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Adverse outcomes of preeclampsia: From mother to baby, pregnancy to postpartum

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Adverse outcomes of preeclampsia: From mother to baby, pregnancy to postpartum

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Editorial: Adverse outcomes of preeclampsia: from mother to baby, pregnancy to postpartum

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Editorial on the Research Topic
Adverse outcomes of preeclampsia: from mother to baby, pregnancy to postpartum

Preeclampsia is a dangerous pregnancy-specific complication that involves new-onset hypertension and signs of systemic vascular dysfunction (Bisson et al.). Beyond pregnancy, women are at greater risk for devastating illnesses including cardiac disease (deMartelly et al., 2021), Alzheimer's disease (Theilen et al., 2016) and stroke (Bellamy et al., 2007; Theilen et al., 2016). Moreover, their offspring present with disturbances in hormonal, metabolic and neurodevelopmental pathways that likely accelerate onset of disease with age (Nahum Sacks et al., 2018; Yang et al., 2023). These observations warrant ongoing research, not only to understand the mechanisms, but because clinicians lack reliable pharmacotherapies to cure preeclampsia. In this editorial, we present pre/clinical original research and literature reviews, including work on biomarkers and molecular pathways that are necessary to identify druggable targets to halt sudden and long-term ramifications.

Preeclampsia has been recognized to foreshadow ensuing chronic disease in women and their baby, which demonstrates the need for advances in earlier prediction and interventions. On this Research Topic, Bisson et al. reviewed a set of studies stressing the requirement to include biomarkers beyond gestational-related blood pressure levels as the latter alone does not strongly predict preeclampsia. Work from this team and their colleagues has contributed to our understanding of the powerful predictive nature of sFlt-1: PlGF ratios in preeclampsia (Bisson et al.) whereby elevations are indicative of a vasoconstrictive milieu within the maternal circulation. Combining this value with blood pressure has a strong association with hospitalizations due to preeclampsia. It is fathomable that strategies to confidently predict preeclampsia as early as possible would allow time to combat and prevent this disease.

Han et al. reviewed use of biomarkers for predicting late-onset, term preeclampsia, which is more prevalent and not as well detected as early-onset preeclampsia. They found that there are currently no molecular biomarkers with sufficient clinical sensitivity and

specificity. Several limitations likely muddled these results but could be overcome standardizing definitions of preeclampsia subtypes as well as sample collection. In a study by Kandel et al. that assessed biomarkers for early- and late-onset preeclampsia, they found reduced galectin-3, which is a marker of cardiovascular disease and heart failure (Chen et al., 2021) in non-pregnant patients, in the former and not latter subtype. However, in another study where preeclampsia subtypes were combined, expression of galectin-3 was increased (Ghorbanpour et al., 2023). Thus, more work is needed to determine if there are biomarkers that can predict timing and severity of preeclampsia.

While biomarkers help to educate clinicians on management options, there are educational opportunities for the patient that could emphasize the seriousness of hypertension in preeclampsia that are associated with lower blood pressure after pregnancy, including: 1) awareness that adversities from preeclampsia can extend past parturition, such as cardiac abnormalities (deMartelly et al., 2021), and dictate a future shortened by hypertensive disease in mother and baby and 2) programs that simplify adoption of routine blood pressure measurement and follow-up appointments. Added opportunities to seek advice, such as with telehealth medicine, are likely needed as online sentiments of emotional support increase and stay high during pregnancy (Hou and Hou).

Beckett et al. reviewed cerebrovascular disease and cognitive impairment in preeclampsia and beyond. The psychological outcomes of pregnancy can be adversely influenced by preeclampsia targeting the cerebrovasculature. Importantly, preeclampsia can quickly advance to eclampsia, which manifests as new-onset seizures and accounts for 13% of maternal deaths worldwide (Nour, 2008; Beckett et al.). Although not fully understood, one mediator may be white matter lesions, which are present in over 60% of women with a history of preeclampsia (Soma-Pillay et al., 2017). Another possible culprit is inflammation. Herrock et al. described how pro-inflammatory cells and cytokines cause widespread endothelial injury. This can disrupt the blood brain barrier and feedforward to promote neuroinflammation, cerebrovascular dysfunction, and white matter lesioning (Beckett et al.).

Neurodevelopmental disorders occur at a greater rate in offspring born from preeclamptic pregnancies. Several studies report that they have increased risk of autism spectrum disorder, attention deficit/hyperactivity disorder and intellectual disability (Ehrenstein et al., 2009; Dang et al., 2016; Alsnes et al., 2017; Nahum Sacks et al., 2018; Gumusoglu et al., 2020). Although these associations exist, less is known about the pathogenic mechanisms. Interluekin-6 (IL-6) is elevated in preeclampsia and contributes to the pro-inflammatory environment that exists in this disease (Bakrania et al., 2020). Despite this, Barron et al. found that in neuroblastoma cells exposed to sera from preeclamptic women, neurite growth and mitochondrial respiration were elevated, in part, due to increased IL-6. Further exploration of the neurodevelopmental implications of this, and other pathogenic factors are critical to properly understand underlying mechanisms.

There is also increased risk for systemic cardiovascular, metabolic and reproductive diseases in preeclampsia-exposed offspring. In a preclinical study, the timing of onset for puberty was significantly earlier in female, but not male, mice originating from preeclamptic BPH/5 mice (Gomes et al.). These data coincide with previous literature that indicate a dominant androgenic profile in female offspring of pregnancies affected by preeclampsia (Ogland et al., 2011; Alsnes et al., 2016). Cardiometabolic characteristics, that are also associated with increased risk of preeclampsia, have also been documented to be elevated in female offspring of BHP/5 mice, but not their male littermates, such as increased food intake, increased body weight, hyperleptinaemia and increased visceral white adipose tissue. Preclinical studies show that overexpression of leptin promotes the pathogenesis of this disease and continued research focuses on whether elevated levels are a novel biomarker for preeclampsia (Wang et al.).

In summary, our editorial highlights original research and literature reviews that focus on the sequelae of preeclampsia. Such adversities impact both mothers and their offspring, not only during, but well beyond pregnancy. There is a desperate need for translational research that utilize human data on biomarkers to design preclinical models assessing their pathogenic potential as targets for pharmacotherapies to prevent outcomes of preeclampsia. Aside from finding a cure, recommendations include a focus on standardizing diagnostic criteria and programs to support *postpartum* care of women and their children.

Author contributions

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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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Placental galectin-3 is reduced in early-onset preeclampsia

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Preeclampsia is a disease of pregnancy responsible for significant maternal and neonatal mortality. Galectin-3 is a β-Galactoside binding protein. This study aimed to characterise galectin-3 in women with preeclampsia and human trophoblast stem cells (hTSCs). Galectin-3 was measured in placental lysates and plasma collected from patients with early-onset preeclampsia (delivered <34 weeks' gestation) and gestation matched controls. Placental galectin-3 protein was significantly reduced in 43 women with early-onset preeclampsia compared to 21 controls. mRNA expression of *LGALS3* (galectin-3 encoding gene) was reduced in 29 women with early-onset preeclampsia, compared to 18 controls (p = 0.009). There was no significant difference in plasma galectin-3 protein in 46 women with early-onset preeclampsia compared to 20 controls. In a separate cohort of samples collected at 36 weeks' gestation, circulating galectin-3 was not altered in 23 women who later developed preeclampsia, versus 182 who did not. In syncytialised hTSCs, hypoxia increased mRNA expression of *LGALS3* (p = 0.01). Treatment with inflammatory cytokines (TNF-α and IL-6) had no effect on *LGALS3* mRNA expression. However, TNF-α treatment caused an increase in mRNA expression of *LGALS3BP* (galectin-3 binding protein encoding gene) in hTSCs (p = 0.03). This study showed a reduction of galectin-3 in placenta from pregnancies complicated by early-onset preeclampsia. *LGALS3* mRNA expression was dysregulated by hypoxia exposure in placental stem cells.

KEYWORDS

placenta, preeclampsia, galectin-3, galectin-3 binding protein, plasma

1 Introduction

Preeclampsia is a pregnancy-specific disorder which affects 2%–8% of mothers (Groot et al., 2016). It is characterised by placental hypoxia, local and systemic inflammation (Roberts and Gammill, 2005).

Galectins are a family of β-Galactoside binding proteins important for successful implantation and maintenance of pregnancy (Jovanović Krivokuća et al., 2021). Galectin-3 (encoded by the *LGALS3* gene) is abundantly expressed at the

TABLE 1 Maternal characteristics and pregnancy outcomes for less than 34 weeks placental samples—measured galectin-3 protein.

	Controls (n = 21)	Preeclampsia (n = 43)	p-value
Maternal age (years) Mean ± SEM	30.14 ± 1.61	31.51 ± 0.83	0.41
Gestation at delivery (weeks) Mean ± SEM	30.11 ± 0.54	30.20 ± 0.36	0.89
Body mass index (kg/m^2) Median (IQR)	28.20 (24.25–34.85)	27.00 (25.00–35.60)	0.99
Parity no. (%)			
0	5 (23.81)	32 (74.42)	0.0006
1	11 (52.38)	7 (16.28)	
≥2	5 (23.81)	4 (9.30)	
SBP at delivery (mmHg) Median (IQR)	120 (111–130)	170 (160–185)	<0.0001
DBP at delivery (mmHg) Median (IQR)	70 (67.50–80.00)	100 (95.00–110.00)	<0.0001
Birthweight (g) Median (IQR)	1,585 (1,278–1,943)	1,253 (866.8–1,479)	0.01
Male no. (%)	11 (52.38)	23 (53.49)	0.93

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure. Unpaired t-test was used for normally distributed data, Mann-Whitney U tests for non-parametric data, and Chi-square tests for categorical variables. BMI data missing for 5/21 control samples, and 10/43 preeclampsia samples. Birthweight data missing for 1/43 PE samples. $p < 0.05$ was considered significant.

maternal-fetal interface and secreted from the cell surface into biological fluids (Blois et al., 2015; Blois et al., 2007; Than et al., 2014; Tirado-Gonzalez et al., 2012). Galectin-3 binding protein (galectin-3BP), encoded by the *LGALS3BP* gene, is a highly glycosylated protein that acts as a ligand for several galectins, including galectin-3 (Lin et al., 2015). Galectin-3 regulates various cellular processes including cellular growth, differentiation, and inflammation (Liu et al., 2002). Dysregulated galectin-3 has been characterised in the pathogenesis of several diseases, such as heart failure, cancer and pulmonary hypertension (Sciacchitano et al., 2018).

In placenta, galectin-3 is expressed in all trophoblastic lineages including cytotrophoblasts and extravillous trophoblasts (Maquoi et al., 1997; Vićovac et al., 1998) and is released in response to hypoxia in BeWo choriocarcinoma cells (Hu et al., 2007). Several studies have shown that elevated galectin-3 levels are associated with preeclampsia (Amarilyo et al., 2011; Božić et al., 2004; Hu et al., 2007; Jeschke et al., 2007). Nikolov et al. (2020) found no change in serum galectin-3 levels between patients with preeclampsia compared to control. These results are likely due to underpowered sample numbers, hence the conflicted results.

Therefore, this study aimed to assess galectin-3 mRNA expression and protein levels in placentas and plasma using two well-defined cohorts, including one with confirmed early-onset preeclampsia, and another collected prior to diagnosis of term preeclampsia. Additionally, we assessed galectin-3 and galectin-3BP levels in an *in vitro* model of preeclampsia where differentiated human trophoblast stem cells (hTSCs) (Okae et al., 2018) were exposed to hypoxia and pro-inflammatory cytokines.

2 Materials and methods

2.1 Placenta and plasma collection at less than 34 weeks' gestation

Ethics approval was obtained from Mercy Health Human Research Ethics Committee (R11/34). Patients presenting to Mercy Hospital for Women (Heidelberg, Victoria) gave written, informed consent for collection of blood during their pregnancy, and placentas following caesarean delivery. Placentas were obtained from patients with established early-onset preeclampsia (<34 weeks' gestation; n = 43) and gestation matched controls (n = 21). Placentas were processed within 30 min of delivery where tissue was sampled from four quadrants of the placenta and washed in

TABLE 2 Maternal characteristics and pregnancy outcomes for less than 34 weeks placental samples—measured galectin-3 mRNA expression.

	Controls (n = 18)	Preeclampsia (n = 29)	p-value
Maternal age (years) Mean ± SEM	31.50 ± 1.64	32.52 ± 0.95	0.56
Gestation at delivery (weeks) Mean ± SEM	30.25 ± 0.59	31.12 ± 0.24	0.13
BMI (kg/m^2) Median (IQR)	28.20 (24.75–35.08)	26.80 (23.75–34.60)	0.34
Parity no. (%)			
0	4 (22.2)	20 (68.97)	0.007
1	9 (50.0)	5 (17.24)	
≥2	5 (27.8)	4 (13.79)	
SBP at delivery (mmHg) Median (IQR)	125 (117.3–130)	180 (169–183)	<0.0001
DBP at delivery (mmHg) Median (IQR)	75 (70.00–80.00)	100 (97.00–110.00)	<0.0001
Birth weight (g) Median (IQR)	1,587 (1,298–2,011)	1,425 (1,314–1,561)	0.22
Male no. (%)	10 (55.56)	20 (68.97)	0.35

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure. Unpaired t-test was used for normally distributed data, Mann-Whitney U tests for non-parametric data, and Chi-square tests for categorical variables. BMI data missing for 4/18 control samples, and 4/29 PE samples. $p < 0.05$ was considered significant.

phosphate buffered saline. Placental tissue was processed with RNA*later*™ stabilization solution and stored at −80°C for future analysis. Preeclampsia was diagnosed in accordance with the American College of Obstetrics and Gynaecology (ACOG) guidelines (2020) (Obstetricians ACo Gynecologists, 2020). Refer to Table 1 and Table 2 for patient characteristics.

Whole blood was collected in a 9 ml ethylenediaminetetraacetic acid (EDTA, BD Vacutainer® K2E) tube, centrifuged and plasma obtained from patients delivering at <34 weeks' gestation with preeclampsia (n = 46), or gestation matched controls (n = 20) who delivered without preeclampsia at term. Plasma was stored at −80°C for further analysis. Refer to Table 3 for patient characteristics.

2.2 Plasma collected at 36 weeks' gestation preceding preeclampsia diagnosis

The Biomarker and Ultrasound Measures for Preventable Stillbirth (BUMPS) study is a large prospective cohort conducted at the Mercy Hospital for Women (Heidelberg, Victoria). This cohort was designed to identify biomarkers for pregnancy complications. Ethics approval was obtained from the Mercy Health Research Committee (approval number: 2019–012). English speaking patients aged 18 years and over, with a singleton pregnancy and normal mid-trimester fetal morphology were eligible to participate. Patient whole blood were collected at 36 (35^{+0}–37^{+0}) weeks' gestation in 9 ml EDTA vacutainers from 182 healthy controls and 23 patients who went on to develop term preeclampsia. Bloods were centrifuged and plasma was stored at −80°C until further analysis. Refer to Table 4 for patient characteristics.

2.3 Culture of first trimester human trophoblast stem cells

First trimester human trophoblast stem cell lines (hTSCs) were imported from the RIKEN BRC through the National BioResource Project of the MEXT/AMED, Japan. Cells were cultured according to the publication by Okae and colleagues (Okae et al., 2018).

2.3.1 Differentiation of human trophoblast stem cells into syncytiotrophoblast or extravillous trophoblasts

First trimester cytotrophoblast stem cell lines (hTSCs) were differentiated into either syncytiotrophoblast or extravillous

TABLE 3 Maternal Characteristics for less than <34 weeks plasma samples.

	Controls (n = 20)	Preeclampsia (n = 46)	p-value
Maternal age (years) Mean ± SEM	33.00 ± 1.103	30.83 ± 0.84	0.21
Gestation at blood collection (weeks) Mean ± SEM	27.61 ± 0.95	29.65 ± 0.39	0.07
Gestation at delivery (weeks) Mean ± SEM	39.06 ± 0.23	29.95 ± 0.43	<0.0001
Parity no. (%)			
0	8 (40.00)	33 (71.74)	0.04
1	7 (35.00)	9 (19.56)	
≥2	5 (25.00)	4 (8.70)	
SBP at delivery (mmHg) Median (IQR)	120.00 (125.00–110.00)	173.50 (180.00–165.00)	<0.0001
DBP at delivery (mmHg) Median (IQR)	75.00 (80.00–70.00)	100.00 (110.00–99.75)	<0.0001
BMI (kg/m^2) Median (IQR)	25.40 (30.05–20.40)	28.50 (35.20–26.00)	0.02
Birth weight (g) Median (IQR)	3,465 (3,873–3,180)	1,277 (1,625–777.50)	<0.0001
Male no. (%)	8 (40.00)	19 (41.30)	0.92

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure. Unpaired t-test was used for normally distributed data, Mann-Whitney U tests for non-parametric data, and Chi-square tests for categorical variables. BMI data missing for 3/46 PE samples; SBP and DBP data missing for 1/20 control samples. $p < 0.05$ was considered significant.

trophoblast cells (EVTs) as described previously (Okae et al., 2018).

2.3.2 Treatment of syncytialised human trophoblast stem cells with interleukin 6, tumor necrosis factor α and hypoxia

Cells were plated at 60,000 cells/well in a 24-well cell culture plate in syncytial [ST(2D)] media and incubated at 37°C, 8% O_2, and 5% CO_2 for 72 h to allow for syncytialisation. Next, cells were incubated in a hypoxic environment or with inflammatory stimuli. Cells in a hypoxic environment were cultured at 1% O_2 whilst normoxic cells were maintained at 8% O_2 for an additional 48 h. To induce inflammation, cells were treated with increasing doses of tumor necrosis factor α (TNFα) or interleukin 6 (IL-6) at 0, 0.1, 1, and 10 ng/ml for 24 h. Experiments were treated in triplicates and repeated separately (n = 5).

2.4 Enzyme linked immunosorbent assay

Galectin-3 levels were measured in plasma and placenta protein lysate using human DuoSet ELISA kits (RnD systems; Catalogue # DY1154, Minnesota, United States) according to the manufacturer's instructions.

2.5 RNA extraction, reverse transcription, and reverse transcriptase polymerase chain reaction

RNA was extracted from hTSCs with GenElute™ mammalian total RNA miniprep kit (Sigma-Aldrich) and quantified using a Nanodrop ND 1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, United States). RNA was converted to cDNA with high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Life Technologies) as per manufacturer's instructions using iCycler iQ5 machine (Biorad) with run conditions: 25°C for 10 min, 37°C for 60 min and 85°C for 5 min.

Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) measured the mRNA expression of genes; *LGALS3* (Assay ID: Hs00173587_m1), *LGALS3BP* (Assay ID: Hs00174774_m1), *TEAD4* (TEA Domain Transcription Factor 4, Assay ID: Hs01125032_m1), *SDC1* (Syndecan 1, Assay ID: Hs00896423_m1) and *HLAG* (Human Leukocyte Antigen G,

TABLE 4 Patient characteristics and pregnancy outcomes for the Mercy Hospital for Women cohort who provided a blood sample at 36 weeks' gestation.

	Controls (n = 182)	PE (n = 23)	p-value
Maternal age (years) Median (IQR)	32.00 (29.75–35.00)	34.00 (32.00–37.00)	0.02
Booking BMI (kg/m^2) Median (IQR)	24.55 (22.29–28.12)	27.88 (24.14–30.66)	0.02
Parity no. (%)			
0	89 (48.90)	19 (82.61)	0.007
1	72 (39.56)	4 (17.39)	
≥2	21 (11.54)	0 (0.00)	
Smoking status no. (%)			
Current smoker	170 (93.40)	21 (91.30)	0.93
Ex-smoker	6 (3.30)	1 (4.35)	
Never smoked	6 (3.30)	1 (4.35)	
GDM no (%)	20 (11.11)	6 (26.10)	0.04
Onset of labour no. (%)			
Spontaneous	80 (43.96)	7 (30.43)	0.29
Induced	72 (39.56)	13 (56.52)	
No labour	30 (16.48)	3 (13.05)	
Caesarean section no. (%)	59 (32.42)	12 (52.17)	0.06
Gestation at delivery (weeks) Median (IQR)	39.42 (38.85–40.42)	38.57 (37.85–39.28)	<0.0001
Birth weight (g) Median (IQR)	3,490 (3,185–3,820)	3,150 (2,550–3,450)	0.0002
Male no. (%)	91 (50.00)	11 (47.83)	0.84

BMI, body mass index; GDM, gestational diabetes mellitus. Unpaired t-test was used for normally distributed data, Mann-Whitney U tests for non-parametric data, and Chi-square tests for categorical variables. BMI and GDM data missing for 2/182 control samples. $p < 0.05$ was considered significant.

Assay ID: Hs03045108_m1) using Fluorescein amidite (FAM) labelled Taqman gene expression assays (Life Technologies) on the CFX 384 (Biorad, Hercules, CA) with Taqman fast advanced universal PCR mastermix (Applied Biosystems). The run conditions were: 95°C for 20 s followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. All data was normalized to the housekeeping gene *YWHAZ* (Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta, Assay ID: Hs01122454_m1) for inflammatory stimuli treated cells and the geometric mean of *TOP1* (topoisomerase-1, Assay ID: Hs00243257_m1) or *CYC1* (cyclin-1, Assay ID: Hs00357717_m1) for hypoxic and EVT cells. Samples were run in duplicate, and an average cycle threshold (Ct) value was used. Results were normalised to the Ct mean of each control group and expressed as a fold change with respect to the control.

2.6 Statistical analysis

Maternal characteristics were compared for patients with preeclampsia compared to controls using a Mann-Whitney U test for continuous data, and Chi-square test for categorical data. Data was initially assessed for normal distribution using Anderson-Darling test, D'Agostino and Pearson test, Shapiro-Wilk test, and Kolmogrov-Smirnov test. For data

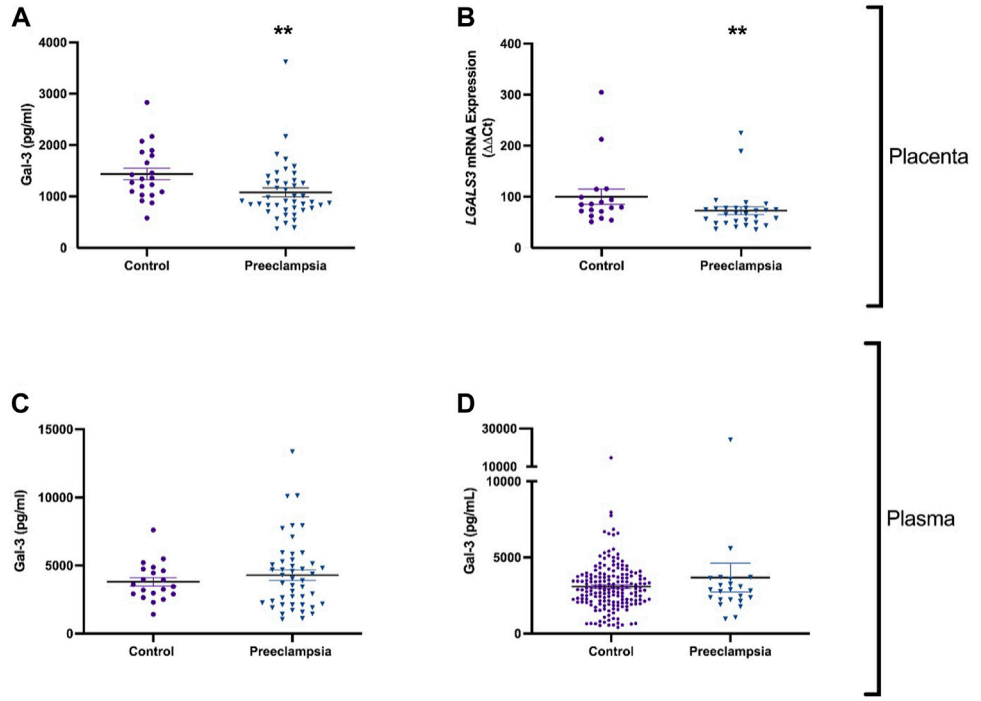

FIGURE 1
Galectin-3 is reduced in the placenta, but not in plasma of patients with early-onset preeclampsia or before the diagnosis of preeclampsia. Galectin-3 protein concentration in placental lysates from 43 patients with early-onset preeclampsia and 21 controls **(A)**. mRNA expression of galectin-3 gene *LGALS3* in placenta from 29 women with preeclampsia and 18 controls **(B)**. Circulating Galectin-3 in 46 patients with early-onset preeclampsia and 20 controls **(C)**. Galectin protein in maternal plasma from 23 women who later developed preeclampsia, and 182 healthy controls **(D)**. Data points represents individual patients (control; purple and preeclampsia; blue). Data are expressed as median (Interquartile range). $^{**}p < 0.01$.

containing two groups, Mann-Whitney test was used for unpaired non-parametric data. For analysis comparing more than three groups, one-way analysis of variance (ANOVA; parametric) or Kruskal Wallis test (non-parametric) was used. *In vitro* experiments were performed in either duplicate or triplicate and repeated five times. The *in vivo* experiments were normalised to controls and data was expressed as percentage control. $p < 0.05$ was considered significant. All statistical analyses were performed using GraphPad Prism 9.3.1 (GraphPad Software, LLC).

3 Results

3.1 Placental galectin-3 is reduced in the patients with early-onset preeclampsia

Galectin-3 protein and mRNA expression were measured in placenta of patients with early-onset preeclampsia who delivered before 34 weeks' gestation. Galectin-3 protein was significantly decreased in placenta from pregnancies ($p = 0.002$) complicated by preeclampsia ($n = 43$) compared to gestation matched controls (Figure 1A; $n = 21$). mRNA expression of LGALS3 was significantly reduced ($p = 0.009$) in placentas from women with preeclampsia ($n = 29$) compared to controls (Figure 1B; $n = 18$).

3.2 Circulating galectin-3 in established early-onset preeclampsia and preceding a diagnosis ofpreeclampsia at term gestation

Given that galectin-3 protein and expression levels were dysregulated in preeclamptic placental lysates, we measured circulating galectin-3 in patients with established early-onset disease relative to gestation matched controls and at 36 weeks prior to any potential term preeclampsia diagnosis. There was no significant difference in circulating galectin-3 levels in women with early-onset preeclampsia ($n = 46$) compared to controls ($n = 20$, Figure 1C). Galectin-3 levels were not different in women who later developed preeclampsia ($n = 23$) versus controls ($n = 182$) (Figure 1D).

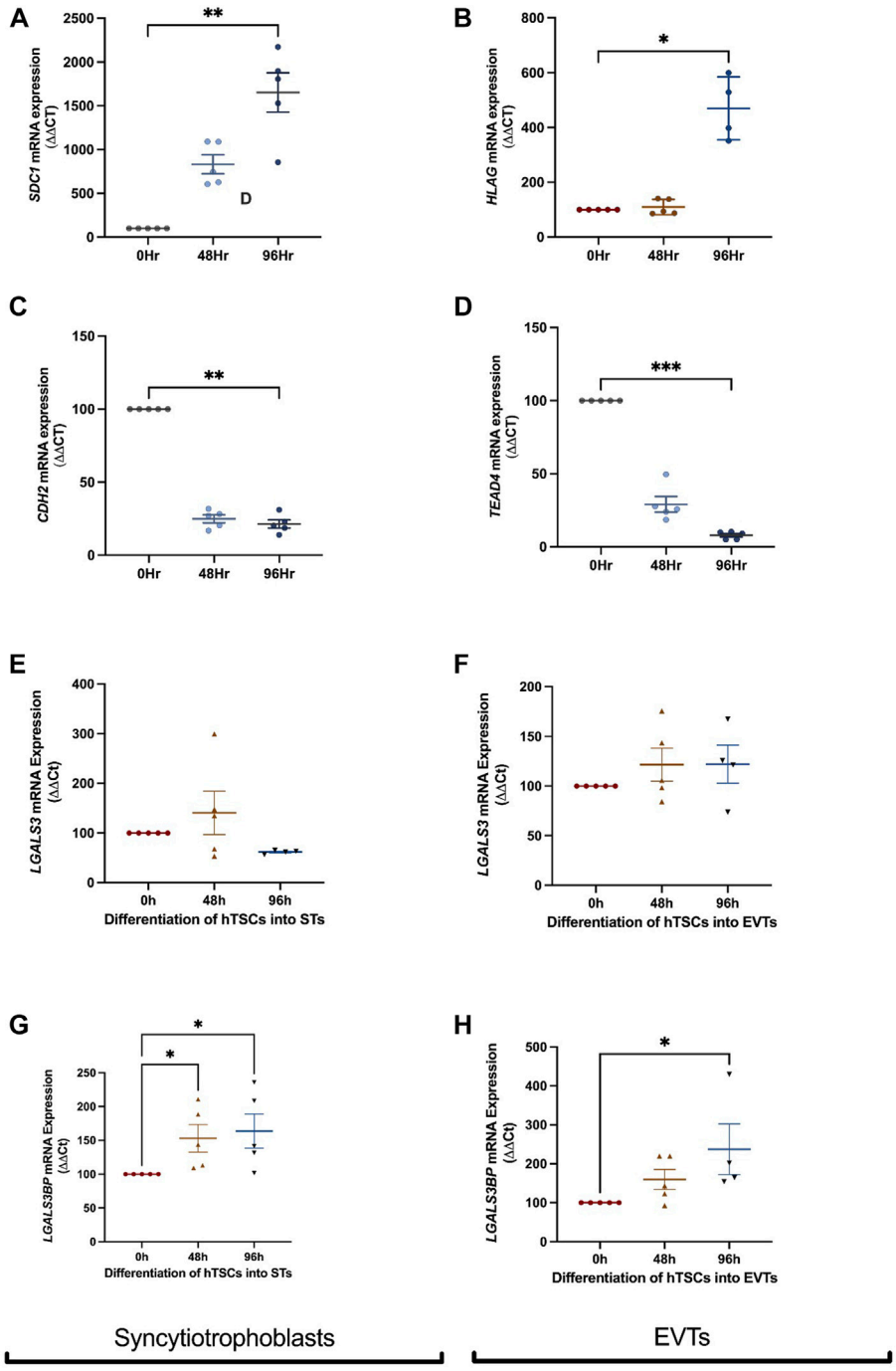

FIGURE 2
LGALS3 and LGALS3BP mRNA expression in first trimester placental stem cells differentiated into syncytiotrophoblast and extravillous trophoblasts. First trimester placental cytotrophoblast cells were differentiated into either syncytiotrophoblast or extravillous trophoblast (EVT) cells over 96 h. Syncytiotrophoblast differentiation was confirmed by increased expression of *SDC1* (syncytiotrophoblast marker) **(A)** and decreased expression of *CDH2* (cell border marker) **(C)** across time. LGALS3 **(E)** and LGALS3BP **(G)** mRNA expression with syncytiotrophoblast differentiation across 96 h. EVT differentiation was confirmed by increased expression of HLAG (EVT marker) **(B)** and reduced expression of TEAD4 (cytotrophoblast marker) **(D)** across time. LGALS3 **(F)** and LGALS3BP **(H)** mRNA expression with EVT differentiation over 96 h. All experiments were repeated $n = 5$ times in duplicate. Data is expressed as mean ± SEM; $*p < 0.05$, $**p < 0.01$.

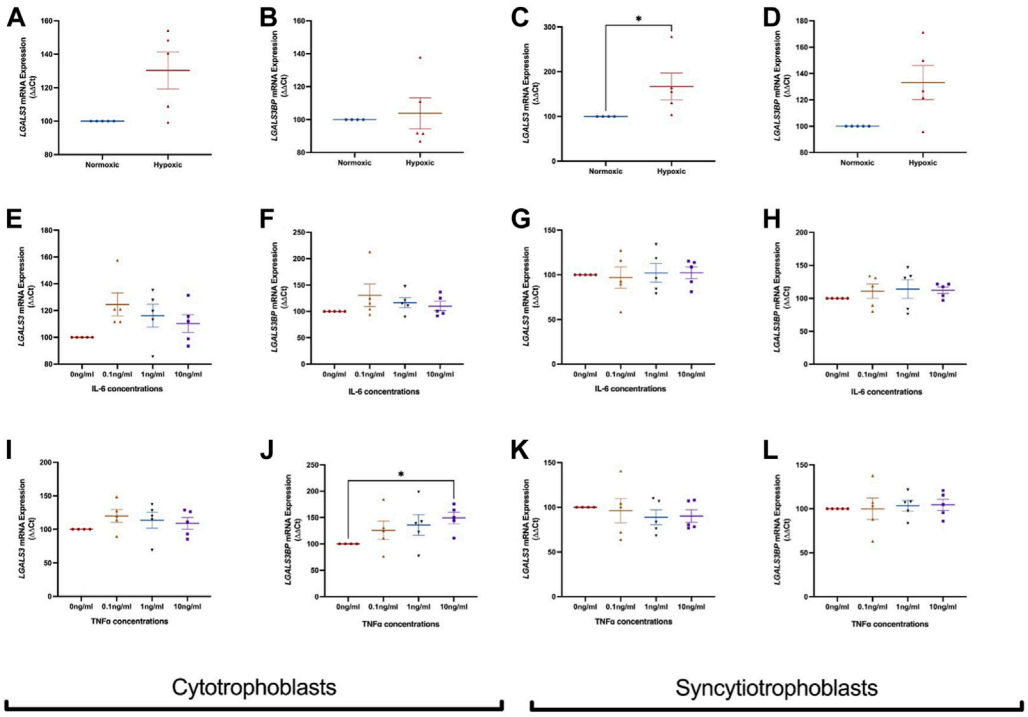

FIGURE 3
Effect of hypoxia and inflammation on LGALS3 and LGALS3BP mRNA expression in cytotrophoblast and syncytiotrophoblast cells. First trimester cytotrophoblast and syncytiotrophoblast cells were cultured in hypoxia or exposed to inflammatory stimuli (IL-6 or TNFα) at 0, 0.1, 1, or 10 ng/ml. mRNA expression of LGALS3 **(A)** and LGALS3BP **(B)** in cytotrophoblast cells after 1% hypoxia or 8% normoxia exposure. mRNA expression of LGALS3 **(C)** and LGALS3BP **(D)** in syncytiotrophoblast cells following 1% hypoxia and 8% normoxia conditions. LGALS3 **(E)** and LGALS3BP **(F)** mRNA expression following IL-6 treatment in cytotrophoblast cells. LGALS3 **(G)** and LGALS3BP **(H)** mRNA expression following IL-6 treatment in syncytiotrophoblast cells. LGALS3 **(I)** and LGALS3BP **(J)** mRNA expression in cytotrophoblast cells following TNFα treatment. LGALS3 **(K)** and LGALS3BP **(L)** mRNA expression in syncytiotrophoblast cells following TNFα treatment. All experiments were repeated $n = 5$ times with triplicate repeats. Data is expressed as mean ± SEM; $^*p < 0.05$.

3.3 *LGALS3* and LGALS3BP mRNA expression in differentiated first trimester cytotrophoblast into syncytiotrophoblast or extravillous trophoblast cells

To characterise the expression of galectin-3 and galectin-3BP in the placenta, cytotrophoblast were differentiated into either syncytiotrophoblast or EVT across 96h, and mRNA was measured at 0, 48, and 96 h time points. Syncytialisation was confirmed by increased SDC1 (syncytiotrophoblast marker) expression (Figure 2A, $p = 0.001$) and reduction in cell border marker, CDH2 (E-cadherin 2) mRNA expression with syncytiotrophoblast differentiation over 48 and 96 h (Figure 2C, $p = 0.005$). In addition, LGALS3 mRNA expression did not change as cytotrophoblast cells were differentiation into syncytiotrophoblast cells (Figure 2E). However, *LGALS3BP*mRNA expression was increased following differentiation into syncytiotrophoblast cells (Figure 2G, $p = 0.01$).

Differentiation of hTSCs into EVTs was confirmed by an increase in HLAG (EVT marker) expression after 96 h (Figure 2B, $p = 0.03$) and reduction in cytotrophoblast marker TEAD4 mRNA expression after 96 h (Figure 2D, $p = 0.0006$). There were no differences in LGALS3 mRNA expression following cytotrophoblast differentiation into EVT cells (Figure 2F). However, LGALS3BP mRNA expression was significantly increased (Figure 2H, $p = 0.02$) following EVT differentiation.

3.4 *LGALS3* and *LGALS3BP* mRNA expression in first trimester placental stem cells exposed to either hypoxia or inflammatory stimuli

Next, LGALS3 and LGALS3BP mRNA expression was measured in cytotrophoblast and syncytiotrophoblast cells exposed to hypoxia (1% O2) or normoxia (8% O2). In cytotrophoblast cells, LGALS3 (Figure 3A) and LGALS3BP (Figure 3B) mRNA expression was unchanged in a hypoxic environment. Hypoxia increased mRNA expression of LGALS3 in syncytiotrophoblast cells (Figure 3C, $p = 0.016$), but not LGALS3BP expression (Figure 3D).

We exposed cytotrophoblasts and syncytiotrophoblast to pro-inflammatory cytokines IL-6 and TNFα to determine if inflammation dysregulates *LGALS3* and LGALS3BP expression. Treatment of IL-6 did not alter mRNA expression of *LGALS3* (Figure 3E) and LGALS3BP (Figure 3F) in cytotrophoblast cells. mRNA expression of *LGALS3* (Figure 3G) and LGALS3BP (Figure 3H) was also not changed in syncytiotrophoblast cells.

When cytotrophoblast (Figure 3I) and syncytiotrophoblast (Figure 3K) cells were treated with TNFα, no change in *LGALS3* mRNA expression was observed. However, there was increased LGALS3BP expression in the presence of TNFα in cytotrophoblast cells (Figure 3J, $p = 0.03$). TNFα did not alter LGALS3BP expression in syncytiotrophoblast (Figure 3L).

4 Discussion

This study identified reduced levels of placental galectin-3 in women with early-onset preeclampsia but no changes within the circulation in established early-onset disease or before development of preeclampsia. The cell studies revealed increased LGALS3 expression in hypoxia treated syncytiotrophoblast cells and LGALS3BP expression in TNF-α treated cytotrophoblast cells.

Galectin-3 is a carbohydrate binding lectin that plays a crucial role in many diseases (Simovic Markovic et al., 2016). Our study revealed reduced placental galectin-3 in established early-onset preeclampsia (delivered at <34 weeks' gestation). In a mouse model of pregnancy with galectin-3 knockdown, reduction in galectin-3 was accompanied by reduced fetal weight, delay in fetal development, and increased placental inflammation (Freitag et al., 2020). Although our study did not detect changes in galectin-3 in plasma from women with preeclampsia, a reason for this may be due to a reduction of galectin-3 production in the placenta, which reduces the amount secreted into the maternal circulation. In contrast to our findings, Ruikar et al. (2022) and others have demonstrated elevated levels of galectin-3 protein in placentas from preeclamptic pregnancies (Jeschke et al., 2007). A possible reason for the discrepancy in findings is Ruikar et al. (2022) measured galectin-3 in preterm preeclamptic placentas, compared with term controls, whilst our study looked at early-onset preeclamptic placentas compared to gestation matched controls. Several studies have suggested differences in the aetiology of early-onset and term preeclampsia, therefore the underlying abnormalities contributing to early-onset preeclampsia may not be comparable to term disease (Gathiram and Moodley, 2016; Phillips et al., 2010). Further studies are required to validate these findings and determine how placental galectin-3 protein differs in early-onset and late-onset preeclampsia in both phenotypes.

This study is the first to evaluate circulating galectin-3 in plasma from women with preeclampsia. In our established disease cohort, circulating plasma galectin-3 was not altered in women with early-onset preeclampsia. Other studies have measured galectin-3 in serum. A recent study measured serum galectin-3 in early-onset preeclampsia also found no significant differences between preeclampsia and controls (Nikolov et al., 2020). However, Pankiewicz et al. (2020) reported higher serum galectin-3 levels in patients with preeclampsia. It is important to note that their study involved samples from both early-onset and late-onset preeclampsia, while our established disease cohort all delivered early-onset (<34 weeks).

In total, there are at least 15 known galectins, and other studies have examined many of them in reproductive tissues from healthy and pathological pregnancies. Galectin-1 likely overlaps with galectin-3 as both are reported to increase with trophoblast invasion and syncytilisation (Jeschke et al., 2007). Although galectin-1 differs from galectin-3 through support of immune tolerance and by influencing the secretion of hCG (Blois and Barrientos, 2014). Galectin-1 is increased in the placenta of patients diagnosed with severe preeclampsia compared to gestation matched controls (Than et al., 2008). As galectin-1 mediates a variety of immune cell interactions and responds to acute inflammation (Rabinovich et al., 2000), the increased expression could be part of a placental response to increased maternal inflammation, which could influence the maternal-fetal tolerance. Placental specific galectin-13 is a well-studied protein that has a role in damage signalling as it is elevated with the onset of preeclampsia. As galectins can be secreted from inflamed tissues following cellular stress, future research would benefit from measuring multiple galectins with galectin-3 to understand their diverse roles in placentas complicated by preeclampsia.

There is limited literature to suggest that LGALS3BP is expressed in all trophoblast subpopulations. Our *in vitro* differentiation studies identified higher LGALS3BP expression in syncytiotrophoblast and EVT compared to cytotrophoblast cells. mRNA expression of LGALS3 was not altered in cytotrophoblast, syncytiotrophoblast or EVT. While the binding protein is only increased in syncytiotrophoblast and EVTs, all trophoblast cell types can produce the ligand galectin-3. Several studies have reported the interaction between galectin-3 and galectin-3 binding protein initiates pathologic, proinflammatory signalling cascades in diseases such as cancer, and venous thrombosis (DeRoo et al., 2015; Newlaczyl and Yu, 2011; Silverman et al., 2012). A study conducted by Silverman et al. reported that interaction between galectin-3 and galectin-3 binding protein resulted in transcriptional upregulation of IL-6 in bone marrow mesenchymal stem cells *via* galectin-3 binding protein/galectin-3/Ras/mEK/ERK signalling pathway (Silverman et al., 2012). Given inflammation and placental hypoxia play crucial role in pathophysiology of preeclampsia (Roberts and Gammill, 2005), we examined the effect of pro-inflammatory cytokines (IL-6 and

TNFα) and hypoxia on LGALS3 and LGALS3BP mRNA expression in placental cytotrophoblast and syncytiotrophoblast cells. In our study, treatment of pro-inflammatory cytokine TNF-α caused an increase in LGALS3BP expression in cytotrophoblast, whilst IL-6 had no effect on LGALS3BP mRNA expression. A study by Gleissner et al. (2017) measured galectin-3BP concentrations in plasma from patients with cardiovascular disease and found increased galectin-3BP were associated with enhanced markers of inflammation, including TNFα. This study shares similarities to ours as we have observed increased LGALS3BP mRNA expression with increased inflammation, indicating the possible involvement of galectin-3BP in inflammation associated with preeclampsia. In addition, previous proteomic analysis by Kolla et al. (2012) reports elevated levels of circulating galectin-3 binding protein in patients at high risk of developing preeclampsia. Given galectin-3 binding protein binds to other galectins and there are other galectins expressed in the fetal-maternal interface (Jovanović Krivokuća et al., 2021), further studies should investigate the role of galectin-3 binding protein in the presence of other galectins in preeclampsia.

Previous studies have reported increased galectin-3 levels in human placenta cell line from choriocarcinoma BeWo cells when cultured in hypoxic conditions (Hu et al., 2007). Our data also suggests that the galectin-3 gene is increased with hypoxia. This is different to our data in preeclamptic placentas where we found decreased galectin-3 protein and mRNA expression. Therefore, the dysregulated galectin-3 that was observed in early-onset preeclampsia is unlikely a result of hypoxia.

Collectively, this study observed dysregulated levels of galectin-3 in early-onset preeclamptic placental lysates and hypoxia, but not inflammation. While results suggest a potential association between galectin-3 binding protein and inflammation, further studies are needed to understand the relationship between galectin-3 binding protein and galectin-3 in preeclampsia.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the Mercy Health Human Research Ethics Committee (R11/34). The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualisation, LB, TK-L, ST, and MK; Methodology, LB, MK, T-VN, PC, and NH; Formal analysis, MK and LB; Investigation, MK; Resources, TK-L, NH, ST, SW, and TM; Writing—original draft preparation, MK, LB, and TK-L; Writing—review and editing, all co-authors; Funding Acquisition, TK-L, ST, and SW.

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Conflict of interest

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

Publisher's note

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Investigating pregnant women's health information needs during pregnancy on internet platforms

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Artificial intelligence gives pregnant women another avenue for receiving healthcare information. With the advancement of information and communication technology, searching online for pregnancy information has become commonplace during COVID-19. This study aimed to explore pregnant women's information-seeking behavior based on data mining and text analysis in China. Posts on maternal and infant-related websites were collected during 1 June 2020, and 31 January 2021. A total of 5,53,117 valid posts were obtained. Based on the data, we performed correlation analysis, topic analysis, and sentiment analysis. The correlation analysis showed the positive effects of population, population with a college education or above, and GDP on post counts. The topic analysis extracted six, nineteen, eighteen, thirteen, eleven, sixteen, thirteen, sixteen, nineteen, and fourteen topics in different months of pregnancy, reflecting different information needs in various pregnancy periods. The results of sentiment analysis show that a peak of the posts emerged in the second month of pregnancy and the proportion of emotionally positive posts reached its peak in the sixth month of pregnancy. The study provides important insights for understanding pregnant women's information-seeking behavior.

KEYWORDS

pregnancy, health information, text analysis, topic analysis, sentiment analysis

1 Introduction

Artificial intelligence (AI) creates opportunities for enabling pregnant women to receive healthcare information. Pregnancy is a crucial period in a woman's life accompanied by physical change, psychological change, and role transformation. Information-seeking can play an important role in addressing the issue of a healthy delivery. Access to advantageous and concerned information contributes to health-related decisions and the life of both pregnant women and unborn children (Kamali et al., 2018). Childbirth-related information is considerable for performing beneficial interventions and suggestions for pregnant women (Kamali et al., 2018). For example, health-related information will enable women to prepare for pregnancy, concentrate on balanced nutrition and medication use during pregnancy, and make decisions on exercise intensity and mode.

Extant research on health information has addressed the crucial role of research contexts, such as the user group and the domain of information subject in determining information needs (Pian et al., 2020; Reifegerste et al., 2020). The development of information technology and the spread of the mobile Internet enable pregnant women to seek information in a more conveniently and fairly way. Centered on the information needs of maternal health, recent studies have shown that pregnant women's information-seeking behavior is crucial to enriching the knowledge of childbirth and maternal health and improving maternal health outcomes (Kamali et al., 2018; Ahmadian et al., 2020; Jin et al., 2020; Kassim, 2021). For example, Kamali et al. (2018) found that pregnant women need information such as psychological and physical complications after delivery and pregnancy nutrition in the descriptive study. The qualitative study conducted by Kassim (2021) found that the unavailability of health facilities and limited chances of accessing professional health care could lead to the results that pregnant women seek information from non-professional and informal sources. Ahmadian et al. (2020) identified commonly searched topics during pregnancy using the questionnaire. However, researchers have not treated the topics of information-seeking and pregnant women's emotions in much detail by employing a relatively large amount of data.

The objective of this research is to explore pregnant women's information-seeking behavior during the whole pregnancy, including the factors that contribute to the information-seeking behavior, the topics that cause pregnant women's attention at different months of pregnancy, and the change in pregnant women's emotions at different stages of pregnancy. By collecting and analyzing the posts in the "pregnant section" under "Mama.cn" from 1 June 2020, to 31 January 2021, and 5,53,117 valid posts, the current work provides a comprehensive study.

2 Materials and methods

2.1 Data collection

With the advancement of Internet technology, pregnant women's behavior of seeking online health information has become a universal trend worldwide because of insufficient information received from healthcare providers and the natural advantage of the Internet to ask questions anonymously (Al-Dahshan et al., 2021). As one of the largest maternal and child health websites in China, "Mama.cn" has integrated websites, APPS, new media, micro-network celebrities, and other media resources, covering hundreds of millions of pan-maternal and infant groups. Dedicated to serving all kinds of needs of pregnant women, the company has built several service sections including information, social networking, tools, and e-commerce, aiming to build a diversified Internet maternal, and infant service platform with pregnant women as the core. "Mama.cn" is widely popular among people who are preparing for pregnancy, during pregnancy, and childrearing. In August 2019, "Mama.cn" had 16.479 million active users. The number of active users of "Mama.cn" reached 19.31 million in June 2020, ranking first in the parenting subdivision list in China. Therefore, "Mama.cn" was selected as the research data source for this study. This study collected the posts in the "pregnant section" under "Mama.cn" from 1 June 2020, to 31 January 2021, involving data from "the first month of pregnancy" to "the tenth month of pregnancy." The current study extracted the following information from the "pregnant section" under "Mama.cn" posts: username, post time, duration of pregnancy, city, and text. A total of 5,75,970 posts were obtained. Examples of our dataset are presented in Table 1.

We pre-processed the original data before formal analysis by the following procedures. First, the raw information may include missing city tags, irrelevant advertising messages, or posts that did not match the actual time of pregnancy. We filtrated and deleted the above data and finally obtained 5,53,117 texts. Second, the original message may contain distracting information, such as interpunction, emoticons, blank, and hashtags. For excluding data noise and improving data analysis efficiency, we employed regular expressions operations in Python for text filtering.

Measures were performed to ensure data privacy, anonymity, and security. The data collection and analysis did not disclose any privacy issues regarding pregnant women's identifiable and sensitive information (Favaretto et al., 2020). During data collection, only username, post time, duration of pregnancy, city, and text were extracted. In data processing and analysis, only the duration of pregnancy, city, and text data was used, while the personal information of users was not disclosed. By involving as many samples as possible, more anonymity was preserved as a combination of the variables will be repeated among the samples (Leon-Sanz, 2019).

2.2 Methods

2.2.1 Text topic analysis based on latent Dirichlet allocation model

LDA (Latent Dirichlet Allocation) topic model is a topic probability distribution model based on PLSI (Probabilistic Latent Semantic Indexing) model (Blei et al., 2003). The LDA topic model simulates the process of document generation by using an implied random variable that follows a Dirichlet distribution to represent the document's topic mixing ratio.

TABLE 1 The examples of dataset.

No	City	Post time	Duration of pregnancy	Text
1	Dazhou city	31/01/2021	Gestation: 3 weeks + 2 days	I just found out I am pregnant, I feel intermittent pain in belly. What's going on?
2	Linyi city	28/01/2021	Gestation: 6 weeks + 1 day	I just went to the toilet and saw a little brown secretion. Not much rubbing, a little worried
3	Wuhan city	25/01/2021	Gestation: 11 weeks + 5 days	My nuchal translucency test passed at one time. The doctor said that the baby was well behaved and in good upgrowth
4	Yinchuan city	31/12/2020	Gestation: 15 weeks + 3 days	My New Year's resolution is to have a healthy baby! No matter if you are a boy or a girl, stay healthy!
5	Zhongshan city	23/01/2021	Gestation: 29 weeks + 5 days	Sometimes the fetus moves so much. It feels like she is about to jump out from my belly
6	Fuzhou city	27/01/2021	Gestation: 34 weeks + 4 days	34 weeks, I feel pain in public bone, back, and coccyx

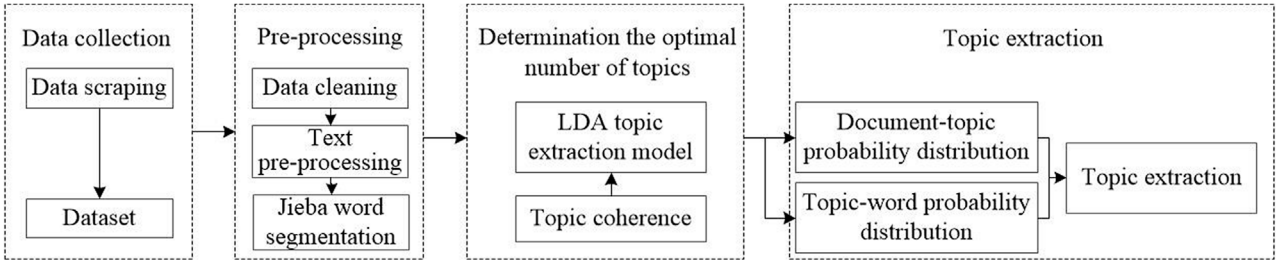

FIGURE 1
The process of topic extraction.

Its model structure is more complete and clearer, and the probability inference algorithm is adopted to process the text, which can greatly reduce the dimension of the text representation, to avoid dimension disaster (Blei, 2012). Therefore, LDA is widely used in text mining, text clustering, language processing, and other aspects. The topic number K contained in the document set is a hyperparameter. Given other hyperparameters, the selection process of topic number K is the process of the model searching for the optimal topic number. When the number of topics is too large, there will be many topics without obvious classification semantic information. When the number of topics is too small, broad topics will be generated with a mixture of two or more distributions (Panichella, 2021). Therefore, the determination of the optimal number of topics is an important issue. A coherence score was used to determine the optimal number of topics, with a higher coherence score indicating better quality of topics (Korenčić et al., 2018; Panichella, 2021; Shah et al., 2021). This study used the open-source LDA tool in the Gensim library. The LDA model was evaluated by topic coherence to determine the optimal number of topics. According to the trained LDA model, the topic words under each topic were obtained and the probability of each text belonging to each topic could be directly predicted. Finally, the corresponding topic name was summarized in accordance with the topic words. Figure 1 presents the process of topic extraction in this study.

2.2.2 Text sentiment analysis based on SnowNLP

In recent years, there has been an increasing interest in sentiment analysis (Wang et al., 2019). Sentiment analysis, also known as opinion mining, is an application of text mining and computational linguistics to mine subjective texts with emotional colors and identify the emotional tendencies contained in them. It is a process of identifying information from texts and analyzing, processing, induction, and reasoning subjective texts with emotional color. Through sentiment analysis, researchers can determine users' emotional orientation in the text. Text-based sentiment analysis methods are mainly divided into three types: sentiment dictionary-based, machine learning-based, and deep learning-based (Xu et al., 2019; Li et al., 2020). The machine learning-based analysis method trains the emotion classifier with emotion-labeled data to achieve emotion classification. Classification accuracy relies on high-quality human-annotated training sets, and large-scale high-quality training data requires a lot of labor costs, and

TABLE 2 The results of descriptive statistical analysis at the provincial level.

Variables	Minimum value	Maximum value	Mean value	Standard deviation	Number (N)
Post counts	410	93449	17819.097	17596.221	31
Population	3648100	126012510	45476733.03	30506939.64	
Population with college education or above (ten thousand)	40	1978	700.6452	443.05752	
Illiteracy rate (%)	0.78	21.11	3.42	3.72	
GDP (100 million yuan)	1902.7	110760.9	32658.5548	26661.80805	

TABLE 3 The results of correlation analysis at the provincial level (N = 31).

	Variable	Correlation coefficient	*p*-value
Post counts	Population	0.889***	<0.001
	Population with college education or above	0.835***	<0.001
	Illiteracy rate	−0.227	>0.05
	GDP	0.819***	<0.001

the results of human subjective data annotation will also affect the classification effect. The deep learning-based analysis is based on feature self-learning and deep neural network. It has a good classification effect when dealing with high-dimensional, unlabeled big data, but it is difficult to accurately classify the semantically ambiguous and short text content in social networks. The sentiment dictionary-based method is an unsupervised method, which uses a sentiment dictionary to discriminate the sentiment polarity of text containing keywords, to achieve sentiment classification for each text. There is no need for complex data labeling in the research process and the accuracy of emotion recognition can be improved by adjusting and expanding the vocabulary of the sentiment dictionary according to the specific research background.

