidualization of ESCs is associated with increased levels of IRE1 and its downstream targets, XBP1s and CCAAT/enhancer-binding protein homologous protein (CHOP). Treatment of DSCs with the IRE1 inhibitor, STF-083010, downregulates the expression of CHOP and prevents the secretion of interleukin-1β (IL-1β) associated with decidualization (87). Furthermore, in a co-culture model designed to mimic implantation, inhibition of IRE1 reduced invasion of trophoblast spheroids (derived from Swan-71 immortalized trophoblasts) into DSCs, suggesting that IRE1 may be required for this process (87). While IRE1-mediated signaling may be required for decidualization, hyperactivation may induce autophagy and disrupt decidualization. For example, the IRE1-XBP1s pathway is negatively regulated by the heat shock cognate 71 kDa (Hsc70). Knockdown of Hsc70 increases XBP1 protein expression, triggers autophagy, and impairs decidualization of ESCs (88). These results suggest that moderate activation of IRE1 supports successful decidualization, whereas too little or too much activation can interfere with this process.

7.5 IRE1 signaling in the placenta during pregnancy complications

Aberrant ER stress and hyperactivation of IRE1 signaling is associated with dysregulated placental development and adverse pregnancy outcomes. This has been most frequently documented in pregnancies complicated by preeclampsia and FGR. Compared to placentas from normotensive pregnancies, placentas from preeclamptic pregnancies (particularly early-onset preeclampsia) exhibit increased IRE1 phosphorylation, XBP1 splicing, and ER

luminal dilation (89, 90). Likewise, expression of *XBP1* is increased in decidual tissues from preeclampsia with and without FGR (86). Endometrial biopsies from patients with recurrent pregnancy loss and implantation failure have elevated expression of IRE1 compared to controls, but surprisingly lower expression of *XBP1*s, suggesting that dysregulated IRE1 activation may contribute to these pregnancy complications (87). However, phosphorylated IRE1 was not measured in the samples from recurrent pregnancy loss and implantation failure, and should be evaluated in future studies to determine whether IRE1 activity may be altered in these pregnancy complications.

IRE1 phosphorylation, along with other markers of ER stress and apoptosis, is also increased when subjecting trophoblasts to hypoxia-reoxygenation *in vitro* to mimic the conditions that placental cells would experience in early-onset preeclampsia (91–93). Therefore, IRE1 signaling likely contributes to the cellular stress response during pathological conditions. Other pregnancy complications associated with ER stress and IRE1 activation include gestational cholestasis, a condition in which bile acids build up in the liver and enter the bloodstream. In a mouse model of gestational cholestasis, increased IRE1 activation is apparent in placental tissue, and this was associated with apoptosis and FGR. Inhibition of IRE1 signaling using 4μ8c prevents trophoblast apoptosis and rescues FGR in mice. Likewise, inhibiting IRE1 signaling prevents cell death in the HTR-8/SVneo cell-line following exposure to deoxycholic acid (94).

Physiological and environmental stressors that increase the risk of pregnancy complications can induce ER stress and activate IRE1 signaling. For example, maternal obesity is a risk factor for various pregnancy complications. Compared to placentas from normal weight pregnancies, obese pregnancies exhibit increased levels of phosphorylated IRE1 and *XBP1*s in placental tissue (95, 96). Palmitate is the most common saturated fatty acid in the body and is detectable at higher levels in the circulation of obese persons. Increased levels of palmitate alter ER morphology, impair invasiveness, and induce ER stress and apoptotic signaling in various human trophoblast cell-lines (97, 98). Other environmental stressors associated with ER stress and IRE1 hyperactivation in trophoblasts include viral infections (e.g., ZIKV) and exposure to toxins such as nicotine and ethanol (99–102). In the latter case, only total IRE1 protein levels were assessed, and therefore the contribution of active IRE1-*XBP1*s signaling to placental function following exposure to toxins remains elusive.

8 PERK

PERK detects and responds to ER stress by reducing the amount of protein in the ER lumen through the attenuation of translation (103). When there is an overaccumulation of misfolded proteins in the ER lumen, PERK homodimerizes and trans-autophosphorylates at Thr⁹⁸⁰, resulting in the activation of its kinase function (104). PERK will subsequently phosphorylate eukaryotic initiation factor 2α (eIF2α), preventing the assembly of ribosomes at the initiator codon of mRNA transcripts and blocking protein synthesis (105).

As part of the PERK signaling branch, phosphorylated eIF2α promotes mRNA translation of the activating transcription factor 4 (ATF4), which can modulate transcription depending on its binding partners. The classical transcriptional target of ATF4 during ER stress is *DDIT3*, which encodes for CHOP, a transcription factor that regulates apoptosis (106). ATF4 can also transcriptionally regulate genes involved in amino acid metabolism and autophagy (107).

8.1 PERK regulates proper protein folding in the mouse placenta

Mice lacking PERK are viable and do not initially differ in weight compared to their wild-type counterparts, suggesting that PERK activity is not required (or is redundant) for placental and embryo development (108). However, other organs with secretory activity are severely impacted by PERK deficiency. For example, mice lacking PERK exhibit progressive loss of pancreatic β cells, neonatal development of diabetes mellitus, and exocrine pancreatic atrophy. Pancreatic cells in these mice have ER lumen dilation and accumulation of electron-dense material in the ER. Mice lacking PERK also have severe skeletal dysplasias and dwarfism associated with reduced hepatic secretion of insulin-like growth factor 1 (108–110). Since the placenta is also a secretory organ, PERK deficiency may affect aspects of placental endocrine function.

To determine a potential contribution of PERK signaling to placental endocrine activity, a conditional mouse model with PERK depletion specifically in the junctional zone has been investigated. The junctional zone secretes numerous hormones and growth factors, and is therefore considered as the endocrine component of the murine placenta (111). There are no differences in litter size, placental weight or fetal weight between wild-type mice and those with junctional zone-specific PERK depletion (as expected, since the global PERK knockout also does not show these differences). However, PERK deficiency exacerbates ER stress in the junctional zone when mice are housed in a reduced oxygen atmosphere, which was done to induce tissue hypoxia and ER stress. When exposed to hypoxia, PERK-deficient junctional zone trophoblasts exhibit dilation of the ER cisternae and accumulation of protein aggregates, indicating possible loss of ER homeostasis through aggregation of misglycosylated secretory proteins in the ER (111, 112). Furthermore, these trophoblasts have a reduced capacity to stimulate maternal physiological adaptations, such as the induction of glycogenolysis in the liver. Therefore, in the placenta, PERK may promote proper protein folding and processing in the ER, whereas PERK-deficient mice may be more susceptible to proteinopathies under ER stress-inducing conditions.

Activation of PERK attenuates translation through the phosphorylation of eIF2α on Ser⁵¹ (113). A mouse model has been generated in which this serine is mutated to alanine, preventing the ability of eIF2α to reduce translation (114). These mice die shortly after birth due to hypoglycemia, resulting from a deficiency in gluconeogenesis and loss of pancreatic β cells. In the placenta, eIF2α mutant mice exhibit increased basal translation,

correlating with reduced placental and fetal growth compared to mice possessing at least one wild-type copy (112). Additionally, these placentas have an accumulation of glycoproteins in the junctional zone and reduced labyrinth zone volume, suggesting that eIF2 α dysfunction may disrupt placental endocrine function (112).

8.2 PERK signaling in human placental and decidual development

As a key regulator of protein translation, PERK signaling may contribute to the control of trophoblast differentiation. For example, PERK signaling is activated in BeWo cells during forskolin-induced differentiation as shown through increased levels of phosphorylated eIF2 α , ATF4, and CHOP. Exposing BeWo cells to a PERK inhibitor, GSK2656157, inhibits cell fusion, but interestingly there is no effect on secreted hCG levels. Similar results are obtained using primary CTBs from term placentas (62). Like IRE1, PERK may promote trophoblast fusion through its interaction with filamin A to regulate cytoskeletal remodeling and ER calcium levels (115). In HTR-8/SVneo cells and JEG-3 cells, another transformed trophoblast cell-line, induction of ER stress using tunicamycin, thapsigargin, or pro-inflammatory cytokines reduces matrix metalloproteinase 2 (MMP2) levels and cell invasion. However, inhibition of PERK restores MMP2 levels (116). Given the importance of PERK signaling for ER homeostasis in other secretory cells, PERK may maintain cellular integrity in the placenta, particularly under ER stress-inducing conditions.

Within the decidua, DSCs from pregnancies deemed healthy express high levels of phosphorylated PERK. Treatment of ESCs and glandular cells with progesterone activates the PERK pathway, as shown by the increased levels of phosphorylated eIF2 α , ATF4, and CHOP (87, 90, 117). Progesterone-driven CHOP expression induces several apoptosis markers (e.g., BAX and cleaved caspase-3) in ESCs and glandular cells. Furthermore, BiP/GRP78, CHOP, and cleaved caspase-3 are upregulated during the secretory phase in stromal and glandular regions of healthy human endometrium, confirming that UPR activation and CHOP-mediated apoptosis increase in response to higher progesterone levels (117). Altogether, these findings indicate that progesterone-driven activation of the PERK/eIF2 α /ATF4 pathway, and subsequent CHOP-mediated apoptosis, may regulate endometrial remodeling during the late secretory phase.

8.3 PERK signaling in the placenta during pregnancy complications

PERK signaling may have important roles in determining cell function and cell fate during placental pathologies. For example, in placentas from preeclamptic pregnancies, increased levels of phosphorylated PERK, phosphorylated eIF2 α , ATF4 and CHOP are evident, and these proteins appear to localize predominantly to

the STB layer (118, 119). Increased levels of phosphorylated PERK are also evident in the decidua (90). Decidual tissue from preeclamptic pregnancies with FGR have increased phosphorylation of eIF2 α and ATF4 levels compared to control pregnancies (86, 120). These findings suggest that dysregulation of PERK signaling in the decidua may impair decidualization and contribute to the development of pregnancy complications.

Maternal serum from preeclamptic pregnancies activates the PERK pathway in placental explants and HTR-8/SVneo cells, as shown through increased eIF2 α phosphorylation and CHOP levels. Cell death was also apparent after explants and cells were exposed to serum from preeclamptic pregnancies, but the specific contribution of PERK signaling to cytotoxicity was not assessed (34). It is possible that the PERK-mediated cell death observed in placentas from preeclamptic pregnancies occurs as a result of histone deacetylase (HDAC) deficiency (121). HDACs are essential for trophoblast differentiation and are often downregulated in placentas from preeclamptic pregnancies (121–124). HDAC2 silencing in HTR-8/SVneo cells increases pyroptosis, which was prevented when PERK was knocked down (121). Therefore, PERK activation may underlie the increased incidence of cell death in placental pathologies.

To recapitulate the ER stress-inducing conditions that may induce PERK signaling and affect trophoblast function and viability, several *in vitro* models of cell stress have been devised. For example, exposure of BeWo cells to hypoxia/reoxygenation (fluctuating between room air and 1% O₂) leads to increased levels of phosphorylated eIF2 α levels and reduced cell number (39). IL-1 β , a pro-inflammatory cytokine that is increased in preeclampsia, induces apoptosis in BeWo cells through the PERK pathway, which is inhibited by progesterone (118). PERK inhibition can also reduce apoptosis in BeWo cells treated with endocannabinoid 2-arachidonoylglycerol (125). Cadmium, an environmental pollutant and carcinogen, reduces 11 β -HSD2 expression in JEG-3 cells, which is restored either by knocking down PERK expression or by treating cells with antioxidants such as melatonin or N-acetylcysteine (126, 127). Thus, signaling through PERK may regulate trophoblast survival under various ER stress-inducing conditions.

9 ATF6

ATF6 is the third ER transmembrane sensor that is activated in response to ER stress (128). There are two isoforms of ATF6: ATF6 α and ATF6 β . Signaling through ATF6 α generally results in a more potent but transient transcriptional response, whereas ATF6 β is a comparatively weaker transcriptional modulator (129). During ER stress, the cytosolic domain of ATF6 translocates to the Golgi apparatus to be cleaved by site 1 and site 2 proteases (130). These proteases remove the luminal and transmembrane portions of ATF6, yielding the ATF6 transcription factor. ATF6 binds to the ER stress response element motif to transcriptionally regulate chaperones and ERAD components that enhance protein folding and contribute to clearance of misfolded proteins, respectively (131, 132).

9.1 ATF6 in mouse placental and decidual development

While various aspects of the UPR have been studied in the context of placental development, the role of ATF6 remains largely unexplored, highlighting a gap in understanding its potential contributions to trophoblast function and placental health. Knockout of either ATF6 α or ATF6 β in mice produces viable progeny, but double ATF6 α/β knockout mice die early during development (around the time of implantation), suggesting that ATF6 α and ATF6 β may share an overlapping function that is essential for decidualization and early embryonic development in mice (133, 134). In the mouse uterus, expression of ATF6 α is primarily localized to luminal and glandular epithelial cells, specifically near the blastocyst implantation site, suggesting a potential role in uterine receptivity (135). Since ATF6 α is also present in primary and secondary decidualization zones, it is plausible that ATF6 α could contribute to the progression of decidualization. Indeed, artificial decidualization of pseudopregnant mice increases ATF6 α expression in decidual cells (135). Altogether, increased ATF6 α levels in the uterus seem to correlate with the implantation window and initial stages of decidualization in mice, but whether ATF6 α directly contributes to these processes requires further exploration.

9.2 ATF6 signaling in human placental and decidual development

ATF6 α is present in the human placenta, and staining is particularly strong in nuclei clustered in syncytial knots (90). Exposure of primary CTBs to an ATF6 α inhibitor, AEBSF, reduces cell fusion and hCG secretion (62, 136). It is possible that ATF6 α promotes STB formation by regulating expression of BiP/GRP78 (137). Additionally, since ATF6 α transcriptionally regulates *XBP1*, there may be contributory or compensatory mechanisms through which ATF6 α influences STB formation by supporting the IRE1-XBP1s axis (131). ATF6 α is also detected in EVT, suggesting its participation in the invasion of EVTs into the decidua and interactions with decidual cells (86). In cancer cells, ATF6 α promotes cell invasion and metastasis (138). Therefore, it is possible that ATF6 α may regulate genes involved in EVT migration and invasion, but this requires further investigation.

Decidual cells from healthy pregnancies exhibit high levels of ATF6 α localized primarily to the cytoplasm, indicating the presence of primarily inactive forms of ATF6 α (90). Decidualization of ESCs is associated with an upregulation of ATF6 α . Inhibition of ATF6 using AEBSF reduces the decidualization-associated increase in IL-1 β secretion, which was also reported when using an IRE1 inhibitor (87, 139). It is unclear whether the mechanistic control of inflammatory cytokine production by ATF6 α overlaps with the IRE1 signaling pathway.

9.3 ATF6 signaling in the placenta during pregnancy complications

In early-onset preeclampsia with FGR, ATF6 α protein levels are increased in placental tissue compared to placentas from pregnancies deemed healthy (39, 86). It is unclear if ATF6 α activation is also increased, as the levels of active ATF6 α , or nuclear localization of the protein, were not assessed. A hallmark of placentas from preeclampsia is reduced secretion of placental growth factor, which is a pro-angiogenic molecule important for maintaining endothelial integrity. Interestingly, reduced placental growth factor in preeclampsia is associated with nuclear localization of ATF4 and ATF6 β (but not ATF6 α or XBP1) in the STB layer (140, 141). Combined ATF4 and ATF6 β silencing in BeWo cells exposed to the ER stressor thapsigargin or hypoxia/reoxygenation increased expression of placental growth factor (141), suggesting that ATF4 and ATF6 β negatively regulate placental growth factor expression. Therefore, interfering with ATF4 and ATF6 β may be a feasible means to enhance placental growth factor production in compromised pregnancies.

10 Future applications: targeting ER stress to alleviate placental dysfunction

As highlighted in the preceding sections, placental development and function require a fine balance of ER stress and UPR activation to maintain cellular homeostasis. Too much or too little UPR activation can impair placental and decidual function and lead to adverse pregnancy outcomes. Therefore, therapeutic approaches targeting ER stress pathways may be promising avenues to restore proper placental function and improve fetal and maternal outcomes (34, 142). As one example, tauroursodeoxycholic acid (TUDCA), a bile acid derivative that is currently being evaluated for the treatment for neurodegeneration, alleviates ER stress by acting as a chemical chaperone and reducing the expression of ER stress-related proteins (143). In a rat model of advanced maternal age, placental insufficiency accompanied by increased ER stress markers (p-eIF2 α and CHOP) was observed in aged, pregnant rats. TUDCA treatment administered via drinking water throughout pregnancy reduced ER stress in the placenta and improved placental blood flow and fetal growth (38, 144–146). TUDCA treatment of pre-implantation embryos can increase the rate of blastocyst formation and enhance the success of implantation and pregnancy in mice (147). Another example includes phenylbutyric acid, a fatty acid that is naturally produced through fermentation by colonic bacteria, which ameliorates ER stress by acting as a chemical chaperone (148). Phenylbutyric acid reduces high glucose-induced ER stress in BeWo cells, suggesting that this compound may be useful to reduce placental ER stress in pregnancies with gestational diabetes (149). Additionally, phenylbutyric acid can also reduce hypoxia-induced

apoptosis in HTR-8/SVneo cells by assisting in protein folding and reducing PERK activation (150). Overall, the use of chemical chaperones to facilitate protein folding and prevent UPR hyperactivation may be a promising avenue to treat placental pathologies and improve fetal outcomes.

