ADVANCE IN TRANSLATIONAL RESEARCH OF PRETERM BIRTH AND RELATED PREGNANCY

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ADVANCE IN TRANSLATIONAL RESEARCH OF PRETERM BIRTH AND RELATED PREGNANCY

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Editorial: Advance in translational research of preterm birth and related pregnancy

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Editorial on the Research Topic
Advance in translational research of preterm birth and related pregnancy

Spontaneous preterm delivery accounts for approximately 8% of all live births worldwide (GBD 2016 Causes of Death Collaborators., 2017), which is the leading cause of perinatal mortality and the second major cause of death in children under 5 years old. Survivals of preterm birth are often faced with short- and long-term morbidity (Slomski, 2021). Adverse environmental exposure and changes in fertility policies may lead to a gradual increase in the premature birth rate (Liu et al.,2019; Deng et al., 2021). Infection-induced inflammation is thought to be the primary cause of rupture of membranes and uterine contraction which consequently results in preterm birth (Kim et al., 2015; Bian et al., 2021; Xu et al., 2021; Xie and Ying, 2020; Pan et al., 2020). However, due to the lack of sufficient understanding of the underlying mechanism, current diagnosis and treatment are often based on clinical symptoms, which sometimes yields inconsistent outcomes.

With the emergence of new technologies such as multi-omics analysis (Wang et al., 2022), searching for novel marker genes and signaling pathways related to premature delivery has become a research hotspot (Wang et al., 2018). As a result, a number of novel and pivotal signalers have been revealed, which may be of potential value for the determination of gestational age and prediction of delivery time. Metabolites and humoral factors derived from the fetus *per se* or fetal appendage appear to be particularly appealing (Chen et al., 2020; Liang et al., 2020; Wang et al., 2018). These findings offer deep insight into the mechanism of preterm birth and expand new visions for the prediction of preterm birth.

While uterine contraction inhibitors and antibiotics are important drugs for the treatment of preterm labor (Sharp and Alfirevic, 2014), the effect of hormones on promoting fetal lung maturation in premature infants has been verified over a wider

range (WHO ACTION Trials Collaborators et al., 2020). Besides, the protective effect of aspirin or progestogens for preventing preterm birth was also found (EPPPIC Group, 2021; Hoffman et al., 2020). Faced with the complex mechanism and heterogeneity of clinical manifestations of spontaneous preterm birth, further optimization and clarification of clinical treatment remain highly warranted, which requires the translational investigation of the mechanism underlying preterm birth.

Therefore, this Research Topic aims to provide the readers with research evidence of effective treatment and novel mechanisms of spontaneous preterm birth, with emphasis on the inflammation of maternal and fetal tissues. Through the hard work of contributors, reviewers, and editors, a total of 9 articles were eventually accepted for publication, including 3 reviews and 7 original studies.

In terms of the pathogenesis of preterm birth, especially inflammation and infection, Saito Reis et al. demonstrated that fetal DNA related to genders, fragment size, and methylation status might cause inflammation in the fetal membranes *via* increased activation of NF-κB, MMP activity, and cytokine secretion, which broadens the theory of the role of fetus in the initiation of labor. A retrospective cohort study of singleton pregnancies with preterm premature rupture of membranes (PPROM) by Matulova et al. investigated the correlation between acute inflammation of the amnion and fetus birth weight, and their findings further supported that the severity of acute inflammation of the amnion may alter the growth of the fetus. Stranik et al. utilized ultrasound-guided transabdominal intra-amniotic administration of a triggering agent to establish a rat model of intra-amniotic inflammation, and they characterized that the concentration of interleukin (IL)-6 in the amniotic fluid may be of critical value to study intra-amniotic inflammatory complications and precisely mimic different specific clinical scenarios. Kacerovsky et al. analyzed 217 women's cervical secretions and amniotic fluid and found that the presence of intra-amniotic infection, sterile intra-amniotic inflammation, or colonization of the amniotic fluid was associated with a higher prevalence and/or load of *Ureaplasma* spp. DNA in the cervical fluid than the absence of intra-amniotic complications in PPROM at <34 weeks, which confirmed the important role of vaginal microbiota in preterm birth-related diseases and its significance in clinical diagnosis and treatment (Keelan et al., 2015). Meanwhile, in terms of antibiotic treatment, there was consistent inconsistency in outcome selection and reporting in studies about antibiotics in PROM. Therefore, Liu et al. formed an initial core outcome set for antibiotics in PROM through a systematic review and semi-structured interview, which could improve the research quality of PROM and provide a reference for research on infection in pregnant women.

In addition, the mechanism of other related treatments has also attracted our attention. Yin et al. explored the feasibility of measuring the uterine and peripheral artery diameters after the administration of different doses of ephedrine using CT and showed that the peripheral artery contracts under the action of ephedrine, whereas the common clinical dose of ephedrine has no significant effect on the diameter of the uterine artery, which will provide a reference for the scientific and rational use of ephedrine in the clinic and, ultimately, improve the safety of patients and fetuses undergoing cesarean section required for premature delivery. The review by Devvanshi et al. highlighted the prospects of exosomes as therapeutic tools and early diagnostic markers at the immune level in adverse pregnancies such as PPROMs. A systematic review and meta-analysis of randomized evidence by Xinyu et al. indicated that prophylactic use of motherwort injection may reduce the risk of uterine hemorrhage in women after abortion, which suggested the potential effect of traditional Chinese medicine. Hsu et al. demonstrated that pregnancy stress was significantly lower in pregnant women who were receiving tocolytic treatment than in those who were receiving non-tocolytic treatment among both who used complementary medicine. This finding can be used as a reference for future studies on pregnant women's health.

In conclusion, this Research Topic has provided new experimental data and updated reviews related to translational research of preterm birth and related pregnancy. These studies further advance our understanding of spontaneous preterm birth pathogenesis. The evidence gathered from this Research Topic is also expected to be translated into more accurate and effective clinical approaches to predict and treat preterm birth in the future.

Author contributions

HX wrote the manuscript. HY revised the manuscript. All the authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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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Acute Histological Chorioamnionitis and Birth Weight in Pregnancies With Preterm Prelabor Rupture of Membranes: A Retrospective Cohort Study

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Aim: To assess the association between the birth weight of newborns from pregnancies with preterm prelabor rupture of membranes (PPROM) and the presence of acute histological chorioamnionitis (HCA) with respect to the: i) fetal and maternal inflammatory responses and ii) acute inflammation of the amnion.

Material and Methods: This retrospective cohort study included 818 women with PPROM. A histopathological examination of the placenta was performed. Fetal inflammatory response was defined as the presence of any neutrophils in umbilical cord (histological grades 1–4) and/or chorionic vasculitis (histological grade 4 for the chorionic plate). Maternal inflammatory response was defined as the presence of histological grade 3–4 for the chorion-decidua and/or grade 3 for the chorionic plate and/or grade 1–4 for the amnion. Acute inflammation of the amnion was defined as the presence of any neutrophils in the amnion (histological grade 1–4 for the amnion). Birth weights of newborns were expressed as percentiles derived from INTERGROWTH-21st standards for the i) estimated fetal weight and ii) newborn birth weight.

Results: No difference in percentiles of birth weights of newborns was found among the women with the women with HCA with fetal inflammatory response, with HCA with maternal inflammatory response and those without HCA. Women with HCA with acute inflammation of the amnion had lower percentiles of birth weights of newborns, derived from the estimated fetal weight standards, than women with HCA without acute inflammation of the amnion and those with the absence of HCA in the crude (with acute inflammation: median 46, without acute inflammation: median 52, the absence of HCA: median 55; $p = 0.004$) and adjusted ($p = 0.02$) analyses. The same subset of

pregnancies exhibited the highest rate of newborns with a birth weight of ≤25 percentile. When percentiles were derived from the newborn weight standards, no differences in birth weights were observed among the subgroups.

Conclusion: Acute inflammation of the amnion was associated with a lower birth weight in PPROM pregnancies, expressed as percentiles derived from the estimated fetal weight standards.

Keywords: amnion, intergrowth, neutrophils, placenta, preterm delivery

INTRODUCTION

Preterm prelabor rupture of the membranes (PPROM) is defined as the rupture of fetal membranes with leakage of amniotic fluid before the onset of regular uterine activity prior to 37 weeks of gestational age (Mercer, 2003; Mercer, 2005). PPROM represents a phenotype of spontaneous preterm delivery that complicates approximately 3–4% of all pregnancies (Mercer, 2003; Mercer, 2005). Despite its predominantly non-infectious nature, PPROM might be complicated by the presence of acute inflammatory changes in the amniotic fluid and/or acute inflammatory lesions of the placenta (Cobo et al., 2012; Musilova et al., 2015).

The presence of acute inflammatory lesions of the placenta is characterized by diffuse infiltration of neutrophils in any of the structures of the placenta (fetal membranes, the placental disc, and the umbilical cord) and collectively are called acute histological chorioamnionitis (HCA) (Kim et al., 2015). Depending on the primary source of infiltrating neutrophils, HCA can be divided into the following: i) maternal inflammatory response, when extravasating maternal neutrophils infiltrate fetal membranes and/or the chorionic plate and ii) fetal inflammatory response, when extravasating fetal neutrophils invade the vessels of the chorionic plate and/or in structures of the umbilical cord (Kim et al., 2015).

The fetal inflammatory response is known to be the most severe form of HCA that is associated with adverse neonatal outcomes (Kim et al., 2015). It is also considered a histopathological counterpart of the fetal inflammatory response syndrome (Pacora et al., 2002). Apart from the fetal inflammatory response, a specific subtype of HCA, infiltration of the amnion by neutrophils has been shown to be related to very intense inflammatory responses, measured by various markers in the amniotic fluid and umbilical cord blood, regardless of the concurrent presence or absence of funisitis (Park et al., 2009). Therefore, two subtypes of HCA–with a fetal inflammatory response and acute inflammation of the amnion, should be of utmost clinical interest since they are associated with the most severe inflammatory responses.

It is obvious that placental lesions other than HCA (maternal and fetal vascular malperfusion, placental hemorrhage, and chronic villitis) mainly lead to impaired placental functions, which can be followed by an alteration of fetal growth (Salafia et al., 1989; Salafia et al., 1992; Salafia et al., 1995; Tyson and Staat, 2008; Mifsud and Sebire, 2014; Novac et al., 2018; Aviram et al., 2019). Collectively, these lesions represent underlying pathologies for conditions known as either small-for-gestational-age (SGA) or fetal growth restriction (FGR).

Nevertheless, some studies have provided evidence for the relationship between impaired fetal growth and HCA (Williams et al., 2000; Levy et al., 2021). This unexpected association is further supported by the following observations: i) lower birth weight is associated with upregulation of genes encoding proinflammatory transcription factor activator protein-1 (Ross et al., 2019); ii) the presence of HCA is found in approximately 10% of SGA pregnancies (DiGiulio et al., 2010); iii) a higher number of placental macrophages and increased placental inflammatory profile are found in pregnancies with impaired fetal growth or FGR (Street et al., 2006; Street et al., 2008; Sharps et al., 2020); iv) elevated concentrations of inflammatory markers in umbilical cord blood may be found in SGA newborns (Amarilyo et al., 2011; Lausten-Thomsen et al., 2014).

However, there is a shortage of information on whether the presence of HCA, particularly its most severe forms, with fetal inflammatory response and acute inflammation of the amnion, is related to impaired fetal growth in pregnancies complicated by PPROM. To fill this knowledge gap, a study on women with singleton pregnancies complicated by PPROM was conducted with the following goals: i) to assess birth weight, expressed as percentiles, and to compare the rates of the percentiles of birth weight that are less than or equal to the first, 10th, and 25th percentiles with respect to the presence of HCA with fetal and maternal inflammatory responses and the absence of HCA; ii) to assess birth weight, expressed as percentiles, and to compare the rates of the percentiles of birth weight that are less than or equal to the first, 10th, and 25th percentiles with respect to the presence of HCA with and without acute inflammation of the amnion and the absence of HCA; and iii) to assess birth weight, expressed as percentiles, and to compare the rates of the percentiles of birth weight that are less than or equal to the first, 10th, and 25th percentiles with respect to the severity of acute inflammation of the amnion.

METHODS

This study was a retrospective cohort study conducted in pregnant women with PPROM admitted to the Department of Obstetrics and Gynecology, University Hospital Hradec Kralove in the Czech Republic between May 2008 and March 2021 who met the following criteria: i) singleton pregnancy; ii) gestational age at admission between 24 + 0 weeks and 36 + 6 weeks; iii)

maternal age ≥18 years; iv) available histopathological results of the placenta. The exclusion criteria were as follows: i) pregnancy-related complications such as gestational diabetes, gestational hypertension, or preeclampsia; ii) chronic diseases such as pregestational diabetes and chronic hypertension; iii) structural or chromosomal abnormalities of the fetus.

Gestational age was determined based on the first-trimester ultrasound scan. The diagnosis of PPROM was established based on visual confirmation of amniotic fluid pooling in the posterior vaginal fornix by a sterile speculum examination. If uncertainty persisted after the clinical examination, the leakage of amniotic fluid was confirmed or ruled out using a test to determine the presence of insulin-like growth factor-binding protein in the vaginal fluid (Actim PROM test; Medix Biochemica, Kauniainen, Finland).

Women with PPROM at less than 34 weeks of gestation were treated with antibiotics and corticosteroids to accelerate lung maturation. Tocolytics were used only when regular uterine activity appeared during the course of corticosteroids, but not for longer than 48 h. Women with PPROM beyond 34 weeks of gestation were treated with antibiotics only. The women included in this study were managed using two different approaches. Between May 2008 and December 2013, women were treated actively (except those at < 28 gestational weeks). Labor was induced, or an elective cesarean section was performed after finalizing corticosteroid treatment but no later than 72 h after the rupture of the membranes, depending on the gestational age, fetal status, and maternal serum C-reactive protein concentrations. Since January 2014, the performance of transabdominal amniocentesis to assess the status of the intra-amniotic environment (microbial invasion of the amniotic cavity and intra-amniotic inflammation) has been a routine part of the clinical management of women with PPROM (Musilova et al., 2017). Thus, women admitted between January 2014 and May 2021 were managed differently. Women with intra-amniotic infection (the presence of both microbial invasion of the amniotic cavity and intra-amniotic inflammation) beyond the 28th gestational week were managed actively (labor was induced, or an elective cesarean section was performed after finalizing corticosteroid treatment within 72 h of membrane rupture for pregnancies before 34 weeks of gestation and within 24 h of membrane rupture for those beyond 34 weeks). The remaining women with PPROM were managed expectantly (Musilova et al., 2017).

After delivery, the placenta, fetal membranes, and umbilical cord were fixed in 10% neutral buffered formalin. Tissue samples were obtained from the placenta (at least two samples), fetal membranes (one sample from the free margin of membranes, one from the central part of the membranes, and one from the membranes with a marginal part of the placenta), and umbilical cord (usually one sample), which were routinely processed and embedded in paraffin. Sections of the tissue blocks were stained with hematoxylin and eosin.

The degree of neutrophil infiltration was evaluated separately in the free membranes (amnion and chorion-decidua), chorionic plate, and umbilical cord based on the criteria provided by Salafia et al. (Salafia et al., 1989). Histopathological examinations were performed by a single pathologist (HH) who was blinded to the clinical status of the women.

The collection of clinical samples and information was approved by the Ethics Committee of the University Hospital of Hradec Kralove, Czech Republic (19 March 2008; No. 200804 SO1P, which was renewed in July 2014 and January 2019, decisions No. 201407 S14P and No. 201902 S16P, respectively). Written informed consent was obtained from all the participants. Biological samples (amniotic fluid, cervical fluid, and umbilical cord blood) from the women included in this study were used in our previous studies and are presented in the publications. A total of 528 women from this cohort were included in our previous publication, where an association between birth weight and microbial invasion of the amniotic cavity and/or intra-amniotic inflammation was evaluated (Matulova et al., 2021). All methods used in this study were carried out in accordance with the relevant guidelines and regulations.

Birth Weight Percentiles

All newborns were weighed immediately after birth using a calibrated electronic scale. Birth weights were converted to percentiles derived from the INTERGROWTH-21st standards (Villar et al., 2014a; Villar et al., 2014b; Papageorghiou et al., 2014; Stirnemann et al., 2017) for the: i) estimated fetal weight (Papageorghiou et al., 2014; Stirnemann et al., 2017) and ii) newborn birth weight (Villar et al., 2014a).

Clinical Definitions

HCA was diagnosed based on the histological grade 3-4 for the chorion-decidua and/or grade 3-4 for the chorionic plate and/or grade 1–4 for the umbilical cord and/or grade 1–4 for the amnion (Salafia et al., 1989). Based on the type of the inflammatory response, women with the presence of HCA were further subdivided into those with: i) fetal inflammatory response—the presence of histological grade 1–4 for the umbilical cord (any neutrophils present in the umbilical cord) and/or histological grade 4 for the chorionic plate (chorionic vasculitis) and ii) maternal inflammatory response—the presence of histological grade 3-4 for the chorion-decidua and/or grade 3 for the chorionic plate and/or grade 1–4 for the amnion. Based on the presence or absence of acute inflammation of the amnion, women with the presence of HCA were further divided into those: i) with acute inflammation of the amnion–the presence of histological grade 1-4 for the amnion and ii) without acute inflammation of the amnion–the presence of histological grade 3-4 for the chorion-decidua and/or grade 3-4 for the chorionic plate and/or grade 1–4 for the umbilical cord (Salafia et al., 1989). Severity of acute inflammation of the amnion: grade 1—one focus of at least five neutrophils; grade 2—more than grade 1 focus or at least one focus of 5–20 neutrophils; grade 3—multiple and/or confluent grade 2 foci; and grade 4—diffuse and dense acute inflammation (Salafia et al., 1989).

TABLE 1 | Maternal and clinical characteristics of women with preterm prelabor rupture of membranes and short-term neonatal outcomes with respect to the presence of HCA with fetal and maternal inflammatory responses and the absence of HCA.

Characteristic	The presence of HCA		The absence of HCA (n = 324)	p-value
	With fetal inflammatory response (n = 343)	With maternal inflammatory response (n = 151)		
Maternal age [years, median (IQR)]	31 (27–36)	31 (27–35)	30 (27–34)	**0.05**
Primiparous [number (%)]	153 (45%)	81 (54%)	196 (61%)	**<0.0001**
Pre-pregnancy body mass index [kg/m^2, median (IQR)]	23.0 (20.6–26.7)	23.7 (21.1–26.7)	22.4 (20.3–26.0)	0.23
Gestational age at sampling [weeks + days, median (IQR)]	32 + 4 (30 + 0–34 + 4)	33 + 3 (31 + 2–35 + 0)	34 + 4 (32 + 3–35 + 4)	**<0.0001**
Gestational age at delivery [weeks + days, median (IQR)]	33 + 1 (30 + 5–35 + 0)	33 + 6 (32 + 0–35 + 3)	34 + 5 (33 + 0–35 + 5)	**<0.0001**
Latency from PPROM to AMC [hours, median (IQR)]	5 (3–11)	5 (3–9)	4 (3–8)	**0.02**
Latency from AMC to delivery [hours, median (IQR)]	64 (25–128)	53 (22–101)	26 (13–60)	**<0.0001**
Active management of PPROM [number (%)]	106 (31%)	55 (36%)	131 (40%)	**0.01**
CRP levels at admission [mg/L, median (IQR)]	6.2 (3.1–11.3)	5.8 (3.2–8.6)	5.0 (2.4–9.1)	**0.006**
WBC count at admission [x10^9 L, median (IQR)]	12.3 (10.4–15.3)	12.0 (10.4–14.8)	12.1 (10.0–14.6)	0.10
Smoking [number (%)]	69 (20%)	24 (16%)	45 (14%)	**0.03**
Administration of corticosteroids [number (%)]	272 (79%)	106 (70%)	189 (58%)	**<0.0001**
Administration of antibiotics [number (%)]	338 (99%)	150 (99%)	316 (98%)	0.32
Spontaneous vaginal delivery [number (%)]	217 (63%)	104 (69%)	240 (74%)	**0.003**
Cesarean section [number (%)]	125 (36%)	45 (30%)	80 (25%)	**0.001**
Forceps delivery [number (%)]	1 (1%)	2 (1%)	4 (1%)	0.19
Sex of the newborn (female) [number (%)]	171 (50%)	62 (41%)	145 (45%)	0.18
Birth weight [grams, median (IQR)]	1960 (1460–2390)	2090 (1680–2520)	2290 (1930–2610)	**<0.0001**
Apgar score <7; 5 min [number (%)]	15 (4%)	6 (4%)	4 (1%)	**0.02**
Apgar score <7; 10 min [number (%)]	5 (2%)	3 (2%)	1 (1%)	0.16
Transient tachypnea of newborns [number (%)]	13 (4%)	1 (1%)	8 (3%)	0.28
Respiratory distress syndrome [number (%)]	117 (34%)	44 (29%)	70 (22%)	**0.0003**
Bronchopulmonary dysplasia [number (%)]	38 (11%)	10 (7%)	5 (2%)	**<0.0001**
Need for intubation [number (%)]	37 (11%)	11 (7%)	10 (3%)	**0.0001**
Intraventricular hemorrhage (grades I-II) [number (%)]	54 (16%)	18 (12%)	51 (16%)	0.99
Intraventricular hemorrhage (grades III-IV) [number (%)]	7 (2%)	2 (1%)	0 (0%)	**0.02**
Retinopathy of prematurity [number (%)]	20 (6%)	5 (3%)	3 (1%)	**0.0005**
Necrotizing enterocolitis [number (%)]	10 (3%)	2 (1%)	1 (0%)	**0.007**
Early-onset sepsis [number (%)]	30 (9%)	3 (2%)	4 (1%)	**<0.0001**
Late-onset sepsis [number (%)]	13 (4%)	3 (2%)	5 (2%)	0.07
Compound neonatal morbidity [number (%)]	165 (48%)	55 (36%)	100 (31%)	**<0.0001**
Neonatal death [number (%)]	6 (2%)	2 (1%)	2 (1%)	0.18

p-value: comparison among the women with the presence of HCA with fetal inflammatory response, women with the presence of HCA with maternal inflammatory response, and women with the absence of HCA. Continuous variables were compared using a nonparametric Jonckheere-Terpstra test for trend and presented as median (interquartile range). Categorical variables were compared using Cochran-Armitage test for trend a presented as number (%). Statistically significant results are marked in bold.