SnowNLP, a Python library for Chinese natural language processing, is used to analyze the sentiment of texts. The tool is based on a sentiment dictionary to analyze the sentiment orientation of texts. SnowNLP employs a sentiment dictionary to realize the sentiment tendency analysis of the text. The main functions include part-of-speech tagging, sentiment analysis, keyword extraction, and text summarization (He et al., 2020; Zhang et al., 2021).

3 Correlation analysis

The correlation analysis is performed using SPSS24.0. The results of descriptive statistical analysis at the provincial level are presented in Table 2. The data of posts, population, population with a college education or above, illiteracy rate, and GDP of the province are from mainland China. More precisely, population, population with a college education and above, and illiteracy rate are all data from the 2020 census.

Table 3 presents the correlation analysis results at the provincial level. Post counts was found to positively related to population (ß = 0.889, $p < 0.001$), population with college education or above (ß = 0.835, $p < 0.001$), and GDP (ß = 0.819, $p < 0.001$). However, a significant relationship between post counts and illiteracy rate ($p > 0.05$) was not found in this study. This result is consistent with previous research which indicates that the illiteracy rate had a small and insignificant correlation with computer and Internet penetration rates statistically (Chinn and Fairlie, 2010).

4 Topic analysis of information needs

4.1 Emerged topics in different months of pregnancy

4.1.1 Information needs in the first month

As mentioned above, topic analysis was divided based on the stages of pregnancy, corresponding to the period from "the first month of pregnancy" to "the tenth month of pregnancy". Table 4 presents the topics identified in the first month of pregnancy, relative weight, and LDA keywords. Six topics emerged in the first month of pregnancy in which the first frequent topic. "Test strip," accounts for 20.53% of all topics. "Pregnancy tests consultation," "early pregnancy inspection," and "early

TABLE 4 Topics in the first month of pregnancy.

	Topic name	Rate (%)	LDA keywords
1	Test strip	20.53	Last menstrual period, ovulation, pregnancy test paper, deepen, detect, intercourse, color, one deep and one shallow, obvious, ovulatory period
2	Pregnancy tests consultation	16.59	Yes or no, pregnancy, take a look, give a hand, pray, really, two lines, duration
3	Early pregnancy inspection	14.73	Hospital, detect, normal, low progesterone, HCG doubled, draw blood, worry, B ultrasound, brown secretion, blood test
4	Early pregnancy reaction	14.02	Eat, feeling, early pregnancy, collywobbles, everyday, night, emesis, symptom, not good, uncomfortable
5	Appeals and desire	12.54	Baby, hope, mother, good pregnancy, healthy, earnestly hope, love, must, finally
6	Question for help	10.96	Pregnancy, have you ever, discern, circumstance, affect, find, why, need, question

TABLE 5 Topics in the second month of pregnancy.

	Topic name	Rate (%)	LDA keywords
1	Precautions for early pregnancy	8.59	Early stages of pregnancy, purchase, affect, fetus, catch a cold, recommend, create profile, skin care product, attention, nuchal translucency, clothes, prepare
2	Early pregnancy inspection	8.35	Check, B ultrasound, gestational sac, report, show, ectopic pregnancy, *in utero*, yolk, transvaginal ultrasound, germ, recheck
3	Symptoms of early pregnancy	7.39	Feeling, collywobbles, normal, symptom, 6 weeks, 7 weeks, why, once in a while, lower abdominal pain
4	The gender of baby	7.04	Take a look, give a hand, boy, girl, discern, everyone, make out, whether or not
5	Fetal heart and embryo bud	6.54	Fetal heart, embryo bud, hope, healthy, good pregnancy, bless, happy, antenatal care, all the best, *in utero*, rest assured
6	Vomiting during pregnancy	6.40	Vomiting during pregnancy, uncomfortable, reaction, nausea, serious, stomach, anesis, loss of appetite, dizziness, retch
7	Early pregnancy indicators	6.31	Low progesterone, HCG doubled, normal, doctor, draw blood, recheck, decline, blood test, relatively low
8	Calculation of pregnancy period	6.30	Month, day, last menstrual period, count pregnancy period, the last time, intercourse, detect, menstrual cycle, the first day, ovulatory period, pattern
9	Appeals and desire	5.70	Baby, mother, hope, cheer, healthy, love, expectation, grow up, safety, happy, birth
10	Prenatal diet	5.34	Eat, hungry, food, drink, folic acid, loss of appetite, like, not allowed, eat nothing, meat

TABLE 6 Topics in the third month of pregnancy.

	Topic name	Rate (%)	LDA keywords
1	Nuchal translucency and filling	13.05	Hospital, nuchal translucency, create a profile, need, appointment, prepare, expense, antenatal care, several weeks, empty stomach, draw blood
2	Vomiting during pregnancy	12.51	Vomiting during pregnancy, reaction, serious, food, everyday, stomach, nausea, hungry, loss of appetite, retch, dizziness
3	The gender of baby	8.65	Take a look, girl, boy, everyone, give a hand, curious, make out, discern, checklist
4	Symptom of early pregnancy	7.94	Feeling, collywobbles, normal, bloat, symptom, why, back pain, buttock, lower abdomen, once in a while
5	Prenatal diet	5.80	Eat, drink, prefer, meat, unthink, unable, fruit, spicy, sour, nutrition, appetite, breakfast
6	Fetal heart and embryo bud	5.72	Fetal heart, embryo bud, check, B ultrasound, Last menstrual period, doctor, show, gestational sac, recheck, upgrowth, yolk
7	Threatened miscarriage	5.12	Brown secretion, bleeding, hospital, restroom, find, suddenly, fetus protection, abortion, in hospital
8	Fetus protection	4.45	Doctor, inspection, low progesterone, HCG, progesterone, recheck, take medicine, fetus protection, draw blood, take an injection, suggestion
9	Share and exchange	4.24	Whether or not, expectant mother, expected date of confinement, the same kind, experience, early pregnancy, the same month, exchange, inform, Wechat group
10	Household affairs	4.22	Husband, cry, mother-in-law, work, at home, think, really, not good, marriage, afterwards, mood

pregnancy reaction," accounting for 16.59%, 14.73%, and 14.02%, respectively, were the second, third, and fourth most frequent topics. Among them, early pregnancy reaction refers to pregnant women's body response during the early pregnancy period. The next two frequent topics are "appeals and desire" and "question for help," at 12.54% and 10.96%, respectively.

TABLE 7 Topics in the fourth month of pregnancy.

	Topic name	Rate (%)	LDA keywords
1	The gender of baby	18.98	Take a look, boy, girl, everyone, give a hand, curious, nuchal translucency, discern, the first pregnancy, the second pregnancy, want, son, daughter
2	Down's syndrome	9.18	Inspection, hospital, non-invasive prenatal testing, Down's syndrome, screening, risks, amniocentesis, suggestions, draw blood, four-dimensional
3	Household affairs	7.50	Husband, child, mother-in-law, mood, work, at home, the first child, home, not good
4	Nuchal translucency	7.42	Nuchal translucency, once, doctor, baby, the first time, successfully, cooperate, finally, twice, make out
5	Appeals and desire	7.25	Baby, hope, mother, healthy, smoothly, cheer, happy, antenatal care, successfully, love, anticipate, bless
6	Abnormality in antenatal care	6.91	Doctor, inspect, worry, B ultrasound, placenta, problem, fetus, bleeding, secreta, upgrowth
7	Prenatal diet	6.85	Eat, food, dislike, drink, hungry, not allowed, unthink, meat, have a meal, specially, loss of appetite
8	Fetal movement	6.83	Feel, belly, move, night, fetal movement, sleep, lie, recently, seem, somewhile, always
9	Question for help	6.26	Pregnant, normal, pain, have you ever, discern, fetal heart, circumstance, why, suddenly, cause, question
10	Belly size and weight	6.25	Pregnant, 3 months, big stomach, almost 4 months, gain, weight, many kilograms, obviously pregnant

4.1.2 Information needs in the second month

Nineteen topics are identified in the second month of pregnancy. The most frequent ten topics in the second month of pregnancy, relative weight, and LDA keywords are presented in Table 5. The results show that "precautions for early pregnancy," "early pregnancy inspection," and "symptoms of early pregnancy" emerged to be the top three frequent topics, accounting for 8.59%, 8.35%, and 7.39%, respectively. The next five frequent topics are "the gender of baby," "fetal heart and embryo bud," "vomiting during pregnancy," "early pregnancy indicators," and "calculation of pregnancy period," at 7.04%, 6.54%, 6.40%, 6.31%, and 6.30%, respectively. The following two frequent topics are "appeals and desire" and "prenatal diet," at 5.70% and 5.34%, respectively.

4.1.3 Information needs in the third month

Eighteen topics are extracted in the third month of pregnancy. Table 6 presents the top ten topics in the third month of pregnancy. The results indicate that "nuchal translucency and filling," "vomiting during pregnancy," "the gender of baby," and "symptom of early pregnancy" emerged to be the four most frequent topics, accounting for 13.05%, 12.51%, 8.65%, and 7.94%, respectively. The next four frequent topics are "prenatal diet," "fetal heart and embryo bud," "threatened miscarriage," and "fetus protection," at 5.80%, 5.72%, 5.12%, and 4.45%, respectively. The following two most frequent topics are "share and exchange" and "household affairs," at 4.24% and 4.22%, respectively.

4.1.4 Information needs in the fourth month

Thirteen topics are identified in the fourth month of pregnancy. Table 7 presents the top ten topics in the fourth month of pregnancy. "The gender of baby" accounts for 18.98% of all topics. "Down's syndrome," "household affairs," "nuchal translucency," and "appeals and desire," accounting for 9.18%, 7.50%, 7.42%, and 7.25%, respectively, were the second, the third, the fourth, and the fifth most frequent topics. The next five frequent topics are "abnormality in antenatal care," "prenatal diet," "fetal movement," "question for help," and "belly size and weight," at 6.91%, 6.85%, 6.83%, 6.26%, and 6.25%, respectively.

4.1.5 Information needs in the fifth month

Eleven topics are extracted in the fifth month of pregnancy. Table 8 indicates the top ten topics in the fifth month of pregnancy. The top two frequent topics are "the gender of baby" and "Down's syndrome," at 12.73% and 11.97%. "Fetal movement," "prenatal diet," "pregnant women's physical discomfort," and "inspection of a large row of deformities" emerged to be the third, fourth, fifth, and sixth frequent topics, accounting for 9.93%, 9.26%, 9.11%, and 8.68%, respectively. The next four most frequent topics are "ponderal growth," "experience sharing," "household affairs," and "appeals and desire," accounting for 8.09%, 7.34%, 6.95%, and 6.26%, respectively.

4.1.6 Information needs in the sixth month

Sixteen topics are identified in the sixth month of pregnancy. Table 9 shows the top ten topics in the sixth month of pregnancy. The top two topics are "the gender of baby" and "four-dimensional ultrasound," accounting for 18.65% and 17.38% of all topics. The following four topics, "pregnant women's physical discomfort," "prenatal diet," "household affairs," and "fetal movement," comprise 7.53%, 6.33%, 6.03%, and 5.73%, respectively. "Appeals and desire," "ponderal growth," "glucose tolerance test," and "sleep during pregnancy" accounted for 5.55%, 5.21%, 4.79%, and 4.04%, respectively.

4.1.7 Information needs in the seventh month

Thirteen topics are extracted in the seventh month of pregnancy. Table 10 shows the top ten most frequent topics,

TABLE 8 Topics in the fifth month of pregnancy.

	Topic name	Rate (%)	LDA keywords
1	The gender of baby	12.73	Take a look, girl, boy, the second pregnancy, give a hand, curious, discern, the first pregnancy, son, daughter
2	Down's syndrome	11.97	Non-invasive prenatal testing, low risk, Down's syndrome, smoothly, high risk, DNA, amniocentesis, threshold, suggestion, hope
3	Fetal movement	9.93	Feel, fetal movement, obvious, fetal heart, the first time, seem, normal, once in a while
4	Prenatal diet	9.26	Eat, emesis, pregnancy, food, prefer, everyday, drink, calcium tablet, constipation, meat, hungry, DHA
5	Pregnant women's physical discomfort	9.11	Night, sleep, legs, buttocks, pain, get up, lie, special, feel ill, uncomfortable, not good, difficulty in sleeping
6	Inspection of a large row of deformities	8.68	Inspection, doctor, hospital, four-dimensional ultrasound, Down's syndrome, placenta, appointment, antenatal care, Nuchal translucency
7	Ponderal growth	8.09	Pregnant, over 4 months, big belly, weight, gain, kilogram, 5 months, first trimester, without getting fat
8	Experience sharing	7.34	Pregnancy, expected date of confinement, sign in, catch a cold, recommendation, the same kind, exchange, chat, prepare, share
9	Household affairs	6.95	Husband, child, mother-in-law, work, the first child, cry, unthink, in bad mood, look after a baby
10	Appeals and desire	6.26	Baby, mother, hope, cheer, healthy, love, happy, anticipate, bless, birth

TABLE 9 Topics in the sixth month of pregnancy.

	Topic name	Rate (%)	LDA keywords
1	The gender of baby	18.65	Take a look, boy, girl, four-dimensional ultrasound results, everyone, give a hand, curious, guess, son, daughter
2	Four-dimensional ultrasound	17.38	Inspection, fetus, four-dimensional ultrasound, worry, problem, normal, umbilical cord, recheck, relatively small
3	Pregnant women's physical discomfort	7.53	Fetal heart, restroom, bleeding, secreta, pain, feel ill, catch a cold, constipation, serious, afford no relief
4	Prenatal diet	6.33	Eat, prefer, pregnancy, drink, food, calcium tablet, hungry, meat, DHA, breakfast, nutrition, anemia
5	Household affairs	6.03	Husband, mother-in-law, work, at home, cry, unthink, everyday, marriage, boring, insist, work
6	Fetal movement	5.73	Feel, fetal movement, obvious, sometimes, frequent, severe, kick, immovability, belly
7	Appeals and desire	5.55	Baby, mother, hope, girl, boy, healthy, love, cheer, anticipate, birth, bless, all the best, safety
8	Ponderal growth	5.21	Pregnant, over 5 months, kilogram, weight, big belly, fat, gain, control, 6 months
9	Glucose tolerance test	4.79	Hospital, prepare, appointment, glucose tolerance test, expense, drink sugar water, blood glucose, empty stomach, high, normal
10	Sleep during pregnancy	4.04	Night, difficulty in sleeping, everyday, stay awake, uncomfortable, lie, always, tired, sleeplessness, often

rates, and LDA keywords. The results present that "the gender of baby" and "pregnant women's physical discomfort" emerged to be the first and the second most frequent topic, accounting for 21.67% and 13.34% of all topics, respectively. The following four topics are "sleep during pregnancy," "ponderal growth," "glucose tolerance test," and "prenatal diet," accounting for 7.19%, 6.63%, 6.58%, and 6.49%, respectively. "Items for childbirth," "household affairs," "appeals and desire," and "fetal movement" then comprised 6.36%, 6.09%, 5.82%, and 5.77%, respectively.

4.1.8 Information needs in the eighth month

Sixteen topics are identified in the eighth month of pregnancy. Table 11 presents the top ten most frequent topics. As shown in the results, the top two topics are "the gender of baby" and "emotion sharing", accounting for 10.36% and 10.31%. The following four topics, "prenatal care," "sleep during late pregnancy," "prenatal diet," and "appeals and desire," account for 7.94%, 7.15%, 6.93%, and 6.76%, respectively. The next four topics are "ponderal growth," "pregnant women's physical discomfort," "household affairs," and "preparation for delivery," at 6.47%, 6.43%, 6.26%, and 5.88%, respectively.

4.1.9 Information needs in the ninth month

Nineteen topics are extracted from the ninth month of pregnancy. Table 12 presents the top ten topics, rates, and LDA keywords. The results show that "prenatal care," "the gender of baby," and "emotion sharing" emerged to be the top three topics, accounting for 10.78%, 7.96%, and 7.13%, respectively. The next four most frequent topics are "items for

TABLE 10 Topics in the seventh month of pregnancy.

	Topic name	Rate (%)	LDA keywords
1	The gender of baby	21.67	Four-dimensional ultrasound results, give a hand, take a look, guess, boy, girl, the second pregnancy, the first pregnancy, want, curious
2	Pregnant women's physical discomfort	13.34	Mid-pregnancy, pain in the legs, pain in the buttocks, uncomfortable, tired, anesis, serious, method, constipation, feel ill
3	Sleep during pregnancy	7.19	Night, sleep, everyday, not good, morning, get up, stay awake, restroom, always, sleeplessness, midnight
4	Ponderal growth	6.63	Big belly, pregnant, kilogram, weight, gain, quick, 6 months, small, fat
5	Glucose tolerance test	6.58	Glucose tolerance, drink sugar water, blood glucose, empty stomach, high, normal, accused of sugar, check, doctor, draw blood
6	Prenatal diet	6.49	Eat, food, prefer, pregnancy, hungry, calcium tablet, fruit, emesis, nutrition, breakfast, meat
7	Items for childbirth	6.36	Expected date of confinement, prepare, purchase, need, goods, maternity package, hospital, clothes, recommend, price, share
8	Household affairs	6.09	Husband, mother-in-law, work, at home, everyday, cry, play with mobile phone, look after a baby
9	Appeals and desire	5.82	Baby, mother, hope, love, healthy, cheer, anticipate, happy, birth, successfully
10	Fetal movement	5.77	Feel, belly, fetal movement, special, normal, recently, obvious, more and more frequent

TABLE 11 Topics in the eighth month of pregnancy.

	Topic name	Rate (%)	LDA keywords
1	The gender of baby	10.36	Take a look, boy, girl, give a hand, the second pregnancy, four-dimensional ultrasound results, curious, daughter, want, the first pregnancy
2	Emotion sharing	10.31	Nervous, anxiety, uncomfortably, smoothly, cheer, emotion, unthink, insist, at home, work, boring
3	Prenatal care	7.94	Inspect, B ultrasound, amniocentesis, too large, too small, position of the fetus, normal, four-dimensional ultrasound, cord around neck, recheck, breech position
4	Sleep during late pregnancy	7.15	Night, sleep, later pregnant trimester, difficulty in sleeping, get up, restroom, stay awake, daytime, wake up in midnight
5	Prenatal diet	6.93	Eat, prefer, drink, hungry, anemia, food, pregnant women, emesis, constipation, meat, nutrition, breakfast, calcium tablet
6	Appeals and desire	6.76	Baby, mother, hope, love, birth, healthy, anticipate, term delivery, father, meet, safety, all the best
7	Ponderal growth	6.47	Expected date of confinement, kilogram, weight, awaiting delivery, control, fat belly, count down, gain
8	Pregnant women's physical discomfort	6.43	Pain, recently, feel ill, upset stomach, why, lie, later pregnant trimester, tired, sometimes, walk, sit, pain in public bone
9	Household affairs	6.26	Husband, child, mother-in-law, look after the first child, cry, home, marriage, unthink, come back
10	Preparation for delivery	5.88	Buy, hospital, need, clothes, pregnant women, breast pump, goods, recommend, child, prepare, price

childbirth," "sleep during late pregnancy," "fetal movement," and "pregnant women's physical discomfort" which comprised 6.27%, 6.16%, 6.10%, and 6.05%, respectively. "Prenatal diet," "household affairs," and "expected date of confinement" emerged to be the last three topics, at 5.72%, 5.40%, and 4.87%.

4.1.10 Information needs in the tenth month

Fourteen topics are identified in the tenth month of pregnancy. Table 13 presents the top ten topics in the tenth month. "Appeals and desire" emerged to be the most frequent topics, accounting for 21.17% of all topics. The following five topics, "delivery," "expected date of confinement," "pregnant women's physical discomfort," "full term," and "prenatal care," comprised 13.16%, 7.62%, 6.86%, 6.46%, and 6.19%, respectively. The next four topics are "sleep during late pregnancy," "nutrition and weight during pregnancy," "household affairs," and "good things to recommend," accounting for 5.96%, 5.64%, 5.28%, and 5.03%, respectively.

4.2 Summary of topic analysis about information needs

To more vividly show the main topics that pregnant women pay attention to during the whole pregnancy, we conducted a word cloud analysis on the LDA keywords of the topics during

TABLE 12 Topics in the ninth month of pregnancy.

	Topic name	Rate (%)	LDA keywords
1	Prenatal care	10.78	Doctor, fetal heart, monitor, prenatal care, B ultrasound, fetus, relatively small, normal, biparietal diameter, cord around neck, amniocentesis
2	The gender of baby	7.96	Boy, girl, give a hand, take a look, name, curious, guess, four-dimensional ultrasound results, shape of belly
3	Emotion sharing	7.13	Cheer, anticipate, sign in, count down, insist, the last month, emotion, finally
4	Items for childbirth	6.27	Prepare, purchase, package for delivery, hospital, need, preparation for delivery, goods, clothes, price, delivery, recommendation
5	Sleep during late pregnancy	6.16	Night, sleep, not good, sleeplessness, later pregnant trimester, restroom, daytime, tantalization, last night, midnight, awake
6	Fetal movement	6.10	Belly, fetal movement, recently, whether or not, uterine constraction, terrible, frequent, sometimes, belly firmness
7	Pregnant women's physical discomfort	6.05	Pain, feel ill, lie, walk, pubis, tired, later pregnant trimester, sit, turn over, get up, buttocks, back pain
8	Prenatal diet	5.72	Eat, pregnant women, prefer, drink, hungry, food, emesis, not allowed, morning, nutrition
9	Household affairs	5.40	Husband, mother-in-law, at home, work, look after, unthink, marriage, child, cook, accompany
10	Expected date of confinement	4.87	Expected date of confinement, pregnant, in advance, over 8 months, day, count, puerperal period, chat

TABLE 13 Topics in the tenth month of pregnancy.

	Topic name	Rate (%)	LDA keywords
1	Appeals and desire	21.17	Earnestly hope, eutocia, meet, no tear, no side out, safety, throes, super quick, uterine contraction, healthy
2	Delivery	13.16	Uterine contraction, hospital, bleed, stomachache, amniorrhea, the opening of the cervix, boy, girl, have sons and daughters
3	Expected date of confinement	7.62	Expected date of confinement, time, reaction, anxious, no action, steady, delay, 2 days
4	Pregnant women's physical discomfort	6.86	Belly, pain, feel, fetal movement, pubis, walk, lie, become hard, frequent, waist, sometimes
5	Full term	6.46	Full term, get ready, anticipate, cheer, give birth, count down, finally, nervous, time, insist
6	Prenatal care	6.19	Doctor, inspect, in hospital, amniocentesis, B ultrasound, prenatal care, fetal heart, normal, monitor, worry, fetus, umbilical cord
7	Sleep during late pregnancy	5.96	Night, later pregnant trimester, everyday, difficulty in sleeping, feel ill, tired, tantalization, sleeplessness
8	Nutrition and weight during pregnancy	5.64	Eat, pregnancy, nutrition, weight, grow, pregnant, biparietal diameter, drink, striae gravidarum, control, fat
9	Household affairs	5.28	Husband, child, mother-in-law, look after the first child, at home, expense, work, cry, confinement in childbirth
10	Good things to recommend	5.03	Purchase, compare, recommend, need, choose, paper diaper, clothes, body, prefer, pregnant women, share

pregnancy. The results are presented in Figure 2. In word cloud statistics, word frequency is distributed by font size. As shown in Figure 2, the fonts of words such as "pregnancy," "child," and "fetus" are prominent, indicating that the topic of pregnancy is centered on pregnant women and babies. Secondly, the fonts of words such as "hospital," "normal," "doctor," and "healthy" are also clearly displayed, indicating that obstetric examination is an important topic that pregnant women continue to pay attention to during pregnancy, which can help pregnant women to keep abreast of their physical status and fetal upgrowth. Then, words such as "pain," "belly," "good," "eat," "hungry," "drink," and "food" appeared frequently, reflecting pregnant women's concerns about their physical condition and diet during pregnancy. Words such as "cheer," "hope," "happy," "love," "boy," and "girl" reflect pregnant women's good wishes for their babies and their curiosity about their babies' gender.

5 Sentiment analysis

Sentiment analysis is performed to further understand the changes in pregnant women's information-seeking behavior during pregnancy. As discussed earlier, we use Python to call the third-party library SnowNLP to calculate the sentiment value

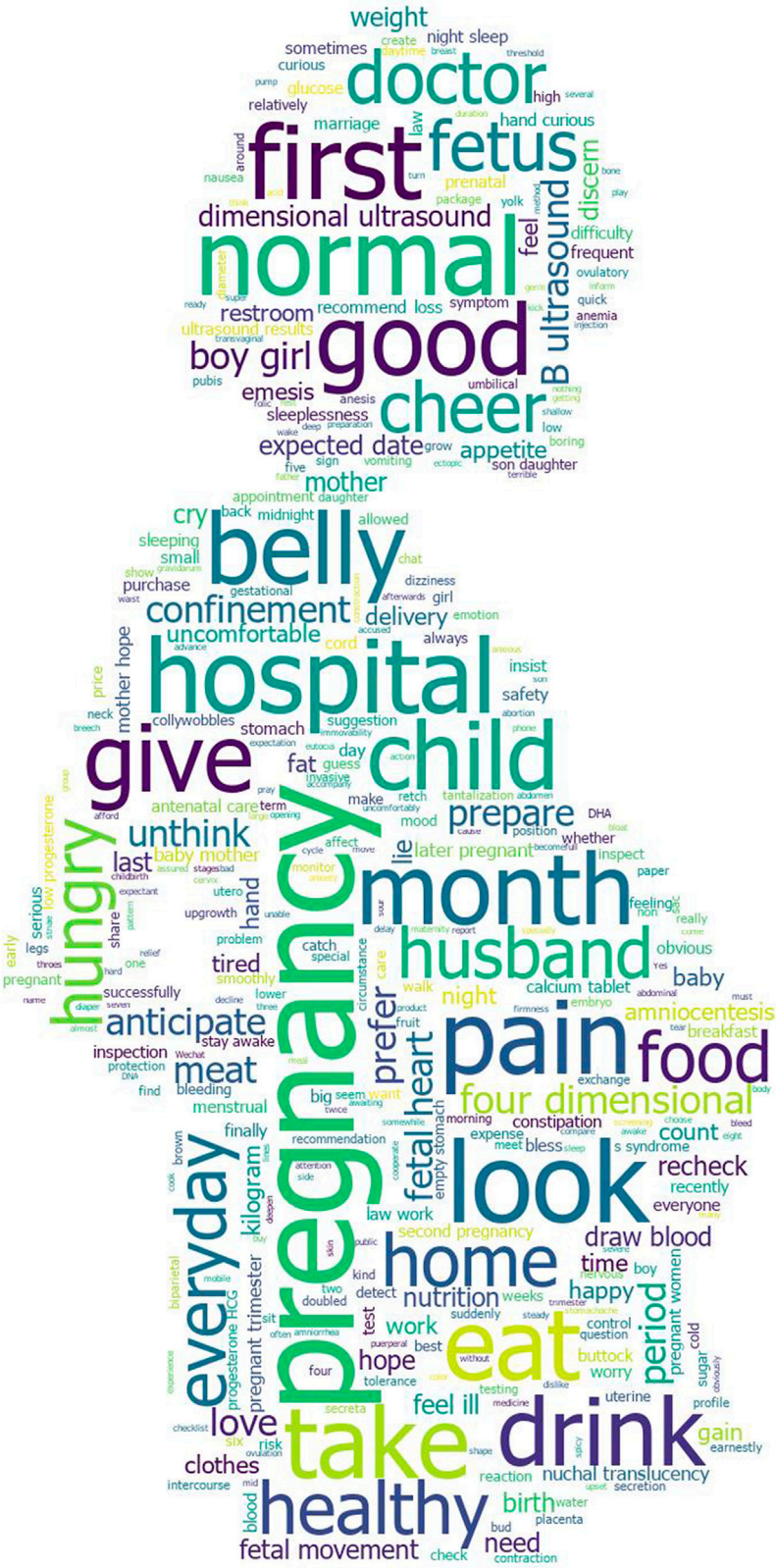

FIGURE 2
The results of word cloud analysis.

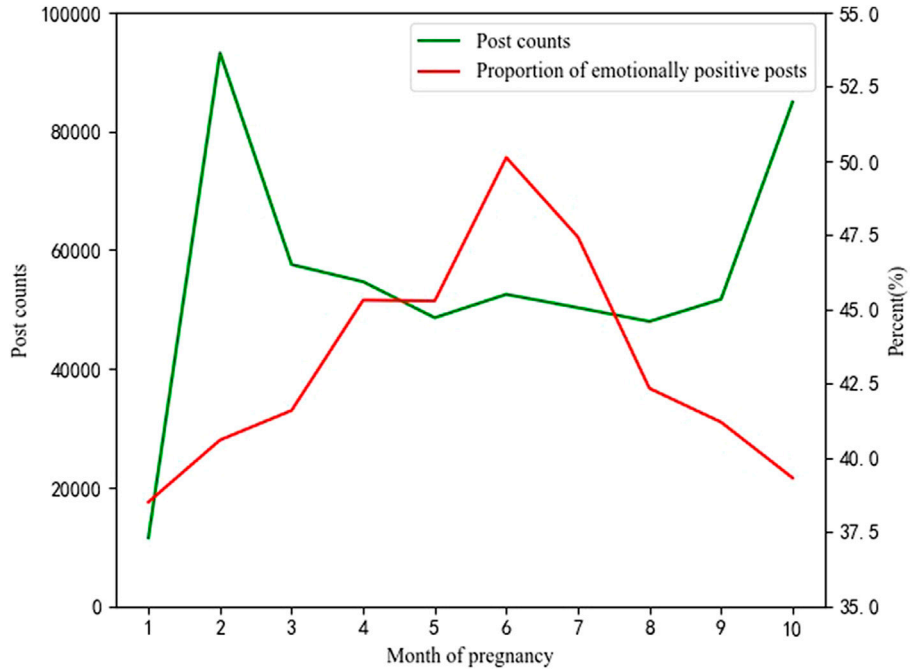

FIGURE 3
The results of sentiment analysis.

of each post text, and the range of sentiment value results is [0, 1]. Among them, a sentiment with a value greater than 0.5 is positive, and a sentiment less than or equal to 0.5 is negative. The closer the value is to 1, the more positive the emotion; the closer the value is to 0, the more negative the emotion. Figure 3 presents the posts with a sentiment value greater than 0.5 in each pregnancy month.

By combining the outcomes of topic analysis and sentiment analysis, the results show that in the first month of pregnancy, the number of posts is relatively small, mainly focusing on topics such as "test strip" and "pregnancy tests consultation," and the proportion of emotionally positive posts is also relatively low. On the one hand, many pregnant women have not found out that they are pregnant in the first month of pregnancy; on the other hand, the first month of pregnancy is often unstable and at a loss for pregnant women, so their emotions are relatively negative.

The number of posts in the second month of pregnancy is the most, but the proportion of posts with positive emotions is also relatively low. In the second month of pregnancy, most pregnant women have already guessed or confirmed pregnancy, but new pregnant women have little knowledge about pregnancy. Therefore, posts about "precautions for early pregnancy," "early pregnancy inspection," "symptoms of early pregnancy" and other related early pregnancy topics surged. However, due to the uncertainty of the baby's status and the lack of relevant knowledge of pregnant women, the proportion of emotionally positive posts in the second month of pregnancy is relatively low. After the first 2 months of relevant inspections and understanding of pregnancy knowledge, pregnant women have entered a relatively mature stage. At the same time, the status of the baby gradually stabilized, so the number of posts from the second month of pregnancy to the third month dropped significantly, and it continued to be stable until the ninth of pregnancy.

The proportion of emotionally positive posts from the third month to the ninth month of pregnancy is higher than that in other months, and there is an upward trend from the third month to the sixth month of pregnancy. The proportion is the highest in the sixth month of pregnancy, and then gradually decreases. After the third month of pregnancy, the baby's state gradually stabilizes, the pregnant women's belly gradually bulges, and the pregnant women can even feel the baby's fetal movement, but there is generally no obvious physical discomfort, so the pregnant women's emotions are relatively more positive. Since the seventh month of pregnancy, the baby's weight increases, the pregnant women's belly increases, the body gradually becomes clumsy, and the body also has various discomforts such as soreness and difficulty sleeping, so pregnant women show more negative emotions.

The number of posts in the tenth month of pregnancy surged again, second only to the second month of pregnancy, and the proportion of emotionally positive posts also dropped sharply, only higher than in the first of pregnancy. The tenth month of pregnancy is the month when the baby is about to be born. On the one hand, the pregnant women's body aches and sleep

problems are more prominent. On the other hand, pregnant women are faced with the uncertainty of childbirth, and a state of fear and anxiety appears. It can also be seen from the results of the topic analysis that in the current month, "appeals and desire" ranked first among the topics that pregnant women paid attention to, accounting for 21.17%. In addition, "expected date of confinement" and "pregnant women's physical discomfort" are also the main contents of concern for pregnant women.

6 Discussion and conclusion

6.1 Summary of findings

The purpose of the current study was to investigate pregnant women's information-seeking behavior. By a combination of descriptive analysis, topic analysis, and sentiment analysis, the current work expands our knowledge by proving important findings. The correlation analysis showed that more pregnant women contribute to more posts. Moreover, pregnant women with a college education or above are more likely to seek information about pregnancy on internet platforms. The more economically developed cities have higher Internet usage. Therefore, pregnant women will be more probable to use Internet platforms to seek information.

Furthermore, the topics from the first month to the tenth month of pregnancy were extracted in topic analysis. The findings show that the topics in different months of pregnancy relate to the present stages of pregnancy. The current paper identified six, nineteen, eighteen, thirteen, eleven, sixteen, thirteen, sixteen, nineteen, and fourteen topics in different months of pregnancy. The specific topics in different stages show the changes in pregnant women's attention.

In addition, the sentiment analysis showed the variation of pregnant women's emotions in information-seeking. The results of sentiment analysis show a peak of the posts in the second month of pregnancy. The proportion of emotionally positive posts reached its peak in the sixth month of pregnancy. Pregnant women's emotional sentiment deeply interacts with the results of topic analysis.

6.2 Practical and theoretical implications

Our study presents theoretical and practical significance. First, this is one of the first studies to understand pregnant women's information-seeking using the methods of data mining and text analysis. Previous studies on the information needs of maternal health revealed the topics that pregnant women pay attention to; however, the existing work is limited in the descriptive analysis and self-reported questionnaire data (Kamali et al., 2018; Ahmadian et al., 2020; Jin et al., 2020; Kassim, 2021). This study is unique by employing enormous quantities of data and the research data covers a long period. By visualizing the posts of every province, the geographical distribution of pregnant women's posts was clearly displayed. The current study enriches our understanding of the relationships among pregnant women's information-seeking, regional economic development level, and educational level.

Second, this study provides comprehensive research, involving abundant analysis. Compared with previous research (Kamali et al., 2018), the current work divides the data from the first month of pregnancy to the tenth month of pregnancy and analyzes the large amounts of data according to the pregnancy period. This study provides important insights for understanding the change of emotions during different pregnant stages and connecting the changes of emotions with the topics that cause pregnant women's attention. The current work provides the perspectives for future research by the subdivision of data in different pregnant stages.

Third, the findings of this study have several practical implications. The findings indicate that pregnant women pay attention to different topics during various months of pregnancy. The maternal and infant-related websites should provide customized information recommendations for pregnant women according to their stages of pregnancy. For example, information such as precautions and inspection for early pregnancy should be recommended for pregnant women in the second month of pregnancy. Moreover, the proportion of emotionally positive posts reached its peak in the sixth month of pregnancy and is relatively low in the first and the tenth of pregnancy. The relevant government management departments and hospitals should concern about anxiety during early pregnancy and before delivery. The popularization of knowledge about pregnancy and childbirth would be useful for improving pregnant women's emotions.

6.3 Limitations and future research

The study is subject to several inevitable limitations. First, the data source of this study is "Mama.cn" mainly located in China. What is now needed in the future is a cross-national study involving data for countries at different levels of development. The present study lays the groundwork for future research into pregnant women's information-seeking behavior around the world. Future studies are encouraged to improve the generalizability of the current work by involving data from different countries and understanding the role of cultural identity in determining pregnant women's information-seeking. Second, the data such as personal attributes and specific family environments are not included in the paper since such data cannot be obtained from the website. It would be interesting to investigate the effect of family-related variables on pregnant women's emotional sentiment in future work.

Data availability statement

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

Author contributions

KH provided the conceptualization, data collection, initial analysis, review and editing. TH worked on the results, methodology, and writing. All authors contributed to this study, read and agreed to the submitted version of the manuscript.

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Conflict of interest

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

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Bioinformatics analysis combined with clinical sample screening reveals that leptin may be a biomarker of preeclampsia

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Introduction: Preeclampsia (PE) is a gestational hypertensive disease with unclear pathogenesis. This study aimed to identify the genes that play an important role in determining the pathogenesis of PE using bioinformatics analysis and fundamental researches.

Materials and methods: Datasets from the Gene Expression Omnibus (GEO) database were used to screen for differentially expressed genes (DEGs). The NCBI, SangerBox, and other databases were used to analyze the functions of the DEGs. Targetscan7, miRWalk, ENCORI, DIANA TOOLS, CircBank databases, and the Cytoscape tool were used to construct the lncRNA/circRNA-miRNA-*LEP* network. SRAMP, RPISeq, RBPsuite, and catRPAID were used to analyze the RNA modifications of *LEP*. Immune cell infiltration was analyzed using the dataset GSE75010. Placental tissues from normal pregnant women and PE patients were collected, screened for gene expression using reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blotting. The results were further verified in HTR-8/SVneo cell line hypoxia model and PE mouse model.

Results: Our analyses revealed that *LEP* was significantly upregulated in eight datasets. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses indicated that *LEP* was involved in the JAK/STAT signaling pathway, angiogenesis, and placental development. Immune cell infiltration analysis showed that M1 and M2 macrophages differed between normal pregnancies and those in PE patients. A competing endogenous RNA (ceRNA) network was constructed, and proteins interacting with *LEP* were identified. RNA modification sites of *LEP* were also identified. Finally, the overexpression of *LEP* in PE was confirmed in clinical samples, HTR-8/SVneo cell line and PE mouse model.

Conclusion: Our results indicate that *LEP* overexpression is associated with PE and may be a potential diagnostic marker and therapeutic target.

KEYWORDS

leptin, preeclampsia, immune infiltration, bioinformatics, competing endogenous RNA, N 6-methyladenosine

1 Introduction

Preeclampsia (PE) is a hypertensive disease associated with pregnancy and is characterized by new-onset hypertension after 20 weeks of gestation, with or without proteinuria, which can affect multiple organs (listed, 2020). It is characterized by placental dysplasia and endothelial dysfunction (Rana et al., 2019; Han and Dong, 2021). PE is one of the leading causes of maternal, fetal, and neonatal deaths, affecting 2%–8% of pregnancies (Ives et al., 2020). PE can be divided into two subtypes: early-onset (<34 weeks) and late-onset (>34 weeks). Abnormal placental development is more strongly associated with early-onset PE, whereas late-onset PE is usually secondary to maternal microvascular disease or is associated with heredity (Dahlia Raymond and Peterson, 2011). Unfortunately, the pathogenesis of PE remains unclear, and there is no gold standard for treatment, apart from the delivery of the placenta. It is crucial to elucidate the pathogenesis of PE and identify sensitive biomarkers for predicting this disease.

Leptin (encoded by the *LEP* gene) is a polypeptide hormone secreted primarily by adipose tissue, and the placenta is also the body's leptin producing tissue. In addition to increased maternal fat mass, placental leptin production is one of the key sources of increased maternal circulating leptin levels. Placental leptin regulates placental functions *via* autocrine or paracrine signaling, and is considered an essential signaling molecule in the reproductive system. It regulates gonadotropin production, blastocyst formation, implantation, normal placental formation, and communication between the fetus and the placenta. In addition, leptin regulates proliferation, protein synthesis, invasion, and apoptosis of placental cells, and plays a crucial role in the early stages of pregnancy (Perez-Perez et al., 2018). Nonn et al. (2021) reported elevated angiotensin IV (Ang IV) levels in the maternal circulation during pregnancy. Ang IV-induced reduction in basal mitochondrial respiration in trophoblastic cells may alter placental metabolism by increasing leptin levels. This study also suggested that the mechanisms underpinning hypertensive disease in pregnancy may be related to changes in leptin and cellular metabolism (Nonn et al., 2021). Cai et al. found that miR-519d targets *LEP* and downregulates its expression, promoting the proliferation and migration/invasion of HTR-8/SVneo cells, which may impede the development of PE (Cai et al., 2021). Huang et al. showed that miR-18b-3p was decreased and *LEP* was increased in placental tissue of PE rats. *LEP* was the direct target gene of miR-18b-3p. And human umbilical cord mesenchymal stem cells (hucMSCs) upregulated miR-18b-3p and targeted leptin, thereby reducing the levels of inflammatory factors in the placental tissues of PE rats (Huang et al., 2021). In light of these data suggesting that leptin is involved in the development of PE, this study aimed to further analyze *LEP* expression and explore additional therapeutic targets for PE.

In this study, we identified differentially expressed genes (DEGs) by comparing gene expression profiles in placental tissues from women who experienced normal pregnancy with tissues from PE patients. We then conducted Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses, immune cell infiltration, and protein-protein interaction (PPI) network analyses. We also constructed a competing endogenous RNA (ceRNA) network based on the screened microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs), and predicted the N^6-methyladenosine (m6A) modification sites of *LEP* and related m6A-binding proteins. Clinical samples were collected, and HTR-8/SVneo cell line hypoxia model and PE mouse model were established, and then screened for gene expression using reverse transcription quantitative polymerase chain reaction (RT–qPCR) and western blotting. The identification and analysis of the *LEP* gene will clarify the role of *LEP* in the pathophysiology of PE and its potential association with PE, and further understand the pathogenesis of PE. This study aimed to provide more theoretical basis for clarifying that *LEP* may be a potential diagnostic marker and therapeutic target for PE.

2 Materials and methods

2.1 Data resources

All datasets [GSE10588 (Saei et al., 2021), GSE74341 (Kang et al., 2021), GSE66273 (Qi et al., 2021), GSE54618 (Saei et al., 2021), GSE4707 (Saei et al., 2021), GSE44711 (Kang et al., 2021), GSE35574 (Saei et al., 2021), and GSE24129 (Ma et al., 2021)] containing normal pregnancy and PE placental tissue sequencing data were downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/). We then performed bioinformatic analyses on these data.

2.2 Analysis of DEG

To identify DEGs, we sorted the DEGs from all eight datasets (GSE10588, GSE74341, GSE66273, GSE54618, GSE4707, GSE44711, GSE35574, and GSE24129) in ascending order of logFC values. DEGs were selected based on $p < 0.05$. Differential expression referred to significantly altered (upregulated or downregulated) gene expression at a genomic level. An interactive Venn diagram of the upregulated genes in each

dataset was prepared using Evenn (http://www.ehbio.com/test/venn/#/). Simultaneously, omicstudio (https://www.omicstudio.cn/index) was used to plot the volcano maps. Additionally, shinyGEO (https://gdancik.Shinyapps.io/shinyGEO/) was used to quantify *LEP* expression in each dataset.

2.2.1 Functional annotation of *LEP*

To analyze *LEP* function in PE, NCBI (https://www.ncbi.nlm.nih.gov/) and SangerBox (http://www.sangerbox.com/) were used to perform single-gene KEGG pathway analysis and GO analysis. The Gene Ontology Biological Process (GO_BP), Gene Ontology Molecular Function (GO_MF), and Gene Ontology Cellular Component (GO_CC) terms for *LEP* were explored.

2.2.2 Analysis of immune cell infiltration

The GSE75010 (Leavey et al., 2016) dataset was selected to analyze immune cell abundance in samples from 157 patients with either normal pregnancy or that with PE. For the analysis, 80 PE patients were divided into *LEP*–high and *LEP*–low expression groups, and gene set enrichment analysis (GSEA) and single sample GSEA (ssGSEA) were performed. CIBERSORT algorithm was used to determine the ratios of the immune cells. The R package "clusterProfiler" was used for GSEA analysis, and ssGSEA analysis was performed using the R package "GSVA".

2.2.3 Construction of a lncRNA/circRNA-miRNA-*LEP* regulatory network

Upstream binding miRNAs of *LEP* were predicted using several target gene prediction programs, including miRWalk (http://mirwalk.umm.uni-heidelberg.de/), miRDB (http://mirdb.org/), miRabel (http://bioinfo.univ-rouen.fr/), and TargetScan7 (http://www.targetscan.org/). Only the 24 predicted miRNAs that appeared in all four programs were included in the subsequent analyses. These 24 miRNAs were also scored using TargetScan by entering the human gene symbol "*LEP*", followed by each of the 24 miRNA names one at a time, and recording the scores that were obtained. LncRNAs targeting the screened miRNAs were predicted and analyzed using ENCORI (https://starbase.sysu.edu.cn/) and DIANA TOOLS (http://snf-515788.vm.okeanos.grnet.gr/). CircBank (http://www.circbank.cn/searchMiRNA.html) was used to predict the circRNAs. Cytoscape software was used to visualize the lncRNA/circRNA-miRNA-*LEP* regulatory network.

2.2.4 Construction of a PPI network

To construct a PPI network, we performed a search on the STRING website (https://string-db.org/) using "LEP" in the "protein name" module and "*Homo sapiens*" in the organism module. We set the following key parameters: meaning of network edges ("evidence"), the minimum required interaction score ["medium confidence (0.400)"], and the maximum number of interactors to show ("no more than 20 interactors" in the 1st shell). *LEP*-binding proteins were also analyzed using GeneMANIA (https://genemania.org/) to determine the interaction between *LEP*-related proteins. The SangerBox portal was used to perform KEGG and GO analyses of *LEP*-related genes.

2.2.5 RNA methylation of LEP

SRAMP (http://www.cuilab.cn/sramp/) was used to predict *LEP* m6A modification sites and their positions in the RNA secondary structure. The *LEP* FASTA mRNA sequence without introns was used for this analysis, with the parameters "Analyze RNA secondary structure" and tissue "Generic (default)". The query sequence was shown as RNA. ENCORI (https://starbase.sysu.edu.cn/) and RBPsuite (http://www.csbio.sjtu.edu.cn/bioinf/RBPsuite/) were used to identify proteins that can interact with *LEP*. RPISeq (http://pridb.gdcb.iastate.edu/RPISeq/) was used to predict the probability of proteins binding to *LEP*. The protein and RNA sequences were inserted in plain text format, and RF classifier and SVM classifier prediction scores were obtained. RBPsuite (http://www.csbio.sjtu.edu.cn/bioinf/RBPsuite/) and catRPAID (http://service.tartaglialab.com/page/catrapid_group) were used to identify the RNA regions of *LEP* most likely to be bound by IGF2BP3.

2.3 Clinical sample collection

All samples were collected from women who underwent cesarean section at the Department of Obstetrics and Gynecology of the Tianjin Medical University General Hospital between August 2021 and July 2022. This study was approved by the Medical Ethics Committee of the Tianjin Medical University General Hospital and the approval number is IRB2020-KY-008. Samples were collected with verbal informed consent from all patients. The pregnant women were divided into two groups, namely normal pregnancy and PE. The normal pregnancy group comprised pregnant women with no history of hypertension or other clinicopathological changes. Inclusion in the PE group was based on the following criteria: 1) diagnosis of systolic blood pressure of 140 mmHg or higher, or diastolic blood pressure of 90 mmHg or higher, on 2 occasions at least 4 h apart after 20 weeks of gestation; and urine protein levels of 300 mg or more every 24 h, or protein/creatinine ratio 0.3 mg/dL or higher, or test paper reading ++; or 2) in the absence of proteinuria, new-onset hypertension associated with any of the following changes: thrombocytopenia, renal insufficiency, liver function impairment, pulmonary edema, or new-onset headache unresponsive to medication that cannot be explained by other diagnoses or visual symptoms (listed, 2020). Exclusion criteria included multiple pregnancies, hypercoagulable state, gestational diabetes, chronic hypertension, autoimmune diseases, kidney and liver disease, and the use of aspirin or anticoagulants during pregnancy. Placental tissue was extracted immediately after cesarean section and placental villus tissue was

dissected at 4°C. After cleaning with cold PBS, samples were quickly stored in liquid nitrogen.

2.4 Culture and treatment of HTR-8/SVneo cell line

The HTR-8/SVneo cell line was obtained from BeNa Culture Collection (BNCC, Beijing, China). The HTR-8/SVneo cell line was cultured with RPMI-1640 medium (Gibco BRL, Grand Island, NY, United State) supplemented with 10% fetal bovine serum (Gibco, Australia), and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were incubated at 37°C in 5% CO_2. For hypoxia, HTR-8/SVneo cell line was cultured in a 3-gas incubator with 1% oxygen, 5% carbon dioxide, serum-free for 24 h to simulate the process of PE.

2.5 Construction of the PE mouse model and sample collection

All mouse experiments were conducted in accordance with protocols approved by the Tianjin medical university animal care and use committee and followed guidelines for animal welfare. Eight-week-old C57BL/6J female and male mice were purchased from Beijing Hufukang Biotechnology Co., LTD. Mating was performed at a 2:1 ratio of male to female. We validated the overexpression of *LEP* in PE using a PE mouse model constructed by Han et al. The PE mouse model was established by injecting placenta-derived extracellular vesicles (pcEVs), which was obtained from normal pregnant mice, into pregnant C57BL/6J mice to increase circulating pcEV levels, and induced preeclampsia-like changes such as hypertension and proteinuria. In addition, two other models were used to complement this PE mouse model in this study. C57BL/6 J non-pregnant mice developed hypertension and proteinuria after injection of pcEVs to increase circulating pcEV levels. Enhanced clearance of circulating pcEVs of PE pregnant mouse model prevented clinical phenotypes of PE induced by pcEVs (Han et al., 2020). Therefore, we refer to this PE model to validate the overexpression of *LEP* in PE. In our study, on day 17–18 of gestation, pregnant mice were injected with PBS buffer through the tail vein as the control group or pcEVs as the PE group. Each pregnant mouse was injected with 100 μL. The intervention concentration (1 × 10^7 pcEVs/mouse) was referenced to the concentration used by (Han et al., 2020). Blood pressure was measured 30 min after injection through the tail vein of the mice. Then, the mice were sacrificed under anesthesia, the placenta and fetus were dissected, and the collected placenta and fetus were weighed.

The detailed steps for obtaining pcEVs are as follows, placentas from normal pregnant C57BL/6J mice between 17, 18 days were washed with ice-cold sterile PBS, cut into small pieces, and frozen in liquid nitrogen. Placentas were gently added to 1 ml PBS and homogenized at 4°C. The placenta homogenates were centrifuged at 1,500 × *g* for 20 min at 4°C to remove intact cells. The supernatant was centrifuged at 13,000 × *g* at 4°C for 2 min to remove large cell debris, and then centrifuged at 4°C at 100,000 × *g* for 60 min (twice) to collect pcEVs and resuspend in PBS (Han et al., 2020).

2.6 RT-qPCR

Total RNA was extracted using the Trizol reagent (Thermo Fisher Scientific, Inc. United State), and cDNA was obtained by reverse transcription using the TransScript® First-Strand cDNA Synthesis SuperMix (Transgen Biotech Corporation, China). The cDNA was amplified using the Hieff UNICON universal Blue qPCR SYBR Green Master Mix kit (YEASEN Corporation, China). The reaction volume was 20 μL, including 10 μL of Universal Blue qPCR SYBR Green Master Mix, 7.6 μL of nucleic acid-free water, 0.2 μL of each primer, and 2 μL of cDNA product. The PCR cycling conditions were as follows: 95°C for 2 min for 1 cycle, 95°C for 10 s, 60°C for 30 s for 40 cycles, followed by the melting curve stage. *GAPDH* was used as an internal reference and relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method. The following primer sequences were used for amplification:

GAPDH
Forward: 5′- AAGGTGAAGGTCGGAGTCAAC-3′,
Reverse: 5′- GGGGTCATTGATGGCAACAAT -3′,
LEP
Forward: 5′- TGCCTTCCAGAAACGTGATCC -3′,
Reverse: 5′- CTCTGTGGAGTAGCCTGAAGC -3’.
LINC00473
Forward: 5′- TCATTTCCCTACCTGCTCCT-3′,
Reverse: 5′- CAGTGTCTGCACATCGCTAAT-3’.

2.7 Western blot analysis

Total protein was extracted from each 50 mg placenta sample using 300 μL of protein lysis buffer, which was composed of RIPA buffer (Solarbio, China), PMSF (Solarbio, China), protease inhibitor (MedChemExpress, China), and DNA enzyme inhibitor (Solarbio, China). The total protein concentration was determined using a bicinchoninic acid (BCA) assay (Solarbio, China). Protein samples (30 μg) were electrophoresed on a 15% sodium dodecyl sulfate polyacrylamide gel at 80 V for 30 min and 120 V for 60 min. After electrophoresis, the protein samples were transferred to polyvinylidene fluoride (PVDF) membrane at 80 V, for 100 min, and the blot was washed thrice with TBST for 5 min. After blocking non-specific binding with 5% milk for 2 h at room temperature, the membranes were washed thrice with TBST for 5 min. The membranes were incubated in mouse anti-leptin (Sino Biological, China), rabbit anti-leptin (ABclonal, China)

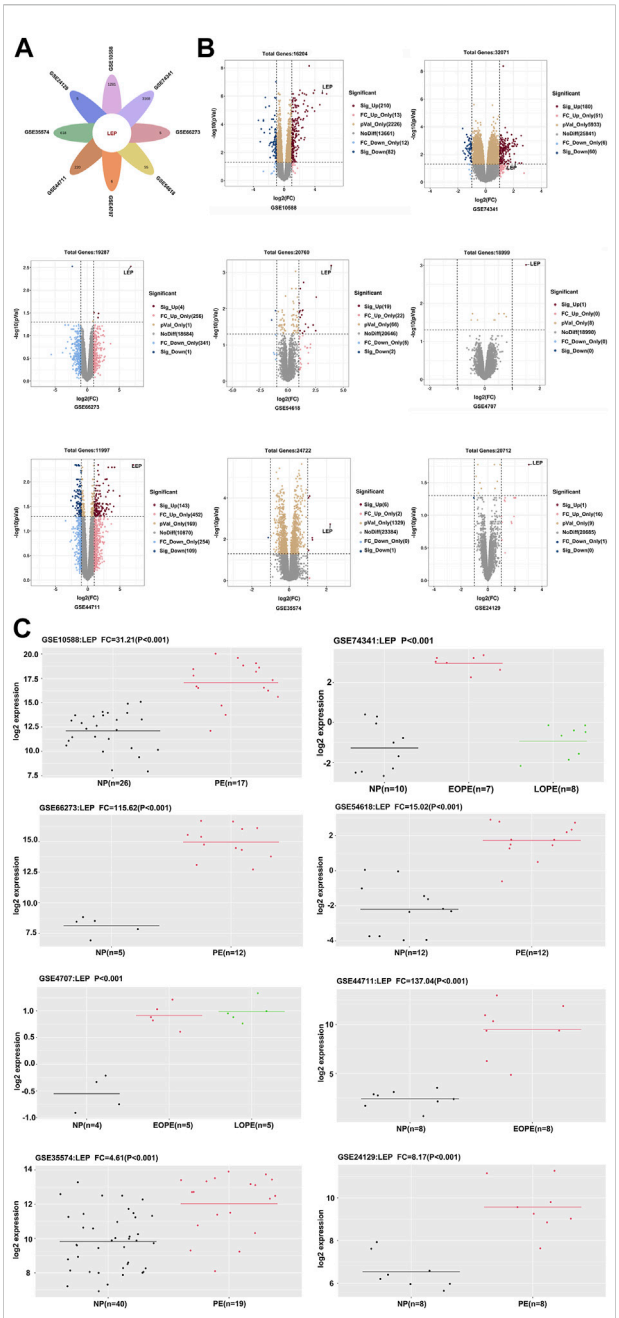

FIGURE 1
Identification of differentially expressed genes. **(A)** Venn diagram showing one upregulated gene, *LEP*. **(B)** Volcano plot of gene expression profile data in GSE10588, GSE74341, GSE66273, GSE54618, GSE4707, GSE44711, GSE35574, and GSE24129 datasets. **(C)** The quantity of *LEP* expression in the GSE10588, GSE74341, GSE66273, GSE54618, GSE4707, GSE44711, GSE35574, and GSE24129 datasets.

or rabbit anti-GAPDH (Cell Signaling Technology Inc. United State) and mouse anti-β actin (ZSGB-BIO, China) primary antibodies at 4 ˚C with low agitation overnight. The next day, the primary antibody was recovered and the membrane was washed thrice with TBST for 5 min. The blots were incubated with horseradish peroxidase (HRP)-labeled goat anti-mouse/rabbit IgG (ZSGB-BIO, China) at room temperature for 1 h, then washed thrice with TBST for 10 min. Protein expression was visualized using the chemiluminescence with GAPDH used as a loading control. The relative expression of leptin was calculated as the ratio of the optical density values of leptin to GAPDH. ImageJ was used to measure the gray values.