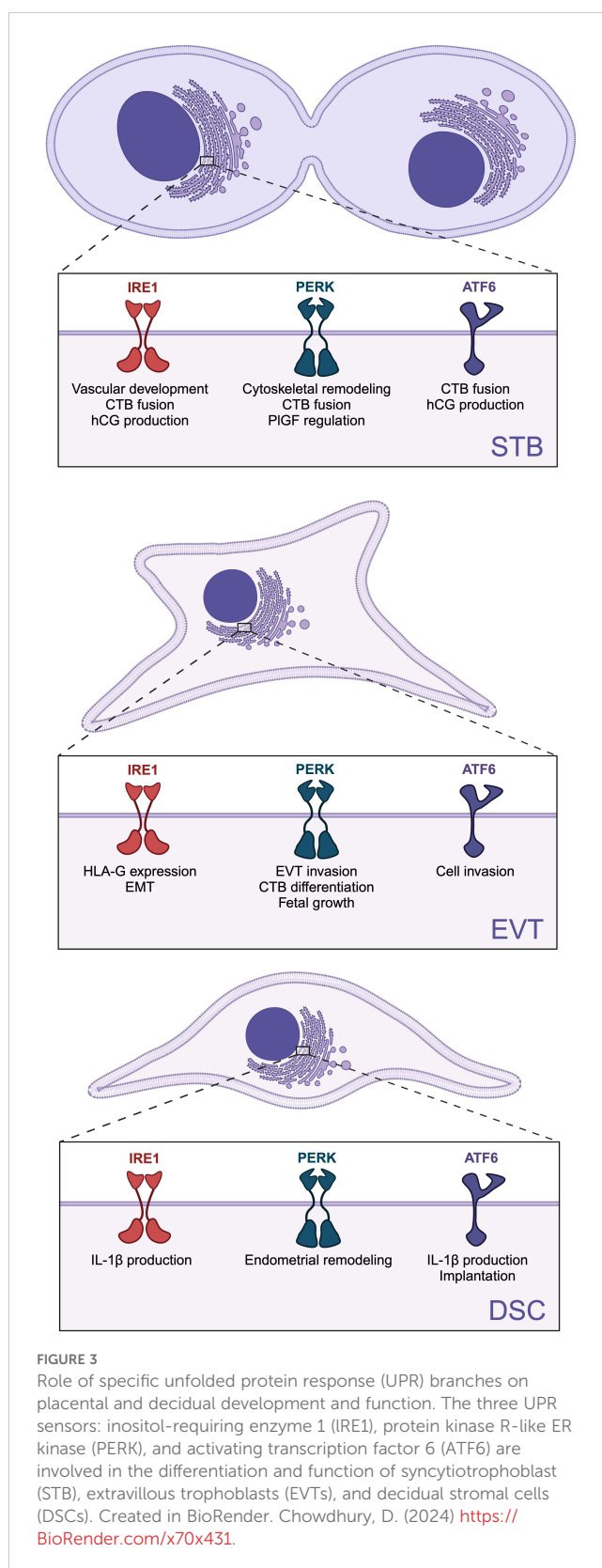
Future studies could also explore whether selectively targeting one or more UPR pathways is an effective way to improve placental function (39, 151, 152). There are several small molecule inhibitors that specifically target these pathways and are being tested in humans for their efficacy in disease settings like cancer (153–155), but it remains uncertain whether these inhibitors can be safely used during pregnancy. Unintended off-target effects and safe delivery strategies need to be considered. Conversely, since UPR pathways are essential for various aspects of placental and decidual function, it is possible that transiently stimulating one or more pathways may be beneficial to restore ER homeostasis and promote cell survival. More research is required to determine whether ER stress-related pathways can be safely modulated to fine-tune placental ER homeostasis.

11 Conclusion and perspectives

In this review, we highlighted the importance of ER homeostasis and UPR signaling for placental and decidual development and function. Although there is resounding evidence of increased ER stress and UPR hyperactivation during pregnancy pathologies, each UPR signaling branch contributes to basic processes needed for placental and decidual function, and these functions may shift depending on cellular conditions (Figure 3). Therefore, tweaking the activity of UPR branches to restore ER homeostasis holds promise as a treatment approach to improve placental function in compromised pregnancies.

Despite significant advances in understanding the role of UPR pathways in placental and decidual development, several gaps remain. It is apparent that UPR functions extend beyond merely responding to stress, as UPR sensors participate in homeostatic mechanisms to facilitate the high demand for protein synthesis and processing in secretory cells. However, the precise roles of each UPR branch in placental and decidual functions are still elusive. Determining their specific contributions can be challenging because there is significant overlap and compensation between the branches and other stress-related pathways. For instance, *Ern1*-deficient mouse placentas exhibit increased PERK and ATF6 activation, which may mask additional ways that IRE1 regulates placental development and function (52). As another example, kinases associated with the integrated stress response, which are not connected to the UPR, can activate eIF2 α independently of PERK (156). Therefore, phosphorylation of eIF2 α does not necessarily guarantee that the PERK branch of the UPR is active. Additionally, the source of ER stress and UPR activation during placental and decidual development remains elusive. The high demand for protein and lipid production could be contributing to ER stress and UPR activation during placental and decidual development.

Another challenge with assessing ER stress, particularly in the context of placentation, is the limited tools that are available to



confirm UPR branch activation. Many studies rely on detecting a handful of changes in gene expression or protein levels of UPR markers in bulk tissue or cell-lines. As the field evolves and better cell models and tools are developed, a more comprehensive

understanding of each UPR branch is likely to emerge. For instance, the use of more complex cellular models of the decidua and placenta, such as decidual and trophoblast organoid cultures or “on-a-chip” platforms, offer promising avenues for better representing cellular interactions at the maternal-fetal interface. The use of these models in combination with more precise detection of UPR activation, such as reporter plasmids to measure activation of specific UPR branches in combination with omics technologies for more comprehensive coverage of downstream UPR targets, may provide a deeper understanding of the cellular response to stress at the maternal-fetal interface. The use of CRISPR-mediated gene targeting to disrupt each ER sensor in trophoblast and decidual models will also be enlightening. Collectively, uncovering the role of UPR branches holds potential for a greater understanding of ER homeostasis during normal and pathological placentation.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fendo.2024.1525189/full#supplementary-material>

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Umbilical vein remodeling is associated with pregestational maternal overweight

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Introduction: Excess weight during pregnancy is a condition that can affect both mother and fetus, through the maternal-fetal interface, which is constituted by the placenta and umbilical cord. The umbilical vein is responsible for transporting oxygen and nutrients to the fetus, and its proper functioning depends on the integrity of its structure. The remodeling of the umbilical vein represents one of the causes of inadequate transport of nutrients to the fetus, being potentially harmful. This study aims to evaluate whether maternal overweight alters the structural characteristics of the umbilical vein.

Methods: Umbilical cords were collected from eutrophic and overweight pregnant women and were processed according to histological routine. We analyzed morphometry parameters, collagen and elastin fibers deposition, glycosaminoglycan level, and cell proliferation.

Results: Veins from overweight pregnant women were found to have greater total area, wall area, wall thickness, and diameter. There was higher collagen labeling in the perivascular region of the overweight group and a higher amount of type III collagen in the vascular smooth muscle. The proliferation of muscle and perivascular cells was higher in overweight pregnant women. A positive, although weak, correlation was observed between BMI and vessel thickness and with type III collagen deposition in vascular smooth muscle.

Discussion: With this study, we show that being overweight can structurally alter the umbilical vein, causing vascular remodeling of the vessel, through hypertrophy and hyperplasia.

KEYWORDS

collagen fibers, elastin fibers, glycosaminoglycans, KI-67, umbilical cord

1 Introduction

Pregestational maternal obesity is associated with unfavorable maternal and fetal outcomes, also favoring the developmental programming of obesity, diabetes, and cardiovascular diseases in early and later life (1, 2). Given the high incidence of obesity affecting pregnancies worldwide, it is imperative to understand the mechanisms involving the deleterious intrauterine environment upon obesity.

The umbilical cord (UC) connects the growing fetus to the placenta, supporting an environment required for a successful pregnancy (3), while it is an easily accessible structure reflecting changes in the intrauterine environment. In this regard, the UC has become an interesting target to investigate, considering its accessibility, almost exclusive fetal derivation, and the influence of maternal and fetal circulation on this component.

The UC circulation is composed of two arteries and a unique vein, all of them surrounded by a mucoid substance known as Wharton's Jelly (WJ). The umbilical vein carries oxygenated blood and other maternal nutrients to the fetus, while the two umbilical arteries carry low-oxygen blood and fetal metabolites to the placenta (4).

Structurally, the UC vessels display peculiar characteristics, differentiating them from most vessels in the body, since in these vessels, the tunica adventitia is replaced by perivascular cells located around the vessel, known as the perivascular Wharton's Jelly (PVWJ). This vascular layer has a high density of components including the mesenchymal cells and extracellular matrix components (ECM), standing out type I, III, and V collagen (5), proteoglycans, glycosaminoglycans (GAGs) and elastic fibers (6). This vascular arrangement builds up a strong structure, intending to protect these vessels from compression due to fetal movements and to guarantee the essential flow of substances (7).

Several physiological or pathological conditions may elicit alterations in the vascular architecture, leading to a process known as vascular remodeling. Interestingly, obesity favors vascular remodeling in several vessels (8). The overall mechanisms involved in vascular remodeling include proliferation and differentiation of vascular smooth muscle cells (VSMCs) degradation, disruption of elastic fibers, and ECM component deposition (9, 10).

The present work was designed to evaluate whether pregestational maternal overweight modifies the umbilical vein structure, eliciting its vascular remodeling.

2 Materials and methods

2.1 Participants

This is a case-control study. Pregnant women were recruited during prenatal visits at UNIMED Hospital from Jataí, Brazil. This study was approved by the Ethical Committee of the Federal University of Jataí and all participants provided written consent. Maternal body composition was assessed between 4–10 weeks of

pregnancy. Eligibility criteria included age ≥ 18 years, live newborn delivery; newborn with no fetal malformations on ultrasound and/or postnatal exam, and gestational age ≥ 37 weeks. Exclusion criteria included smoking, pre-eclampsia, diabetes, hypertension, artificial reproductive techniques, HIV, syphilis, infections with possible vertical transmission, and deliveries occurring outside the study hospital. The participants were grouped by their pregestational body mass index (BMI) into two groups: control (n=19), where BMI was between 18.5 and 24.9 kg/m²; and overweight (n=16), where BMI was greater than 25.0 kg/m².

2.2 Sample collection and processing

The UC samples were collected immediately at delivery, washed in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 24 hours. For histological analysis, the tissues were dehydrated, clarified and embedded in paraffin. Sections with a thickness of 5 μ m were adhered to glass slides, deparaffinized, rehydrated and stained for morphometric measurements with hematoxylin-eosin; picosirius for collagen deposition; resorcin-fuchsin for elastic fibers; alcian blue for glycosaminoglycans precipitation.

The collection of segments near the placental region was standardized due to the direction of fetal blood flow. The images were recorded using a light microscope with a digital camera (Nikon Eclipse E-200, Tokyo, Japan) and software (TCapture). The slides stained with picosirius were also submitted to polarized light microscopy (Zeiss Axioskop 2). All the images were evaluated using the ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

2.3 Morphometric and histochemical analysis

Vascular measurements were defined as: total vein area (TVA), lumen area (LA), vein wall area (VWA), vein wall thickness (VWT), vein diameter (VD) and wall/lumen ratio. Three images of each vessel were obtained, and the average between them was used as the final value for that sample (40x magnification). For TVA and LA measurements, the contour of the inner and outer vessel wall was evaluated. The subtraction of these measurements (TVA - LA) resulted in the VWA. For VWT, the distance between the outer and inner border of the vessel's muscular layer was measured in four different regions in the same vessel. The wall/lumen ratio was calculated by a simple division of wall area (mm²) and lumen area (mm²) for each sample. For the VD, two perpendicular measurements of each vein were used. Histochemical analysis of collagen, elastic fibers and glycosaminoglycans was performed by an average of ten images taken of perivascular region and smooth muscle layer (400x magnification). The tool "threshold" was used to set a limit of stain and standardize the protocol. The collagen birefringence analysis was conducted to evaluate type I and type III collagen fibers, according to the hue scale of the image, with values

set from 0 to 40 for type I (yellowish red) and 40 to 120 for type III (greenish yellow) (11).

2.4 KI-67 immunohistochemistry

Deparaffinized slides were subjected to antigen retrieval with sodium citrate buffer pH 6.0 (#ab64236, Abcam, USA) at a high temperature (95°C) to expose the epitopes. Next, blocking steps were performed with human serum (1:50 in PBS/BSA 1%) and CAS-Block™ (#008120, ThermoFisher, USA). Incubation with a specific KI-67 primary antibody (#ab15580, Abcam, USA, 1:300) was performed at 4°C overnight. Anti-Rabbit IgG biotinylated secondary antibody (#ab6012, Abcam, USA, 1:250) was used. ImmunoCruz® ABC Kit (#sc-516216, Santa Cruz Biotechnology, USA) was used for signal amplification and background reduction, according to the manufacturer’s instructions. Slides were revealed using the ImmPACT™ NovaRED™ Peroxidase (HRP) Substrate kit (Vector Labs, USA). The ImageJ plugin “Cell Counter” was used to count the number of Ki67 positive cells in 10 image fields in the same region, for each sample. Images were deconvoluted using the tool “Color Deconvolution” with “Colour_1” and “Colour_2” used for cell counting and staining intensity, respectively. Then, it was measured the intensity of the staining using the tool “Threshold”, according to the instructions from the “Semi-quantitative determination of protein expression using immunohistochemistry staining and analysis (Crowe and Yue, 2019). The intensity value detected was divided by the number of cells (stained or not) from its respective field, the average was calculated for each sample and presented as percentage (12).

2.5 Data analysis

Statistical analysis was performed using GraphPad Prism 8 (Graph Pad Software Inc). Clinical characteristics and histological analysis results were described for the entire sample and represented by mean ± SEM. The distribution of the variables was tested using the Shapiro-Wilk test. Correlation analysis was performed between the variables using Pearson’s linear coefficient. Differences between groups were compared using the Student t-test (parametric data) or Mann-Whitney test (non-parametric data) and values were considered statistically significant when p < 0.05.

3 Results

The current study comprises 35 pregnant women, who attended the inclusion and exclusion criteria, divided into control (n=19) and overweight group (n=16). The mean age among mothers and gestational age were similar between groups. BMI was reduced in control compared to the overweight group (p<0.0001). The newborn’s weight and the maternal weight gain during the pregnancy were similar between groups (Table 1).

TABLE 1 General data in the groups.

Parameter	Groups	Mean ± SEM	P value
Age (years)	Control	28.83 ± 1.34	0.516
	Overweight	30.06 ± 1.31	
Gestational Age (weeks)	Control	38.62 ± 0.14	0.214
	Overweight	38.92 ± 0.19	
BMI (kg/m²)	Control	22.27 ± 0.44	< 0.0001*
	Overweight	28.02 ± 0.71	
Newborn weight (kg)	Control	3.26 ± 0.15	0.273
	Overweight	3.45 ± 0.08	
Maternal weight gain (kg)	Control	12.31 ± 0.72	0.889
	Overweight	12.48 ± 0.98	

The difference between groups was assessed using the Mann-Whitney test. *p<0.05 vs. control. BMI, body mass index; SEM, standard error of the mean. Bold values indicate p<0.05.

3.1 Morphometric measurements of umbilical vein

Morphometric measurements are shown in Table 2 and the umbilical veins from groups are represented in Figure 1. Umbilical vein morphometric analysis revealed a significant increase in total area, vein wall area, diameter and thickness in the overweight group, compared to the control. The total lumen area, the wall/lumen ratio and diameter did not show significant differences when the two groups were compared between the groups.

3.2 Collagen and elastin deposition

Total collagen and elastin deposition was evaluated around the PVWJ and between the vascular smooth muscle (VSM) cells from the umbilical vein. Greater deposition of total collagen in the PVWJ from umbilical veins was observed in the overweight group, compared to the control. However, total collagen deposition between the VSM was similar between groups (Figure 2). Polarized microscopy was performed to access type I and III

TABLE 2 Values of umbilical cord vein morphometry analysis of control and overweight pregnant women.

Parameter	Groups	Mean ± SEM	P value
Total area (mm²)	Control	3.10 ± 0.22	0.04 *
	Overweight	4.16 ± 0.45	
Lumen areal (mm²)	Control	0.71 ± 0.11	0.385
	Overweight	0.86 ± 0.13	
Wall areal (mm²)	Control	2.43 ± 0.17	0.04 *
	Overweight	3.29 ± 0.36	
Wall/lumen area	Control	4.02 ± 0.37	0.519
	Overweight	4.40 ± 0.45	
Wall thickness (mm)	Control	0.48 ± 0.02	0.035 *
	Overweight	0.58 ± 0.04	
Diameter (mm)	Control	1.98 ± 0.07	0.034 *
	Overweight	2.31 ± 0.13	

The difference between groups was assessed using the Mann-Whitney test. *p<0.05 vs. control. SEM, standard error of the mean. Bold values indicate p<0.05.

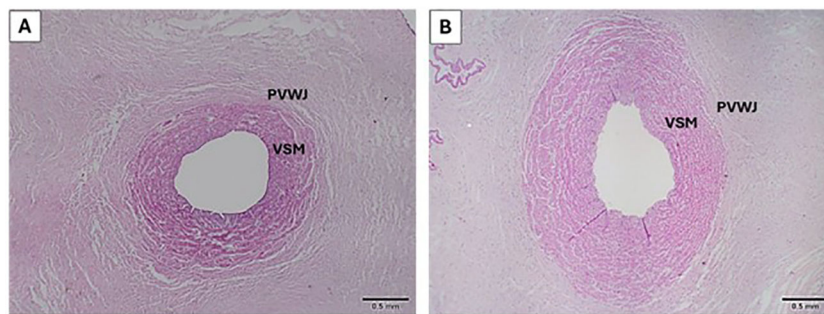


FIGURE 1
Representatives images of umbilical veins from control (A) and overweight (B) groups. PVWJ, Perivascular Wharton's Jelly; VSM, Vascular Smooth Muscle. H&E 40x.

collagen quantification. In the PVWJ region, type I and III collagens were similar between groups. Interestingly, collagen III was more abundant in VSM in the umbilical veins from the overweight group, compared to the control, and no differences were observed related to type I collagen in VSM between groups (Table 3, Figure 2).

Elastin deposition was smaller between VSM from the overweight group, compared to the control. No differences were observed in elastin deposition around PVWJ from the umbilical vein (Table 4, Figure 3).

3.3 Glycosaminoglycans deposition

The distribution of GAGs was evaluated in the perivascular and smooth muscle regions of the umbilical vein (Figure 4). There was no difference in GAGs deposition between the groups, as shown in Table 5.

3.4 Cell proliferation

Cell proliferation, accessed with positive labeling for the KI-67 antibody, was observed in the PVWJ and VSM from umbilical veins. In the overweight group, both regions showed a higher percentage of labeled cells when compared to the control group (Table 6), indicating greater cell proliferation in these regions in the group (Figure 5).

3.5 BMI and fetal weight correlation

The correlation test showed a positive association between BMI and vein thickness ($r=0.357$; $p=0.035$) and type III collagen in vascular smooth muscle ($r=0.567$; $p=0.022$), as shown in Figure 2 and Table 7. Fetal weight negatively correlated with the lumen area ($r=0.4038$; $p=0.027$) and positively correlated with the wall/lumen area ($r=0.4050$; $p=0.023$), Table 8. For those other parameters, no significant correlations were found.

4 Discussion

In the present study, it was demonstrated that the umbilical vein undergoes extensive remodeling in overweight women, increasing the caliber of this vessel through hypertrophy and hyperplasia. Furthermore, greater collagen deposition was observed in the perivascular region, with a predominance of type III collagen in the smooth muscle region, but without changes in glycosaminoglycan deposition. Additionally, a correlation between BMI and vein thickness and type III collagen in smooth muscle was established.