AMC, amniocentesis; CRP, C-reactive protein; HCA, acute histological chorioamnionitis; IQR, interquartile range; PPROM, preterm prelabor rupture of membranes; WBC, white blood cells.

Statistical Analysis

The normality of the data was tested using the Anderson-Darling test. Continuous variables were compared using the nonparametric Jonckheere-Terpstra test for trend, or Mann-Whitney U test, as appropriate, and presented as medians [interquartile range (IQR)]. Categorical variables were compared using the Cochran-Armitage test for trend, and presented as numbers (%). Spearman's partial correlation was used to adjust the results for the following potential confounders: various methods for managing PPROM, maternal age, nulliparity, smoking, the interval between PPROM and amniocentesis, the interval between amniocentesis and delivery, administration of corticosteroids, mode of delivery. Differences were considered significant at $p < 0.05$. All p-values were obtained using two-tailed tests. All statistical analyses were performed using GraphPad Prism version 8.4.3 and the Statistical Package for the Social Sciences (SPSS), version 28.0.0.0, for Windows (SPSS Inc., Chicago, IL, United States).

RESULTS

A total of 918 women with singleton pregnancies complicated by PPROM were eligible for the study, and 100 women were excluded for the following reasons: i) gestational diabetes mellitus (n = 53); ii) gestational hypertension (n = 19); iii) preeclampsia (n = 5); iv) pre-gestational diabetes mellitus (n = 12); v) chronic hypertension (n = 5); vi) combination of the above-mentioned diseases (n = 6). The remaining 818 women were included in the analysis.

In total, HCA was observed in 494 (60%) women. Among women with the presence of HCA, fetal and maternal inflammatory responses were identified in 343 (69%) and 151 (31%) women, respectively. Remaining 324 (60%) women had the absence of HCA. The demographic and clinical characteristics of the study population, as well as short-term neonatal outcomes, with respect to the presence of fetal and maternal inflammatory responses and the absence of HCA are shown in **Table 1**. Among

TABLE 2 | Maternal and clinical characteristics of women with preterm prelabor rupture of membranes and short-term neonatal outcomes with respect to the presence of HCA with and without acute inflammation in the amnion and the absence of HCA.

Characteristic	The presence of HCA		The absence of HCA (n = 324)	*p*-value
	With acute inflammation of the amnion (n = 279)	Without acute inflammation of the amnion (n = 215)		
Maternal age [years, median (IQR)]	31 (27–35)	32 (28–35)	30 (27–34)	0.07
Primiparous [number (%)]	117 (42%)	117 (54%)	196 (61%)	<**0.0001**
Pre-pregnancy body mass index [kg/m^2, median (IQR)]	23.0 (20.6–27.1)	23.5 (21.0–26.3)	22.4 (20.3–26.0)	0.22
Gestational age at sampling [weeks + days, median (IQR)]	31 + 5 (28+6-34 + 0)	34 + 0 (32+0-35 + 2)	34 + 4 (32 + 3 – 35 + 4)	<**0.0001**
Gestational age at delivery [weeks + days, median (IQR)]	32 + 2 (29+4-34 + 2)	34 + 2 (32+4-35 + 4)	34 + 5 (33 + 0 – 35 + 5)	<**0.0001**
Latency from PPROM to AMC [hours, median (IQR)]	6 (3–12)	5 (3–8)	4 (3–8)	**0.001**
Latency from AMC to delivery [hours, median (IQR)]	65 (29–142)	46 (19–96)	26 (13–60)	<**0.0001**
Active management of PPROM [number (%)]	97 (35%)	64 (30%)	131 (40%)	0.13
CRP levels at admission [mg/L, median (IQR)]	6.1 (2.9–11.4)	5.9 (3.3–9.0)	5.0 (2.4–9.1)	**0.03**
WBC count at admission [x10^9 L, median (IQR)]	12.5 (10.7–15.3)	12.1 (9.9–14.6)	12.1 (10.0–14.6)	0.06
Smoking [number (%)]	55 (20%)	38 (18%)	45 (14%)	0.06
Administration of corticosteroids [number (%)]	229 (82%)	149 (69%)	189 (58%)	<**0.0001**
Administration of antibiotics [number (%)]	275 (99%)	213 (99%)	316 (98%)	0.31
Spontaneous vaginal delivery [number (%)]	171 (61%)	150 (70%)	240 (74%)	**0.0008**
Cesarean section [number (%)]	107 (38%)	63 (29%)	80 (25%)	**0.0003**
Forceps delivery [number (%)]	1 (1%)	2 (1%)	4 (1%)	0.25
Sex of the newborn (female) [number (%)]	133 (48%)	100 (47%)	145 (45%)	0.47
Birth weight [grams, median (IQR)]	1850 (1300–2260)	2220 (1870–2580)	2290 (1930–2610)	<**0.0001**
Apgar score <7; 5 min [number (%)]	18 (6%)	3 (1%)	4 (1%)	**0.0003**
Apgar score <7; 10 min [number (%)]	6 (2%)	2 (1%)	1 (1%)	**0.03**
Transient tachypnea of newborns [number (%)]	7 (3%)	7 (3%)	8 (3%)	0.96
Respiratory distress syndrome [number (%)]	108 (39%)	53 (25%)	70 (22%)	<**0.0001**
Bronchopulmonary dysplasia [number (%)]	39 (14%)	9 (4%)	5 (2%)	<**0.0001**
Need for intubation [number (%)]	39 (14%)	9 (4%)	10 (3%)	<**0.0001**
Intraventricular hemorrhage (grades III-IV) [number (%)]	47 (17%)	25 (12%)	51 (16%)	0.75
Intraventricular hemorrhage (grades III-IV) [number (%)]	7 (3%)	2 (1%)	0 (0%)	**0.003**
Retinopathy of prematurity [number (%)]	21 (8%)	4 (2%)	3 (1%)	<**0.0001**
Necrotizing enterocolitis [number (%)]	11 (4%)	1 (1%)	1 (0%)	**0.0005**
Early-onset sepsis [number (%)]	24 (9%)	9 (4%)	4 (1%)	<**0.0001**
Late-onset sepsis [number (%)]	13 (5%)	3 (1%)	5 (2%)	**0.02**
Compound neonatal morbidity [number (%)]	145 (52%)	75 (35%)	100 (31%)	<**0.0001**
Neonatal death [number (%)]	6 (2%)	2 (1%)	2 (1%)	0.09

p-value: comparison among the women with the presence of HCA with acute inflammation in the amnion, women with the presence of HCA without acute inflammation in the amnion, and women with the absence of HCA. Continuous variables were compared using a nonparametric Jonckheere-Terpstra test for trend and presented as median (interquartile range). Categorical variables were compared using Cochran-Armitage test for trend a presented as number (%). Statistically significant results are marked in bold.
AMC, amniocentesis; CRP, C-reactive protein; HCA, acute histological chorioamnionitis; IQR, interquartile range; PPROM, preterm prelabor rupture of membranes; WBC, white blood cells.

women with the presence of HCA, 279 (57%) and 215 (43%) women were with and without acute inflammation of the amnion was observed in 279 (34%) women. The demographic and clinical characteristics of the study population, as well as short-term neonatal outcomes, with respect to the presence of HCA with and without inflammation of the amnion and the absence of HCA are shown in **Table 2**.

Birth Weight Expressed as Percentiles Derived From the Estimated Fetal Weight Standards

A difference in the percentiles of birth weights of newborns was identified among the women with the presence of HCA with fetal (median 48, IQR 24–72) and maternal (median 52, IQR 28–78) inflammatory responses and those with the absence of HCA (median 55, IQR 31–76) in the crude analysis (p = 0.02; **Figure 1A**) but not in the analysis adjusted for potential confounders (p = 0.14).

Women with the presence of HCA with acute inflammation of the amnion had lower percentiles of birth weights of newborns (median 46, IQR 21–71) than women with the presence of HCA without acute inflammation of the amnion (median 54, IQR 30–76) and those with the absence of HCA (median 55, IQR 31–76) in the crude (p = 0.004; **Figure 1B**) and adjusted (p = 0.02) analyses (**Table 3**).

A difference in the percentiles of birth weights of newborns was found among the women with the presence of HCA with acute inflammation of the amnion, when the women were divided into four subgroups based on the severity of acute inflammation of the amnion [grade I (median 50, IQR 25–77), grade II (median 50, IQR 20–72), grade III (median 41, IQR 13–68), and grade IV (median 37, IQR 9–63)] in the crude (p = 0.004; **Figure 1C**) and adjusted (p = 0.03) analyses.

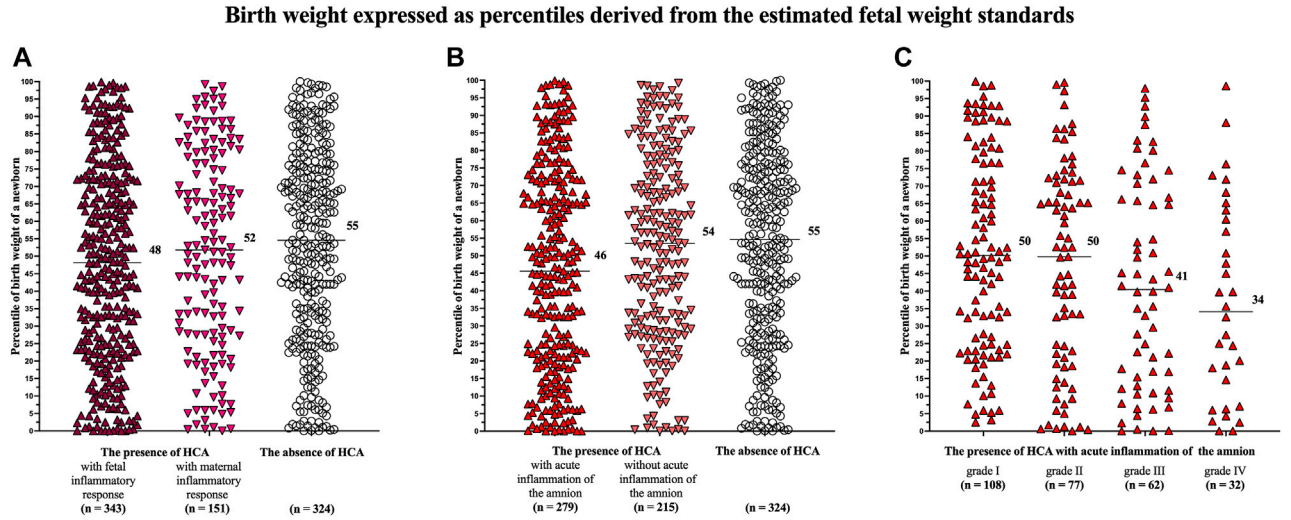

FIGURE 1 | Comparison of the birth weights of newborns, expressed as percentiles derived from the estimated fetal weight standards, among the women with the presence of HCA with fetal and maternal inflammatory responses and those with the absence of HCA **(A)**; among the women with the presence of HCA with and without acute inflammation of the amnion and those with the absence of HCA **(B)**; and among the women with of acute inflammation of the amnion divided based on its severity (grades I–IV) **(C)**. A difference was observed among the women with the presence of HCA with fetal and maternal inflammatory responses and those with the absence of HCA only in the crude analysis ($p = 0.02$) but not in the adjusted analysis ($p = 0.14$) **(A)**. A difference was observed among the women with the presence of HCA with and without acute inflammation and those with the absence of HCA in both the crude and adjusted analyses ($p = 0.004$, $p = 0.02$) **(B)**. A difference was observed among the women with acute inflammation of the amnion divided into the subgroups based on its severity in both the crude and adjusted analyses ($p = 0.004$, $p = 0.03$) **(C)**. Medians are marked. Abbreviation: HCA, acute histological chorioamnionitis.

TABLE 3 | Comparisons of the percentiles of birth weights of newborns derived from the estimated fetal weight standards among the subgroups of women with the presence of HCA with and without acute inflammation of the amnion and those with the absence of HCA.

	HCA with acute inflammation of the amnion	HCA without acute inflammation of the amnion	The absence of HCA
HCA with acute inflammation of the amnion	X	***p* = 0.01 adj. *p* = 0.03**	***p* = 0.002 adj. *p* = 0.03**
HCA without acute inflammation of the amnion	***p* = 0.01 adj. *p* = 0.03**	x	$p = 0.31$
The absence of HCA	***p* = 0.002 adj. *p* = 0.03**	$p = 0.31$	x

p-value: a comparison between two subgroups (a nonparametric Mann-Whitney U test). adj. p-value: a comparison between two subgroups after the adjustment for gestational potential confounders (a Spearman partial correlation). Statistically significant results are marked in bold.
HCA, acute histological chorioamnionitis.

A difference in the rates of the birth weights of newborns that were less than or equal to the 25th percentiles were observed among women with the presence of HCA with fetal and maternal inflammatory responses and those with the absence of HCA ($p = 0.05$; **Table 4**), as well as and among women with the presence of HCA with and without acute inflammation of the amnion and those with the absence of HCA ($p = 0.0009$; **Table 4**). However, after adjustment for potential confounders, only the latter result remained significant ($p = 0.29$; $p = 0.02$). Differences in the rates of the birth weights of newborns that were less than or equal to the first, 10th, and 25th percentiles were observed among women with HCA with acute inflammation of the amnion, when the women were stratified into the four subgroups based on the severity of acute inflammation of the amnion in the crude (first percentile: $p = 0.02$; 10th percentile: $p = 0.003$; 25th percentile: $p = 0.03$; **Table 4**) and adjusted analyses (first percentile: $p = 0.02$; 10th percentile: $p = 0.01$; 25th percentile: $p = 0.03$; **Table 4**).

Birth Weight Expressed as Percentiles Derived From the Newborn Birth Weight Standards

There were no differences in the percentiles of birth weights of newborns among women with HCA with fetal (median 52, IQR 33–67) and maternal (median 55, IQR 32–70) inflammatory responses and those with the absence of HCA (median 52, IQR 34–71 $p = 0.56$; **Figure 2A**), as well as among women with the presence of HCA with (median 52, IQR 33–68) and without (median 55, IQR 32–69) acute inflammation of the amnion and those the absence of HCA (median 52, IQR 34–71 $p = 0.41$; **Figure 2B**).

TABLE 4 | The rate of newborns with birth weights, expressed as percentiles derived from the estimated fetal weight standards, that were less than or equal to the first, 10th, and 25th percentiles according to: (a) the presence of HCA with fetal and maternal inflammatory responses and the absence of HCA; (b) the presence of HCA with and without acute inflammation of the amnion and the absence of HCA; and (c) severity of acute inflammation of the amnion (grades I-IV).

	≤1 percentile		≤10 percentiles		≤25 percentiles	
a)						
The presence of HCA with fetal inflammatory response (*n* = 343)	12 (4%)	*p* = 0.44	37 (11%)	*p* = 0.51	91 (27%)	***p* = 0.05**
The presence of HCA with maternal inflammatory response (*n* = 151)	5 (3%)		16 (11%)		34 (23%)	
The absence of HCA (*n* = 324)	8 (3%)		30 (10%)		65 (20%)	
b)						
The presence of HCA with acute inflammation of the amnion (*n* = 279)	10 (4%)	*p* = 0.57	38 (14%)	*p* = 0.82	89 (32%)	***p* = 0.0009**[a]
The presence of HCA without acute inflammation of the amnion (*n* = 215)	7 (3%)		15 (7%)		36 (17%)	
The absence of HCA (*n* = 324)	8 (3%)		30 (10%)		65 (20%)	
c)						
Acute inflammation of the amnion—grade I (*n* = 108)	0 (0%)	***p* = 0.02**[a]	7 (7%)	***p* = 0.003**[a]	28 (26%)	***p* = 0.028**[a]
Acute inflammation of the amnion—grade II (*n* = 77)	4 (5%)		12 (16%)		24 (31%)	
Acute inflammation of the amnion—grade III (*n* = 62)	4 (7%)		11 (18%)		24 (38%)	
Acute inflammation of the amnion—grade IV (*n* = 32)	2 (6%)		8 (25%)		14 (44%)	

Variables are presented as number (%) and were compared using Cochran-Armitage test for trend. Statistically significant results are marked in bold.
HCA, acute histological chorioamnionitis.
[a]*Result remains significant after the adjustment for potential confounders.*

FIGURE 2 | Comparison of the birth weights of newborns, expressed as percentiles derived from the newborn birth weight standards, among the women with the presence of HCA with fetal and maternal inflammatory responses and those with the absence of HCA **(A)**; among the women with the presence of HCA with and without acute inflammation of the amnion and those with the absence of HCA **(B)**; and among the women with acute inflammation of the amnion divided based on its severity (grades I-IV) **(C)**. No difference was observed among the women with the presence of HCA with fetal and maternal inflammatory responses and those with the absence of HCA in the crude analysis ($p = 0.56$) **(A)**. No difference was observed among the women with the presence of HCA with and without acute inflammation of the amnion and those with the absence of HCA in the crude analysis ($p = 0.41$) **(B)**. No difference was observed among the women with acute inflammation of the amnion divided into the subgroups based on the severity of acute inflammation of the amnion ($p = 0.85$). Medians are marked. Abbreviation: HCA, acute histological chorioamnionitis.

No difference in the percentiles of birth weights of newborns was found among women with the presence of HCA with acute inflammation of the amnion, when the women were divided into four subgroups based on the severity of acute inflammation of the amnion [grade I (median 53, IQR 34–73), grade II (median 52, IQR 28–66), grade III (median 48, IQR 30–66), and grade IV (median 50, IQR 41–65); $p = 0.85$; **Figure 2C**].

No differences were observed in the rates of the birth weights of newborns that were less than or equal to the first, 10th, and 25th percentiles among women with the presence of HCA with fetal and maternal inflammatory responses and women with the absence of HCA (**Table 5**) and among those with the presence of HCA with and without acute inflammation of the amnion and women with the absence of HCA (**Table 5**), except the 10th percentile in the subgroups of women with the presence of HCA

TABLE 5 | The rate of newborns with birth weights, expressed as percentiles derived from the birth weight standards, that were less than or equal to the first, 10th, and 25th percentiles according to: (a) the presence of HCA with fetal and maternal inflammatory responses and the absence of HCA; (b) the presence of HCA with and without acute inflammation of the amnion and the absence of HCA; and (c) severity of acute inflammation of the amnion (grades I–IV).

	≤1 percentile		≤10 percentiles		≤25 percentiles	
a)						
The presence of HCA with fetal inflammatory response (n = 343)	0 (0%)	p = 0.11	11 (3%)	**p = 0.05**	55 (16%)	p = 0.92
The presence of HCA with maternal inflammatory response (n = 151)	0 (0%)		8 (5%)		29 (19%)	
The absence of HCA (n = 324)	2 (1%)		21 (6%)		51 (16%)	
b)						
The presence of HCA with acute inflammation of the amnion (n = 279)	0 (0%)	p = 0.11	12 (4%)	p = 0.20	51 (18%)	p = 0.41
The presence of HCA without acute inflammation of the amnion (n = 215)	0 (0%)		7 (3%)		33 (15%)	
The absence of HCA (n = 324)	2 (1%)		21 (6%)		51 (16%)	
c)						
Acute inflammation of the amnion—grade I (n = 107)	0 (0%)	-	5 (5%)	p = 0.61	20 (19%)	p = 0.43
Acute inflammation of the amnion—grade II (n = 77)	0 (0%)		4 (5%)		16 (21%)	
Acute inflammation of the amnion—grade III (n = 62)	0 (0%)		2 (4%)		12 (19%)	
Acute inflammation of the amnion—grade IV (n = 32)	0 (0%)		1 (3%)		3 (10%)	

Variables are presented as number (%) and were compared using Cochran-Armitage test for trend. Statistically significant results are marked in bold.
HCA, acute histological chorioamnionitis.
No result remains significant after the adjustment for potential confounders.

with fetal and maternal inflammatory responses and women with the absence of HCA (p = 0.05). However, the result did not remain significant after adjusting for potential confounders (p = 0.08). No differences were observed in the rates of the birth weights of newborns that were less than or equal to the first, 10th, and 25th percentiles among women with the presence of HCA with acute inflammation of the amnion, when the women were divided into four groups based on the severity of acute inflammation of the amnion (**Table 5**).

DISCUSSION

The principal findings of this study are as follows: i) HCA with acute inflammation of the amnion was associated with lower birth weight, expressed as percentiles derived from the estimated fetal weight standards; ii) HCA with acute inflammation of the amnion was related to the highest rate of newborns with a birth weight equal to or below the 25th percentile derived from the estimated fetal weight standards; iii) alteration of fetal growth, expressed as percentiles derived from the estimated fetal weight standards, associated with HCA with acute inflammation of the amnion was dependent on the severity of acute inflammation; and iv) when percentiles were derived from the birth weight standard, no difference in percentiles of the birth weights of newborns was observed among those with the presence of HCA with fetal and maternal inflammatory responses and those with the absence of HCA, as well as among those with the presence of HCA with and without acute inflammation of the amnion and those with the absence of HCA.

Neutrophils are not usually present in the placental tissue and fetal membranes (Kim et al., 2015). Following chemotactic stimulus and its gradient, neutrophils migrate towards the amniotic cavity i) from the intervillous space of the placenta into the chorionic plate and/or ii) from the decidua into fetal membranes (Kim et al., 2015). To develop acute inflammation of the amnion, neutrophils need a strong chemotactic stimulus because they must transmigrate through the entire chorionic layer (Park et al., 2009; Goldstein et al., 2020). This process is time-consuming and requires more than 36 h to develop acute inflammatory changes in the amnion from the first exposure to an inflammatory stimulus (Redline, 2006; Goldstein et al., 2020).

Besides the placentas from pregnancies complicated by PPROM and spontaneous preterm labor, the presence of HCA with acute inflammation of the amnion has also been reported in preterm pregnancies with impaired fetal growth (Salafia et al., 1995). Salafia et al. observed HCA with acute inflammation of the amnion in 37% of the pregnancies with appropriate-for-gestational-age newborns, 38% of the pregnancies with "asymmetric intrauterine growth restriction" and 8% of the pregnancies with "symmetric intrauterine growth restriction" (Salafia et al., 1995).