2.8 Statistical analysis

Statistical and image analyses were performed using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, United State). Data are expressed as mean ± standard error of the mean (SEM). Comparisons between two groups were performed using an independent sample *t*-test. The criterion for statistical significance was set at $p < 0.05$.

3 Results

3.1 Identification of LEP overexpression in PE

We selected eight datasets of PE data (GSE10588, GSE74341, GSE66273, GSE54618, GSE4707, GSE44711, GSE35574, and GSE24129) from the GEO database, based on the literature. Our analyses revealed that *LEP* was significantly overexpressed in all eight databases (Figure 1A). *LEP* expression was higher in the PE group compared with the normal pregnancy group. In datasets GSE74341 and GSE4707, PE was divided into early-onset and late-onset PE, and dataset GSE74341 showed that *LEP* expression was higher in early-onset PE than in late-onset PE (Figure 1C). And *LEP* is labeled in the volcano maps of each dataset ($p < 0.05$) (Figure 1B). Together, these results show that *LEP* is significantly upregulated in PE.

3.2 Functional annotation of LEP

Next, we focused on the relationship between *LEP* and PE development. Single-gene KEGG and GO analyses of *LEP* were performed to evaluate its biological functions (Tables 1,2). Single-gene KEGG pathway analysis showed that *LEP* is associated with the Janus kinase/signal transducer and activator of transcription (JAK/STAT) and adipocytokine signaling pathways, HIF-1-alpha transcription factor network, and developmental biology. The GO_BP annotation showed that *LEP* is involved in angiogenesis, placental development, response to estradiol, regulation of blood pressure, female pregnancy, positive regulation of tyrosine phosphorylation of STAT protein, positive regulation of receptor signaling pathway *via* JAK/STAT, positive regulation of MAPK

TABLE 1 Single-gene KEGG Pathway in NCBI and SangerBox database.

Pathway	Source
Amp-activated protein kinase (ampk) signaling	WikiPathways
Adipogenesis	WikiPathways
Antipsychotics pathway (metabolic side effects), pharmacodynamics	PharmGKB
Developmental biology	Reactome
Differentiation of white and brown adipocyte	WikiPathways
HIF-1-Alpha transcription factor network	Pathway Interaction Database
Incretin synthesis, secretion, and inactivation	Reactome
Leptin and adiponectin	WikiPathways
Leptin-Insulin Signaling Overlap	WikiPathways
Metabolism of proteins	Reactome
Nonalcoholic fatty liver disease	WikiPathways
Peptide hormone metabolism	Reactome
Signaling pathways	Reactome
Signaling by leptin	Reactome
Signaling events mediated by ptp1b	Pathway Interaction Database
Spinal cord injury	WikiPathways
Synthesis, secretion, and deacylation of ghrelin	Reactome
Synthesis, secretion, and inactivation of glucagon-like peptide-1 (GLP-1)	Reactome
Transcription factor regulation in adipogenesis	WikiPathways
Transcriptional regulation of white adipocyte differentiation	Reactome
Cytokine-cytokine receptor interaction	SangerBox database
Neuroactive ligand-receptor interaction	SangerBox database
Ampk signaling pathway	SangerBox database
JAK/STAT signaling pathway	SangerBox database
ADipocytokine signaling pathway	SangerBox database
Non-alcoholic fatty liver disease (nafld)	SangerBox database

cascade, and other biological processes; while GO_CC annotation revealed links to extracellular region, extracellular space, and cytoplasm. The GO_MF annotations were mainly related to peptide receptor binding and hormone activity.

3.3 Analysis of immune cell infiltration

157 samples from GSE75010 were selected to study the infiltration of immune cells into normal pregnancy and PE placental tissues. Among the 22 immune cell types assessed, the number of naive B Cells, resting NK cells, activated NK cells, M1 macrophages, M2 macrophages, and eosinophils was significantly different between the two groups (Figures 2A,B). Next, 80 PE patients were divided into *LEP* high and low expression groups for GSEA. The results revealed that angiogenesis, placental development, and response to estradiol were enriched (Figure 2C). Concurrent ssGSEA revealed differences in activated dendritic cells, central memory CD8 T Cells, macrophages, memory B Cells, and T follicular helper cells between the two groups (Figure 2D).

3.4 Identification of differentially expressed miRNAs (DEMs)

We screened the miRWalk, miRDB, TargetScan, and miRabel databases and identified 24 miRNAs that potentially

TABLE 2 Single-gene GO analysis in SangerBox database.

GO id	Name space	Name	Rref database
GO:0000122	Biological_process	Negative regulation of transcription by RNA polymerase II	GO_REF:0000107
GO:0001525	Biological_process	Angiogenesis	PMID:19910644
GO:0001525	Biological_process	Angiogenesis	PMID:21771332
GO:0001542	Biological_process	Ovulation from ovarian follicle	GO_REF:0000107
GO:0001666	Biological_process	Response to hypoxia	GO_REF:0000107
GO:0001890	Biological_process	Placenta development	PMID:17957153
GO:0001936	Biological_process	Regulation of endothelial cell proliferation	PMID:11460888
GO:0002021	Biological_process	Response to dietary excess	GO_REF:0000107
GO:0002021	Biological_process	Response to dietary excess	GO_REF:0000107
GO:0003300	Biological_process	Cardiac muscle hypertrophy	GO_REF:0000107
GO:0005576	Cellular_component	Extracellular region	Reactome:R-HSA-1183003
GO:0005615	Cellular_component	Extracellular space	GO_REF:0000024
GO:0005737	Cellular_component	Cytoplasm	GO_REF:0000107
GO:0006006	Biological_process	Glucose metabolic process	GO_REF:0000107
GO:0006111	Biological_process	Regulation of gluconeogenesis	GO_REF:0000107
GO:0006114	Biological_process	Glycerol biosynthetic process	GO_REF:0000107
GO:0006635	Biological_process	Fatty acid beta-oxidation	GO_REF:0000107
GO:0006909	Biological_process	Phagocytosis	GO_REF:0000107
GO:0006909	Biological_process	Phagocytosis	GO_REF:0000107
GO:0007565	Biological_process	Female pregnancy	GO_REF:0000107
GO:0007623	Biological_process	Circadian rhythm	GO_REF:0000107
GO:0008203	Biological_process	Cholesterol metabolic process	GO_REF:0000107
GO:0008206	Biological_process	Bile acid metabolic process	GO_REF:0000107
GO:0008217	Biological_process	Regulation of blood pressure	GO_REF:0,000107
GO:0008340	Biological_process	Determination of adult lifespan	GO_REF:0000107
GO:0008343	Biological_process	Adult feeding behavior	GO_REF:0000024
GO:0010507	Biological_process	Negative regulation of autophagy	PMID:25060689
GO:0010888	Biological_process	Negative regulation of lipid storage	GO_REF:0000107
GO:0014068	Biological_process	Positive regulation of phosphatidylinositol 3-kinase signaling	GO_REF:0000024
GO:0014068	Biological_process	Positive regulation of phosphatidylinositol 3-kinase signaling	GO_REF:0000024
GO:0014068	Biological_process	Positive regulation of phosphatidylinositol 3-kinase signaling	PMID:24340098
GO:0014823	Biological_process	Response to activity	GO_REF:0000107
GO:0019953	Biological_process	Sexual reproduction	PMID:8589726
GO:0021954	Biological_process	Central nervous system neuron development	GO_REF:0000107
GO:0030073	Biological_process	Insulin secretion	GO_REF:0000107
GO:0030217	Biological_process	T-cell differentiation	GO_REF:0000024

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TABLE 2 (*Continued*) Single-gene GO analysis in SangerBox database.

GO id	Name space	Name	Rref database
GO:0030300	Biological_process	Regulation of intestinal cholesterol absorption	GO_REF:0000107
GO:0032008	Biological_process	Positive regulation of TOR signaling	PMID:25060689
GO:0032099	Biological_process	Negative regulation of appetite	GO_REF:0000024
GO:0032310	Biological_process	Prostaglandin secretion	PMID:19688109
GO:0032355	Biological_process	Response to estradiol	GO_REF:0000107
GO:0032615	Biological_process	Interleukin-12 production	GO_REF:0000107
GO:0032760	Biological_process	Positive regulation of tumor necrosis factor production	GO_REF:0000107
GO:0032814	Biological_process	Regulation of natural killer cell activation	PMID:12504075
GO:0032817	Biological_process	Regulation of natural killer cell proliferation	PMID:12504075
GO:0033197	Biological_process	Response to vitamin E	GO_REF:0000107
GO:0033210	Biological_process	Leptin-mediated signaling pathway	GO_REF:0000024
GO:0033210	Biological_process	Leptin-mediated signaling pathway	GO_REF:0000024
GO:0033686	Biological_process	Positive regulation of luteinizing hormone secretion	GO_REF:0000107
GO:0035360	Biological_process	Positive regulation of peroxisome proliferator activated receptor signaling pathway	GO_REF:0000107
GO:0035556	Biological_process	Intracellular signal transduction	GO_REF:0000107
GO:0035630	Biological_process	Bone mineralization involved in bone maturation	GO_REF:0000107
GO:0035904	Biological_process	Aorta development	GO_REF:0000107
GO:0038108	Biological_process	Negative regulation of appetite by leptin-mediated signaling pathway	GO_REF:0000024
GO:0042102	Biological_process	Positive regulation of T-cell proliferation	PMID:25060689
GO:0042269	Biological_process	Regulation of natural killer cell mediated cytotoxicity	PMID:12504075
GO:0042307	Biological_process	Positive regulation of protein import into nucleus	GO_REF:0000107
GO:0042445	Biological_process	Hormone metabolic process	GO_REF:0000107
GO:0042531	Biological_process	Positive regulation of tyrosine phosphorylation of STAT protein	GO_REF:0000107
GO:0042593	Biological_process	Glucose homeostasis	GO_REF:0000107
GO:0042755	Biological_process	Eating behavior	GO_REF:0000107
GO:0043066	Biological_process	Negative regulation of apoptotic process	GO_REF:0000107
GO:0043270	Biological_process	Positive regulation of ion transport	GO_REF:0000107
GO:0043410	Biological_process	Positive regulation of MAPK cascade	GO_REF:0000024
GO:0043410	Biological_process	Positive regulation of MAPK cascade	PMID:24340098
GO:0044320	Biological_process	Cellular response to leptin stimulus	PMID:17344214
GO:0045471	Biological_process	Response to ethanol	GO_REF:0000107
GO:0045765	Biological_process	Regulation of angiogenesis	PMID:11460888
GO:0045906	Biological_process	Negative regulation of vasoconstriction	GO_REF:0000107
GO:0046325	Biological_process	Negative regulation of glucose import	PMID:24340098
GO:0046427	Biological_process	Positive regulation of receptor signaling pathway *via* JAK/STAT	PMID:17344214
GO:0046628	Biological_process	Positive regulation of insulin receptor signaling pathway	GO_REF:0000107

(Continued on following page)

TABLE 2 (*Continued*) Single-gene GO analysis in SangerBox database.

GO id	Name space	Name	Rref database
GO:0046850	Biological_process	Regulation of bone remodeling	GO_REF:0000024
GO:0046881	Biological_process	Positive regulation of follicle-stimulating hormone secretion	GO_REF:0000107
GO:0048639	Biological_process	Positive regulation of developmental growth	PMID:17957153
GO:0050796	Biological_process	Regulation of insulin secretion	GO_REF:0000107
GO:0050810	Biological_process	Regulation of steroid biosynthetic process	GO_REF:0000107
GO:0050892	Biological_process	Intestinal absorption	PMID:24340098
GO:0050901	Biological_process	Leukocyte tethering or rolling	GO_REF:0000107
GO:0050999	Biological_process	Regulation of nitric-oxide synthase activity	PMID:15899045
GO:0051541	Biological_process	Elastin metabolic process	GO_REF:0000107
GO:0051726	Biological_process	Regulation of cell cycle	PMID:17344214
GO:0051897	Biological_process	Positive regulation of protein kinase B signaling	GO_REF:0000024
GO:0060587	Biological_process	Regulation of lipoprotein lipid oxidation	GO_REF:0000107
GO:0060612	Biological_process	Adipose tissue development	GO_REF:0000107
GO:0061037	Biological_process	Negative regulation of cartilage development	GO_REF:0000107
GO:0070093	Biological_process	Negative regulation of glucagon secretion	GO_REF:0000107
GO:0071298	Biological_process	Cellular response to L-ascorbic acid	GO_REF:0000107
GO:0071300	Biological_process	Cellular response to retinoic acid	GO_REF:0000107
GO:0072604	Biological_process	Interleukin-6 secretion	PMID:1968809
GO:0072606	Biological_process	Interleukin-8 secretion	PMID:19688109
GO:0090335	Biological_process	Regulation of brown fat cell differentiation	GO_REF:0000024
GO:0098868	Biological_process	Bone growth	GO_REF:0000024
GO:0120162	Biological_process	Positive regulation of cold-induced thermogenesis	PMID:27986616
GO:1900015	Biological_process	Regulation of cytokine production involved in inflammatory response	PMID:19688109
GO:1900745	Biological_process	Positive regulation of p38MAPK cascade	PMID:24340098
GO:1904651	Biological_process	Positive regulation of fat cell apoptotic process	GO_REF:0000107
GO:1990051	Biological_process	Activation of protein kinase C activity	PMID:24340098
GO:2000379	Biological_process	Positive regulation of reactive oxygen species metabolic process	GO_REF:0000107
GO:2000486	Biological_process	Negative regulation of glutamine transport	GO_REF:0000107
GO:2000491	Biological_process	Positive regulation of hepatic stellate cell activation	GO_REF:0000107
GO:0014068	Biological_process	Positive regulation of phosphatidylinositol 3-kinase signaling	PMID:21873635
GO:0046427	Biological_process	Positive regulation of receptor signaling pathway *via* JAK/STAT	PMID:21873635
GO:1990051	Biological_process	Activation of protein kinase C activity	PMID:21873635
GO:0006112	Biological_process	Energy reserve metabolic process	PMID:21873635
GO:0051428	Molecular_function	Peptide hormone receptor binding	PMID:21873635
GO:0006629	Biological_process	Lipid metabolic process	PMID:21873635
GO:0032008	Biological_process	Positive regulation of TOR signaling	PMID:21873635

(Continued on following page)

TABLE 2 (*Continued*) Single-gene GO analysis in SangerBox database.

GO id	Name space	Name	Rref database
GO:0005615	Cellular_component	Extracellular space	PMID:21873635
GO:0007260	Biological_process	Tyrosine phosphorylation of STAT protein	PMID:21873635
GO:0032868	Biological_process	Response to insulin	PMID:21873635
GO:1900745	Biological_process	Positive regulation of p38 MAPK cascade	PMID:21873635
GO:0038108	Biological_process	Negative regulation of appetite by leptin-mediated signaling pathway	PMID:21873635
GO:0005179	Molecular_function	Hormone activity	PMID:21873635

target *LEP* mRNAs (Figure 3A). These included hsa-miR-212-5p, hsa-miR-4661-3p, hsa-miR-1182, hsa-miR-3936, hsa-miR-1224-3p, hsa-miR-6890-3p, hsa-miR-147a, hsa-miR-5699-5p, hsa-miR-1304-5p, hsa-miR-4683, hsa-miR-668-3p, hsa-miR-4267, hsa-miR-6870-5p, hsa-miR-942-5p, hsa-miR-7855-5p, hsa-miR-3907, hsa-miR-619-5p, hsa-miR-7151-5p, hsa-miR-33a-3p, hsa-miR-6868-3p, hsa-miR-3173-5p, hsa-miR-6756-3p, hsa-miR-1245b-3p, and hsa-miR-5692a. Additionally, these 24 miRNAs were scored (Figure 3B). KEGG and GO analyses were performed on the selected miRNAs to explore their functions (Tables 3,4). KEGG analysis showed significant differences in the relevance of the TGF-beta, PI3K-Akt, MAPK, and JAK/STAT signaling pathways. GO analysis revealed comparisons with the stress-activated MAPK cascade, immune system process, blood coagulation, and *in utero* embryonic development.

3.5 Identification of differentially expressed lncRNAs and circRNAs, and the construction of a ceRNA network

We screened the ENCORI and DIANA TOOLS databases and identified 11 lncRNAs that might target miRNAs (Figure 3C), including hsa-miR-147a/LINC00473; hsa-miR-212-5p/LIFR-AS1, EXTL3-AS1, KCNQ1OT1, and NEAT1; hsa-miR-668-3p/SNHG12, TTN-AS1, KCNQ1OT1, NEAT1, MEG3, and FTX; hsa-miR-942-5p/AC159540.1 and LINC01011. We also screened the ENCORI and CircBank databases and identified 6 circRNAs that may target miRNAs, including hsa-miR-147a/hsa_circ_0000175; hsa-miR-212-5p/hsa_circ_0004333, hsa_circ_0075961, hsa_circ_0001699, hsa_circ_0001731, and hsa_circ_0081673 (Figure 3D). Based on the miRNAs, lncRNAs, and circRNAs screened, a ceRNA network containing 4 miRNAs, 11 lncRNAs, and 6 circRNAs was constructed (Figure 3E). In addition, we selected LINC00473 in the ceRNA network to detect its expression; LINC00473 was highly expressed in placentas from preeclamptic women compared with those from normal pregnant women. This finding is consistent with the results of the microarray analysis (Figure 3F). The clinical information of the normal pregnant women and patients with PE is listed in Table 5.

3.6 Construction of a PPI network

The STRING database analysis identified 20 leptin-binding proteins. As shown in Figure 4A, LEPR, GHRL, GCG, IAPP, PPARG, STAT3, SOCS3, JAK2, NPY, PTPN1, PPARGC1A, CEBPA, CCK, CRP, PRKAA2, PRL, INS, ADIPOQ, RXRA, and PRKAG1 were predicted to interact with leptin. Using GeneMANIA, we constructed a PPI network, which showed that LEPR, HK3, CD33, GHRL, SOCS3, CEBPA, PTPN1, CLU, SH2B1, FABP4, ZBTB17, PTGDS, GRN, IGFBP4, ARNT, PRKAA2, PRKAG2, JAK2, PRKAB2, and MED8 interact with leptin (Figure 4B). We then performed KEGG and GO analyses of the proteins predicted from the STRING database. KEGG analysis showed significant differences in the relevance of the PI3K-Akt and JAK/STAT signaling pathways. GO_BP classification revealed that proteins co-expressed with leptin were mainly involved in placental development, signal transduction, growth hormone signaling pathway *via* JAK/STAT, receptor signaling pathway *via* JAK/STAT, leptin-mediated signaling pathway, and female pregnancy. GO_CC annotations were mainly associated with the RNA polymerase II transcription regulator complex (Figures 4C,D). The protein KEGG analysis revealed that STAT3, PRL, SOCS3, LEPR, and JAK2 were associated with leptin in the JAK/STAT signaling pathway, suggesting that leptin may interact with these proteins to activate JAK/STAT signaling in PE. KEGG and GO analyses of leptin-binding proteins from geneMANIA are shown in Supplementary Figure S1.

3.7 RNA modification of LEP

Using SRAMP, a sequence-based m6A modification site predictor, we identified m6A modification sites in the *LEP* mRNA sequence, and displayed it on a high-confidence RNA

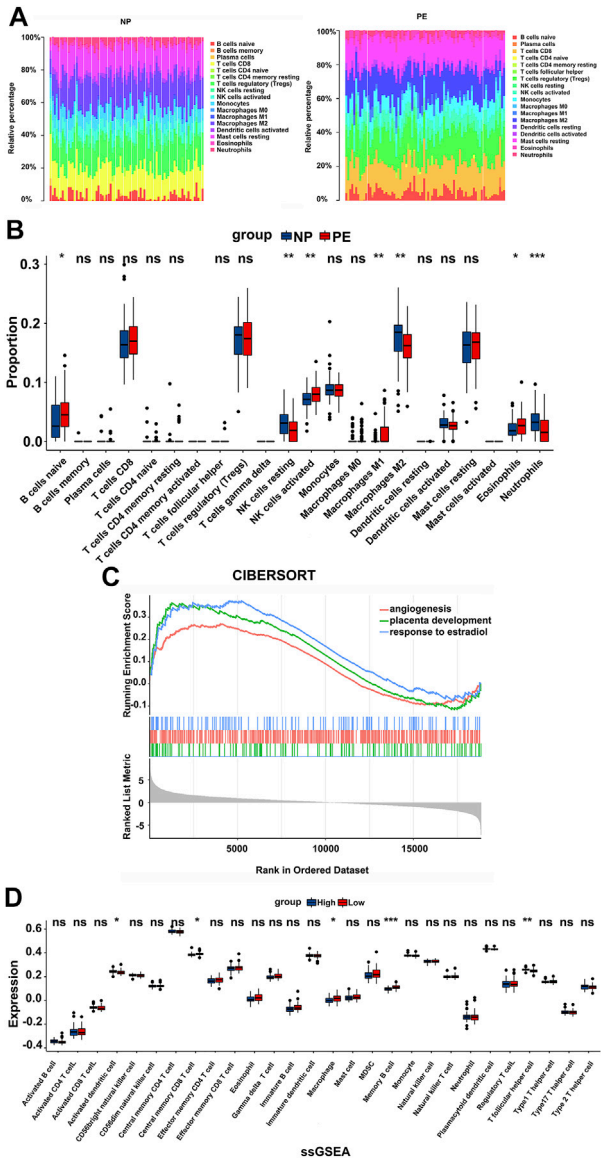


FIGURE 2
Placental immune cell infiltration analysis. **(A,B)** Differences in placental immune cell infiltration between normal pregnancy and PE. **(C)** A cohort of 80 PE patients were divided into *LEP* high and low expression groups for GSEA. Angiogenesis: NES: 1.27; FDR: 0.16; *p*-value: 0.016. Placenta development: NES: 1.51; FDR: 0.13; *p*-value: 0.007. Response to estradiol: NES: 1.54; FDR: 0.11; *p*-value: 0.005; **(D)** ssGSEA of the *LEP* high and low expression groups, to analyze immune cell infiltration. ns (not significant), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

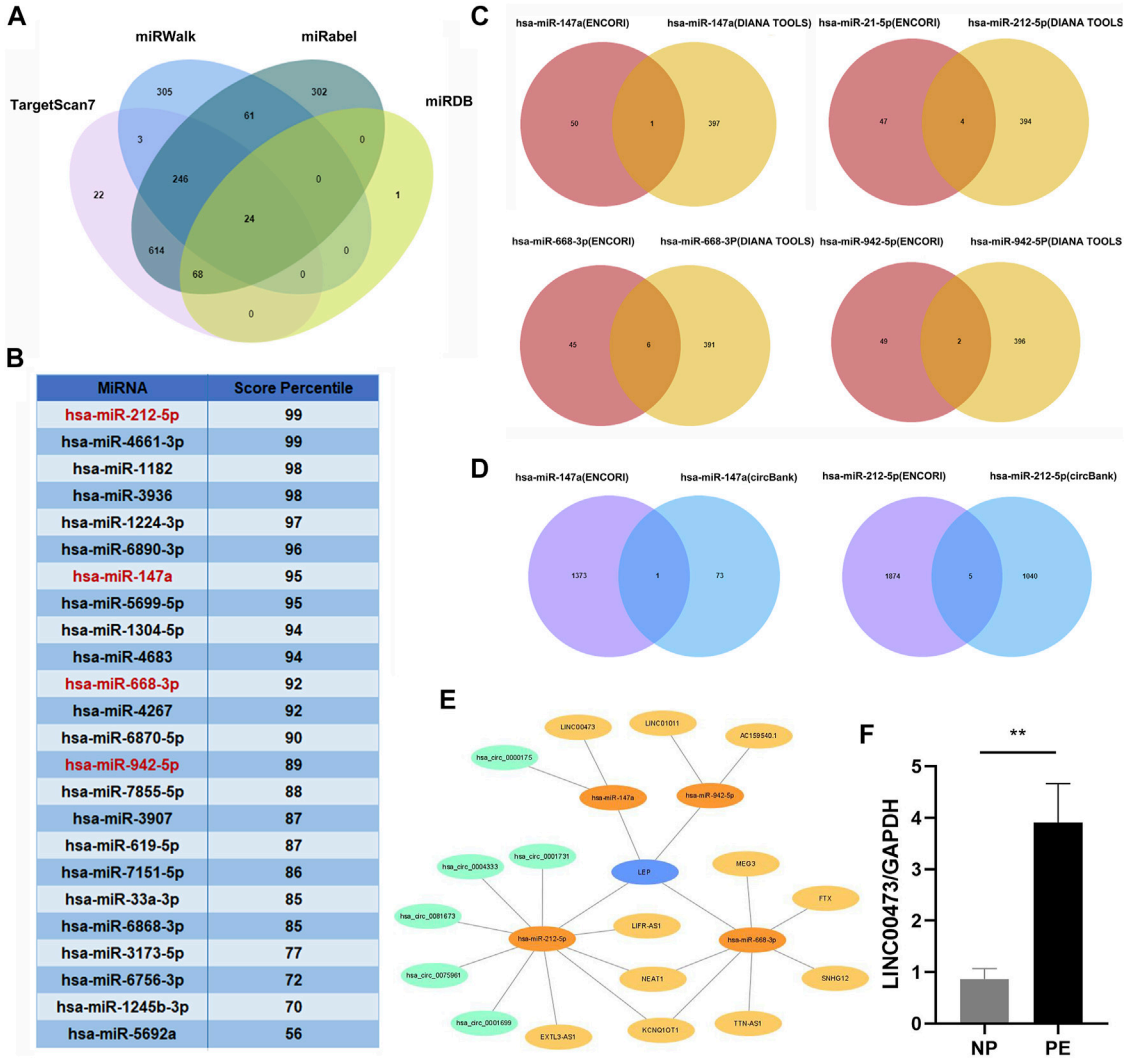
secondary structure (Figures 5A,B). We then used ENCORI and RBPsuite to identify an m6A-modified protein that interacts with *LEP*, IGF2BP3. Using RPISeq, we predicted the probability of IGF2BP3 associating with *LEP*. Predictions with probabilities >0.5 are considered "positive," indicating that the corresponding RNA and protein are likely to interact. For IGF2BP3, the RF classifier score was 0.8, and the SVM classifier score was 0.82 (Figure 5C). The IGF2BP3 motif was analyzed using the RBPsuite online database (Figure 5D). We used catRPAID to screen for *LEP* RNA regions that were most likely to be bound by IGF2BP3. The results revealed interaction profile peaks around the 500–600 nt site, indicating that IGF2BP3 may bind to *LEP* at this site (Figure 5E). We also used RBPsuite to explore the binding sites of IGF2BP3 on *LEP*, and the results indicated that IGF2BP3 may bind *LEP* at the 1,600–1,800 nt and 500–600 nt sites (Figure 5F).

3.8 Validation of LEP/leptin overexpression in clinical samples, the HTR-8/SVneo cell line, and a PE mouse model

To further explore the expression of *LEP* in PE, we collected placental tissue samples and examined the expression of *LEP* in these specimens using RT-qPCR. Our results, shown in Figure 6A, reveal that *LEP* expression in PE placental tissue samples (n = 22) was significantly higher than its expression in normal pregnancy placental tissue samples (n = 21), which is consistent with the bioassay results. To confirm that *LEP* is upregulated at the protein level, western blotting was used to determine protein expression in placental tissue samples from normal pregnant women (n = 20) and PE patients (n = 23). The clinical information of the normal pregnant women and patients with PE is listed in Table 6. As shown in Figure 6B, leptin protein expression was significantly higher in the placental tissues of patients with PE than in normal pregnant women. In addition, the overexpression of *LEP* in PE was validated in the HTR-8/SVneo cell line and in a PE mouse model. In the hypoxia model of HTR-8/SVneo cell line, we found that *LEP* was upregulated at mRNA level under hypoxic conditions by RT-qRCR (Figure 6C). In the mouse model of PE, we found that the blood pressure (systolic and diastolic) in the PE group was significantly higher than that in the PBS group (Figure 6D), and the placental and fetal weights were significantly lower in the PE group than those in the PBS group (Figure 6E). It was also observed in appearance that the PE group was significantly smaller than the PBS group (Figure 6F). Importantly, our results indicate that *LEP* is overexpressed in the placentas of preeclamptic pregnant mice by western blot (Figure 6G).

4 Discussion

In this study, a screen of datasets in the GEO database revealed that *LEP* is significantly upregulated in PE. Using TargetScan, miWalk, miRDB, miRabel, ENCORI, CircBank, and other websites to identify *LEP*-targeting miRNAs, lncRNAs, and circRNAs, we constructed a lncRNA/



MiRNA	Score Percentile
hsa-miR-212-5p	99
hsa-miR-4661-3p	99
hsa-miR-1182	98
hsa-miR-3936	98
hsa-miR-1224-3p	97
hsa-miR-6890-3p	96
hsa-miR-147a	95
hsa-miR-5699-5p	95
hsa-miR-1304-5p	94
hsa-miR-4683	94
hsa-miR-668-3p	92
hsa-miR-4267	92
hsa-miR-6870-5p	90
hsa-miR-942-5p	89
hsa-miR-7855-5p	88
hsa-miR-3907	87
hsa-miR-619-5p	87
hsa-miR-7151-5p	86
hsa-miR-33a-3p	85
hsa-miR-6868-3p	85
hsa-miR-3173-5p	77
hsa-miR-6756-3p	72
hsa-miR-1245b-3p	70
hsa-miR-5692a	56

FIGURE 3
Construction of a lncRNA/circRNA-miRNA-*LEP* regulatory network. **(A)** The upstream miRNAs of *LEP* were predicted by miRWalk, miRDB, Targetscan and miRabel databases and the intersecting miRNAs were selected (24 miRNAs). **(B)** The 24 miRNAs targeting *LEP* were scored in the Targetscan database. **(C)** The upstream lncRNAs targeting miRNAs were predicted using the ENCORI and DIANA TOOLS databases and the intersecting lncRNAs were selected (11 lncRNAs). **(D)** The upstream circRNAs targeting miRNAs were predicted using the ENCORI and CircBank databases and the intersecting circRNAs were selected (6 circRNAs) **(E)** The lncRNA/circRNA-miRNA-*LEP* regulatory network was constructed using Cytoscape. **(F)** RT-qPCR analysis of LINC00473 expression in PE placental tissue samples (n = 10) compared with that in normal pregnancy placental tissue samples (n = 10). * $p < 0.05$, ** $p < 0.01$.

circRNA-miRNA-*LEP* regulatory network that may regulate the abnormal expression of *LEP* in PE. We also performed single-gene KEGG, GO, and immune cell infiltration analyses, and by constructing a PPI network, we explored proteins interacting with *LEP* and their associated pathways and biological functions. We predicted m6A modification sites of *LEP* and related m6A-binding proteins, and importantly, we verified the overexpression of *LEP* mRNA and protein levels in clinical samples, the HTR-8/SVneo cell line and a PE mouse model using RT-qPCR and western blotting.

Leptin expression is higher in women experiencing normal pregnancies than in non-pregnant women, suggesting that leptin supports implantation and placental growth. Serum leptin levels increase weeks or months before the onset of PE, which may indicate that leptin itself is involved in the early-onset of PE. And studies have shown that serum leptin levels can be used as a biomarker to distinguish between early- and late-onset PE (Taylor et al., 2015; Hao et al., 2020; Liu et al., 2020). Leptin plays several roles in the regulation of pregnancy-related functions, while metabolic disorders and dynamic imbalances

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TABLE 3 miRNA KEGG analysis (DIANA TOOLS).

KEGG pathway	*p*-value	Genes	miRNAs
TGF-beta signaling pathway	1.13E-07	53	20
ErbB signaling pathway	7.46E-06	60	22
N-Glycan biosynthesis	1.53E-05	30	17
FoxO signaling pathway	1.53E-05	83	21
Mucin type O-Glycan biosynthesis	1.62E-05	18	15
Adrenergic signaling in cardiomyocytes	5.90E-05	83	23
Signaling pathways regulating pluripotency of stem cells	7.58E-05	81	21
Hippo signaling pathway	8.01E-05	92	23
Ras signaling pathway	0.000154,077	124	24
Regulation of actin cytoskeleton	0.000185,611	122	24
Rap1 signaling pathway	0.000224,072	122	23
Glutamatergic synapse	0.001,802,716	66	21
Glycosaminoglycan biosynthesis - chondroitin sulfate/dermatan sulfate	0.002,242,769	12	10
Adherens junction	0.002,242,769	48	16
Thyroid hormone signaling pathway	0.002,242,769	69	21
Long-term depression	0.003,659,698	39	21
Estrogen signaling pathway	0.005,232,062	53	20
Oxytocin signaling pathway	0.005,232,062	86	23
Axon guidance	0.00627,603	74	23
PI3K-Akt signaling pathway	0.00627,603	176	24
Sphingolipid signaling pathway	0.006,351,843	65	20
Phosphatidylinositol signaling system	0.008,158,926	43	19
Melanogenesis	0.010,385,807	57	17
Focal adhesion	0.011,193,156	111	24
MAPK signaling pathway	0.012,068,648	133	22
Bacterial invasion of epithelial cells	0.016,363,283	45	20
JAK/STAT signaling pathway	0.017,172,374	85	23
Dilated cardiomyopathy	0.020,923,949	52	21
Pantothenate and CoA biosynthesis	0.02,187,599	13	9
Dorso-ventral axis formation	0.02,187,599	19	12
Endocytosis	0.02,187,599	108	23
Morphine addiction	0.02,368,039	51	18
T-cell receptor signaling pathway	0.024,226,069	60	22
Circadian entrainment	0.026,972,795	52	21
Ubiquitin mediated proteolysis	0.031,051,668	76	23
Long-term potentiation	0.044,819,592	39	19

TABLE 4 miRNA GO Category (DIANA TOOLS).

GO category	*p*-value	Genes	miRNAs
Organelle	1.25E-46	325	1
Cellular nitrogen compound metabolic process	1.33E-36	195	1
Ion binding	5.61E-30	217	1
Biosynthetic process	8.70E-25	159	1
Molecular_function	7.87E-15	430	1
Nucleic acid binding transcription factor activity	3.69E-12	53	1
Transcription, DNA-templated	1.43E-07	93	1
Cytosol	1.56E-07	94	1
Catabolic process	2.39E-07	69	1
RNA binding	3.00E-07	71	1
Neurotrophin TRK receptor signaling pathway	5.46E-07	17	1
Gene expression	7.36E-07	27	1
Enzyme binding	9.39E-07	51	1
Biological_process	3.20E-06	405	1
Nucleoplasm	4.30E-06	47	1
Cell death	7.95E-06	39	1
Nucleobase-containing compound catabolic process	1.14E-05	37	1
Symbiosis, encompassing mutualism through parasitism	1.25E-05	24	1
Cellular protein metabolic process	1.31E-05	22	1
Cellular protein modification process	1.67E-05	71	1
DNA binding	2.38E-05	125	1
Cellular_component	2.38E-05	411	1
Transcription from RNA polymerase II promoter	2.95E-05	32	1
Viral process	4.64E-05	21	1
Fc-epsilon receptor signaling pathway	5.36E-05	11	1
Protein complex	8.15E-05	108	1
Response to stress	0.00011875	70	1
Toll-like receptor 10 signaling pathway	0.000148,087	7	1
Poly(A) RNA binding	0.000206,746	61	1
Toll-like receptor TLR1:TLR2 signaling pathway	0.00022013	7	1
Toll-like receptor TLR6:TLR2 signaling pathway	0.00022013	7	1
Toll-like receptor 5 signaling pathway	0.000337,865	7	1
Activation of signaling protein activity involved in unfolded protein response	0.000390,124	7	1
Virion assembly	0.000559,594	5	1
Toll-like receptor 9 signaling pathway	0.000603,341	7	1
Toll-like receptor 2 signaling pathway	0.001,511,812	7	1

(Continued on following page)

TABLE 4 (*Continued*) miRNA GO Category (DIANA TOOLS).

GO category	*p*-value	Genes	miRNAs
Epidermal growth factor receptor signaling pathway	0.001,564,322	12	1
Stress-activated MAPK cascade	0.001,940,873	6	1
TRIF-dependent toll-like receptor signaling pathway	0.003,796,881	6	1
Toll-like receptor signaling pathway	0.004,187,716	8	1
Translation factor activity, nucleic acid binding	0.004,187,716	9	1
Small molecule metabolic process	0.004,187,716	62	1
MyD88-independent toll-like receptor signaling pathway	0.006,760,211	6	1
Viral protein processing	0.007,430,104	3	1
Mitotic cell cycle	0.007,570,654	15	1
Toll-like receptor 4 signaling pathway	0.007,635,799	7	1
MyD88-dependent toll-like receptor signaling pathway	0.009,441,203	7	1
Odontogenesis of dentin-containing tooth	0.010,274,693	8	1
Innate immune response	0.010,274,693	26	1
MRNA metabolic process	0.011,989,448	10	1
Toll-like receptor 3 signaling pathway	0.012,073,024	6	1
Enzyme regulator activity	0.012,073,024	28	1
Viral life cycle	0.012,215,227	7	1
Immune system process	0.012,215,227	47	1
Antigen processing and presentation of exogenous peptide antigen *via* MHC class II	0.012,228,456	8	1
Regulation of transcription, DNA-templated	0.012,489,378	93	1
Intrinsic apoptotic signaling pathway	0.013,169,422	6	1
Double-stranded DNA binding	0.013,169,422	11	1
in utero embryonic development	0.013,869,985	17	1
Intracellular transport of virus	0.016,044,445	3	1
Blood coagulation	0.016,044,445	16	1
Membrane organization	0.016,735,745	20	1
Entry into host cell	0.017,330,871	2	1
Transcription regulatory region DNA binding	0.018,173,983	16	1
Cellular response to potassium ion	0.020,863,254	3	1
Cytoplasmic stress granule	0.020,863,254	6	1
Cytoskeletal protein binding	0.027,511,888	25	1
Cell proliferation	0.029,665,962	24	1
Cellular component disassembly involved in execution phase of apoptosis	0.030,387,763	4	1
Methionine adenosyltransferase complex	0.038,551,825	2	1
RNA metabolic process	0.043,481,388	10	1
Positive regulation of macroautophagy	0.045,017,736	4	1

TABLE 5 Clinical characteristics of samples for the validation of LINC00473.

Characteristics	NP (n = 10)	PE (n = 10)	p-value
Age (year)	34.40 ± 3.44	30.60 ± 3.53	0.025
Pre-pregnancy BMI	23.20 ± 2.38	28.13 ± 5.51	0.023
Gestation, (week)	38.84 ± 0.81	36.52 ± 3.75	0.085
Sbp (mmHg)	125.10 ± 9.37	161.60 ± 16.14	0.000
Dbp (mmHg)	72.30 ± 7.82	96.60 ± 10.09	0.000
Birth weight (g)	3,428.50 ± 428.31	2,673.00 ± 1,058.19	0.059

NP, normal pregnancy; PE, preeclampsia; BMI, body mass index; Sbp, Systolic blood pressure; Dbp, Diastolic blood pressure.

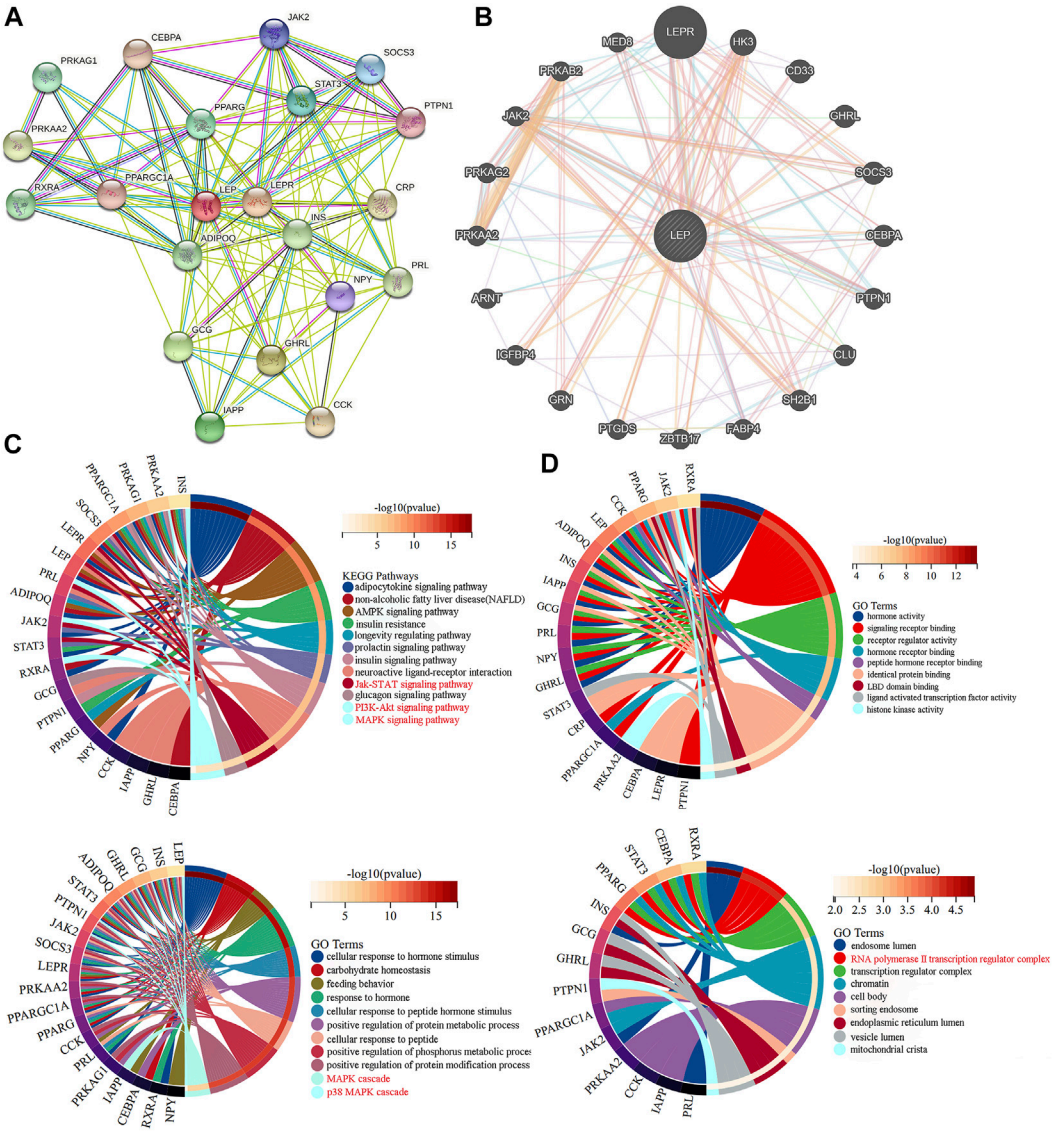
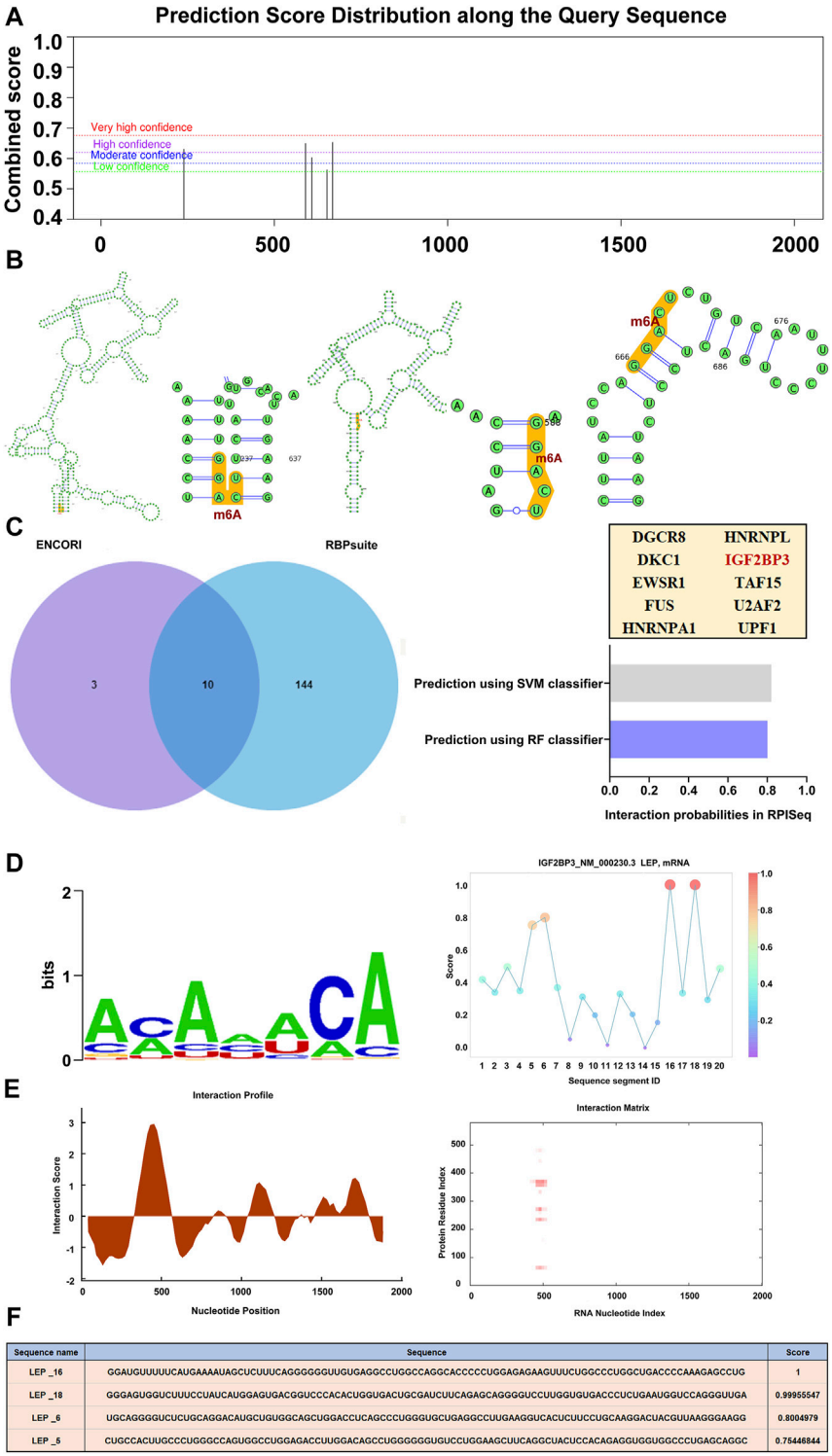


FIGURE 4
PPI networks exploring leptin-protein interactions. Leptin-binding proteins were identified using **(A)** the STRING tool, and **(B)** GeneMANIA. **(C,D)** KEGG and GO analyses of leptin-binding proteins from the STRING tool.



Sequence name	Sequence	Score
LEP _16	GGAUGUUUUUCAUGAAAAUAGCUCUUUCAGGGGGGUUGUGAGGCCUGGCCAGGCACCCCCUGGAGAGAAGUUUCUGGCCCUGGCUGACCCCAAAGAGCCUG	1
LEP _18	GGGAGUGGUCUUUCCUAUCAUGGAGUGACGGUCCCACACUGGUGACUGCGAUCUUCAGAGCAGGGGUCCUUGGUGUGACCCUCUGAAUGGUCCAGGGUUGA	0.99955547
LEP _6	UGCAGGGGUCUCUGCAGGACAUGCUGUGGCAGCUGGACCUCAGCCCUGGGUGCUGAGGCCUUGAAGGUCACUCUUCCUGCAAGGACUACGUUAAGGGAAGG	0.8004979
LEP _5	CUGCCACUUGCCCUGGGCCAGUGGCCUGGAGACCUUGGACAGCCUGGGGGGUGUCCUGGAAGCUUCAGGCUACUCCACAGAGGUGGUGGCCCUGAGCAGGC	0.75446844

FIGURE 5
RNA modification of *LEP*. **(A,B)** The m6A modification site and its location in the RNA secondary structure (high confidence). **(C)** m6A-modified proteins that interact with *LEP* (IGF2BP3) were predicted using ENCORI and RBPsuite. The probability that IGF2BP3 interacts with *LEP* were predicted using RPISeq, which indicates RF classifier and SVM classifier scores. **(D,E)** The IGF2BP3 motif was analyzed using RBPsuite and catRPAID to screen for regions of the *LEP* mRNA most likely to be bound by IGF2BP3. **(F)** The binding site of IGF2BP3 on *LEP* was explored with RBPsuite.

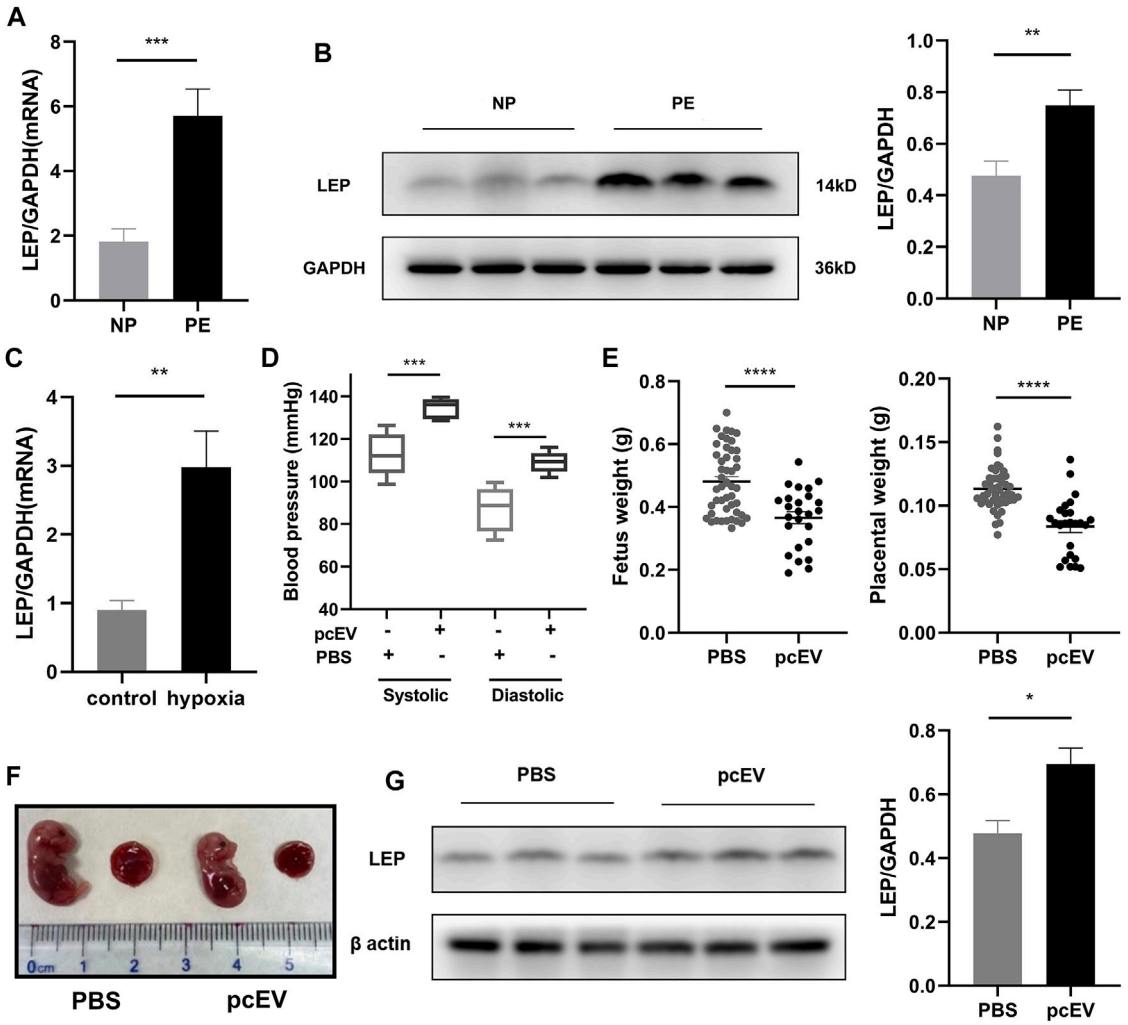


FIGURE 6
Validation of *LEP*/leptin expression in clinical samples, the HTR-8/SVneo cell line, and a PE mouse model. **(A)** RT-qPCR analysis of *LEP* expression in PE placental tissue samples (n = 22) compared with normal pregnancy placental tissue samples (n = 21). **(B)** Western blot analysis of leptin expression in PE placental tissue samples (n = 23) compared with normal pregnancy placental tissue samples (n = 20). **(C)** RT-qPCR analysis of *LEP* expression under normal oxygen concentration and hypoxia, control: normal oxygen concentration for 24 h, hypoxia: hypoxia condition for 24 h.**(D)** Blood pressure in PBS group (n = 5) compared with that in the pcEV group (n = 8). **(E)** Fetus weight and placental weight in PBS group (n = 47) compared with that in the pcEV group (n = 25). 5 litters in each group. **(F)** The appearance of fetus and placenta in PBS group compared with that in the pcEV group. **(G)** Western blot analysis of leptin expression in PBS group (n = 5) compared with that in the pcEV group (n = 8). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

related to leptin during pregnancy play a decisive role in the occurrence and development of PE (de Knegt et al., 2021). Furthermore, using rat models, Ibrahim et al. proved that leptin can increase blood pressure and affect endothelial function, exhibiting pro-inflammatory properties (Ibrahim et al., 2013). In this study, we found that *LEP* was highly expressed in patients with PE, and we speculate that *LEP* may be a potential biomarker for the prevention, diagnosis, and treatment of PE. In the subsequent study, we can use siRNA or plasmid to knock down or over-express *LEP* in HTR-8/SVneo cell line, and experimentally verify whether it will change the migration and invasion ability of HTR-8/SVneo cells. At present, impaired trophoblast cell migration and invasion ability is considered to be one of the important mechanisms in the development of PE (Jiang et al., 2020). Thus, the role of *LEP* in the occurrence and development of preeclampsia was explored by targeting *LEP*.