The umbilical vein plays a crucial role during pregnancy, being responsible for transporting blood rich in oxygen and nutrients from the placenta to the fetus (13). This vein connects the placenta to the fetal liver, where blood is distributed throughout the fetal body through fetal circulation. This ensures that the fetus receives the nutrients and oxygen necessary for proper development (14). The blood flow in the umbilical vein is influenced by the helical pattern of the umbilical vessels (15) with an internal pressure of around 2 to 9 mmHg, with an acceleration of 10-22 cm/s. Under normal conditions, the vascular wall responds to shear stress exerted by the blood through the production of vasoactive factors and cytokines (16).

Vascular remodeling occurs as a body response to different stimuli, both physiological and pathological. This process involves changes in the structure and function of blood vessels, such as arteries and veins, and can occur because of the maternal environment, including high blood pressure (17), hyperglycemia (18) and obesity. The vascular remodeling contributes to congenital malformations and reproductive losses, but the mechanisms related to these associations are still unclear (19).

Vascular smooth muscle cells alteration is related to vascular remodeling, since they may switch from contractile to synthetic phenotype (20). This plasticity is necessary to adapt to the different conditions of the vascular tissue upon signs of stress or injury (21). Recent studies have demonstrated that the synthetic phenotype is complex and may acquire characteristics of different mesenchymal lineages, including osteoblastic, chondrocyte and adipocytic (22). This implies that local factors present in the pathological

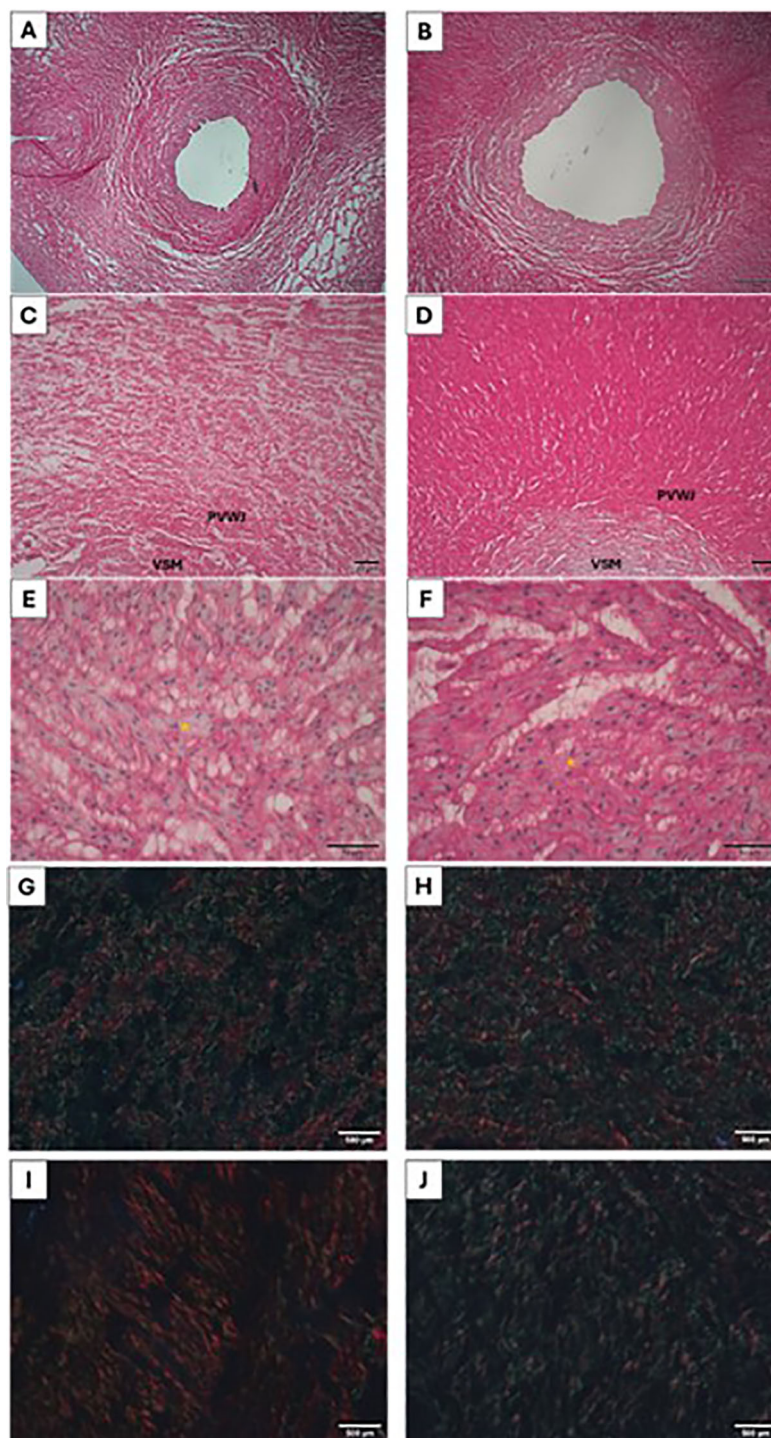


FIGURE 2

Micrograph of the umbilical vein for collagen analysis in the control (A, C, E, G, I) and overweight (B, D, F, H, J) groups. In (A, C), it is possible to observe less collagen deposition (red) in the perivascular region when compared to (B, D) (Picrosirius 40x and 100x). Collagen deposition between smooth muscle cells (red), nuclei stained with hematoxylin (yellow asterisks) [(E, F), (Picrosirius 400x)]. Type I (red) and type III (green) collagen deposition in the perivascular (G, H) and vascular smooth cells (I, J) regions, under polarized light (Picrosirius 200x).

environment probably drive part of this inadequate differentiation. The phenotypic switching contributes to changes in the primary function of these cells from regulation of vessel tone, blood pressure, and blood flow through cells focused on producing extracellular

matrix components, assuming a proliferative and migratory phenotype (23).

In pregnancies complicated by obesity, there may be dysregulation in proliferative control in umbilical cord cells. The percentage of cells

TABLE 3 Collagen deposition in the perivascular Wharton’s Jelly and around Smooth Muscle Cells.

Parameter	Groups	Mean ± SEM	P value
Total collagen PVWJ (% area)	Control	57.74 ± 0.02	<0.0001 *
	Overweight	71.08 ± 0.50	
Total collagen VSM (% area)	Control	13.74 ± 1.39	0.192
	Overweight	17.13 ± 2.12	
Collagen I PVWJ (% area)	Control	14.89 ± 2.05	0.509
	Overweight	12.66 ± 2.64	
Collagen III PVWJ (% area)	Control	6.32 ± 1.41	0.200
	Overweight	9.43 ± 1.91	
Collagen I VSM (% area)	Control	6.87 ± 1.36	0.122
	Overweight	4.34 ± 0.82	
Collagen III VSM (% area)	Control	3.48 ± 0.50	0.031 *
	Overweight	5.14 ± 0.54	

The difference between groups was assessed using the Mann-Whitney test. *p<0.05 vs. control. PVWJ, Perivascular Wharton’s Jelly; SEM, standard error of the mean; VSM, Vascular Smooth Muscle. Bold values indicate p<0.05.

TABLE 4 Elastin deposition in the perivascular Wharton’s Jelly and around Smooth Muscle Cells.

Parameter	Groups	Mean ± SEM	P value
Elastin PVWJ (% area)	Control	4.94 ± 0.90	0.375
	Overweight	3.93 ± 0.67	
Elastin VSM (% da area)	Control	0.71 ± 0.25	0.043 *
	Overweight	0.15 ± 0.07	

The difference between groups was assessed using the Mann-Whitney test. *p<0.05 vs. control. PVWJ, Perivascular Wharton’s Jelly; SEM, standard error of the mean; VSM, Vascular Smooth Muscle. Bold values indicate p<0.05.

positive for the cell proliferation marker, KI-67, was higher in the overweight group. In addition to the association with increased vessel caliber resulting from the need to adapt to the adverse environment, factors that are elevated in obesity, such as leptin, induce the proliferation of VSM cells, using the nuclear factor kappa B (NF-κB) and kinase pathways. regulated by extracellular signals (ERK) 1/2 (24). Hence, PVWJ displays some paracrine actions, and releases

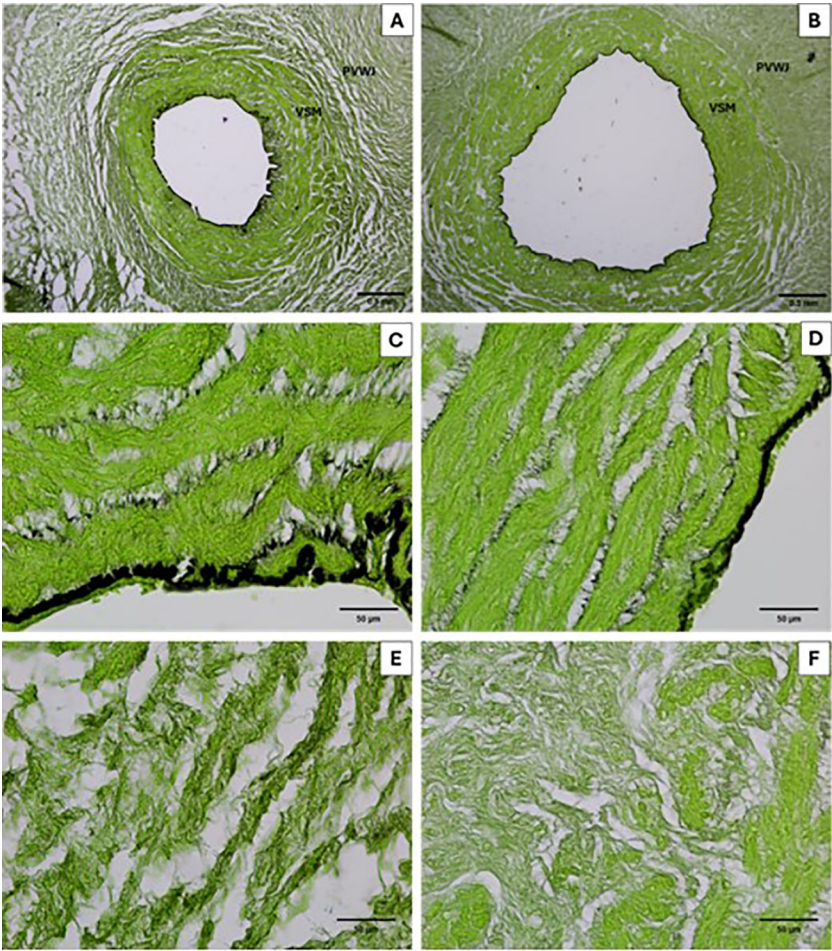


FIGURE 3 Micrograph of the umbilical vein for elastin analysis in the control (A, C, E) and overweight (B, D, F) groups. In (A, C), it is possible to observe more elastin deposition (black) in the vascular region when compared to (B, D) (Resorcin-fuchsin 40x and 400x). No difference were observed in the perivascular region (E, F).

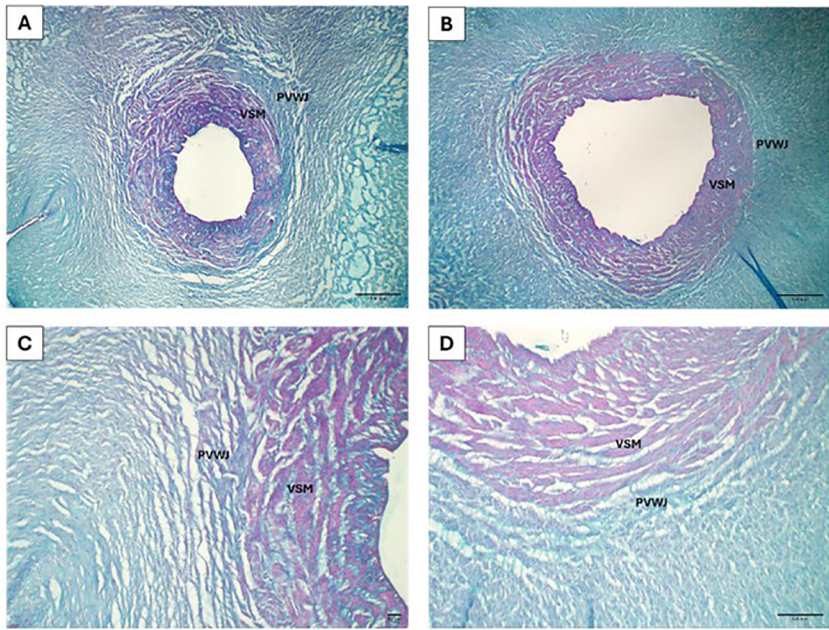


FIGURE 4 Micrograph of the umbilical vein for glycosaminoglycans analysis in the control (A, C) and overweight (B, D) groups. Veins from the control (A) and overweight (B) groups are observed, with the glycosaminoglycans stained in blue (Alcian blue, 40x). The delimitation between PVWJ and VSM is evident in the control (C) and overweight (D) groups (Alcian blue, 100x).

several substances including growth factors, cytokines, chemokines, exosomes, and microparticles (25), and in theory, these molecules may directly modulate VSM cell proliferation (26). This may occur even due to the interaction of endothelium and PVWJ, since it was demonstrated that the secretome of human endothelial cell-differentiated mesenchymal stem cell enhanced proliferation and wound healing *in vivo*, in a type-2 diabetes model (27). It was shown that maternal obesity negatively affects PVWJ, suggesting that epigenetic mechanisms may impact PVWJ mesenchymal stromal/stem cells since the methylation of CpG sites was enhanced in samples from obese women (27). However, new, more in-depth studies are necessary to highlight the molecular mechanisms involved in the VSM cell proliferation observed in the umbilical vein.

The presence of arterial stiffness is commonly reported during obesity, both in humans (28–30) and in murine studies (31, 32). The vascular mechanical properties are dependent on the quantity and organization of extracellular matrix components, mainly in relation to collagen and elastin (33).

Collagen is crucial to the normal function and longevity of blood vessels, providing the structure and strength necessary to withstand the demands of the circulatory system (34). Total collagen deposition was increased in the perivascular region of the umbilical vein, indicating a response to structural changes resulting from obesity, in addition to an increase in the proliferation of cells in this region. These findings are consistent with the literature, in which changes in vessel conformation in response to stress signals increase the deposition of extracellular matrix components and promote cell proliferation (35). Furthermore, type III collagen is increased in the VSM of the umbilical vein from overweight pregnant women. A similar observation was done in the umbilical vein of pregnant women with pre-eclampsia (36). Type III collagen has different characteristics from type I, as it does not have high density, its fibers are thinner and have less resistance, allowing the tissue to undergo greater distension (37). In this sense, we can infer that vascular adaptation occurs in the face of remodeling induced by

TABLE 5 Glycosaminoglycans deposition in the perivascular Wharton's Jelly and around Smooth Muscle Cells.

Parameter	Groups	Mean ± SEM	P value
Alcian Blue PVGJ (% area)	Control	56.32 ± 7.38	0.921
	Overweight	54.57 ± 10.12	
Alcian Blue VSM (% area)	Control	30.25 ± 3.98	0.228
	Overweight	22.65 ± 4.73	

The difference between groups was assessed using the Mann-Whitney test. PVWJ, Perivascular Wharton's Jelly; SEM, standard error of the mean; VSM, Vascular Smooth Muscle. Bold values indicate $p < 0.05$.

TABLE 6 Cell proliferation in umbilical veins from control and overweight groups.

Parameter	Groups	Mean ± SEM	P value
PVWJ (% stained cells)	Control	0.07 ± 0.02	0.008 *
	Overweight	1.72 ± 0.54	
VSM (% stained cells)	Control	0.14 ± 0.04	0.002 *
	Overweight	0.38 ± 0.06	

The difference between groups was assessed using the Mann-Whitney test. * $p < 0.05$ vs. control. PVWJ, Perivascular Wharton's Jelly; SEM, standard error of the mean; VSM, Vascular Smooth Muscle. Bold values indicate $p < 0.05$.

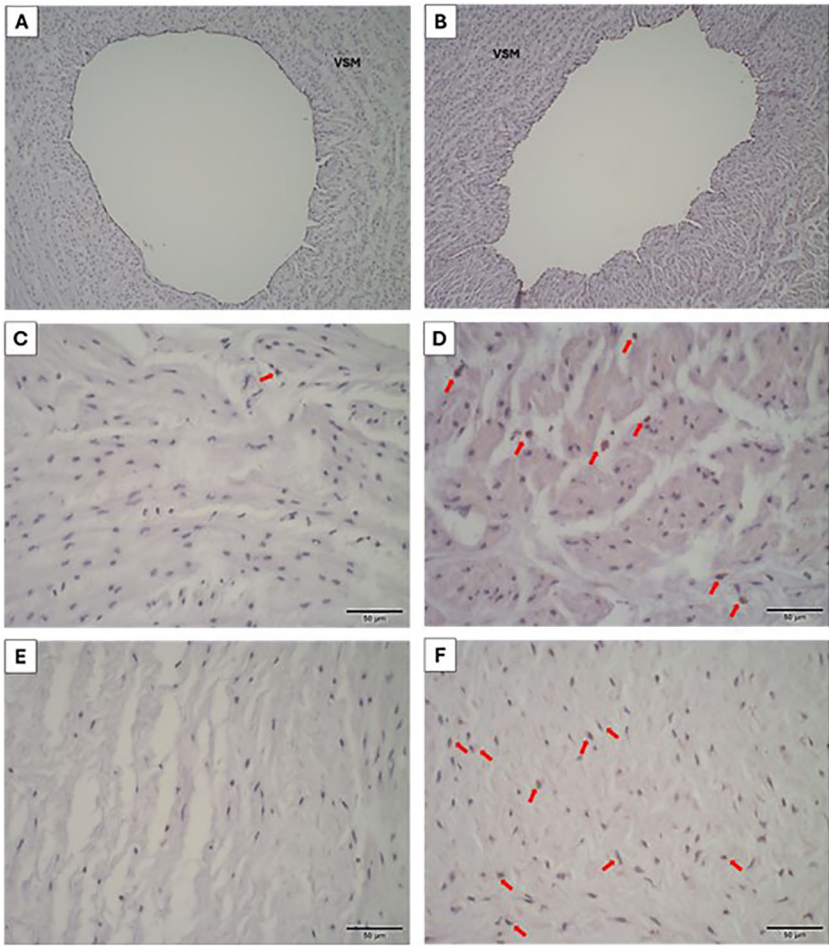


FIGURE 5
Cell proliferation in the umbilical vein from control (A, C, E) and overweight (B, D, F) groups. Positive staining (red arrows) for KI-67 in the VSM (C, D) and PVWJ (E, F) regions (IHC, 400x).

TABLE 7 Correlations values between BMI and umbilical vein histological parameters from the control and overweight groups.