In this study, newborns from pregnancies with HCA with acute inflammation of the amnion had the lowest birth weight, expressed in percentile derived from the estimated fetal weight standard. This interesting finding was further supported by the fact that the alteration of fetal growth was dependent on the severity of acute inflammation of the amnion. The lowest percentiles and the highest rates of birth weight equal to or less than the 10th and 25th percentiles were found in the women with the most severe form of acute inflammation of the amnion (grade IV). The mechanistic explanation for this observation is unclear. It can be hypothesized that the placenta is affected by various lesions (maternal or fetal vascular malperfusion or chronic villitis), which are responsible for impaired fetal growth, might produce endogenous "danger signals" (e.g., heat shock protein 70, high mobility group box-1, or S100B) (Gazzolo et al., 2006; Zenerino et al., 2017; Lai et al., 2020). These signals lead to the production of chemotactic stimuli such as interleukin (IL)-8, which is followed by the migration of maternal neutrophils into the placenta and/or fetal membranes. When the production of chemotactic stimuli persists long enough, neutrophils can get a sufficient temporal period to reach and

infiltrate the amnion. This hypothesis is supported by the following observations: i) placentas from pregnancies with FGR had a higher expression of mRNA for IL-8 (Hahn-Zoric et al., 2002); ii) maternal blood lymphocytes from pregnancies with FGR produce higher levels of IL-8 after stimulation with trophoblast (Raghupathy et al., 2012); iii) maternal serum concentrations of IL-8 are higher in pregnancies with SGA fetuses (Wang et al., 2015); and iv) malarial infection in pregnancy, usually leading to FGR, is associated with a higher expression of IL-8 mRNA in the placenta (Moormann et al., 1999). This hypothesis suggests that placental changes leading to impaired fetal growth are associated with the development of the acute inflammation of the amnion rather than the fetal inflammatory response, as shown in this study. We cannot fully exclude a contribution of fetal membranes on the production of the chemotactic stimuli along with the placenta for the following reasons: i) transporter proteins in the fetal membranes along with nutritional transport system suggests that fetal membranes play an equal role to that of the placenta in drug and nutrients transports (Ganguly et al., 2021; Kammala et al., 2022); ii) endogenous activities in the fetal membranes on cellular level can generate danger signals (Menon and Peltier, 2020; Sheller-Miller and Menon, 2020; Shahin et al., 2021; Shepherd et al., 2021; Tantengco et al., 2021); iii) fetal membranes function can be independent of the placenta and placental involvement (Menon, 2016); iv) fetal growth restriction can increase apoptosis in the chorionic trophoblast cells of fetal membranes and expression of parathyroid-related protein expression in the fetal membranes (Curtis et al., 2000; Murthi et al., 2005); and v) fetal membranes are not the mere extension of the placenta and have their own identity, function and hence, their compromise alone without the placental involvement can be detrimental (Collins et al., 1993; Menon and Moore, 2020). Therefore, functions of fetal membranes might be impaired in pregnancies complicated by the alteration of fetal growth.

The cutoff value of the 10th percentile is widely accepted by obstetricians and pediatricians as a threshold for identifying fetuses/newborns with SGA (DiGiulio et al., 2010; Figueras and Gratacos, 2014). This was the reason why this cutoff, along with the first percentile (to identify newborns with extremely impaired growth) and 25th percentile (to reveal a mild alteration of growth not fulfilling a threshold for SGA), were selected and used in this study. According to the abovementioned results, the highest rate (47%, 89/190) of newborns with having birth weight equal to or less than the 25th percentile was identified in the subset of women with HCA with acute inflammation of the amnion. This observation seems to be clinically relevant because it suggests that underlying placental pathologies associated with impaired fetal growth might be lasting enough to develop acute inflammatory lesions in the amnion but not the restriction of fetal growth that reaches the threshold of the 10th percentile due to subsequent/co-incidental development of PPROM.

Two INTERGROWTH-21st standards (for estimated fetal weight and newborn birth weight) were used in this study to derive percentiles for the birth weights of newborns. Two different growth charts were employed because it is still under debate which standard should be preferred (Marsal et al., 1996; Salomon et al., 2007; Kiserud et al., 2017; Stirnemann et al., 2017; Nicolaides et al., 2018). The main advantage of using the standard for estimated fetal weight is that reference ranges of the estimated fetal weight are representative of the whole population. On the other hand, the reference ranges of birth weight standards, particularly for newborns delivered preterm, suffer from the overrepresentation of pathological pregnancies resulting in iatrogenic or spontaneous preterm delivery in whom impaired placentation might be expected (Nicolaides et al., 2018). This limitation of birth weight standard might be seen mainly in the subset of newborns delivered before 33 weeks of gestation(Villar et al., 2014a). These methodological differences between the charts were observed in this study. For example, the subgroups of women with fetal inflammatory response and acute inflammation of the amnion, with the lowest gestational age at delivery, had lower medians of the percentiles of birth weight derived from the estimated fetal weight standard than when they were derived from the birth weight standard. Taking this into consideration, clinicians should be aware that using a growth chart based on birth weight might not reveal all cases with mildly impaired fetal growth, mainly in lower gestational ages. In light of this fact, it is not surprising that no differences among the assessed subgroups were observed when percentiles derived from the birth weight standard were used. In addition, no differences were observed in the rates of the birth weight equal to or lower than the first, 10th, and 25th percentiles among the subgroups of women with PPROM.

This study has two main strengths. First, the study was conducted on a large homogeneous cohort of Caucasian women with a thoroughly defined phenotype of spontaneous preterm delivery. Second, the assessment of HCA was performed by a single, experienced perinatal pathologist who was blinded to the clinical status of the women. However, there are certain limitations to this study. First, this study covers a 13 years-long interval. During this time, two different management strategies (active management and expectant management) of PPROM pregnancies were used. Therefore, attention was paid to this important confounding factor in this study, and the results were adjusted for the same. Second, no data regarding ultrasonographically estimated fetal weight from the time of delivery or shortly before delivery were available. This shortcoming prevented us from assessing the relationship between the estimated fetal weight, expressed in percentiles derived from the estimated fetal weight standards and the presence of acute inflammatory lesions in the placenta. Third, the histopathological assessment of placental lesions other than HCA (maternal and fetal vascular malperfusion, placental hemorrhage, and chronic villitis) were not systematically performed in the placentas from PPROM pregnancies. Last, the risk factors of the development of fetal and maternal inflammatory responses were not assessed and evaluated in this study.

In conclusion, in this large retrospective cohort study of singleton pregnancies with PPROM, acute inflammation of the amnion was associated with a lower birth weight but only when

the percentiles of birth weight were derived from the standards for estimated fetal weight.

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 Ethics Committee of the University Hospital of Hradec Kralove, Czechia. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JMa and MK: drafting the paper JMa, MK, JS, JM, IM, RS, HB, and BJ: conception and design of the work HH: analysis of the samples JMa, MK, HH, IM, and BJ: analysis of the data JMa, BJ, IM, JS, JM, RS, HH, and HB: revising the draft of the paper critically for important intellectual content JMa, MK, BJ, IM, JM, JS, HH, RS, and HB: provided approval for publication.

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Prevalence and Load of Cervical Ureaplasma Species With Respect to Intra-amniotic Complications in Women With Preterm Prelabor Rupture of Membranes Before 34 weeks

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Objectives: To determine the prevalence and load of *Ureaplasma* spp. DNA in the cervical fluid of women with singleton pregnancies complicated by preterm prelabor rupture of membranes (PPROM) with respect to intra-amniotic infection, sterile intra-amniotic inflammation, and colonization of the amniotic fluid.

Methods: A total of 217 women with PPROM between gestational ages 24 + 0 and 33 + 6 weeks were included in this study. Paired amniotic and cervical fluid samples were collected at the time of admission via transabdominal amniocentesis and using a Dacron polyester swab, respectively. Microbial invasion of the amniotic cavity was diagnosed using a combination of culture and molecular biology methods. Intra-amniotic inflammation was determined based on the concentration of interleukin-6 in the amniotic fluid. Based on the presence or absence of these conditions, the women were stratified into the following subgroups: intra-amniotic infection (with both), sterile intra-amniotic inflammation (with inflammation only), colonization (with microorganisms only), and negative amniotic fluid (without either). The *Ureaplasma* spp. DNA load in the cervical fluid was assessed using PCR.

Results: *Ureaplasma* spp. DNA in the cervical fluid was found in 61% (133/217) of the women. Women with negative amniotic had similar prevalence of *Ureaplasma* spp. DNA in cervical fluid (55%) to those with sterile intra-amniotic inflammation (54%) but lower than those with intra-amniotic infection (73%) and colonization (86%; $p < 0.0001$). Women with negative amniotic fluid had a lower load of *Ureaplasma* spp. DNA in their cervical fluid

(median: 4.7×10^3 copies of DNA/ml) than those with intra-amniotic infection (median: 2.8×10^5 copies DNA/ml), sterile intra-amniotic inflammation (median: 5.3×10^4 copies DNA/ml), and colonization (median: 1.2×10^5 copies DNA/mL; $p < 0.0001$).

Conclusion: In conclusion, in PPROM at <34 weeks, the presence of intra-amniotic infection, sterile intra-amniotic inflammation, or colonization of the amniotic fluid was associated with a higher prevalence and/or load of *Ureaplasma* spp. DNA in the cervical fluid than the absence of intra-amniotic complications.

Keywords: microbial invasion of the amniotic cavity, genital *mycoplasma*, intra-amniotic inflammation, non-invasive sample, preterm delivery

INTRODUCTION

Preterm prelabor rupture of the membranes (PPROM) is defined as rupture of the fetal membranes with leakage of amniotic fluid before the onset of regular uterine activity before 37 weeks of gestation (Mercer, 2003; Mercer, 2005). PPROM is one of the "great obstetrical syndromes," with considerable medical and socio-economic impacts (Romero, 1996; Romero et al., 2006; Di Renzo, 2009; Brosens et al., 2011). PPROM remains under intensive debate among scientists, researchers, and clinicians given the necessity: 1) to fully unravel the underlying pathophysiological mechanisms to make the prevention of PPROM possible (Moore et al., 2020); 2) to understand the underlying mechanisms affecting the interval between PPROM and delivery to optimize the timing of induction of lung maturity (Battarbee et al., 2020); 3) to characterize the causality and consequences of microbial invasion of the amniotic cavity (the presence of microorganisms and/or their DNA in the amniotic fluid) and intra-amniotic inflammation (elevation of inflammatory mediators in the amniotic fluid) (Menon and Richardson, 2017); and 4) to identify risk factors and reliable biomarkers of microbial invasion of the amniotic cavity and intra-amniotic inflammation to enable a individualized therapeutic approach (Stranik et al., 2021).

The presence of microbial invasion of the amniotic cavity and intra-amniotic inflammation complicates approximately 23–41% and 17–58%, respectively, of pregnancies with PPROM (Romero et al., 2015; Kacerovsky et al., 2020a; Kacerovsky et al., 2021b). Based on these two intra-amniotic complications, the following scenarios can occur in PPROM pregnancies: 1) intra-amniotic infection (presence of both), 2) sterile intra-amniotic inflammation (intra-amniotic inflammation only), 3) colonization of the amniotic fluid (microbial invasion of the amniotic cavity only), and 4) negative amniotic fluid (absence of both) (Musilova et al., 2015; Romero et al., 2015).

There is plethora of evidence that *Ureaplasma* spp. are the most common microorganisms recovered from the amniotic fluid obtained from PPROM pregnancies. Therefore, these low-virulence bacteria with sizes comparable to those of viruses represent the most common cause of intra-amniotic infection or colonization of the amniotic cavity in PPROM (Kacerovsky et al., 2021b). Because they are commonly found in the cervical/vaginal niche of women with PPROM, the lower genital tract is considered the main source of amniotic fluid *Ureaplasma* spp.

The presence of *Ureaplasma* spp. in the cervical/vaginal niche in PPROM pregnancies is related to a higher risk of ascension and their subsequent presence in amniotic fluid (Musilova et al., 2016). Nevertheless, there is a shortage of information on whether their prevalence and loads in the cervical niche might be prone to a specific subtype of intra-amniotic complications, such as intra-amniotic infection, sterile intra-amniotic inflammation, and colonization of the amniotic cavity.

To fill this knowledge gap, this study aimed to determine the prevalence of *Ureaplasma* spp. DNA in the cervical fluid of the subgroups of women with PPROM with intra-amniotic infection, sterile intra-amniotic inflammation, colonization of the amniotic cavity, and negative amniotic fluid. The secondary aim was to compare the microbial load of *Ureaplasma* spp. DNA in the cervical fluid among subgroups of women with PPROM. The final aim was to compare the relative abundance of *Ureaplasma* spp. DNA in the cervical fluid among subgroups of women with PPROM.

MATERIALS AND METHODS

In this retrospective study, we included pregnant women admitted to the Department of Obstetrics and Gynecology of the University Hospital Hradec Kralove, Czech Republic, between May 2015 and May 2021, who met the following criteria: 1) age ≥18 years; 2) singleton pregnancy; 3) gestational ages between 24 + 0 and 33 + 6 weeks; 4) PPROM; and 5) amniocentesis to assess the intra-amniotic environment. The exclusion criteria were as follows: 1) pregnancy-related complications (e.g., fetal growth restriction, gestational, gestational hypertension, and preeclampsia); 2) chronic medical complications (e.g., pregestational diabetes mellitus and chronic hypertension); 3) congenital or chromosomal fetal abnormalities; 4) signs of fetal hypoxia; 4) significant vaginal bleeding; and 5) preterm labor with intact membranes.

PPROM was diagnosed based on visual confirmation of amniotic fluid pooling in the posterior vaginal fornix by sterile speculum examination. If uncertainty about amniotic fluid leakage remained after the clinical examination, the presence of insulin-like growth factor-binding protein in the vaginal fluid was assessed (Actim PROM test; Medix Biochemica, Kauniainen, and Finland).

Body fluid samples (amniotic fluid first, cervical fluid second) were collected at the time of admission before the administration of corticosteroids, antibiotics, or tocolytics. Transabdominal amniocentesis to obtain an amniotic fluid sample is a standard part of the department's clinical management of women with PPROM to assess the intra-amniotic environment. Women with PPROM received corticosteroids to accelerate lung maturation and intravenous antibiotics. Women with intra-amniotic inflammation received clarithromycin for 7 days unless delivery occurred. Those without intra-amniotic inflammation were treated with benzylpenicillin for 7 days unless delivery occurred. In the case of penicillin allergy, women were treated with clindamycin for 7 days unless delivery occurred. Once the final results regarding microbial invasion of the amniotic cavity from cultivation or PCR were known, the attending clinician modified the women's antibiotic therapies accordingly. Tocolysis was used only in women who developed regular uterine activity during the course of corticosteroids or within 24 h after their administration. The women were managed expectantly, except those with intra-amniotic infection (the presence of both microbial invasion of the amniotic cavity and intra-amniotic inflammation) beyond the 28th gestational week. This subset of women with PPROM was managed actively (labor was induced or an elective cesarean section was performed after finalizing corticosteroid treatment within 72 h of membrane rupture).

This study was approved by the institutional review board of the University Hospital Hradec Kralove (June 2014, No. 201408 S07P). All study participants were Caucasian, and informed consent was obtained from all participants.

Amniotic fluid samples, cervical fluid samples, and the clinical and demographic data of some women from this cohort were used in our previous studies (Musilova et al., 2017a; Musilova et al., 2017b; Hornychova et al., 2018; Kacerovsky et al., 2018; Musilova et al., 2018; Janku et al., 2019; Kacerovsky et al., 2020a; Kacerovsky et al., 2020b; Musilova et al., 2020; Soucek et al., 2020; Spacek et al., 2020; Kacerovsky et al., 2021a; Kacerovsky et al., 2021b; Matulova et al., 2021; Musilova et al., 2021; Stranik et al., 2021)

Amniotic Fluid Sampling

Ultrasonography-guided transabdominal amniocentesis was performed before administration of corticosteroids, antibiotics, or tocolytics. The details of amniotic fluid sampling have been previously described (Kacerovsky et al., 2020a; Stranik et al., 2021).

Cervical Fluid Sampling

Cervical fluid samples were collected using Dacron polyester swabs. The details of the procedure have been described previously. Pellets were used to assess the bacterial DNA and *Ureaplasma* spp. DNA (Musilova et al., 2016; Kacerovsky et al., 2020a; Kacerovsky et al., 2021c).

Assessment of IL-6

Amniotic fluid samples obtained from May 2015 to November 2018 were assessed using a Milenia QuickLine IL-6 lateral flow immunoassay and Milenia POC-Scan Reader (Milenia Biotec, GmbH, Giessen, Germany) (Kacerovsky et al., 2014). The measurement range was 50–10,000 pg/ml. Samples obtained between December 2018 and May 2021 were evaluated using an automated electrochemiluminescence immunoassay method with a Cobas e602 immunoanalyzer, which is part of the Cobas 8,000 platform (Roche Diagnostics, Basel, Switzerland) (Musilova et al., 2020). The measurement range was 1.5–5,000 pg/ml, which could be extended to 50,000 pg/ml with a 10-fold dilution of the sample.

Detection of *Ureaplasma* spp., *Mycoplasma hominis*, and *Chlamydia trachomatis* in the amniotic fluid.

To assess the amniotic fluid, a commercial AmpliSens® *C. trachomatis*/*Ureaplasma*/*M. hominis*-FRT kit (Federal State Institution of Science, Central Research Institute of Epidemiology, Moscow, Russia) was used to detect DNA from *Ureaplasma* spp., *M. hominis*, and *Ch. trachomatis* in a single PCR tube for each fluid. The details of this procedure have been described previously (Kacerovsky et al., 2020a; Kacerovsky et al., 2020b; Stranik et al., 2021).

Detection of bacteria other than *Ureaplasma* spp., *Mycoplasma hominis*, or *Chlamydia trachomatis* in the amniotic fluid.

The detection of bacteria other than *Ureaplasma* spp., *M. hominis*, and *Ch. trachomatis* in the women's amniotic fluid using aerobic/anaerobic cultivation and non-cultivation methods has been described previously (Kacerovsky et al., 2020a; Kacerovsky et al., 2020b; Stranik et al., 2021).

Detection of *Ureaplasma* spp. and *Mycoplasma hominis* in the Cervical Fluid

To assess the cervical fluid, a commercial AmpliSens® *C. trachomatis*/*Ureaplasma*/*M. hominis*-FRT kit (Federal State Institution of Science, Central Research Institute of Epidemiology, Moscow, Russia) was used to detect DNA from *Ureaplasma* spp., *M. hominis*, and *Ch. trachomatis* in a single PCR tube for each fluid. The details of this procedure have been described previously. The load of *Ureaplasma* spp. (copies/ml) was determined using an absolute quantification technique with an external calibration curve. Plasmid DNA (pCR3, Invitrogen, Carlsbad, CA, United States) was used to prepare a calibration curve.

The relative abundance of *Ureaplasma* spp. DNA in the cervical fluid and total bacterial DNA detection were performed using quantitative RT-PCR–BactQuant (Liu et al., 2012). To quantify the bacterial load, we used the forward primer CCTACGGGDGGCWGCA, reverse primer GGA CTACHVGGGTMTCTAATC, and hydrolysis probe FAM-BHQ1 CAGCCGCGGTA. A calibration curve was generated using 10-fold dilutions of linearized and normalized plasmids containing the cloned target sequence of the 466-bp region in the V3-V4 domain of 16S rRNA at a concentration of 10^7 copies/μl (Generi Biotech,

Hradec Kralove, Czech Republic) (Kacerovsky et al., 2019). The relative abundance of *Ureaplasma* spp. in the cervical microbiota was calculated [(*Ureaplasma* spp. DNA load/total bacterial DNA load) × 100] and expressed as percentages. The PCR conditions used in the BactQuant assay were the same as those used for *Ureaplasma* spp.

Clinical Definitions

Microbial invasion of the amniotic cavity was determined based on a positive PCR analysis for *Ureaplasma* spp., *M. hominis, or Ch. trachomatis* in the amniotic fluid, their combination, positivity for the 16S rRNA gene in the amniotic fluid, aerobic/anaerobic cultivation of the amniotic fluid, or a combination of these parameters. Intra-amniotic inflammation was defined as a concentration of IL-6 in the amniotic fluid that was ≥745 pg/ml when measured using a lateral flow immunoassay point-of-care test (Chaemsaithong et al., 2016a; Chaemsaithong et al., 2016b) or ≥3,000 pg/ml when measured using an automated electrochemiluminescence immunoassay method (Musilova et al., 2020). Intra-amniotic infection was defined as the presence of microbial invasion of the amniotic cavity and intra-amniotic inflammation. Women with intra-amniotic infection were further subdivided into those with and without *Ureaplasma* spp. in the amniotic fluid. Sterile intra-amniotic inflammation was defined as the presence of intra-amniotic inflammation without microbial invasion into the amniotic cavity. Colonization of the amniotic cavity was defined as the presence of microbial invasion in the amniotic cavity in the absence of intra-amniotic inflammation. Women with colonization were further subdivided into those with and without *Ureaplasma* spp. in the amniotic fluid. Negative amniotic fluid was defined as amniotic fluid without microbial invasion of the amniotic cavity or intra-amniotic inflammation.

Statistical Analyses

The demographic and clinical characteristics of the patients were compared using the non-parametric Kruskal–Wallis test for continuous variables and chi-square test for categorical variables, and the results are presented as medians (interquartile range [IQR]) and numbers (%), respectively. The normality of the data was tested using the Anderson–Darling test. The non-parametric Kruskal–Wallis or Mann-Whitney *U* tests were used, as appropriate, to compare the loads of bacteria and *Ureaplasma* spp. DNA in the cervical fluid and the relative abundance of *Ureaplasma* spp. DNA in the cervical fluid. Chi-squared or Fisher's exact tests were used, as appropriate, to compare the prevalence of *Ureaplasma* spp. DNA in the cervical fluid. Differences were considered statistically significant at $p < 0.05$. All *p*-values were determined using two-tailed tests, and all statistical analyses were performed using GraphPad Prism 8.4.3 for Mac OS X (GraphPad Software, San Diego, CA, United States).