The JAK/STAT pathway is a major intracellular signal transduction pathway. It is a critical downstream regulator of cytokines, hormones, and growth factors. Four members of the JAK family (JAK1, JAK2, JAK3, and TYK2) and seven members of the STAT family (STAT1-4, STAT5A/B, and STAT6) have

TABLE 6 Clinical characteristics of samples for the validation of *LEP*.

Characteristics	NP (n = 21)	PE (n = 24)	p-value
Age (year)	33.57 ± 4.31	32.92 ± 3.91	0.596
Pre-pregnancy BMI	22.29 ± 2.74	27.48 ± 5.79	0.001
Gestation, (week)	38.84 ± 0.85	36.17 ± 3.26	0.001
Sbp (mmHg)	122.67 ± 8.96	158.96 ± 15.29	0.000
Dbp (mmHg)	74.33 ± 8.11	100.63 ± 11.72	0.000
Birth weight (g)	3,491.43 ± 566.37	2,713.26 ± 947.20	0.002

NP, Normal pregnancy; PE, Preeclampsia; BMI, Body mass index; Sbp, Systolic blood pressure; Dbp, Diastolic blood pressure.

been identified in mammals. Depending on the cytokine or growth factor that stimulates signaling, different combinations of JAKs and STATs are activated with a high degree of specificity (Dodington et al., 2018). At present, JAK/STAT signaling pathway has been studied in PE. Qu et al. found that hypoxia-inducible factor (HIF)-3α regulates the growth of EVT by up-regulating Fms-like tyrosine kinase receptor (Flt) 1/JAK/STAT signaling during hypoxia, which affects the progression of PE (Qu et al., 2021). However, there are few studies on the involvement of *LEP* in JAK/STAT signaling in PE. Previous studies have shown that leptin regulates placental amino acid transport by activating JAK/STAT signaling (JAK2 or STAT3) (Alijotas-Reig et al., 2017). STAT3 may play an important role in mediating trophoblast invasion (Zhang et al., 2018). Therefore, *LEP* may be involved in the development of PE through the JAK/STAT signaling pathway. In this study, the *LEP* single gene, miRNA, and PPI network-related gene KEGG analyses all showed that *LEP* might be associated with JAK/STAT signaling in PE. Therefore, we speculate that *LEP* may play an important role in the genesis and development of PE *via* this signaling pathway, which is a hypothesis worthy of further investigation.

Previous studies report that JAK/STAT signaling pathway has a certain relationship with immune infiltration. Wang et al. demonstrated that levamisole (LMS) inhibited T-cell activation and downregulated related molecules by inhibiting the activation of JAK/STAT signaling pathway (Wang et al., 2022). Zhou et al. found that the expansion of renal CD8+TRM cells may mediate and maintain renal inflammation and injury in lupus nephritis (LN), and the maintenance of renal CD8+TRM cell effector function depends on JAK/STAT signaling in LN kidneys (Zhou et al., 2020). All these studies have shown that JAK/STAT signaling pathway can regulate the occurrence and development of diseases by acting on immune cells through different signaling molecules. Therefore, we performed an immune cell infiltration assay in the hope that further studies can be conducted. An abnormal response of the maternal immune system to the placenta may be the first pathogenic step in PE, followed by a systemic inflammatory response involving the endothelium (Rambaldi et al., 2019). It is well known that not only does the number of macrophages change in patients with PE, but they also have a different state of polarization compared to patients with normal pregnancy. The total number of macrophages in the placenta of PE patients increased, while the number of M1 and M2 macrophages increased and decreased, respectively (Yao et al., 2019). Ji et al. showed that chemerin, by activating the CMKLR1/Akt/CEBPα axis, promotes the polarization of macrophages to an M1 subtype and inhibits the migration, invasion, and angiogenesis of trophoblast cells, thus participating in the initiation and development of PE (Ji et al., 2021). This finding is consistent with our results from the database screen. Our analysis of immune cell infiltration showed an increased percentage of M1 and a decreased percentage of M2 macrophages in PE patients compared to normal pregnant women. There were also differences in macrophages between the high and low *LEP* expression groups, suggesting that the level of *LEP* expression may be related to the change of macrophage infiltration in placenta. In our analysis of GSE75010, the expression of some other immune cells also changed. Our results suggest that the differences in immune cell infiltration in PE placentas may be related to differences in *LEP* expression.

The ceRNA networks constructed using bioinformatic tools are useful for exploring the role of mRNAs in disease. Recent studies have shown that lncRNAs and circRNAs can positively or negatively regulate miRNAs to influence the expression of downstream mRNAs and play an important role in the development of PE (Chen, 2016; Song et al., 2017). Zhang et al. constructed a lncRNA-related ceRNA network that regulates the expression of key genes in early-onset PE, including 21 lncRNAs, 3 mRNAs, and 69 miRNAs (Zhang et al., 2020). In their study, Yu et al. revealed that *SNHG16* expression is downregulated in PE placentas. *SNHG16* regulates trophoblast cell migration and invasion *via* the miR-218-5p/*LASP1* axis (Yu et al., 2021). Data from Ou et al. confirmed that hsa_circ_0111277 is upregulated in PE placenta, and that circ_

0111,277 acted as a sponge for hsa-miR-494-3p in trophoblast cells by regulating the HTRA1/notch-1 signaling pathway, which inhibited the migration and invasion of these cells (Ou et al., 2020). However, there are still relatively few studies that have focused on the lncRNA/circRNA-miRNA-mRNA regulatory networks in PE. Therefore, we investigated lncRNA/circRNA-miRNA-*LEP* networks that may regulate *LEP* expression in PE, which is a likely target for developing new therapeutic strategies for PE. However, although our study describes a lncRNA/circRNA-miRNA-*LEP* regulatory network in PE, there is still a lack of studies on the pathological process of PE regulated by the lncRNA/circRNA-miRNA-*LEP* regulatory network, which may be a new challenge.

The m6A RNA modification is the most common internal modification in eukaryotic genes and plays a unique role in regulating mRNA metabolism, including mRNA splicing, output, localization, translation, and stability. M6A-modified mRNA also plays a vital role in many biological processes such as embryonic development, cell proliferation, and tumor formation (Zhou et al., 2021). Recently, studies on the m6A modification of RNA have become more extensive. Hou et al. found that LINC00460/DHX9/IGF2BP2 complex may regulate the expression of high mobility group AT-hook 1 (HMGA1) by recognizing the m6A modification site of HMGA1, thereby enhancing its mRNA stability and promoting the metastasis of colorectal cancer (Hou et al., 2021). Zhang et al. found that IGF2BP1 recognized and stabilized the mRNA of PEG10 in an m6A-dependent manner, enhancing the expression of PEG10, thereby accelerating the cell cycle and promoting EC progression (Zhang et al., 2021). Gu et al. found that increased *METTL3* expression and m6A RNA methylation were associated with increased *HNRNPC1/C2* expression in placental trophoblasts in PE, suggesting that abnormal m6A modification may be one of the causes of trophoblast cell dysfunction in PE (Gu et al., 2021). Guo et al. found that *ALKBH5* which is an m6A demethylase was significantly upregulated, and *PPARG* expression downregulated in PE placentas. *ALKBH5* interference reduced m6A levels on *PPARG*, increased the stability of *PPARG*, and promoted *PPARG* translation. Moreover, *ALKBH5* interference significantly promoted the proliferation, migration, and epithelial-to-mesenchymal transition of HTR-8/SVneo cells, as well as the inhibition of apoptosis and oxidative stress (Guo et al., 2022). Wang et al. found that *HSPA1A* may be involved in the pathophysiology of PE, and showed that m6A modification significantly upregulated the expression of *HSPA1A* and its protein, suggesting that m6A plays a key role in gene expression regulation and is involved in the pathophysiological processes underpinning PE (Wang et al., 2020). Furthermore, studies have indicated that m6A may play an important role in blood pressure regulation (Mo et al., 2019). Based on numerous studies of m6A in PE and other diseases, we sought to explore whether m6A affects the translational stability of *LEP* in PE. Currently, few studies which have focused on the RNA modification of *LEP* in PE, and our study found that there is an m6A modification site on *LEP*, as well as an m6A binding protein, IGF2BP3, that may interact with *LEP*. RNA modification often relies on consensus motifs to form secondary structures that bind to RNA-modifying proteins known as writers, readers, and erasers. Therefore, the IGF2BP3 motif was analyzed. In addition, we found that IGF2BP3 is likely to bind *LEP* at the 500–600 nt site. Interestingly, the effect of m6A modification on targeted mRNAs depends primarily on the different m6A binding proteins. A study by Wang et al. revealed that IGF2BP3 affects the stability of TMBIM6 by participating in the m6A modification of TMBIM6 (Wang et al., 2021). Therefore, our study suggests that IGF2BP3 may bind *LEP* mRNAs to influence the development of PE. We hypothesize that RNA modification of *LEP* might stabilize the transcript and promote the expression of leptin.

Notably, the expression of *LEP*/leptin, and LINC00473 in clinical PE tissues were detected by both RT-qPCR and western blot analyses. We confirmed that the expression of *LEP*/leptin, LINC00473 in PE was significantly higher than that in normal pregnancy. Together, these data confirmed that overexpression *LEP*/leptin may indeed play a role in PE. In addition, the high expression of *LEP* in PE was verified in both the hypoxic HTR-8/SVneo cell line and the PE mouse model. By investigating the expression of *LEP* in PE *in vitro* and *in vivo*, we further found that *LEP* may play a role in the pathogenesis of PE. The study by Han et al. (2020) successfully generated a mouse model of PE. Injection of pcEVs into pregnant C57BL/6J mice to increase circulating pcEV levels leads to preeclampsia-like changes such as hypertension, proteinuria, and other pathological changes such as vascular injury and constriction. Our results also showed that pregnant C57BL/6J female mice with increased circulating pcEVs by pcEVs injection developed hypertension and fetal growth restriction. These provided a basis for us to validate the high expression of *LEP* in PE using this PE mouse model. However, this study does have some limitations. Although our investigations involved related pathway analyses, we didn't conduct in-depth research or further study on the predicted miRNAs, lncRNAs, and circRNAs. Further *in vitro* and *in vivo* experiments are needed to explore whether the upstream predicted lncRNAs or circRNAs binds to miRNAs and whether miRNAs binds to *LEP*, and then to investigate the regulatory effect of upstream non-coding RNAs (ncRNAs) on *LEP* in the case of knockdown or overexpression, and its effect on the occurrence and development of PE. It would be worthwhile exploring these regulatory RNAs in future studies to better understand the etiology and pathological mechanisms of PE. In addition, validation of *LEP* as a possible biomarker for PE by using clinical samples collected in the third trimester with a clear

diagnosis of normal pregnancy or PE at the time of collection has certain limitations. This requires us to conduct prospective studies, such as examining the levels of *LEP* in peripheral blood during pregnancy, to predict PE.

5 Conclusion

In this study, database screening identified the *LEP* gene to be upregulated in PE and bioinformatics tools were used to predict the corresponding miRNAs, lncRNAs, and circRNAs, and construct a lncRNA/circRNA-miRNA-*LEP* regulatory network. We then investigated the function of *LEP* by KEGG, GO, and immune cell infiltration analyses, in addition to predicting m6A modification sites and corresponding binding proteins. Finally, we verified the high expression of *LEP* in clinical samples, the hypoxic HTR-8/SVneo cell line and the PE mouse model at an mRNA and protein level. These data lay the foundation for further research on the role of leptin in the pathogenesis of PE, which could lead to a better theoretical basis for predicting, preventing, and treating PE in clinical settings.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ Supplementary Material.

Ethics statement

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

Author contributions

YW analyzed the data, conducted experiments, recruited patients, collected samples, and drafted the manuscript. XB and XG collected the samples and analyzed the data. XLG, YC, HL, and WF analyzed the data. CH developed the hypothesis, designed the study, and drafted the manuscript.

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Conflict of interest

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

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Supplementary material

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Maternal pre-eclampsia serum increases neurite growth and mitochondrial function through a potential IL-6-dependent mechanism in differentiated SH-SY5Y cells

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Introduction: Pre-eclampsia (PE) is a common and serious hypertensive disorder of pregnancy, which affects 3%–5% of first-time pregnancies and is a leading cause of maternal and neonatal morbidity and mortality. Prenatal exposure to PE is associated with an increased risk of neurodevelopmental disorders in affected offspring, although the cellular and molecular basis of this increased risk is largely unknown.

Methods: Here, we examined the effects of exposure to maternal serum from women with PE or a healthy uncomplicated pregnancy on the survival, neurite growth and mitochondrial function of neuronally differentiated human SH-SY5Y neuroblastoma cells, which are commonly used to study neurite growth. Neurite growth and mitochondrial function are two strongly linked neurodevelopmental parameters in which alterations have been implicated in neurodevelopmental disorders. Following this, we investigated the pleiotropic cytokine interleukin-6 (IL-6) levels as a potential mechanism.

Results: Cells exposed to 3% (v/v) PE serum for 72 h exhibited increased neurite growth ($p < 0.05$), which was validated in the human neural progenitor cell line, ReNcell® VM ($p < 0.01$), and mitochondrial respiration (elevated oxygen consumption rate ($p < 0.05$), basal mitochondrial respiration, proton leak, ATP synthesis, and non-mitochondrial respiration) compared to control serum-treated cells. ELISA analysis showed elevations in maternal IL-6 in PE sera ($p < 0.05$) and placental explants ($p < 0.05$). In support of this, SH-SY5Y cells exposed to 3% (v/v) PE serum for 24 h had increased phospho-STAT3 levels, which is a key intracellular mediator of IL-6

Abbreviations: ADHD, attention-deficit/hyperactivity disorder; ASD, autism spectrum disorder; ATP, adenosine triphosphate; BDNF, brain-derived neurotrophic factor; BMI, body mass index; BP, blood pressure; BSA, bovine serum albumin; DMEM, dulbecco's modified eagle's mixture; E, embryonic day; EGF, epidermal growth factor; FBS, fetal bovine serum; FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; FGF2, fibroblast growth factor 2; ID, intellectual disability; IL-6, interleukin-6; LDH, lactate dehydrogenase; MIA, maternal immune activation; OCR, oxygen consumption rate; PBS, phosphate-buffered saline; PE, pre-eclampsia; PFA, paraformaldehyde; RA, retinoic acid; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; STAT3, signal transducer and activator of transcription 3; TBS, Tris-buffered saline.

signalling ($p < 0.05$). Furthermore, treatment with anti-IL-6 neutralizing antibody blocked the effects of PE serum on neurite growth ($p < 0.05$), and exposure to IL-6 promoted neurite growth in SH-SY5Y cells ($p < 0.01$).

Discussion: Collectively these data show elevated serum levels of maternal IL-6 in PE, which increases neurite growth and mitochondrial function in SH-SY5Y cells. This rationalizes the further study of IL-6 as a potential mediator between PE exposure and neurodevelopmental outcome in the offspring.

KEYWORDS

pre-eclampsia, neurodevelopmental disorder, autism spectrum disorder, interleukin-6, inflammation, neurite growth, mitochondria

1 Introduction

Pre-eclampsia (PE) is a hypertensive disorder of pregnancy affecting approximately 5% of primiparous pregnant women. PE involves new-onset hypertension on or after 20 weeks' gestation and one of proteinuria, organ dysfunction or uteroplacental dysfunction (Brown et al., 2018; Barron et al., 2021). Well-recognized as a leading cause of maternal and neonatal morbidity and mortality, PE also has adverse consequences for the long-term health and neurodevelopmental trajectories of exposed offspring (Wu et al., 2009; Andraweera and Lassi, 2019; Li et al., 2021). This includes an increased risk of neurodevelopmental disorders, particularly autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHD), and intellectual disability (ID) (Maher et al., 2018; Sun et al., 2020; Greca et al., 2021; Wang et al., 2021). In addition, recent neuroimaging studies have revealed alterations in brain structure, function and metabolites of children prenatally exposed to PE (Rätsep et al., 2016; Figueiró-Filho et al., 2017; Mak et al., 2018; Katsuki et al., 2021; Xing et al., 2021).

For these reasons, there has been significant interest in using rodent models to examine the brain and behavior of offspring prenatally exposed to a PE-like state *in utero*. These have yielded valuable insights into the effects of exposure to PE-like environment on mammalian neurodevelopment, which include alterations in neurogenesis and gliogenesis, regional brain volumes, forebrain transcriptional profile, and pronounced behavioral deficits (Liu et al., 2016; Ijomone et al., 2020; Gumusoglu et al., 2021; Rains et al., 2021). However, there is a need to understand whether exposure to PE affects neuronal development at a single cell level, particularly in human cells, in order to understand the potential mechanisms involved. For example, some studies have reported that exposure to PE serum increases neurite growth and branching in embryonic day (E)18 rat primary cortical neurons (Curran et al., 2018); yet others have shown that secreted factors from the PE placenta *reduce* neurite growth in E18 cortical neurons, alter neurotransmitter receptor expression and enhance astrogliogenesis (Scott et al., 2018). Thus, there is a need for further studies that explore the physiological effects, and molecular mechanisms, of PE exposure on developing neurons.

This study aimed to assess the effects of PE exposure on neurite growth and mitochondrial function, two important neurodevelopmental parameters known to be implicated in neurodevelopmental disorders, particularly ASD (Gu et al., 2013; Hashimoto et al., 2016; Barron et al., 2021). These are two tightly linked processes: neurites are rich in mitochondria; mitochondrial-derived reactive oxygen species derived are key regulators of neurodevelopmental processes, including neurite growth; and a significant proportion of cellular ATP, generated by mitochondria in the neurite and growth cone, is used for actin polymerization, the chief mechanism responsible for neurite growth (Smith and Gallo, 2018; Wilson et al., 2018). Therefore, the experiments described here examined whether serum from women with PE or women with healthy uncomplicated pregnancies (controls) differentially affect neurite growth and mitochondrial function in neuronally-differentiated–SH-SY5Y cells, a human neuroblastoma cell line commonly used to study neurite growth *in vitro* (Kovalevich and Langford, 2013). The use of human sera was chosen to identify whether there are maternal circulating factors in PE that affect neuronal development.

While the physiological mechanisms underlying the association between PE and offspring neurodevelopment are yet to be discerned, one candidate mechanism may be the sustained maternal immune activation (MIA) which is a prominent feature of PE (Sharma et al., 2007; Cornelius, 2018; Aggarwal et al., 2019; Barron et al., 2021). MIA is known to adversely affect neurodevelopment both directly *via* the effects of cytokines on neurodevelopmental processes in the fetal brain (Jarskog et al., 1997; Nolan et al., 2011; Crampton et al., 2012), and indirectly *via* non-canonical mechanisms through which MIA-induced alterations of maternal physiology create a sub-optimal *in utero* environment for the fetus (Shi et al., 2005; Zuckerman and Weiner, 2005; Brown et al., 2014; Straley et al., 2017; Barron et al., 2021).

Specifically, the cytokine interleukin-6 (IL-6) may play a significant role in this association. Elevated maternal IL-6 is associated with altered structural and functional brain connectivity in the offspring (Spann et al., 2018; Rasmussen et al., 2019), and the adverse effects of MIA on offspring brain and behavior in animal models are dependent on maternal or placental IL-6 (Smith et al., 2007; Gumusoglu et al., 2017; Wu et al., 2017). The phenotypic effects of IL-6 signaling, acting through phospho-activation of the transcription factor signal transducer and activator of transcription 3 (STAT3) at Tyr_{705}, are pleiotropic, although in neurons it typically exerts a neurogenic, neuritogenic and neurotrophic effect–several studies have identified a role for IL-6-STAT3 signaling in promoting neuronal differentiation and survival, and enhancing neurite outgrowth, axon regeneration and synaptogenesis, in various neuronal models (März et al., 1997; Bissonnette et al., 2004; Miao et al., 2006; Zhou and Too, 2011; Yang et al., 2012; Leibinger et al., 2013; Su et al., 2020; Kummer et al., 2021; Mirabella et al., 2021). Importantly, STAT3 is also known to stimulate mitochondrial activity (Gough et al., 2009; Zhou and Too, 2011; Yang et al., 2015; Luo et al., 2016; Su et al., 2020).

TABLE 1 Maternal clinical characteristics for all control and patient mothers enrolled in the current study. Mean ± SD. Mean Arterial blood pressure was calculated as MAP = (2 × diastolic) + systolic/3.

	Uncomplicated controls (n = 18)	Pre-eclampsia (n = 18)	p-value
Maternal age (years)	35.2 ± 4.5	36.5 ± 6.6	0.6909
Maternal BMI (kg/m^2)	25.2 ± 5.2	30.8 ± 7.9	0.1731
Mean arterial blood pressure in 1st trimester (mm Hg)	78.8 ± 6.5	88 ± 6.2	0.0310*
Gestational age at delivery (weeks)	38.7 ± 1	36.5 ± 1.5	0.0160*
Fetal birthweight (g)	3,339.7 ± 368.2	2,836.7 ± 882.4	0.2266

Maternal IL-6 is reportedly elevated in PE (Aggarwal et al., 2019; Gencheva et al., 2021), it crosses both the placental and blood-brain barriers (Zaretsky et al., 2004; Banks, 2005) and is increased in the umbilical cord blood of neonates exposed to PE (Tosun et al., 2010). For these reasons, we measured IL-6 in serum and placental explant supernatants in PE and hypothesized that elevated IL-6 in PE would increase neurite growth and mitochondrial respiration in neuronally differentiated SH-SY5Y cells.

2 Materials and methods

2.1 Patient enrolment and serum collection

Pre-eclampsia patients and controls were recruited from Cork University Maternity Hospital, Cork, Ireland, as part of the COMRADES Study, a non-interventional cohort study of nulliparous singleton pregnancies with the aim of characterizing the immune cell profile of women with PE. PE cases (n = 18) were defined as sustained hypertension (with systolic blood pressure (BP) ≥ 140 or diastolic BP ≥ 90 on at least 2 occasions at least 4 h apart) with significant quantified proteinuria (>300 mg protein on 24 h collection, urine protein creatinine >30 mg/mmol or +3 Dipstick Proteinuria) as per International Society for the Study of Hypertension in Pregnancy guidelines (Brown et al., 2018). Matched selected controls (n = 18) were taken from healthy pregnant women who had uncomplicated pregnancies which were defined as pregnancies not affected by PE, preterm birth or fetal growth restriction and delivered at >37 weeks. All control blood pressure readings were <140 and/or <90 mmHg prior to the delivery. Controls were matched with the PE cases for maternal age, body mass index (BMI) and gestational age. All women were delivered by prolabor elective Caesarean section for reasons such as breech presentation. Fasting blood samples were taken the morning of the scheduled elective Caesarean section. Serum samples were collected in BD EDTA Vacutainer tubes, placed on ice, and centrifuged once at 2,400 g for 10 min, followed by once at 2,000 g for 10 min, at 4°C according to a standardized protocol. Serum samples were stored at −80°C until analysis. The COMRADES study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all the procedures were approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals [ECM4 (ff) 04/12/18], and all women provided written informed consent. Clinical data from women with pre-eclampsia and matched healthy controls are shown in Table 1.

2.2 Cell culture, differentiation and treatments

Human neuroblastoma SH-SY5Y cells (ATCC) were cultured in Dulbecco's modified Eagle's (DMEM)/Nutrient Mixture F-12 Ham's medium, supplemented with 2 mM·L-glutamine, 1% penicillin-streptomycin, and 10% fetal bovine serum (FBS) (all from Sigma Aldrich) and maintained in a T75 culture flask (Sarstedt) at 37°C and 5% CO_2. Media was changed every 3 days and cells were passaged and/or plated for experiments once they were ~80% confluent. In all experiments except where otherwise indicated, 10 μM retinoic acid (RA, Sigma Aldrich) was added daily for the experimental duration to induce partial neuronal differentiation, concomitant with other experimental interventions.

For some experiments, full neuronal differentiation was achieved by adapting a 12-day protocol described by Taylor-Whiteley et al., 2019 (Taylor-Whiteley et al., 2019). Briefly, SH-SY5Y cells were cultured in Dulbecco's modified Eagle's (DMEM) high glucose medium, which included 2 mM L-glutamine, and supplemented with 1% penicillin-streptomycin, 1 mM sodium pyruvate and 10% FBS (all from Sigma Aldrich). Cells were seeded in a 24-well plate at 10,000 cells per well in complete high glucose media +10% FBS and treated daily with 10 μM RA for 5 days. After 5 days, cells were washed once in serum-free, high-glucose media and then the media was changed to serum-free, high-glucose media. Cells were then treated daily with 50 ng/mL brain-derived neurotrophic factor (BDNF, Peprotech) for a further 7 days before analysis.

The human neural progenitor cell line ReNcell® VM (Sigma) was used to validate findings. Cells were cultured in ReNcell® Maintenance Medium supplemented 20 ng/mL EGF (epidermal growth factor) and FGF2 (fibroblast growth factor 2) (all from Sigma Aldrich) and maintained in a T75 culture flask (Sarstedt) at 37°C and 5% CO_2. Cells were seeded at 7,500 cells per well in a laminin-coated 96-well plate. 24 h after seeding, cells were washed, and media changed to ReNcell® Maintenance Medium without EGF and FGF2—the restriction of growth factors initiates spontaneous differentiation to neurons. Cells were differentiated for 7 days.

For all experiments, except where otherwise indicated, treatments were 2 h after plating, and analyses were performed 72 h after first treatment. Final concentrations used were: 10 μM RA or 50 ng/mL BDNF added daily during differentiation; 3% (v/v) maternal serum from women with PE or normotensive pregnant women, added once (Curran et al., 2018); 20 ng/mL recombinant IL-6 (Peprotech), added daily (Qian et al., 2014; Sackmann et al., 2017; Marko et al., 2020); and 0.5 μg/mL anti-IL-6 function-blocking antibody (R&D Systems, MAB 206), incubated with sera or IL-6

for 1 h at room temperature before respective treatments. Of the total n = 18 control and n = 18 PE sera, smaller samples were selected randomly or based on availability, and sample sizes for each experiment are detailed in the figure legends. All experiments involved equal numbers of control and PE sera, where one PE serum sample was equal to one independent replicate (n).

2.3 Quantification of IL-6 in maternal serum and placental explant supernatants

IL-6 was examined using the U-PLEX Biomarker Group 1 Human Assays K15067L-1 immunoassay (Mesoscale Diagnostics, United States). All standards and serum and placenta explant supernatant samples were run in duplicate. Plates were prepared according to manufacturer's instructions and analyzed on the Meso QuickPlex SQ 120. Results were generated as calculated concentration means on the Mesoscale (MSD) Discovery Workbench 4.0 assay analysis software. The MSD analysis software determines individual cytokine concentrations from electro-chemiluminescent signals *via* backfitting to the calibration curve. IL-6 concentration is presented in pg/mL.

2.4 Neurite length measurements

For neurite growth measurements, cells were plated at a density of 12,500 cells/cm^2 and live-cell imaging was performed after 72 h using either fluorescent microscopy following 1 h incubation with the vital cell dye Calcein-AM (Sigma Aldrich) at 0.4 μg/mL, or phase contrast, at × 20 magnification using an Olympus I ×71 inverted microscope. Five non-overlapping fields were acquired per well with a DP72 camera, and neurites were traced to calculate neurite length using ImageJ. In all cases the analyses were performed in a blinded fashion.

2.5 Scratch wound assay

A scratch wound assay experiment was used to assess cell migration. SH-SY5Y cells were grown until confluent for 72 h. A single, straight scratch was made through the cell monolayer using a P200 pipette tip and the media was then changed. The wound was imaged using phase contrast microscopy at ×10 magnification on an Olympus I ×71 inverted microscope at three distinct locations in each well at the following timepoints post-scratch: 0 h, 24 h, 48 h, and 72 h. The mean wound width was measured at each time point using ImageJ, and this was used to calculate the rate of wound closure as a measure of cell migration.

2.6 Oxidative stress measurement

Oxidative stress was assessed using the fluorescent cell dye CellROX™ Green Reagent (Invitrogen), according to manufacturer's guidelines. Briefly, cells were incubated with 5 μM CellROX™ Green Reagent at 37°C for 30 min, then washed once in PBS and imaged live in PBS at ×20 magnification using FITC fluorescent channel, on an Olympus I X71 inverted microscope. Five non-overlapping fields were acquired per well with a DP72 camera. The mean fluorescence intensity of five cells per field minus adjacent background was measured using ImageJ.

2.7 Cytotoxicity assay

Cytotoxic cell damage was determined using the CyQUANT™ LDH Cytotoxicity Assay Kit (Invitrogen), which measures cytotoxicity based on extracellular lactate dehydrogenase (LDH) activity, according to manufacturer's guidelines. Briefly, media was collected at the end of each experiment and centrifuged to remove any remaining cells or debris, and the supernatant was collected and used for the assay. 50 μL of the medium was combined with 50 μL of the reaction mixture in a flat-bottomed, 96-well plate and incubated for 30 min at room temperature in darkness. The reaction was terminated with 50 μL of stop solution and absorbance at 680 nm measured and subtracted from absorbance at 490 nm.

2.8 Mitochondrial respiration

Mitochondrial function and metabolism was assessed using the Seahorse XF96 Mito Stress Test (Agilent Technologies). Optimal seeding density for SH-SY5Y cells for 3 days was determined to be 40,000 cells per well. For all subsequent experiments, cells were seeded at 40,000 cells/well in a XF96 culture plate, with 4 corner wells left empty for background correction. One hour before the assay, media was changed to Seahorse XF DMEM media, supplemented with 2 mM L-glutamine, 1 mM pyruvate and 10 mM glucose, and cells were allowed to equilibrate at 37°C and 0% CO_2 for 1 h. After calibration, oxygen consumption rate (OCR) was measured by the Seahorse XF96 Analyzer and recorded with XF Wave software 1.4.2. at 12 distinct timepoints over the course of an 80-min run: three times at basal respiration; three times following injection of 2.5 μM oligomycin to inhibit complex V; three times following injection of 2 μM of the ionophore carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) to uncouple the H^+ gradient at the inner mitochondrial membrane; and three times following injection of 0.5 μM each of rotenone and antimycin A, to inhibit complexes I and III, respectively. After completion of the assay, cells were lysed in 1X RIPA buffer and total protein quantified by bicinchoninic acid (BCA) assay, and OCR values normalized to protein concentration per well. From normalized OCR values, the following respiratory parameters were calculated: basal respiration, proton leak, maximal respiration, non-mitochondrial respiration, ATP production and spare respiratory capacity.

2.9 Mitochondrial superoxide, biomass, and membrane potential

Mitochondrial superoxide, mitochondrial biomass and mitochondrial membrane potential were measured using the fluorescent dyes MitoSOX™ Red (2.5 μM, Invitrogen), MitoGreen (200 nM, Promocell) and MitoTracker™ Deep Red FM (50 nM, Invitrogen), respectively. For all three dyes, cells were seeded at 37,500 cells/cm^2 for 72 h, and then incubated with the dye at 37°C for 30 min, as per manufacturers' guidelines. The dye was then removed, and cells detached with trypsin-EDTA and analyzed live in fluorescence-activated cell sorting (FACS) buffer containing PBS, 2% FBS and 2 mM EDTA. Mean fluorescence intensity was determined by FACS, using the BD LSRII Flow Cytometer (BD Biosciences). 20,000 events were measured for MitoSOX™ Red and MitoGreen, and 10,000 for MitoTracker™ Deep Red FM to determine the geometric mean representing mean fluorescence intensity.

2.10 Immunocytochemistry

Cells seeded at 12,500 cells/cm^2 for 72 h were fixed for immunostaining in 4% PFA and preserved in 0.02% PBS-Triton × (PBS-T). Non-specific binding was blocked by incubating the cells in 5% BSA at room temperature for 1 h. Cells were then incubated at 4°C overnight with a primary antibody against βIII tubulin (1:1,000 (0.5 μg/mL), R&D Systems MAB1195). After overnight incubation, cells were washed in PBS-T and incubated at room temperature for 2 h with goat anti-mouse alexa fluor 594 secondary antibody (1:500, Invitrogen A11005). Cells were washed in PBS-T, counterstained with DAPI and imaged at ×20 magnification on an Olympus IX71 inverted microscope using the appropriate fluorescent filter (DAPI or TXRED). Five non-overlapping fields were acquired per well with a DP72 camera and mean fluorescence intensity was determined using ImageJ.

2.11 Western blot

Confluent cells were lysed in 1X radioimmunoprecipitation assay (RIPA) buffer, centrifuged at 14,000 × g for 10 min, and supernatants were stored at −80°C prior to Western blot analysis. Protein concentration was determined using a PierceTM bicinchoninic acid (BCA) assay (ThermoFisher), and xxμg protein from each cell lysate was separated by SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) and transferred onto a methanol-activated PVDF membrane (Millipore). The membrane was blocked in 5% BSA for 1 h at room temperature and incubated at 4°C overnight with primary antibody against βIII tubulin (1:1,000 (0.5 μg/mL), R&D Systems MAB1195), STAT3 (1:1,000 (0.05 μg/mL), Cell Signaling Technology mAb No. 9139), p-STAT3 (1:2000 (0.05 μg/mL), Cell Signalling Technology mAb No. 9145) or GAPDH (1:1,000 (0.2 μg/mL), Santa Cruz Biotechnology sc-47724). After overnight incubation, the membrane was washed in 0.1% TBS-Tween (TBS-T) and incubated at room temperature for 1 h with goat anti-rabbit secondary antibody (1:5,000, Cell Signaling Technology mAb No. 7074) or HRP-conjugated mouse IgG_κ light chain binding protein (1: 2000 Santa Cruz Biotechnology Product No. sc-516102). Membrane was washed in TBS-T and developed using Pierce™ ECL Western Blotting Substrate (Thermo Scientific) and the Fujifilm LAS3000 luminescent image analyzer.

2.12 Statistical analysis

All statistical analyses were performed using Graphpad Prism 9. Statistical significance was set at $p < 0.05$, and the statistical tests applied to the data were Student's unpaired two-tailed t-test, one- and two-way ANOVA or mixed effects model as appropriate, with any statistically significant main effects further analysed using Fisher's least significant difference (LSD) post-hoc test. All data are expressed as the mean with standard error of the mean (SEM) where indicated. Where data followed a non-parametric distribution, Mann-Whitney test was used. Results from t-tests are reported as $t_x = y$, $p = z$, where x is the degrees of freedom, y is the t-statistic, and z is the p-value; results from F-tests are reported as $F_{a,b} = c$, $p = d$, where a is the between-groups degrees of freedom, b is the within-groups degrees of freedom, c is the F-statistic, and d is the p-value.

3 Results

3.1 Exposure to PE serum increases neurite growth in differentiated SH-SY5Y cells

SH-SY5Y cells were differentiated with 10 μM RA for 72 h (Supplementary Figures S1A–E). To examine the effects of maternal PE serum, RA-differentiated SH-SY5Y cells were co-treated with 3% (v/v) maternal serum from women with PE or women with healthy uncomplicated pregnancies, that were matched for maternal and gestational age and maternal BMI. Neurite growth was examined at 72 h post serum treatment. Exposure to PE serum significantly increased neurite growth compared to controls ($t_{24} = 2.230$, $p < 0.05$) (Figures 1A, D). There was no significant change in oxidative stress ($U = 65$, $Med_1 = 82.24$, $n_1 = 8$, $Med_2 = 90.21$, $n_2 = 8$, $p = 0.713$) (Figures 1B, E) or cytotoxicity ($U = 26$, $Med_1 = 96.42$, $n_1 = 8$, $Med_2 = 93.78$, $n_2 = 8$, $p = 0.574$) (Figure 1C), as measured by CellROX™ Green Reagent fluorescent intensity or extracellular LDH activity, respectively. To validate these findings, we next used a 12-day RA + BDNF differentiation protocol which promotes longer and more complex neurite growth. Similarly, RA + BDNF-differentiated cells treated with PE serum significantly increased neurite growth relative to controls ($t_6 = 2.776$, $p < 0.05$) (Figure 1F), without changes in oxidative stress ($t_6 = 0.028$, $p = 0.978$) (Figure 1G) or cytotoxicity ($t_8 = 1.797$, $p = 0.110$) (Figure 1H).

Lastly, to confirm this phenotype in a more neuronal model, the human neural progenitor cell line ReNcell® VM was differentiated for 7 days by restriction of the growth factors EGF and FGF2, and exposed to maternal serum for the last 3 days *in vitro*. As in SH-SY5Y cells, PE serum increased neurite growth in differentiating ReNcell® VM cells ($t_8 = 3.542$, $p < 0.01$) (Figures 1I, J). Collectively, these data show exposure to PE serum increases neurite growth which is not secondary to any changes in oxidative stress or viability in differentiated human neuroblastoma and human neural progenitor cells.

3.2 PE serum increases the oxygen consumption rate in differentiated SH-SY5Y cells

As PE serum has previously been shown to induce alterations in mitochondrial function in endothelial cells (McCarthy and Kenny, 2016), we next determined whether exposure to PE serum affects mitochondrial function in SH-SY5Y cells. To do this we performed bioenergetic state analysis of the oxygen consumption rate (OCR) in RA-differentiated SH-SY5Y cells treated with 3% (v/v) serum from women with PE or women with healthy uncomplicated pregnancies for 72 h. Cells treated with PE serum had significantly elevated OCR relative to those treated with control serum ($F_{1,96} = 10.01$, $p < 0.01$) (Figure 2A). This equated to significant increases in basal respiration ($U = 710$, $Med_1 = 4.149$, $n_1 = 42$, $Med_2 = 4.985$, $n_2 = 49$, $p < 0.05$), proton leak ($U = 723$, $Med_1 = 0.9654$, $n_1 = 42$, $Med_2 = 1.199$, $n_2 = 49$, $p < 0.05$), non-mitochondrial respiration ($U = 758$, $Med_1 = 2.404$, $n_1 = 42$, $Med_2 = 2.932$, $n_2 = 49$, $p < 0.05$), and ATP synthesis ($U = 727$, $Med_1 = 3.117$, $n_1 = 42$, $Med_2 = 3.901$, $n_2 = 49$, $p < 0.05$) (Figure 2B). This effect was not accompanied by changes in mitochondrial superoxide ($t_5 = 0.3103$, $p = 0.769$) (Supplementary

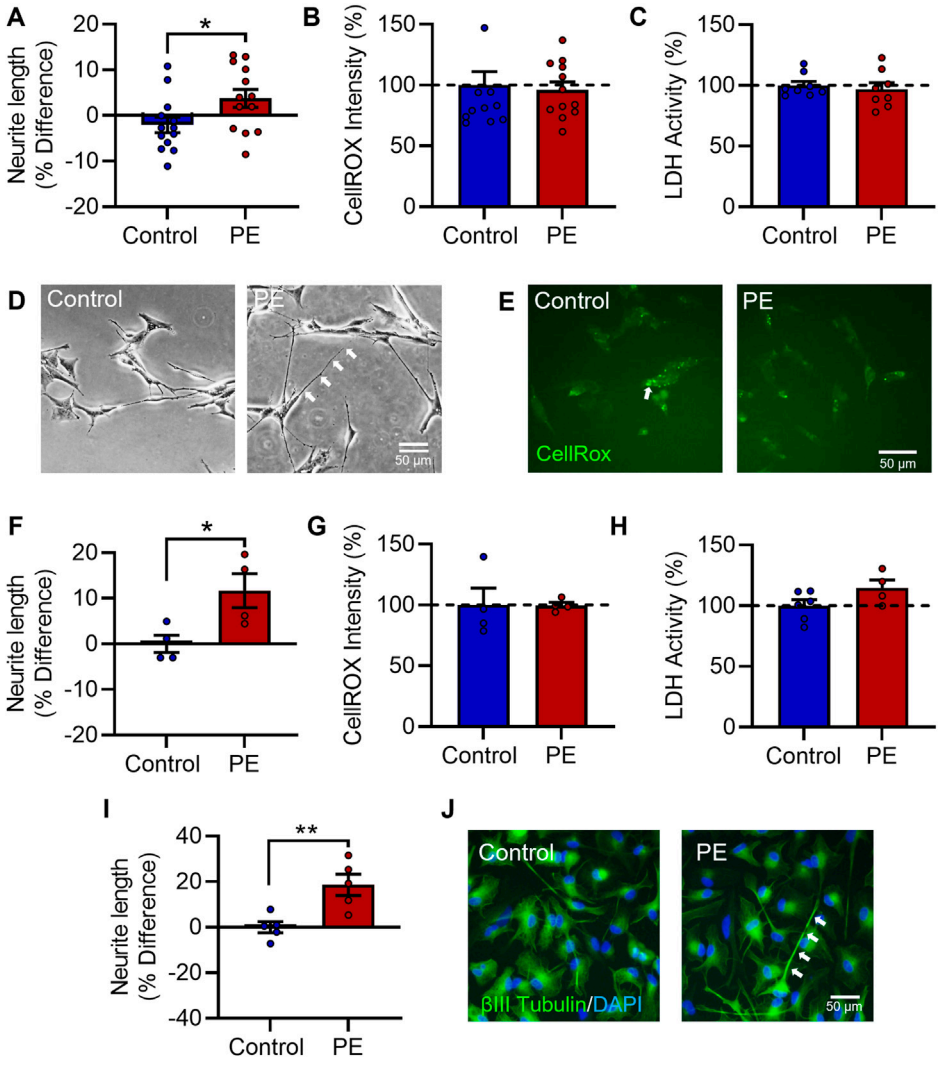

FIGURE 1
Pre-eclampsia serum increases neurite growth in differentiated SH-SY5Y Cells. RA-differentiated SH-SY5Y cells were treated with 3% (v/v) serum from pre-eclamptic patients (PE) or normotensive pregnant controls for 72 h. **(A–C)** Graphs of **(A)** neurite growth, **(B)** CellROX™ Green Reagent fluorescent intensity as a measure of oxidative stress, and **(C)** extracellular LDH activity as a measure of cytotoxicity. **(D,E)** Representative photomicrographs of **(D)** neurite growth, imaged under phase contrast, and **(E)** CellROX™ green reagent fluorescent intensity, 72 h after serum treatment. **(F,G)** SH-SY5Y cells were neuronally differentiated with 10 µM RA daily for 5 days and 50 ng/mL BDNF daily for 7 days, with 3% (v/v) serum from pre-eclamptic patients (PE) or normotensive pregnant controls for the last 3 days *in vitro*, and assessed for **(F)** neurite growth, **(G)** oxidative stress, and **(H)** cytotoxicity. **(I,J)** Graph and representative photomicrographs of neurite growth in serum-exposed ReNcell® VM cells stained for βIII tubulin (green) reactivity and bisbenzimide (blue). Data are mean +SEM from thirteen, twelve, or eight serum samples per group for **(A–C)**, respectively (n = 13; n = 12; n = 8); four per group for F-H (n = 4); and five per group for **(I,J)** (n = 5). Student's unpaired t-test for A, **(F–I)**, Mann-Whitney test for **(B–C)** (* $p < 0.05$, ** $p < 0.01$ vs. control).

Figure S2A), biomass ($t_5 = 1.233$, $p = 0.276$) (Supplementary Figure S2B), or membrane potential ($t_5 = 1.498$, $p = 0.1945$) (Supplementary Figure S2C), measured with the fluorescent mitochondrial dyes MitoSOX™ Red, MitoGreen and MitoTracker™ Deep Red FM, respectively. Taken together these data indicate exposure to maternal PE serum leads to elevations in mitochondrial and non-mitochondrial oxygen consumption.

3.3 Elevated levels of maternal IL-6 in PE

We next sought to gain insight into the molecular basis of increased neurite growth and altered mitochondrial function following exposure to PE serum. Due to previous reports of elevated IL-6 in PE (Aggarwal et al., 2019; Gencheva et al., 2021) and the known effects of IL-6 on neuronal development (März et al., 1997; Kummer et al., 2021; Mirabella et al., 2021), we postulated that IL-6 may be involved in mediating these effects. We therefore examined the levels of IL-6 in maternal sera and placental explant secretions using an immunoassay in a cohort of women with PE and uncomplicated controls. The levels of IL-6 were significantly elevated by 79% in women with PE compared to serum from healthy pregnant women (0.4975 ± 0.0357 pg/mL vs. 0.2775 ± 0.0720 pg/mL, $t_6 = 2.737$, $p < 0.05$) (Figure 3A). Similarly, levels of IL-6 were also elevated in placental explants from women with PE when compared to controls respectively (3,560.7 ± 330 pg/mL vs. 2,178.3 ± 413 pg/mL, $t_8 = 2.614$, $p < 0.05$) (Figure 3B).

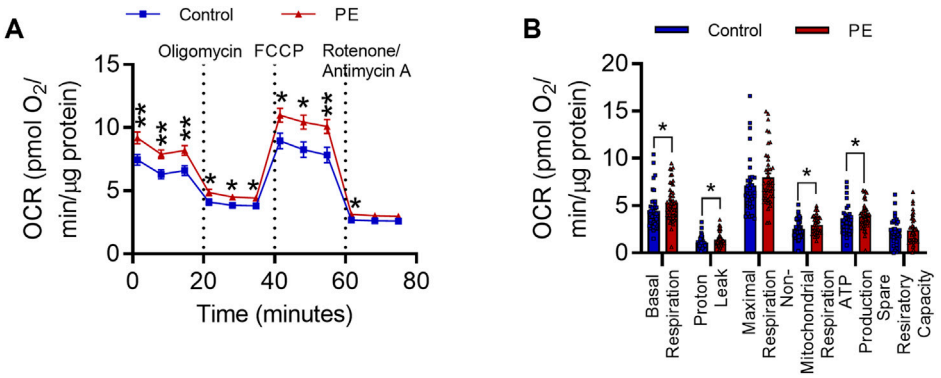

FIGURE 2
Pre-eclampsia serum alters mitochondrial function in differentiated SH-SY5Y Cells. **(A)** Oxygen consumption rate during 80-min Seahorse XF Mito Stress Test. Mean OCR values are normalized to protein content per well. **(B)** Graph representing individual parameters of respiration, calculated from the values plotted in I. Data are mean +SEM from $N = 10$ serum samples for each group with $n = 1–5$ wells per sample for I and J, or, expressed as percentage of the control. [$*p < 0.05$, $**p < 0.01$ vs. control. Mixed effects model and *post-hoc* Fisher's least significant difference (LSD) test for **(A)**, mann-whitney test for **(B)**].

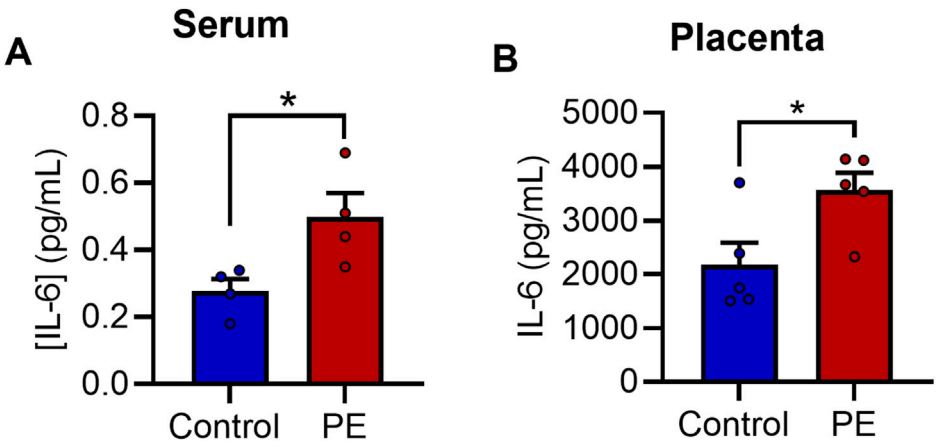

FIGURE 3
IL-6 is elevated in pre-eclampsia serum. Evaluation of [IL-6] in **(A)** maternal serum samples and **(B)** placental explant secretions. Data are mean +SEM from four samples per group for A and five for B ($n = 4–5$). ($*p < 0.05$ vs. control. Student's unpaired *t*-test).

3.4 IL-6 signaling is stimulated in pre-eclampsia serum-treated RA-differentiated SH-SY5Y cells and is required for increased neurite growth

IL-6 activates the JAK/STAT signaling pathway resulting in phosphorylation of the transcription factor signal transducer and activator of transcription 3 (STAT3) at Tyr_{705} (Carpenter and Lo, 2014). To examine whether exposure to PE serum stimulated the IL-6 signaling pathway, RA-differentiated SH-SY5Y cells were treated with 3% (v/v) maternal serum for 24 h and were then assessed for phosphorylation of STAT3 at Tyr_{705} by Western blot. Expression of p-Tyr_{705} STAT3 relative to total STAT3 was significantly increased by 50% in cells treated with PE serum vs. control serum ($t_6 = 2.499$, $p < 0.05$) (Figures 4A, B).

Several studies have identified a role for IL-6-STAT3 signaling in enhancing neurite outgrowth in various neuronal models (März et al., 1997; Bissonnette et al., 2004; Miao et al., 2006; Zhou and Too, 2011; Yang et al., 2012; Leibinger et al., 2013; Su et al., 2020; Kummer et al., 2021; Mirabella et al., 2021). Next, to determine whether IL-6 signaling is necessary for the increased neurite growth caused by PE serum, RA-differentiated SH-SY5Y cells were treated with 3% (v/v) serum for 72 h in the presence of a function-blocking anti-IL-6 antibody (anti-IL-6). Anti-IL-6 attenuated the neuritogenic effects of PE serum—2-way ANOVA revealed a main effect for PE vs. control sera ($F_{1,16} = 5.519$, $p < 0.05$) (Figures 4C, D), while *post-hoc* analyses showed a significant difference specifically between control and PE groups in the absence of anti-IL-6 ($p < 0.05$), but in its presence ($p = 0.252$), suggesting that IL-6 is required for the increased neurite growth seen in cells exposed to PE serum.

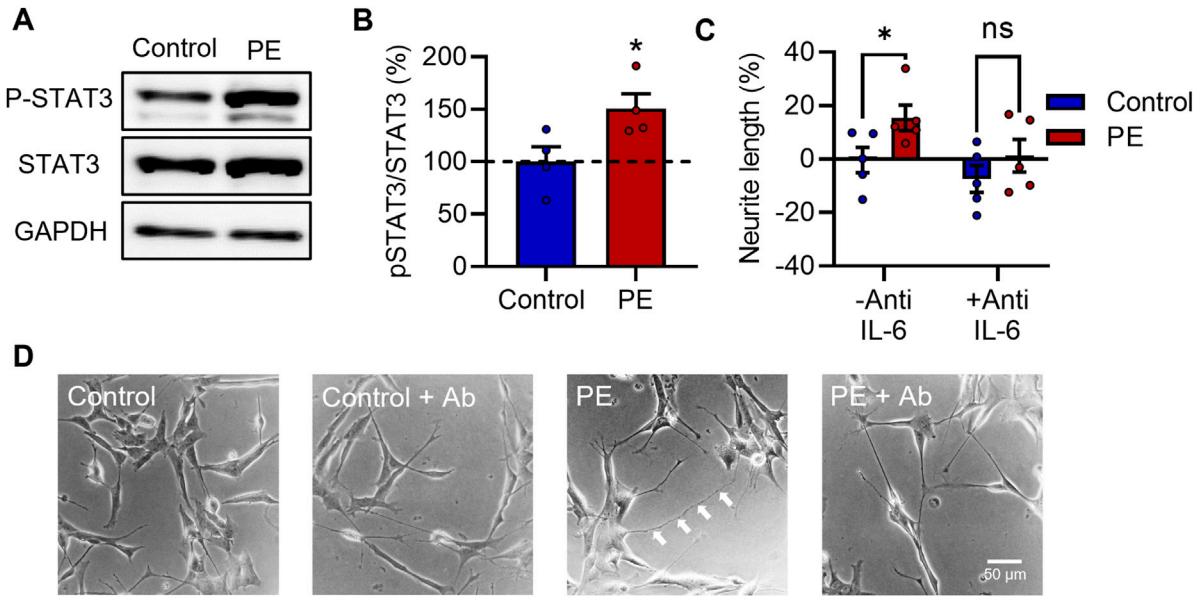

FIGURE 4
IL-6 signaling is stimulated in pre-eclampsia serum-treated RA-differentiated SH-SY5Y cells and is required for increased neurite growth. **(A, B)** Protein expression of p-Tyr$_{705}$ STAT3 relative to total STAT3 in RA-differentiated SH-SY5Y cells treated with 3% (v/v) serum for 24 h. **(C, D)** Graph and representative photomicrographs of neurite growth 72 h after serum treatment with or without an anti-IL-6 function-blocking antibody. Data are mean +SEM from four serum samples per group for B, or five for C (n = 4–5). (*p < 0.05 vs. control. Student's unpaired t-test for B, 2-way ANOVA and *post-hoc* Fisher's least significant difference (LSD) test for **(C)**.

3.5 IL-6 increases neurite growth in differentiated SH-SY5Y cells

To investigate whether IL-6 alone is sufficient to induce the increased neurite growth and elevated OCR seen in PE serum-treated cells, RA-differentiated SH-SY5Y cells were treated with 20 ng/mL IL-6 daily for 72 h. IL-6 treatment increased neurite growth (t_3 = 4.445, p < 0.05) (Figures 5A, D), did not affect oxidative stress (t_3 = 0.3762, p = 0.732) (Figures 5B, E) and decreased cytotoxic cell membrane damage (t_3 = 43.897, p < 0.05) (Figure 5C) after 72 h. To validate these findings in the more differentiated model, SH-SY5Y cells differentiated according to the 12-day RA/BDNF paradigm were treated with 20 ng/mL IL-6 daily for the last 3 days of differentiation. IL-6 treatment in this differentiation paradigm similarly increased neurite growth, although this was not statistically significant (t_3 = 1.643, p = 0.1989) (Figure 5F), and did not affect oxidative stress (t_3 = 0.4987, p = 0.652) (Figure 5G) or cytotoxicity (t_3 = 1.422, p = 0.250) (Figure 5H). IL-6-induced neurite growth was completely prevented by anti-IL-6, with a significant main effect for IL-6 ($F_{1,4}$ = 34.08, p < 0.01) (Figures 5I, J); *post-hoc* analyses showed IL-6 increased neurite growth in the absence of anti-IL-6 (p < 0.01), but not in its presence (p = 0.750). Collectively these data show that elevations in maternal IL-6 in PE (Figure 6). Likely mediates the neurite growth promoting effects of maternal PE serum on neurite growth in SH-SY5Y cells.

4 Discussion

Pre-eclampsia is a hypertensive disorder of pregnancy which is associated with an increased risk of neurodevelopmental disorders in affected offspring, although the mechanisms involved in this association are largely unknown. This study sought to characterize the effects of serum from women with pre-eclampsia on neuronal development at the single-cell level using neuronally-differentiated SH-SY5Y cells.

Before commencing human sera experiments, we initially validated the model of RA-induced neuronal differentiation by assessing the effects of RA on SH-SY5Y cells. RA treatment has previously been shown to increase protein expression of the neuronal markers MAP2, NeuN, and NSE (Lopes et al., 2010; Schneider et al., 2011), and here we observed significantly increased expression of the marker βIII tubulin. Similarly, the RA-induced elongation of neurites seen here is in line with previous reports (López-Carballo et al., 2002; Lopes et al., 2010; Teppola et al., 2016). RA-treated cells also exhibited a reduced capacity to migrate, which has been observed in a related SK-N-SH neuroblastoma cell line (Messi et al., 2008), and is demonstrative of a functional loss of neuroblastoma phenotype. Overall, these results provide evidence that cells exposed to RA are differentiating towards a neuronal phenotype. In all subsequent experiments, SH-SY5Y cells were differentiated either with RA for 72 h, or more prominently differentiated with RA and BDNF for 12 days.