Correlation	Spearman (r)	P Value
BMI vs. Total area (UV)	0.1999	0.249
BMI vs. Lumen area (UV)	0.01952	0.911
BMI vs. Wall thickness (UV)	0.2501	0.147
BMI vs. Ratio wall/lumen (UV)	0.1638	0.347
BMI vs. Thickness (UV)	0.3571	0.035 *
BMI vs. Diameter (UV)	0.2473	0.152
BMI vs. Picrosirius PVWJ	0.4072	0.054
BMI vs. Picrosirius VSM	0.3509	0.101
BMI vs. Type I Collagen PVWJ	-0.3134	0.237
BMI vs. Type III Collagen PVWJ	0.4604	0.073
BMI vs. Type I Collagen VSM	-0.1336	0.954
BMI vs. Type III Collagen MLV	0.5674	0.022 *

(Continued)

TABLE 7 Continued

Correlation	Spearman (r)	P Value
BMI vs. GAGs PVWJ	0.1189	0.547
BMI vs. GAGx VSM	0.01139	0.622

The difference between groups was assessed using the Mann-Whitney test. *p<0.05 vs. control. PVWJ, Perivascular Wharton's Jelly; r, Spearman correlation coefficient; UV, Umbilical Vein; VSM, Vascular Smooth Muscle. Bold values indicate p<0.05.

TABLE 8 Correlations values between fetal weight and umbilical vein histological parameters from the control and overweight groups.

Parameter	Spearman (r)	P value
Fetal weight x Total area (mm ²)	-0.1785	0.336
Fetal weight x Lumen area (mm ²)	-0.4038	0.027*
Fetal weight x Wall thickness (mm ²)	-0.1218	0.513
Fetal weight x Wall/lumen area	0.4050	0.023*
Fetal weight x Wall thickness (mm)	-0.0781	0.716
Fetal weight x Diameter (mm)	-0.1892	0.308

The difference between groups was assessed using the Mann-Whitney test. *p<0.05 vs. control. r, Sperman correlation coefficient; UV, Umbilical Vein. Bold values indicate p<0.05.

excess weight, in the same way that occurs in the brachial arteries of overweight individuals (38).

A comparison between the saphenous vein and the umbilical vein showed that the first one is stiffer, considering a greater amount of elastin and collagen (39). With this information in mind, the umbilical vein from the control group showed greater stiffness than the overweight group. This fact impacts on the capacity of the vessel to influence the blood flow. In other conditions such as preeclampsia, reduced elastin expression was observed in umbilical cord veins (40). Defective elastin is associated with disorganized elastic fiber deposition and VSM proliferation, causing arterial stiffening (41, 42).

Another extracellular matrix parameter analyzed was the glycosaminoglycans distribution. These molecules form an important network for the diffusion of substances, due to their hydrophilic characteristic, facilitating vessel nutrition (43). However, these polysaccharides also have an important effect on vascular mechanics, contributing to the elastic capacity of the tissue (44). In our data, there were no differences in the number of GAGs in the vascular wall or extracellular matrix. Analysis of the umbilical vein from preeclamptic women displayed similar results (45).

The alterations mentioned above may contribute to several undesirable scenarios regarding fetal health. For example, intrauterine growth restriction may occur due to decreased umbilical vein capacity to provide oxygen and nutrients. Yet, reduced nutritional/oxygen apport elicits commitment to organ development. Yet, fibrosis can increase the risk of hypoxia in the fetus, resulting in perinatal asphyxia, and impacting neurological development (46). Another complication due to low oxygenation and undernutrition is fetal stress, a condition that may be monitored during prenatal examination, leading to interventions that include early birth (47).

Furthermore, this study has limitations associated with the number of samples and collection origin, requiring a larger cohort, with a delimitation between overweight and obesity to infer the influences of each degree separately. Furthermore, here the components of the extracellular matrix were analyzed separately, and tests that demonstrate the interaction dynamics of these components can be used to better understand these findings.

In summary, our data show that maternal excess weight structurally alters the umbilical cord vessels, opening a range of possibilities to be explored in the field of umbilical cord histopathology associated with excess weight. Our findings have clinical relevance, as the context of obesity is common among pregnant women (48).

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 humans were approved by Ethical Committee of the Federal University of Jataí. The studies were conducted in accordance with the local legislation and institutional

requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

KS: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. LH: Data curation, Formal analysis, Investigation, Software, Writing – review & editing. FC: Data curation, Formal analysis, Investigation, Software, Writing – review & editing. GS: Formal analysis, Investigation, Methodology, Writing – review & editing. GC: Data curation, Investigation, Methodology, Writing – review & editing. JL: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. FA: Investigation, Methodology, Resources, Supervision, Writing – review & editing. FG: Conceptualization, Formal analysis, Resources, Supervision, Validation, Writing – review & editing. NL: Conceptualization, Resources, Supervision, Writing – review & editing, Formal analysis, Investigation, Methodology, Validation. PS: Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

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

The handling editor AED declared a past collaboration with the author FRG.

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Epigenetics of maternal-fetal interface immune microenvironment and placental related pregnancy complications

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Epigenetic regulation of placental development and pregnancy-related disease processes has recently been a hot research topic. Implantation and subsequent placental development depend on carefully orchestrated interactions between fetal and maternal tissues, involving a delicate balance of immune factors. Epigenetic regulation, which refers to altering gene expression and function without changing the DNA sequence, is an essential regulatory process in cell biology. Several epigenetic modifications are known, such as DNA methylation, histone modifications, non-coding RNA regulation, and RNA methylation. Recently, there has been increasing evidence that epigenetic modifications are critical for the immune microenvironment at the maternal-fetal interface. In this review, we highlight recent advances in the role of epigenetics in the immune microenvironment at the maternal-fetal interface and in epigenetic regulation and placenta-associated pregnancy complications.

KEYWORDS

epigenetics, DNA methylation, histone modification, non-coding RNA, RNA methylation, placental interface, immune microenvironment

1 Introduction

Pregnancy is a physiological state in which the maternal immune system smoothly tolerates hemizygous fetal tissues, and several immune mechanisms at the maternal-fetal interface act synergistically to protect the fetus from rejection (1). The percentage and function of immune cells *in utero* changes dynamically at different stages of pregnancy (2). During normal pregnancy, dynamic interactions between trophoblast cells and decidual immune cells are required to provide a suitable immune microenvironment for successful embryo implantation and normal fetal development (3). The most important immune cells in the human decidua include uterine natural killer (uNK) cells, different T-cell subpopulations, dendritic cells, and macrophages (4). In addition, humoral factors such as sex hormones and cytokines secreted by several immune and non-immune cells play an important role in immune tolerance and pregnancy maintenance (5). An in-depth understanding of the regulatory mechanisms of the immune microenvironment in fetal

and maternal tissues will help to understand better the pathophysiological characteristics of pregnancy-related complications such as infertility, miscarriage, and pre-eclampsia (PE), and at the same time, provide a foundation for improving adverse perinatal outcomes (6).

Epigenetics is defined as protein expression changes that occur without alterations in the DNA sequence. It can be stably transmitted during cell proliferation and organismal development and is closely linked to various pathophysiological processes (7). Epigenetics is a cutting-edge discipline that has gradually developed while studying many life phenomena that do not correspond to traditional genetics. The regulatory modes of action of this process include but are not limited to, DNA methylation, histone modification, non-coding RNA (ncRNA) modification, and RNA methylation. These modes of regulation act individually or interact with each other (8, 9).

The pivotal role of epigenetic regulation in developing the maternal-fetal immune microenvironment has garnered significant scientific interest, with many recent studies published. This paper aims to provide a review of the epigenetic mechanisms of the immune microenvironment at the maternal-fetal interface and an overview of recent advances in epigenetic regulation and placenta-associated pregnancy complications.

2 An overview of epigenetic inheritance

“Epigenetics” describes altering gene expression and function without altering the DNA sequence, resulting in heritable phenotypes. The concept of epigenetic inheritance was first proposed by Waddington in the journal *Endeavour* in 1942 (10). The primary focus of genetic research is the inheritance or transmission of genotypes, whereas the process by which genotypes produce phenotypes is the domain of epigenetics. The process of epigenetic inheritance encompasses a range of mechanisms, including DNA methylation, histone modification, the regulation of ncRNA RNAs, and RNA methylation (Figure 1). These mechanisms influence the functions and properties of genes, primarily by regulating their transcription or translation processes (11).

2.1 DNA methylation

DNA methylation is prevalent in the human genome, and the normal methylation state of DNA is essential for the typical structure and function of the organism's cells. DNA methylation, as a significant modality in the regulation of epigenetic mechanisms, refers to the process by which the 5th carbon atom on the CpG dinucleotide cytosine of a DNA sequence receives a methyl group from the methyl donor S-adenosyl methionine (SAM) using covalent bonding under the action of the enzyme DNA methyltransferase (DNMT). Methyl donor S-adenosyl methionine (SAM) is used to obtain a methyl group, and the product of this

reaction is called 5-methylcytosine (5-mC) (12). DNA methylation is associated with gene silencing, where methylation of CpG sequences in the promoter or non-promoter regions can inhibit promoter binding to transcription factors or be recognized and bound by methyl CpG binding domain (MBD), thereby directly or indirectly inhibiting gene expression (13). DNA methylation occurs mainly at CpG islands in eukaryotes and is mainly regulated by DNMTs and methyl-binding proteins. Five DNMT proteins have been identified in mammals, but only DNMT1, DNMT3A, and DNMT3B have methyltransferase activity (14). DNMT1 can maintain the methylation status of hemimethylated DNA, while DNMT3A and DNMT3B preferentially act on unmethylated and hemimethylated DNA, and the TET protein (ten-eleven translocation, TET) can convert 5-methylcytosine to 5-hydroxymethylcytosine in order to induce DNA demethylation (15). At the maternal-fetal interface, DNA methylation exhibits significant differences in immune cells, which are crucial for maintaining pregnancy immune tolerance and placental development (16–18). However, abnormal DNA methylation regulation may affect embryonic development ability and interfere with the immune microenvironment of embryo attachment and maternal-fetal interface, leading to adverse pregnancy outcomes. Studies have shown that the DNA methylation pattern in human placenta has a reliable and significant correlation with preeclampsia (PE) and gestational diabetes (GDM) (19). Especially in PE patients, the DNA methylation status of non-imprinting genes in the placenta undergoes significant changes, with genes involved in cell adhesion, proliferation, invasion, and other functions showing particularly prominent methylation abnormalities (20).

2.2 Histone modifications

Histone modification refers to acetylation, phosphorylation, methylation, ubiquitination, and other modifications at histone amino acid sites (21). These modifications affect the compactness and accessibility of chromatin in different ways, affecting gene expression and, consequently, all aspects of biological physiology and developmental processes. At the same time, histone modification involves many modification sites and enzymes. Any abnormal link can lead to the occurrence of diabetes in pregnancy, recurrent abortion, PE, and other diseases.

2.2.1 Histone acetylation

Acetylation of histones involves introducing acetyl groups to lysine residues in histone tails. This process is carried out by histone acetyltransferase (HAT), which adds the acetyl group to catalyze histone acetylation, and histone deacetylase (HDAC), which removes the acetyl group for deacetylation (22). Under normal conditions, histone acetylation is associated with transcription activation, whereas histone deacetylation is associated with gene silencing (23, 24). The level of acetylation is determined by the balance between HAT and HDAC activity and plays an essential role in chromatin remodeling and transcriptional regulation (25). Specifically, histone deacetylases 8 and 9 (HDAC8, HDAC9) are

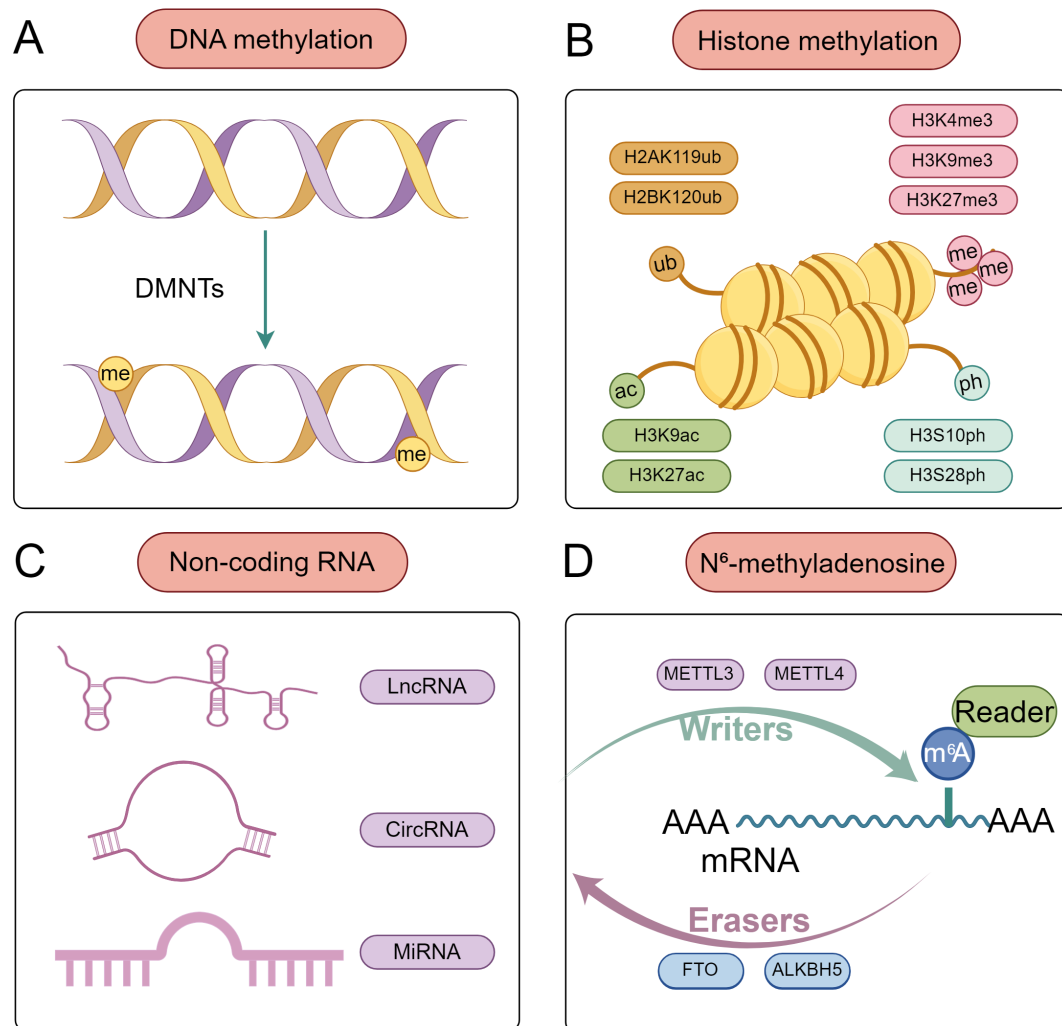


FIGURE 1

Common epigenetic modifications at the maternal-fetal interface. **(A)** DNA methylation. DNA methyltransferases (DNMTs) catalyze the covalent addition of a methyl group ($-\text{CH}_3$) to the fifth carbon atom of cytosine (**C**) in DNA molecules, forming 5-methylcytosine (5mC). **(B)** Histone modifications. These include acetylation, phosphorylation, methylation, and ubiquitination, which play critical regulatory roles at the maternal-fetal interface by modulating chromatin structure and gene transcription activity. **(C)** Non-coding RNA. MiRNA mainly inhibits gene expression by binding to the 3' untranslated region of mRNA; lncRNA is ncRNA with a length exceeding 200 nucleotides; CircRNAs are highly conserved circular ncRNAs that can act as miRNA sponges, bind to RNA binding proteins, or encode proteins. **(D)** N⁶-methyladenosine is the most common form of RNA methylation, which includes three regulatory systems: Writer, Eraser, and Readers. The Writer is responsible for catalyzing the addition of methyl groups to RNA, thereby promoting RNA methylation and ensuring its stability, such as METTL3 and METTL4. Eraser is responsible for mediating the demethylation modification process of RNA, such as FTO and ALKBH5. Readers can recognize and bind to the m⁶A site in mRNA.

involved in regulating M1/M2 polarization of macrophages, while HDAC1 and HDAC2 can promote differentiation and fusion of placental trophoblast cells by regulating the development of trophoblast cells (26, 27).

2.2.2 Histone methylation

Histone methylation is an important form of histone modification that involves adding one, two, or three methyl groups to certain amino acids in histones, known as mono-, bi-, and trimethylation, respectively. Although methylation can occur at many sites in histones, it mainly occurs at lysine (K) and arginine (R) residues in the tail (28–30). Here, we focus on histone lysine

methylation because of its importance and range of vital functions. Histone lysine methylation mainly occurs on histones H3 and H4, and the six loci of H3K4, H3K9, H3K27, H3K36, H3K79, and H4K20 have been studied more (31, 32). Usually, H3K4, H3K36, and H3K39 are involved in transcriptional activation, while H3K9, H3K27, and H4K20 are involved in transcriptional repression or silencing, and thus the biological effects will vary according to different methylation sites and methylation levels (33). At the maternal-fetal interface, the process of sensitization of trophoblast cells is closely related to the enhancement of various histone modifications, including H3K4 trimethylation (H3K4me3), H3K9 acetylation (H3K9ac), and H3K27 acetylation (H3K27ac). The

activation of natural killer cells (NK cells) is closely related to H3K4 monomethylation (H3K4me1) and H3K27 acetylation (H3K27ac) (33).

2.2.3 Histone phosphorylation

Histone phosphorylation is achieved by adding phosphate groups to amino acid residues in histone tails (34). Several protein kinases catalyze this histone modification, are closely related to the cell cycle, and can affect the transcriptional activation of DNA by mediating the recruitment of DNA damage repair proteins (35). Almost all histones can be phosphorylated at specific sites, and together with other post-translational modifications, they regulate a variety of biological processes (36).

2.3 Non-coding RNA

The human genome is highly active at the transcriptional level, but only about 1.9% of the gene sequence is transcribed into proteins, while the rest is transcribed into ncRNAs (37). ncRNAs mainly consist of microRNAs (miRNAs), long-stranded non-coding RNAs (lncRNAs), and cyclic RNAs (circRNAs) (38). Among them, miRNAs are between 19 and 23 nucleotide units in length and mainly play a role in silencing gene expression by antisense inhibition of the 3' untranslated regions (3'UTR) of mRNAs (39); lncRNAs are a type of ncRNAs with a length of >200 nucleotide units (40); circRNAs are a type of highly conserved ncRNAs that form a closed-loop structure with covalent bonds, and their mechanism of action mainly includes acting as miRNA sponges, binding to RNA-binding proteins to regulate transcription, and encoding proteins (41). **Supplementary Table 1** summarizes the association of Maternal-fetal interface immune cells with ncRNAs.