RESULTS

Overall, 217 women with singleton pregnancies and PPROM between gestational ages 24 + 0 and 33 + 6 weeks were included in the study. Intra-amniotic infection, sterile intra-amniotic inflammation, colonization of the amniotic cavity, and negative amniotic fluid were observed in 20% (44/217), 11% (24/217), 10% (21/217), and 59% (128/217) of women, respectively. The demographic and clinical data of the women with PPROM, as well as short-term neonatal outcomes are summarized in **Table 1**.

The most common microorganisms in the amniotic fluid were *Ureaplasma* spp., which were found in 59% (26/44) and 71% (15/21) of women with intra-amniotic infection and colonization of the amniotic cavity, respectively. All microbial findings in the amniotic fluid that were identified in the women with intra-amniotic infection are shown in **Table 2**.

Ureaplasma spp. DNA in the cervical fluid was identified in 61% (133/217) of the women. All of the women with *Ureaplasma* spp. DNA in their amniotic fluid ($n = 41$) also had *Ureaplasma* spp. DNA in their cervical fluid.

Prevalence of *Ureaplasma* spp. DNA in the Cervical Fluid

The prevalence of *Ureaplasma* spp. DNA in the cervical fluid varied among the subgroups of women with intra-amniotic infection (73% [32/44]), sterile intra-amniotic inflammation (54% [13/24]), colonization (86% [18/21]), and negative amniotic fluid (55% [70/128]; $p = 0.01$; **Figure 1**).

Women with negative amniotic fluid had a lower prevalence of *Ureaplasma* spp. DNA in their cervical fluid compared to the women with intra-amniotic infections ($p = 0.05$) and colonization ($p = 0.008$); however, they had a similar prevalence to those with sterile intra-amniotic inflammation ($p = 1.00$).

Microbial Load of *Ureaplasma* spp. DNA in the Cervical Fluid

The load of *Ureaplasma* spp. DNA in the cervical fluid varied among the subgroups (median [IQR]; intra-amniotic infection: 2.8×10^5 copies DNA/mL [8.9×10^4–1.1×10^6]; sterile intraamniotic inflammation: 5.3×10^4 copies DNA/mL [7.8×10^3–9.2×10^5]; colonization 1.2×10^5 copies DNA/ml [5.2×10^4–1.1×10^6]; and negative amniotic fluid: 4.7×10^3 copies DNA/mL [9.3×10^2–4.6×10^4]; $p < 0.0001$; **Figure 2A**), as well as when the women with *Ureaplasma* spp. in the amniotic fluid were excluded (median [IQR]; intra-amniotic infection: 1.1×10^5 copies DNA/mL [7.3×10^3–6.7×10^5]; colonization 1.3×10^5 copies DNA/mL [1.1×10^5–1.6×10^5]; $p = 0.003$; **Figure 2B**).

Women with negative amniotic fluid had a lower load of *Ureaplasma* spp. DNA in the cervical fluid compared to those with intra-amniotic infection ($p < 0.0001$; without *Ureaplasma* spp. in amniotic fluid: $p = 0.04$), sterile intra-amniotic inflammation ($p = 0.004$), and colonization ($p = 0.0001$; without *Ureaplasma* spp. in amniotic fluid: $p = 0.04$).

Relative Abundance of *Ureaplasma* spp. in the Cervical Fluid

To assess the relative abundance of *Ureaplasma* spp. in the cervical fluid between the subgroups of women with PPROM, the amount of bacterial DNA in the cervical fluid was first evaluated. No difference in the concentration of bacterial DNA

TABLE 1 | Demographical and clinical characteristics and short-term neonatal outcomes of pregnancies with preterm prelabor rupture of membranes prior to 34 weeks of gestation according to the presence of intra-amniotic infection, sterile intra-amniotic inflammation, colonization of the amniotic cavity, and negative amniotic fluid.

Characteristic	Intra-amniotic infection (*n* = 44)	Sterile intra-amniotic inflammation (*n* = 24)	Colonization of the amniotic cavity (*n* = 21)	Negative amniotic fluid (*n* = 128)	*p*-value
Maternal age [years, median (IQR)]	31 (25–36)	31 (29–36)	34 (28–37)	31 (27–34)	0.42
Primiparous [number (%)]	21 (48%)	7 (29%)	7 (33%)	74 (58%)	**0.02**
Smoking [number (%)]	7 (16%)	3 (13%)	2 (10%)	18 (14%)	0.91
Pre-pregnancy body mass index [kg/m^2, median (IQR)]	23.3 (20.4–27.3)	25.5 (20.6–28.9)	23.6 (20.4–26.2)	24.3 (21.4–28.6)	0.40
Gestational age at sampling [weeks + days, median (IQR)]	28 + 5 (25+2–31 + 5)	26 + 6 (24+0–30 + 1)	31 + 5 (31+0–33 + 0)	31 + 5 (30+0–33 + 1)	<**0.0001**
Gestational age at delivery [weeks + days, median (IQR)]	29 + 4 (26+4–32 + 0)	30 + 0 (27+6–32 + 2)	32 + 5 (31+4–33 + 6)	32 + 5 (30+6–33 + 5)	<**0.0001**
Latency from PPROM to amniocentesis [hours, median (IQR)]	4 (3–10)	5 (4–13)	5 (2–12)	5 (3–9)	0.87
Latency from amniocentesis to delivery [days, median (IQR)]	76 (42–169)	188 (52–632)	96 (65–159)	79 (22–285)	0.15
Presence of *Ureaplasma* spp. in amniotic fluid [number (%)]	26 (59%)	0 (0%)	15 (71%)	0 (0%)	<**0.0001**
CRP levels at admission [mg/L, median (IQR)]	18.0 (4.9–36.5)	6.4 (2.5–9.2)	3.2 (1.5–7.0)	4.8 (2.9–8.9)	<**0.0001**
WBC count at admission [× 10^9 L, median (IQR)]	15.4 (11.2–18.6)	12.1 (10.0–15.2)	12.2 (10.2–14.4)	12.6 (10.5–15.4)	**0.02**
Administration of corticosteroids [number (%)]	42 (96%)	22 (92%)	20 (95%)	125 (98%)	0.52
Administration of antibiotics [number (%)]	44 (100%)	24 (100%)	21 (100%)	127 (99%)	0.87
Spontaneous vaginal delivery [number (%)]	23 (52%)	11 (46%)	13 (62%)	75 (59%)	0.42
Cesarean section [number (%)]	11 (25%)	13 (54%)	7 (33%)	53 (41%)	0.35
Forceps delivery [number (%)]	0 (0%)	0 (0%)	1 (5%)	0 (0%)	**0.03**
Birth weight [grams, median (IQR)]	1285 (820–1873)	1345 (983–1733)	1910 (1715–2050)	1915 (1563–2198)	<**0.0001**
Apgar score <7; 5 min [number (%)]	7 (16%)	2 (8%)	0 (0%)	4 (3%)	**0.01**
Apgar score <7; 10 min [number (%)]	3 (7%)	0 (0%)	0 (0%)	2 (2%)	0.15
Transient tachypnea of newborns [number (%)]	2 (5%)	1 (4%)	1 (5%)	14 (11%)	0.12
Respiratory distress syndrome [number (%)]	29 (65%)	14 (58%)	4 (19%)	43 (34%)	<**0.0001**
Bronchopulmonary dysplasia [number (%)]	15 (34%)	5 (21%)	0 (0%)	7 (6%)	<**0.0001**
Need for intubation [number (%)]	5 (11%)	2 (8%)	1 (5%)	4 (3%)	**0.03**
Intraventricular hemorrhage (grades I-II) [number (%)]	5 (11%)	5 (21%)	5 (24%)	24 (19%)	0.38
Intraventricular hemorrhage (grades III-IV) [number (%)]	3 (7%)	0 (0%)	0 (0%)	0 (0%)	**0.003**
Retinopathy of prematurity [number (%)]	5 (11%)	4 (17%)	0 (0%)	2 (2%)	**0.001**
Necrotizing enterocolitis [number (%)]	2 (5%)	1 (4%)	0 (0%)	1 (1%)	0.08
Early-onset sepsis [number (%)]	5 (11%)	2 (8%)	2 (10%)	1 (1%)	**0.002**
Late-onset sepsis [number (%)]	5 (11%)	0 (0%)	0 (0%)	0 (0%)	**0.0001**
Compound neonatal morbidity [number (%)]	31 (70%)	17 (71%)	8 (38%)	70 (56%)	**0.04**
Neonatal death [number (%)]	2 (5%)	1 (4%)	0 (0%)	2 (2%)	0.21

Abbreviations; CRP, C-reactive protein; IQR, interquartile range; PPROM, preterm prelabor rupture of membranes; WBC, white blood cells
Continuous variables were compared using a nonparametric Kruskal–Wallis test. Categorical variables were compared using chi-square test. Statistically significant results are marked in bold. Continuous variables are presented as median (interquartile range) and categorical as number (%).

was identified between the subgroups (median [IQR]; intra-amniotic infection: 5.7×10^6 copies DNA/ml [2.9×10^5–3.1×10^7]; sterile intra-amniotic inflammation: 5.8×10^6 copies DNA/ml [4.4×10^5–5.4×10^7]; colonization: 5.8×10^6 copies DNA/ml [9.0×10^5–6.1×10^7]; and negative amniotic fluid: 4.3×10^6 copies DNA/ml [5.9×10^5–2.0×10^7]; $p = 0.89$; **Figure 3**).

The relative abundance of *Ureaplasma* spp. in the cervical fluid differed among the subgroups (median [IQR]; intra-amniotic infection: 14.2% [2.6–100.0]; sterile intra-amniotic inflammation: 0.8% [0.4–5.9]; colonization: 4.4% [0.5–9.4]; and negative amniotic fluid: 0.3% [0.1–0.9]; $p < 0.0001$; **Figure 4A**). However, after excluding the women with *Ureaplasma* spp. in the amniotic fluid, the difference reached borderline statistical significance (median [IQR]; intra-amniotic infection: 0.3% [0.2–7.7]; colonization: 9.5% [0.4–23.7]; $p = 0.06$; **Figure 4B**).

Women with negative amniotic fluid had a lower relative abundance than those with intra-amniotic infection ($p < 0.0001$), sterile intra-amniotic inflammation ($p = 0.05$), or colonization ($p = 0.008$). After excluding the women with *Ureaplasma* spp. in the amniotic fluid, there was no difference (intra-amniotic infection: $p = 0.36$; colonization: $p = 0.06$).

DISCUSSION

The principal findings of this study in women with PPROM before 34 weeks were as follows: 1) the total prevalence of *Ureaplasma* spp. DNA in the cervical fluid was 61%; 2) the presence of intra-amniotic infection and colonization was related to higher rates of *Ureaplasma* spp. DNA in the

TABLE 2 | The microbial species identified in the amniotic fluid from pregnancies with preterm prelabor rupture of membranes prior to 34 weeks of gestation complicated with intra-amniotic infection and colonization of the amniotic cavity.

Intra-amniotic infection (*n* = 44)	Colonization of the amniotic cavity (*n* = 21)
Atopobium vaginae, Dialister micraerophilus, Fusobacterium nucleatum, Ureaplasma spp. (*n* = 1)	*Corynebacterium tuberculosterericum, Dermabacter hominis, Staphylococcus epidermidis* (*n* = 1)
Aerococcus christensenii, Gardnerella vaginalis, Ureaplasma spp. (*n* = 1)	*Gardnerella vaginalis, Ureaplasma* spp. (*n* = 1)
Campylobacter ureolyticus, Streptococcus anginosus, Streptococcus oralis (*n* = 1)	*Mycoplasma hominis, Ureaplasma* spp. (*n* = 1)
Chlamydia trachomatis, Ureaplasma spp. (*n* = 2)	*Ureaplasma* spp. (*n* = 13)
Fusobacterium nucleatum, Ureaplasma spp. (*n* = 1)	*Escherichia coli* (*n* = 1)
Streptococcus anginosus, Ureaplasma spp. (*n* = 1)	*Gardnerella vaginalis* (*n* = 1)
Ureaplasma spp. (*n* = 20)	*Lactobacillus iners* (*n* = 1)
Haemophilus influenzae (*n* = 5)	*Sneathia sanguinegens* (*n* = 1)
Anaerococcus tetradius (*n* = 1)	*Streptococcus agalactiae* (*n* = 1)
Enterococcus faecalis (*n* = 1)	
Fusobacterium nucleatum (*n* = 1)	
Gardnerella vaginalis (*n* = 1)	
Lactobacillus jensenii (*n* = 1)	
Parvimonas micra (*n* = 1)	
Peptoniphilus spp. (*n* = 1)	
Sneathia sanguinegens (*n* = 1)	
Streptococcus agalactiae (*n* = 1)	
Streptococcus anginosus (*n* = 1)	
Streptococcus intermedius (*n* = 1)	
Non-identifiable bacteria by sequencing (*n* = 1)	

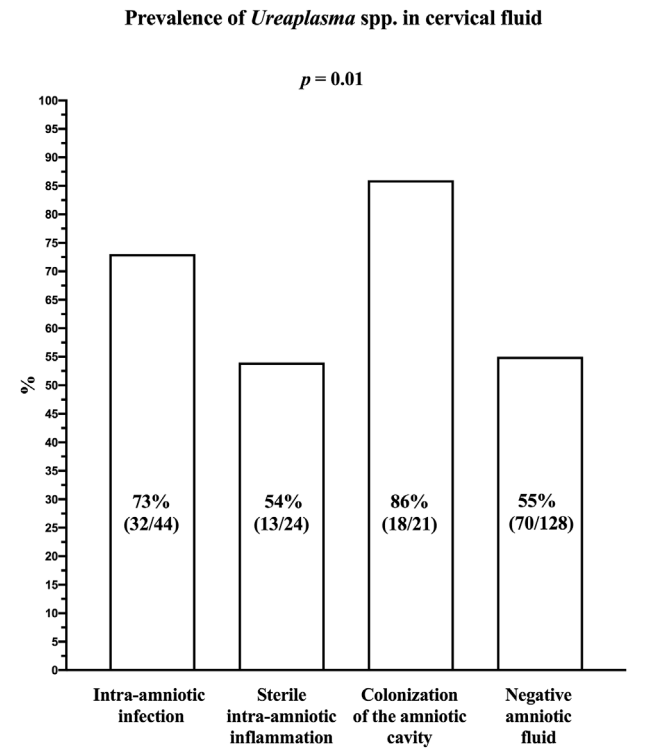

FIGURE 1 | Prevalence of *Ureaplasma* spp. DNA in the cervical fluid of women with preterm prelabor rupture of membranes before 34 weeks of gestation according to the presence of intra-amniotic infection, sterile intra-amniotic inflammation, colonization of the amniotic cavity, and negative amniotic fluid.

cervical fluid compared to those with negative amniotic fluid results; and 3) the presence of intra-amniotic infection, sterile intra-amniotic inflammation, or colonization of the amniotic fluid was associated with higher microbial loads of *Ureaplasma* spp. DNA in the cervical fluid compared to those with negative amniotic fluid; and 4) the presence of sterile intra-amniotic inflammation was related to a higher relative abundance of *Ureaplasma* spp. DNA in the cervical fluid compared to that in negative amniotic fluid.

Ureaplasma spp. are considered commensal microorganisms of the cervical/vaginal niche owing to: 1) their high prevalence in pregnant or non-pregnant women, and 2) the absence of a difference in the prevalence between women of reproductive ages with and without symptoms of infection of the urogenital tract (Marovt et al., 2015; Sweeney et al., 2017). In pregnant women, the presence of *Ureaplasma* spp. in the cervical/vaginal niche has been shown to be associated with adverse pregnancy outcomes (Abele-Horn et al., 2000; Kafetzis et al., 2004; Kataoka et al., 2006). Accordingly, *Ureaplasma* spp. in the cervical/vaginal niche is more frequently observed in pregnancies complicated by PPROM than in uncomplicated pregnancies (Larsen and Hwang, 2010; Donders et al., 2017; Sprong et al., 2020). Its prevalence in PPROM pregnancies varies between 53 and 73% (Kwak et al., 2014; Kwak et al., 2015; Musilova et al., 2016). The observations from this study (61%) are in agreement with previously published findings.

Interestingly, in this study, women with intra-amniotic infection and colonization of the amniotic cavity had higher rates of *Ureaplasma* spp. DNA in their cervical fluid than did women with sterile intra-amniotic inflammation and negative amniotic fluid. In other words, a higher proportion of *Ureaplasma* spp. DNA in the cervical fluid was found in the subgroups of women with microbial invasion of the amniotic cavity. This observation is consistent with the findings of our previous study, in which women with microbial invasion of amniotic inflammation had a higher rate of *Ureaplasma* spp.

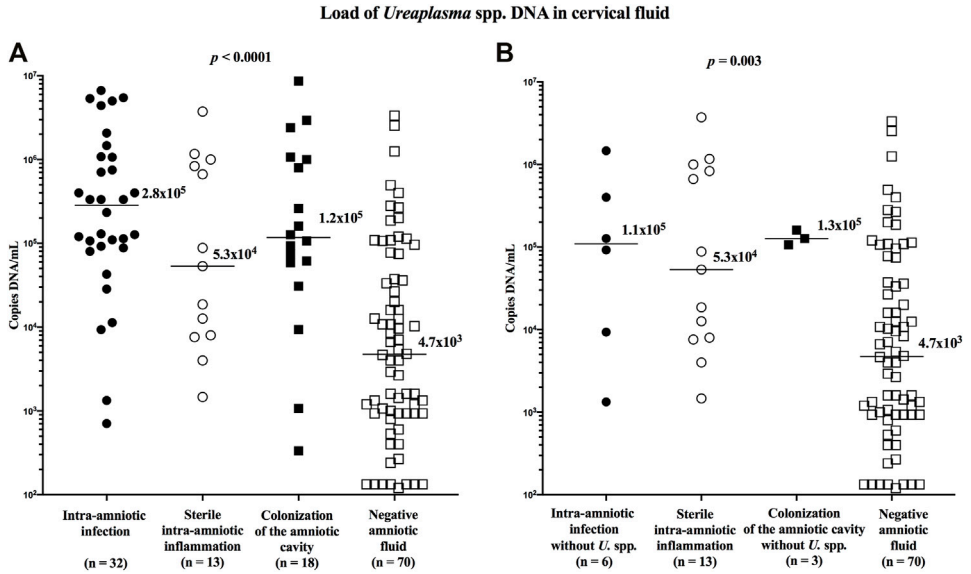

FIGURE 2 | Comparison of the loads of *Ureaplasma* spp. DNA in the cervical fluid among groups of the women with intra-amniotic infection, sterile intra-amniotic inflammation, colonization of the amniotic fluid, and negative amniotic fluid **(A)** and among the same groups when women with the presence of *Ureaplasma* spp. in amniotic fluid were excluded **(B)**. The median values are marked.

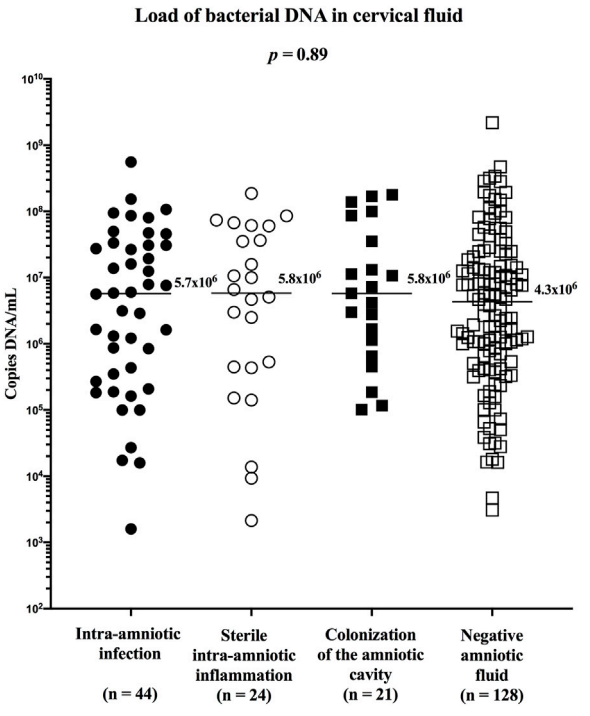

FIGURE 3 | Comparison of the load of bacterial DNA in the cervical fluid among groups of women with intra-amniotic infection, sterile intra-amniotic inflammation, colonization of the amniotic fluid, and negative amniotic fluid.

DNA in the cervical fluid than did those without this complication (Musilova et al., 2016). Notably, in this study, no difference in the frequency of *Ureaplasma* spp. in the cervical fluid between women with sterile inflammation and negative amniotic fluid was identified. This observation differs from that recently reported in women with preterm labor and intact membranes. In that study, the prevalence of *Ureaplasma* spp. DNA in the group of women with sterile intra-amniotic inflammation was comparable to that in women with intra-amniotic infection; however, it was 2-fold higher than that in women with negative amniotic fluid (Kacerovsky et al., 2021c). The diversity in observations between the studies on different phenotypes of spontaneous preterm delivery supports the hypothesis that pathophysiological pathways leading to the development of sterile intra-amniotic inflammation might differ between PPROM and preterm labor with intact membranes.