Differentiated SH-SY5Y cells were then exposed to serum from women either with PE or a healthy uncomplicated pregnancy for 72 h. When compared to control serum-treated cells, those exposed to PE serum exhibited increased neurite growth and elevated mitochondrial function. This increased neurite growth is in line with observations from the one other study that performed a similar experiment in rat primary cortical neurons (Curran et al., 2018), demonstrating that the neurite growth induced by PE serum is conserved across *in vitro* models. The effect on OCR, however, is in contrast to that seen in

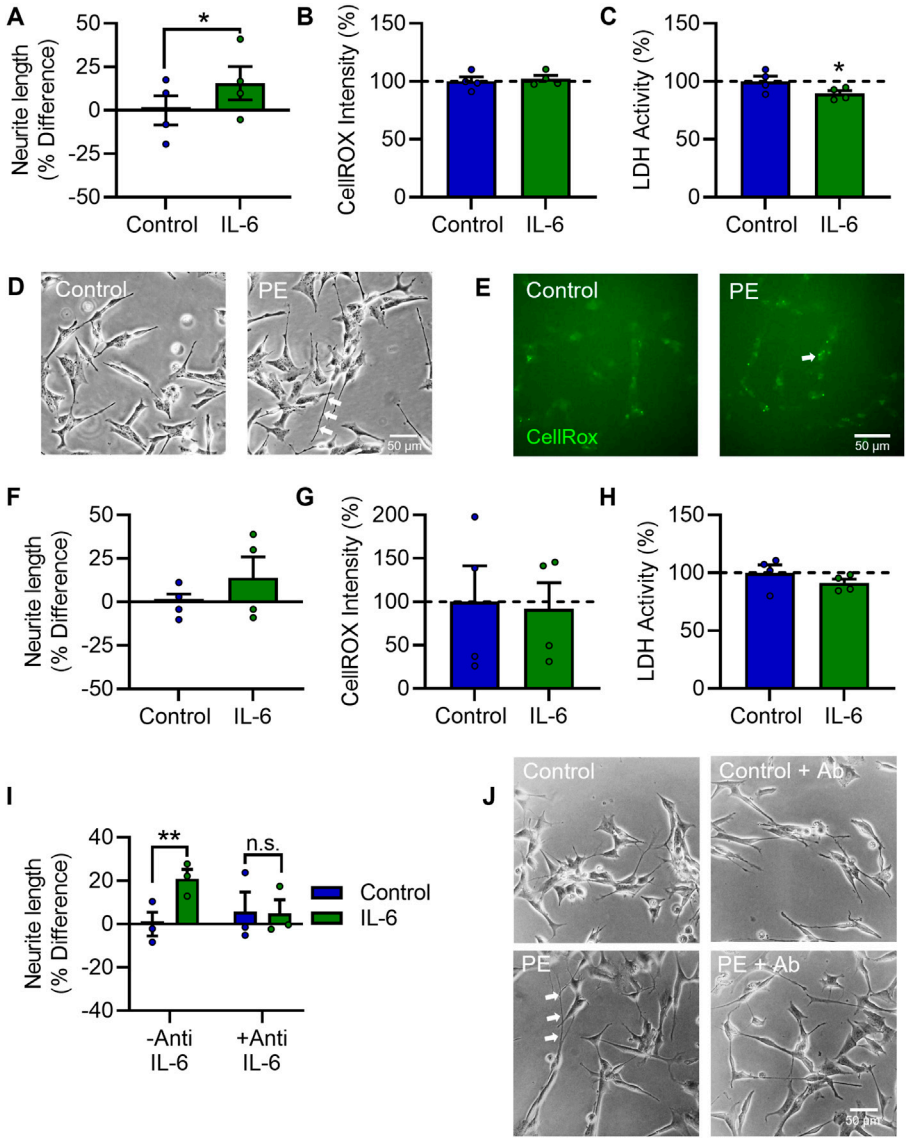

FIGURE 5
IL-6 increases neurite growth and enhances mitochondrial activity in differentiated SH-SY5Y Cells. RA-differentiated SH-SY5Y cells were treated with 20 ng/mL IL-6 daily for 72 h. **(A–C)** Graphs of **(A)** neurite growth, **(B)** CellROX™ Green Reagent fluorescent intensity as a measure of oxidative stress, and **(C)** extracellular LDH activity as a measure of cytotoxicity. **(D, E)** Representative photomicrographs of **(D)** neurite growth, imaged under phase contrast, and **(E)** CellROX™ Green Reagent fluorescent intensity, 72 h after serum treatment. **(F–H)** Graphs of **(F)** neurite growth, **(G)** oxidative stress, and **(H)** cytotoxicity in cells differentiated with 10 µM RA daily for 5 days and 50 ng/mL BDNF daily for 7 days, with or without 20 ng/mL IL-6, added daily for the last 3 days *in vitro*. **(I,J)** Graph and representative photomicrographs of neurite growth 72 h after IL-6 treatment with or without an anti-IL-6 function-blocking antibody. Data are mean +SEM from four independent experiments for **(A–C)**, **(F–H)**, or three for I (n = 3–4), expressed as percentage of the control. (*p < 0.05; **p < 0.01 vs. control. Student's paired t-test for A–C, F–H, 2-way ANOVA and *post-hoc* fisher's least significant difference test (LSD) for .I.

human umbilical vein endothelial cells, where OCR was decreased following exposure to PE serum (McCarthy and Kenny, 2016). This illustrates how the effects of PE serum may be target cell-type specific, which is perhaps unsurprising considering that serum is a complex milieu of various ligands and that each cell type expresses a distinct pattern of receptors. However, these results suggest the presence of circulating maternal factors in PE which can directly affect neuronal development and the metabolism of neuronal-like cells differently to circulating factors of a healthy pregnancy.

PE is often accompanied by intra-uterine growth restriction (IUGR), which may obscure the relationship between PE and fetal brain development. However, a number of studies have demonstrated that, when restricting the study population to IUGR-exposed offspring, stratifying the data into average- or small-for-gestational-age offspring, or using mediation analyses, that PE still exerts an independent influence on the risk for neurodevelopmental disorders, due to some specific physiological feature (s) of PE (Many et al., 2003; Morsing and Maršál, 2014; Lahti-Pulkkinen et al., 2020; Basso et al., 2022). Importantly, none of the subjects in the control or PE group in the current study experienced IUGR, which could otherwise have confounded the interpretation of our results. Thus, any molecular factors observed to play a mechanistic role are likely to be due to pathophysiological changes intrinsic to PE, and not secondary to comorbid IUGR.

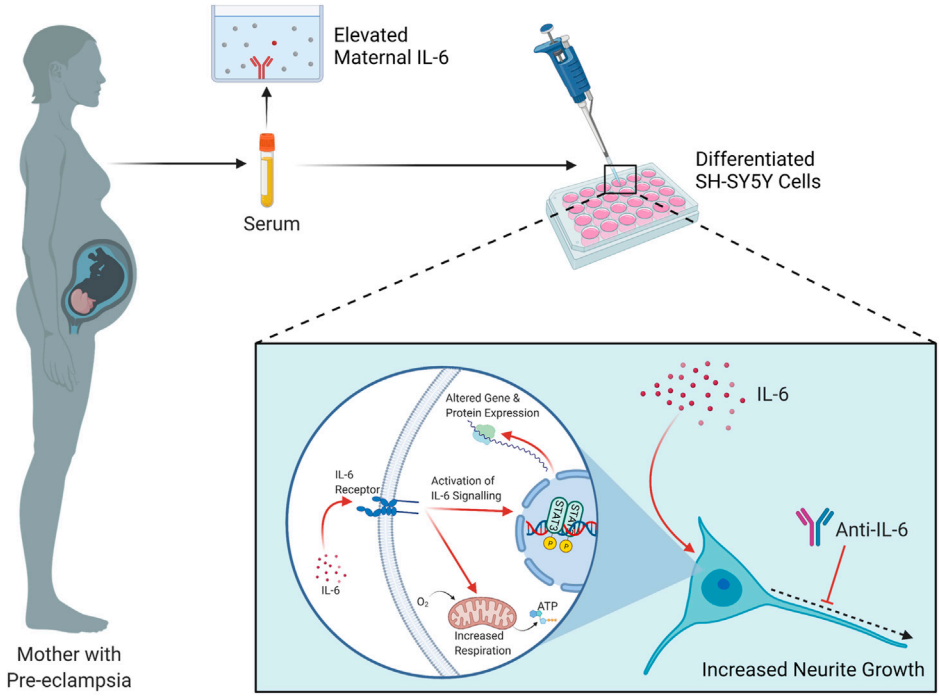

FIGURE 6
Summary of findings and proposed mechanism. Elevated IL-6 is detected in the circulation of pregnant women with Pre-eclampsia, compared to healthy pregnant controls. When applied to RA- or RA/BDNF-differentiated SH-SY5Y cells, this serum induces increases in neurite growth and mitochondrial oxygen consumption. It is proposed that the elevated IL-6 in maternal serum in PE activates the IL-6 signaling pathway in these cells, terminating in the phosphorylation and consequent induction of the transcription factor STAT3 which alters the gene expression profile of the cell, contributing in part to this respiratory and neurite growth increase phenotype. Schematic created with Biorender.com.

Considering IL-6/STAT3 signaling is known to have the capacity to modulate neurite growth and mitochondrial activity, we then investigated levels of IL-6 in patient sera and this was found to be elevated in PE, in agreement with previous reports in women with PE from other cohorts (Sharma et al., 2007; Aggarwal et al., 2019; Gencheva et al., 2021). Thus, it was of interest whether the IL-6 signaling pathway, which culminates in phospho-activation of STAT3, is stimulated in differentiated SH-SY5Y cells exposed to PE serum. Phosphorylation of STAT3 at Tyr_{705} was significantly higher in cells exposed to PE serum relative to control serum, which suggests increased activity of the IL-6 signaling pathway in these cells following exposure to PE serum. IL-6-STAT3 signalling activates several target genes that regulate cell survival and apoptosis, proliferation and differentiation, inflammation, as well as mitochondrial-associated genes (Carpenter and Lo, 2014), all of which can significantly affect neuronal development. Thus, developing neurons in the brain of offspring exposed to elevated maternal IL-6 in the context of PE may be driven towards an altered pattern of gene and protein expression, ultimately influencing their neurite growth, respiration, and developmental trajectory. Importantly, the increased neurite growth of differentiated SH-SY5Y cells was attenuated by IL-6 neutralization, demonstrating that IL-6 is *necessary* for this effect.

Differentiated SH-SY5Y cells were next treated with IL-6 for 72 h, and this induced a similar effect to PE serum. IL-6 treatment increased both neurite growth, a phenotype comparable to the difference between cells exposed to PE vs. control serum. These effects agree with previous studies from different neuronal models wherein neurite growth and mitochondrial activity were increased by IL-6 and/or STAT3 activity (März et al., 1997; Miao et al., 2006; Zhou and Too, 2011; Yang et al., 2012; Leibinger et al., 2013; Luo et al., 2016; Yang and Rincon, 2016; Su et al., 2020). Thus, IL-6 alone is also *sufficient* to augment neurite growth and mitochondrial respiration in differentiated SH-SY5Y cells. While we have shown here that PE serum, *via* IL-6, increases neurite growth and mitochondrial respiration, it is still unclear whether these are independent effects, or whether the increase in neurite growth is driving the elevated oxygen consumption due to an increased demand for ATP.

While the current study has shown that elevated IL-6 in PE increases neurite growth and mitochondrial respiration, it is likely to be one among several biomolecules altered in PE that can affect neurodevelopmental processes. The foetal brain is likely to be exposed to altered levels of various proteins, lipids, metabolites, microRNAs and other compounds in the context of PE, many of which could influence the developing brain, and this will be important to investigate in future work. Perhaps the most well-characterised molecular change in PE is an increase in placental-derived sFlt-1, which could impair feto-placental angiogenesis and the development of the fetal neurovascular unit (Torres-Vergara et al., 2022; Vogtmann et al., 2022), but whether sFlt-1 in PE directly affects neuronal development, as we shown here for IL-6, is less well known.

The approach described in this study of exposing cells to PE maternal serum as they develop neurites has allowed us to probe the cellular and molecular mechanisms of the consequences of PE exposure on developing neural cells. A significant strength of this work is the use of human sera, as circulating factors in animal or cell

models of PE may differ from the serum profile of women with idiopathic pre-eclampsia. Significant strength is added to this study by the fact that the main result–increased neurite growth caused by PE serum–was replicated in differentiating human neural progenitor cells. Despite these advantages, there are however limitations and opportunities for future development of this work. Firstly, there are always inherent difficulties in extrapolating results from *in vitro* models to whole systems and processes like human neurodevelopment, albeit our aim was to study effects on single cells. Secondly, as we have shown that factors within maternal serum in PE can affect the parameters we investigated in this study, in future work it will be of equal interest to characterize the effects of PE placental secretions on neuron development. Additionally, there is one important question regarding the role of IL-6 signaling in this study–although the concentration of IL-6 is substantially higher in PE than control serum, it is still considerably lower than the concentration of recombinant IL-6 required to elicit the response in differentiated SH-SY5Y cells. There are a number of explanations for this, such as that there are other ligands elevated in the PE serum, such as IL-10 or IL-11, which also activate STAT3 signaling; that there are other circulating factors that sensitize the cells to the effects of IL-6; that other factors, acting through independent mechanisms have cumulative small effects that are only detectable when combined; or that IL-6 in the serum is acting partially through *trans*-signaling, an alternative and potent mechanism that involves binding of IL-6 to a soluble form of the IL-6 receptor (sIL-6Rα), which is absent when treating with IL-6 alone (Garbers et al., 2018). However, the key point remains: exposure to maternal PE serum elevates pSTAT3 signaling and changes neural cellular function, in an IL-6-dependent mechanism.

Overall, this study has shown that there are circulating factors in the serum of women with PE that increase neurite growth and mitochondrial respiration, two important neurodevelopmental parameters, in differentiated SH-SY5Y cells; that IL-6 is elevated in their sera and placenta, that this induces STAT3 phosphorylation in these cells; and that IL-6 alone is both necessary and sufficient for this phenomenon. We therefore propose that the elevated IL-6 is responsible, at least partially, for these effects (Figure 6). This may have important implications for our understanding of the physiological relationship between pre-eclampsia and neurodevelopment *in vivo*, considering IL-6 is able to permeate both the human placenta and the blood-brain barrier (Zaretsky et al., 2004; Banks, 2005), and IL-6 is correspondingly elevated in the circulation of human neonates born to pre-eclamptic pregnancies (Tosun et al., 2010) and the brains of rat pups exposed to a pre-clinical model of PE (Giambrone et al., 2019). This suggests IL-6 as a potential pathway for early therapeutic intervention, not to prevent the progression of PE in the mother, but to attenuate its deleterious effects on the fetal brain, although further preclinical and clinical studies will be required to discern this. These data provide important insights into our understanding of the consequences of pre-eclampsia exposure and its effects on neurodevelopmental processes which may influence neurodevelopmental trajectories in exposed offspring.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Clinical Research Ethics Committee of the Cork Teaching Hospitals [ECM4 (ff) 04/12/18]. The patients/participants provided their written informed consent to participate in this study.

Author contributions

AB performed all experiments and data analysis described above, except where otherwise indicated herein, and wrote the first draft of the manuscript. SM, CJM, and FM carried out patient recruitment, and sample collection and preparation, and SM also performed IL-6 quantification in serum samples. AM performed flow cytometric detection and analysis of mitochondrial dyes. GO'K and CMM supervised and designed the study and made significant contributions to the manuscript. All authors edited the final manuscript.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.1043481/full#supplementary-material

SUPPLEMENTARY FIGURE S1
RA Promotes Neuronal Differentiation in SH-SY5Y Cells. SH-SY5Y cells were treated with 10 μM RA daily for 72 h. βIII Tubulin protein expression measured by immunoblotting relative to GAPDH expression. **(C)** Photomicrographs of SH-SY5Y cells stained for βIII Tubulin by immunocytochemistry, with or without RA. **(D)** Representative photomicrographs and **(E)** graph of neurite growth following 72 h treatment with RA. Cells are stained with the vital cell dye Calcein-AM. **(F)** Graph and **(G)** representative photomicrographs of cell migration measured by wound width at 24 h, 48 h, and 72 h post-scratch relative to initial wound width. Data are mean + SEM from four independent experiments (n = 4) for B and F, or eight independent experiments (n = 8) for E,

all expressed as percentage of the control. [*$p < 0.05$; **$p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ vs. control. One-way ANOVA and *post-hoc* Dunnett's multiple comparisons test for B; Student's unpaired *t*-test for E; two-way ANOVA and *post-hoc* Fisher's least significant difference (LSD) for F].

SUPPLEMENTARY FIGURE S2
Pre-eclampsia serum does not affect mitochondrial superoxide, biomass or membrane potential in differentiated SH-SY5Y Cells. RA-differentiated SH-SY5Y cells were treated with 3% (v/v) serum from pre-eclamptic patients (PE) or normotensive pregnant controls for 72 h Mean fluorescence intensity of **(A)** MitoSOX™ Red, **(B)** MitoGreen, or **(C)** MitoTracker™ Red FM, as measures of mitochondrial superoxide, biomass and membrane potential, respectively. Data are mean + SEM from four serum samples per group for A-C ($n = 4$), expressed as percentage of the control. (Student's unpaired *t*-test).

SUPPLEMENTARY FIGURE S3
Comparison of ReNcell® VM neural progenitor cells at day 1 vs. day 7 *in vitro*. ReNcell® VM neural progenitor cells were differentiated for 7 days by removal of EGF and FGF2 from the culture medium. Blue = DAPI, green = βIII tubulin, red = GFAP, PhC = phase contrast microscopy.

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Setting a stage: Inflammation during preeclampsia and postpartum

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Preeclampsia (PE) is a leading cause of maternal and fetal mortality worldwide. The immune system plays a critical role in normal pregnancy progression; however, inappropriate inflammatory responses have been consistently linked with PE pathophysiology. This inflammatory phenotype consists of activation of the innate immune system, adaptive immune system, and increased inflammatory mediators in circulation. Moreover, recent studies have shown that the inflammatory profile seen in PE persists into the postpartum period. This manuscript aims to highlight recent advances in research relating to inflammation in PE as well as the inflammation that persists postpartum in women after a PE pregnancy. With the advent of the COVID-19 pandemic, there has been an increase in obstetric disorders associated with COVID-19 infection during pregnancy. This manuscript also aims to shed light on the relationship between COVID-19 infection during pregnancy and the increased incidence of PE in these women.

KEYWORDS

preecclampsia, immune cells, chronic inflammation, pregnancy, postpartum

Introduction

Preeclampsia (PE) is a multisystem obstetric disorder that presents as new onset of hypertension in conjunction with evidence of end-organ dysfunction beyond the 20th week of gestation (ACOG, 2022). PE is a leading cause of maternal-perinatal morbidity and mortality each year and occurs in about 7% of pregnancies (English et al., 2015). In conjunction with hypertension, PE patients may present with proteinuria, HELLP (Hemolysis, Elevated Liver enzymes, and Low Platelets), or visual disturbances (Roos et al., 2012; ACOG, 2022). The only cure for PE is delivery of the fetal-placental unit, making PE a leading cause of premature birth (Staff et al., 2022). Moreover, the offspring of PE pregnancies are at higher risks for stillbirth, FGR (Fetal Growth Restriction), and additional neonatal complications (Fox et al., 2019). Therefore, the management of PE pregnancies is a delicate balance between maternal and fetal health, which could be attributed to the lack of any major developments or changes in treatments for PE in the last 50 years (Bell, 2010).

PE is a state of chronic inflammation with activation of antigen presenting cells (APC's), T helper (Th) cells, B cells, and Natural killer cells which contribute to the presentation of PE symptoms during pregnancy (LaMarca et al., 2016; Aneman et al., 2020). Th cells secrete cytokines that activate innate immune cells, induce production of anti-angiogenic factors, and increase sodium conductance in the nephron (LaMarca et al., 2016). B cells produce agonistic antibodies against the angiotensin II type 1 receptor (AT1-AA) which activate the

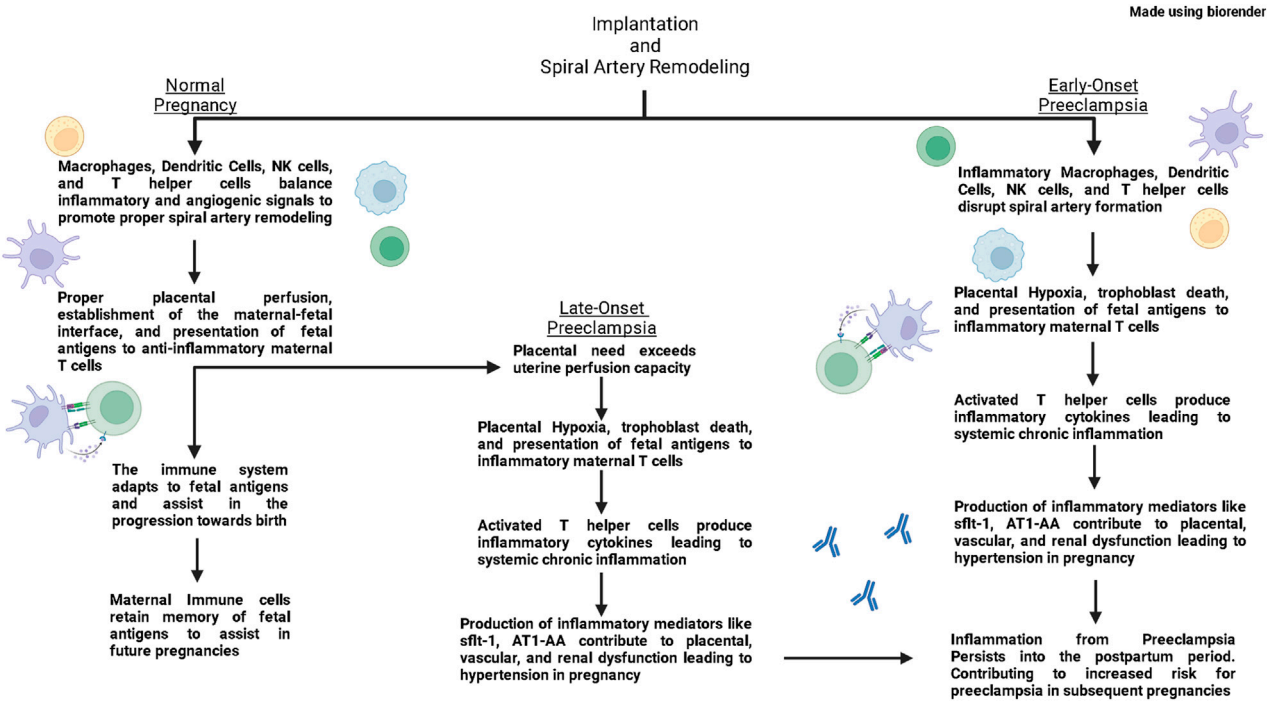

FIGURE 1
In normal pregnancies, macrophages, dendritic cells, Natural Killer (NK) cells, and T helper (Th) cells produce both inflammatory and anti-inflammatory signals that support vascular remodeling and promote spiral artery remodeling. As pregnancy progresses, antigen presenting cells present fetal antigens to Th cells and polarize them towards a regulatory Th cell phenotype (Tregs). These Tregs then quell immune responses in the placenta and maintain an anti-inflammatory environment through the second trimester. As a normal pregnancy reaches its end, maternal immune cells produce inflammatory signals that help signal the uterus for delivery. In the postpartum period, maternal immune cells retain memory of fetal antigens to help in future pregnancies. In early-onset preeclampsia, inflammatory macrophages, dendritic cells, NK cells, and Th cells disrupt spiral artery formation leading to placental hypoxia. Hypoxia leads to cell death which leads to placental dysfunction and release of fetal antigens. Antigen Presenting Cells present these fetal antigens and polarize Th cells toward an inflammatory phenotype. Inflammatory Th cells then induce production of inflammatory and anti-angiogenic factors from the placenta and other immune cells which lead to placental, vascular, and renal dysfunction contributing to hypertension in pregnancy. These inflammatory cells from preeclampsia retain memory postpartum, and increase the risk for developing PE in subsequent pregnancies. In late-onset preeclampsia, the spiral artery formation occurs normally, however eventually the needs of the placenta out-weigh the perfusion capacity of the uterus, leading to placental hypoxia and a similar inflammatory environment as seen in early-onset preeclampsia.

Renin-Angiotensin system and activate killer cells to target AT1-AA marked cells (Campbell et al., 2018). Moreover, innate immune cells contribute to the inflammatory cytokine milieu while contributing to tissue damage in the kidney and placenta (Aneman et al., 2020). Over the COVID-19 pandemic, there has been an increase in the incidence of PE in women that contracted COVID-19 during their pregnancy (Jamieson and Rasmussen, 2022). COVID-19 infection during pregnancy leads to an immune reaction in the decidua; however, this immune reaction appears to change depending on the trimester in which the COVID-19 infection occurs (Juttukonda et al., 2022). Furthermore, studies in women that contract COVID-19 during pregnancy have shown that there are increased markers of placental dysfunction or placental damage (Jaiswal et al., 2021; Schwartz et al., 2022). Inflammation and placental damage following COVID-19 infection could be contributing factors toward the increased incidence of PE in women with a history of COVID-19 infection during their pregnancies (Villar et al., 2021).

While symptoms of PE usually end upon delivery of the placenta, the inflammation persists into the mother's postpartum period. Research has demonstrated that patients have an increased risk of recurrent PE, hypertension, coronary heart disease and stroke following a PE pregnancy (Wu et al., 2017; Opichka et al., 2021), thereby illustrating the long-term cardiovascular complications caused by PE. Furthermore, the immune system and inflammation has been repeatedly implicated in the pathophysiology of cardiovascular disease during pregnancy and outside of pregnancy (Harrison et al., 2021; Stefanska et al., 2021). Therefore, this review aims to provide insight into the potential role of immune cells and immune mediators in contributing to PE and cardiovascular disease postpartum of PE. This review aims to highlight clinical studies that have elucidated contributions of the immune system in physiological pregnancy and PE; while also presenting basic science studies that have provided mechanistic insight into how immune mediators impact pregnancy (Figure 1).

Placental ischemia—The initiating event in preeclampsia pathophysiology

During normal pregnancies, fetal trophoblasts invade the maternal myometrium with the aid of uterine immune cells (Staff

et al., 2022). Trophoblasts and immune cells secrete proteases and angiogenic factors to progressively remodel uterine spiral arteries into high-capacity, low-resistance vessels (Albrecht and Pepe, 2020). In PE pregnancies, there is a breakdown in placental perfusion which ultimately results in placental ischemia. Placental ischemia induces the production of inflammatory modulators and anti-angiogenic factors (Zhang, 2018) which contribute to vascular dysfunction in the placenta and peripheral vessels. Furthermore, these circulating placental factors cause endothelial dysfunction and oxidative stress which contribute to hypertension and multi-organ dysfunction as seen in PE (Rana et al., 2019).

PE presents as systolic pressure ≥140 mmHg or diastolic pressure ≥90 mmHg with an accompanying organ dysfunction including elevated 24-h urine protein value of ≥300 mg, urine protein to creatine ratio of ≥0.3, thrombocytopenia, impaired liver function, pulmonary edema, or new-onset headache with or without visual disturbances (Karrar and Hong, 2022). PE can be stratified by the timing of symptom presentation. While these categories of PE are unified by placental ischemia, they are believed to stem from different etiologies. PE that develops prior to 34 weeks of gestation is considered early-onset (EO) PE, PE that develops after 34 weeks of gestation is considered late-onset (LO) PE, and while PE that develops after delivery of the fetal-placental unit is considered postpartum PE (PP-PE) (Raymond and Peterson, 2011; Hauspurg and Jeyabalan, 2022). EOPE is characterized by insufficient invasion of trophoblasts into the endometrium resulting in poor spiral artery remodeling leading to placental ischemia and PE phenotype (Tamas et al., 2022). EOPE is considered the most severe form of PE and causes the highest rates of morbidity and mortality in mother and neonate compared of the PE subsets (Madazli et al., 2014). EOPE mothers are the most likely to have placental pathologies and higher PE recurrence rates compared to LOPE mothers (Li et al., 2014; Fillion et al., 2021). Mothers with both placental pathologies (syncytial knots, placental infarcts, etc.) and severe PE symptoms are more likely to have strokes or develop cardiovascular disease postpartum (Veerbeek et al., 2015). LOPE is associated with normal placentation in early pregnancy, with placental dysfunction occurring later in pregnancy. LOPE is projected to occur when the needs of the placenta are above the perfusion capacity of the uterus leading to placental ischemia and the symptoms of PE (Staff et al., 2022). Even though LOPE is less associated with neonatal morbidity (Staff et al., 2016; Wadhwani et al., 2020), LOPE still predisposes mothers and offspring to develop cardiovascular disease later in life. Moreover, children born of LOPE pregnancies are less likely to be growth restricted. However, they still have increased incidence of cardiovascular disease and stroke in later life compared to children born of normal pregnancies (Kajantie et al., 2009; Touwslager et al., 2012), thereby suggesting that cardiovascular disease in PE offspring is caused by more than just FGR. LOPE is often associated with increased maternal BMI, high weight gain during pregnancy, and other metabolic disorders (Robillard et al., 2019; Robillard et al., 2022). Obesity and metabolic disorders are considered states of chronic inflammation (Esposito and Giugliano, 2004; Khanna et al., 2022), and these metabolic and inflammatory signals may contribute to the increase risk of cardiovascular disease in offspring of women with LOPE. Lastly, there are rare cases where patients develop a PE phenotype after delivery, which is known as PP-PE. Patients with PP-PE have similar placental vasculopathies that resemble LOPE by demonstrating a similar role for placenta dysfunction (Ditisheim et al., 2020; Hauspurg and Jeyabalan, 2022). But, more studies are needed to elucidate the mechanisms that induce postpartum PE.

Dynamic shifts in the immune system during normal pregnancy

Implantation and the first trimester of pregnancy

In a normal pregnancy, the maternal immune system must balance immune tolerance of the semi-allogenic fetus with defending the mother from disease. However, disruption of this balance has been associated with developing PE. During pregnancy, women undergo time-dependent shifts in activation of the innate and adaptive immune systems, but dysregulation of the immune system can cause adverse outcomes in both the mother and fetus (Cornelius et al., 2019). In the first trimester, macrophages (Mφ) and Natural Killer (NK) cells are two of the most prevalent cells in the uterus (Jena et al., 2019). Uterine subsets of both NK (uNK) cells Mφ (uMφ) are pivotal in uterine remodeling during pregnancy (Gustafsson et al., 2008; Jetten et al., 2014) (Bazhenov et al., 2019). The uNK cell population have high concentrations of intracellular killer granules, but do not release cytolytic granules and are not efficient in cell killing compared to conventional NK cells (Faas and de Vos, 2017). uNK cells have higher expression of Killer cell Immunoglobulin-like Receptors than conventional NK cells; however, uNK cells lack FcγRIII (CD16), which may contribute to their reduced killing capacity in normal pregnancies (Koopman et al., 2003). CD16 recognizes the FC region of IgG antibodies and is responsible for antibody induced cellular cytotoxicity (Yeap et al., 2017). uMφ are unique compared to peripheral macrophages (Houser et al., 2011); however, uMφ resemble alternatively activated M2s phenotype which produces lower levels of the inflammatory IL-1 while producing higher levels of the anti-inflammatory IL-10 compared to inflammatory M1s (Mizuno et al., 1994; Gough et al., 2001). These uMφ are also important in anti-microbial defense in the uterus and clearing cellular debris during pregnancy (Thiruchelvam et al., 2013).

During embryo implantation, the uterine immune cells help to induce vascular remodeling while initiating processes to promote fetal tolerance. uNK cells produce copious amounts of interferon gamma (IFN-γ) which destabilizes vascular endothelial cells and initiates uterine artery remodeling (Ashkar et al., 2000). This uNK cell derived IFN-γ also activates uMφs which allow them to assist in vascular remodeling. These uNK cells and Mφs migrate and localize to fetal trophoblasts (Helige et al., 2014). The uNK cells and Mφs then produce proteases to destabilize vascular smooth muscle (Naruse et al., 2009; Smith et al., 2009) and angiogenic factors (Ferrante et al., 2013; El-Azzamy et al., 2018) that promote growth of new blood vessels and maturation of existing blood vessels. During this critical stage of implantation, uterine stromal cells and uterine dendritic cells (uDCs) work to prevent excessive vascularization during implantation. Uterine stromal cells are also important cytokine producers and cross-talk with uDCs during pregnancy. Uterine stromal cells produce granulocyte-colony stimulating factor

(G-CSF) which appears to increase IL-1β from uDCs (Shao et al., 2020). In the short term, this may contribute to uDCs producing soluble Flt1, which binds soluble vascular endothelial growth factor (VEGF) and serves as a control for angiogenesis during pregnancy (Plaks et al., 2008).

IFN-γ from uNK cells also induces uMφ′s to express HLA-II, which is an important in inducing T helper (Th) cell activation (Xie et al., 2005). T helper (Th) cells are CD4+ T cells that are crucial for both directing immune responses and helping to orchestrate transitions in pregnancy. Th1 cells are characterized by the expression of the transcription factor Tbet, and help with uterine remodeling during the first trimester (Faas and De Vos, 2018). Th1s and uNK cells also produce Tumor Necrosis Factor (TNF)-α which prevents excessive invasion of trophoblasts into the uterus through activating the NF-κB pathway (Todt et al., 1996; Torchinsky et al., 2003). Inhibiting IFN-γ in early pregnancy prevents successful uterine artery remodeling therefore causing fetal demise (Ashkar et al., 2000), suggesting roles for Th1s and uNK cells to promote vascular remodeling in pregnancy through IFN-γ production. Overall, uNK cells, uMφs, uDCs, and Th1s cells carefully collaborate to promote the formation of the maternal-fetal interface during the first trimester. Moreover, inflammatory and anti-inflammatory mechanisms are important during implantation in the first trimester.

Second trimester of pregnancy

The transition from early to mid-pregnancy is accompanied by a shift from a pro-inflammatory to an anti-inflammatory uterine environment. DCs are regulate vascularization during the first trimester however DCs are also professional APC's that internalize antigens and present them on major histocompatibility complex-II in order to activate Th cells. In pregnancy, DCs help transition from the early pregnancy pro-inflammatory Th1 environment to the middle pregnancy anti-inflammatory Th2 environment. Cross-talk between uterine stromal cells and uDCs eventually promotes uDCs to express co-stimulators of Th cell activation CD80 and CD86 (Shao et al., 2020). Moreover, these uDCs polarize Th cells towards a Th2 phenotype *in vitro*. Negishi et al. (2012) found that DCs that express Dendritic Cell Inhibitory Receptor 2 (33D1+) DCs slowly increase in number during the first trimester and these 33D1+ DCs promote the switch from Th1s to Th2s in middle pregnancy. Further supporting a role for uDCs to induce the pivot from the inflammatory environment needed for implantation towards the anti-inflammatory Th2 environment needed for pregnancy tolerance. Mφs are another important APC that help with clearing cellular debris and help polarize Th cells towards the appropriate Th subtype to promote fetal tolerance in pregnancy. Specifically, T cell immunoglobulin mucin-3 (Tim-3$^+$) uMφ help polarize Th cells towards anti-inflammatory Th2 and Treg phenotypes (Li et al., 2022). Tim-3$^+$ blockade lead to decreased fetal viability and fetal growth restriction, further implicating Mφ in promoting fetal health during pregnancy. Together these studies show that uDCs and uMφ help to promote an anti-inflammatory environment in the transition towards the second trimester of pregnancy.

Th2 cells begin to increase near the end of the first trimester and continue to predominate over Th1 cells through the rest of the pregnancy (Saito et al., 1999). Th2 cells are producers of the cytokine IL-4, which prevents Th1 cells proliferation and promotes a Th2 profile in normal pregnancies (Lazarski et al., 2013). IL-4 also inhibits IFN-γ production, which reduces NK cell activation and promotes fetal tolerance (Zissler et al., 2016). Th2 cells also promote B cells to produce protective "asymmetric" antibodies during pregnancy (Zenclussen et al., 2001). These asymmetric antibodies bind fetal antigens on the placenta, but do not activate killer cells. Asymmetric antibodies effectively block the fetal antigens from being found by the maternal immune system. Therefore asymmetric antibodies protect paternal antigens and the fetus from killer immune cells while promoting immune tolerance in pregnancy (Gutierrez et al., 2005).

In normal pregnancy, paternal antigen-specific Treg cells expand in circulation and in the uterus to promote fetal tolerance (Kahn and Baltimore, 2010; Shima et al., 2015; Huang et al., 2020). Tregs are known as important producers of IL-10 during pregnancy (Cheng and Sharma, 2015). IL-10 reduces antigen presentation co-stimulatory molecules by APCs which downregulates the ability of APCs to produce IL-12, which is an important stimulator of NK cells and Th1s (Moore et al., 2001; Zundler and Neurath, 2015). Treg cells also constitutively express CTLA4 (Cytotoxic T-Lymphocyte Associated-protein), which reduces the ability of APCs to activate new Th cells and prevent new inflammatory responses (Qureshi et al., 2011). Therefore, Tregs use IL-10 and CTLA4 to prevent new Th1 polarization. IL-10 also protects nitric oxide production pathways (Gunnett et al., 2002) and prevents vascular dysfunction from endogenous vasoconstrictors (Didion et al., 2009).

Third trimester of pregnancy and parturition

As pregnancy reaches its end, parturition is characterized by a drastic shift towards an inflammatory phenotype (Leimert et al., 2021). Two to four weeks prior to parturition are characterized by a shift from pregnancy maintenance to preparation for labor (Stelzer et al., 2021). During this period, IL-6 and IL-1β promote the production of prostaglandins, endothelin-1, and cyclooxygenases which induce uterine contractions during labor (Ashkar et al., 2000). This inflammatory phenotype may in part be due to the progressive decrease in circulating progesterone and Progesterone Induced Blocking Factor (PIBF) that occurs as normal pregnancies progress to parturition (Polgar et al., 2004; Hudic et al., 2016). Progesterone and PIBF both inhibit lymphocyte proliferation and inflammatory cytokine production, which potentially implicates them as important anti-inflammatory agents in the earlier portion of the pregnancy (Fedotcheva et al., 2022). Near the 37th week of pregnancy, PIBF decreases triggering increases in IL-1β and IL-6 along with decreases in the anti-inflammatory cytokines IL-1Ra and IL-9 (Polgar et al., 2004; Jarmund et al., 2021). Uterine levels of IL-6 appear to be important to induce the onset of labor, as IL-6 deficient mice experience delayed labor compared to controls (Gomez-Lopez et al., 2016). As IL-6 is produced by many cell types, multiple cellular sources of IL-6 likely contribute to the IL-6 needed for parturition. Among

major IL-6 producers are Mφs which are sensitive toward changes in estrogen and progesterone during pregnancy (Mendoza-Cabrera et al., 2020). At term, uMφs invade the decidua and cervix and produce reactive oxygen species and TNF-α which lead promote cervical ripening (Hamilton et al., 2012). At this time, uterine levels of the inflammatory Th9 and Th17 subsets increase (Gomez-Lopez et al., 2016). However there has not been a direct link drawn between uMφs and the increase in inflammatory Th subsets. Moreover, the precise mechanisms that induce the shift towards parturition have not been fully elucidated and more studies are needed to tease at the role of the immune system during parturition.

The chronic inflammatory environment in preeclampsia

The immune system during PE is in a state of chronic inflammation (Cornelius et al., 2019; Aneman et al., 2020; Stefanska et al., 2021). Patients with PE have activation of both the innate and adaptive arms of the immune system which induce a feed-forward mechanism for inflammation (Murray et al., 2021). Cytolytic NK cells, macrophages, dendritic cells induce tissue damage and vascular dysfunction in PE while activating Th cells through antigen presentation (Deer et al., 2023). Moreover, patients with PE have activated Th cells and activated B cells producing agonistic antibodies against the angiotensin II type 1 receptor (AT1-AA) which lead to antigen specific mechanisms of cell destruction and tissue damage (LaMarca et al., 2016). The communication between immune cells during pregnancy in preeclampsia may also lead to immune memory which could contribute to inflammation postpartum (van Rijn et al., 2016; Kieffer et al., 2017; Brien et al., 2019). This prolonged inflammatory state contributes to hypertension, endothelial dysfunction, and fetal complications (Murray et al., 2021). Because PE presents after the 20th week of gestation, most studies that have investigated inflammation in PE have been limited to studying middle gestation and late gestation periods.

Dendritic cells and macrophages in preeclampsia

While the initiating factor for uterine inflammation in PE is not fully understood, Mφ and DCs play a role to expand the inflammatory environment in PE. PE placentas have increased invading DCs and macrophages compared to normal placentas (Huang et al., 2008). These uMφ also produce high levels of TNF-α and IFN-γ which contribute to apoptosis of fetal trophoblasts in pregnant mice (Blois et al., 2004). Excessive debris from dying trophoblasts could lead to more internalization and presentation of fetal antigens by Mφ and DCs leading to inflammatory Th subsets in PE. Moreover, PE placentas have shown increased chemokines CCL2, CCL4, CCL7, and CCL20 which promote recruitment of innate immune cells and T cells which could contribute to placental dysfunction in PE. There has been some dispute in the phenotype of uMφs in PE, with some studies showing lower levels of M2s in the placenta while others show higher levels of M2s during PE (Reister et al., 1999; Katabuchi et al., 2003; Schonkeren et al., 2011). These discrepancies have been equated to location of Mφs, citing less localization M2s near trophoblasts and spiral arteries in PE (Faas et al., 2014). Illustrating an important distinction in macrophage location during the pathophysiology of PE. However, there have been few studies to investigate the role of Mφs in the induction of improper spiral artery remodeling in PE; therefore, much more research is needed to better understand how Mφs impact the progression of PE pregnancies.

DCs have been similarly underserved in research of PE pregnancies. In PE there is a higher conventional myeloid DCs to plasmacytoid DC ratio in the circulation (Darmochwal-Kolarz et al., 2003; Wang et al., 2013; Li et al., 2019). These studies also associated the rise in myeloid DCs with increased circulating Th1 and Th17 populations, postulating that myeloid DCs are related to the increased inflammatory Th cell phenotype of PE. However, these studies do not provide as much insight into the placental DC populations in PE. A study by Zhang et al. (2017) showed that there were increased mature dendritic cells in the decidua of PE women, and these DCs had higher expression of a DC specific long non-coding RNA strand that assists in DC maturation by phosphorylating STAT-3. Moreover, a study by Panda et al. (2012) showed that circulating DCs expressed higher levels of TLR3, TLR4, and TLR9 compared to DCs from control patients. These DCs also secreted higher levels of TNF-α, IFN-α, IL-6, and IL-12 measured by flow cytometry. The authors also found that PE DCs were less able to mount inflammatory responses to TLR activation, suggesting that the dysregulation of TLR signaling also impaired DC responses to pathogens. Interestingly, women with PE have increased circulating memory T cells during pregnancy (Chaiworapongsa et al., 2002). Which could suggest that antigen presentation to Th cells leads to development of Th1's and Th17s during PE but also leads to memory T cell production that persists after a PE pregnancy. However, there is limited research available investigating how DCs become altered in PE and how they are involved in placental dysfunction in PE.

T helper cells and B cells in preeclampsia

Patients with PE have an increase in Th1/Th2 ratio and an increase Th17/Treg ratio in the placenta and in the circulation (Eghbal-Fard et al., 2019; Romao-Veiga et al., 2022) (Wallace et al., 2014). The location of where Th cells become activated is unknown, but Th cells induce dysfunction at the placental level as well as in other organs and the vasculature (Murray et al., 2021). Th cells are important mediators to spread immune signals throughout the body; and, Th1 cells and Th17 subsets are potent inducers of inflammatory actions and promote cell mediated cytotoxicity (Wang et al., 2020). Interestingly, adoptive transfer of placental Th cells from placentas of PE women induce hypertension in pregnant athymic nude rats (Harmon et al., 2019). Th cell adoptive transfer also lead to increased plasma sflt-1, IL-17, and TNF-α, while also leading to increased expression of pre-pro-endothelin-1 mRNA which is the precursor to the potent vasoconstrictor endothelin-1. This study suggested that placental Th cells contribute to vascular and renal dysfunction during PE. Animal models of PE have been important to help in delineating the

effects of T cells on the placenta, the vascular, and the kidney in PE. Adoptive transfer of Th1 cells from the Reduced Uterine Perfusion Pressure (RUPP) model of PE induce hypertension and AT1-AA, placental mitochondrial oxidative stress and FGR, as well as renal oxidative stress during pregnancy (Zenclussen, 2006; LaMarca et al., 2008a). Together these studies illustrate that Th cells activated in response to placental ischemia contribute to dysfunction in the vasculature, placenta, and the kidney during pregnancy. Moreover, adoptive transfer of Th17 cells induce hypertension, placental NK cell activation, circulating NK cell activation, and FGR in pregnant rats (Shields et al., 2018). Further showing that Th17 cells are also able to contribute to placental and vascular dysfunctions in pregnancy with the help of NK cells.

Our lab has shown that Th cells stimulated by placental ischemia from rats or humans induce hypertension, mitochondrial dysfunction, sflt-1, AT1-AA, and other features of PE in pregnant rats (Deer et al., 2021; Reeve et al., 2022). However, blockade of communication between Th cells and B cells prevents the features of PE induced by Th cell adoptive transfer (Cornelius et al., 2015). This indicates that Th cell-B cell communication could be an important feature contributing to the pathophysiology of PE. This is attributed to T cell-B cell communication as an integral component in stimulating B cells to transform into Memory B cells which retain antigen memory long after antigen exposure (Palm and Henry, 2019). There has been much less investigation into the role of B cells in the pathophysiology of PE. B cells can be divided into B1 and B2 subsets. B1 cells are innate-like B cells that are responsible for T cell independent antibody responses while B2 cells are classical B cells that are responsible for T cell dependent antibody responses (Mahajan et al., 2021). In the context of PE, Jensen et al. implicated B1 cells as potential producers of AT1-AA *in vitro* after exposing isolated placental B1 cells to patient serum (Jensen et al., 2012). Our lab recently showed that adoptive transfer of RUPP B2 cells into pregnant rats were able to induce hypertension, AT1-AA production, and NK cell activation during pregnancy (Herrock et al., 2022),therefore, implicating both B1 and B2 subsets in the pathophysiology of PE.

Soluble immune factors in preeclampsia

Th1 cells are important producers of inflammatory cytokines IFN-γ and TNF-α which are both increased in women with PE (Sheibak et al., 2020). Inhibiting TNF-α in RUPP rats attenuated hypertension, Endothelin-1, oxidative stress, and NK cell activation (LaMarca et al., 2008b; Cunningham et al., 2020). Other studies have infused TNF-α into pregnant animals to see the effects of total TNF-α during pregnancy (Bobek et al., 2015; Ampey et al., 2019; Jayaram et al., 2021). TNF-α alone induces symptoms of PE in animal models including hypertension, FGR, oxidative stress, Endothelin-1 and AT1-AA in pregnancy (LaMarca et al., 2005; Jayaram et al., 2021). But inhibition of TNF-α in multiple models of PE can attenuate hypertension, maternal inflammation, and fetal morbidity (Irani et al., 2010; Travis et al., 2021). PE patients also have increased Th17s and IL-17 (Ding et al., 2019; El Shahaway et al., 2019). IL-17 can cause more Th17 cells which make more IL-17 in a positive-feedback loop which contributes to the inflammation in PE (Travis et al., 2020). Th17s and IL-17 have been connected with oxidative stress, NK cell activation, anti-angiogenic factors, and AT1-AA production in pregnancy, all of which contribute to hypertension and FGR (Cornelius et al., 2013; Travis et al., 2020). Alternatively, IL-17 inhibition prevents hypertension and oxidative stress in placental ischemic rats (Travis et al., 2020) and elicits natural killer cell activation and hypertension further supporting a roll for Th17s and IL-17 in PE (Travis et al., 2019).

Women with PE have activated B cells producing AT1-AA which causes AT1 receptor activation leading to vasoconstriction in afferent arterioles, renal dysfunction and hypertension during pregnancy (Wallukat et al., 1999). AT1-AA acts by binding the AT1 receptor and stimulating it similar to angiotensin II but also work synergistically with endogenous angiotensin II to further increase blood pressure, oxidative stress, production of sflt-1 and Endothelin, indicating that it may exacerbate the activity of endogenous ANGII in PE patients (Brewer et al., 2013). AT1-AA also causes natural killer cell activation, which may be one mechanism responsible for multi-organ dysfunction in PE (Cunningham et al., 2018; Zhai et al., 2022). AT1-AA is also able to induce oxidative stress through NADPH oxidase and activate NF-κB pathway which contribute to vascular stress in PE (Dechend et al., 2003). Moreover, AT1-AA is also increased in the RUPP model of placental ischemia and our lab has shown that directly inhibiting AT1-AA can prevent the PE-like phenotype associated with placental ischemia (Cunningham et al., 2018).

Immune memory after normal pregnancy

Adaptive immune memory

After the immune adaptations that happen in pregnancy, the maternal immune system retains memory of paternal antigens in order to facilitate future pregnancies. Following activation, T cells and B cells transform into effector cells and memory cells. While the effector cells are responsible for the short-term immune response, the memory cells retain long-term antigen memory in case of future infections. Paternal antigen memory Treg cells remain after a healthy pregnancy, and persist at least one-year postpartum (Kieffer et al., 2017). Upon initiation of another pregnancy with the same partner, memory Tregs rapidly expand in the circulation upon initiation of a new pregnancy (Tilburgs et al., 2008). These memory Tregs then progressively migrate towards the placenta and promote tolerance of the new fetus (Rowe et al., 2012; Gomez-Lopez et al., 2020). It has been suggested that insufficient memory Treg expansion could contribute to infertility (Jasper et al., 2006) suggesting an important role for Tregs in pregnancy success. Moreover, first-time pregnancies have higher incidence of PE (ACOG, 2022), lower Treg expansion (Rowe et al., 2012), and increased sflt-1 (Bdolah et al., 2014) compared to second-time pregnancies. Furthermore, if a subsequent pregnancy is with a different father than the first, there is no Treg induced protection against PE (Need, 1975). These trends could be explained by the lack of paternal-antigen Tregs, implicating paternal-antigen Tregs to promote fetal tolerance in pregnancy.

Innate trained immunity

"Trained Immunity" is a relatively new concept in immunology and appears to be unique to the innate immune system. In simple terms, innate immune cells can make epigenetic modifications after encountering an antigen (Netea et al., 2016). This style of memory is in contrast to adaptive immunity, which involves gene recombination events to retain long-term antigen memory. A study by Novakovic et al. (2016) showed that murine Mφs retain epigenetic modifications following *in vitro* exposure to LPS. This study also importantly showed that these epigenetic modifications may be reverse, showing the increased volatility of trained immunity compared to adaptive immunity. Trained immunity is associated with a three-month to one-year lifetime, but there have been cases of trained immunity lasting up to five years (Nankabirwa et al., 2015).

In the context of pregnancy, trained immunity is still in its infancy in terms of investigation.Gamliel et al. (2018) showed that a subset of "pregnancy trained" uNK cells were more readily able to produce IFN-γ and VEGF compared to untrained uNK cells. As stated above, both IFN-γ and VEGF are crucial in implantation and establishing the maternal-fetal interface. Therefore, this study provides crucial insight into direct applications of trained immunity during physiological pregnancy. However, this is one of the only studies to show direct mechanisms of trained immunity in human pregnancy.

Immune memory and inflammation after a preeclamptic pregnancy

Adaptive immune memory

A recent study also showed that women with PP-PE have increased inflammatory markers and placental pathologies (Brien et al., 2019). Brien et al. found that patients with PE or PP-PE had increased CD4+ and CD8+ T cells in circulation at time of PE presentation. However, only PP-PE patients had increased circulating Natural Killer T (NKT) cells. NKT cells are an innate-like lymphoid cells that stem from the thymus but share markers of T cells and NK cells (Hashemi et al., 2017). While NKT cells have been implicated in the pathophysiology of PE, this study further implicates NKT cells and inflammation in the pathophysiology of PP-PE. Histological analysis of placentas revealed increased syncytial knots, a morphological marker of dysfunction, suggesting that placental dysfunction is important in PP-PE. This study shows that inflammation may be important in the pathophysiology of PP-PE as well. Therefore, therapies targeting inflammatory mediators may be helpful in treating PE in pregnancy or postpartum.

Following a PE pregnancy, there is a persistent inflammatory profile. Vitoratos et al. (2010) found that PE women had increased plasma TNF-α during pregnancy and this increase in TNF-α persisted three months postpartum. Interestingly, they found that plasma IL-6 was not changed during pregnancy, but plasma IL-6 increased postpartum in PE women compared to NP indicating that inflammation may worsen after a PE pregnancy. It was later found that previously PE women have an exaggerated inflammatory response to the flu vaccine which included increased C reactive protein and IL-18 which are both involved in Th1 responses (van Rijn et al., 2016). This indicates that there are alterations to the normal inflammatory response in women that previously had PE that lasts well into their postpartum period. However, circulating memory Th cells are decreased in patients with a previous PE pregnancy (Kieffer et al., 2019), suggesting that Th dysfunction continues into the postpartum period after PE. Studies of Treg cells in idiopathic pre-term labor patients revealed that decreased functional Tregs is associated with recurrent pregnancy loss (Gomez-Lopez et al., 2020), which could be caused by a sustained inflammatory environment. This is mirrored in subsequent pregnancies following PE, where Tregs do not undergo peripheral expansion nor do they migrate to the uterus (Tsuda et al., 2018). This suggests that dysfunctional memory Tregs may be important in PE recurrence. Interestingly, there is an inverse relationship between memory Tregs and Memory B cells in previously PE patients where lower Tregs is associated with higher Memory B cells (Zeng et al., 2013). Memory B cells from a previously PE pregnancy could continue to secrete AT1-AA and contribute to PE in subsequent pregnancies. Rieber-Mohn et al. found that AT1-AA was increased in serum eight years postpartum of a PE pregnancy (Rieber-Mohn et al., 2018), supporting a role for Memory B cells and AT1-AA not only in PE pathophysiology but also in consequences observed in postpartum PE. Future studies in humans or animal models of PE could investigate the role of memory immune cells to contribute to PE in future pregnancies.

Innate trained immunity

Unfortunately, there is even less information investigating trained immunity in PE than normal pregnancy. However, an elegant study by Huang et al. showed that both uneventful and PE pregnancies lead to methylation changes in both Th and NK cell populations (Huang et al., 2021). These DNA methylation events are distinct between physiological pregnancies or PE pregnancies, further implicating immunological memory in the increased risk to develop PE following a PE pregnancy. Please refer to the cited article by Huang et al. to get a more thorough understanding of this fascinating immunological adaption to pregnancy.