2.4 RNA methylation

RNA methylation is the most common type of RNA modification, including N6 methyladenosine (m6A), 5-methylcytosine (m5C), N7 methylguanine (m7G), and N1 methyladenosine (m1A) (42). Among them, m6A is the most abundant RNA modification, mainly located in the 3' untranslated region (3' UTR) near the mRNA protein coding sequence and stops codons, regulating gene expression and biological functions by controlling RNA metabolism, alternative splicing, degradation, and translation processes (43, 44). The m6A methylation modification regulatory system mainly includes three categories: Writer, Eraser, and Readers. The writer mainly promotes RNA methylation and maintains its stability, with the most common molecules being METTL3 and METTL4. Eraser is responsible for mediating the demethylation modification process of RNA, such as molecular FTO and ALKBH5. Readers can recognize and bind to the m6A site in mRNA, shortening the half-life of mRNA. As an important post-transcriptional regulatory mechanism, RNA methylation can significantly affect the stability,

intracellular localization, transport process, splicing mode, and translation efficiency of RNA (45). Research has shown that this epigenetic modification exhibits abnormal expression patterns in various reproductive system diseases, including pathological states such as PE, spontaneous abortion, endometriosis, and premature ovarian failure. The root cause of many pregnancy complications lies in poor implantation. Zhan Hong Zheng et al. (46) found that mice with Mettl3 deficiency were completely infertile due to implantation and decidualization failure, revealing that the m6A modification in the 5' UTR of Pgr mRNA mediated by METTL3 is closely related to the signal transduction of normal progesterone. In addition, the elevated level of RNA demethylase ALKBH5 in placental villus tissue of patients with recurrent spontaneous abortion (RSA) may be due to its ability to shorten the half-life of CYR61 mRNA in an m6A dependent manner and reduce the level of total m6A modification, affecting the invasiveness of the trophoblast and leading to miscarriage (47).

3 The role of epigenetic inheritance in the immune microenvironment at the maternal-fetal interface

The maternal-fetal interface consists mainly of decidua from the mother and trophoblast cells from the embryo. The interactions between decidual immune cells and trophoblast cells form a vast network of cellular connections. The decidual immune system is constituted by different subpopulations of maternal immune cells, including decidual natural killer (dNK) cells, macrophages, and T cells, among others. Modern reproductive immunology suggests that imbalances in the immune microenvironment at the maternal-fetal interface may be involved in the development of pregnancy-related diseases. Increasing evidence suggests that epigenetic modifications are critical to the immune microenvironment at the maternal-fetal interface, and studies on the correlation between the dynamic function and compositional changes of vital immune cells (NK cells, macrophages, and T cells) and trophoblasts at the maternal-fetal interface and epigenetics are described in detail below (Figure 2).

3.1 The role of epigenetics in maternal-fetal interface NK cells

3.1.1 Maternal-fetal interface NK cells

NK cells are intrinsic immune cells in the human body and are found in peripheral blood and endometrium (48). Among them, peripheral blood (pb) NK cells have two main subpopulations (CD56bright CD16dim and CD56dim CD16bright), and up to 90% of pNK cells are predominantly CD56dim CD16bright, and the primary function of pNK cells is highly cytotoxic (49). Compared to pbNK cells, dNK cells have a different phenotype; they resemble the CD56brightCD16dim pbNK subpopulation but have their unique characteristics, are mainly responsible for the production of cytokines and have poor cytotoxicity (50). Vento-

Tormo et al. (51) have identified three significant subpopulations of dNK cells. In early pregnancy, NK cells are present in large numbers at the maternal-fetal interface, accounting for 70% to 80% of the decidual lymphocytes, whereas in the human decidua, 10% to 15% of the cells are decidual lymphocytes (52). Dnk cells are multifunctional, secreting various growth factors, angiogenic factors, chemokines, and cytokines. These not only synergistically promote the metamorphosis process of the endometrium and the remodeling of the spiral arteries of the uterus, thus laying a solid foundation for the growth of placental blood vessels and the construction of the placenta, but also exhibit a unique immune-regulatory mechanism. NK cells guide the migration of trophoblasts toward the fetal membranes and spiral arteries while preventing trophoblasts from being destroyed, thus regulating trophoblast invasion into the uterine wall and ensuring that the whole process is safe and smooth (52, 53).

3.1.2 Maternal-fetal interface NK cells and DNA methylation

Decidual NK cells promote angiogenesis and trophoblast invasion and are closely associated with placental development (54). Epigenetic mechanisms such as DNA demethylation and histone acetylation have been shown to dynamically regulate gene transcriptional activity in immune cells, including CD8 (+) T cells and NK cells (18). However, research on the DNA methylation regulatory network in NK cells at the maternal-fetal interface is still limited. It is worth noting that the DNA methylation status is closely related to the activation level of NK cells: activated NK cells exhibit low methylation characteristics of CpG sites, while primitive NK cells maintain relatively high methylation levels, indicating that the methylation status of NK cells has significant plasticity and reproductivity (55). Hu et al. (17) found that DNA methylation in chorionic extravillous trophoblast (EVT) cells is regulated by dNK and the soluble molecules it secretes, which in turn affects EVT differentiation, adhesion, and migration. This discovery extends the function of dNK from classical immune regulation to epigenetic-mediated regulation of trophoblast development. In addition, Ana Sofia Cerdeira et al. (56) successfully induced dNK-like cell generation through pharmacological demethylation intervention, providing evidence for the core role of epigenetic mechanisms in dNK development. In summary, DNA methylation is not only a key epigenetic switch for the dynamic regulation of NK cell function but also affects the immune microenvironment and placental development at the maternal-fetal interface through dNK-mediated methylation remodeling. Future research needs to reveal the specific regulatory network of DNA methylation in dNK cells and its impact on pregnancy outcomes.

3.1.3 Maternal-fetal interface NK cells and histone modification

Histone modifications are essential to promote normal NK cell development, and the histone H2A deubiquitinase MYSM1 regulates NK cells by controlling transcription factors, the lack of which severely impairs NK cell development (57). Giuseppe Sciumè et al. (58) found that activation of NK cells induced enhancer

regions to H3K4me1 and H3K27ac histone modifications. Wiedemann et al. (59) used a multi-omics approach to analyze the complex interactions between cytokine signaling pathways in NK cells and found that IFN - α can enhance the epigenetic modification of the H3K4me3 promoter site. Ma et al. (60) demonstrated that SPA-VSMC has the potential to transform into CD56 dNK by identifying a small portion of dNK modified with H3K4me2 in the myosin heavy chain 11 (MYH11) promoter region through immunofluorescence-DNA *in situ* hybridization-neighbor-joining and chromatin immunoprecipitation experiments, which is the first demonstration that SPA-VSMC has the potential to transform into CD56 dNK. Zhang et al. (61) showed that ubiquitin C-terminal hydrolase L1 (UCHL1) can regulate decidualization through the JAK2/STAT3 signaling pathway. When UCHL1 is deficient, it can cause damage to decidualization during pregnancy in mice, leading to miscarriage, and is associated with a decrease in the number of decidual natural killer cells (dNKs). Through the above research, we can gain a deeper understanding of the epigenetic regulatory mechanisms of NK cells, as well as the pathogenesis of related diseases such as miscarriage.

3.1.4 Maternal-fetal interface NK cells and non-coding RNAs

In recent years, multiple studies have revealed the important role of NK cells in pregnancy and their epigenetic regulatory mechanisms. Gamliel et al. (62) discovered a unique phenotype and transcriptional features of NKG2C high NK cell population in the decidua of women with multiple pregnancies. These cells showed increased accessibility at the *Ifng* and *Vegfa* loci and exhibited more vigorous IFN - γ and VEGF-A secretion. It provides important clues for understanding how microenvironmental changes during pregnancy induce epigenetic reprogramming of NK cells. In terms of miRNA regulation of NK cells, research (63) found through comparative analysis that 36 miRNAs were expressed explicitly in decidual NK cells. In comparison, two miRNAs were only expressed in peripheral blood NK cells, revealing molecular differences in NK cells from different sources. Another study (64) through miRNA profiling analysis of decidual NK cells from patients with unexplained recurrent spontaneous abortion (URSA), 50 differentially expressed miRNAs were identified, of which 49 were upregulated, and 1 was downregulated, indicating a close association between abnormal miRNA expression and URSA. Specifically, researchers have found through *in vitro* experiments that upregulated miR-30e can reduce the cytotoxicity of peripheral blood and decidual NK cells by targeting PRF1, inhibit Th1 tolerance phenotype, and induce Th2 immune dominance, thereby promoting the formation of a micro immune tolerance environment at the maternal-fetal interface (65). In addition, miR-185-5p is involved in the occurrence of RSA by interfering with VEGF expression and angiogenesis in decidual NK cells (66). Fang et al. (67) found that a decrease in miR-29a-3p levels in villous-derived exosomes of patients with URSA can increase IFN - γ levels in decidual NK cells. *In vivo*, experiments in mice revealed that vEXO carrying

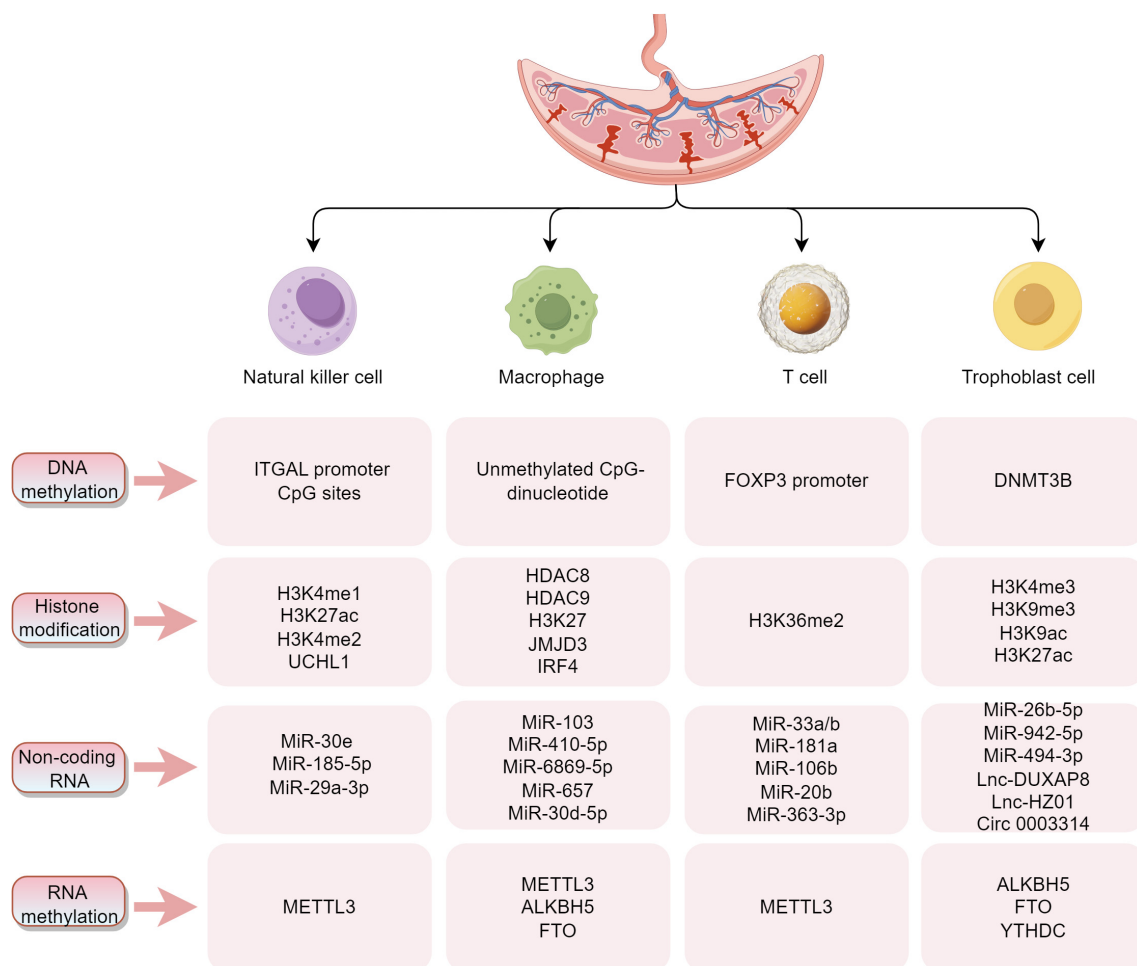


FIGURE 2

Summarizes the key molecules and modification sites associated with epigenetic regulation in four critical cell types (NK cells, macrophages, T cells, and trophoblasts) at the maternal-fetal interface. These epigenetic modifications, encompassing DNA methylation, histone modifications, non-coding RNAs (ncRNAs), and RNA methylation, play pivotal roles in regulating immune tolerance at the maternal-fetal interface and supporting placental development.

miR-29a-3p can reduce embryonic resorption in RPL mice. However, despite significant progress in these studies, the epigenetic regulatory mechanisms of decidual NK cells have not been fully elucidated. Further in-depth research is needed regarding the proportion, functional status, and relationship with epigenetic regulatory mechanisms of decidual NK cells.

3.1.5 Maternal-fetal interface NK cells and RNA methylation

In METTL3 deficient NK cells, the absence of m6A modification leads to a decrease in SHP-2 protein expression, inhibiting the activation of AKT and MAPK signaling pathways, and significantly reducing NK cell responsiveness to IL-15. In addition, the absence of METTL3 disrupts the homeostasis of NK cells. It inhibits their infiltration and function in the tumor microenvironment, ultimately accelerating the development of mouse tumors and shortening their survival period (68). Although the function of RNA methylation (especially m6A

modification) in immune cells is gradually being revealed, research on its role in natural killer cells (NK cells) at the maternal-fetal interface is still relatively limited. Mother-fetal interface NK cells (NK cells) are crucial in early pregnancy embryo implantation, placental formation, and maternal-fetal immune tolerance. However, their functional regulatory mechanisms still need further exploration.

3.2 The role of epigenetics in maternal-fetal interface macrophages

3.2.1 Maternal-fetal interface macrophages

Decidual macrophages are the second largest immune cell population (10%-20%) in the uterine decidua in early human pregnancy, second only to the percentage of dNK cells (60%-80%) (69). Notably, macrophages can be categorized into classically activated (M1) and alternatively activated (M2) phenotypes based

on their function and the types of cytokines they produce (70). M1 macrophages are pro-inflammatory and are characterized by the production of higher levels of IL-12 and IL-23, as well as lower levels of IL-10, thus killing intracellular microbes and inducing Th1 immunity. In contrast, M2 macrophages produce higher levels of IL-10 and lower levels of IL-12 and IL-23 and perform functions such as anti-inflammatory and tissue remodeling, as well as the removal of apoptotic cells and debris (71). At the maternal-fetal interface, the number and ratio of M1/M2 macrophages change during different gestation periods. Specifically, M1-type macrophages predominate during the precomputation period, followed by a transition to a mixed population of M1 and M2 types; after the establishment of the placenta, M2-type macrophages dominate (72). Evidence suggests that an imbalance of M1/M2 macrophages leads to a pro-inflammatory microenvironment in the endometrium, which is not conducive to inducing fetal tolerance and further leads to pregnancy-related disorders such as recurrent miscarriages (73, 74). M1 and M2 macrophages play different roles at different gestational stages; thus, the maintenance of a normal pregnancy requires that the M1/M2 macrophage is in a state of equilibrium.

3.2.2 Maternal-fetal interface macrophages and DNA methylation

A study (16) conducted genome-wide methylation analysis on maternal monocytes, fetal monocytes, decidual macrophages, and fetal placental macrophages (Hofbauer cells) using Illumina Infinium Human Methylation 27 BeadChip technology and found significant differences in DNA methylation among these cell populations. Notably, genes related to immune response are highly methylated in fetal cells, while maternal cells exhibit different methylation patterns. Meanwhile, excessive pro-inflammatory reactions at the maternal-fetal interface may jeopardize pregnancy maintenance. At the same time, DNA methylation may play a protective role in this process by regulating the anti-inflammatory function of macrophages. In premature birth studies, genome-wide placental DNA methylation analysis combined with methylated DNA immunoprecipitation sequencing (MeDIP-seq) technology revealed the gene functional characteristics of differentially methylated regions (DMRs), with a significant enrichment of Fc- γ receptor-mediated macrophage phagocytosis related pathways (75). This discovery suggests that DNA methylation may be involved in the mechanism of premature birth by regulating the phagocytic function of macrophages. In addition, unmethylated CpG dinucleotides derived from microorganisms can activate Toll-like receptor 9 (TLR-9), and these DNA activators exert phagocytic and clearance functions through the production of intrauterine immune cells (such as macrophages), thereby affecting the immune microenvironment at the maternal-fetal interface (76). These studies collectively reveal the important role of DNA methylation in regulating macrophage function at the maternal-fetal interface and maintaining pregnancy, providing a new perspective for a deeper understanding of the epigenetic mechanisms of pregnancy-related diseases.

3.2.3 Maternal-fetal interface macrophages and histone modification

It was found that the expression of HDAC8 was reduced in decidual macrophages from patients with RSA, and the knockdown of HDAC8 inhibited M2 macrophage activation and promoted apoptosis of differentiated THP-1 (dTHP-1) macrophages through ERK pathway (26). Meng et al. (71) reported that nuclear factor- κ B ligand (RANKL) receptor activator, secreted by human embryonic trophoblasts and maternal decidual stromal cells, polarizes decidual macrophages to an M2 phenotype, which is mediated by activation of Akt/signal transducer and activator of transcription factor 6 (STAT6) signaling, and correlates with the up-regulation of the histone H3 lysine 27 demethylases, Jmjd3 and IRF4, in decidual macrophages. Liu et al. (77) found that Hdac9/HDAC9 deficiency promotes macrophage polarization toward M2 macrophages, while Hdac9/HDAC9 ablation significantly enhanced phagocytosis of fluorescent microspheres in M2 Raw 264.7 cells, but reduced the capacity of THP-1-derived M1 macrophages. These studies collectively reveal the key role of histone modifying enzymes in macrophage polarization and functional regulation.

3.2.4 Maternal-fetal interface macrophages and non-coding RNAs

3.2.4.1 miRNAs

NcRNA can regulate macrophage function and M1/M2 polarization, closely related to the occurrence and development of various pregnancy-related diseases. Some *in vitro* experimental studies have shown that specific miRNAs affect macrophage polarization by regulating key signaling pathways. For example, miR-103 and miR-410-5p inhibit the STAT1-mediated signaling pathway, preventing polarization of M1 macrophages and preventing RSA (78, 79). MiR-6869-5p induces M2 polarization in gestational diabetes by regulating PTPRO (80). On the contrary, miR-657 promotes gestational glucose by targeting FAM46C (81). In addition, miRNA-30d-5p derived from placental exosomes induces M2 polarization by inhibiting HDAC9 expression (82). The low-level miR-455-3p in decidual stromal cells inhibits the invasion of trophoblast cells by promoting macrophage polarization (83). MiR-146a-5p has been shown to increase embryo resorption rate and promote M2 polarization of macrophages in URSA (84). The article also briefly introduces the relationship between some miRNAs and macrophages (85).