Abnormal microbiota in the cervical/vaginal niche have been shown to be associated not only with a higher frequency of *Ureaplasma* spp. but also with higher loads of these bacteria in that niche (Donders et al., 2017). Kwak *et al.* reported that a higher load of *Ureaplasma* spp. in the vaginal niche is associated with a higher frequency of histological chorioamnionitis in women with PPROM (Kwak et al., 2014). In line with their report, a difference in the microbial load of *Ureaplasma* spp. DNA in the cervical fluid among the subgroups with intra-amniotic infection, sterile intra-amniotic inflammation, colonization, and negative amniotic fluid (with the lowest levels found in those with negative amniotic fluid) was found in this study. However, this observation was not in concordance with our previous study, where no difference in the load of *Ureaplasma* spp. DNA in the cervical fluid was identified in women with and without

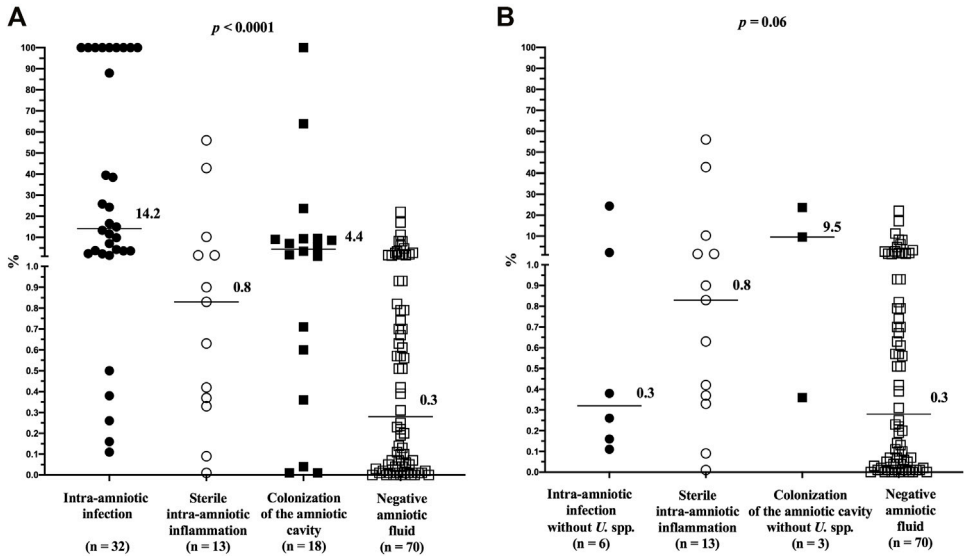

FIGURE 4 | Comparison of the relative abundance of *Ureaplasma* spp. DNA in the cervical fluid among groups of women with intra-amniotic infection, sterile intra-amniotic inflammation, colonization of the amniotic fluid, and negative amniotic fluid **(A)** and among the same groups when women with the presence of *Ureaplasma* spp. in their amniotic fluid were excluded **(B)**. The median values are marked.

microbial invasion of the amniotic cavity (Musilova et al., 2016). The reason for this difference is not fully clear; however, it can be explained by the differences between the studies, including: 1) the sample sizes of women with *Ureaplasma* spp. DNA (previous: $n = 40$; current: $n = 133$) that were studied; and 2) the gestational ages considered during sampling (previous: 24 + 0 to 36 + 6; current: 24 + 0 to 33 + 6).

The relative abundance of *Ureaplasma* spp. DNA in the cervical fluid might represent a more precise tool with which to characterize the association between the composition of the cervical microbiome and intra-amniotic complications, as opposed to the assessment of the absolute number of copies of *Ureaplasma* spp. DNA that are present. In this study, women with PPROM and sterile intra-amniotic inflammation had an approximately 2.5-fold higher relative abundance of *Ureaplasma* spp. DNA compared to women with negative amniotic fluid results. This observation should be considered in terms of the pathophysiology of the development of sterile intra-amniotic inflammation in PPROM.

Taken together, the results from this study suggest that 1) the subgroup of women with PPROM and negative amniotic fluid differs from those with intra-amniotic complications in terms of frequency, loads, and relative abundance of *Ureaplasma* spp. DNA in the cervical fluid; and 2) differences exist between PPROM and preterm labor with intact membranes with regard to the association between *Ureaplasma* spp. DNA in the cervical fluid and intra-amniotic complications. Collectively, these observations further support that pathophysiological pathways leading to the development of intra-amniotic complications might differ between PPROM and preterm labor with intact membranes.

This study has several strengths. First, a relatively large cohort of women with a well-defined clinical phenotype for spontaneous preterm delivery (PPROM) was assessed. Secondly, the presence of *Ureaplasma* spp. DNA in the cervical fluid was evaluated with a specific PCR procedure, which allowed us to identify even very low loads of *Ureaplasma* spp. DNA. Third, thorough assessment of microbial invasion of the amniotic cavity, with the use of a combination of non-specific PCR (16S rRNA) followed by sequencing, specific PCR for *Ureaplasma* spp., *Mycoplasma hominis*, and *Chlamydia trachomatis*, and aerobic/anaerobic cultivation allowed us to precisely dissect the subgroups of women with sterile intra-amniotic inflammation.

This study has limitations that are worth mentioning. First, in women with PPROM, amniotic fluid leaks from the amniotic cavity through the cervix into the posterior vaginal fornix. This means that the leaking amniotic fluid can contaminate the cervical fluid. Therefore, the fluid obtained with a Dacron swab from the endocervical canal of women with PPROM should be considered not just as cervical fluid but as a compound fluid, composed of both cervical fluid and amniotic fluid components. Its composition, as well as the ratio between amniotic and cervical fluids, might vary among women with PPROM and depend on various clinical parameters and scenarios (e.g., amount of residual amniotic fluid, location of the membrane rupture site, position of the fetus, and interval between PPROM and sampling) that occur at the time of sampling. Therefore, if the leaked amniotic fluid contains *Ureaplasma* spp. DNA (in cases of intra-amniotic infection and colonization of the amniotic cavity caused by *Ureaplasma*

spp.), it can affect the total microbial load of the *Ureaplasma* spp. DNA and its relative abundance in the fluid obtained from the endocervical canal. Unfortunately, the presence of this phenomenon is inevitable in PPROM and could affect the assessment of microbial loads and the relative abundance of *Ureaplasma* spp. DNA in the cervical fluid among subgroups of women with PPROM. On the other hand, it is highly unlikely that the presence of *Ureaplasma* spp. DNA in leaked amniotic fluid could affect the assessment of the prevalence of *Ureaplasma* spp. in the cervical fluid, since the cervical/vaginal niche is considered to be a primary source of *Ureaplasma* spp.

Second, biovars, serovars, and other types of *Ureaplasma* spp. were not assessed and considered in this study. This fact should be taken as a shortcoming of this study. However, there is evidence of the possibility of horizontal gene transfer in *Ureaplasma* spp., allowing them to carry markers of multiple serovars (Xiao et al., 2011; Sweeney et al., 2017). Thus, the assessment of serovars could have a limited diagnostic value. Third, during the study period, the methods to assess the concentrations of IL-6 in amniotic fluid were modified. Therefore, the concentrations of IL-6 in this study were determined using two different approaches (lateral flow immunoassay point-of-care test and automated electrochemiluminescence). This fact prevents us from assessing the association between the intensity of the intra-amniotic inflammatory response, measured by IL-6 concentrations in amniotic fluid, and the loads of *Ureaplasma* spp. DNA in the cervical fluid.

In conclusion, in PPROM at < 34 weeks, the presence of intra-amniotic infection, sterile intra-amniotic inflammation, or colonization of the amniotic fluid was associated with a higher prevalence and/or load of *Ureaplasma* spp. DNA in cervical fluid than in the absence of intra-amniotic complications.

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 Institutional review board of the University Hospital Hradec Kralove. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MK, IM—writing of the manuscript MK, JM, JS, JM, IM, RK, RB, and PB -data acquistion MK, IM, BJ, and JM - data analysis MK, IM, BJ, PB, RK, RB, and JM—interpretation of data RK, RB, PB, JS, JMa, JMl, and BJ—critical revising of the manuscript MK, RK, RP, PB, JS, JMa, JMl, BJ, and IM—approval for publication.

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Development of a Rat Model of Intra-Amniotic Inflammation *via* Ultrasound-Guided Administration of a Triggering Agent in the Gestational Sac to Enable Analysis of Individual Amniotic Fluid Samples

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Objectives: To develop a rat model of intra-amniotic inflammation, characterized by the concentration of interleukin-6 in the amniotic fluid, induced by an ultrasound-guided transabdominal administration of lipopolysaccharide into individual gestational sacs.

Methods: An ultrasound-guided transabdominal intra-amniotic administration of lipopolysaccharide or phosphate-buffered saline (PBS) as control was performed in rats on embryonic day 18. Only accessible gestational sacs with precise recording of their positions were injected. Twenty-four hours later, individual amniotic fluid samples were collected from the gestational sacs of laparotomized animals. The gestational sacs were divided into four subgroups: (i) with lipopolysaccharide: injected gestational sacs from rats undergoing lipopolysaccharide administration; (ii) without lipopolysaccharide: non-injected gestational sacs from rats undergoing lipopolysaccharide administration; (iii) with PBS: injected gestational sacs from rats undergoing PBS administration; and (iv) without PBS: non-injected gestational sacs from rats undergoing PBS administration. The concentration of interleukin-6 in individual amniotic fluid samples was assessed using ELISA.

Results: In the group of five animals receiving lipopolysaccharide, 24 (33%) and 48 (77%) gestational sacs were and were not injected, respectively. The amniotic fluid was obtained from 21 (88%) injected and 46 (95%) non-injected sacs. In the control group of five animals receiving phosphate-buffered saline, 28 (35%) and 52 (75%) gestational sacs were and

were not injected, respectively. The amniotic fluid was obtained from 18 (64%) injected and 50 (96%) non-injected sacs. No labor occurred, and only one fetal death was observed in a gestational sac injected with lipopolysaccharide. Differences in concentrations of interleukin-6 in the amniotic fluid were found among the subgroups of the gestational sacs (with lipopolysaccharide: median 762 pg/ml; without lipopolysaccharide: median 35.6 pg/ml; with PBS: median 35.6 pg/ml; and without PBS: median 35.6 pg/ml; $p < 0.0001$). Concentrations of interleukin-6 in the amniotic fluid from the gestational sacs with lipopolysaccharide were significantly higher than those in the three remaining subgroups ($p < 0.0001$). No differences in concentrations of interleukin-6 in the amniotic fluid were identified between the three remaining subgroups.

Conclusion: The ultrasound-guided transabdominal intra-amniotic administration of lipopolysaccharide with a subsequent collection and analysis of amniotic fluid samples is feasible in rats. The intra-amniotic administration of lipopolysaccharide led to the development of intra-amniotic inflammation without leading to fetal mortality or induction of labor.

Keywords: animal model, preterm birth, preterm delivery, lipopolysaccharide, minimally invasive, amniocentesis

INTRODUCTION

Spontaneous preterm delivery accounts for approximately 8% of all live births worldwide and is the leading cause of perinatal mortality and morbidity (Blencowe et al., 2012; Walani, 2020). It represents one of the "great obstetrical syndromes," resulting from a multifactorial etiology and complex pathogenesis (Romero et al., 2014a). Intra-amniotic inflammation plays a crucial role in the pathogenesis of preterm delivery and is characterized by the elevation in various inflammatory mediators in the amniotic fluid (Romero et al., 2007). This intra-amniotic complication may be identified in up to 40% of pregnancies with spontaneous preterm delivery (Romero et al., 2014c; Musilova et al., 2015; Kacerovsky et al., 2021; Stranik et al., 2021). Based on the triggering stimulus, two different clinical phenotypes of intra-amniotic inflammation can be distinguished: (i) intra-amniotic infection and (ii) sterile intra-amniotic inflammation when microorganisms and/or their nucleic acids are present or absent in the amniotic fluid, respectively (Romero et al., 2014c; Stranik et al., 2021). Regardless of the nature of intra-amniotic inflammation, its presence remains a serious clinical issue because of its association with adverse pregnancy and neonatal outcomes (Soucy-Giguere et al., 2018).

Animal models represent an important tool in the research on intra-amniotic inflammatory complications in spontaneous preterm births (Nielsen et al., 2016). Compared with human studies, they provide an opportunity for a broad range of study designs, enabling deep and comprehensive insights into the pathogenesis of intra-amniotic inflammatory complications and their association with spontaneous preterm delivery (Spencer et al., 2021). The development of an animal model of intra-amniotic inflammation provides various routes for the administration of triggering agents leading to an intra-amniotic inflammatory response (Elovitz and Mrinalini, 2004). The possible routes of application involve two main approaches: (i) systemic, mainly intraperitoneal application, which is far from a real clinical scenario, and (ii) localized, including vaginal, intracervical, intrauterine, and intra- or extra-amniotic routes (Elovitz and Mrinalini, 2004; Stranik et al., 2020). The intra-amniotic administration of a triggering agent is an ideal approach for the development of a well-defined, local, and intra-amniotic inflammatory response, with the opportunity to study intra-amniotic inflammatory complications and precisely mimic different specific clinical scenarios.

Rodents, particularly mice and rats, are the most frequently used experimental animals to study spontaneous preterm delivery and intra-amniotic inflammatory complications (Nielsen et al., 2016). In these small animals, the administration of triggering agents *via* the intra-amniotic route requires an invasive approach, i.e., laparotomy to access the uterus (Rounioja et al., 2003; Gisslen et al., 2019; Stranik et al., 2020). However, the surgical nature of such an approach is associated with additional stressful stimuli for the animals, particularly for control groups, which might affect the results of the experiment (Rinaldi et al., 2015). To reduce these adverse effects and potential bias, a non-invasive ultrasound-guided transabdominal intra-amniotic administration of triggering agents was introduced by Gomez–Lopez et al. in 2016 for mice (Gomez-Lopez et al., 2016). Unfortunately, the limited amount of amniotic fluid in the gestational sac of mice leads to the use of pooled amniotic fluid from various gestational sacs and prevents the use of individual amniotic fluid samples from a particular gestational sac (Garcia-Flores et al., 2018; Brown et al., 2019; Motomura et al., 2020; Shynlova et al., 2021). This shortcoming can be overcome by using larger animals, such as rats, which have higher volumes of amniotic fluid. In addition, rats are more resistant to labor induction following the administration of an inflammatory agent into the gestational sac (Cookson et al., 2018; Dedja et al., 2018). This prerequisite makes this animal ideal to thoroughly study the intrauterine effects and consequences of intra-amniotic

inflammatory complications on the fetus, placenta, and fetal membranes. Collectively, these results suggest that rats are an optimal animal model of intra-amniotic inflammatory complications. However, a rat model based on a non-invasive ultrasound-guided transabdominal intra-amniotic administration of a triggering agent has yet to be developed.

Therefore, the main aim of this study was to establish a rat model of intra-amniotic inflammation, characterized by the concentration of interleukin (IL)-6 in the amniotic fluid, induced by an ultrasound-guided transabdominal administration of *Escherichia coli* (*E. coli*) lipopolysaccharide (LPS) into individual gestational sacs.

MATERIALS AND METHODS

Animals

Pregnant Wistar rats were purchased from Velaz (Prague, Czechia) and housed in the vivarium of the Faculty of Medicine at Hradec Kralove under standard conditions (12 h light/dark cycles, a steady temperature of 22 ± 2°C, a relative air humidity of 50 ± 10%, and water and pellets ad libitum). Embryonic day (E) 1 was defined as the morning when vaginal plug formation occurred. All procedures were performed in accordance with the Act on the Protection of Animals against Cruelty, Act No. 246/1992 Coll., with the approval of the Animal Welfare Committee of the Faculty of Medicine in Hradec Kralove, Charles University and Czech Ministry of Education, Youth and Sports (No. 41058/2016-MZE-17214).

Ultrasound-Guided Intra-Amniotic Administration of LPS

The intra-amniotic administration was performed on E18. Dams were sedated by the inhalation of 5% isoflurane (Isoflurin, Vetpharma AH, S.L., Barcelona, Spain) with oxygen at 2 L/min in the induction chamber. Anesthesia was maintained with 1.5–2.0% isoflurane and oxygen at 2 L/min. The animals were positioned and fixed on a heating pad from the Vevo Imaging Station (FUJIFILM VisualSonics Inc., Toronto, ON, Canada). The body temperature was maintained at 37 ± 1°C and measured using a rectal thermometer (FUJIFILM VisualSonics Inc., Toronto, ON, Canada). The heart rate and respiratory rate were monitored using electrodes embedded in the heating pad. Fur was removed using a depilatory cream. The ultrasound transducer MX400 (Vevo 3100; FUJIFILM VisualSonics Inc., Toronto, ON, Canada) was placed in a mechanical holder and stabilized at a position that displayed the target gestational sac.

Under ultrasound guidance, the intra-amniotic administration of 10 μg of *E. coli* LPS (serotype O55:B5, Sigma-Aldrich, Prague, Czechia) in 100 μL of phosphate-buffered saline (PBS) was performed using a 27 G × 40 mm needle (B. Braun Melsungen, Germany) with an Omnican® 50 syringe (B. Braun, Melsungen, Germany) stabilized in a mechanical holder. The control animals were injected with 100 μL of PBS. Only the accessible gestational sacs with precise recordings of their positions were manipulated (**Figure 1**). Following the procedure, the animals were kept under a heat lamp for recovery and then returned to their cages.

The gestational sacs were divided into four subgroups based on selective intra-amniotic administration: (i) **gestational sacs with LPS:** injected gestational sacs from rats undergoing LPS administration; (ii) **gestational sacs without LPS:** non-injected gestational sacs from rats undergoing LPS administration; (iii) **gestational sacs with PBS:** injected gestational sacs from rats undergoing PBS administration; and (iv) **gestational sacs without PBS:** non-injected gestational sacs from rats undergoing PBS administration.

Amniotic Fluid Collection

Twenty-four hours after the intra-amniotic administration, on E19, the animals were anesthetized and prepared for an ultrasound examination in the same manner as for the intra-amniotic administration. The position of the gestational sac and vitality of the pups were assessed using the ultrasound transducer MX250S (15–30 MHz). The uterine horns were exposed using a midline abdominal incision. Before any manipulation of the uterine horns, the injected sacs were identified with respect to the localization recorded a day before administration. After identification, both the uterine horns were removed from the abdominal cavity. Using a sterile 30 G × 13 mm needle (B. Braun Melsungen, Germany), the amniotic fluid was aspirated from all sacs and stored in polypropylene tubes at –70°C until analysis (**Figure 1**). The placentas, membranes, and fetal tissues were harvested, snap-frozen in liquid nitrogen, and stored at –70°C for further analyses. The animals were sacrificed *via* exsanguination under anesthesia.

Assessment of Amniotic Fluid IL-6

Concentrations of IL-6 in the amniotic fluid samples were assessed using the Rat IL-6 Quantikine ELISA Kit (R&D Systems Inc., Minneapolis, MN, United States) according to the manufacturer's instructions. The sensitivity of the kit was 36 pg/ml, and the inter-assay and intra-assay coefficients were <9% and <10%, respectively. The absorbance was measured at 450 nm using a Multiskan RC ELISA reader (Thermo Fisher Scientific, Waltham, MA, United States).

Statistical Analyses

The normality of the data was tested using the Anderson–Darling test. The concentrations of IL-6 in the amniotic fluid were not normally distributed, therefore, nonparametric Kruskal–Wallis or Mann–Whitney *U* tests were used for analyses. All *p* values were determined using two-tailed tests, and all statistical analyses were performed using GraphPad Prism v8 for Mac OS X (GraphPad Software, San Diego, CA, United States).

RESULTS

Animal Characteristics

In total, ten rats were included in the study, of which five received LPS and five were administered PBS only.

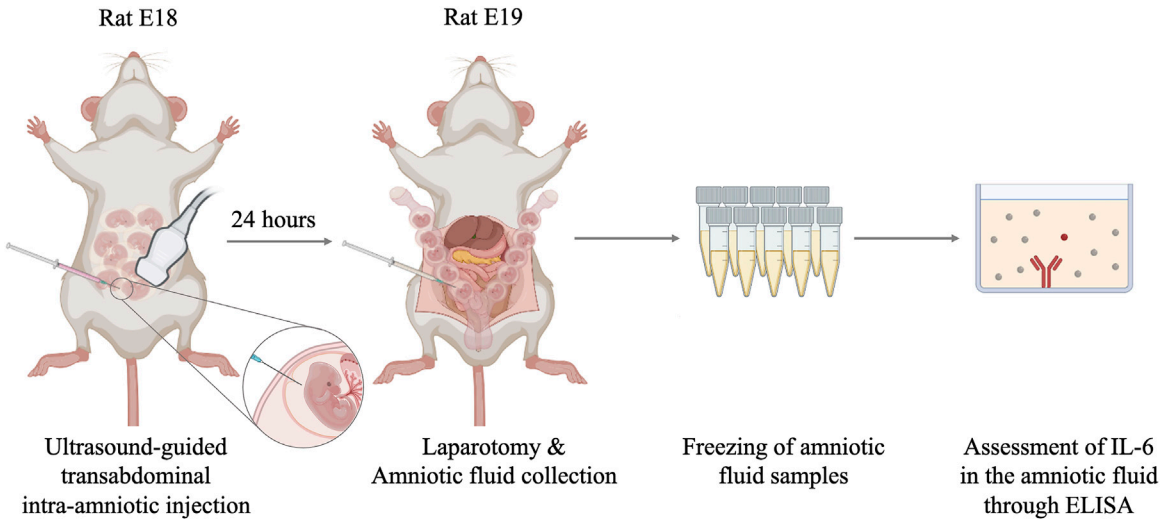

FIGURE 1 | Graphic illustration of the experimental design. IL: interleukin.

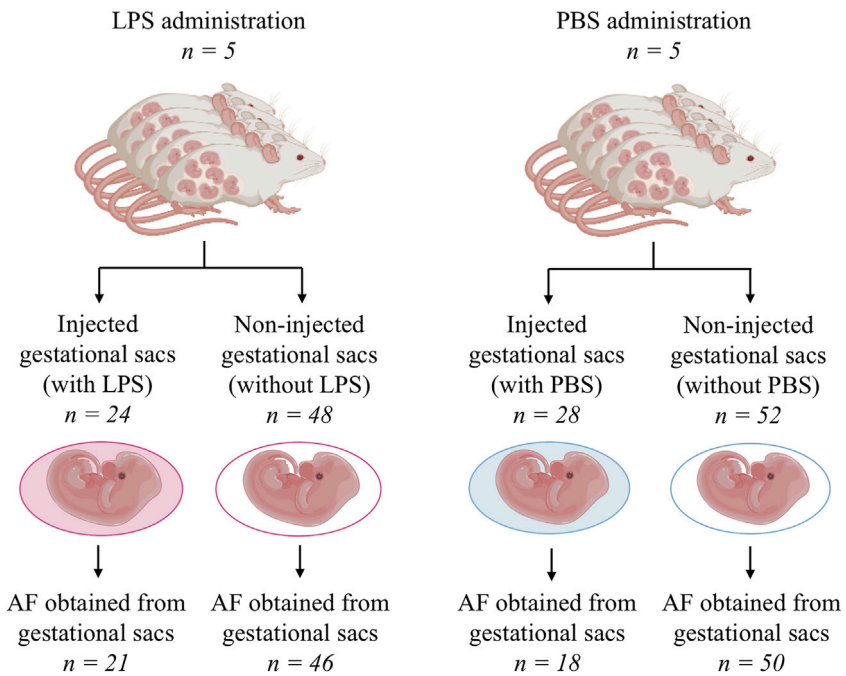

FIGURE 2 | Schema of the flow through the animal experiment. AF, amniotic fluid; LPS, lipopolysaccharide; and PBS, phosphate-buffered saline.