Adverse outcomes associated with risk factors for preeclampsia and COVID-19

There are many factors that predispose patients to develop PE in pregnancy. Cardiometabolic disease like obesity, type 2 diabetes (T2D), and hypertension increase the risk for adverse outcomes of pregnancy, including PE (Alston et al., 2022). Each of these diseases have adverse effects on the health of pregnancy, but importantly they are all recognized as states of chronic inflammation (Harrison et al., 2021). Moreover, patients with autoimmune disease like Systemic Lupus Erythematosus, Rheumatoid Arthritis, and Type 1 Diabetes are at an increased risk for developing PE (Tamas et al., 2022). There is also an association between infectious diseases in pregnancy and increased incidence of PE, such as seen in those diagnosed with COVID-19 (Nourollahpour Shiadeh et al., 2017; Lokki et al., 2018).

SARS-CoV-2 infection has been shown to significantly increase complications of pregnancy including PE (Jamieson and Rasmussen, 2022). In 2019, the novel, zoonotic virus SARS-CoV-2 became a global pandemic (Sun et al., 2020). SARS-CoV-2 acts by binding ACE-2 (Angiotensin Converting Enzyme-2) in order to invade host cells (Seyed Hosseini et al., 2020). The virus takes over the host cell, replicates, and eventually spreads to other cells in the body. While its uncertain if pregnant patients have higher risk of contracting COVID (Jamieson and Rasmussen, 2022), pregnant patients with COVID have higher rates of morbidity and mortality (Sayad et al., 2022). Importantly, pregnant patients with COVID have higher rates PE, eclampsia, and preterm birth compared to control (Villar et al., 2021; Wei et al., 2021). Placental samples from a cohort of women with COVID in pregnancy and fetal demise have showed copious placental fibrosis, histiocytic intervillositis, and trophoblast necrosis which has been attributed to COVID induced placental destruction (Jaiswal et al., 2021; Schwartz et al., 2022). Strangely, COVID patients also have increased serum levels of AT1-AA (Rodriguez-Perez et al., 2021). AT1-AA in COVID patients could contribute to post-senescence hypertension in some former COVID patients (Angeli et al., 2022); but, AT1-AA could also contribute to the increased risk for PE in pregnant patients. Moreover, the Renin Angiotensin Aldosterone System (RAAS) must compensate for increased fluid loads during pregnancy (Irani and Xia, 2011; Xia and Kellems, 2011), but dysregulation of RAAS by COVID infection could also contribute to hypertension during pregnancy. Additionally, patients diagnosed with COVID during pregnancy should be monitored postpartum to examine the effects of their uniquely challenging pregnancy.

Conclusion

In conclusion, developing PE is a major contributor to cardiovascular and immune complications in pregnancy and postpartum. After a PE pregnancy, patients have increased incidence of cardiovascular disease, stroke, and PE in a subsequent pregnancy which could be caused by inflammatory mediators. Moreover, developing severe PE is associated with post-traumatic stress disorders that may discourage patients from having future desired pregnancies (Hoedjes et al., 2011). Recent evidence after the COVID pandemic has shed more light on the relationship between placental ischemia, inflammation, and PE suggesting that immune dysregulation could be a major contributor to PE development. Yet, more studies are needed to investigate the mechanisms involved to cause cardiovascular disease or PE recurrence in previously PE patients. Immune memory may contribute to cardiovascular disease or PE recurrence following a PE pregnancy, but more studies could investigate mechanisms of inflammation to induce these features in animal models of PE. Modulators of inflammation have shown promise to lessen maternal disease in models of PE, but longitudinal studies could show efficacy of these agents in the postpartum period. Importantly, investigators must continue to reveal important pathophysiological components that lead to the onset of PE so that we may better serve patients stricken by this terrible disease.

Author contributions

OH: Conceptualization, Writing—Original Draft; ED: Writing—Reviewing and Editing; BL: Conceptualization, Supervision, Writing—Reviewing and Editing.

Conflict of interest

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

Publisher's note

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Potential biomarkers for late-onset and term preeclampsia: A scoping review

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Preeclampsia is a progressive, multisystem pregnancy disorder. According to the time of onset or delivery, preeclampsia has been subclassified into early-onset (<34 weeks) and late-onset (≥34 weeks), or preterm (<37 weeks) and term (≥37 weeks). Preterm preeclampsia can be effectively predicted at 11–13 weeks well before onset, and its incidence can be reduced by preventively using low-dose aspirin. However, late-onset and term preeclampsia are more prevalent than early forms and still lack effective predictive and preventive measures. This scoping review aims to systematically identify the evidence of predictive biomarkers reported in late-onset and term preeclampsia. This study was conducted based on the guidance of the Joanna Briggs Institute (JBI) methodology for scoping reviews. The Preferred Reporting Items for Systematic Reviews and Meta-Analysis extension for scoping reviews (PRISMA-ScR) was used to guide the study. The following databases were searched for related studies: PubMed, Web of Science, Scopus, and ProQuest. Search terms contain "preeclampsia," "late-onset," "term," "biomarker," or "marker," and other synonyms combined as appropriate using the Boolean operators "AND" and "OR." The search was restricted to articles published in English from 2012 to August 2022. Publications were selected if study participants were pregnant women and biomarkers were detected in maternal blood or urine samples before late-onset or term preeclampsia diagnosis. The search retrieved 4,257 records, of which 125 studies were included in the final assessment. The results demonstrate that no single molecular biomarker presents sufficient clinical sensitivity and specificity for screening late-onset and term preeclampsia. Multivariable models combining maternal risk factors with biochemical and/or biophysical markers generate higher detection rates, but they need more effective biomarkers and validation data for clinical utility. This review proposes that further research into novel biomarkers for late-onset and term preeclampsia is warranted and important to find strategies to predict this complication. Other critical factors to help identify candidate markers should be considered, such as a consensus on defining preeclampsia subtypes, optimal testing time, and sample types.

KEYWORDS

late-onset preeclampsia, term preeclampsia, biomarker, prediction model, screening

Introduction

Preeclampsia (PE) is a progressive, multisystem pregnancy disorder that can be a serious, even fatal condition. It affects 2%–4% of pregnancies worldwide and is responsible for nearly 46,000 maternal deaths and around 500,000 fetal and neonatal deaths every year (Magee et al., 2022b). PE not only leads to adverse health outcomes for mothers and babies but also produces a substantial financial burden on the healthcare system. The healthcare cost for pregnancies with PE is significantly higher than uncomplicated pregnancies, including higher inpatient costs, birth costs, and postpartum costs, especially for newborns admitted to Neonatal Intensive Care Unit (NICU) (Fox et al., 2017).

The etiology of PE is still not fully understood, but the understanding of this disorder has been greatly improved. Increasing evidence has shown that PE is a complex and heterogeneous disorder, as reflected in several aspects, such as pathophysiology, clinical phenotypes, screening effectiveness, aspirin prevention performance, and clinical outcomes. Previously, PE was diagnosed as a new onset of hypertension and proteinuria after 20 weeks of gestation. The diagnostic definition was broadened in 2018 by the International Society of the Study of Hypertension in Pregnancy (ISSHP). The latest ISSHP guideline (2021) characterizes PE as hypertension arising *de novo* plus one or more other conditions, including proteinuria, maternal organ dysfunctions, and uteroplacental dysfunction such as fetal growth restriction, abnormal umbilical artery Doppler, and imbalance of angiogenic markers (increased soluble fms-like tyrosine/placental growth factor (sFlt/PlGF) ratio or reduced PlGF) at or after 20 weeks (Magee et al., 2022a).

Previous research divided PE into early and late forms, whereas there is no consistent classification of PE subtypes. The gestational age of 34 or 37 is the cut-off value and is defined by the time of disease onset, diagnosis, or delivery. PE is widely accepted to be subclassified into early-onset (<34 weeks) and late-onset (≥34 weeks), or preterm (delivery <37 weeks) and term (delivery ≥37 weeks) (Poon et al., 2019). Despite the early and late subtypes sharing similar clinical symptoms, recent studies suggest that they have varied pathophysiology (Leavey et al., 2016; Wang et al., 2020; Ren et al., 2021). Moreover, subtyping PE based on etiology has been proposed (Roberts et al., 2021; Than et al., 2022).

A biomarker is an indicator of normal biological processes, pathogenic processes, or biological responses to an exposure or intervention that could be used to predict, diagnose, and monitor diseases (Cagney et al., 2018). This broad definition contains different types of biomarkers, such as molecular, histologic, radiographic, or physiologic characteristics. Useful biomarkers like biochemical markers (PlGF) or biophysical markers (mean arterial pressure, MAP) could improve the effectiveness of risk stratification for PE pregnancies, thereby creating a window of opportunity for clinicians to take preventative actions for high-risk women. Nevertheless, much previous research predicted PE as one type, and some focused more on reporting prediction achievement for the early form of PE. Apart from that, many biomarker studies tried to discover candidate predictors by using blood samples collected during PE diagnosis or placenta samples obtained after delivery, while those altered biomarkers may show better diagnostic value and reflect pathogenesis than the prediction.

The difference in detection rates for predicting PE subtypes presents another challenge. For example, the Fetal Medicine Foundation (FMF) developed a first-trimester screening model which combines maternal factors with biochemical markers (PlGF and pregnancy-associated plasma protein A, PAPP-A) and biophysical markers (uterine artery pulsatility index, UtA-PI, and MAP). This model achieves a high detection rate for predicting preterm PE (75%) at a 10% false positive rate (FPR), making PE screening clinically useful for this subtype. However, the detection rate for predicting term PE is less satisfactory (41%), and the biochemical markers did not improve in predicting term PE (Tan et al., 2018). This may indicate that the same prediction model and biomarkers are not suitable for the late type of PE. Further ongoing research should place a priority on improving prediction for late-onset/term PE since the rate of late type is substantially higher than the early type. The incidences of early-onset and late-onset PE are 30% and 70% in developing countries, as well as 10% and 90% in developed countries (Robillard et al., 2019).

A scoping review is a method of evidence synthesis that systematically identifies the evidence on a particular topic or field. In contrast to systematic reviews, scoping reviews address broader research questions and integrate heterogeneous evidence through a comprehensive search process. Considering the numerous and diverse biomarkers reported in PE prediction studies, this study intends to conduct a scoping review summarising the current state of knowledge of biomarkers for predicting late-onset and term PE. To reflect current challenges in PE prediction, the review will focus on molecular biomarkers tested in maternal blood or urine before late-onset and term PE diagnosis published within the past decade. It will provide an overview of current evidence on predictive biomarkers for late-onset and term PE and provide an in-depth analysis of screening for the late forms of PE. In a preliminary search, no current systematic reviews were found in the Cochrane Database of Systematic Reviews and JBI Evidence Synthesis website. No current or underway scoping reviews specifically addressing biomarkers for late-type PE were identified. The objective is to systematically analyse the recent literature in order to identify potentially useful biomarkers for predicting late-onset and term preeclampsia with the ultimate goal of improving the efficiency of PE screening.

Methods

Study design

This scoping review was conducted by the latest JBI methodology for scoping reviews (Peters et al., 2022). The checklist of Preferred Reporting Items for Systematic Reviews and Meta-Analysis extension for scoping reviews (PRISMA-ScR) was applied to report the review (Tricco et al., 2018). The review protocol was registered in Open Science Framework (Registration DOI https://doi.org/10.17605/OSF.IO/XW9QU).

Research questions

The following research question and selection criteria were defined by the PCC framework ("Participants," "Concept," "Context").

Primary question: Is there any effective molecular biomarker reported in the previous literature that could potentially predict late-onset and term PE?

Secondary questions:

- Which gestations that significantly changed molecular biomarkers have been detected?
- What techniques have been used to study molecular biomarkers?

Eligibility criteria

Publications were chosen if study participants were pregnant women, and molecular biomarkers (including proteins, nucleic acids, and metabolites) for late-onset or term PE were detected. Late-onset and term PE are defined according to disease onset, diagnosis, or delivery gestation ≥34 and 37, respectively. Study sample types are limited to maternal blood, serum, plasma, and urine. Only full-text articles published in English from 2012 to August 2022 were included. At the full-text screening stage, the number of potential biomarkers was considerable. Therefore, the criteria were refined to include biomarkers tested before diagnosing PE or clinical symptoms manifest for predictive purposes.

Search strategy

The following four databases: PubMed, Web of Science, Scopus, and ProQuest, were searched for relevant studies. Search terms contain "preeclampsia," "late-onset," "term," "biomarker," or "marker," and other synonyms combined as appropriate using the Boolean operators "AND" and "OR". Studies identified were limited to those published in English from 2012 to August 2022. More details, such as the electronic search strategy and keywords, can be found in the protocol (DOI https://doi.org/10.17605/OSF.IO/XW9QU).

Data extraction and synthesis

Covidence, a reference manager for screening and data extraction, was used to select published studies for inclusion. All search records were imported into Covidence, and duplicates were identified and removed automatically and manually. One reviewer (LH) screened the titles and abstracts. Two reviewers (LH and OH) independently conducted the full-text screening. Disagreements were resolved through discussion with the third and fourth reviewers (AP and FD). Data were extracted by one author (LH), including publication information, method, and results of biomarkers, and then confirmed by other reviewers (OH, AP and FD).

Results

Search results

A flow diagram (Figure 1) shows the study selection and screening process following the PRISMA guidelines (Page et al., 2021). A total of 4,257 records were found after a systematic search. After removing 1,978 duplicates, 2,279 studies were screened by abstract, and 125 articles with full text were included in the final assessment. Based on our analysis of those included studies, there were two ways to study molecule biomarkers for predicting late-onset and term PE. The first (66/125, 53%) is to study molecular biomarkers alone, such as reporting level change and association with disease. The other (59/125, 47%) investigated molecular biomarkers with a combination of other predictors, such as maternal risk factors and biophysical markers, to build multivariable models.

Sixty-six studies investigated biomarkers alone (summarized in Supplementary Table S1). The majority are protein markers (study number = 48), and others are nucleic acids markers (study number = 9) and metabolic markers (study number = 9). The type of molecular markers varies, including protein, cell-free DNA, mitochondrial DNA, mRNA, microRNA, and metabolites. The most frequently studied biomarkers are proteins, especially angiogenesis factors, PlGF and sFlt-1, and placenta-expressed proteins, such as PAPP-A. Enzyme-linked immunosorbent assay (ELISA) and biochemical analyzer are the most popular methods for protein measurement, and reverse transcription polymerase chain reaction (RT-PCR) is the main approach for mRNA and microRNA study. Nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-mass spectrometry (LC-MS) are two primary techniques for metabolomics study. All protein markers were measured in serum and plasma samples. Five mRNA and microRNA studies used whole blood, and peripheral blood mononuclear cells (PBMC), and only one study analyzed urine samples.

Fifty-nine multivariable model studies combine maternal factors with biochemical and/or biophysical markers to predict late-onset/term PE. Table 1 lists seven longitudinal research studies, and Supplementary Table S2 summarizes the other studies focused on single trimester. Figure 2 displays an overview of the main characteristics of multivariable model studies. As shown in Figure 2A, most studies (61%) were conducted in the first trimester, and 12% were longitudinal studies. Only 7% and 20% were second and third trimester screening models. The study design includes 56% cohort and 44% case-control studies (Figure 2B). Figure 2C shows the type of algorithms used in building multivariable models, which are logistic regression (56%), competing risk models developed by FMF (39%), commercial software (3%), and Cox proportional hazard risk model (2%). According to searching results, reporting PE subtypes' cut-off value varies among studies. Cut-offs of 30, 32, 34, and 37 weeks of gestational age were used to classify late-onset and term PE. Fourteen studies were excluded at the full-text screening stage because the cut-off is 30 or 32 (Figure 1). 49% reported PE ≥ 37 weeks, 44% were PE ≥ 34 weeks, and 7% separated late-type PE into 34–37 weeks and 37 weeks (Figure 2D).

As shown in Table 1, the detection rates at 10% FPR for screening term PE by maternal risk factors alone range from 36% to 41% at 11–13 weeks. Combined maternal factors with angiogenic markers (PlGF, sFlt-1, sEng) at 30–34 weeks are useful for screening term PE by identifying over 50% of term PE. We calculated the average detection rates in 52 studies conducted only in one trimester to compare the prediction performance difference. Case-control

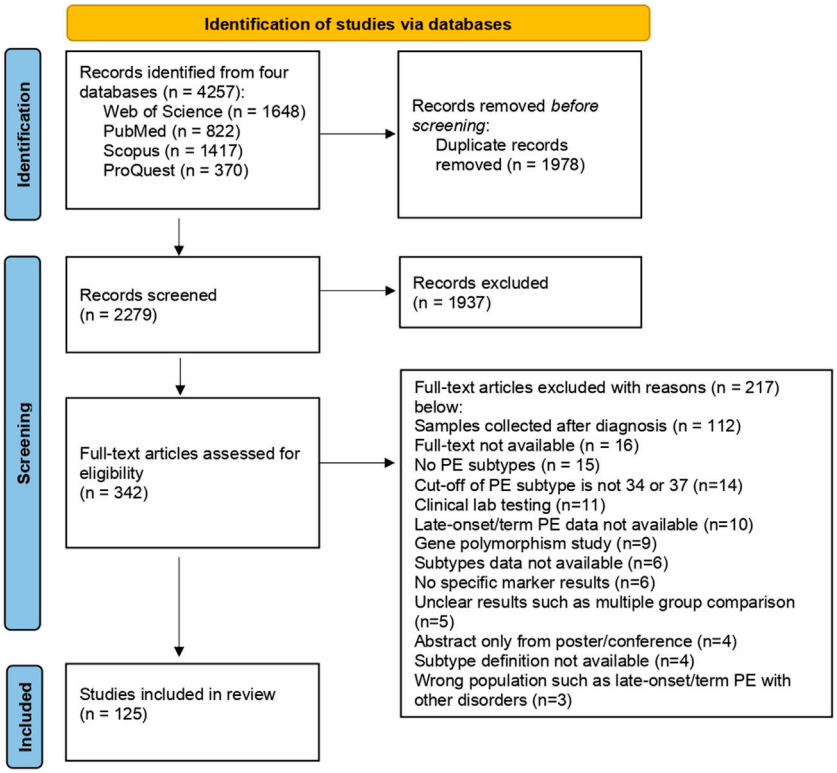

FIGURE 1
PRISMA flow diagram of the study selection process.

studies present a higher detection rate (mean = 51%, SD = 19%) than cohort studies (mean = 39% and SD = 7%) in the first trimester but with greater variance. In cohort studies, the mean detection rates in the first and second trimesters are 39% (SD = 7%) and 50% (SD = 5%) but increase to 61% (SD = 7%), and 76% (SD = 5%) in the early and late third trimesters (Figure 2E).

Discussion

In this scoping review, we explored past research about predictive biomarkers for the late subtype of PE. One hundred twenty-five articles reported individual molecular biomarkers and biomarkers combined with maternal factors and/or biophysical markers. Together these results provide important insights into the possible ways of predicting the late form of PE. To become clinically useful, a potential biomarker should demonstrate clinical reproducibility, validity, and utility (Cagney et al., 2018; Ou et al., 2021). Evidence sufficient to qualify a biomarker depends on factors such as the context of use, potential benefits, and risks associated with its use (Sauer and Porter, 2021). Our results show that current molecular biomarker research for late PE remains at the discovery and validation stage. There is still a wide gap in translating biomarkers into clinical use. Further investigation is necessary to confirm the changed biomarkers and evaluate their efficiency in conjunction with other predictive measures.

Predictive molecular markers

Protein markers

Angiogenic and antiangiogenic factors have been widely investigated as biomarkers for PE for decades. PlGF and its soluble receptor sFlt-1, and sFlt-1/PlGF ratio, are the best clinically available markers for now and are recommended in commercial tests for preterm PE screening and diagnosis (Kmietowicz, 2022). The challenge for preterm PE now is clinical implementation and cost-effectiveness of tests. In this review, PlGF and sFlt-1 are also the most frequently studied biomarkers for late-onset and term PE, including 16 studies alone (Supplementary Table S1) and 50 studies combined with other predictors (Table 1; Supplementary Table S2). According to two longitudinal studies, multivariable models combining maternal factors with PlGF and sFlt-1 individually achieved detection rates (DR) of 54% and 52% at a 10% FPR at 30–34 weeks, which are higher than with maternal factors alone (DR = 37% at 10% FPR) (Tsiakkas et al., 2016a; Tsiakkas et al., 2016b). Moreover, soluble Endoglin (sEng) is another promising angiogenic marker that could identify half of late-onset PE cases at 30–33 weeks when combined with maternal factors (Lai et al., 2013b).

Another source of promising biomarkers is placenta-derived proteins. Placenta dysfunction is considered the main factor of PE development and leads to changes in the release of small molecules into the maternal circulation. Researchers applying

TABLE 1 Summary of longitudinal studies that combined maternal factors with biomarkers.

Study	Country	PE subtypes	Population	Study design	Sample size	Biochemical marker	Algorithm	Screen GA (weeks)	Combined model	DR at 10% FPR	AUC	Note
Tarca et al. (2022)	USA	Term PE with delivery ≥37 weeks	A retrospective analysis of data from 1,150 pregnancies, previously described as part of a case-cohort	Longitudinal case-cohort	1,150	PlGF, sVEGR-1, sEng	Logistic regression	8–15	MF + MAP + PlGF + sVEGFR-1+sEng	36%	0.780	Sensitivity for term PE improved after 32 weeks; models performed similarly to the FMF algorithm when the same biomarker data were used.
								16–19		36%	0.710	
								20–23		41%	0.730	
								24–27		43%	0.770	
								28–31		39%	0.750	
								32–36		51%	0.820	
Andrietti et al. (2017)	UK	Term PE with delivery ≥37 weeks	From prospective screening for adverse obstetric outcomes in women attending routine second and third trimester visits in the UK, Dec 2010 - Aug 2014	Longitudinal prospective cohort		PlGF	Competing risks model	11–13	MF alone	40.5%	0.796	Measurements of UtA-PI, MAP, and PlGF in the first and/or second trimesters have a small or no effect on improving the prediction of PE in the early third trimester.
								11–13	MF + PlGF	42.8%	0.771	
								19–24		40.6%	0.750	
								30–34		55.8%	0.835	
Bredaki et al. (2016)	UK	Term PE with delivery ≥37 weeks	From prospective screening for adverse obstetric outcomes in women attending three routine visits in the UK, Mar 2006 - Apr 2014	Longitudinal prospective cohort	17,071	AFP	Competing risks model	11–13	MF alone	37%	0.721	Measuring serum AFP at 11–13 weeks is not a good predictive marker of PE.
								11–13	MF + AFP	37%	0.754	
					8,583			19–24		38%	0.770	
					8,609			30–34		NA	NA	
Wright et al. (2016a)	UK	Term PE with delivery ≥37 weeks	Prospective screening for adverse obstetric outcomes in women attending three routine hospital visits in the UK	Longitudinal prospective cohort	94,989	PAPP-A	Competing risk model	11–13	MF alone	37%	0.749	Measuring serum PAPP-A and β-hCG did not help predict term PE in the first and second trimesters. DR is slightly improved from 37% (MF only) to 45% at 30–34 weeks.
								11–13	MF + PAPP-A	38%	0.753	
								11–13	MF+ β-hCG	37%	0.748	
					7,597	β-HCG		19–24	MF+ β-hCG	38%	0.727	
					8,088	PAPP-A, β-hCG		30–34	MF + PAPP-A+β-hCG	45%	0.749	
Tsiakkas et al. (2016a)	UK	Term PE with delivery ≥37 weeks	Prospective screening for adverse obstetric outcomes in women attending three routine hospital visits in the UK, Mar 2006 - Dec 2014	Longitudinal prospective cohort	40,212	PlGF	Competing risk model	11–13	MF alone	37%	0.748	Compared to applying maternal factors only (37% at 10% FPR), combining with PLGF could modestly improve the detection rate from 30–34 weeks.
								11–13	MF + PLGF	40%	0.765	
					10,282			19–24		37%	0.757	
					10,400			30–34		54%	0.831	
					4,043			35–37		64%	0.874	

(Continued on following page)

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TABLE 1 (*Continued*) Summary of longitudinal studies that combined maternal factors with biomarkers.

Study	Country	PE subtypes	Population	Study design	Sample size	Biochemical marker	Algorithm	Screen GA (weeks)	Combined model	DR at 10% FPR	AUC	Note
Tsiakkas et al. (2016b)	UK	Term PE with delivery ≥37 weeks	Prospective screening for adverse obstetric outcomes in women attending three routine hospital visits in the UK, Nov 2011 - Dec 2014	Longitudinal prospective cohort	7,066	sFlt-1	Competing risk model	11–13	MF alone	37%	0.748	The combined model with sFlt-1 improved the prediction of term PE at 30–34 and 35–37 weeks.
								11–13	MF + sFlt-1	37%	0.748	
					8,079			19–24		37%	0.748	
					8,472			30–34		52%	0.818	
					4,043			35–37		69%	0.896	
Wright et al. (2016b)	UK	Term PE with delivery ≥37 weeks	Prospective screening for adverse obstetric outcomes in women attending routine hospital visits in the UK, Nov 2011 -Jul 2014	Longitudinal prospective cohort	7,565	sFlt-1	Competing risk model		MF alone	41%	0.750	Screening sFlt-1 at 19–24 improves predicting PE at 30–34.
								19–24	MF + sFlt-1	41%	0.750	
					8,264			30–34		54%	0.825	
								Combined 19–24 and 30–34		64%	0.860	

PE, preeclampsia; GA, gestational age; DR, detection rate; FPR, false positive rate; AUC, area under the ROC curve; PlGF, placental growth factor; sVEGF-R1, soluble vascular endothelial growth factor receptor-1; sEng, soluble Endoglin; MF, maternal factor; MAP, mean arterial pressure; FMF, fetal medicine foundation; UtA-PI, Uterine artery pulsatility index; AFP, alpha-fetoprotein; PAPP-A, pregnancy-associated plasma protein A; β-hCG, beta human chorionic gonadotropin; sFlt-1, soluble fms-like tyrosine kinase-1.

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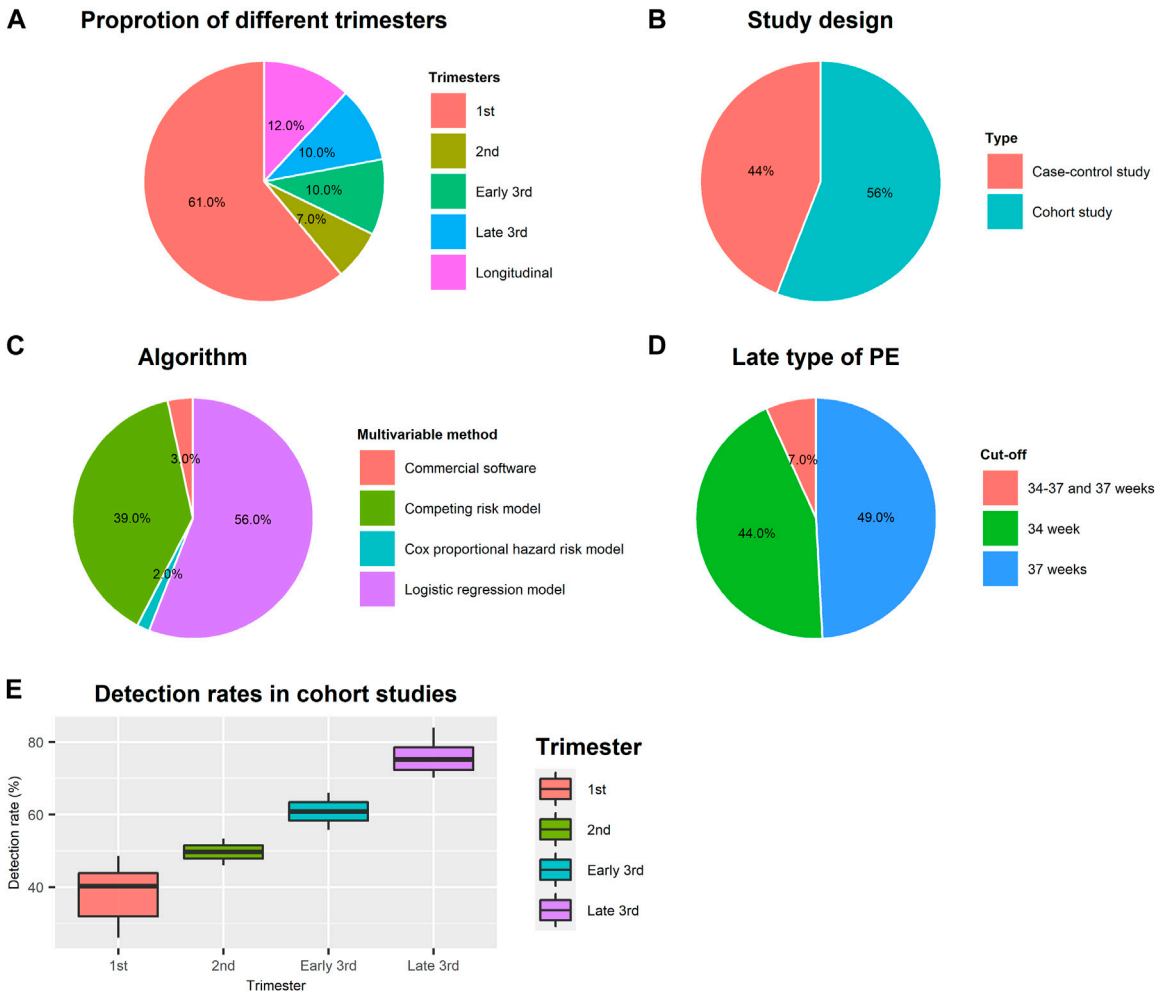

FIGURE 2
Study characteristics of multivariable model studies. **(A)** Proportion of multivariable model studies conducted in different trimesters; **(B)** Proportion of study design; **(C)** Proportion of algorithms used for multivariable model-building; **(D)** Cut-off value of gestational age for defining late type of PE; **(E)** Average detection rates in different gestation age in cohort studies.

fetal chromosomal anomalies screening biomarkers (β-hCG and PAPP-A) to predict PE found that the two markers were less useful for term PE compared to preterm PE (Scazzocchio et al., 2013; Teixeira et al., 2014; Wright et al., 2016a; Scazzocchio et al., 2017). Activin-A and inhibin-A are glycoprotein hormones expressed by the placenta and released into the maternal circulation. In two previous studies, activin-A increased at 30–33 and 36 weeks in late-onset PE and term PE, but not at 11–13 weeks, and the detection rate at 30–33 weeks was 36% at 10% FPR and increased to 50% when combined with maternal factors (Lai et al., 2013a; Wong et al., 2022). Another study proposed that inhibin-A is a better predictor than PlGF for late-onset PE at 12–14 weeks (Keikkala et al., 2021). Other placenta-related biomarkers such as HtrA3 (Wang et al., 2018), A disintegrin and metalloproteinase 12 (ADAM12) (Andres et al., 2022), growth differentiation factor 15 (GDF-15) (Cruickshank et al., 2021), tissue factor pathway inhibitor (TFPI) (MacDonald et al., 2021), and SPINT2 (Murphy et al., 2021) are also reported to change in the maternal circulation of late PE cases.

Nucleic acid markers

Besides proteins, plasma and serum samples contain cell-free DNA (cfDNA) and RNA fragments. Fetal cfDNA testing is widely used for fetal aneuploidy screening and may be helpful for PE. Rolnik et al. found that total cfDNA is only increased in early-onset PE, not late-onset PE. Despite a significant decrease in the median fetal fraction in late-onset PE at 20–24 weeks, its capacity as a marker requires further validation (Rolnik et al., 2015). A recent study reported that cell-free RNA changes could predict the risk of PE before the onset of symptoms but did not specifically address subtypes of PE (Moufarrej et al., 2022).

Busnelli et al. reported that mitochondrial DNA copy number in maternal peripheral blood was lower in late-onset PE in the first trimester. However, this change is more associated with cases with Intrauterine growth restriction (IUGR) (Busnelli et al., 2019). Two studies investigated mRNA in maternal whole blood samples. One article reported that adrenomedullin (ADM) mRNA significantly decreased in maternal circulation up to 10–12 weeks before term PE

onset (Whigham et al., 2019). Another study validated several genes in a test cohort based on bioinformatic analysis of GEO databases and identified three genes (*HDC, MS4A2, SLC18A2*) differentially expressed in late-onset PE (Lin et al., 2022). A few microRNA studies were performed to identify candidate biomarkers associated with late-onset PE and term PE (Winger et al., 2015; Mavreli et al., 2020; Whigham et al., 2020).

Metabolomic markers

Nine studies reported that metabolic markers are significantly changed in maternal serum or plasma, and five multivariable model studies combined metabolic markers with maternal factors and/or other markers. Bahado-Singh et al. reported that the levels of 17 metabolites were significantly altered in late-onset PE cases at 11–13 weeks, and combining those differential metabolites with maternal factors achieved 76.6% sensitivity at 100% specificity in predicting late-onset PE (Bahado-Singh et al., 2013). Later, a study reported that a multivariable model (maternal weight plus UtA-PI and pyruvate, carnitine) yielded a 34.8% detection rate at 17.4% FPR in late-onset PE (Bahado-Singh et al., 2017). Another study found that stearoylcarnitine could modestly improve the combined model consisting of prior risk, MAP, PAPP-A, and PlGF and increase the detection rate from 27% to 32% for late-onset PE (Koster et al., 2015). Kuc et al. (2014) reported that glycylglycine was significantly changed in late-onset PE in the first trimester but did not improve the prediction model (prior risk combined with MAP). Through an untargeted metabolomics analysis in term PE cases, Sovio et al. (2020) identified 100 differential metabolites at 20/28 weeks and validated 33 of them at 36 weeks. 4-hydroxyglutamate and C-glycosyltryptophan showed independent predictive value for term PE.

Although metabolic markers could be potentially applied in prenatal screening, they have poor reproducibility because they are easily influenced by external factors such as diet, lifestyle, and the environment (Monni et al., 2021). Hence, fewer metabolic markers are validated among those studies. Compared to protein markers, data about the prediction efficacy of metabolic markers for the late form of PE is limited.

Combined model screening

In clinical practice, the widely used approach to identify women with a high risk of PE is based on maternal risk factors defined by the American College of Obstetricians and Gynecologists (Gestational hypertension and preeclampsia: ACOG practice bulletin, number 222, 2020) and the National Institute for Health and Care Excellence (NICE, 2019) guidelines. However, the detection rates are low (41% and 34% for preterm and term PE at a 10% FPR). Various multivariable models that combined maternal risk factors with various markers have been developed to improve the PE prediction performance. The FMF first trimester prediction model successfully predicted early-onset and preterm PE with 90% and 75% detection rates at a 10% FPR (O'Gorman et al., 2016). In our study, 59 multivariable model studies reported data on prediction performance for late-onset or term PE (Table 1; Supplementary Table S2). Most of those studies (61%) were conducted in the first trimester, potentially due to the convenience of collecting blood samples. Most pregnant women have their first blood test for fetal chromosomal anomalies screening at 8–12 weeks, making the first trimester an accessible time to recruit pregnant participants and collect blood samples.

Nevertheless, the first-trimester screening may not be suitable for late-onset and term PE. We analyzed detection rates in cohort studies and found the average detection rate of multivariable models in the first trimester is 39% which is similar to using maternal risk factors alone. As shown in Figure 2E, the detection rates are improved with screening at increasing gestation age. A combination of maternal factors and biomarkers at 35–37 weeks' gestation could identify about 76% term PE. This evidence suggests that the optimal time for biomarker screening may be later than the first trimester. However, data from the combined model used in late PE in the second and third trimesters are insufficient. Only 7% of multivariable model studies reported combined screening models in the second trimester, and 20% reported combined screening models in the third trimester.

Considerations in biomarker studies for late-onset and term PE

Subtype definition

PE involves multifactorial etiology and progresses dynamically during pregnancy. It can occur at various gestational ages, even postpartum, and display different grades of severity. Increasing publications encourage researchers to examine PE as a heterogeneous syndrome with subtypes instead of merging into one category (Roberts et al., 2021; Than et al., 2022). There is currently no consistent approach to classify PE subtypes. In this review, we only targeted late-onset and term PE, defined as after 34 and 37 weeks of gestation according to International Federation of Gynecology and Obstetrics (FIGO) guidelines (Poon et al., 2019). The cut-off value (34/37 weeks) is based on the gestation age of disease onset, diagnosis, or delivery. Nevertheless, this classification is limited since the timing of PE onset, diagnosis, and delivery are different, making the prediction results in each study less comparable. While the onset of PE may more accurately reflect disease pathophysiology, delivery time is likely to be related to disease severity, requiring delivery due to maternal and/or fetal symptoms, and possibly varied between healthcare institutions (Dimitriadis et al., 2023). Our analysis of PE subtype definitions in multivariable model studies found that 49% used ≥37 weeks and 44% used ≥34 weeks. 7% of those studies separated late PE into 34–37 weeks and ≥37 weeks. Questions remain about whether a cut-off of 34 or 37 weeks should be utilized and how to define the cut-off time.

ISSHP guidelines suggest PE should not be classified as "mild" or "severe" in an ongoing pregnancy. However, Villa et al. show that sFlt-1 concentration differs between severe and non-severe late-onset PE, which may indicate biomarker level change is related to the disease severity (Villa et al., 2013). Furthermore, researchers recently suggested that PE may be subtyped more accurately based on pathogenesis instead of gestational age at onset or delivery (Than et al., 2022). How we report and categorize PE subtypes is still up for debate and requires further research. Here, we underline the importance and need for consensus on classifying PE in future

studies. Consistent definitions for PE subtypes will directly impact how researchers report the results and biomarker performance.

Timing of biomarker test

Given that PE may involve multiple etiologies, it is unlikely that a single strategy could be used to predict all PE cases. One hypothesis proposes that multiple pathogenetic pathways may result in the same pathologic endpoint, placental dysfunction (Redman et al., 2022). The onset time and severity of placental dysfunction may vary in PE subtypes. In the early type, abnormal placentation probably occurs during early gestation and is the leading cause of pathology. However, for late PE, both placenta and maternal dysfunction, such as maternal cardiac dysfunction (Thilaganathan, 2020), may contribute to disease and progress later during pregnancy. The subclinical period in which the biomarkers begin to present significant change before diagnosis may create a window of opportunity for prediction and prevention. So far, very little is currently known about the subclinical period, which is critical to biomarker tests in the late type of PE.

It is hypothesized that the closer the disease onset, the more significant potential biomarkers change. Lai et al. (2013a, 2013b) measured activin-A and sEng at 11–13 weeks and 30–33 weeks and found these two proteins only present significant change at 30–33 weeks. Researchers from Australia reported a group of placental-derived proteins which are significantly changed in 36 weeks preceding term PE diagnosis from two cohort studies (Cruickshank et al., 2021; MacDonald et al., 2021; Murphy et al., 2021; Andres et al., 2022; Kandel et al., 2022; Wong et al., 2022). Other evidence also shows that gene expression in circulating neutrophils altered 8 weeks before term PE onset (Walsh et al., 2021). Moreover, as mentioned before, PlGF and sFlt-1 started improving the combined screening model at 30–34 weeks. Earlier identification of high-risk pregnancies allows interventive actions. Therefore, exploring the time threshold of biomarkers starting to show significant alteration in late-onset/term PE samples is likely to be a fruitful area of future research, as well as determining the optimal time point of screening benefit using biomarkers.

Other factors

Other factors such as study design, sample type, and statistical method are critical for discovering and identifying potential biomarkers. Many PE studies have detected biomarkers in blood samples collected during PE diagnosis or in placenta samples obtained after delivery. However, this type of biomarker may be less capable of reflecting the change before the disease manifestations. In this review, we excluded these studies (n = 112, Figure 1) and limited the inclusion criteria to biomarkers tested prior to disease diagnosis. Also, we found that the average detection rate of case-control studies with the multivariable model is higher than cohort studies but with a greater standard deviation. The population in a cohort considers the disease frequency a key factor and may better reflect the actual prediction performance.

The current multivariable models are mainly based on logistic regression analysis (56%) and FMF-developed competing risk models (39%). Methods from machine learning algorithms or artificial intelligence for building prediction models have rapidly increased in popularity. A study that applied machine learning to combine maternal factors and clinical laboratory data could effectively predict late-onset PE from the second trimester to 34 weeks (Jhee et al., 2019). Another article utilized artificial intelligence and machine learning methods to evaluate the accuracy of prediction PE, although they only reported data on all PE cases and preterm PE (Ansbacher-Feldman et al., 2022). Machine learning seems to be a powerful tool to improve late-onset and term PE risk assessment by integrating large datasets, including clinical information, ultrasound imaging data, and biomarker tests. Building this type of model requires research and clinical data, which need close collaboration between researchers and clinicians. Additionally, expertise from multiple disciplines, such as data science, biomedical science, and clinical research will be critical.

Strengths and limitations

In this review, heterogeneity and quality assessment have not been conducted to determine the clinical performance of biomarkers and prediction models. Additionally, heterogeneity among the study populations was not specifically accounted for. Therefore, detection rates were only roughly estimated for the combined model in cohort studies. In addition, this review mainly focuses on searching for potential prediction methods for late-onset and term PE and therefore does not summarize the data for early-onset/preterm and compare subtypes. Researching and comparing the differences between subtypes of PE is likely to be a worthwhile area of investigation because this may help to determine the mechanism behind PE subtypes. Our strength is that we specifically address the problem that there is scant attention in the research of late-onset/term PE and characterization of subtypes is essential for better prediction of late-onset/term PE.

Conclusion

This study summarized the current predictive biomarkers for late-onset and term PE. Findings emphasize the necessity for further validation and optimization of prediction strategy for late PE through integrating new biomarkers and algorithms.

For better prediction of late-onset and term PE, we suggest that future studies should 1) use a consistent definition of subtypes PE and stratify cases into subtypes; 2) consider the time when biomarkers start to show a significant change in maternal samples; and 3) engage collaboration with clinicians to obtain more clinical information and data. Further, PE risk assessment may incorporate machine learning into the risk assessment system for monitoring disease progression and adverse outcomes.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: https://doi.org/10.17605/OSF.IO/XW9QU.

Author contributions

LH was responsible for conducting the search, screening, and writing of the manuscript. OH conducted full-text screening, and AP and FD decided on the disagreements. All authors provided critical feedback and helped shape the research and manuscript.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1143543/full#supplementary-material

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Preeclampsia pathophysiology and adverse outcomes during pregnancy and postpartum

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Background: Preeclampsia is a disease with far-reaching consequences that extend beyond the immediate postpartum period and have a significant impact later in life. Preeclampsia exerts an effect on most organ systems in the body. These sequelae are mediated in part by the incompletely elucidated pathophysiology of preeclampsia and the associated vascular changes.

Content: Current research focuses on unraveling the pathophysiology of preeclampsia with the goal of implementing accurate screening and treatment modalities based on disease development and progression. Preeclampsia causes significant short- and long-term maternal morbidity and mortality, not only in the cardiovascular system but also in other organ systems throughout the body. This impact persists beyond pregnancy and the immediate postpartum period.

Summary: The goal of this review is to discuss the current understanding of the pathophysiology of preeclampsia as it relates to the adverse health consequences in patients impacted by this disease, along with a brief discussion of ways to improve overall outcomes.

KEYWORDS

preeclampsia, long-term effect, pregnancy, hypertension, morbidity

1. Background and epidemiology

Hypertensive disorders of pregnancy (HDP) represent a major cause of pregnancy-associated morbidity and mortality. These disorders have far-reaching consequences that extend well beyond the pregnancy and the immediate postpartum period. According to the Centers for Disease Control (CDC), HDP, including preeclampsia, accounts for nearly 7% of all maternal deaths (1). HDP consists of a myriad of diagnoses, including chronic hypertension, gestational hypertension, preeclampsia, preeclampsia with severe features, and eclampsia (2, 3). Similar to chronic hypertension, data suggest that preeclampsia has significant sequelae later in life. Therefore, a thorough understanding of the pathogenesis and prediction of HDP and its implications on short- and long-term health outcomes is crucial to provide optimal care to pregnant patients, especially as they transition out of the immediate postpartum period.

2. Risks and diagnosis

To improve long-term morbidity in the pregnant patient, minimizing the development of disease and early detection are paramount. No perfect prediction model exists to identify all

patients who will develop HDP accurately nor define those at greatest risk of long-term morbidity. However, risk factors for the disease have been identified. As determined by the American College of Obstetrics and Gynecology (ACOG), risk factors for the development of preeclampsia include prior preeclampsia, chronic hypertension, diabetes, renal disease, autoimmune diseases such as lupus, and multifetal gestations (2, 4, 5). Traditionally, the Black race has been identified as a risk factor for the development of HDP, with Black people having much higher rates of preeclampsia compared to White counterparts (6). Recent research into health inequities, however, has questioned whether one's race or ethnicity is a concrete risk factor for HDP or whether race and ethnicity are merely reflective of unequal access to care and unfavorable socio-economic conditions present in the healthcare system and society.

2.1. Diagnosis

Preeclampsia and HDP have well-established guidelines that aid clinicians in diagnosis (Table 1). (2, 7) Chronic hypertension is defined as a diagnosis that predates the pregnancy or blood pressure elevations (≥ 140/90) diagnosed prior to 20 weeks on two occasions at least 4 h apart (8). Though these patients have a diagnosis of chronic hypertension prior to pregnancy, they remain at risk of worsening hypertension and the development of preeclampsia. Gestational hypertension is diagnosed with two elevated blood pressure readings ≥140/90 (defined as mild range blood pressures) after 20 weeks on two occasions at least 4 h apart (2). Patients with this diagnosis lack the overt signs and symptoms of preeclampsia, though they are at increased risk of developing preeclampsia. Patients diagnosed earlier in pregnancy have a higher risk of progression to preeclampsia, with 15–25% of patients ultimately developing preeclampsia (9). This risk of progression increases the earlier a patient is diagnosed and necessitates close patient monitoring.

Preeclampsia itself is defined as newly elevated blood pressure ≥ 140/90 after 20 weeks' gestation in addition to proteinuria defined as 300 mg or more in a 24-h urine specimen, or a protein/creatinine ratio of 0.3 or more, or 2+ protein on urine dipstick (used if other quantitative methods are unavailable) (2). A patient meeting these criteria is diagnosed with preeclampsia without severe features. It is important to recognize that there are other ways to meet the criteria for preeclampsia, all of which upstage the disease process to preeclampsia with severe features. Differentiating these two entities is important for management decisions and pregnancy implications. Preeclampsia with severe features represents a more severe form of the disease and has various diagnostic criteria, which can generally be divided into three main categories: blood pressure values (severe range blood pressures defined as ≥160/110), laboratory values, and symptomatology. Patients with blood pressure elevations of ≥160/110 (with *either* systolic *or* diastolic elevations) with two readings at least

TABLE 1 Diagnostic criteria for HDP.

HDP	Blood pressure criteria	Lab values	Signs and symptoms
Gestational hypertension	BP≥140 or ≥90, >4 h apart after 20 weeks gestation	N/A	N/A
Preeclampsia without severe features	BP ≥140 or ≥90, >4 h apart after 20 weeks gestation	Proteinuria defined as: - At least 300 mg in 24-h urine OR - Protein/creatinine ratio ≥ 0.3	N/A
Preeclampsia with severe features	BP ≥160 or ≥110, >4 h apart after 20 weeks gestation OR BP ≥160 or ≥ 110 after 20 weeks gestation requiring acute treatment OR BP ≥140 or ≥ 90 with lab and/or symptom criteria	Thrombocytopenia (platelets <100×10^9/L) AND/OR Transaminitis (AST/ALT twice normal) AND/OR Acute kidney injury (doubling of patient's baseline creatinine OR > 1.1)	• Intractable headache • Persistent vision changes • Severe right upper quadrant pain • Pulmonary edema
HELLP syndrome (hemolysis, elevated liver enzymes, low platelets)	BP ≥140 or ≥ 90 with lab and symptom criteria* **About 15% of patients HELLP syndrome may not have hypertension*	Thrombocytopenia (platelets <100×10^9/L) AND Transaminitis (AST/ALT twice normal) AND Hemolysis - LDH > 600 U/l - Haptoglobin <25 mg/dl - Bilirubin ≥1.2 mg/dl - Schistocytes on peripheral smear	Variable
Eclampsia	Variable	Variable	Seizures

Adapted from Ref. (2).

4 h apart or continuous severe range blood pressures necessitating rapid treatment are formally diagnosed with preeclampsia with severe features (2). Even without severe range blood pressures and only mild range blood pressures, patients with the following laboratory criteria are diagnosed with preeclampsia with severe features: thrombocytopenia (platelets <100×10^9/L), renal insufficiency (doubling of patient's baseline creatinine or creatinine >1.1 mg/dl), or liver impairment (liver enzymes twice the normal value) (2, 7, 10). Lastly, patients with or without severe range blood pressures and symptoms of pulmonary edema, severe right upper quadrant pain not due to other etiologies, persistent vision changes, and new-onset headache refractory to medications may also be diagnosed with preeclampsia with severe features (2, 7, 10). Patients with pre-existing chronic hypertension may meet the criteria for superimposed preeclampsia with severe features if they exhibit any of the laboratory abnormalities or symptoms (2, 7, 11).

A more severe form of preeclampsia known as HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets) presents increasing rates of mortality and adverse maternal and fetal outcomes (12). Patients demonstrate signs of hemolysis (elevated lactate dehydrogenase [LDH] > 600 IU/l), elevated liver enzymes (more than twice normal lab values), and low platelets (<100×10^9/L) (2, 13). Finally, eclampsia represents the most severe form of the disease and is categorized by new-onset seizures, typically in patients who already carry a diagnosis of preeclampsia (2). Importantly 20–30% of patients diagnosed with eclampsia will be normotensive or have no other disease manifestation, so a high index of suspicion is crucial (14, 15).

Several diagnostic conundrums exist regarding the accurate diagnosis of preeclampsia, most notably in the setting of superimposed disease and patient symptoms. Patients with chronic hypertension may have worsening of their blood pressure during pregnancy, though this does not inherently warrant a diagnosis of preeclampsia. Patient observation and evaluation are required to characterize the diagnosis in this patient subset further. Additionally, patients that meet preeclampsia criteria with persistent symptoms represent a unique cohort. These symptoms require a full workup, and evaluation is vital to rule out other causes of symptomatology as preeclampsia symptoms are vague and non-specific (16).

2.2. Clinical manifestations

Patients with preeclampsia generally present with some degree of blood pressure elevation (17). They may have an unrelenting headache, right upper quadrant pain, or vision changes. Occasionally, patients may complain of increased lower extremity edema, which, while not diagnostic of preeclampsia, should certainly raise concern for disease development. In the absence of symptoms, laboratories drawn for any indication that could be indicative of developing preeclampsia should alert the clinician's suspicion. Patients generally have their blood pressure checked at every appointment, and the overall blood pressure trend is important as patients who ultimately are diagnosed with preeclampsia often have increased blood pressure during their pregnancy (18). In addition to patient symptoms, the fetus can often have pathology consistent with preeclampsia. While not specified in the guidelines in the United States by the American College of Obstetrics and Gynecology (ACOG), international organizations recommend including fetal growth restriction and other signs of uteroplacental insufficiency in the diagnosis of preeclampsia (7). Any change in the fetal growth or well-being should therefore prompt a thorough evaluation by the clinician.

Though a more thorough discussion of diagnostic modalities and criteria is beyond the scope of this article, recognizing signs and symptoms of HDP is important as those diagnosed earlier in pregnancy have a higher risk of progression to more severe disease and possibly a higher risk of long-term morbidity (9).

3. Pathophysiology

Although the pathophysiology of HDP is not fully understood, proposed contributors include placental dysfunction and immunologic changes culminating in poor uteroplacental perfusion (Figure 1). Importantly, the underlying mechanisms thought to contribute to vascular dysfunction in preeclampsia are like those in cardiovascular and atherosclerotic diseases in the non-pregnant individual. These similarities may help explain why preeclampsia is associated with an increased risk of cardiovascular disease later in life.

In normal pregnancy, cytotrophoblasts invade the uterine myometrium and spiral arteries to create a rich network of vascular anastomoses that will ultimately perfuse the placenta and fetus (19). In patients with preeclampsia, cytotrophoblasts do not develop the invasive phenotype required to create these robust anastomoses, which leads to decreased and shallow endovascular invasion of the spiral arteries (20–22). These abnormal blood vessels have narrow caliber, which leads to placental ischemia and ineffective oxygen transfer (23). This is demonstrated in the Stage 1 portion of Figure 1. Additionally, higher levels of various pro-inflammatory molecules are noted in patients with preeclampsia, including natural killer cells and other non-specific markers of inflammation (5, 24). In a normal pregnancy, an "immune tolerance" exists, largely due to changes in the maternal immune system surrounding T cells (24). In pregnancies not impacted by preeclampsia, Th1 cells and Th2 cells exist in harmony to prevent excessive inflammation and fetal rejection. In models of preeclampsia, this balance is disrupted, and many T cells shift to a Th1 phenotype, like those with chronic autoimmune diseases (19, 24). Th1 cells promote inflammation *via* pro-inflammatory cytokines, autoantibodies, and increased oxidative stress, which further worsens the damage and ischemia noted in preeclampsia (24).

The complex process of the development of preeclampsia may be facilitated by a combination of abnormal placentation and ischemia, which results in the release of pro-inflammatory and anti-angiogenic proteins in maternal circulation, ultimately resulting in endothelial dysfunction leading to the clinical syndrome seen in patients with preeclampsia. The two most studied and implicated biomarkers, especially in relation to the development of preeclampsia, are soluble FMS-like tyrosine kinase-1 (sFlt-1) and placental growth factor (PlGF) (5). sFlt-1 is an anti-angiogenic factor that inhibits neovascularization (25). Higher levels of sFlt-1 are found in patients with preeclampsia and the placentas of patients with preeclampsia (25, 26). The levels of PlGF are lower, and the ratio between sFlt-1 and PlGF is elevated in patients with preeclampsia (26, 27). This is demonstrated in the Stage 2 portion of Figure 1.

Overall, the pathogenesis of preeclampsia is extremely complex and likely multifactorial. The proposed main tenets in the development suggest abnormal placentation resulting in inappropriate spiral artery

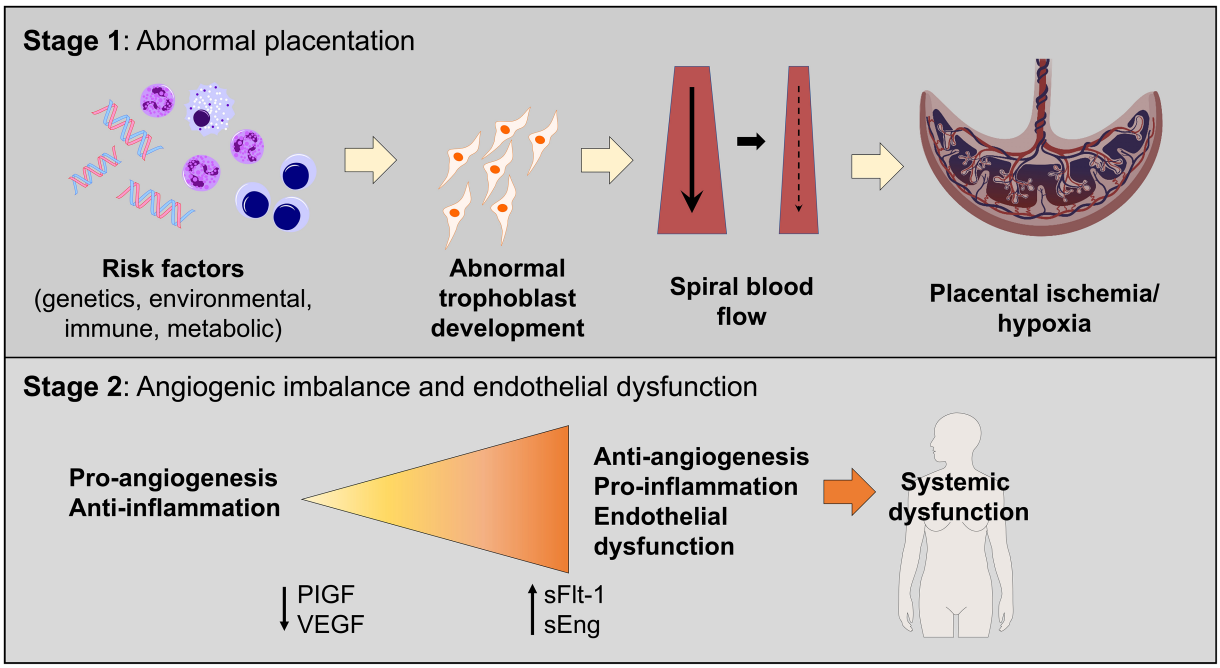

FIGURE 1
Two-staged model of preeclampsia pathogenesis. Stage 1 consists of the preclinical stage and is characterized by abnormal placentation, which leads to the release of soluble factors in maternal circulation, leading to systemic endothelial dysfunction and hypertension (Stage 2).