3.2.4.2 lncRNA

lncRNAs also participate in the regulatory network of macrophage polarization. Wu et al. (86) found that the expression of AOC4P was significantly up-regulated in trophoblast cells of patients with RSA. This molecule inhibits the degradation of EZH2 by regulating TRAF6, thus inhibiting glycolysis in trophoblast cells and actively participating in the polarization process of M2 macrophages. Xiujun Li et al. (87) confirmed through *in vitro* experiments that lncRNA MALAT1 not only regulates the proliferation and angiogenesis of mesenchymal stem cells (MSCs) but also promotes M2 polarization by regulating the expression of

indoleamine 2,3-dioxygenase (IDO). LINC00240 could promote macrophage polarization toward the M2 type by regulating the miR-155/Nrf2 axis (88). The knockdown of LINC00221 negatively regulated the expression of miR-542-3p in trophoblasts to reduce macrophage migration and invasion (89). These studies provide a new perspective for elucidating the regulatory role of ncRNA in macrophage polarization. However, most current research is limited to *in vitro* experiments. The mechanism of action of lncRNA is highly complex, and its function may vary significantly depending on cell type and pathological state. Therefore, in the future, more *in vivo* experiments are needed to verify its biological functions and further explore its regulatory mechanisms and therapeutic potential in diseases by combining clinical samples.

3.2.5 Maternal-fetal interface macrophages and RNA methylation

METTL3 participates in macrophage regulation through different mechanisms as a “writer” in RNA methylation. Low expression of METTL3 can inhibit the degradation of NOD1 and RIPK2 mRNA mediated by YTHDF1 and YTHDF2, thereby upregulating the NOD1 pathway and subsequently promoting LPS-induced macrophage inflammatory response (90). Another study showed that METTL3 could promote oxLDL-induced macrophage inflammatory response by activating STAT1 signaling (91). Zhao et al. (92) co-cultured ALKBH5 overexpressing cell lines with THP-1 and found that the N6 methyladenosine regulatory factor ALKBH5 damaged macrophage recruitment and M2 differentiation by reducing VEGF secretion in stromal cells, leading to miscarriage. Meanwhile, inhibiting VEGF signaling can help discover changes in macrophage polarization in different pregnancy complications (93). In addition, Cox analysis found that M2 macrophages were positively correlated with the m6A regulatory factor FTO and negatively correlated with CBL1 (94).

3.3 The role of epigenetics in maternal-fetal interface T lymphocytes

3.3.1 Maternal-fetal interface T lymphocytes

In early pregnancy, 30% to 45% of the decidual T cells are CD4⁺ T cells at the maternal-fetal interface, and 45% to 75% are CD8⁺ T cells (95). Under normal conditions, a dynamic balance is maintained between CD4⁺ and CD8⁺ T lymphocytes (96). CD4⁺ T cells are mainly helper T cells (Th) and regulatory T cells (Treg) (97). CD4⁺ helper T cells (Th) are classified into Th1, Th2, and Th17 cells according to the type of cytokines they secrete (98). Th1 cytokines promote macrophage activation and cytotoxicity and mainly mediate cellular immune responses (99). Th2 cytokines mediate humoral immune responses, promote eosinophil and mast cell differentiation, inhibit immune inflammation, reduce excessive damage, and have immunotoxic effects (100). After conception, maternal recognition of the fetus and maintenance of pregnancy is achieved through the immune balance between Th17/Treg, Th1/Th2 cells, and the dominant Th2-type cells (101). The large amount of Th2-type

cytokines at the maternal-fetal interface inhibits the production of Th1-type cytokines through a negative feedback effect, suppresses TDTH and CTL cell activity, and thus inhibits rejection (102). Some subpopulations of maternal-fetal interface T cells help EVT invade the endometrium and promote embryo implantation and placenta formation, whereas other subpopulations are closely associated with pregnancy complications (103). The classical pattern of immune regulation in human pregnancy is a shift in maternal immune response from an inflammatory Th1 cytokine pattern to a Th2 pattern, with maternal-fetal interface decidual T cells playing a crucial role in regulating the placental microenvironment and recognizing fetal antigens (104).

3.3.2 T-lymphocytes and DNA methylation at the maternal-fetal interface

Research has shown that the methylation levels of Th1/Th2 pathway genes exhibit dynamic changes at different stages of pregnancy. During normal pregnancy, the methylation levels of Th2 pathway genes change more significantly in the early stages of pregnancy. In contrast, the methylation levels of Th1 pathway CpG genes change more frequently in the late stages of pregnancy, suggesting that the temporal regulation of Th1/Th2 gene methylation is closely related to the maintenance of pregnancy (105). Abnormal DNA methylation can participate in the occurrence of pregnancy-related diseases through multiple pathways. It can disrupt the immune balance at the maternal-fetal interface by regulating the proliferation, differentiation, and cellular activity of Th and Treg cells, thereby promoting pathological processes such as recurrent RSA (106). Comparative analysis reveals that RSA patients exhibit abnormal FOXP3 promoter hypermethylation in decidual tissues, resulting in suppressed FOXP3 expression and subsequent disruption of maternal-fetal immune tolerance, potentially representing a key molecular pathway in RSA (107).

3.3.3 T-lymphocytes and histone modification at the maternal-fetal interface

Histone methyltransferase Nsd2 was found to upregulate CXCR4 expression through H3K36me2 modification, promoting Treg cell recruitment into the decidua to ensure maternal-fetal immune tolerance (108).

3.3.4 T-lymphocytes and non-coding RNA at the maternal-fetal interface

Schjenken et al. (109) found that miR-155 is required to expand regulatory T cells to mediate robust pregnancy tolerance in mice. Notably, the expression of miR-33a/b and miR-181a was significantly down-regulated in patients with RSA and led to a reduction in the number of Treg cells by negatively regulating the expression level of the vital molecule sphingosine-1-phosphate receptor-1 (S1PR1) (110). In patients with PE, miR-106b inhibits Treg differentiation by suppressing TNF- β expression, while miR-20b and miR-363-3p upregulate TH17 cell transcription factor ROR γ t/STAT3 and enhance TH17 activity, respectively, leading to Treg/TH17 imbalance (111). It is worth noting that environmental factors can also interfere with ncRNA networks. For example, Jamie L. McCall

et al. (112) confirmed that prenatal cadmium exposure activates CD4⁺T cells in offspring by upregulating lncRNA Snhg7, suggesting that exogenous toxic substances may participate in the pathological process of pregnancy through epigenetic mechanisms.

3.3.5 T-lymphocytes and RNA methylation at the maternal-fetal interface

METTL3 deficient CD4⁺ T cells disrupt the homeostasis and differentiation of immature T cells (113). By hybridizing Mettl3^{f/f} mice with Foxp3Cre YFP mice and specifically knocking out Mettl3 in Tregs, the results showed that the offspring mice's peripheral lymph nodes and spleen significantly increased and developed severe autoimmune diseases and infertility. Mechanism studies have shown that the absence of Mettl3/m6A leads to an increase in Socs mRNA levels, inhibiting the IL-2-STAT5 signaling pathway, which is crucial for the function and stability of Treg cells (114). In addition, METTL3-dependent m6A methylation plays a critical role in regulating follicular helper T cell (TFH) lineage differentiation. Research has found that the absence of METTL3 in CD4⁺T cells impairs the function of TFH cells, leading to a significant reduction in germinal center (GC) response in METTL3 deficient mice after acute viral infection (115).

3.4 The role of epigenetics in trophoblasts at the maternal-fetal interface

3.4.1 Trophoblast cells at the maternal-fetal interface

EVT are one of the main coordinators of immunity at the maternal-fetal interface (116), actively creating an environment for tolerogenic phenotypes by expressing a unique set of major histocompatibility complex (MHC) molecules (117).

3.4.2 Trophoblasts and DNA methylation

DNA methylation in trophoblast cells continues to change dynamically after implantation and throughout pregnancy, with the differentiation of cytotrophoblasts into syngeneic trophoblasts and the acquisition of an invasive phenotype in EVT, both of which involve extensive DNA methylation changes (118). The study (119) identified ZBTB24 as an epigenetic regulator that modulates E-cadherin expression and, thus, cell viability, differentiation, and migration in trophoblasts by altering DNA methylation in the promoter region. In addition, dNK can regulate trophoblast function by altering gene expression through DNA methylation (120). In summary, these findings reveal the core regulatory role of DNA methylation in nourishing cells and pregnancy maintenance at the maternal-fetal interface.

3.4.3 Trophoblast and histone modification

During the sensitization process of BeWo trophoblast cells, histone markers associated with active transcription (H3K4me3, H3K9ac, and H3K27ac) significantly increased, while the levels of inhibitory histone modifications (H3K9me3 and H3K27me3) remained unchanged (121). This process is consistent with increased mRNA levels of EP300 and P300/CBP-related factors

(PCAF). The binding of RNA polymerase II to specific gene promoters is enhanced during the process of trophoblast differentiation, which is closely related to active histone markers. Meanwhile, the downregulation of pro-inflammatory transcription factors after differentiation of multinucleated syncytiotrophoblasts is associated with reduced enrichment of H3K27Ac and H3K9Ac promoters, as well as enhanced binding of H3K9me3 to histone deacetylase 1 (122). Histone deacetylases 1 and 2 regulate the development of extrachorionic trophoblast cells and promote the differentiation and fusion of human placental trophoblast cells (27). In addition, glycolytic metabolism is involved in regulating the function of trophoblast cells. Yu et al. (123) found that glycolysis exhibited high activity in human trophoblast stem cells and cytotrophoblast cells, whereas it was significantly reduced in syncytiotrophoblast cells. However, when supplemented with the glycolytic derivative acetyl coenzyme A, the fusion function of the syncytiotrophoblast was restored. Of particular note, acetylation of H3K9, H3K18, H3K27, and H4K16 was particularly sensitive to glycolysis during syncytiotrophoblast fusion with the metabolism of acetyl-coenzyme A. These modifications regulated the promoters of relevant genes involved in syncytiotrophoblast fusion and metabolism of syncytiotrophoblast cells. In terms of pathological mechanisms, an *in vitro* experiment found that knocking down ACLY disrupts histone acetylation and IL-10 secretion in nourishing cells, thereby inhibiting M2 polarization of macrophages. This process is involved in the pathogenesis of RSA (124). Sumi et al. (125) found that LPS treatment increased SIRT1 expression in mouse placental tissue and human trophoblast cells and inhibited NLRP3 inflammasome activation in trophoblast cells by reducing oxidative stress, thereby reducing lipopolysaccharide-induced IL-1 β levels. These studies collectively reveal the important role of histone modifications in the differentiation, metabolic regulation, and pathological processes of trophoblast cells.

3.4.4 Trophoblasts and non-coding RNAs

MiRNAs are equally important in regulating trophoblast function, and their abnormal expression is closely related to pregnancy-related diseases. Liang et al. (126) systematically summarized miRNA regulation on trophoblast function and its related signaling pathways, providing a theoretical basis for understanding the mechanisms of pregnancy diseases. Meanwhile, lncRNA also participates in trophoblast dysfunction through various mechanisms. The long noncoding RNA DUXAP8 is significantly upregulated in the placental tissue of PE and can inhibit biological functions such as proliferation, migration, and invasion of trophoblast cells. The research team constructed a pregnant rat PE model to further validate its molecular mechanism by mediating DUXAP8 overexpression through adenovirus vectors. The experimental results showed that DUXAP8 activates the AKT/mTOR signaling pathway, inhibits endoplasmic reticulum autophagy, and promotes the occurrence and development of PE (127). *In vitro* experiments, lnc-HZ01 with m6A RNA methylation modification forms a positive feedback loop with MXD1, which plays a vital role in regulating abortive and BPDE-exposed trophoblast cell function regulation. This loop

increases Inc-HZ01 levels by enhancing the RNA stability of MXD1, which inhibits trophoblast proliferation and induces miscarriage (128). Circ_0003314 inhibits trophoblast function and induces cell apoptosis through the miR-26b-5p/IL1RAP signaling axis (129). In the PE study, circ_0015382 impaired trophoblast function through the miR-942-5p/NDRG1 axis, and its expression level was significantly elevated in the placental tissue of PE patients (130). In addition, Ou et al. (131) found through Pearson correlation analysis of placental tissue from 25 PE patients that the expression level of circ_0111277 was significantly higher than that of normal pregnancy placenta. Mechanism studies have shown that circ_0111277 participates in the occurrence of PE by regulating the miR-494-3p/HTRA1/Notch-1 signaling pathway, inhibiting the invasion and migration ability of trophoblast cells. These research results indicate that ncRNA participates in the precise regulation of trophoblast function through a complex regulatory network, providing potential molecular targets for diagnosing and treating pregnancy-related diseases. However, the exact molecular pathways of these regulatory mechanisms still need to be elucidated through more in-depth *in vivo* experimental studies, including the construction of conditional gene knockout animal models.

3.4.5 Trophoblasts and RNA methylation

In vitro experiments showed that knocking down m6A demethylase ALKBH5 can promote the invasion of HTR-8/SVneo in trophoblast cells by regulating the stability of CYR61 mRNA, while overexpression of ALKBH5 has the opposite effect (132). Meanwhile, Chuanmei Qin et al. (133) found that the expression of ALKBH5 in the villus tissue of RSA patients was elevated compared with the control group and confirmed through *in vitro* experiments that ALKBH5-overexpression-inhibited RSL3-induced cell death in the trophoblast by promoting the expression of iron death-related gene FTL. In addition, the expression of m6A demethylase FTO protein increased in URSA trophoblast cells, inhibiting m6A modification of MEG3 and weakening the stabilizing effect of YTHDC1 protein on MEG3. MEG3 inhibits the expression of the TGF- β 1 gene by binding to the EZH2 protein and TGF- β 1 gene promoter, thereby affecting the invasion and proliferation of trophoblast cells (134). These findings reveal the key role of RNA methylation in regulating the function of trophoblast cells and its potential mechanisms in miscarriage.

4 Epigenetic regulation and placenta-related pregnancy complications

As mentioned above, epigenetic regulation plays a moderately important role in the immune microenvironment at the maternal-fetal interface, and their absence or dysfunction may lead to complications during pregnancy (135). It has been suggested that normal and abnormal pregnancies lead to changes in cell populations, which may be facilitated by epigenetic modifications induced by different pregnancy-associated processes (136). The placenta, a uniquely evolved organ that develops externally to the embryo, undergoes rapid growth and fulfills diverse roles

throughout pregnancy, primarily ensuring a stable and protective milieu for fetal development. Notably, in recent years, shifts in lifestyle have coincided with a steady increase in the incidence of placental-related pregnancy disorders, exerting profound consequences on both maternal and neonatal health (137). Table 1 summarizes the association between epigenetic regulation and placental related pregnancy complications.

4.1 DNA methylation

Abnormal changes in DNA methylation are closely related to maternal health and fetal development. There are extensive DNA methylation changes in early-onset PE, among which TGFBR1 induces disease occurrence through a DNMT3A downregulation mediated DNA methylation-independent pathway (138). Meanwhile, the low methylation status of placental growth factor and Fms-related tyrosine kinase-1 further affects placental function (139). In terms of fetal development, changes in CpG methylation associated with maternal PE, pre-pregnancy weight loss, and type 2 diabetes risk are significantly associated with low birth weight, suggesting that placental DNA methylation may serve as a bridge between maternal metabolic status and the risk of chronic disease in offspring (140). In addition, epigenetic changes are also associated with other pregnancy-related diseases. Researchers recruited three decidua samples from patients with RSA and normal controls. They systematically identified key genes regulated by DNA methylation in the decidua and blood of RSA patients through genome-wide bisulfite sequencing (GWBS) and transcriptome sequencing. They found that 23 genes exhibited significant methylation and expression differences between RSA patients and healthy controls, with hypomethylated differential methylation regions (DMR) and upregulated differential gene expression (DGE) co-enriched in the Rap1, GnRH, and Estrogen signaling pathways. Additionally, 32 genes from these three pathways showed significant differences in DMR between RSA patients and the control group. Hi-MethylSeq analysis further revealed that SGK1 in RSA patients' blood and decidua samples exhibited a high methylation state. At the same time, SGK3 and CREB5 also showed significant changes in methylation levels in the decidua (141). T lymphocyte CD3 methylation may mediate psychiatric symptoms such as depression and anxiety during pregnancy (142). The above research reveals the extensive role of epigenetic modifications in pregnancy-related diseases, providing a new perspective for the mechanism research of related diseases.

4.2 Histone modifications

HDAC 2 expression and activity are down-regulated in monocytes/macrophages of patients with gestational diabetes, suggesting the role of histone deacetylation in metabolic disorders (143). The difference in placental chromatin activity is associated with fetal growth restriction, and H3K27 acetylation may be involved in regulating placental function (144). Men1, a member

TABLE 1 Epigenetic regulation and placenta-associated pregnancy complications.