In the group of animals receiving LPS (n = 5), from a total number of 72 gestational sacs, 24 (33%) and 48 (77%) were and were not injected with LPS, respectively. In the group of animals receiving PBS only, from a total number of 80 gestational sacs, 28 (35%) and 52 (75%) were and were not injected with PBS, respectively (**Figure 2**).

Parturition Initiation

Labor did not occur in any dam within 24 h following administration.

Fetal Mortality

One intrauterine fetal death was observed in the subgroup of the gestational sacs with LPS, and the mortality rate of this subgroup was

4% (1/28). No amniotic fluid was received from the gestational sac owing to anhydramnios. All fetuses from the other subgroups survived.

Amniotic Fluid Collection

In the group of animals receiving LPS, an amniotic fluid volume sufficient for analysis was obtained from 21 (88%) gestational sacs injected with LPS and from 46 (95%) gestational sacs without LPS (**Figure 2**). In the PBS group, an amniotic fluid volume sufficient for analysis was obtained from 18 (64%) gestational sacs injected with PBS and 50 (96%) gestational sacs without PBS (**Figure 2**).

Concentration of IL-6 in the Amniotic Fluid After Intra-Amniotic LPS Administration

Differences in the concentration of IL-6 in the amniotic fluid were found among the subgroups of the gestational sacs (gestational sacs with LPS: median 762 pg/ml; IQR 340.8–1,093 pg/ml; gestational sacs without LPS: median 35.6 pg/ml, IQR 35.6–46.63 pg/ml; gestational sacs with PBS: median 35.6 pg/ml, IQR 35.6–45.38 pg/ml; and gestational sacs without PBS: median 35.6 pg/ml, IQR 35.6–35.6 pg/ml; $p \leq 0.0001$; **Figure 3**).

The concentrations of IL-6 in the amniotic fluid samples obtained from the gestational sacs with LPS were higher than those in the amniotic fluid samples obtained from gestational sacs without LPS and those with or without PBS (**Table 1**). No differences in the concentrations of IL-6 in the amniotic fluid were identified between the gestational sacs without LPS and those with or without PBS (**Table 1**).

DISCUSSION

The principal findings of this study were as follows: i) the ultrasound-guided transabdominal intra-amniotic administration of an agent was a feasible procedure in rats, ii) the ultrasound-guided transabdominal intra-amniotic administration of 10 μg of *E. coli* LPS serotype O55:B5 did not induce labor within 24 h after administration in rats, iii) fetal mortality associated with the ultrasound-guided transabdominal intra-amniotic administration of 10 μg of *E. coli* LPS serotype O55:B5 was at 4%, iv) the collection of individual amniotic fluid samples from the gestational sacs was feasible, v) the ultrasound-guided transabdominal intra-amniotic administration of 10 μg of *E. coli* LPS serotype O55:B5 in rats led to elevated concentrations of IL-6 in the amniotic fluid only in the injected gestational sacs, and vi) there were no elevations in IL-6 concentrations in the amniotic fluid of gestational sacs not injected with LPS.

In small laboratory animals, the administration of a triggering agent under ultrasound guidance has become possible owing to the advent of high-frequency ultrasound devices designed specifically for small animals (Greco et al., 2013; Galaz et al., 2020). Ultrasound-guided procedures have been used to induce intra-amniotic inflammation in mice (Gomez-Lopez et al., 2016; Gomez-Lopez et al., 2018; Faro et al., 2019; Gomez-Lopez et al., 2019; Motomura et al., 2020). The first use of ultrasound guidance for transabdominal injections was reported by Rinaldi et al. (2015), but this approach was used for intrauterine extra-amniotic administration. Ultrasound-guided transabdominal intra-amniotic administration in mice has become the standard method for the research group of Gomez–Lopez (Gomez-Lopez et al., 2016; Gomez-Lopez et al., 2016; Gomez-Lopez et al., 2018; Faro et al., 2019; Gomez-Lopez et al., 2019; Motomura et al., 2020). The present absence of the use of an ultrasound-guided transabdominal intra-amniotic administration of a triggering agent in rats may be owing to multiple reasons, nevertheless, the thicker rat skin impeding needle passage through the abdominal wall might play a substantive role. This obstacle might be resolved by slowly puncturing the skin to allow the tip of the needle to spontaneously slide through the skin, abdominal and uterine walls, and membranes of the gestational sac.

There is evidence that the intra-amniotic administration of LPS to mice causes preterm delivery in most animals (Garcia-Flores et al., 2018; Gomez-Lopez et al., 2018; Faro et al., 2019). In our study, the dams did not deliver within 24 h after the intra-amniotic administration of LPS despite the development of an intra-amniotic inflammation, characterized by the elevation in IL-6 concentrations in the amniotic fluid. This was in line with other rat studies, in which the intra-amniotic administration of LPS via laparotomy did not induce delivery (Cookson et al., 2018; Dedja et al., 2018). The fact that the rat animal model of intra-amniotic inflammation was not associated with the risk of labor induction within 24 h is important from a research standpoint because it provides an opportunity to collect various body fluids

TABLE 1 | Comparisons of interleukin-6 concentrations in the amniotic fluid from individual gestational sacs after an ultrasound-guided transabdominal intra-amniotic administration of either lipopolysaccharide or phosphate-buffered saline in rats.

		Administration of lipopolysaccharide (LPS)		Administration of phosphate-buffered saline (PBS)	
		Gestational sacs with LPS	Gestational sacs without LPS	Gestational sacs with PBS	Gestational sacs without PBS
Administration of LPS	Gestational sacs with LPS	x	$\mathbf{p < 0.0001}$	$\mathbf{p < 0.0001}$	$\mathbf{p < 0.0001}$
	Gestational sac without LPS	$\mathbf{p < 0.0001}$	x	$p = 1.00$	$p = 0.61$
Administration of PBS	Gestational sac with PBS	$\mathbf{p < 0.0001}$	$p = 1.00$	x	$p = 1.00$
	Gestational sac without PBS	$\mathbf{p < 0.0001}$	$p = 0.61$	$p = 1.00$	x

Comparisons were performed using a nonparametric Mann–Whitney U test.
Statistically significant results are marked in bold.

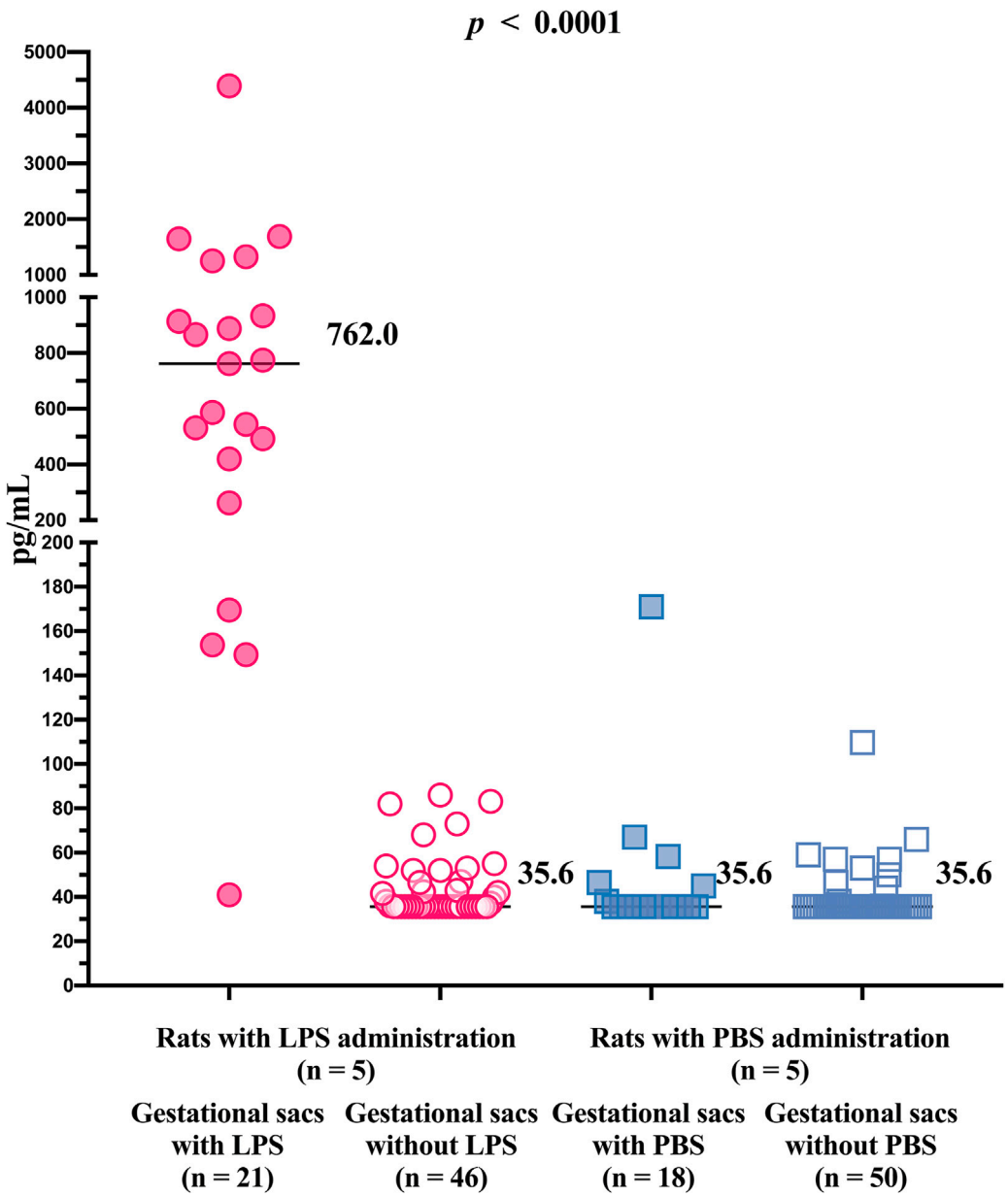

FIGURE 3 | Concentrations of IL-6 in the amniotic fluid from individual gestational sacs of rats after an ultrasound-guided transabdominal intra-amniotic administration of either lipopolysaccharide or phosphate-buffered saline. LPS, lipopolysaccharide; PBS, phosphate-buffered saline; and IL, interleukin.

and tissues from gestational sacs after their exposure to intra-amniotic inflammation. This approach mimics human clinical scenarios (Musilova et al., 2018; Kacerovsky et al., 2020b).

The preservation of the vitality of the fetus after a triggering agent administration represents an important key feature of the animal model of local intra-amniotic inflammation, mimicking the clinical scenario of human pregnancy complicated by intra-amniotic inflammation (Musilova et al., 2015). The presence of a vital fetus allows the possibility of studying the consequences of intra-amniotic inflammation on the fetus *in utero*, for example, via ultrasonography or other diagnostic methods. The intra-amniotic administration of LPS in mice models is related to a significantly high mortality rate, however, such a phenomenon has not been reported in rat studies (Cookson et al., 2018; Gomez-Lopez et al., 2018; Jantzie et al., 2018; Faro et al., 2019). Fetal mortality in mice due to LPS administration is dependent on the

dose and type of LPS (Migale et al., 2015), however, based on the rare occurrence of fetal mortality after the intra-amniotic administration of LPS in rats, this animal model seems to be less sensitive to this complication. These observations are in agreement with those of our study, where only one intrauterine fetal death occurred among 28 fetuses from the gestational sacs with intra-amniotic inflammation (injected with LPS). This intrauterine demise might be considered a direct consequence of intra-amniotic inflammation, however, the fetal trauma associated with the gestational sac puncture, as a cause of death, cannot be completely excluded.

In human clinical practice, intra-amniotic inflammation is identified based on the assessment of various inflammatory markers (such as IL-6, matrix metalloproteinase 8, or glucose) in the sampled amniotic fluid (Nien et al., 2006; Kacerovsky et al., 2014; Kacerovsky et al., 2020a; Oh et al., 2020). Therefore, it is of utmost relevance to have individual amniotic fluid samples available from animal models to study changes in amniotic fluid composition under various research scenarios. Thus far, the analysis of pooled samples has been preferred owing to the low volume of the amniotic fluid obtained from the gestational sacs in small laboratory animals (Awad et al., 2011; Garcia-Flores et al., 2018; Simoes et al., 2018; Motomura et al., 2020). In this study, individual amniotic fluid samples from rats were analyzed. The amount of amniotic fluid obtained from gestational sacs was sufficient to assess IL-6 using a commercially available ELISA kit in a standard manner. Regardless of the limited amount of amniotic fluid obtained from each gestational sac, we believe that this amount would be sufficient for running most antibody-based assays, however, it might restrict the spectrum or number of analytes that can be assessed. It is worth mentioning that intra-amniotic administration/injection is prone to the reduction of the amniotic fluid volume owing to i) a possible leakage of the amniotic fluid from the gestational sac through the site of fetal membrane perforation and ii) an alteration of the amniotic fluid production by the fetal membranes owing to the development of an intra-amniotic inflammatory response.

LPS, a component of the cell wall of Gram-negative bacteria, has been a typical triggering agent that has been inducing inflammation in animal models for decades (Kemp et al., 2010). In clinical scenarios, Gram-negative bacteria are not the most common microorganisms causing intra-amniotic complications (Musilova et al., 2015; Musilova et al., 2017). However, the strong potential of LPS to trigger a severe intra-amniotic inflammatory response leading to the development of intra-amniotic inflammation was the reason for using this triggering agent in the development of this animal model. Systemic LPS administration to pregnant rats was followed by the production of IL-6, IL-1β, and the tumor necrosis factor-α in the amniotic fluid (Urakubo et al., 2001; Beloosesky et al., 2006; Awad et al., 2011). However, no studies have assessed the levels of inflammatory mediators in the amniotic fluid following intra-amniotic administration in rats. In our study, the intra-amniotic injection of 10 μg of *E. coli* LPS serotype O55:B5 per gestational sac triggered a marked elevation in the IL-6 concentration in the amniotic fluid. This elevation was observed only in gestational sacs injected with LPS, whereas no changes in the concentrations of IL-6 were identified in the non-injected gestational sacs from the same dam 24 h after LPS administration. The concurrent presence of injected and non-injected gestational sacs in one uterus offers a scenario clinically relevant to multiple pregnancies, as the presence of intra-amniotic inflammation associated with spontaneous preterm delivery in twins may be observed in only one of them (Oh et al., 2019).

This study has several strengths. First, a minimally invasive approach for the intra-amniotic administration of LPS was used. Second, the intensity of the intra-amniotic inflammatory response in individual gestational sacs was determined *via* the analysis of selected inflammatory mediators in the amniotic fluid. The individual analysis of amniotic fluid samples after the selective intra-amniotic administration of various triggering agents provides the opportunity to study differences in the intensities of intra-amniotic inflammatory responses not only between various dams but also between injected and non-injected gestational sacs originating from one dam.

Nevertheless, the study has some limitations. First, only one dose of LPS was used. We used an already proven dose of 10 μg *E. coli* LPS serotype O55:B5 that induces fetal lung inflammatory injury and does not lead to preterm delivery (Cookson et al., 2018). Second, only one 24-h interval from LPS administration to amniotic fluid sampling was used. The absence of other time intervals did not allow us to describe the temporal relationship between intra-amniotic administration of triggering agents and the development of intra-amniotic inflammatory response. Third, only one thoroughly selected inflammatory mediator was used to determine the intensity of intra-amniotic inflammation. Nevertheless, IL-6 is considered the gold standard marker in the identification of intra-amniotic inflammation, superior to classical markers such as glucose, lactate, and white blood cell counts and is not inferior to modern proteomic markers (Romero et al., 1993a; Romero et al., 1993b; Romero et al., 2014b).

In conclusion, the ultrasound-guided transabdominal intra-amniotic administration of LPS with subsequent collection and analysis of amniotic fluid samples was feasible in rats. The intra-amniotic administration of LPS led to the development of intra-amniotic inflammation without fetal mortality or induction of labor.

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 animal study was reviewed and approved by the Animal Welfare Committee of the Faculty of Medicine in Hradec Kralove, Charles University and Czech Ministry of Education, Youth and Sports (No. 41058/2016-MZE-17214).

AUTHOR CONTRIBUTIONS

Conception of the study: IM, MK, and BJ; drafting the manuscript: IM and JS; experiments and analyses: IM, JS, CtA, CiA, MS, FS, SM, and OS; critical revision of the manuscript: MK, FS, SM, and BJ; funding: MK; revision and approval of the final version of the manuscript: JS, MK, MS, CtA, CiA, FS, SM, OS, BJ, and IM.

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REVIEW
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Immunological Changes in Pregnancy and Prospects of Therapeutic Pla-Xosomes in Adverse Pregnancy Outcomes

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Stringent balance of the immune system is a key regulatory factor in defining successful implantation, fetal development, and timely parturition. Interference in these primary regulatory mechanisms, either at adolescence or prenatal state led to adverse pregnancy outcomes. Fertility restoration with the help of injectable gonadotrophins/progesterone, ovulation-inducing drugs, immunomodulatory drugs (corticosteroids), and reproductive surgeries provides inadequate responses, which manifest its own side effects. The development of a potential diagnostic biomarker and an effectual treatment for adverse pregnancy outcomes is a prerequisite to maternal and child health. Parent cell originated bi-layered-intraluminal nano-vesicles (30–150 nm) also known as exosomes are detected in all types of bodily fluids like blood, saliva, breast milk, urine, etc. Exosomes being the most biological residual structures with the least cytotoxicity are loaded with cargo in the form of RNAs (miRNAs), proteins (cytokines), hormones (estrogen, progesterone, etc.), cDNAs, and metabolites making them chief molecules of cell-cell communication. Their keen involvement in the regulation of biological processes has portrayed them as the power shots of cues to understand the disease's pathophysiology and progression. Recent studies have demonstrated the role of immunexosomes (immunomodulating exosomes) in maintaining unwavering immune homeostasis between the mother and developing fetus for a healthy pregnancy. Moreover, the concentration and size of the exosomes are extensively studied in adverse pregnancies like preeclampsia, gestational diabetes mellitus (GDM), and preterm premature rupture of membrane (pPROMs) as an early diagnostic marker, thus giving in-depth information about their pathophysiology. Exosomes have also been engineered physically as well as genetically to enhance their encapsulation efficiency and specificity in therapy for cancer and adverse pregnancies. Successful bench to bedside discoveries and interventions in cancer has motivated developmental biologists to investigate the role of immunexosomes and their active components. Our review summarizes the pre-clinical studies for the use of these power-shots as therapeutic agents. We envisage that these studies will pave the path for the use of immunexosomes in

clinical settings for reproductive problems that arise due to immune perturbance in homeostasis either at adolescence or prenatal state.

Keywords: pregnancy, pla-xosomes, cancer, exosomes, adverse pregnancy outcome, immune exhaustion, immune-therapy

INTRODUCTION

The semi-allogenic fetus develops and resides within the mother's womb, causing a series of physiological, structural, organismal changes in her body. These profound changes take place proximally in the endometrium and the uterine cavity to protect the fetus from rejection via modulation of the maternal immune system and structural remodeling to provide better nutrition for the growing fetus. Distally the informed changes in maternal physiology are an adaptation process in order to prepare the mother for the rest of the gestational journey. The endocrine signals (progesterone, estrogen, human chorionic gonadotrophin (hCG), genomic (miRNAs), and metabolomic entities (lipids, amino acids, etc.) work in conjunction to progenerate the maternal immune system towards accepting the fetal antigens, which is a kind of stress test for the mother (Bukovsky et al., 2003; Li et al., 2004; Mulac-Jericevic and Conneely, 2004; Rolle et al., 2013; Jabrane-Ferrat, 2019). The fetoplacental communication resembles a webbed structure with every node impersonating an immune cell, required to maintain equilibrium among all cells in the unit. The maternal immune system is renovated, providing a suppressive immune niche for fetal survival, thus establishing a crucial feto-maternal immune crosstalk. Interestingly in cancer, a similar mechanism of reconditioning the immune system for favorable changes is very well studied (Costanzo et al., 2018). Cancer progression is thus a phenomenon of forced changes and has similarities with regulated fetal growth during pregnancy. The host and maternal immune system engage in a contest of strength towards producing a response against developing cancer and the fetus. Ultimately this response modulates the host and maternal immune system resulting in the establishment of cancer and sustenance of the fetus, respectively. This immunomodulation is effectively aided by the signals emanating within the bilayered-intraluminal nanovesicles, which work distally in maintaining the immune crosstalk for their stabilization (Salomon et al., 2014a). Discovered almost 40 years ago in 1989 (Trams et al., 1981; Pan and Johnstone, 1983), the extracellular vesicles named exosomes were characterized later as lipid-bilayered-intraluminal microvesicles (ILVs) (30–150 nm), yielded by invagination of multivesicular bodies (MVBs) derived from endosomes during stress response or for cell-cell communication (Harding et al., 1984). Exosomes are decisive in an aspect because they encapsulate regulatory signals of cellular behavior. Demonstrated in the database, over 9,690 kinds of proteins, more than 3,300 varieties of mRNAs, and 1,010 different types of lipids can exist in an exosome depending upon its origin (Keerthikumar et al., 2016; Kurian and Modi, 2019). Studies have represented that the exosomes are extensively involved in feto-maternal communication facilitating embryo implantation, trophoblast invasion, trophoblast proliferation, angiogenesis, glucose metabolism, and immunological signaling (Salomon et al., 2014b). The mission to these exosomes is assigned by the placenta. Evidential studies have praised the similarities between the placenta and cancer on the behalf of their mechanism for evasion of immune response utilizing exosomes, thus generating a fetal or tumor-sustaining environment (Holtan et al., 2009). Such similarities have puzzled the brilliant scientific mind for ages, hence it is fascinating to connect and observe the underlined mechanisms. This review emphasizes how these factors (immune-exosomes) interact with the immune cells to modify their functions and affect their metabolic rates so as to yield a balanced pro- and anti-inflammatory milieu for successful fetal development and timely parturition. A well-sustained fetal development and timely parturition are based on a well-regulated immune clock implicating a pro-inflammatory milieu in the first and third trimester along with a skewed but required anti-inflammatory milieu in the second trimester (Dekel et al., 2010). Alterations of this stringent immune clock result in pregnancy complications like pre-eclampsia (PE), gestational diabetes (GDM), and preterm birth (PTB) (Erlebacher et al., 2007; Schonkeren et al., 2011; Han et al., 2015). The mass manipulation of the immune system by cancer cells via exosomes, for their survival, can be instigated for the ideas in mending the immune perturbations resulting in pregnancy complications. Therefore, we attempt to explore the role of the immune-exosomes in cancer and pregnancy focusing on taking lessons from the trail followed by cancer-derived immune-exosomes, which can help in the development of future therapeutics for pregnancy complications.