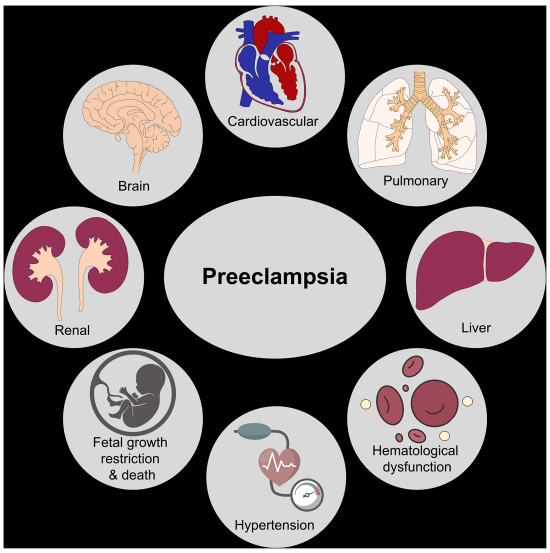

FIGURE 2
Organ systems impacted by preeclampsia. The figure shows various organ systems affected by preeclampsia leading to short-term and long-term maternal/fetal morbidity and mortality.

remodeling, and the resultant tissue hypoxia causes endothelial damage leading to hypertensive pathology. Meanwhile, changes in the maternal immune system in patients with preeclampsia facilitate a low level of chronic inflammation, which continues to perpetuate the cycle of endothelial damage. This combination may result in imbalances in angiogenic and anti-angiogenic factors. The complex interplay between placental pathology, inflammation, and changes in angiogenesis ultimately results in the clinical syndrome known as preeclampsia and contributes to adverse health outcomes in patients during pregnancy and postpartum (28, 29).

4. Adverse outcomes related to preeclampsia

Preeclampsia can result in significant health impairment in patients during pregnancy, immediately postpartum, and beyond. Understanding the risks of preeclampsia and the pathophysiology of the disease process can aid in the prevention of maternal morbidity and mortality. The spectrum of these adverse outcomes is discussed in detail below and illustrated in Figure 2.

4.1. Maternal adverse outcomes associated with HDP

The development of preeclampsia profoundly impacts the cardiovascular system. In the short term, severe cases of preeclampsia can lead to cardiac dysfunction and severe hypertension and are also associated with peripartum cardiomyopathy (30, 31). Studies examining cardiac structure and function in preeclampsia have shown marked diastolic dysfunction and left ventricular remodeling beginning in pregnancy (32). Importantly, this type of remodeling is sometimes irreversible, and though it may be clinically apparent during pregnancy, it does not always regress in the postpartum setting (32). Patients with preeclampsia have a two-fold increase in the subsequent development of both fatal and non-fatal ischemic heart disease (33). These patients demonstrate accelerated cardiovascular

aging, illustrating increased overall arterial stiffness like older, postmenopausal patients (34).

The risk of the development of heart failure over the first 5 years postpartum is elevated in patients with HDP, especially those with pre-existing chronic hypertension (35). Importantly, when controlling for other factors, non-Hispanic Black patients consistently had higher rates of heart failure following HDP compared to their non-Hispanic White counterparts (35). The development of HDP also significantly increases the risk of sustained hypertension in the postpartum period as well as over the course of a patient's life, developing disease earlier in life than those without a preeclampsia history (25, 30, 31). A study in the United Kingdom investigating blood pressure changes in patients with preeclampsia diagnosed prior to 34 weeks illustrated an increase in immediate postpartum and long-term systolic and diastolic blood pressures over 13 years (36). This was in comparison to those diagnosed after 34 weeks, indicating that earlier preeclampsia diagnosis is correlated with worse long-term blood pressure parameters. Studies such as these illustrate the far-reaching cardiac consequences of HDP and further demonstrate some of the racial inequities seen in outcomes.

Beyond the cardiovascular system, preeclampsia significantly impacts the kidneys and is the most common glomerular-based kidney disease in the world (37). In normal pregnancy, there is an increase in the glomerular filtration rate (GFR), resulting in a decrease in the serum creatinine value (38). Thus, normal creatinine values for a non-pregnant person may be pathologic in the state of pregnancy. Because of the altered physiology in pregnancy, a 30 to 40% reduction in GFR occurs prior to a significant elevation of the serum creatinine (39). Histologically, changes have been observed in the kidneys of people with preeclampsia, including endothelial swelling with a decrease in surface area for filtration as a result of increased subendothelial fibrinoid deposits (40). These changes contribute to an increased incidence of acute renal failure in pregnancy, with the rate in the United States increasing from 1.3 to 4.5 per 10,000 births between the years 1998 to 2008 (41).

These changes and renal pathologies can persist postpartum and are associated with significant maternal morbidity (42–44). One study followed patients for 1 year after childbirth, measuring estimated GFR at 3 and 12 months postpartum (45). This study illustrated a significant reduction in renal function as measured by estimated GFR at 3 and 12 months postpartum in patients after a pregnancy complicated by preeclampsia (45). Similarly, a large Norwegian study assessing risks for end-stage renal disease after pregnancy found that patients with a history of preeclampsia, after adjusting for confounders, continued to have an increased risk for the future development of end-stage renal disease (46). This risk increased with the number of pregnancies affected by preeclampsia (46). Compounding this association is the relationship between preeclampsia and the renal disease itself. Many risk factors for preeclampsia are also associated with the risk of developing renal disease later in life, so the true relationship may be difficult to determine fully. Regardless, patients with a history of preeclampsia have an increased risk of chronic renal disease later in life.

Blood cell dyscrasias and hepatic dysfunction are often observed in preeclampsia. Thrombocytopenia is observed in 30–50% of people with preeclampsia, with platelets less than 100×10^9/L being diagnostic for preeclampsia with severe features (2). A combination of altered platelet clearance and hemolysis is thought to contribute to the thrombocytopenia seen in preeclampsia (47). Thrombocytopenia may also be caused from the activation and consumption of platelets caused by the endothelial injury seen in preeclampsia (48). Some studies have shown that certain platelet indices can be used for the prediction and early diagnosis of preeclampsia. However, this evidence is not conclusive and larger studies are needed (49). Hemolysis associated with preeclampsia is associated with an increased risk of poor outcomes, including acute kidney injury, blood transfusion, ICU admission, pulmonary edema, and poor neonatal outcomes (50). While the short-term implications on the hematologic system in patients with preeclampsia are relatively well understood, the long-term impact warrants further study.

Hepatic dysfunction in preeclampsia is marked by microvesicular fat changes and periportal and sinusoidal fibrin deposition in the liver parenchyma (51). These changes are typically transient and do not result in severe disability. Rarely (~1/40,000 to 1/250,000 pregnancies), in the context of preeclampsia, a subcapsular hematoma may form (52). This is potentially catastrophic, with resultant mortality rates ranging from 17 to 59% with an expanding hematoma or hepatic rupture (52). Rarely, patients may require a liver transplant after severe liver involvement in preeclampsia, though this is an exceedingly rare complication (52). With the exception of the more severe complications such as subcapsular hematoma, patients typically do not exhibit long-term hepatic impairment.

The nervous system is also significantly affected in pregnancy and preeclampsia. Eclamptic seizures are the most well-known neurologic sequela of preeclampsia, with an incidence of 0.5–1.5% of deliveries in developing countries but as low as 0.01–0.1% of deliveries in developed countries (53). Patients with eclampsia have an increased risk of disseminated intravascular coagulation, acute renal failure, pulmonary edema, heart failure, cerebrovascular disease, and death (53). Aside from eclampsia, there is an increased risk of a cerebrovascular accident caused by uncontrolled hypertension from preeclampsia (54, 55). Once patients are outside of the acute postpartum setting, their long-term risk of stroke remains elevated, with a two-fold increase in cerebrovascular accidents noted in patients with a history of HDP (56).

Some studies have suggested that patients with preeclampsia demonstrate long-term cognitive decline compared to those with pregnancies not impacted by preeclampsia (57). A retrospective study of 40 women at least 35 years from their antecedent pregnancy investigated neurocognition and dementia between patients who had pregnancies with hypertensive disorders of pregnancy and those without (57). Though not statistically significant, mild cognitive impairment was noted in a higher frequency in those with history of HDP compared to those without (p – 0.10) (57). Alternatively, some studies have questioned whether preeclampsia remains an independent risk factor for neurocognitive problems later in life. A large retrospective cohort study investigated the impact of a history of HDP on long-term cognition, illustrating that preeclampsia history was associated with decreased scores when measuring psychomotor function, memory, and executive function (58). This impact was no longer present once adjusted for age, BMI, education, depression, and hypertension (58). Though studies illustrate conflicting results regarding long-term cognitive impact, there is evidence to suggest that preeclampsia could, at the very least, contribute to neurocognitive dysfunction.

All complications of preeclampsia can worsen or occur for the first time in the immediate postpartum period, with hypertensive disorders

of pregnancy being the leading cause of postpartum readmission (59). The postpartum period is an especially high-risk time, given the transition from at least weekly visits with a physician (antepartum) or continuous inpatient care (intrapartum) to no medical surveillance, usually until 6 weeks postpartum. Of the patients who are diagnosed with new, postpartum preeclampsia, 60% have never had a prior hypertensive diagnosis and present with severely elevated blood pressures and symptoms (60). As a result of this, people who are readmitted postpartum without a prior diagnosis of hypertension are at higher risk of eclampsia, stroke, and overall severe maternal mortality (61).

4.2. Fetal adverse outcomes

Though preeclampsia has significant sequelae in the pregnant person, there are also important implications for the fetus. As many as 1/3 of fetuses of patients with preeclampsia will develop fetal growth restriction (62). Fetal growth restriction itself carries an increased risk of stillbirth and neonatal death, necessitating increased healthcare visits and resultant costs as well as often inpatient admission (63). There is a seven-fold increase in the risk of intrauterine fetal death in preeclampsia with severe features as compared to pregnancies unaffected by hypertensive disorders (64). With these known risks, patients with preeclampsia are extensively monitored during their pregnancy. This monitoring consists of increased physician visits, laboratory evaluation, and fetal ultrasounds (2). Increased surveillance is more burdensome for the patient and costly for the healthcare system overall. If there is a high suspicion of worsening disease or a need for more monitoring, patients may be admitted for prolonged periods of time. Lastly, increased monitoring and the need for diagnostic evaluation can result in iatrogenic preterm delivery and the associated morbidities and costs that accompany preterm birth.

Newborns of pregnancies affected by preeclampsia are at higher risk of being small for gestational age and of having low seven-minute APGAR scores (65). Children born after pregnancies affected by preeclampsia are noted to have higher systolic blood pressures and body mass indices (66). A recent study demonstrated that there is persistent abnormal circulation in the offspring of patients with preeclampsia, including elevated pulmonary artery pressures (67). In addition, a few small studies have demonstrated changes in brain structural and vascular anatomy along with evidence of cognitive changes (67, 68). More studies are needed on the long-term outcomes of those born from pregnancies affected by hypertensive disorders of pregnancy.

5. Prediction of preeclampsia and related adverse outcomes

5.1. Antepartum

Many evidence-based screening guidelines have been developed in an attempt to diagnose preeclampsia and identify those at highest risk of adverse events. Numerous studies have illustrated that prophylactic treatment with low-dose aspirin therapy provides a significant decrease in the risk of preeclampsia (69). Traditionally, low-dose aspirin therapy has been reserved for these patients at high risk of HDP, with initiation as early as 12 weeks gestation. More recent evidence suggests that universal aspirin or higher dose aspirin therapy may be warranted (69). Various countries have different screening protocols to identify patients who will benefit from aspirin therapy. Current screening in the United States consists of the identification of major and moderate risk factors for preeclampsia development (Table 2) (70). Patients with one major and more than one moderate risk factor for preeclampsia are considered candidates for aspirin therapy. While aspirin therapy provides a significant reduction in preeclampsia development, more aggressive screening strategies and diagnostic modalities may aid in the increased reduction of disease.

Validated screening methods can not only aid in early risk stratification for patients who may benefit from aspirin therapy but can also provide information about who are likely to develop preeclampsia (71). The triple test, as released by the Fetal Medicine Foundation, utilizes uterine artery pulsatility, biomarkers (PlGF), and mean arterial pressure in the first trimester to predict those at the highest risk of disease development later in pregnancy with remarkable results (71). With this test alone, a 90% detection rate for early preeclampsia and a 75% detection rate for the preterm disease were achieved with a 10% false-positive rate (71). This strategy has been validated in various populations and represents a new and more specific way to screen for patients at high risk of disease development during pregnancy (71).

Similarly, the utilization of Pregnancy-Associated Plasma Protein (PAPP-A) has demonstrated another route through which screening for preeclampsia may be accomplished (72). A large prospective study in India assessed uterine artery pulsatility and maternal serum PAPP-A in predicting preeclampsia development. This study found that among patients with preeclampsia, PAPP-A levels on average were higher than those without the disease, with a sensitivity of 28%, specificity of 90%, and a detection rate of 79% (72). The negative

TABLE 2 Clinical risk factors and risk stratification of patients.

Major Risk Factors
History of preeclampsia
Multifetal gestation
Chronic hypertension
Pregestational diabetes
Kidney disease
Autoimmune disease
Moderate Risk Factors
Nulliparity
Obesity
Family history of preeclampsia
Black race (representing systemic racism)
Lower income
>35 years of age
Personal history of low birth weight, previous adverse pregnancy outcome
>10 years between pregnancies
In vitro fertilization

Adapted from Davidson et al. (70).

predictive value of PAPP-A has been quoted to be as high as 97.55 with positive predictive value quoted at 2.95 (73). Uterine artery pulsatility was also significantly elevated in those who went on to develop preeclampsia, with a sensitivity of 68%, specificity 53, and 55% detection rate (72). The implementation of these types of first-trimester screening could further aid in the identification of patients who may derive the most benefit from prophylactic aspirin therapy or other antepartum intervention. Given the significant long-term health morbidity and mortality in those ultimately diagnosed with HDP, earlier detection to optimize prevention strategies represents an important way to improve health outcomes for patients.

In addition to early screening for preeclampsia prediction, current research focuses on strategies to predict those most likely to develop adverse outcomes related to their preeclampsia diagnosis (74, 75). Most of these studies focus on the exploitation of the proposed pathogenesis of HDP through the utilization of biomarkers to predict the development of worsening preeclampsia and adverse outcomes. As mentioned earlier in this review, sFlt-1 and PIGF are two biomarkers that have been implicated in the development of preeclampsia and have also been investigated as predictors for adverse outcomes related to preeclampsia (74). Studies have shown that levels of these markers, and specifically the ratio of sFlt-1/PIGF, are different in pregnancies affected by HDP (74, 76). Furthermore, these biomarkers are altered more significantly in patients with early-onset preeclampsia, and those with the early-onset disease generally have more severe long-term sequelae (75).

Given that preeclampsia with severe features in general results in more significant morbidity compared to lesser HDP, utilizing biomarkers may help aid clinicians in triaging patients to more aggressive therapy and monitoring or outpatient management. A cohort study in the United States investigated the predictive value of sFlt-1/PlGF among patients presenting to obstetrical triage for preeclampsia evaluation (77). A ratio of >38 in patients with suspected preeclampsia and patients diagnosed with preeclampsia without severe features while in triage was predictive of the development of preeclampsia with severe features within 2 weeks of presentation (OR 15.6%, confidence interval 8.91–27.40 for restrictive diagnosis, and OR 14.56% with 95% confidence interval 8.30–25.56 for broader diagnosis) (77). Similarly, another large study assessed the value of sFlt-1/PlGF in predicting progression to preeclampsia with severe features and identifying those at the highest risk of adverse maternal outcomes (78). In patients between gestational ages of 23 and 35 weeks, a ratio of >40 (PPV 65% [95% CI 59, 71] and NPV 95% [95% CI 93, 98]) similarly showed an increased risk in progression to severe disease, but also an increased risk in adverse maternal outcomes (78). Blood pressures alone in the antepartum and intrapartum period have a poor positive predictive value (PPV) for the accurate prediction of adverse outcomes (PPV 18–20% with antepartum and intrapartum blood pressures and 22–36% with antepartum blood pressures alone) (79). The development of machine-based learning models has also shown promise in identifying early- and late-onset preeclampsia as well as those at the highest risk of adverse outcomes (80–82); however, it is not readily available for clinical use. The integration of early screening for preeclampsia and biomarker use to aid in the determination of those at the highest risk of disease progression and adverse outcomes is becoming an important tool to improve the healthcare of pregnant persons suffering from preeclampsia.

5.2. Postpartum

Patients in the postpartum period remain at risk for development of HDP in the postpartum period and long-term sequelae related to a prior diagnosis of preeclampsia. In the postpartum period, there are tools that exist to aid in minimizing these adverse outcomes. Studies have illustrated that postpartum blood pressure monitoring in patients with chronic hypertension and HDP is a sustainable and important intervention for patients in the postpartum setting (83, 84). One tertiary care center implemented a postpartum blood pressure monitoring program that included standardized education and assisted follow-up, illustrating a dramatic increase in postpartum visit attendance (33.5% vs. 59.4%, $p < 0.001$) with more patients reporting blood pressures of <140/90 (39.1% vs. 18.5%, $p = 0.004$) (84). Importantly, when incorporating telemedicine, racial disparities in visit compliance were reduced, providing one mechanism through which health equity in the setting of postpartum care can be mitigated (85). Utilizing programs such as these might provide an important mechanism through which postpartum adverse outcomes can be prevented.

The American Heart Association recognizes that adverse pregnancy outcomes (APO's), including HDP, increase the risk of cardiac disease for the pregnant person (86). Though how exactly to incorporate a history of HDP in a formal risk evaluation is not clearly established, this organization recommends that providers caring for patients with a history of HDP recognize this as an important risk factor for future disease and place a strong emphasis on primary prevention of cardiac disease in these patients (86). Recommendations include heart healthy diet, maintaining an appropriate weight, and engaging in recommended amounts of physical activity (86). Even though clear guidelines do not exist regarding how to modify a patient's cardiovascular risk score with this history, the importance of recognition and close follow-up cannot be understated to help prevent long-term morbidity.

6. Conclusion

Preeclampsia and hypertensive disorders of pregnancy are diagnoses that contribute significantly to maternal and fetal morbidity and mortality. A better understanding of how to prevent and treat these disorders is crucial to improving maternal and fetal/child health. An accurate understanding of the development of preeclampsia and disease progression represents an important tenant in improving patient care and preventing adverse outcomes. In addition, future treatment modalities targeting known pathogenic mechanisms are an area ripe for future studies. Patients with preeclampsia carry an increased risk of major morbidity and mortality throughout their life beyond the immediate postpartum period, underscoring the importance of prevention and treatment of this disease. Preeclampsia has a significant impact on overall cardiac health and the development of future cardiovascular disease, development of chronic hypertension, hepatic and hematopoietic dysfunction, and renal and neurologic outcomes. This disease has important immediate and long-term implications for the neonate, further illustrating the importance of accurate treatment and prevention of preeclampsia. Given the enormity of this impact on short-term and long-term health, more research into prevention is needed along with an emphasis on long-term follow-up after a pregnancy complicated by HDP.

Author contributions

SR initiated the study conception and design. CB and SD wrote the initial drafts of the manuscript. EP and SS collected the references and edited the manuscript. PD made the figures for the manuscript and edited the manuscript. All authors have contributed to the writing and editing of the manuscript and reviewed and approved the submitted version.

Conflict of interest

SR reports serving as a consultant to Roche Diagnostics and Thermo Fisher Scientific and has received funding from Roche Diagnostics and Siemens for studies related to the use of angiogenic factors in pregnancy which is unrelated to work for this manuscript.

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

Publisher's note

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Sexually dimorphic pubertal development and adipose tissue kisspeptin dysregulation in the obese and preeclamptic-like BPH/5 mouse model offspring

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Preeclampsia (PE) is a devastating hypertensive disorder of pregnancy closely linked to obesity. Long-term adverse outcomes may occur in offspring from preeclamptic pregnancies. Accordingly, sex-specific changes in pubertal development have been described in children from preeclamptic women, but the underlying mechanisms remain vastly unexplored. Features of PE are spontaneously recapitulated by the blood pressure high subline 5 (BPH/5) mouse model, including obesity and dyslipidemia in females before and throughout pregnancy, superimposed hypertension from late gestation to parturition and fetal growth restriction. A sexually dimorphic cardiometabolic phenotype has been described in BPH/5 offspring: while females are hyperphagic, hyperleptinemic, and overweight, with increased reproductive white adipose tissue (rWAT), males have similar food intake, serum leptin concentration, body weight and rWAT mass as controls. Herein, pubertal development and adiposity were further investigated in BPH/5 progeny. Precocious onset of puberty occurs in BPH/5 females, but not in male offspring. When reaching adulthood, the obese BPH/5 females display hypoestrogenism and hyperandrogenism. Kisspeptins, a family of peptides closely linked to reproduction and metabolism, have been previously shown to induce lipolysis and inhibit adipogenesis. Interestingly, expression of kisspeptins (Kiss1) and their cognate receptor (Kiss1r) in the adipose tissue seem to be modulated by the sex steroid hormone milieu. To further understand the metabolic-reproductive crosstalk in the BPH/5 offspring, *Kiss1/Kiss1r* expression in male and female rWAT were investigated. Downregulation of *Kiss1/Kiss1r* occurs in BPH/5 females when compared to males. Interestingly, dietary weight loss attenuated circulating testosterone concentration and rWAT *Kiss1* downregulation in BPH/5 females. Altogether, the studies demonstrate reproductive abnormalities in offspring gestated in a PE-like uterus, which appear to be closely associated to the sexually dimorphic metabolic phenotype of the BPH/5 mouse model.

KEYWORDS

puberty, testosterone, *Kiss1/Kiss1r*, obesity, adiposity

1 Introduction

Preeclampsia, a leading cause of maternal and fetal morbidity and mortality, is a hypertensive disorder of pregnancy characterized by new-onset hypertension after 20 weeks of gestation (systolic blood pressure >140 mmHg and diastolic blood pressure >90 mmHg), and clinical signs secondary to either hematopoietic, renal, hepatic, pulmonary, or neurological compromising (Burton et al., 2019; ACOG, 2020). The incidence of severe preeclampsia in the United States has increased approximately 6.7-fold over the course of 3 decades (Annath et al., 2013). Correspondingly, the prevalence of obesity, a major risk factor for preeclampsia, has also increased globally in the same period, with a substantial rise in the population of overweight children and adolescents (Ng et al., 2014; Olson et al., 2019). Importantly, transgenerational effects of maternal obesity and preeclampsia have been gradually unraveled, including long-term cardiovascular, metabolic, neurological and endocrine dysfunctions in offspring gestated in an obesogenic and hypertensive uterine environment (Wu et al., 2017; Fox et al., 2019; Lu and Hu, 2019).

While many investigations have focused on mechanisms underlying the risk of adverse cardiometabolic consequences in offspring from obese and preeclamptic mothers, the potential long-term reproductive outcomes remain largely unexplored (Wu et al., 2017; Fox et al., 2019; Lu and Hu, 2019). Sex-specific abnormalities in pubertal development may occur in children born from preeclamptic pregnancies (Ogland et al., 2011; Alsnes et al., 2016). In a population-based study, increased body mass index (BMI) and altered progression of pubertal development were described in daughters from preeclamptic pregnancies, with the latter being positively associated with the severity of maternal disease (Ogland et al., 2011). Notably, the pattern of pubertal development of girls exposed to preeclampsia *in utero* seems to differ from changes in puberty previously reported in girls affected by juvenile obesity born from uncomplicated pregnancies (Ogland et al., 2011). Peripubertal boys and girls exposed to preeclampsia *in utero* have also presented sexually dimorphic alterations in blood concentration of androgens at 11–12 years of age when compared to children born from uncomplicated pregnancies (Alsnes et al., 2016). Importantly, hyperandrogenism has not only been linked to altered female pubertal development, but also to cardiometabolic disorders, including insulin resistance, obesity, hypertension, and preeclampsia (Ogland et al., 2011; Mouritsen et al., 2015; Iwasa et al., 2017; Kumar et al., 2018; Zeng et al., 2020). Nonetheless, the specific pathways in the crosstalk between reproductive and metabolic abnormalities in offspring from preeclamptic mothers remain vastly speculative.

Kisspeptins are a family of small peptides encoded by the *Kiss1* gene with sex-specific roles in reproduction and metabolism (Hussain et al., 2015; Dudek et al., 2018; Harter et al., 2018). Besides the central role of kisspeptins in the activation of the hypothalamic-pituitary-gonadal axis, this family of peptides appear to be important peripheral regulators of metabolism, energy expenditure, thermoregulation and adipogenesis (Hussain et al., 2015; Dudek et al., 2018; Harter et al., 2018; Izzi-Engbeaya et al., 2019; Hudson and Kauffman, 2022; Musa et al., 2022). *In vitro* studies suggest that kisspeptins may induce lipolysis and impair adipocyte glucose uptake in rodents and humans (Hussain et al., 2015; Pruszynska-Oszmalek et al., 2017; Dudek et al., 2018; Hudson and Kauffman, 2022). Interestingly, a sexually dimorphic metabolic phenotype has been reported in mice lacking functional kisspeptin receptor gene (*Kiss1r)* (Tolson et al., 2014; Tolson et al., 2016; Tolson et al., 2019). Namely, global *Kiss1r* knockout (KO) female mice display increased body weight, adiposity, serum leptin concentration and impaired glucose tolerance when compared to wild type littermates. Conversely, global *Kiss1r* KO males have similar body weight and glucose homeostasis as wild type counterparts (Tolson et al., 2014; Tolson et al., 2016; Tolson et al., 2019). Studies in gonadectomized rats suggest that both sex steroid hormones and nutrition modulate adipose tissue *Kiss1* (Brown et al., 2008). Therefore, similar to the central nervous system, we speculate that kisspeptins are also the "missing link" in the crosstalk between reproduction and metabolism in peripheral tissues (Hussain et al., 2015; Dudek et al., 2018; Harter et al., 2018; Izzi-Engbeaya et al., 2019; Hudson and Kauffman, 2022; Musa et al., 2022).

The Blood Pressure High Subline-5 (BPH/5) mouse is a well-established translational model of superimposed preeclampsia (Davisson et al., 2002; Sones et al., 2021). The BPH/5 mouse resulted from an eight-way cross of the mouse strains LP, SJL, BALB/c, C57BL/6, 129, CBA, RF, and BDP, followed by multiple generations of brother-sister matings (Schlager, 1973). BPH/5 females are spontaneously obese, dyslipidemic, hyperleptinemic, hyperphagic and hypertensive, a phenotype exacerbated during pregnancy (Davisson et al., 2002; Reijnders et al., 2019; Sones et al., 2021). Even though long-term adverse outcomes occur in both BPH/5 male and female offspring, a sexually dimorphic cardiometabolic phenotype has been elucidated (Sutton et al., 2017; Beckers et al., 2021). Interestingly, while the obese phenotype is perpetuated in BPH/5 female offspring, BPH/5 males do not present increased food intake, body weight, or reproductive white adipose tissue (rWAT) mass in comparison to controls (Sutton et al., 2017; Beckers et al., 2021). Importantly, the influence of obesity in the preeclamptic-like phenotype of the BPH/5 mouse model has been clearly established (Reijnders et al., 2019; Beckers et al., 2022). Of interest, maternal weight loss *via* a pair-feeding paradigm not only attenuates the BPH/5 mouse maternal inflammatory milieu and pregnancy outcomes, but also improves long-term consequences in the offspring in a sex-specific manner (Reijnders et al., 2019; Beckers et al., 2022).

In this study, the BPH/5 mouse model was utilized to further investigate long-term reproductive outcomes of *in utero* exposure to maternal obesity and a preeclampsia-like syndrome. It was hypothesized that BPH/5 progeny would present abnormal pubertal development when compared to C57BL/6 (C57) control mice. Furthermore, the sexually dimorphic obese phenotype of the BPH/5 mouse was further explored, and a potential link between nutrition, rWAT kisspeptin expression and sex steroid hormone profile was explored, utilizing the well-established BPH/5 pair-feeding paradigm (Reijnders et al., 2019; Beckers et al., 2022).

2 Materials and methods

2.1 Animal husbandry

Experiments were performed using virgin BPH/5 and control C57 males and females from in-house colonies. The normotensive C57 strain was used in the eight-way cross that originated the BPH/5 (Davisson et al., 2002). Peripubertal (3 weeks of age) and adult

(8–12 weeks of age) mice were housed in a climate-controlled environment (12-h light-dark cycle, 70.5–71°F) and fed a standard chow diet (Purina 5001 rodent chow: 23% crude protein, 4.5% crude fat, 6% crude fiber, and 8% ash, Neenah, WI) and *ad libitum* water. For studies using estrous cycle-staged females, vaginal cytology samples were collected daily from single-housed virgin BPH/5 and C57 adult females for at least two complete estrous cycles, in accordance with previous reports, and sample collection was performed during the first day of cytological proestrus (n = 3–8/group) (Caligioni, 2009; Cora et al., 2015). In accordance with the American Veterinary Medical Association Guidelines for the Euthanasia of Animals, the animals were humanely euthanized *via* carbon dioxide inhalation, followed by immediate cervical dislocation and exsanguination as secondary methods to ensure death (Leary et al., 2020). All animal procedures were approved by the Institutional Animal Care and Use Committee at Louisiana State University School of Veterinary Medicine and are in accordance with the PHS Guide for the Care and Use of Laboratory Animals.

2.2 Assessment of puberty

Peripubertal BPH/5 and C57 males and females were examined daily for signs of puberty starting at 2 weeks of age, utilizing clinical parameters previously described (Novaira et al., 2014). In females, visual assessment of vaginal opening was performed. In males, balanopreputial separation from the glans penis was assessed *via* gentle manual preputial retraction (Supplementary Figures S1B–D). Mice were weaned at 21 days of age, and body weight was recorded using a Gram scale. Post-weaning, littermates were housed in groups by sex. Four BPH/5 and C57 litters were used, with all males and females included in the study. Exclusion criteria included litters with less than four pups at weaning, and male or female mice singly housed post-weaning. Once clinical signs of puberty were noted, namely, vaginal opening or balanopreputial separation, age was recorded, and humane euthanasia was performed. Post-mortem, body weight was recorded, and vaginal opening or balanopreputial separation were confirmed. Testes in males, the female reproductive tract, including the uterus, uterine tubes and ovaries, and rWAT were dissected immediately after euthanasia and wet weights were recorded with an analytical balance (Ohaus EX324N/AD NTEP, Columbia, MD).

2.3 Pair-feeding protocol

Weight loss was induced in a cohort of non-pregnant adult BPH/5 females using a feeding paradigm previously established in this mouse model (Reijnders et al., 2019; Beckers et al., 2022). Briefly, the diet was restricted to 3 g of rodent standard chow (Purina 5001, Neenah, WI) per day for a total of 7 days. With this diet, pair-fed BPH/5 females (BPH/5 PF) are expected to consume 25% less calories than ad libitum-fed counterparts (BPH/5 AL), matching the food intake of lean, ad libitum-fed, C57 controls (Reijnders et al., 2019; Beckers et al., 2022). The non-pregnant pair-fed females were humanely euthanized the day after completion of the 7-day diet and sample collection was performed.

2.4 Histology

Testicles from adult BPH/5 and C57 mice were fixed in 10% formalin, paraffin embedded, sectioned and stained using hematoxylin and eosin (n = 3/group). Tissue architecture was evaluated by a Diplomate of the American College of Veterinary Pathologists that was blinded to the study design. Adipocyte histomorphometry was performed using the Zeiss Zen software (version 3.7), as previously described (Parlee et al., 2014). Specifically, five microscopic fields were randomly selected from each sample/subject by an observer blinded to the study design, and the number of adipocytes per field was calculated. Furthermore, five adipocytes were randomly selected from each microscopic field and the adipocyte diameter was recorded (n = 75 adipocytes/group).

2.5 Liquid chromatography

Blood was collected from peripubertal and adult BPH/5 and C57 mice *via* cardiac puncture, allowed to clot at room temperature for 60 to 90 min, and centrifuged at 3,500 rpm for 20 min. The serum was collected and cryopreserved at −80°C until further analysis. Serum was submitted for liquid chromatography with tandem mass spectrometry to assess 17β-estradiol and testosterone concentration at the Mayo Clinic Immunochemical Core (Rochester, MN).

2.6 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Samples of rWAT were collected from adult BPH/5 and C57 mice immediately after humane euthanasia. Aliquots were flash-frozen and cryopreserved at −80°C until further analysis. Genomic DNA was eliminated, and total RNA was extracted using TRIzol according to the manufacturer's instructions (Thermo Fisher Scientific, Wilmington, United States). The RNA ratio of absorbance and concentration were assessed using a NanoDrop Spectrophotometer (NanoDrop 200, ThermoFisher Scientific, Wilmington, United States) and 1,000 ng cDNA was synthetized using a commercial kit for reverse transcription (qScript cDNA, Quanta Biosciences, Gaithersburg, United States). Quantification of gene expression levels was performed by qRT-PCR using SYBR Green (PerfeCTa SYBR Green FastMix, Quanta Biosciences, Gaithersburg, United States). Each sample was run in triplicates and mRNA expression was normalized to 18 s and analyzed using the ddCT method (Livak and Schmittgen, 2001). Sequence-specific amplification was confirmed by a single peak during the dissociation protocol following amplification and by product size using gel electrophoresis. Gene targets and primer sequences are listed in Supplementary Table S1.

2.7 Statistical analysis

Data analyses were performed using GraphPad Prism, version 9.4 (GraphPad Prism Software, Inc., La Jolla, CA, United States). Student's t-tests were used for comparisons between age- and sex-

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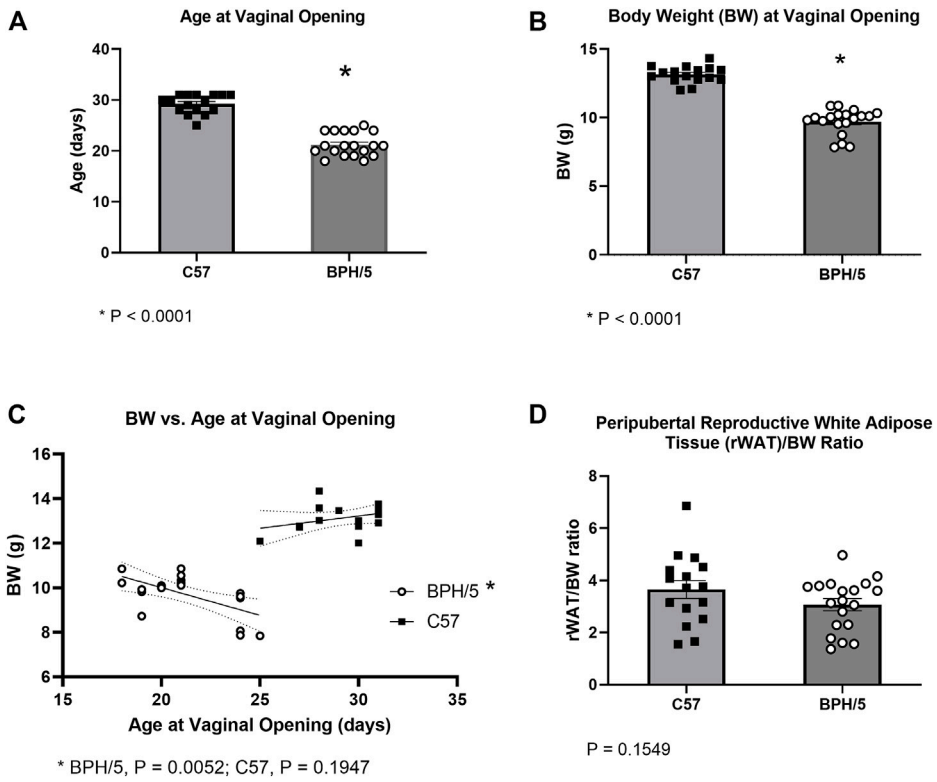

FIGURE 1
Precocious pubertal development occurs in the preeclamptic-like BPH/5 female offspring. **(A)** Age at the day of vaginal opening in BPH/5 and C57 female offspring (n = 16–19/group, Student's t-test, mean ± SEM, *p < 0.0001). **(B)** Body weight (BW) at the day of vaginal opening in peripubertal BPH/5 and C57 female offspring (n = 16–19/group, Student's t-test, mean ± SEM, *p < 0.0001). **(C)** A negative correlation occurs between BW and age at vaginal opening in BPH/5 females (n = 19, Simple linear regression with 95% confidence bands of best-fit line, p = 0.0052), while a significant correlation was not seen in C57 controls (n = 16, Simple linear regression with 95% confidence bands of best-fit line, p = 0.1974). **(D)** Reproductive white adipose tissue weight corrected to BW (rWAT/BW ratio) in peripubertal BPH/5 and C57 females at the day of vaginal opening (n = 16–19/group, Student's t-test, mean ± SEM, p = 0.1549).

matched BPH/5 and C57. Welch's corrections were performed for inequality of variances. One-way ANOVA and *post hoc* Tukey's test were used for age-matched group comparisons, and two-way ANOVA and *post hoc* Tukey's test were used for investigations of male mice potential age and strain interactions on testosterone concentration. Furthermore, a simple linear regression was performed between age at vaginal opening and BW within the groups of peripubertal BPH/5 and peripubertal C57 females. Logarithmic transformation was performed for data that did not meet the normality criteria. Normality of residuals from the models were accessed and confirmed *via* Shapiro-Wilk tests. Data are presented as mean ± SEM. Significance was set at $p < 0.05$.

3 Results

3.1 Pubertal development is altered in the preeclamptic-like BPH/5 mouse offspring in a sex-dependent manner

A total of four BPH/5 and four C57 litters were included in the cohort utilized for peripubertal investigations. Since one C57 litter contained a single male, only the females from that litter were included in further studies. Age at the time of vaginal opening (females) and balanopreputial separation (males) were used as clinical signs of onset of puberty. The BPH/5 female offspring presented precocious pubertal development in comparison to C57 counterparts. Mean age at vaginal opening was 21.21 ± 2.28 days in BPH/5 females (mean ± SD), compared to 29.25 ± 1.88 days in C57 females (Figure 1A, $p < 0.0001$). Since age at weaning was fixed at 21 days, many BPH/5 females achieved puberty before weaning, while pre-weaning vaginal opening was not observed in C57 progeny. Body weight and adiposity were further investigated in peripubertal BPH/5 females. At the onset of puberty, BPH/5 females had lower body weight than C57 controls (Figure 1B, $p < 0.0001$). While a negative correlation between body weight and age at vaginal opening occurred in BPH/5 females (Figure 1C, $p = 0.0052$), a significant correlation was not observed in the C57 group (Figure 1C, $p = 0.1947$). Remarkably, despite the lower body weight of BPH/5 females during onset of puberty, the corrected rWAT mass (rWAT/body weight ratio) was not different between BPH/5 and C57 at the day of vaginal opening (Figure 1D, $p = 0.1549$).

In males, age at balanopreputial separation, a testosterone-dependent event, was not different between BPH/5 and C57, with mean ages of 29.64 ± 1.20 and 29.71 ± 1.11 days, respectively

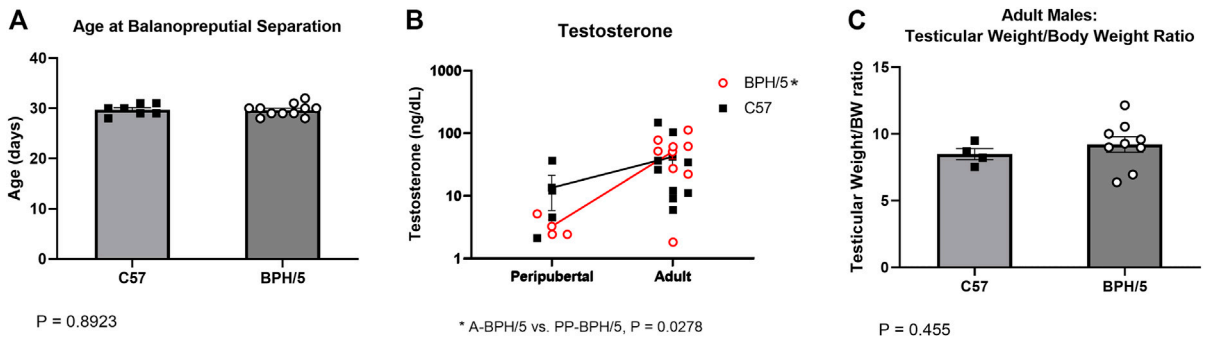

FIGURE 2
Precocious pubertal development does not occur in BPH/5 male offspring. **(A)** Age at the day of balanopreputial separation in peripubertal BPH/5 and C57 male offspring (n = 7–11/group, Student's t-test, mean ± SEM, p = 0.8923). **(B)** Serum testosterone concentration in peripubertal and adult BPH/5 and C57 males (n = 3–9/group, Two-way ANOVA, *post hoc* Tukey's test, mean ± SEM, *p = 0.0278 in adult BPH/5 vs. peripubertal BPH/5). **(C)** Combined testicular weight (i.e., both testes) corrected by body weight (Testicular weight/BW ratio) in adult BPH/5 males and age matched C57 (n = 8–17/group, Student's t-test, mean ± SEM, p = 0.455).

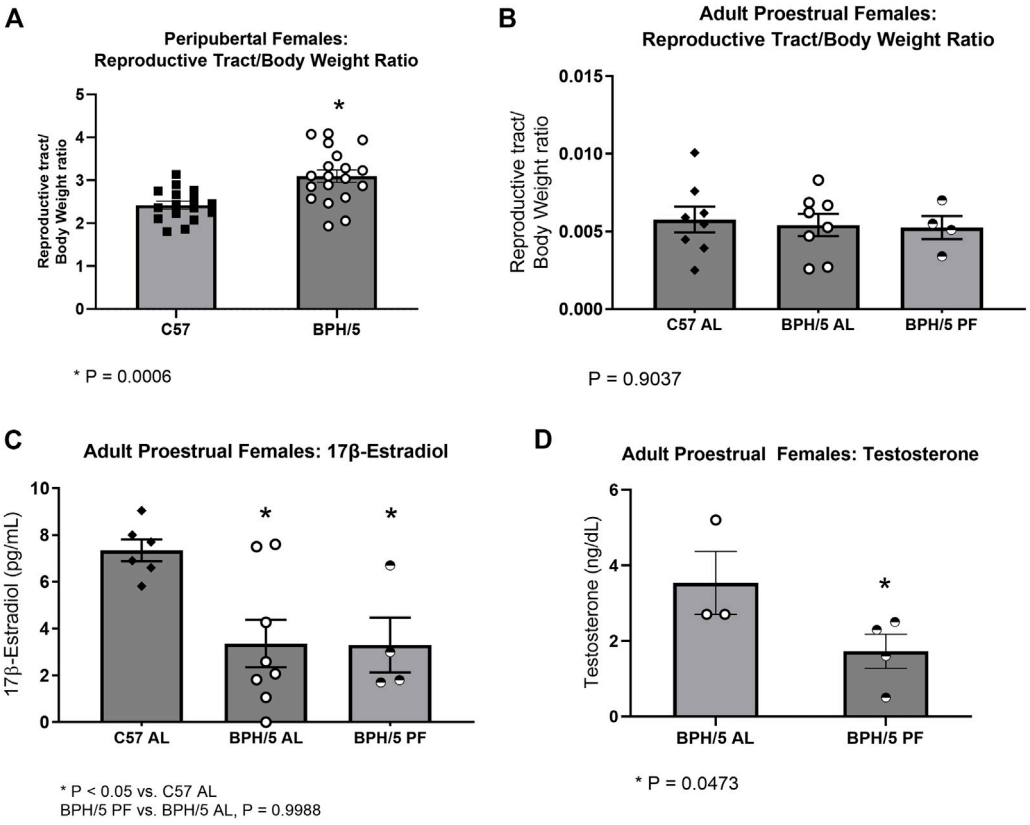

FIGURE 3
Female BPH/5 offspring present hyperandrogenism during adulthood, which is attenuated by dietary weight loss. **(A)** Reproductive tract wet weight corrected to body weight (BW) in peripubertal BPH/5 and C57 females (n = 16–19/group, Student's t-test, mean ± SEM, *p = 0.0006). **(B,C)** As previously reported (Sutton et al., 2017), adult ad libitum-fed BPH/5 females (BPH/5 AL) present lower serum 17β-estradiol and similar uterine wet weight to ad libitum-fed C57 (C57 AL) during proestrus. In this study, **(B)** reproductive tract/body weight ratio and **(C)** serum 17β-estradiol were unchanged in proestrual pair-fed BPH/5 females (BPH/5 PF) vs. BPH/5 AL females (n = 4–8/group, One-way ANOVA and *post hoc* Tukey's test, mean ± SEM, *p < 0.05 vs. C57 AL). **(D)** Serum testosterone concentration was below the assay detection limit of 2 ng/dL in proestrual C57 AL. Serum testosterone concentration was detected in proestrual BPH/5 AL females and was attenuated in BPH/5 PF females (n = 3–4/group, Student's t-test, mean ± SEM, *p = 0.0473).

(Figure 2A, p = 0.8923). Accordingly, serum testosterone concentration was not different between age matched BPH/5 vs. C57 males, ranging from 2.1 (min) to 36 (max) ng/dL (median = 4.5 ng/dL) in peripubertal animals, and 1.8 (min) to 146 ng/dL (median = 34.0 ng/dL) in adults (Figure 2B, $p > 0.05$). Nonetheless, testosterone concentration was significantly higher in adult BPH/5 males when compared to peripubertal BPH/5 (p = 0.0278), but not in adult C57 males when compared to peripubertal C57 (p = 0.3849). Corrected testicular weight was not different between adult BPH/5 and C57 males, and histopathological abnormalities were not noted (Figure 2C, p = 0.455).

3.2 Female BPH/5 offspring present hyperandrogenism during adulthood, which is attenuated by dietary weight loss

It has been previously shown that adult BPH/5 females present aberrant estrous cycles and decreased 17β-estradiol during proestrus (Sutton et al., 2017). Additionally, adult BPH/5 females present increased uterine wet weight during diestrus, which may be associated with tissue inflammation (Sutton et al., 2017). Interestingly, peripubertal BPH/5 females also present a higher reproductive tract wet weight at the day of vaginal opening (Figure 3A, p = 0.0006). To further characterize the endocrine profile of BPH/5 offspring, serum concentration of 17β-estradiol was investigated in peripubertal animals, but was below the assay detection limit (3 pg/mL) in all peripubertal BPH/5 and C57 females investigated. In addition to estrogens, androgens have an important role in the sex steroid hormone control of female pubertal development. There is also compelling evidence of a link between hyperandrogenism and metabolic disorders such as hyperphagia and obesity (Iwasa et al., 2017; Leeners et al., 2017). Similar to 17β-estradiol, however, serum testosterone concentration was below the assay detection limit (2 ng/dL) in all peripubertal animals studied.

We have previously shown attenuation of the obesogenic and inflammatory phenotype of BPH/5 females *via* dietary weight loss by either one or 2 weeks of pair-feeding to C57 counterparts (Sutton et al., 2017; Reijnders et al., 2019). Curiously, pair feeding adult BPH/5 females appear to be associated with regularization of estrual cyclicity. Namely, while irregular estrous cycles are commonly seen in ad libitum-fed BPH/5 females, approximately 80% of pair-fed BPH/5 females present regular 4–5 days cycles. Herein, the influence of dietary weight loss in the BPH/5 female sex steroid hormone profile was investigated. In accordance with previous reports, reproductive tract wet weight/body weight ratio was not different between ad libitum-fed BPH/5 and C57 females during proestrus and was not changed by dietary weight loss (Sutton et al., 2017) (Figure 3B, p = 0.9037). Likewise, hypoestrogenism was unchanged by controlled food intake. Specifically, the previously described lower serum 17β-estradiol concentration in BPH/5 during proestrus (Figure 3C, $p < 0.05$) was not altered by 7 days of pair feeding (Sutton et al., 2017) (Figure 3C, p = 0.9988). Interestingly, however, while serum testosterone concentration was lower than the assay detection limit (2 ng/dL) in adult C57 females, it ranged from 2.7 (min) to 5.2 (max) ng/dL (median = 2.7 ng/dL) in adult BPH/5 females during proestrus and was attenuated by 1 week of pair feeding (Figure 3D, p = 0.0473).

3.3 Sexually dimorphic adipocyte hypertrophy and altered kisspeptin/receptor expression occurs in reproductive white adipose tissue of adult BPH/5 offspring

Striking phenotypic similarities exist between the previously described global *Kiss1r* KO mice (Figure 4A) and the BPH/5 mouse model (Figure 4B), in a sex-dependent manner (Tolson et al., 2014; Tolson et al., 2016; Tolson et al., 2019). Specifically, adult BPH/5 females present markedly increased body weight, adiposity and serum leptin concentration in comparison to adult C57 females. Conversely, adult BPH/5 males have similar body weight than their control counterparts, adult C57 males. When investigating BPH/5 rWAT histomorphometry, adult BPH/5 females displayed increased mean adipocyte diameter when compared to males (Figure 4C, $p < 0.0001$) and, consequently, a lower number of adipocytes per microscopic field (Figure 4D, $p < 0.0001$), findings suggestive of adipocyte hypertrophy in females (Figures 4E, F) (Parlee et al., 2014). Considering the potential role of kisspeptins in adipocyte function, the expression of *Kiss1* and *Kiss1r* in the rWAT of BPH/5 male and female offspring was investigated. Both *Kiss1* and *Kiss1r* were downregulated in the rWAT of adult BPH/5 females when compared to age-matched males (Figures 4G, H, p = 0.0095 and 0.003, respectively). Of note, *Kiss1* downregulation was also seen in BPH/5 females when compared to sex- and age-matched C57 controls (Figure 5A, p = 0.0002), while rWAT *Kiss1* and *Kiss1r* were not different between adult BPH/5 and A-C57 males (Supplementary Figure 2, $p > 0.05$).

3.4 Reproductive white adipose tissue kisspeptin downregulation is ameliorated in BPH/5 females by dietary weight loss

While there is evidence that kisspeptins may function as regulators of lipolysis and adipogenesis, particularly in females, the upstream mechanisms modulating rWAT *Kiss1* expression remain largely unknown. The expression of *Kiss1*/*Kiss1r* seems to be directly associated with the sex steroid hormone milieu in the brain and multiple peripheral tissues, including the adipose tissue in gonadectomized rats, and the uterus and placenta of the BPH/5 mouse model (Hou and Gorski, 1993; Brown et al., 2008; Cejudo Roman et al., 2012; Baba et al., 2015; Hussain et al., 2015; Stephens et al., 2015; Dudek et al., 2018; Harter et al., 2018; Schaefer et al., 2021; Gomes et al., 2022). However, there is also evidence of a direct role of nutritional status on adipose tissue *Kiss1* expression, with upregulation promoted by high fat diet, and downregulation promoted by fasting (Brown et al., 2008). Therefore, the rWAT *Kiss1*/*Kiss1r* was also investigated in adult BPH/5 females subjected to 7 days of pair feeding to age- and sex-matched C57. In agreement with previous findings, rWAT *Kiss1* downregulation was attenuated in pair-fed BPH/5 females (BPH/5 PF), when compared to ad libitum-fed BPH/5 counterparts (BPH/5 AL, Figure 5A, p = 0.0054). While rWAT *Kiss1r* was not significantly lower in BPH/5 AL vs. C57 AL (Figure 5B, p = 0.1083), it was lower in BPH/5 PF females when compared to C57 AL (Figure 5B, p = 0.0186).