Epigenetic modifications	Molecules	Mechanisms	Diseases	Reference
DNA Methylation	DNMT3A Downgrade	Mediates the non-dependent induction of DNA methylation by TGFBR1	Early onset severe pre-eclampsia	(138)
	Placental growth factor and Fms-associated tyrosine kinase-1 hypomethylation	—	Pre-eclampsia	(139)
	DNA methylation at the CPG site	—	Low birth weight	(140)
	Placental DNA methylation sites associated with birth weight	—	Perinatal cardiometabolic status of the mother, chronic disease in later life of the offspring	(140)
	CD3 methylation in pregnancy	—	Psychiatric symptoms such as depression and anxiety	(142)
Histone modification	Deletion of Men1, a member of the histone H3K4 methyltransferase complex	Disruption of terminal differentiation of stromal cells	Embryo resorption and pregnancy failure	(145)
	H3K18la downgrade	Influence on endometrial tolerance	Abortion	(146)
	Knockout of KAT8	Adjustment of H4K16ac/CDX2 axis	Vulnerable to embryo implantation failure induced miscarriage	(148)
	KDM5CK upward	Regulate the expression of TGFβ2 and RAGE	Recurrent spontaneous abortion	(149)
	H3K4me3 and H3K9ac downgrades	Regulated by Gal-2 and PPARγ	Pre-eclampsia	(150, 151)
	Down-regulation of HDAC 2 expression and activity in monocytes/macrophages	—	Gestational diabetes	(153)
	Up-regulation of miR-153-3p	Mediated inhibition of trophoblast function by the IDO/STAT3 pathway	Unexplained recurrent miscarriage	(152)
Non-coding RNA	Up-regulation of miR-185-5p	Reduced VEGF Expression and Angiogenesis in dNK Cells	Recurrent spontaneous abortion	(66)
	Up-regulation of miR-515-5P	Reducing HDAC2 levels harms trophoblast cell biological behavior	Recurrent spontaneous abortion	(153)
	Up-regulation of miR-23a	Inhibition of HDAC2 and activation of NF-κB impede trophoblast migration and invasion and promote apoptosis	Pre-eclampsia	(154)
	Down-regulation of miR-199a-5p	Reduction of VEGFA expression to inhibit trophoblast invasion	Pre-eclampsia	(155)
RNA methylation	Upregulation of METTL3	Increase the level of m6A RNA methylation and hnRNPC1/C2 expression in trophoblasts	Pre-eclampsia	(157)
	Upregulation of METTL14	Increase the level of m6A RNA methylation and FOXO3a expression and inhibit trophoblast proliferation and invasion	Pre-eclampsia	(159)
	Upregulation of RBM15	RBM15 suppresses hepatic insulin sensitivity of offspring of gestational diabetes mellitus mice via m6A-mediated regulation of CLDN4	Gestational diabetes Mellitus	(160)
	Down-regulation of m6A methylation level	ALKBH5 regulates CYR61 mRNA stability through m6A dependent mechanism and affects trophoblast function	Recurrent spontaneous abortion	(132)
	Down-regulation of METTL3	METTL3 mediated ZBTB4 m6A RNA methylation modification inhibits trophoblast invasion ability	Recurrent spontaneous abortion	(161)

of the H3K4 methyltransferase complex, is crucial for endometrial decidual transformation. Research has shown that abnormal differentiation of uterine stromal cells in mice with Men1 deficiency can lead to embryo resorption and pregnancy failure (145). Intriguingly, novel histone acetylation modifications, including H3K18la, have been identified as critical regulators of embryo implantation, with reduced levels potentially contributing to pregnancy loss. Optimal lactate concentrations facilitate endometrial cell proliferation and apoptosis regulation by enhancing histone acetylation, creating a favorable environment for successful embryo implantation (146, 147). Qianying Yang et al. (147) detected relatively high levels of lactate in the fetal membranes and endometrial tissues of pregnant sheep and found that lactate can induce H3K18 acetylation and regulate the balance of redox homeostasis and apoptosis in the endometrium to ensure successful implantation. RSA is closely related to the abnormal proliferation and differentiation of early trophoblast cells. Histone acetyltransferase KAT8 activates downstream gene CDX2 through H4K16ac, regulating trophoblast cell proliferation. Its absence increases the risk of embryo implantation failure. Clinical analysis shows that decreased expression of KAT8, CDX2, and H4K16ac is associated with RSA (148). Meanwhile, H3K4-specific demethylase KDM5C affects the function of trophoblast cells by regulating H3K4me3 demethylation at the promoter of TGF β 2 and RAGE genes. Overexpression of KDM5C can lead to decreased proliferation and invasion ability of trophoblast cells. *In vivo* experiments further confirmed that overexpression of KDM5C leads to a significant increase in mouse embryo absorption rate (149). In addition, the expression of H3K4me3 and H3K9ac in the placenta of patients with PE is reduced, and these modifications are regulated by substances such as Gal-2 and PPAR γ , suggesting the potential role of epigenetic modifications in gestational hypertension (150, 151). In conclusion, histone modification plays an important role in embryo implantation, placental development, and pregnancy maintenance by regulating gene expression and cell function. Its abnormal expression may lead to complications such as pregnancy diabetes, RSA, and PE.

4.3 Non-coding RNA

Abnormal expression of ncRNA can affect pregnancy outcomes by regulating the function of trophoblast cells. In RSA, miR-153-3p is highly expressed in extracellular vesicles derived from decidual macrophages, inhibiting the proliferation and migration of trophoblast cells through the IDO/STAT3 pathway (152). Meanwhile, miR-185-5p expression was elevated in the villi of RPL patients, and further *in vitro* experiments showed that miR-185-5p leads to pregnancy failure by reducing VEGF expression in dNK cells and decreasing angiogenesis (66). In PE, multiple *in vitro* experiments have found that miRNA participates in disease development by regulating trophoblast cell proliferation, migration, and apoptosis. For example, miR-515-5P inhibits the

proliferation, migration, and invasion of HTR-8/SVneo in trophoblast cells by reducing HDAC2 levels (153). MiR-23a inhibits HDAC2 and activates NF- κ B, hindering the migration of trophoblast cells and promoting apoptosis (154). MiR-199a-5p inhibits trophoblast cell invasion by suppressing VEGFA expression (155). In addition, Fu et al. (156) have outlined the role of ncRNAs in pregnancy-related complications, which will not be described in detail here. The results indicate that ncRNA significantly contributes to the pathogenesis of RSA and PE by modulating crucial molecular pathways.

4.4 RNA methylation

N6-methyladenosine (m6A) RNA modification is a pivotal regulatory mechanism in pregnancy-related disorders. In PE, *in vitro* studies have found the methylation level of m6A RNA in the placental trophoblast is significantly increased, and the methyltransferase METTL3 participates in the pathogenesis by regulating the expression of hnRNPC1/C2 (157). METTL14 upregulates FOXO3a expression through m6A dependent mechanism, inhibiting proliferation and invasion of trophoblast cells (158). At the same time, the m6A-modified circRNA in PE placenta generally increases, while the m6A modification of circPAPP2 is enhanced, but its expression is reduced. Its stability is regulated by IGF2BP3 (159). In gestational diabetes (GDM), the researchers found that the overall mRNA m6A methylation level in the fetal liver of the mouse was significantly increased by constructing a GDM mouse model, and *in vitro* experiments revealed that RBM15 mediated m6A modification affected the insulin sensitivity of offspring by regulating the expression of CLDN4, leading to metabolic syndrome (160). Compared with normal early pregnant women, the m6A methylation level in placental villus tissue of RM patients was significantly reduced, while ALKBH5 expression was not explicitly regulated. *In vitro* experiments have shown that knocking down ALKBH5 promotes trophoblast invasion, while overexpression inhibits invasion. It may be that ALKBH5 governs the stability of CYR61 mRNA through a dependent mechanism, thereby affecting the function of the trophoblast (132). In addition, *In vitro* studies have found that reduced m6A modification mediated by METTL3 can increase ZBTB4 expression, impair the invasive ability of the trophoblast layer, and lead to adverse pregnancy outcomes (161). Suqi Wu et al. (162) further elaborated on the role of m6A modification in maternal-fetal crosstalk and its potential mechanisms in pregnancy-related diseases. These findings suggest that m6A modification plays an important role in pregnancy-related diseases by regulating different target genes. However, the complex regulatory network of m6A modification and its specific functions under different pathological conditions still needs further exploration. Future research should combine multi-omics analysis and *in vivo* experiments to deeply reveal the dynamic regulatory mechanism of m6A modification at the maternal-fetal interface.

5 Conclusion and prospect

Successful pregnancy depends on the dynamic balance of multiple immune factors in the maternal-fetal interface immune microenvironment (3). The production of maternal autoimmune antibodies and the imbalance of immune regulation at the maternal-fetal interface are important factors affecting embryo implantation and leading to RSA. In recent years, epigenetic research has revealed the key roles of DNA methylation, histone modification, ncRNA and RNA methylation in immune regulation at the maternal-fetal interface. Especially DNA methylation, as the most promising and sensitive biological marker in epigenetic modifications, its abnormalities are not only closely related to the occurrence of various immune-related diseases but can also affect the immune tolerance at the maternal-fetal interface, leading to the maternal immune system producing a defensive rejection response to the embryo (13, 54). Research on DNA methylation in oncology has shown that it occurs earlier than gene mutations and can directly obtain relevant genetic material from tissues, providing the possibility for early prevention and treatment of diseases. The pathogenesis of immune-type RSA is similar to that of tumors, characterized by immune cell dysfunction and immune response suppression. With the deepening of epigenetic research, DNA methylation is expected to become a key breakthrough point for targeted diagnosis and precise treatment of immune type RSA, promoting the rapid development of reproductive medicine.

The epigenetic study of immune regulation at the maternal-fetal interface provides a new perspective for analyzing pregnancy's physiological and pathological mechanisms. By revealing the role of epigenetic modifications in the maternal-fetal interface immune microenvironment, researchers can gain a deeper understanding of the pathogenesis of pregnancy-related diseases and identify effective targets for immunotherapy. For example, dynamic changes in DNA methylation and histone modification may become key nodes in regulating maternal-fetal interface immune tolerance. In addition, the role of ncRN (such as miRNA and lncRNA) in regulating immune cell function at the maternal-fetal interface is gradually revealed. These findings lay a theoretical foundation for developing intervention strategies for pregnancy-related diseases based on epigenetics.

The fundamental causes of many placental diseases are still unclear. However, it is now widely believed that immune dysregulation at the placental interface can lead to many diseases (135, 163). Meanwhile, compared to genetic changes, epigenetic abnormalities are more straightforward to reverse. Drugs targeting specific epigenetic mechanisms involved in gene expression regulation, and even some nutrients, may become emerging tools for disease prevention or treatment. In this review, some evidence suggests that epigenetic modifications of inhibitory compounds or regulation of noncoding RNA expression through genetic tools may alleviate placental pregnancy-related diseases caused by immune dysregulation at the placental interface. These will provide new ideas for precise prevention and targeted treatment of diseases related to placental pregnancy.

Although significant progress has been made in the study of epigenetics in the maternal-fetal interface immune microenvironment, some limitations remain. Existing research has focused chiefly on single epigenetic modifications (such as DNA methylation or specific noncoding RNA). In contrast, research on the interactions and synergistic regulatory mechanisms between multiple epigenetic modifications is still insufficient. In addition, most studies rely on *in vitro* cell experiments or animal models, which make it difficult to fully simulate the complex immune microenvironment of the human maternal-fetal interface. However, a great deal of further research is still needed. Examples include (1) exploring how dysregulation of immunoregulation at the placental interface leads to epigenetic alterations in specific genes; (2) identifying human susceptibility genes in epigenetic alterations induced by dysregulation of immunoregulation at the placental interface; (3) demonstrating whether epigenetic alterations can be used as a biomarker for the early detection of, and therapeutic targeting for, pregnancy-associated diseases induced by dysregulation of immunoregulation at the placental interface; (4) developing novel inhibitors targeting epigenetic modifications; and (5) In-depth study of the interaction network and synergistic regulatory mechanism among various epigenetic modifications.

Author contributions

XQM: Visualization, Writing – original draft, Writing – review & editing. XC: Visualization, Writing – original draft, Writing – review & editing. XFM: Conceptualization, Supervision, Writing – review & editing. MC: Supervision, Writing – review & editing. YZ: Conceptualization, Supervision, Validation, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2025.1549839/full#supplementary-material>

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Happy feet: the key roles of podosomes and invadopodia in trophoblast invasion at the maternal-fetal interface

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Cells move by forming specialized projections or invasive feet known as podosomes in normal invasive cells and invadopodia in transformed and cancer cells. An understanding of invasive projections of trophoblasts at the maternal-fetal interface and their formation is important for developing novel therapies for pregnancy complications where invasion is abnormal, in instances where over- or under-invasion of cells manifests as serious pregnancy pathologies such as accreta or preeclampsia. Podosomes and invadopodia have distinctive morphological and molecular features that are used to distinguish them from each other. Despite this, there is still debate and uncertainty around how to definitively classify them. Analyses of novel models of cell invasion have demonstrated the existence of hybrid structures that are neither true podosomes nor invadopodia but which display features of both. This raises the question as to whether the classification of invasive structures needs redefining.

KEYWORDS

cell invasion, cell migration, invasive projection, podosome, invadopodia, cancer, pregnancy, trophoblast cell

Introduction

In order for cells to invade into tissue they must interact with and digest the extracellular matrix. This normal physiological process can lead to serious disease pathologies when it is defective with cancer being the best known and researched example. Understanding cell invasion is thus critically important to gain insights for developing treatments for diseases where it is abnormal. Invasive cells form specialized projections which make physical contact with the extracellular matrix and secrete proteases, thus enabling them to adhere, migrate and invade. Known as podosomes in non-transformed cells and invadopodia in transformed and cancer cells, these invasive

projections each have distinctive characteristics (1–4). Despite a growing body of knowledge on podosomes and invadopodia, there are still many unknowns. There is much debate about whether they are different structures, whether one is a precursor of the other or whether they are merely two representations of the same structure altered by the *in vivo* environment where it forms (5–9). In order to address this, it is important to study novel models of cell invasion. This will help to define if podosomes and invadopodia are two extremes of a continuum of structures or if they are of independent origin and will help to refine the current theories on how invasive projections are classified. These vitally important insights will impact current studies targeting the control of cell invasion as a therapeutic strategy.

Podosomes and invadopodia

Morphological features

Cells make contact with their surroundings via several types of specialized structures which enable them to either adhere to, or move through, the extracellular environment (10). Focal adhesions and focal contacts are points of adherence which attach cells to the substratum and can act as mechanosensors (11–13). They anchor stable cross-linked bundles of actin filaments at sites of integrin clustering. In order for cells to be able to move and invade they must form structures that can be rapidly assembled and dis-assembled and which can digest the extracellular matrix. For this purpose,

actin-rich membrane protrusions known as podosomes and invadopodia are formed. These invasive feet extend from cells and are associated with sites where there is digestion of the extracellular matrix by proteases secreted by the cells. The similarities and differences between these two structures are the subject of much discussion although there are morphological and molecular features that distinguish them (See Figure 1) including their length, duration of lifespan, number per cell and the proteins and lipids present in and secreted by the projections (1, 2, 12–14). Functionally it has been suggested that podosomes act to promote directed cell movement while invadopodia primarily function to digest extracellular matrix (15–17).

Podosomes are formed by normal invasive cells including monocytes, osteoclasts, endothelial cells and vascular smooth muscle cells and digest matrix via mechanisms involving membrane type-matrix metalloproteinase (MT1-MMP) and the urokinase receptor uPAR (6, 18, 19). They are typically less than 2 μm in length, have a lifespan measured in minutes and 20–100 form per cell (1, 2). Podosome adhesions are dot-like in nature and have a core of actin and associated proteins embedded in a ring of adhesion plaque proteins including paxillin, talin or vinculin (1, 6). Invadopodia is the name given to invasive projections formed by cancerous and transformed cells (2). They use matrix metalloproteinase 2 (MMP2), MMP9 and MT1-MMP to digest the extracellular matrix and are able to degrade matrix to a greater extent than podosomes (2, 20). Invadopodia are typically greater than 2 μm in length, have a lifespan of hours and less than 10 are usually present on a cell (1). The rigidity of the extracellular matrix

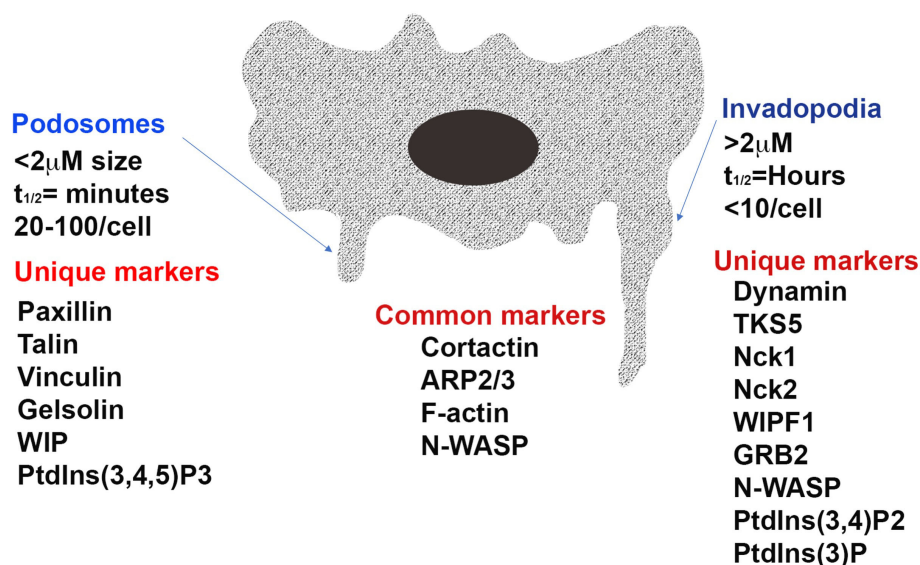


FIGURE 1

The structure of the invasive projections of a cell. Distinct and common features of a podosome and invadopodia of an invasive cell is depicted in Image 1. As shown the structure of invasive projections of podosomes and invadopodia are characterized by high actin polymerization activity and short, disorganized actin cores surrounded by a ring of adhesion structures. While the podosome cores are dynamic and have a turnover rate of minutes, invadopodia can remain stable for several hours. Invadopodia also extend into and invade the extracellular matrix to a greater depth through aggressive matrix degradation. ARP2/3, actin related protein 2/3 complex; N-WASP, Neural Wiskott-Aldrich syndrome protein; WIPF1, WASP-interacting protein; TKS5 tyrosine kinase substrate with five SH3 domains; Nck, non-catalytic region of tyrosine kinase; GRB2, growth factor receptor-bound protein 2; PtdIns(3,4,5)P3, phosphatidylinositol-3,4,5-triphosphate; PtdIns(3,4)P2, phosphatidylinositol-3,4-bisphosphate; PtdIns(3)P, phosphatidylinositol-3-phosphate.

is an important determinant of invadopodia formation with more rigid matrices promoting their formation (21). Invadopodia appear as puncta with small clusters of a few large actin-rich dots (15).

Molecular features

Many proteins, including tyrosine kinases, proteases and adapter proteins are involved in the assembly and maturation of invasive projections. Figure 1 depicts the structure of invasive projections of a cell with specific structural and common molecular features of a podosome and an invadopodia. Many proteins have been found in, or associated with, both structures. As described in Table 1, while many studies have focused on determining components that are unique to either podosomes or invadopodia in various cell types, in order to definitively identify these structures, to date, no specific marker has been identified that distinguishes invadopodia from podosomes. Despite this, some distinct patterns of protein localization have been demonstrated. The presence of vinculin has been proposed to be a marker for a podosome (5) while the localization patterns of the adaptor proteins Nck1 and Grb2 have been reported to distinguish invadopodia from podosomes (22).