Further, we envisage that bringing about modification at the immune level with the use of exosomes as immunomodulatory effectors may prove as therapeutic tools, as have been studied in building up a strong tolerogenic niche for cancer survival.

Conjunction of Bio-Molecules in a Healthy Pregnancy

Hormones, miRNAs and metabolites impact various immune cells and alter their lineages resulting in modification of their effector functions. This causes disbalance of pro- and anti-inflammatory milieu leading to adverse pregnancy outcomes.

Hormones: The Catalysts of Pregnancy

Progesterone, in most mammals, is essential for successful implantation and maintenance of gestation. Progesterone acts through its two nuclear progesterone receptor (PR) isoforms, PRA and PRB (Li et al., 2004; Mulac-Jericevic and Conneely, 2004). The A isoform is responsible for fertility in mice and B is involved in the development of the mammary gland (Mulac-Jericevic et al., 2000;

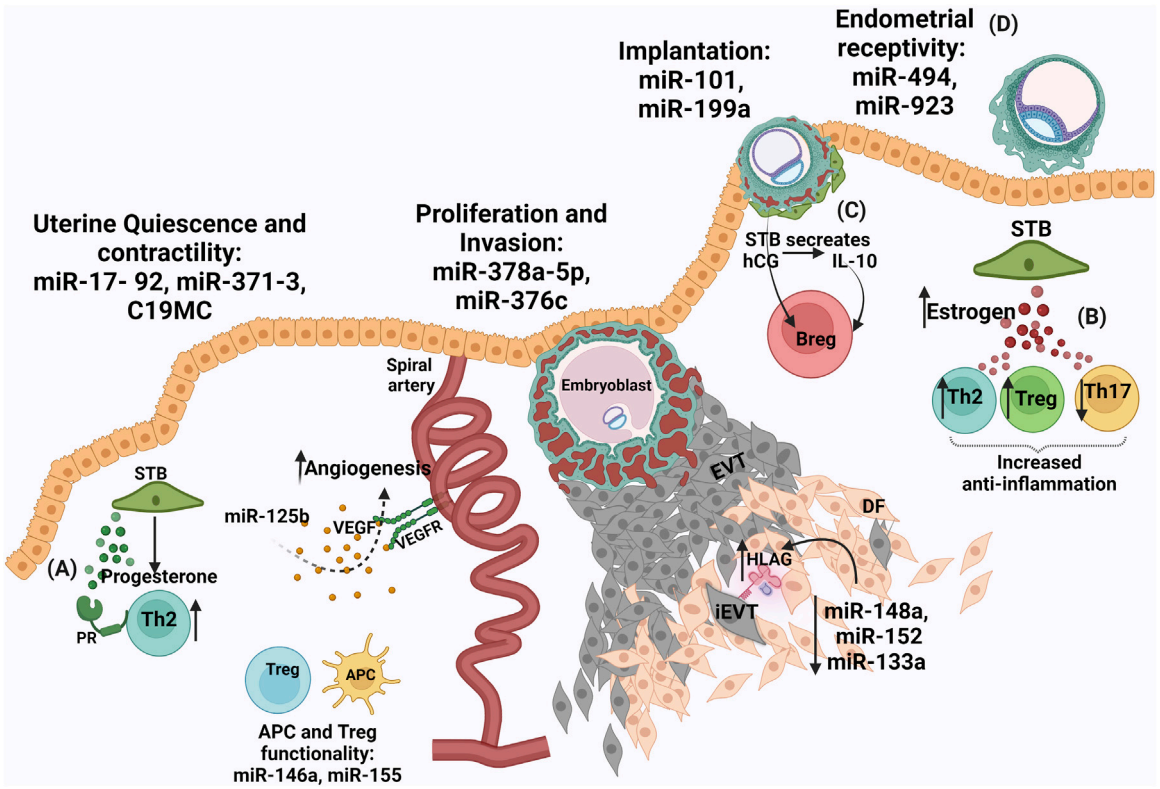


FIGURE 1 | Conjunction of biomolecules in healthy pregnancy **(A)** Progesterone from syncytiotrophoblasts (STB) causes Treg expansion to form a tolerogenic zone **(B)** Increased estrogen levels from STB aids an anti-inflammatory response **(C)** Human chorionic gonadotrophin hormone (hCG) released from STB induces interleukin-10 (IL-10) which causes expansion of regulatory B cells and an assured immune environment **(D)** miRNAs are involved during placentation for endometrial receptivity: miR-30 family, miR-494, miR-923, implantation: miR-101 and miR-199a, proliferation and invasion: miR-378a-5p and miR-376c, uterine quiescence and contractility: miR-17- 92, miR-371–3, C19MC, APC and Treg functionality. miR0146a and miR-155 are involved in both pro- and anti-angiogenic functions. Reduced expression of miR-148a, miR-152, miR-133a in the extravillous trophoblasts (EVTs) ensures high levels of HLA-G to aid in a conducive environment by regulating NK cell cytotoxicity.

Mulac-Jericevic et al., 2003; Conneely et al., 2003). It also lays a tolerant immunological environment in the endometrium, to shield the fetus expressing paternal antigens from the maternal immune attack responses. In peripheral blood, both PR isoforms are expressed on NK cells (Arruvito et al., 2008). During a healthy pregnancy, a significantly upregulated expression (approx. 97%) of PRs on γδ-TCR positive T-cells has been reported. However, in non-pregnant individuals the expression of PRs on γδ-TCR positive T-cells was reported to be as low as 14% (Polgar et al., 1999). Interestingly, the increased progesterone levels during a healthy pregnancy have been reported to induce progesterone-induced blocking factor (PIBF), which suppresses NK cytotoxic activity in the decidua thus, aiding successful pregnancy (Kandzija et al., 2019). Progesterone is crucial as a mediator to induce the naïve T cells to differentiate into Th2-type cells and inhibit activities of T effector cells, especially Th1 (Piccinni et al., 1995) (**Figure 1A**). Lower levels of PR on peripheral blood lymphocytes and serum PIBF have been associated with women having recurrent miscarriages (RM) (Liang et al., 2021). Lymphocyte immunotherapy has shown an improvement in outcomes for RM and is reported to induce increased PR expression on maternal lymphocytes (Hudic et al., 2020). In preeclamptic rat models, administering PIBF displayed normalized Th1/Th2 ratio, it suppressed inflammation, adjusted blood pressure to normal, and prevented fetal growth restriction. PIBF is detectable in the serum after 14 days of embryo transfer *in vitro* fertilization (IVF) patients PIBF concentration in serum increase with gestational age in normal pregnancy. However, a lower-than-normal threshold can help predict spontaneous pregnancy termination (Lim et al., 2020). Dydrogesterone treatment on peripheral blood mononuclear cells (PBMCs) isolated from women with a history of unexplained RSM induces Th2 responses by elevating IL-4 and IL-6 while suppressing Th1/Th-17 cytokines such as IFN-γ (Interferon-Gamma), TNF-α, and IL-17. Dydrogesterone treatment to women at risk of preterm delivery also resulted in increased PIBF production, IL-10 concentrations, and lower concentrations of IFN-γ (Lim et al., 2020).

Estrogens are extensively produced by the fetoplacental unit and required in maintaining pregnancy as well as for fetal maturation. The receptors for estrogens, similar to progesterone receptors are of two types, estrogen receptors (ER) -alpha (α) and -beta (β) (Bukovsky et al., 2003). These receptors are differentially expressed on subsets of immune cells such as lymphocytes, macrophages (MØ), and dendritic cells

(DCs) (Kadel and Kovats, 2018). Increased expression of estrogen is associated with healthy pregnancies (Levitz and Young, 1977). Estrogen expression by the placenta raises the level of the hormone in circulation during gestation. Elevated expression levels of ER-alpha are found on T cells whereas, ER-beta elevated expression is reported on B-cells (Phiel et al., 2005). Estrogen exposed immune cells executes paired responses such that it can enhance NK cell cytotoxicity and interferon-gamma (IFN-γ) production but can also suppress granzyme B and FasL to increase and reduce inflammation, respectively (Hao et al., 2007). A dose-dependent effect of estrogen is observed on monocytes, where lower levels of estrogen result in an increase of pro-inflammatory interleukins (IL) IL-1, IL-6, and TNF- α and the higher level of estrogen reduces these pro-inflammatory cytokines (Bouman et al., 2005). In adaptive immunity, a higher concentration of estrogen promotes Th2 responses, expands regulatory T (Tregs) cells, and causes suppression of Th-17 in mice (**Figure 1B**) (Arruvito et al., 2007; Mao et al., 2010). Estrogen also aids angiogenesis by upregulating VEGF and VEGFR1, during normal pregnancy (Storment et al., 2000). Lower levels of estrogen in this aspect result in dysfunctional angiogenesis contributing to PE (Cantonwine et al., 2019). Short intramuscular-administration of estrogen in pre-eclamptic women reduces mean arterial blood pressure (Babic et al., 2018). Genistein, a phytoestrogen that works by binding G-protein ER (GPER) is used to treat PE. Lower levels of estrogen resulted in insulin resistance and thus are also associated with GDM pregnancies (Fernandez et al., 2016).

hCG, driven by the endocrine and immune system, induces maternal immune cells via lectin-glycan interactions to promote the attachment of the embryo to aid its invasion. Signals from embryo to endometrial immune environment lay a healthy embryo–endometrial relationship, producing pregnancy-induced immune tolerance in favor of the fetus. This stability deciphers the acceptance of the embryo for successful implantation (Schumacher et al., 2009; Schumacher et al., 2013; Schumacher and Zenclussen, 2019). hCG, via hCG receptors, stimulates IL-10 which is shown to increase CD19 + CD24 (high+) CD27 $^{+}$ regulatory B-cells population (**Figure 1C**). These regulatory B-cells enhance the positive effects of the immune environment in pregnancy (Rolle et al., 2013). In baboon endometrial stromal cells and glycodelin in the glandular epithelium, hCG was found to be directly involved in the induction of α-smooth muscle actin (SMA) expression. This suggests that the primate blastocyst, prior to implantation mediate changes in the uterine environment. Concomitant signals between the embryo and maternal endometrium form a cross-talk, which directs the event of successful embryo implantation (Fazleabas et al., 1999). A study demonstrated that hCG hormone is encapsulated in placental-derived exosomes and amnion-derived exosomes forming a no-contact bridge between maternal and embryo thus, providing distal effects. hCG from chorionic trophoblast cells is found to be involved in DC differentiation, maturation, and function regulation at the maternal-fetal interface (Fitzgerald et al., 2018).

Pregnancy Involved miRNAs

Shown to be multiplayer, miRNAs are involved in inhibition of mRNA and promotion of translation (Taganov et al., 2007; Vasudevan et al., 2007; Olsen and Ambros, 1999). In humans, endometrial receptivity associated miRNAs are miR-30 family, miR-494, and miR-923, whereas, miR-101 and miR-199a aid the embryo implantation (Altmäe et al., 2013; Chakrabarty et al., 2007). miRNAs regulating placental functions like uterine quiescence and contractility are miR-17- 92, miR-371-3, chromosome 19 miRNA cluster (C19MC), miR-200 whereas, miR-378a-5p and miR-376c are involved in proliferation and invasion of trophoblast (**Figure 1D**) (Renthal et al., 2010). The myeloid cell differentiation has been reported to be regulated by miR-20a, miR-17-5p, and miR-106a. Clusters namely, C19MC, miR-371-3 (both located on chromosome 19), and C14MC (located on chromosome 14) are reported, out of which the C19MC is the most extensively researched (Flor et al., 2012). C19MC is expressed in trophoblast and placenta-derived stromal cells. miRNAs from this cluster are expressed in human embryonic stem cells and function in cell proliferation, invasion, and differentiation processes. C19MC expression is recorded in extravillous trophoblasts (EVTs) and several malignancies. Several miRNAs are involved in both pro- and anti-angiogenic functions (Donker et al., 2012). Members of the miRNA-17-92 cluster (miR-17, miR -18a, miR -19a, miR -19b, miR-20a, and miR -92a) have been shown to have anti-angiogenic effects on the endothelial cell *in vitro*, and inhibition of these leads towards pro-angiogenesis (Doebele et al., 2010). This regulation potential towards angiogenesis by miRNAs is exploited by cancer cells (Alpini et al., 2011). miRNAs are also involved in generating tolerance, such that HLA-G expressed mainly by the EVTs of the placenta could be downregulated by miRNA (miR-148a, and miR-152) binding to its 39-untranslated region thus, masking trophoblast antigenicity and shielding it from the attack of NK cells (Manaster et al., 2012). Favorably, the expression of these miRNAs have been reported to be expressed at low levels in the placenta, thus aiding the higher expression of HLA-G to create a tolerogenic realm. Modulating the immune cells, miR-155 is required for DC differentiation and DC endocytic capacity. 109 miRNAs are reported to influence macrophage (MØ) differentiation and exhibit both pro-inflammatory and anti-inflammatory phenotypes (Ferretti and La Cava, 2014). miR-146a, miR-155, and miR-223 miRNAs are involved in Treg cell differentiation. miR-17–92, a polycistronic miRNA mediates the regulation and differentiation of antigen-specific IL10-producing natural Tregs (Tregs) (de Kouchkovsky et al., 2013; Herberth et al., 2014). The maternal blood isolated at the 34th week of gestation and umbilical cord blood isolated at the time of birth had a higher miR-223, expression which was correlated with the lower number of Treg cells implying the increase in inflammation required for parturition. miR-146a enhances the suppressive capacity of Treg cells and in turn, limits Th1 responses (Zhou et al., 2015). miR-146a regulates TLR signaling and produces cytokines by decreasing the inflammatory response. However, decreased expression of miR-146a-5p was present in decidual tissue from patients with recurrent spontaneous abortions (Zhao et al., 2018a). *In-vitro* culturing of bovine embryos, revealed an increase expression of miR-25, miR-302c, miR-196a2, and miR-181a in embryos that demonstrated ceased development from morula to blastocyst stage, as compared to the embryos that

successfully attained blastocyst stage. Thus, indicating a correlation between miRNA expression pattern and embryo development (Kropp et al., 2014). A study concluded that miR-34 is involved in cervical remodelling in normal labor whereas (Hassan et al., 2010), mir-223-3p is associated with preterm labor regulating the immune system. In preterm labor, mir-223-3p regulates inflammasome activity and MØ activation via NLRP3 and Pknox1 thus, regulating IL-1beta production (Bauernfeind et al., 2012; Haneklaus et al., 2012). miRNAs are unstable species thus, are encapsulated in exosomes to increase their stability and provide a targeted delivery. For embryo implantation miR-17, miR-106a and miR-200c are the most abundant miRNAs in placental exosomes (Yang et al., 2011; Ng et al., 2013). Exosomal C19MC family provides anti-viral responses by executing autophagy and thus may protect developing fetus from infections (Dumont et al., 2017).

Immune Metabolome

A healthy pregnancy requires degradation of stored energy to facilitate fetal development and achieve timely parturition, thus causing a shift of an anabolic state in the first and second trimester to a catabolic state in the third trimester. These shifts primarily regulate the physiological immune responses in normal pregnancy whereas, alteration in these can lead to pregnancy complications.

NK Cells

mTOR signaling-dependent regulation of glycolysis and mitochondrial functions are enhanced and most importantly studied in NK cell activation. In response to IL-2/IL-12/IL-5, the NK cells are activated, which leads to upregulation of nutrient receptors like CD71 and CD98 causing increased expression of GLUT-1 in an mTOR-dependent manner. This energy is required by NK cells to interact with villous trophoblasts and produce required responses for trophoblast invasion, proliferation, and tolerance (Jabrane-Ferrat, 2019). This provided the nutrition and energy which are essential at the initial stage of pregnancy (Donnelly et al., 2014; Slattery et al., 2021).

Macrophages

Differentiated phenotypes of MØ have varied glycometabolism pathways. Pro-inflammatory type-1 macrophage (M1) provide spontaneous responses against invading pathogens inside the body receiving their power by anaerobic glycolysis. However, anti-inflammatory M2 responses are long-lasting and generated by mitochondrial oxidative phosphorylation (Van den Bossche et al., 2016). In response to lipopolysaccharide (LPS) and IFN-γ exposures, the MØ mitochondrial oxidative phosphorylation is downregulated, which triggers a shift towards type-1 macrophage (M1) polarization by anaerobic glycolysis and pentose phosphate pathway (PPP). Adding, hexokinases and GLUT1 are positively regulated by accumulated TCA cycle metabolites and increased HIF-1α (Tannahill et al., 2013). M1 are responsible for regulating the trophoblast invasion and proliferation by providing optimal inflammation during the early phase of pregnancy. However, prolonged dysfunction of mitochondrial oxidative phosphorylation is responsible for the generation of pro-inflammatory conditions like PE, gestational diabetes mellitus (GDM), and preterm birth (PTB). Thus, researchers have targeted the metabolic programming for the reversal of M1 to M2 polarization for therapeutic purposes. For instance, a study showed reconstruction of dysregulated mitochondrial oxidative phosphorylation by inhibiting iNOS thereby, reverting polarized M1 into M2 ultimately reducing the inflammation (Van den Bossche et al., 2016).

Dendritic Cells

The activation of DCs and stimulation of DCs via LPS leads to inactivation of mitochondrial oxidative phosphorylation and thus a prompt response is generated to increase glycolysis rate for increasing the ATP production (Brombacher and Everts, 2020). The inhibition of mitochondrial oxidative phosphorylation occurs due to endogenous synthesis of NO by iNOS enzyme and stabilized HIF-1α via mTOR signalling. Amino acids like leucine, glutamine, (required for mTORC1 activity), and arginine (fuel for NO production) also affect mTOR signalling (Everts et al., 2012; Lawless et al., 2017). When DCs interact with T cells for antigen transfer, uptake of glucose and amino acids increases, yielding nutrient competitive surrounding and this competition cause prolonged T cell responses. However, these prolonged T cell responses are regulated on the type of T cell subset demand during the course of pregnancy. Extended inflammatory Th cell responses have been associated with pregnancy complications like GDM (Lawless et al., 2017).

T Cells

Stimulatory responses by T cells are produced via switching between glucose and lipid metabolism, whereas the quiescent state of T cells is provided via oxidative phosphorylation (Warburg, 1956). T cell proliferation consumes a high concentration of ATP which is produced via conversion of pyruvate into lactate during glycolysis. Thus, producing essential bio-macromolecules for executing physiological processes of a cell such as growth and division (Pearce et al., 2013). Moreover, T cell stimulation requires increased absorption, this happens by the interaction between its co-stimulatory molecule (CD28) and TCR on APC. This interaction increases GLUT1 expressions via mTOR signalling resulting in increased glucose uptake by respective cells resulting in the execution of their effector responses (Macintyre et al., 2014). In the T cell subset, Th1, Th2, and Th17 closely rely on mitochondrial metabolism with Th17 being the fastest and longest consumer of glucose in a HIF-1α dependent manner (Dang et al., 2011). In addition, Treg cells have multiple metabolic pathways such as glycolysis, lipid oxidation, and oxidative phosphorylation regulating their responses. A regulated balance between these metabolic pathways for pro and anti-inflammatory cells exists (Michalek et al., 2011) however, mitochondrial metabolism could be targeted to decrease inflammatory T-cell responses. Similarly, to receive Treg prominent responses, its respective metabolic pathways could be targeted in creating therapeutics for chronic inflammation-associated pregnancies. The transport of these metabolic signals to the target cell could be via simple diffusion or carrier-mediated (Hardy et al., 2009; Weiler et al., 2017). During pregnancy, the role of exosomes in carrying immuno-metabolic signals to the target cell is still unclear and