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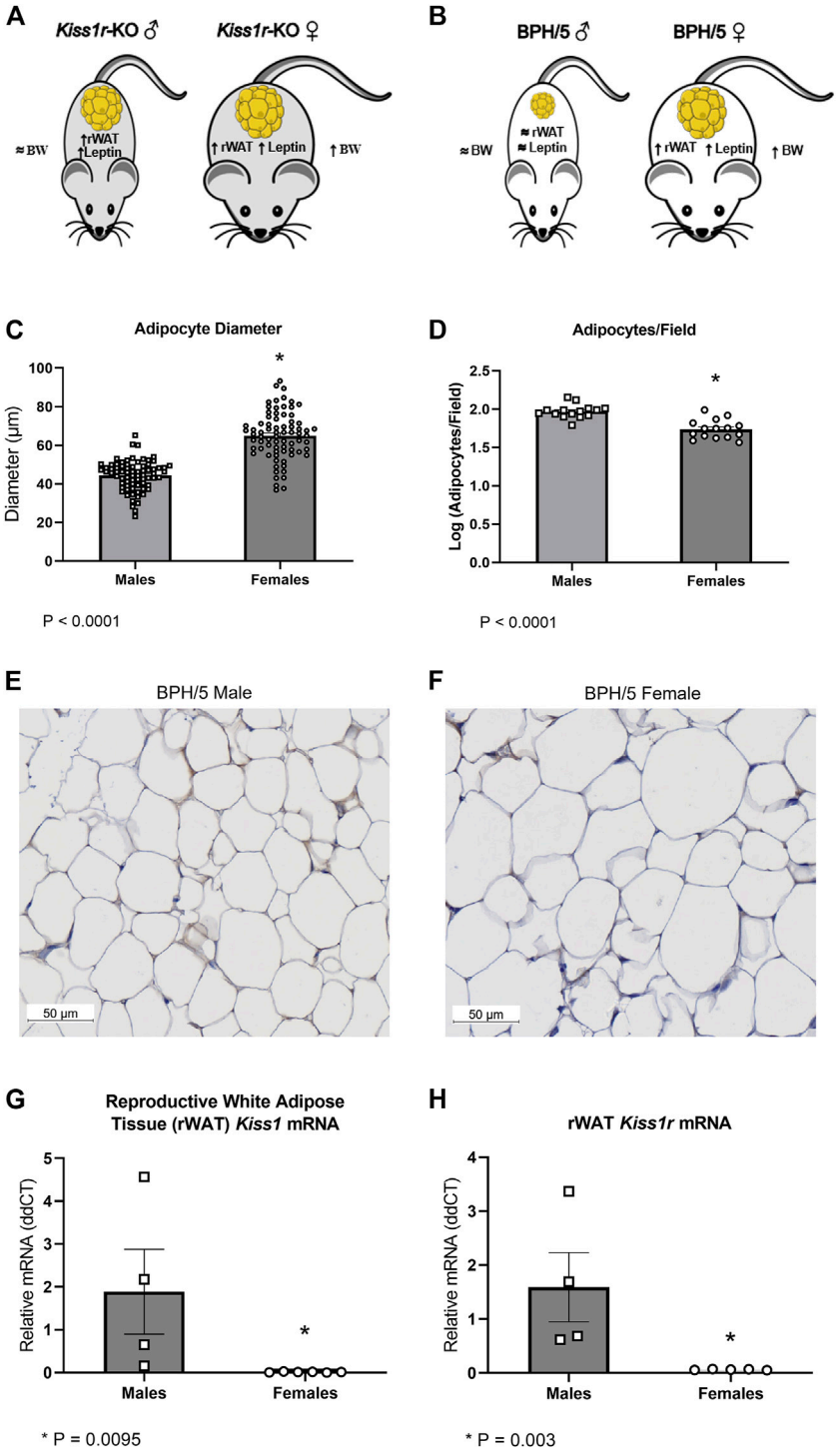

FIGURE 4
Sexually dimorphic adipocyte hypertrophy and altered kisspeptin/receptor expression occurs in reproductive white adipose tissue of adult BPH/5 offspring. **(A)** Previously described sexually dimorphic metabolic phenotype global *Kiss1r* knockout (KO) mice (Tolson et al., 2014; Tolson et al., 2016). **(B)** Sexually dimorphic metabolic phenotype of adult BPH/5 offspring. **(C)** Average adipocyte diameter (µm) in the reproductive white adipose tissue (rWAT) of BPH/5 males and BPH/5 females ($n = 75$ adipocytes/group, Student's t-test, mean ± SEM, $*p < 0.0001$). **(D)** Number of adipocytes per microscopic field in the rWAT of adult BPH/5 males and females ($n = 15$ fields/group, Student's t-test, mean ± SEM, $*p < 0.0001$). **(E,F)** Representative photomicrographs of rWAT of adult **(E)** BPH/5 males and **(F)** BPH/5 females (scale bar = 50 µm). **(G)** *Kiss1* relative mRNA expression in rWAT of adult BPH/5 males and females ($n = 4–6$/group, Student's t-test, mean ± SEM, $*p = 0.0095$). **(H)** *Kiss1r* relative mRNA expression in rWAT of adult BPH/5 males and females ($n = 4–6$/group, Student's t-test, mean ± SEM, $*p = 0.003$).

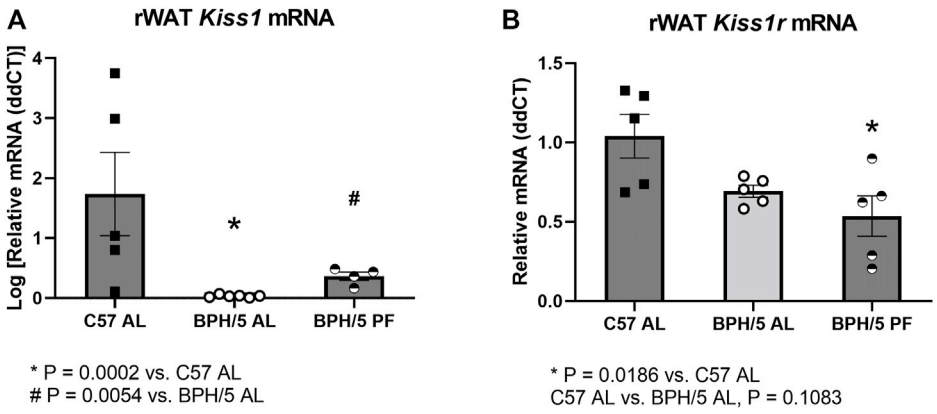

FIGURE 5
Reproductive white adipose tissue kisspeptin downregulation is ameliorated in BPH/5 adult females by dietary weight loss. **(A)** *Kiss1* is downregulated in ad libitum-fed adult BPH/5 female (BPH/5 AL) rWAT when compared to C57 AL. The downregulation is attenuated in BPH/5 female rWAT by pair feeding (BPH/5 PF) to mirror C57 AL food intake (n = 4–6/group, One-way ANOVA and post-hoc Tukey's test, mean ± SEM, *p = 0.0002 vs. C57 AL, # p = 0.0054 vs. BPH/5 AL). **(B)** rWAT *Kiss1r* expression was not different between BPH/5 AL and C57 AL, while rWAT *Kiss1r* was lower in BPH/5 PF when compared to C57 AL (n = 5/group, One-way ANOVA and post-hoc Tukey's test, mean ± SEM, *p = 0.0186 vs. C57 AL, p = 0.1083 in BPH/5 AL vs. C57 AL.

4 Discussion

In this study, the BPH/5 mouse model was used to further investigate transgenerational reproductive and metabolic outcomes of a preeclampsia-like syndrome. Herein, we provide evidence of sex-specific abnormalities of pubertal development in offspring prenatally exposed to an obesogenic and hypertensive uterine environment. In accordance with previous studies, the day of vaginal opening and balanopreputial separation were selected as clinical signs of onset of puberty (Lapatto et al., 2007; Caligioni, 2009; Novaira et al., 2014). While BPH/5 female offspring presented marked precocious pubertal development, age at balanopreputial separation was not different between BPH/5 male offspring and C57 controls. Accordingly, serum testosterone concentration and testicular wet weight were also similar between age matched BPH/5 and C57 males. As one could anticipate based on their younger age, BPH/5 females had lower body weight than C57 at the day of vaginal opening. Notably, however, peripubertal BPH/5 females presented similar rWAT/body weight ratio as age-matched C57, further highlighting the role of adiposity in pubertal development.

The correlation between body weight, body composition and puberty has long been recognized (Baker, 1985). Specifically, a minimum body weight and fat mass are required for onset and progression of pubertal development (Baker, 1985; Rosenfield et al., 2009). In peripubertal girls, normal age at onset of puberty ranges from 8–13 years old, and the normal progression of pubertal development is breast development, termed thelarche, followed by axillary and pubic hair growth (pubarche) and onset of menses (menarche) (Rosenfield et al., 2009; Ogland et al., 2011). There is a rising incidence of early onset of puberty in girls, which has been closely associated to juvenile obesity (Jasik and Lustig, 2008; Rosenfield et al., 2009). Notably, however, while obesity *per se* is associated with hastened thelarche, daughters from preeclamptic pregnancies may present an abnormal progression of pubertal development, with pubarche preceding thelarche, particularly in offspring from severe preeclampsia (Jasik and Lustig, 2008; Ogland et al., 2011). To date, the mechanisms and clinical significance of those findings are not known.

An adverse intrauterine environment has an important role in postnatal offspring outcomes of preeclampsia. Prenatal starvation and fetal growth restriction may lead to altered *in utero* metabolic programing, excessive compensatory growth, obesity, and adverse cardiovascular outcomes later in life (Jain and Singhal, 2012; Jensen et al., 2015). Correspondingly, children born from preeclamptic pregnancies often have higher BMI postnatally, and higher risk of developing obesity (Ogland et al., 2011; Yang et al., 2021). High rate of BMI increase from 6 to 12 years of age has been associated with increased risk of cardiovascular disease (Yuan et al., 2020; Yang et al., 2021). Additionally, the risk of preeclampsia is reportedly 3-fold higher in obese women (i.e., body mass index >30 kg/m^2) when compared to lean counterparts (Mbah et al., 2010). Since daughters from preeclamptic women are more prone to develop preeclampsia (Arngrimsson et al., 1990), it is speculated that fetal growth restriction, excessive compensatory growth and obesity in girls born from preeclamptic mothers may contribute to self-perpetuation of this syndrome.

Adverse fetal programing seems to be recapitulated by the preeclamptic-like BPH/5 mouse model. When reciprocal breeding crosses of BPH/5 and C57 pairs were performed, only embryos gestated in BPH/5 dams presented delayed embryonic development (Sones et al., 2016). Additionally, BPH/5 offspring are affected by intrauterine growth restriction, evidenced by fetal demise, smaller litter sizes, and marked decrease in birth weight when compared to C57 controls (Davisson et al., 2002). Postnatally, excessive compensatory growth from birth to early adulthood has been reported in BPH/5 females, but not males (Sutton et al., 2017; Beckers et al., 2021). Specifically, while BPH/5 females are smaller

than C57 controls at postnatal day 1, no difference in body weight is seen at 3 weeks of age, and higher body weight is displayed by 8-week-old BPH/5 females (Sutton et al., 2017). It is therefore speculated that higher growth rate and adiposity in BPH/5 females from birth to early adulthood may trigger earlier onset of puberty. Leptin, a metabolic hormone mainly derived from white adipose tissue and placenta, has an important role in metabolism and pubertal development, providing cues to the hypothalamic-pituitary-gonadal axis (Kiess et al., 1999; Messager et al., 2005; De Bond et al., 2016). Hyperleptinemia has been demonstrated in non-pregnant and pregnant BPH/5 females, but does not seem to occur in males, which is in agreement with the sexually dimorphic reproductive phenotype reported herein (Sutton et al., 2017; Reijnders et al., 2019; Beckers et al., 2021). Nonetheless, further investigation of serum leptin concentration and hypothalamic leptin signaling in the peripubertal BPH/5 mouse is warranted.

Hypoestrogenism and hyperandrogenism have been described in women carrying preeclamptic pregnancies and in daughters gestated in a preeclamptic uterus (Ogland et al., 2011; Alsnes et al., 2016; Berkane et al., 2018; Kumar et al., 2018; Keya et al., 2019). A protective metabolic role of estrogens in females is widely recognized, with low estrogens directly associated with increased body weight and adiposity (Leeners et al., 2017). Notably, high testosterone levels in females may further disrupt mechanisms that prevent hyperphagia and adiposity, leading to exacerbated food intake, leptin resistance, and obesity (Iwasa et al., 2017; Leeners et al., 2017). Additionally, hyperandrogenism seems to be associated with pubarche superseding thelarche in girls born from preeclamptic women (Mouritsen et al., 2015; Alsnes et al., 2016). Although 17β-estradiol and testosterone were below the assay detection limit in peripubertal females in this study, BPH/5 females presented higher reproductive tract/body weight ratios at the day of vaginal opening. It remains to be determined if the increased reproductive tract weight of peripubertal BPH/5 females is due to a sex steroid hormone imbalance or uterine inflammation, as suspected in adult BPH/5 (Sutton et al., 2017). Considering the previously reported irregular estrous cyclicity of adult BPH/5 females and hypoestrogenism during proestrus, this estrous cycle stage was selected for further sex steroid hormone profile characterization in BPH/5 female offspring (Sutton et al., 2017). Interestingly, hyperandrogenism accompanies hypoestrogenism in proestrual BPH/5 mice. The source of excessive androgens in girls born from preeclamptic mothers and in preeclamptic-like BPH/5 females is yet to be fully elucidated. Abnormal in-utero programing of steroidogenic enzyme activity, particularly aromatase, the rate-limiting enzyme in the conversion of androgens to estrogens, is speculated in preeclamptic offspring, and may also occur in the BPH/5 mouse model (Mouritsen et al., 2015; Alsnes et al., 2016; Berkane et al., 2018; Noyola-Martinez et al., 2019). Importantly, a metabolic-endocrine interplay is emphasized by the attenuation of hyperandrogenism in proestrual BPH/5 females after 1 week of dietary restriction.

Kisspeptins are considered the "gatekeepers" of the hypothalamic-pituitary-gonadal axis, since hypothalamic kisspeptin signaling is critical for pubertal development and reproduction in males and females (de Roux et al., 2003; Funes et al., 2003; Seminara et al., 2003; Lapatto et al., 2007; Tassigny et al., 2007). Besides their role in the central nervous system, kisspeptins seem to have important roles in peripheral metabolic tissues, including sex-specific regulation of adiposity (Hussain et al., 2015; Dudek et al., 2018; Wang et al., 2018; Izzi-Engbeaya et al., 2019; Hudson and Kauffman, 2022). Altered adipose tissue *Kiss1* expression has been previously associated with obesity in rats and humans (Brown et al., 2008; Cockwell et al., 2013). Furthermore, studies using *Kiss1r* KO mice have shown that impaired kisspeptin signaling leads to increased body weight, adiposity, and hyperleptinemia in adult female mice, along with decreased energy expenditure and impaired glucose tolerance (Tolson et al., 2014; Tolson et al., 2016; Tolson et al., 2019). Conversely, *Kiss1r* KO males display normal body weight and glucose homeostasis when compared to WT littermates (Tolson et al., 2014; Tolson et al., 2019). Herein, we have shown a sexually dimorphic *Kiss1/Kiss1r* dysregulation in the rWAT of preeclamptic-like BPH/5 offspring and hypothesize that rWAT kisspeptin downregulation is linked to obesity in BPH/5 females.

A series of *in vitro* studies using mouse (3T3-L1) and rat adipocytes have shown that kisspeptin-10 inhibits cell proliferation and viability, reduces the intensity of intracellular glucose uptake and triglyceride synthesis, and stimulates basal lipolysis (Pruszynska-Oszmalek et al., 2017). Histomorphometry of adult BPH/5 rWAT is suggestive of adipocyte hypertrophy in females when compared to males (Parlee et al., 2014). Further mechanistic investigations are warranted to confirm if lower levels of kisspeptin-10 in BPH/5 female offspring are associated with adipocyte glucose uptake, lipogenesis, and adipocyte engorgement. Although the upstream regulators of adipose tissue kisspeptin expression are poorly understood, studies suggest that adipose tissue Kiss*1* expression is influenced by nutrition and the sex steroid hormone milieu (Brown et al., 2008). Namely, exogenous administration of testosterone and 17β-estradiol to gonadectomized male and female rats, respectively, increased adipose tissue *Kiss1* expression (Brown et al., 2008). Additionally, while 6 h of fasting led to adipose tissue *Kiss1* upregulation, *Kiss1* downregulation occurred in rats after 19 days of high fat diet (Brown et al., 2008). Accordingly, both serum testosterone and rWAT *Kiss1* were ameliorated in a cohort of BPH/5 females subjected to dietary weight loss compared to ad libitum-fed counterparts. Hence, we speculate that rWAT *Kiss1* downregulation in BPH/5 females may result from an abnormal sex steroid hormone profile from puberty onward, somewhat "closing the circle" between reproduction and adipose tissue regulation in a preeclampsia-like syndrome. While mice globally lacking either functional *Kiss1* or *Kiss1r* have impaired pubertal development (i.e., hypogonadotropic hypogonadism), the BPH/5 mouse may be a suitable additional model to further study the crosstalk between reproductive hormones, adipose tissue kisspeptin dysregulation and adiposity (de Roux et al., 2003; Funes et al., 2003; Seminara et al., 2003; Tassigny et al., 2007; Tolson et al., 2019).

•In summary, beyond the long-term adverse cardiometabolic outcomes, offspring born to obese and preeclamptic-like BPH/5 dams also display sex-specific reproductive abnormalities during pubertal development and early adulthood. In BPH/5 female offspring, the crosstalk between metabolic and reproductive abnormalities seem to involve altered levels of sex steroid hormones and adipose tissue kisspeptin signaling, which may be consequences of abnormal fetal programming. Excitingly, the preeclamptic-like BPH/5 mouse model closely recapitulates clinical findings of children exposed to a hypertensive uterine environment and may be a suitable model to unravel the mechanisms underlying this altered phenotype.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at Louisiana State University School of Veterinary Medicine.

Author contributions

Conceptualization, VG and JS; methodology, VG and JS; validation, VG; sample collection, VG, KB, KC, CL, JF, and RA; data acquisition, VG, KC, CL, JF, and RA; investigation, VG, KC, and CL; resources, JS; data curation, VG and C-CL; writing: original draft preparation, VG; writing: review and editing, VG, KC, C-CL, and JS; visualization, VG, C-CL, and JS; supervision, JS; project administration, JS; funding acquisition, VG and JS. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1070426/full#supplementary-material

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Preeclampsia history and postpartum risk of cerebrovascular disease and cognitive impairment: Potential mechanisms

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Hypertensive disorders of pregnancy such as preeclampsia, eclampsia, superimposed preeclampsia, and gestational hypertension are major causes of fetal and maternal morbidity and mortality. Women with a history of hypertensive pregnancy disorders have increased risk of stroke and cognitive impairments later in life. Moreover, women with a history of preeclampsia have increased risk of mortality from diseases including stroke, Alzheimer's disease, and cardiovascular disease. The underlying pathophysiological mechanisms are currently not fully known. Here, we present clinical, epidemiological, and preclinical studies focused on evaluating the long-term cerebrovascular and cognitive dysfunction that affect women with a history of hypertensive pregnancy disorders and discuss potential underlying pathophysiological mechanisms.

KEYWORDS

preeclampsia, stroke, white matter lesions, postpartum, vascular dementia, Alzheimer's disease, blood-brain barrier

1 Introduction

The diagnosis of preeclampsia, a hypertensive disorder of pregnancy, has increased over 25% in the last 20 years (Ananth et al., 2013). Preeclampsia is characterized by new-onset hypertension presenting after the 20th week of gestation, with one or more of the following symptoms: proteinuria, low platelet count, kidney or liver abnormalities, or cerebral or visual symptoms (Gynecologists and Pregnancy, 2013). Based on the diagnosis criteria, one can appreciate that preeclampsia is a multi-organ disorder of pregnancy, affecting organs such as the lungs, kidneys, liver, and brain. Preeclampsia is the leading cause of fetal and maternal morbidity and mortality and affects 3%–8% of pregnancies (Tranquilli et al., 2012) and higher percentage of African American pregnancies. Preeclampsia can quickly progress to eclampsia, characterized by new-onset seizures or unexplained coma during pregnancy or early postpartum period. The Preeclampsia Foundation reports that eclampsia is one of the top five causes of maternal and infant morbidity and mortality, responsible for 13% of all maternal deaths worldwide (Nour, 2008). Despite these statistics, low dose aspirin is the only recommended prophylactic treatment for women at high risk of developing preeclampsia (Gynecologists and Pregnancy, 2013), and early delivery of the placenta and fetus still remains the primary course of intervention in these patients (Sibai et al., 2005; Sibai, 2006). Importantly, while the vast majority of preeclampsia cases occur in the antepartum period,

preeclampsia can be diagnosed in the postpartum period as well. Magnesium sulfate has shown efficacy in preventing seizures in women with preeclampsia with severe features; however, the effect of magnesium sulfate on other preeclampsia features such as increased anti-angiogenic factors or blood pressure remains mixed (Euser and Cipolla, 2009). Thus, there remains an urgent need for the development of novel therapeutic options for preeclampsia and eclampsia.

1.1 Preeclampsia is associated with neurological symptoms during pregnancy

Cerebral and visual symptoms are included as potential diagnosis criteria if presenting along with new-onset hypertension in pregnancy. Cerebrovascular complications are common findings in preeclampsia/eclampsia [(pre)eclampsia] patients. Indeed, of all preeclampsia-related deaths, cerebrovascular events are the main cause in about 40% of cases, with stroke and edema being most prevalent (MacKay et al., 2001). During pregnancy, preeclampsia patients are four times more likely to have a stroke compared to normotensive patients (James et al., 2005) with hemorrhagic stroke being more common than ischemic strokes. These disproportional findings have been reported in small studies in France (Sharshar et al., 1995) and Taiwan (Jeng et al., 2004). Importantly, preeclampsia is one of the most common risk factors for antepartum and postpartum stroke (Lanska and Kryscio, 1998; James et al., 2005). The underlying pathophysiological mechanisms contributing to stroke and other cerebrovascular complications during or after (pre)eclampsia-complicated pregnancies have not been fully elucidated. Nevertheless, some of the cerebrovascular changes and potential underlying mechanisms in hypertensive disorders of pregnancy have been covered in a review paper from our group (Jones-Muhammad and Warrington, 2019). In the current review, we focus on cerebrovascular and cognitive changes that occur in the early and late postpartum periods in women with a history of (pre)eclampsia and other hypertensive disorders of pregnancy, and discuss some potential mechanisms from clinical and preclinical studies.

1.2 A history of (pre)eclampsia is associated with increased incidence of stroke

Women with a history of preeclampsia are at higher risk of having a stroke during the non-pregnant, postpartum period, than women with a history of normotensive pregnancies (Tranquilli et al., 2012), and a history of (pre)eclampsia is associated with a two-fold increase in the likelihood of stroke later in life (Wu et al., 2017). Additionally, a 4-5 fold increased risk of stroke has been reported for women with a history of (pre)eclampsia compared to normotensive pregnant women (Hammer and Cipolla, 2015). Of note, hemorrhagic strokes are the most frequent type of stroke affecting (pre)eclampsia patients. In a small study of 27 preeclampsia patients, 25 had hemorrhagic stroke while only two had ischemic strokes (Martin et al., 2005). This increased risk of hemorrhagic stroke has also been documented with other hypertensive disorders of pregnancy such as chronic hypertension and gestational hypertension (Bateman et al., 2006). It should be noted that not only is overall risk of stroke increased, but a history of preeclampsia is associated with an increased risk of fatal strokes as opposed to non-fatal stroke.

In addition to hemorrhagic strokes, women with a history of preeclampsia also have an increased risk of ischemic stroke. In a population study, Brown et al. (2006) showed that women with a history of preeclampsia were over 60% more likely to suffer from ischemic stroke during the postpartum period than women without a history of preeclampsia (OR: 1.63; 95% CI: 1.02–2.62 after adjustment for age, race, education, and number of pregnancies). Another study looked specifically at the incidence and causes of stroke during the peripartum and postpartum periods and showed that although the incidence of intraparenchymal hemorrhages (4.6 per 1,00,000 deliveries) was similar to ischemic stroke (4.3 per 1,00,000 deliveries), eclampsia was the leading cause of intraparenchymal hemorrhage (44%) and ischemic stroke (47%) (Sharshar et al., 1995). These findings suggest that (pre)eclampsia may increase the risk of stroke after childbirth due to hemodynamic dysfunction. In a previous review article, the relative risk for fatal stroke events after preeclampsia was found to be greater than non-fatal stroke events (RR: 2.98; 95% CI: 1.11–7.96 and RR: 1.76; 95% CI: 1.40–2.22) (Bellamy et al., 2007). Keskinkılıç et al. (2017) reported that (pre)eclampsia/Hemolysis Elevated Liver enzymes Low Platelet count (HELLP)-related intracranial hemorrhage accounted for 41.3% of hypertension-related maternal death in Turkey. Likewise, similar results were reported in a nationwide study of pregnancy-related intracerebral hemorrhage (ICH) in Japan; 26.3% (n = 10) of ICH in pregnant women were related to preeclampsia. The prevalence of preeclampsia-related ICH mortalities was 40% (n = 4) during the acute postpartum period (Yoshimatsu et al., 2014). Taken together, these studies highlight the elevated risk for later-in-life hemorrhagic and ischemic stroke in women with a history of pregnancy-related hypertensive disorders.

1.3 Visual changes have been reported in preeclampsia patients

Headache and visual changes are two of the most common preceding symptoms to a (pre)eclampsia episode. Multiple case studies over the past 20 years have reported visual changes in (pre)eclampsia patients during the antepartum and postpartum period (Kesler et al., 1998; Garg et al., 2013; Bereczki et al., 2016; Borovac et al., 2016). Headaches during the peripartum and postpartum period, when associated with elevated blood pressure, tend to reflect an elevation in cerebellar perfusion pressure, cerebral edema, and encephalopathy (Gestational Hypertension and Preeclampsia, 2020). Cortical blindness is an acute complication with an incidence of 1%–15% in patients with severe preeclampsia (Borovac et al., 2016). In one case study, a (pre)eclampsia patient presented with double vision at the 32nd week of gestation. On subsequent postpartum fluid-attenuated inversion recovery (FLAIR) coronal magnetic resonance imaging (MRI), vasogenic edema in the occipital cortex was found to be the main cause of the cortical blindness. The patient eventually fully recovered with no edematous lesions visible on the 6 months follow-up MRI scan (Borovac et al., 2016). Two other case studies reported transient

cortical blindness presentation during the antepartum and postpartum period. In one case study, the patient's vision gradually returned to normal after cesarean delivery was performed, but in the other case study, vision loss did not improve until a month after delivery. The cause for the vision loss in these studies was found to be vascular endothelial damage seen on MRI scans (Kesler et al., 1998; Garg et al., 2013). A prospective study performed over a 14 year period also reported cortical blindness to be a further complication of (pre)eclampsia (Cunningham et al., 1995). The data on (pre)eclampsia-induced cortical blindness demonstrate the negative changes in the structural integrity of the visual pathway leading to transient damage.

1.4 A history of (pre)eclampsia is associated with cognitive changes

Previous studies have shown that childbirth is associated with increased vulnerability to psychiatric episodes such as depression, anxiety, and psychosis after delivery (Postma et al., 2014; Bergink et al., 2015). Furthermore, preeclampsia has an added risk of psychiatric episodes in postpartum mothers, increasing the incidence rate ratios (IRR) from 2.93 (95% CI: 2.53–3.40) in normotensive primiparous women to 4.21 (95% CI: 2.89–6.31) in preeclampsia patients (Bergink et al., 2015). Furthermore, the highest incidence rate ratios were reported in women with a history of preeclampsia plus a somatic co-morbidity, IRR = 4.81 (95% CI: 2.72–8.50). Several studies have demonstrated higher anxiety and depression scores in preeclampsia patients several years after pregnancy (Postma et al., 2014; Fields et al., 2017). One study measured anxiety using self-report inventories on a basis of physiological symptoms such as inability to relax, rapid movement of hands, feeling of heart racing, and/or catastrophic thinking (Fields et al., 2017). Using the Beck Depression Inventory, previously normotensive pregnant women had a score of 2.0, while women with a history of preeclampsia had a score of 4.0 (Fields et al., 2017). Additionally, normotensive women had a score of 1.5, while preeclamptic women had a score of 3.0. Although the data did not reach statistical significance in either of these tests, the score was doubled for preeclampsia patients compared to normotensive patients, demonstrating a trend for higher depression and anxiety in preeclampsia patients. In another study, the Hospital Anxiety and Depression Scales were used to measure the severity of emotional disorders and reported that women with (pre)eclampsia had significantly higher scores compared to normotensive controls on both scales (Postma et al., 2014). These studies demonstrate increased susceptibility for further psychiatric care during the postpartum period in (pre)eclamptic women even decades after pregnancy when compared to their normotensive counterparts.

Cognitive impairment can range from daily absent-mindedness to errors in motor function. Very few studies have assessed the frequency of cognitive failures in postpartum women with a history of (pre)eclampsia. Two specific studies have shown a significantly higher score on cognitive failure questionnaires (CFQs) for formerly (pre)eclamptic women compared to normotensive patients (Aukes et al., 2007; Postma et al., 2014). CFQs were administered to formerly eclamptic (n = 30), preeclamptic (n = 31), and normotensive (n = 30) participants years after pregnancy. CFQ scores were found to be significantly higher in eclamptic women vs. normotensive controls with distractibility being the highest sub-category (Aukes et al., 2007). Similarly, Postma et al. (2014) demonstrated that women with (pre)eclampsia had a higher incidence of cognitive difficulties in their daily life than normotensive women during a long-term follow up study. Furthermore, (pre)eclamptic women scored significantly higher than normotensive women on the cognitive failure questions with forgetfulness and distractibility being the highest sub-categories in (pre)eclampsia patients. Visuomotor speed, tested using trail making test, was worse in eclamptic women than preeclamptic women, but both patient groups scored significantly worse than normotensive controls. Likewise, in another postpartum study, slower processing speed was also deemed a postpartum residual effect for hypertensive pregnancy disorders such as (pre)eclampsia, gestational hypertension, and chronic hypertension (Mielke et al., 2016). The Digit Symbol Substitution Task and Trail Making Test- Part A were used to assess processing speed. Women with hypertensive pregnancy disorders scored worse on all measures of speed processing (Digital Symbol Substitution Test mean score of hypertensive pregnancy = 41.2; normotensive pregnancy = 43.4), Trail Making Test mean seconds hypertensive (pregnancy = 45.1; normotensive pregnancy = 42.4) (Mielke et al., 2016). Furthermore, women with eclampsia or preeclampsia with pulmonary edema had lower scores on the MoCA cognitive test at the time of discharge compared to normotensive pregnant women or preeclampsia patients without severe symptoms (Bergman et al., 2021c). A recent study demonstrated that 15 years after a hypertensive pregnancy disorder (gestational hypertension or preeclampsia), significant impairments in working memory was observed in women with a history of hypertension during pregnancy (Adank et al., 2021). These studies demonstrate the increased risk for a spectrum of cognitive impairments that can affect previously (pre)eclamptic women during the postpartum period.

1.5 Women with a history of (pre)eclampsia have increased risk of cerebral white matter lesions

Increased cerebrovascular vulnerability after (pre)eclampsia can lead to accumulated brain damage, manifested as brain lesions and changes in brain volume. White matter lesions (WMLs) are abnormalities seen as hyperintense areas on MRI, most commonly in the elderly community (Soma-Pillay et al., 2017). The etiology of white matter lesions in relation to (pre)eclampsia is unknown, but the presence of these WMLs has been observed in many cohort studies over the past several years (Wiegman et al., 2014; Siepmann et al., 2017) (Mielke et al., 2016) (Aukes et al., 2012; Soma-Pillay et al., 2017). These WMLs have also been reported to have a causal relationship to Alzheimer's disease as well (Abheiden et al., 2015; Soma-Pillay et al., 2017). WMLs have been observed in women with a history of hypertensive pregnancy disorders months to years after index pregnancy.

1.5.1 Months postpartum

In a longitudinal study performed in South Africa, cerebral WMLs were identified and their location determined in previously preeclamptic women (n = 94) (Soma-Pillay et al., 2017). MRI was performed after delivery, at 6 months, and at 1 year postpartum. At delivery, 61.7% of previously preeclamptic women had identifiable WMLs, with the majority of lesions found in the frontal lobe (60%).

Other locations of lesions included parietal lobe (28%) and occipital lobe (12%). At 6 months postpartum, 56.4% of previously preeclamptic women had identifiable WMLs. At 1 year postpartum, 47.9% had identifiable WMLs, with the majority of lesions (67%) located in the frontal lobe. Interestingly, the number of medications needed to control blood pressure during pregnancy was significantly associated with increased WMLs at 1 year postpartum.

1.5.2 Years postpartum

The distribution and severity of WMLs years after hypertensive pregnancy (Index since pregnancy: Eclampsia: 7.6 ± 4.7 years; Preeclampsia: 5.2 ± 4.1 years; Normal pregnant: 5.0 ± 3.3 years) (Wiegman et al., 2014) were assessed, revealing that women with a history of (pre)eclampsia were more likely to have WMLs [(pre) eclampsia = 34.4%; normotensive pregnancy = 21.3%], and these lesions were more severe than in women with normotensive pregnancy history [(pre)eclampsia = 0.07 mL; normotensive pregnancy = 0.02 mL]. Majority of lesions were observed in the frontal lobe followed by parietal, insular, and temporal lobes. Other studies have reported similar results in terms of the presence and severity of WMLs years after hypertensive pregnancy disorders (Aukes et al., 2012; Wiegman et al., 2014; Mielke et al., 2016; Siepmann et al., 2017).

In addition to WMLs, changes in regional and global brain volumes have been reported in women with a history of hypertensive pregnancy disorders. One specific study reported that women with previous hypertensive pregnancies were more likely to have smaller brain volumes than those with normotensive pregnancies years after pregnancy (Mielke et al., 2016). This same study reported a trend for greater mean WML volumes in prior hypertensive women compared to normotensive women, although the data did not reach statistical significance. The prevailing hypothesis for the formation of WMLs is that they may form as a result of vasogenic edema stemming from increased blood pressure. Vasogenic edema can lead to cytotoxic edema, or cell swelling. The presence of edema is a key finding in the pathogenesis of hypertensive pregnancy disorders (Aukes et al., 2007). For this reason, white matter lesions may be found in previously hypertensive patients years after pregnancy, and can indicate residual cognitive impairments, while also increasing risk for stroke later in life (Kitt et al., 2021). In a study of the Framingham offspring assessing WMLs and hippocampal volume, WML volume increased the odds of detecting mild cognitive impairment (MCI) at baseline by 48% [OR: 1.48; 95% CI: 1.03–2.12]. Similar odds were found in the amnestic group with MCI (Bangen et al., 2017). MCI has been considered a transitional state between the normal aging process and Alzheimer's disease (AD). Thus, these data support the hypothesis that WMLs have a causal relationship to AD through the transitional state of MCI.

In a retrospective case-control study, there was no significant difference between women with or without AD who reported previous hypertensive pregnancy disorders; however, there was a significant difference in a sub-analysis of women with early onset AD (20.4%) reporting hypertensive pregnancy disorders vs. late-onset AD (5.2%). Early-onset AD has been associated with a different pathophysiology than late-onset AD including: increased cognitive deterioration, WMLs, and higher genetic load (Abheiden et al., 2015). Other studies have shown a link between history of preeclampsia and risk of mortality from AD (Theilen et al., 2016). Other studies showed no increased risk of AD (Basit et al., 2018) or dementia (Nelander et al., 2016), although an increased risk of vascular dementia was reported in women with a history of preeclampsia (Basit et al., 2018). Furthermore, when women with a history of preeclampsia was divided based on whether they had a small for gestational age infant, the risk of vascular dementia was seven-fold higher in those with a small for gestational age infant. This relationship was not changed when AD risk was considered (Basit et al., 2018). The previous studies suggest there is evidence of long-term presence of WMLs in previously hypertensive disorders of pregnancy, and these WMLs can cause cognitive impairments that are more likely to be seen in early-onset AD patients. More long-term studies should be performed in order to determine the increased risk of residual cognitive changes due to WMLs in previously (pre)eclamptic patients.

1.6 Conundrum: Why antepartum changes are predominantly posterior cerebral and postpartum changes are predominantly anterior cerebral?

In the postpartum period, white matter lesions tend to occur predominantly in the frontal lobe compared to any other brain region following (pre)eclampsia (Postma et al., 2014). During pregnancy, however, neurological findings in (pre)eclampsia patients occur mostly in the posterior cortical or subcortical region (Sharshar et al., 1995) including the parietal-occipital lobe (Topuz et al., 2008; Aygün et al., 2010; Mitas and Rogulski, 2012) (Figure 1). While not as frequent, anterior/frontal lobe abnormalities have also been reported in (pre)eclampsia patients during pregnancy (Riskin-Mashiah and Belfort, 2005). It is not known why the affected regions are different during antepartum versus postpartum periods. A histological study of postmortem vasculature revealed that posterior cerebral vessels (vertebral and basilar arteries) were structurally different from the anterior cerebral vessels, with posterior cerebral vessels having features of outward remodeling (Roth et al., 2017). While the study did not assess sex differences or directly assess the contribution of prior hypertensive pregnancy disorder, the presence of structural differences between anterior and posterior vessels is enough to hypothesize that structural differences could potentially explain the conundrum of posterior cerebral abnormalities in antepartum and anterior cerebral abnormalities in postpartum periods. It is necessary that preclinical and clinical studies consider these regional differences when designing studies.

1.7 Does eclampsia confer increased risk of postpartum cerebrovascular abnormalities?

The experience of seizures during pregnancy or in pregnant epileptic patients, carries increased risks. One prospective study compared dynamic cerebral autoregulation in pregnant women with eclampsia, preeclampsia with severe features, preeclampsia patients without severe features, and normotensive controls and reported decreased dynamic cerebral autoregulation in eclampsia

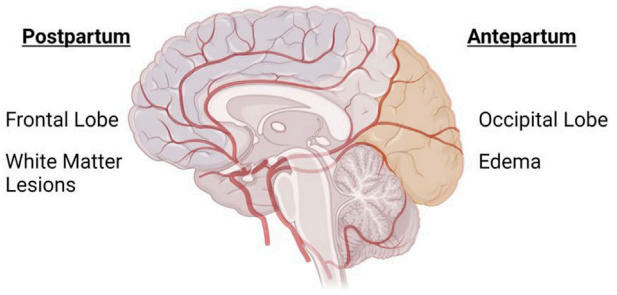

FIGURE 1
Anterior and Posterior cerebrum are differentially affected in antepartum and postpartum periods in preeclampsia. Image shows an overlay of the cerebral vasculature over the brain, highlighting the different areas of the brain that are primarily affected during (pre) eclampsia-complicated pregnancy. During pregnancy, the posterior cerebrum (occipital and parietal lobes) is most commonly affected, while white matter lesions are more prevalent in the anterior cerebrum/frontal lobe of women with a history of (pre)eclampsia. Created with BioRender.com.

patients [3.9(3.1–5.2)] compared to preeclampsia patients with severe features [5.6(4.4–6.8)], preeclampsia patients without severe features [6.8(5.1–7.4)], and normotensive controls [7.1(6.1–7.9)] (Bergman et al., 2022). While there have not been many studies assessing the specific risks of eclampsia to postpartum cerebrovascular injury, there is some evidence that eclampsia poses a higher risk of long-term cerebrovascular complications. For example, during pregnancy, 25/27 eclampsia patients had evidence of vasogenic edema on MRI scans; and out of 6 of these patients with infarction on imaging during pregnancy, 5 had hyperintense lesions at 6–8 weeks follow-up (Zeeman et al., 2004). Interestingly, 5 out of 6 patients with evidence of infarctions during pregnancy had experienced multiple seizures, suggesting that the severity of seizures may proportionally influence the level of damage. A case study reported an eclamptic patient who experienced vision loss, severe headache, and hemiparesis 4 days after delivery (Garg et al., 2013). While her cerebral vasoconstriction reversed, her vision did not improve up to 1 month later. Moreover, Postma et al. (2014) showed that while formerly (pre)eclampsia patients perform worse on the motor function domain of neurocognitive tasks and reported more cognitive failures than controls, there was no difference in scores between prior preeclampsia and eclampsia patients. Thus, the experience of seizures in eclampsia does not seem to impact motor function any more than preeclampsia in the postpartum years, but has worse cerebrovascular effects. Studies should be designed to assess postpartum sequelae in eclampsia patients in countries where eclampsia rates are still high.

1.8 Multiple pregnancies, maternal comorbidities, and onset of preeclampsia

Because a single pregnancy complicated by (pre)eclampsia increases the risk of significant neurological complications postpartum, one can speculate that having multiple pregnancies complicated by (pre)eclampsia would lead to worse outcomes for the mother. Indeed, women who had preeclampsia multiple times had an increased risk of having a stroke (Brouwers et al., 2018). Interestingly, women with two prior pregnancies affected by preeclampsia were ten times more likely to use blood pressure medication at follow-up (Magnussen et al., 2009). Studies designed to directly address this possibility are required.

Overweight [body mass index (BMI) 25–30] and obese (BMI > 30) pregnant women have a higher risk of developing preeclampsia [1.44(1.28–1.62) for overweight and 2.14(1.85–2.47) in obese] and postpartum hemorrhage [1.16(1.12–1.21) and 1.39(13.2–1.46)] (Sebire et al., 2001). Other studies have reported similar increased risk of preeclampsia, gestational hypertension, and gestational diabetes in obese and morbidly obese pregnant women (Weiss et al., 2004). Taken together, studies are needed to directly compare incidence of cerebrovascular abnormalities in women with a history of preeclampsia/eclampsia with no comorbidities versus those with preeclampsia/eclampsia and different comorbid conditions during pregnancy.

Women with early-onset preeclampsia are at a higher incidence of higher blood pressure, BMI, and abnormal lipid profiles at 9–16 years from their index pregnancy (Bokslag et al., 2017). Studies have not assessed whether early-onset preeclampsia confers greater risk of cerebrovascular abnormalities in later life compared to late-onset preeclampsia. Because early-onset preeclampsia is generally associated with severe symptoms, one could hypothesize that women who had early-onset preeclampsia would have worse cerebrovascular complications later in life compared to women with a history of late-onset preeclampsia. Studies should be designed to address this possibility.

1.9 Potential mechanisms contributing to increased risk of cerebrovascular impairments following (pre)eclampsia

Because preeclampsia is a hypertensive disorder, increased blood pressure, especially sudden, acute spikes, results in increased transmission of pressure to the delicate cerebral micro-vessels, leading to increased blood-brain barrier (BBB) permeability (Aygün et al., 2010; Cipolla et al., 2011). During pregnancy, (pre) eclampsia patients present with impaired cerebral blood flow autoregulation, edema, and features consistent with BBB disruption on imaging studies (Apollon et al., 2000; Demirtaş et al., 2005; Aygün et al., 2010; Mitas and Rogulski, 2012). It is possible that if vascular repair mechanisms are impaired, complete recovery of the cerebral vasculature does not occur and women enter the postpartum period with sub-clinical damage to the cerebral micro-vessels. Furthermore, if cerebral blood velocity remains elevated, there is opportunity for continuous and cumulative damage to cerebral blood vessels and neural cells. Indeed, there is evidence that this might be the case. Giannina et al. (1997) showed that at 6 and 12 weeks postpartum, cerebral and ophthalmic vessels from preeclampsia patients were still subjected to higher blood velocities compared to those from women who had normal pregnancies. This demonstrates that vessels are susceptible to pressure-induced damage in the early postpartum period. Left

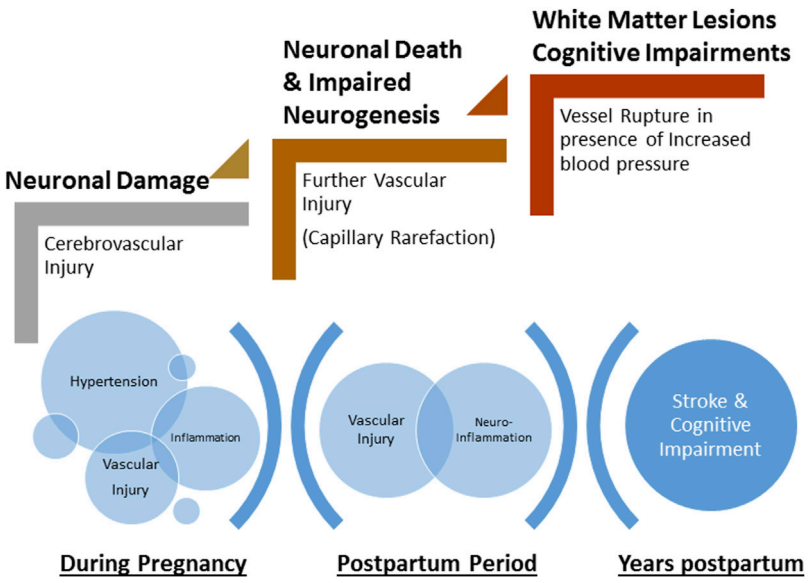

FIGURE 2
Schematic of the progressive damage to neurons and cerebrovasculature during pregnancy and the postpartum period. Hypertension during pregnancy and associated inflammation contributes to cerebrovascular injury. Incomplete repair of neurovasculature and persistent inflammation lead to further injury including capillary rarefaction and neuronal death during early postpartum period. Microscopic damage and late-life hypertension exacerbate neurovascular damage leading to rupture of vessels (stroke) and cognitive impairments.

unrepaired, vascular injury can be exacerbated with time, contributing to long-term neurological complications (Figure 2).

Furthermore, (pre)eclampsia is considered an inflammatory disease, as affected women present with increased levels of circulating pro-inflammatory factors (Szarka et al., 2010; Cipolla et al., 2012; Pinheiro et al., 2013; Ferguson et al., 2017) and increased endothelial cell activation compared to normotensive pregnancies (Bergink et al., 2015). Endothelial cell activation increases vulnerability of the blood-brain barrier and induces functional changes to neurotransmitter metabolism and synaptic adaptability (Bergink et al., 2015). Acute disruption of the BBB was also observed in patients with posterior reversible encephalopathy syndrome in response to abrupt increases in blood pressure (Aygün et al., 2010). These pathophysiological changes can lead to changes in the structural integrity of the blood vessels and weaken the neurological circuitry. Thus, any structural damage to the cerebral micro-vessels could result in leakage of plasma constituents into the brain parenchyma, inducing neuroinflammation. Previous studies have shown that plasma from women diagnosed with preeclampsia increases the permeability of isolated cerebral veins from non-pregnant rats (Amburgey et al., 2010; Schreurs and Cipolla, 2013; Schreurs et al., 2013) and induces neuroinflammation (Cipolla et al., 2012). Taken together, leakage of plasma constituents into the cerebral parenchyma, as occurs with BBB disruption, can induce neuroinflammation and the cerebral and visual symptoms associated with (pre)eclampsia.

The blood-brain barrier (BBB) is formed by the close association of endothelial cells that line the blood vessels, connected *via* tight junctions. Smooth muscle cells or pericytes surround the endothelial cells and are further contacted by astrocytic end feet (Figure 3). Under physiological conditions, blood constituents such as albumin is kept within the vessels; however, following damage to the BBB, albumin and blood cells, can leak out of the vessels into the brain parenchyma, causing neuronal damage. Studies have shown that extracellular vesicles (exosomes) from preeclampsia patients can induce increased permeability in human endothelial cell culture monolayers and increase permeability to Evans blue dye in C57BL/6 non-pregnant mice (Leon et al., 2021). Furthermore, when endothelial cells were treated with plasma from preeclampsia patients, increased permeability was observed (Bergman et al., 2021a). Other studies that have assessed markers of neuronal injury or neuroinflammation in women with preeclampsia or eclampsia during pregnancy have shown increased pro-inflammatory cytokines in cerebrospinal fluid from preeclampsia and eclampsia patients compared to normal pregnant women (Bergman et al., 2021b). Increased plasma concentration of different markers of glial or neuronal damage were found in women with preeclampsia compared to normal pregnant women (Friis et al., 2022). Taken together, clinical and preclinical studies demonstrate increased BBB permeability in preeclampsia with evidence of involvement of extracellular vesicles, pro-inflammatory cytokines, and other factors during pregnancy.

In the postpartum period, circulating inflammatory factors are no longer different between women who had early preeclampsia vs. normal pregnancy at 1–3 years postpartum (van Rijn et al., 2016). Importantly, an inflammatory challenge, induced in response to influenza vaccination, resulted in an exaggerated immune response in women with a history of early preeclampsia compared to those with a normal pregnancy (van Rijn et al., 2016). Together, these studies suggest that while increased pro-inflammatory factors are no longer different in women a year after early preeclampsia diagnosis,

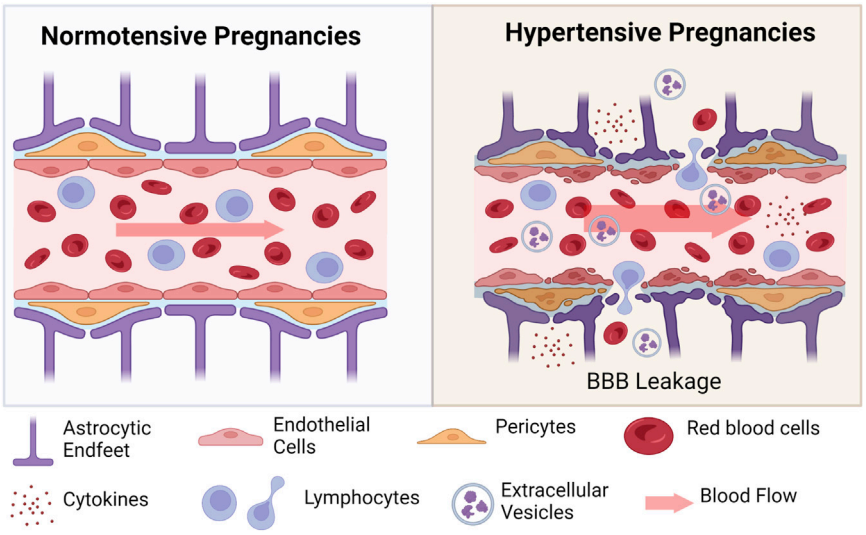

FIGURE 3
Schematic of changes at the blood-brain barrier in hypertensive pregnancies. During hypertensive pregnancies, all cells of the neurovascular unit are impacted. Endothelial cells, pericytes, and astrocytic end feet are damaged under conditions of increased blood flow (indicated by wider arrow). Damage to these cells results in extravasation of blood cells (red blood cells and various lymphocytes) into the surrounding tissue. Inflammatory cytokines and anti-angiogenic factors are also secreted into the cerebral tissues. Created with BioRender.com.

an inflammatory challenge induces a heightened response. This observation is in line with the idea of a "second hit" phenomenon in which pregnancy is considered a stressor for women, leading to subclinical damage, exacerbated by later physiological challenges such as postpartum hypertension, obesity, diabetes, or other condition (Ferguson et al., 2017). The case for the second hit phenomenon contributing to some of the increased cerebrovascular risk factors in postpartum preeclampsia patients has been made (Ferguson et al., 2017). Thus, these later life challenges result in the manifestation of neurological disorders such as stroke, cognitive impairment, and vascular dementia.

1.10 Leveraging pre-clinical studies to uncover underlying mechanisms

Clinical studies have demonstrated that preeclampsia is associated with reduced utero-placental perfusion, thought to be a result of incomplete remodeling of the maternal uterine spiral arteries (Roberts and Gammill, 2005). During normal pregnancy, spiral arteries within the maternal uterus become less resistant and more compliant to allow increased blood flow to the developing placenta and fetus. This process is incomplete in preeclampsia, leading to placental and fetal hypoxia. Consequently, the ischemic placenta releases numerous factors (pro-inflammatory and anti-angiogenic) into the maternal circulation, resulting in the hallmark features of the disorder (Reviewed in (Warrington et al., 2013). To model reduced uterine perfusion, the reduced uterine perfusion pressure (RUPP) model was developed in the rat (Granger et al., 2006) and mouse (Intapad et al., 2014; Fushima et al., 2016; Jones-Muhammad et al., 2021). Preclinical studies geared at identifying postpartum cerebrovascular and cognitive changes following a (pre)eclampsia-like pregnancy are sparse. Using the rat RUPP model, persistent vascular dysfunction in isolated mesenteric arteries at 1 month and 3 months postpartum (Brennan et al., 2016) was reported. Our group later reported that at 2 months postpartum, dams subjected to the RUPP procedure during pregnancy had features of posterior cortical edema and neuroinflammation compared to sham control rats (Clayton et al., 2018). In both studies, blood pressure was no longer different between sham controls and rats subjected to RUPP in the postpartum period, demonstrating that persistent hypertension was not the underlying contributor to the vascular dysfunction observed. To our knowledge, there are no reports of cognitive function or cerebrovascular function in RUPP rats or mice in the postpartum period, although these studies are ongoing in the authors' laboratory.

Another rodent model of preeclampsia has been used specifically for the study of superimposed preeclampsia. Superimposed preeclampsia is diagnosed in women with chronic hypertension who then go on to develop other preeclampsia symptoms after the 20th week of gestation. The pregnant Dahl salt sensitive (Dahl-SS/Jr) rat has been described as a model of spontaneous superimposed preeclampsia as it displays clinical features of preeclampsia including hypertension, proteinuria, placental hypoxia, increased sFlt-1 and TNF-alpha, and fetal growth restriction (Gillis et al., 2015). These rats also display features of disruption of cerebral endothelial of tight junctions and increased BBB permeability (Maeda et al., 2021). Our group later showed that in the postpartum period, after 2 pregnancies, Dahl-SS/Jr rats have increased pial vascular-associated microglia/macrophages compared to the normotensive Sprague Dawley rats (Warrington et al., 2022), hinting to morphological and cellular changes that are consistent with neuroinflammation.

While several pharmacological and genetic models have been described in the literature (Marshall et al., 2018), these studies focus

primarily on the antepartum changes or changes in the offspring. In order to determine whether specific pathways or circulating factors contribute to the postpartum cerebrovascular sequelae, investigators ought to perform further investigations in postpartum dams after the circulating factors are removed. Using a rat model of experimental preeclampsia induced by cholesterol diet (2%) at day 7 of gestation, investigators demonstrated that 5 months postpartum, rats in the experimental preeclampsia group displayed impairments in memory and had impaired vascular reactivity to vasoactive substances (Johnson et al., 2022).

The anti-angiogenic protein, Soluble fms-like tyrosine kinase -1 (sFlt-1), has been shown to be elevated in pregnant women (Maynard et al., 2003; Rana et al., 2012). This factor sequesters the pro-angiogenic factors, vascular endothelial growth factor and placental growth factor that are important for placental vascular formation and growth during the entirety of pregnancy. Increased sFlt-1 in pregnant animals induce hypertension and fetal growth restriction and reflect pathogenesis closely associated with preeclampsia (Maynard et al., 2003; Lu et al., 2007). Moreover, reduction of sFlt-1 levels in preeclampsia patients has shown promise in delaying early delivery (Thadhani et al., 2011). While animal models of elevated sFlt-1 mimics some of the clinical characteristics, none of the studies have looked beyond pregnancy to assess postpartum neurological changes induced specifically by increased sFlt-1 during pregnancy.

2 Conclusion and perspectives

The studies presented in this review have highlighted the increased risk of neurological complications in women with a history of hypertensive disorders of pregnancy, specifically, (pre) eclampsia. During pregnancy, cerebral and visual symptoms are reported, and is now used as a possible diagnosis criterion if combined with new onset hypertension. In contrast to what was believed where delivery of the placenta and fetus was a “cure” for preeclampsia, emerging epidemiological and clinical studies now show increased risk for long-term neurological disorders after a pregnancy complicated by (pre)eclampsia. New research should endeavor to establish the long-term pathophysiological link between previously hypertensive pregnancy disorders and long-term residual complications. Furthermore, various interventions and therapeutics are warranted to determine whether specific pathways can be targeted to prevent the long-term neurovascular and cognitive changes in women with a history of hypertensive pregnancy disorders.

Author contributions

Manuscript Draft (AB), Editing and reviewing (AB, MM, and JW), Approval of Final Manuscript Version (AB, MM, and JW).

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Conflict of interest

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

Publisher’s note

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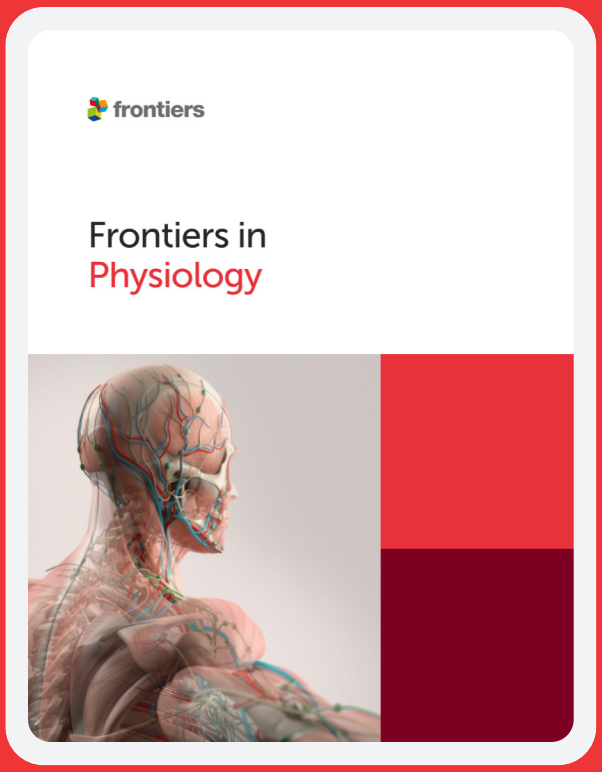

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