Key proteins in the formation of both podosomes and invadopodia are those associated with actin nucleation, actin binding, kinases and scaffold proteins that regulate the actin organization within the structures (2, 23). Podosomes are rich in F-actin (localized to their core) and proteins that regulate the actin cytoskeleton assembly and disassembly. These include actin related protein 2/3 complex (ARP2/3), cortactin, gelsolin, Neural Wiskott-Aldrich syndrome protein (N-WASP) and WASP-interacting protein (WIP). Invadopodia are also rich in actin filaments and proteins that regulate components of the actin cytoskeleton. In the initial stages, tyrosine kinase substrate with five SH3 domains (TKS5) co-localizes with cortactin in precursors of invadosomes, suggesting that TKS5 recruits cortactin in a crucial initiation step of formation (24). TKS5 also recruits and interacts with many other proteins both directly and indirectly, including growth factor receptor-bound protein 2 (GRB2) and the actin regulators Nck1, Nck2 and N-WASP (25). Cortactin is also associated with and/or regulated by many other proteins. These include the ARP2/3 complex, WASP-interacting protein (WIPF1), and dynamin. All of these are key players in the formation of both podosomes and invadopodia (5). Cortactin can be phosphorylated by PAK1, regulating its interactions with the aforementioned proteins (15). The co-localization of cortactin with phosphotyrosine is a key marker of matrix-degrading invadopodia (26). In regards to matrix degradation, TKS4 has been shown to regulate the localization of MMPs, while cortactin controls the secretion of these proteases (21, 23, 27).

While they share some proteins, the way these proteins function may differ between podosomes and invadopodia. For example, it has been shown that invadopodia formation is dependent on the actin cytoskeleton and does not require microtubules or vimentin

intermediate filaments (28). Podosome function on the other hand has been found to require intact microtubules (1, 29).

As well as proteins, the membranes that surround podosomes and invadopodia have differing lipid composition. Specifically, podosomes and invadopodia differ in their predominant phosphatidylinositol (PI) phosphate composition. Podosomes are rich in phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P₃), while invadopodia exhibit a higher abundance of phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P₂) and phosphatidylinositol-3-phosphate (PtdIns(3)P) (2, 30). These lipids play a key role in the recruitment and activation of the signaling intermediates which are localized to invasive projections (30). Furthermore, invadopodia formation has been demonstrated to be dependent on the availability of cholesterol, with invadopodia having the properties of cholesterol-rich lipid rafts (31).

The matrix-associated surface of podosomes is rich in integrins which anchor the structure to the extracellular matrix (6). There are two main classes of proteases present in podosomes and invadopodia that facilitate ECM degradation (27). These include zinc-regulated metalloproteinases MMP2, MMP9 and MT1MMP (21), and the ADAM (a disintegrin and metalloproteinase) family, including ADAM12, ADAM 15 and ADAM19 (1). MMPs as well as the ADAM family are markers of a highly invasive phenotype and are highly expressed in invadopodia-forming cells (32).

The formation of invasive projections has been shown to be dependent on the activity of several different signaling pathways. Formation of invadopodia has been shown to depend on the activity of the tyrosine kinases Tsk5 (33) and c-Abl (34). The adaptor protein Tks5, a substrate for Src kinase, localizes to invadopodia and has been shown to be necessary for invadopodia formation and invasive behavior in several cancer cell lines (33). Abl kinase has recently been shown to be an essential regulator of invadopodia assembly and function (34).

Novel models of cell invasion

It was initially hypothesized that cell type determined which structures were produced, with podosomes exclusive to normally invasive cells (i.e. macrophages, endothelial cells, smooth muscle cells), and invadopodia only formed by highly invasive carcinoma cells. However, a study showed that podosomes were formed by non-invasive oral squamous cell carcinoma (SCC) cells, but after these cells underwent epithelial-mesenchymal transition and became invasive, they began to produce invadopodia (35). These podosomes that were found on non-invasive SCC cells were atypical from classically described podosomes, in that they all formed *de novo*, where usually new podosomes form by fragmenting off pre-existing podosomes. They also had a longer lasting life span, with life span being more comparable to that of invadopodia (35).

Another example of podosomes behaving similarly to invadopodia is seen in osteoclasts during bone remodeling. During this process, osteoclasts turn from a migratory phenotype to one with resorptive activity, remaining stationary where sites of

TABLE 1 Depicts protein characterization identified in podosomes and invadopodia in various cell types.

Feature/Protein	Description	Cell Type Studied	Found In	Reference
Cortactin	Actin-binding protein involved in cytoskeletal organization	MDA-MB-231 breast cancer cells	Invadopodia	(70)
N-WASP	Regulates actin polymerization and cytoskeletal dynamics	MDA-MB-231 breast cancer cells	Invadopodia	(71)
Arp2/3 Complex	Actin nucleation factor that promotes branching	MDA-MB-231 breast cancer cells	Both podosomes and invadopodia	(72)
TKS5	Scaffolding protein essential for invadopodia formation	MDA-MB-231 breast cancer cells	Both podosomes and invadopodia	(73)
MT1-MMP	Matrix metalloproteinase involved in ECM degradation	MDA-MB-231 breast cancer cells	Invadopodia	(74)
Fascin	Actin-bundling protein associated with invasive potential	MDA-MB-231 breast cancer cells	Invadopodia	(75)
Integrins (e.g., $\beta 1$, αV)	Cell adhesion receptors mediating ECM interactions	Endothelial cells	Both podosomes and invadopodia	(76)
Paxillin	Focal adhesion protein involved in signal transduction	Osteoclasts	Podosomes	(77)
Vinculin	Actin-binding protein that stabilizes cell adhesion	Osteoclasts	Podosomes	(78)
Talin	Links integrins to the actin cytoskeleton	Osteoclasts	Podosomes	(79)
MMP-2	Matrix metalloproteinase involved in ECM remodeling	Trabecular meshwork cells	Podosomes	(80)
MMP-14	Matrix metalloproteinase critical for ECM degradation	Trabecular meshwork cells	Both podosomes and invadopodia	(81)
Cdc42	Small GTPase regulating actin cytoskeleton	MDA-MB-231 breast cancer cells	Invadopodia	(82)
Caldesmon	Actin-binding protein regulating actomyosin interactions	A7r5 aortic smooth muscle cells	Podosomes	(83)
α -Actinin	Actin cross-linking protein stabilizing cytoskeletal structures	Osteoclasts, MDA-MB-231 breast cancer cells	Both podosomes and invadopodia	(84)
Fibronectin	ECM glycoprotein involved in cell adhesion and migration	Trabecular meshwork cells	Podosomes	(85)
Versican	ECM proteoglycan involved in cell adhesion and proliferation	Trabecular meshwork cells	Podosomes	(86)
WASP	Actin nucleation-promoting factor	MDA-MB-231 breast cancer cells	Both podosomes and invadopodia	(87)
Tyrosine Kinase Substrate	Adapter protein involved in invadopodia signaling	MDA-MB-231 breast cancer cells	Both podosomes and invadopodia	(88)
Actin Filaments	Structural filaments forming the cytoskeleton	Osteoclasts, MDA-MB-231 breast cancer cells	Both podosomes and invadopodia	(89)
Src Kinase	Tyrosine kinase involved in invadopodia formation	MDA-MB-231 breast cancer cells	Invadopodia	(90)
Dynamin	GTPase involved in vesicle trafficking and invadopodia turnover	MDA-MB-231 breast cancer cells	Invadopodia	(91)
β -Actin	Cytoskeletal protein essential for invadopodia structure	Fibrosarcoma	Both podosomes and invadopodia	(92)
Filamin A	Actin-binding protein that cross-links filaments	MCF-7-ErbB2, MDA-231, MDA-231-ErbB2, and BT-20 Breast cancer cells	Invadopodia	(93)

bone-matrix degradation are situated. It is well documented that MMP14 localizes to both podosomes and invadopodia, and it had recently been found that MMP14 also localizes to osteoclast podosomes, but the mechanism used is similar to that of invadopodia (36). In light of these findings, it has been suggested that podosomes may be a precursor structure to invadopodia, and it is only when it is required that invadopodia form (36). Following from this, it was also suggested that invadopodia represent a physiological form of podosomes that are actively associated with the localized degradation of the matrix.

Another suggestion is that cells may have an innate ability to form both podosomes and invadopodia, and the formation of one or the other may be dependent on the matrix the cells are situated on, as well as the signaling pathways activated at the time (35). Previous studies demonstrated the presence of invadopodia and focal adhesions in both 2D and 3D *in vitro* environments (37–39). Normally, physiological migration and invasion of cells is tightly regulated, and if the formation of invadopodia requires specific signaling pathways, formation will not occur if culture conditions are incorrect.

Trophoblast cell invasion

A critical event early in human pregnancy is the transformation of the uterine spiral arterioles to create a high flow, low resistance vasculature. Successful remodeling of the maternal uterine vasculature facilitates increased maternal blood flow to the placenta thus ensuring normal fetal growth and development (40). This requires invasion of trophoblast cells which move from

the placenta into the maternal uterine vessels and surrounding matrix and integrate into the vessel walls (41, 42).

As depicted in Figure 2, the trophoblast cells that carry out the remodeling process are characterized by a gain of invasive ability. This phenotypic change enables them to move away from the tips of the placental villi and migrate into the maternal tissue (43–45). In order to become invasive, a subset of trophoblast cells must begin to secrete matrix metalloproteinases (MMPs) (44, 45). The ability to express the gelatinases MMP-2, MMP-9 and MMP-12 (46, 47) confers these trophoblast cells with the capacity to degrade elastin, collagens and laminin, thus enabling them to invade through the extracellular matrix of the uterine decidua stroma and integrate into the walls of the spiral arterioles. The remodeling process occurs in a very defined time frame and space, commencing early in the first trimester of pregnancy and continuing into the second trimester before ceasing at around 16–20 weeks of gestation with cells invading only as deep as the first third of the myometrium (42, 45, 48).

As depicted in Table 2, the invasion of trophoblast cells during human pregnancy is often considered analogous to the invasion of malignant cancer cells as both are highly invasive, the adhesion molecules and proteases involved are similar and the cells use similar strategies to evade the host immune system (49). However, the major difference is that trophoblast cell invasion is very tightly controlled and regulated by the complex interplay of growth factors, cytokines, endocrine factors, oxygen concentrations and haemodynamics at the maternal-fetal interface. These act both temporally and spatially to initially promote and then limit the extent of trophoblast cell invasion (43, 50). While the similarities between invasive trophoblast cells and cancerous cells have been

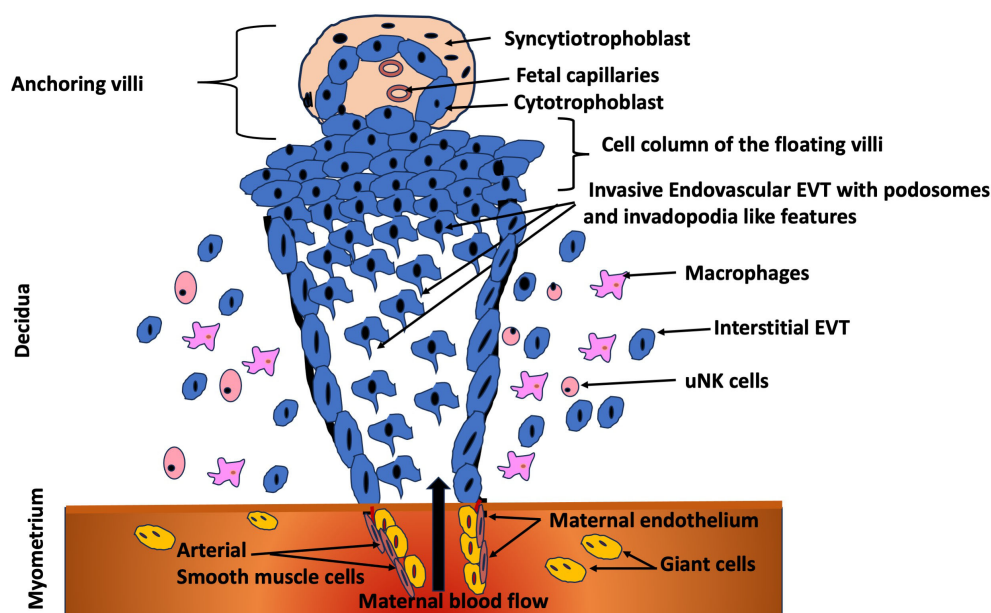


FIGURE 2

Trophoblast invasion at the maternal-fetal interface. Extravillous trophoblasts (EVT) originating from the trophoblastic shell of the anchoring villi proliferate in the cell column, migrate to the decidua and subsequently invade the myometrial stroma (41), by displaying podosomes and invadopodia-like features. Subsequently, invade the lumen of the arterioles to replace the endothelium of the maternal vessels (94). Figure adapted and modified from Lunghi et al. (2007) (95).

TABLE 2 Invasive projections of trophoblasts and cancer cells.

Feature	Trophoblast Projections	Cancer Cell Projections
Types of projections	Podosomes, invadopodia-like structures	Invadopodia, filopodia (thin, exploratory projections), lamellipodia (broad ruffling edges), microtentacles for migration and invasion.
Function	Penetrate maternal penetrate the decidua and remodel spiral arteries to establish a physiological placental interface	Degrade ECM for invasion and metastasis of aggressive cancer
Actin Organization	Organized, often podosome-like (actin core with adhesion ring)	Invadopodia: actin-rich core, MMP-rich zone
MMP Expression	Regulated expression of MMP-2, MMP-9	Over expression of MMP-2, MMP-9, MT1-MMP
Stability	Transient, tightly regulated	Longer-lived, more stable in invasive cells
Regulation	Hormonal (e.g., hCG), cytokines, maternal signals including modulation by maternal immune cells	Oncogenes (e.g., Src, Ras), hypoxia, inflammation
Polarity	Directed, guided invasion	Multidirectional and unguided invasion
Outcome	Controlled invasion for placental anchoring and nutrient exchange for physiological adaptation	Uncontrolled invasion leading to metastasis.

described, particularly their ability to digest matrix via secretion of MMPs, only one recent study (51) has examined whether trophoblast cells form podosomes similar to other non-cancerous cells or whether they form invasive projections that are invadopodia-like. The evidence suggests that trophoblast cells form atypical invasive projections that are neither podosomes or invadopodia but that exhibit features of both structures (51). Furthermore, the focal adhesion molecules that are mission control for trophoblast invasion, regulates adhesion, signaling, cytoskeletal changes, and ECM interaction to ensure that invasion is precise, regulated, and adaptive (52–54). Focal adhesion molecules also allow trophoblasts to “sense” mechanical cues, oxygen levels, and ECM stiffness that are essential for them adapt to their invasion depth. In low oxygen, for example, certain pathways (e.g. hypoxia inducible factor-1, HIF-1 α) are triggered, modulating focal adhesion signaling and promoting invasion early in pregnancy (55). Unlike cancer cells, trophoblasts need to attach just enough to anchor maternal decidua, but also stay mobile enough to invade. Focal adhesion dynamics control this balance through turnover and recycling of adhesions.

Freshly isolated primary first trimester trophoblast cells grown on Matrigel for 48 hours to promote acquisition of the invasive phenotype leads to expression of mRNA for key proteolytic enzymes, known to be associated with invadopodia. Expression of mRNAs for the gelatinases MMP2, MMP9, MT1-MMP (MMP14 precursor) and MMP14 have all been detected (56). These enzymes are secreted by mature invadopodia and enable them to digest extracellular matrix to a greater extent than podosomes. In addition, expression of mRNAs for the membrane bound metalloproteinases ADAM8, ADAM12, ADAM15 and ADAM19 can also be detected (57). While a role for ADAMs in invadopodia is less well defined, ADAM12, ADAM15 and ADAM19 have been shown to interact with the Tks5 adaptor protein which localizes to invadopodia and ADAM8 has also been detected in podosomes (51). This suggests that components associated with invadopodia rather than podosomes are present in first trimester trophoblast.

Invasive projections as therapeutic targets

Invasive projections represent a new and exciting therapeutic target. Recent studies highlight the proteomic and transcriptomic profiling of invasive cells, with an emphasis on proteins associated with invadopodia and their structures (58) (59). Inhibition or stimulation of invasive projection formation can be the basis for disease therapy where cell invasion is abnormal. Potentially, invasive projections present two paths that can be exploited in the therapeutic arena. First, matrix degradation *in vitro* by invasive projections can, and is, being used to test the effect of lead compounds and to screen drug libraries (60). Secondly, invasive projections can be a target for therapy where disruption or stimulation of their formation is beneficial. The development of novel therapies is hindered by a lack of knowledge of podosome or invadopodia-specific components. Thus, there is an urgent need to define the composition of invasive projections on cells of differing origins and invasive potentials (14).

Over-invasion

In cancer biology, inhibition of invadopodia formation has been proposed as a strategy for treating breast, lung and pancreatic cancers (2, 61–63). This approach is considered appealing as cell viability is not affected by targeting invadopodia thus therapies based on this strategy would be anticipated to have fewer side effects than current therapies (64). In addition, a therapy targeting invadopodia formation will only target the cancer cell population thus vastly improving drug selectivity (65).

Abnormal trophoblast cell invasion is associated with very serious pregnancy complications that can be life-threatening for the mother and have serious health consequences for the baby which is often born premature. Over invasion results in complications

including placenta percreta, increta and accreta, where cells invade into maternal tissues beyond the uterine wall (66), and gestational trophoblastic diseases such as choriocarcinoma and invasive mole with highly invasive, metastatic tumors (67). It could be envisaged that a therapy targeting a unique property of the invasive projections formed by trophoblast cells could be employed to treat these conditions.

Under-invasion

Switching cell invasion on could also be beneficial in some instances. Transient activation of invasive projection formation has been suggested as potentially useful in the treatment of skeletal anomalies, where mutations in podosome proteins cause several distinct craniofacial defects (2, 68). Several serious pregnancy complications are characterized by under invasion of trophoblast cells. Shallow trophoblast cell invasion is associated with pre-term delivery, pre-eclampsia and fetal growth restriction (41, 45), and possibly some instances of miscarriage (69). These represent pathologies where there are currently no effective therapies other than delivery of the baby which is undesirable pre-term. Just as for pregnancy complications with over-invasive cells, targeting the invasive projections formed by trophoblast cells could be a new and novel therapeutic strategy.

Conclusions

Though sharing similarities with cancer cell invasion, trophoblast cell invasion is highly regulated compared to the dysregulated invasion seen in cancer. Trophoblast cells form invasive structures with unique properties (51) but little is known about their molecular characteristics or regulation. Evidence suggests the structures formed by trophoblasts are atypical, being neither a true podosome nor invadopodia. Comparing trophoblast cell and cancer cell invasion, as well as other novel models of cell invasion, will contribute to a better understanding of the classification and regulation of podosomes and invadopodia, which will ultimately inform and improve disease treatment strategies that aim to modify cell invasion. The identification of the similarities and differences could provide novel targets for the diagnosis and treatment of pathological pregnancies, cancer and

other diseases where cell invasion is abnormal, enabling the translation of basic research discoveries into clinical applications.

Author contributions

PM: Conceptualization, Project administration, Supervision, Writing – original draft, Writing – review & editing, Formal analysis, Funding acquisition, Resources, Validation, Visualization. EO: Writing – review & editing, Investigation. SB: Supervision, Writing – review & editing. RK: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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