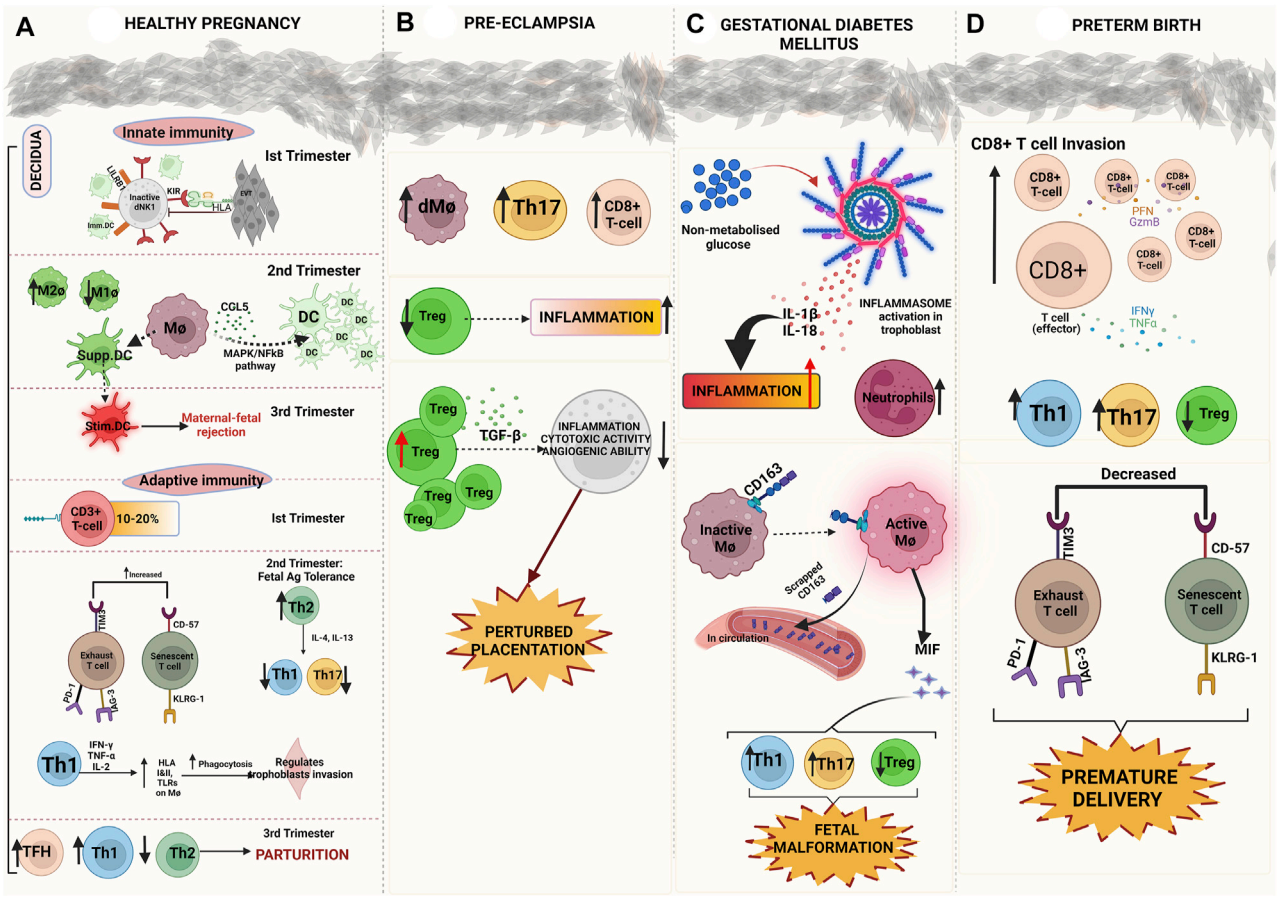


FIGURE 2 | Immununological changes in pregnancy across gestation and in complications. **(A)** Healthy pregnancy: Innate immune responses: HLA disparity on extravillous trophoblasts (EVTs) causes NK suppression (first Trimester), increased M2Ø expression, production of DCs recruiting chemokine CCL5, accumulation of immature immunosuppressive DCs in decidua (second Trimester) for progression of pregnancy, differentiation of immunosuppressive DCs into stimulatory DCs contributing to parturition (third Trimester). Adaptive immune responses: 10–20% $CD3^+$ Tcells exists in first Trimester, increased exhausted and senescent T cell population, increased Th2, decreased Th1 and Th17, pro-inflammatory cytokines (IFN-γ, TNF-α, IL-2) stimulate MØ to regulate trophoblasts invasion by phagocytosis (second Trimester), Increased expression of Th1, TFh, and decreased expression of Th2 promotes parturition as the gestation ends (third Trimester) **(B)** Immune dysregulation in pre-eclampsia (PE)- Increased expression of decidual MØ, T helper 17 cells, $CD8^+$ cytotoxic T cells, increased suppression of regulatory T cells (Tregs) all these events lead to inflammation. Hyper-activation of Tregs causes decreased cytotoxic and angiogenic responses from NK cells leading to perturbed placentation causing PE **(C)** Gestational Diabetes mellitus (GDM) Increased levels of non-metabolized glucose induces inflammasome activation causing cytokine storm (IL-1β, IL-18) and increased neutrophilia leading to hyper-inflammation (MØ) activation causes cleavage of CD163 surface marker and its release into circulation, MØ releases MØ migration inhibitory factor (MIF) that further stimulates Th1 and Th17 promoting inflammation in GDM pathogenesis **(D)** Preterm birth (PTB) Increased $CD8^+$ cytotoxic T cells infiltrate the decidua, increased Th1, Th17, and decreased Treg expression promote inflammation. Decreased expression of exhausted and senescent T cells are involved in PTB pathogenesis.

requires more attention. Although, the communication in the immune cells during pregnancy is crucial for fetal protection.

Immune System in Pregnancy: Simply Complex

During the first trimester of pregnancy MØ, DCs and NK cells infiltrate the decidual tissue surrounding the invading trophoblast cells (Ashkar et al., 2000; Shimada et al., 2006). The events of implantation and placentation along the first and early second trimester of pregnancy display a close resemblance to "an open wound" which requires strong inflammatory responses (Dekel et al., 2010). In the first trimester, the human decidua has been reported to demonstrate a high number of immune cells, such as NK cells (70%), MØ (20–25%), DCs (1.7%), T lymphocytes (3–10%) with relatively lower expression of B cells in the decidua (Bulmer et al., 1988; King et al., 1997; Aluvihare et al., 2004; Zenclussen, 2005; Wicherek et al., 2009; Benner et al., 2020).

Innate Immune Cell Cross-Talk During Pregnancy

DC-mediated NK cells activation induces innate immune response whereas, NK-mediated DC editing and maturation activate adaptive immune response (Ferlazzo and Morandi, 2014). DCs and NK cells have been successful in establishing a

reciprocal cross-talk in the decidual tissue across the pregnancy, in a direct or in an indirect manner by either cell-cell contact or by cytokine secretions, respectively (**Figure 2A**).

It was shown that over 60% immature DCs (imDCs) in the decidua were in close vicinity of NK cells (Kämmerer et al., 2003). Displaying a pregnancy-specific interaction, this clustering phenomenon between DCs and NK cells have been observed at the maternal-fetal interface (Tirado-González et al., 2012). The progression towards the second trimester occurs when IL-1β and TNF-α induce MØ and produce DC-recruiting chemokines through the MAPK and NFκB pathways (Li et al., 2011). CCL2 is the main chemoattractant for MØ and CCL5 is the main chemoattractant for immature DCs (imDCs). This results in the accumulation of MØ and DCs in decidual tissue (**Figure 2A**). Overexpression of anti-inflammatory genes, such as TGF-β is also reported (Dekel et al., 2014). In the second trimester, decidual MØ differentiates into immunosuppressive DC-like cells. There is an interesting shift of such immunosuppressive DC-like cells to immunostimulatory DC-like cells in the third trimester of pregnancy. This demonstrated a conclusive shift of maternal-fetal immunotolerance to maternal-fetal immune-rejection (**Figure 2A**) (Wang et al., 2016). Decidual MØ is believed to initiate childbirth through increased expression of inflammatory mediators to promote uterine contraction, parturition, and placental detachment (Bollapragada et al., 2009). In humans and rats, the MØ population was found to be increased in the decidua and also recruited to the cervix during ripening prior to the parturition (Päzolt and Henkert, 1990; Sakamoto et al., 2005). MØ subtypes work altogether to execute an optimal trophoblast invasion and spiral artery remodelling during healthy pregnancy. This occurs to meet the nutritional and respiration demands of the growing fetus. During the invasion of EVTs into the uterine stroma, a combinational profile of M1/M2 is established (Jaiswal et al., 2012). For the sustenance of the uterus and hence to avoid its rejection, a shift towards a predominantly M2 phenotype is observed (**Figure 2A**) (Mor et al., 2011).

On the basis of CD-11c expression, MØ are classified into two distinct groups in the decidual tissue during the first trimester (Houser et al., 2011). $CD11c_{high}$ and $CD11c_{low}$ secrete pro-and anti-inflammatory cytokines thereby helping in maintaining immune homeostasis during the first trimester while retaining defense against invading pathogens at the maternal-fetal interface (Houser et al., 2011). Conversely, gene expression profiling and surface marker phenotyping demonstrate that the term MØ resembles M2 skewed cells (Gustafsson et al., 2008; Repnik et al., 2008; Xu et al., 2016). Term MØ in decidua exhibit an immunomodulatory property with low expression of CD80/CD86 and produce major volumes of the immunosuppressive cytokine IL-10 (Heikkinen et al., 2003). Along with IL-10, trophoblast-derived macrophage colony-stimulating factor (M-CSF) in maternal monocytes have been proven to induce this M2 regulatory phenotype (Svensson et al., 2011). Soluble HLAG5 has been found to induce MØ by polarizing them to bear immunomodulatory phenotype exhibiting increased numbers of activated MØ (CD163 high) but decreased CD86 expression (Lee et al., 2015). Interestingly, in placental MØ pro-M2 genes like CCL2, CCL13, CCL14, and CD209 are hypomethylated to induce an M2-like phenotype and M1 phenotype is repressed by the hypermethylation of genes such as TLR-9, IL1B, IL-12 receptor β-2, and CD48 (Kim et al., 2012). To regulate angiogenesis in the feto-placental vasculature, a hallmark of organogenesis, placental MØ secretes VEGF and fibroblast growth factors (FGFs) like FGF2 (Demir et al., 2004; Loegl et al., 2016). Phenotypically characterized as M2-like, placental MØ can induce a pro-inflammatory response when activated via TLRs (Young et al., 2015; Thomas et al., 2021) and function to impart host defense within the placenta thus, triggering the local inflammation required for the initial development of the placenta (Young et al., 2015).

Adaptive Immune Responses in Pregnancy

T cells constitute 45–60% of the total leukocytes in the endometrium in the early proliferative phase but the percentage decreases at the time of pre-conception creating a conducive environment for implantation (Gomez-Lopez et al., 2010; Bulmer et al., 1991). $CD3^+$ T lymphocytes are present around 10–20% (**Figure 2A**) of the endometrial stromal leukocytes in the first trimester. Among the entire T cell population, $CD4^+$ T cells (30–45%) and $CD8^+$ T cells (45–75%) along with Th2 and Th17 cells accounting for 5 and 2% of $CD4^+$ T cells, respectively (Bulmer et al., 1991; Nancy and Erlebacher, 2014). Nearly 5–30% of $CD4^+$ T cells are found to be Th1 (CCR4-CXCR3+CCR6−) cells and nearly 5% CD25hi FOXP3+ Treg cells are $CD4^+$ T cells (Nancy and Erlebacher, 2014).

In the early phase of pregnancy, the inflammatory priming of PBMCs occurs at the implantation site (Germain et al., 2007). Circulating syncytiotrophoblast's microparticle (STBM) stimulates the production of various inflammatory cytokines, like IL-12, TNF-α along with mild-level of IL-18, from monocytes leading to the establishment of mild systemic inflammation (Sargent et al., 2006). On the surface of $CD4^+$ T cells, chemokine receptor expressions (especially CCR molecules) determine their trafficking patterns which include the recognition of target tissue, timing, and signals to receive (Knieke et al., 2012). To keep track of the number and movement of trophoblast and prevent excessive trophoblast invasion, Th1 cells secrete cytokines IL- 2, TNF- α, and IFN-g (**Figure 2A**) (Torchinsky et al., 2003). TNF-α has been reported to act as a protector of the fetoplacental unit and regulates trophoblast invasion, by altering trophoblast cell adhesion to laminin and inhibiting the mobility of trophoblast cells studied through *in vitro* approaches (Todt et al., 1996). TNF-α hikes the trophoblast-derived plasminogen activator inhibitor-1 (PAI-1) levels and neutralizes the invasive capacity of trophoblasts (Bauer et al., 2004; Renaud et al., 2005). It has been stated that IFN-γ is involved in vascular remodelling during the peri-implantation phase and IFN-γ mRNA expression has been visualized at the implantation sites of healthy pregnant women and the murine model

(Delassus et al., 1994; Jokhi et al., 1994). IFN-γ has a critical role of regulating EVT invasion, by increasing apoptosis of EVT and/or decreasing protease activity. Contrary to the physiological roles of IFN-γ, it impels pro-inflammatory functions as it increases expression of HLA class I and II antigen and TLR in innate immune cells (Podaný et al., 1975) which in turn promotes various functions like isotype commutation, chemokine secretion, (MØ) activation, and increased phagocytosis (Raphael et al., 2015). Pregnant women in the third trimester when compared to the non-pregnant counterparts have a higher percentage of peripheral blood follicular T helper cells (Tfh), despite co-expressing markers, including programmed death (PD)-1, ICOS, or CXCR3. Pregnant women also reveal a notably higher percentage of CXCR3C Tfh cells than non-pregnant women, which may produce IL-6, IL-10, and IL-21, and particularly, PD-1/CXCR3 (Monteiro et al., 2017). Th9 cells, a subpopulation of Th2 cells differ by altered phenotypical and functional aspects, which subjects to PPARγ involved in fatty acid storage and glucose metabolism (Micossé et al., 2019). In the presence of TGF-β, Th-17 cells produce IL-9 which have an inflammation-inducing function. In mouse, IL-9 was reported to be present in the non-pregnant uterus. However, during pregnancy, high level of IL-9 remained in both the placenta and uterus pointing again to its role in local inflammatory immune responses which might pose a threat to the developing offspring (Habbeddine et al., 2014). IL-22 secreted by Th22 cells has been found to be relevant for physiologic immune regulation and pathologic allograft rejection, therefore could potentially harm the pregnancy (Jia and Wu, 2014). At the maternal-fetal junction, IL-22 promotes proliferation, reduces apoptosis of trophoblast cells, and positively affects their viability (Wang et al., 2013). IL-22 plays an important role in protecting trophoblast cells from pathogens and producing inflammatory immune responses following intrauterine infection (Graham et al., 2011; Dambaeva et al., 2018). IL-22 receptors (IL-22R) are located on placental villi, a subunit of IL-22R, IL-22R1, allows binding of IL-22 from dNK cells and decidual stromal cells (Dambaeva et al., 2018). The downstream IL-22/IL-22R1 pathway is said to be involved in the trophoblasts survival and maintenance of pregnancy. In a successful pregnancy, IL-22, Th17/Th2 and Th17/Th0 subsets were highly prevalent, and the mRNA expression of GATA-3, ROR-C, AHR, IL-4, IL-17A, and IL-22 were recorded at the site of implantation. However, mRNA expression of T-bet and IFN-γ was detected away from the site of implantation. Hence, for a successful pregnancy, the pertinent association of IL-22 and IL-4 production at the implantation site is proved (Logiodice et al., 2019).

Immune Tolerance in Pregnancy

In healthy pregnancy, the earlier defined Th1/Th2 paradigm shifted to Th1/Th2/Th17/Treg paradigm when the advancement in the understanding of feto-maternal immune cross-talk for building a fetal alloantigen tolerogenic environment became clearer. The shift of pro-inflammatory milieu to anti-inflammatory milieu majorly occurs during the second trimester of pregnancy where fetal tolerance is at its maximum while at the end of the third trimester of pregnancy shows the generation of fetal rejecting environment to induce parturition (Chaouat and Voisin, 1979; Saito et al., 2010). In the early pregnancy development of fetal tolerant surroundings takes place when the maternal immune system encounters paternal antigens on the fetus, which causes phenotypic suppression of maternal immune cells. This suppression of immune cells is contributed from both fetal and maternal side. It has been reported that even fetal immune cells in response to maternal antigens cause inactivation of inflammation producing fetal immune cells and expansion of anti-inflammation producing fetal immune cells. In addition, the construction of fetal trophoblasts is in such a way that they escape maternal immune cell attack. The cytotrophoblasts, and STB along with STBM do not express any variety of HLA or NOD-like receptor family CARD domain containing 5 (NLRC5) (Tilburgs et al., 2017). Thus, during healthy pregnancy, the alloreactivity of $CD3^+CD4^+$ T helper cell is suppressed in the absence of HLA class I and II antigens on villous trophoblasts. In contrast to villous trophoblast, EVTs expressed HLA C, a classical MHC class I molecule, and a non-classical MHC class I molecules HLA E, F, and G and MHC transcriptional activators such as NLRP2 (Tilburgs et al., 2017; Tilburgs et al., 2010). At the maternal-fetal junction, HLA-C histo-incompatibility has been recorded to induce a tolerogenic microenvironment (Tilburgs et al., 2009). Prior to implantation, paternal antigen-specific Treg cells accumulate and increase in number in the uterus after implantation. Intriguing results from (Mohr et al., 2019) showed how seminal plasma initiates the expansion of Treg cells specific to paternal antigens imparting tolerance to paternal alloantigen (Shima et al., 2015; Robertson et al., 2009). As the pregnancy progresses, the cellular responses of innate and adaptive immunity work in collaboration to strengthen and extend fetal tolerance. DCs drives differentiation of naïve T cells into Th2 and Tregs in response to fetal antigen exposure. Increased Th2 response causes secretion of anti-inflammatory cytokines like IL-4, IL-5, IL-6, IL-10, IL-13, and TGF-β thereby decreasing the local inflammation. IL-4 and IL-13 work in a paracrine manner and represses Th1 and Th17 immunities, respectively, and brings forth allograft tolerance (**Figure 2A**) (Mitchell et al., 2017). Another subset of T cells like $CD8^+$ Tc cells upon indirect recognition of fetal antigens, undertake the fate of clonal deletion (Erlebacher et al., 2007) whereas, $CD4^+CD25$ + Fox3+ Treg expansion has been found to establish and maintain an allogeneic pregnancy in both mice and humans(Zenclussen et al., 2005). Treg cells play a crucial role in the production of paternal antigen-specific tolerance (Rowe et al., 2012). Another physiological phenomenon of inducing tolerance during pregnancy is T cell exhaustion and senescence which are known to occur because of excessive stimulation of T cells. This causes T cells to lose their proliferative and cytokine secreting properties however, the exact mechanism leading to this is still unknown. T cell exhaustion and senescence is characterized by increased surface expression of inhibitory receptors like PD-1,

TIM-3, CTLA4, LAG-3 and CD57, KLRG-1, respectively (**Figure 2A**) (Sugita et al., 2013). PD-1/PD-L1 (CD274) axis engages in the suppression of autoreactive immune effectors and to achieve T cell homeostasis. Through negative costimulatory interactions, the PD1/PD-L1 pathway can also suppress Th22 and Th9 cells (Wang et al., 2020a). Primarily, identified as a Th1-specific receptor, Tim-3 is present on the surface of the cell. These domains engage galectin-9 (Gal-9) to transduce an apoptotic signal which ultimately results in inhibition of Th1 responses (Zhu et al., 2005; Miyanishi et al., 2007). The interaction of Tim-3 and its ligand Gal-9, causes intracellular calcium influx which commence the supersession of Th1 and Th17 cells (Seki et al., 2008; Oomizu et al., 2012). Conversely, Tim-3 enhances Th2 immunity at the maternal-fetal junction thereby safeguarding the decidual stromal cells from inflammatory damages and apoptosis mediated by TLR (Wang et al., 2015a; Wu et al., 2015). Therefore, Tim-3 signalling during pregnancy may operate as a self-control mechanism in TLR-triggered inflammation (Wang et al., 2015a). CD-57 expression is indicative of shortened telomere inside the cell implying that the cell has lost the ability to proliferate conferring a suppressed state of immune cell which is required for preventing fetal rejection (Slutsky et al., 2019). Later in pregnancy, paternal antigen-specific tolerance disappears post-delivery which is earlier present during pregnancy (Rowe et al., 2012). In a study, cytokine analysis of serum from pregnant women revealed the increased levels of IL-1b, IL-6, IL-8, IL-12p70, L13, IL-15, IP-10, and FLT3-ligand in relation to gestational weeks while, serum IFN alpha-2, IL-1RA, IL-3, IL-9, IL-12p40, and soluble CD40L levels were increased with the advancement of the trimester (Holtan et al., 2015). As interpreted, the optimal increase in pro-inflammation in the third trimester of pregnancy is associated with the preparation for the healthy delivery.

Immune Dysregulation Causing Pregnancy Complications

Immune tolerance built by various diverse cellular interactions is the cornerstone for successful gestation and healthy outcomes. The breakdown of this mechanism is proved to be one of the causes for the pathophysiology observed in adverse pregnancy outcomes. Various studies have been performed to understand the immune dysregulation in the context of pregnancy complications like PE, GDM, and PTB.

Pre-Eclampsia

PE is indicated as a state of hypertension and proteinuria any time after 20 weeks of gestation and is categorized as early-onset PE (EOP) that presents before 34 weeks and late-onset PE (LOP) that initiates after 34 weeks of gestation. A hallmark of PE is a deficiency of EVT infiltration and spiral artery remodelling, which results in a placental microenvironment that is ischemic towards increasing oxidative stress (Cartwright et al., 2017). Hyper-activation of pro-inflammatory cells (M1, Th1, Th17, cytotoxic dNK cells) or hyper-activation of anti-inflammatory cells (M2, Th2, Treg, suppressive dNK cells) causes alterations in the process of placental formation leading to pre-eclampsia. M1 have been reported to have elevated levels than M2 in the decidua of patients with PE, with a total increase in the MØ numbers in PE patients when compared to healthy controls (Schonkeren et al., 2011). Uterine M1 by the action of TNF-α has been reported to inhibit trophoblast invasion and disrupt spiral artery remodelling (Renaud et al., 2005). Similarly, the cytotoxic capacity of $CD8^+$ T cells has been involved in controlling trophoblast invasion. In a human study, $CD3^+$ and $CD8^+$ T cells were significantly increased in the maternal decidua of PE patients compared to normotensive controls, indicating that an inflammatory environment aids in the progression of the disease (Milosevic-Stevanovic et al., 2019). Higher Th17/Treg ratios in umbilical cord blood, peripheral blood, and decidua have been reported to be associated with preeclamptic women when compared to healthy pregnant and non-pregnant controls (**Figure 2B**) (Milosevic-Stevanovic et al., 2019). In addition, animal studies have shown that depletion of Tregs in early gestation results in the generation of an uncontrolled pro-inflammatory milieu that causes preeclampsia-like phenotype (Care et al., 2018). This is suggestive of an exacerbated pro-inflammatory response that disturbs the trophoblastic properties of migration, invasion, and proliferation thus causing PE. However, contradicting studies have also been reported to be involved in PE pathogenesis. Increased expression of cytotoxic $CD8^+$ T cells in PE patients' decidua basalis, has also been reported by few studies and is suggestive of their role in the pathophysiology of PE (Milosevic-Stevanovic et al., 2019). Moreover, the increased number of dNK cells, decidual Treg cells, and TGFβ-1 in pre-eclamptic women is connected with a profound notion that excess anti-inflammation or increased suppression of cytotoxic and angiogenic properties of dNK cells can also result in insufficient trophoblasts proliferation, migration, and invasion. Thus, indicating the need for a balanced spatio-temporal relationship between inflammation and anti-inflammation for adequate spiral artery remodeling (**Figure 2B**) (Zhang et al., 2019). Another important aspect of PE pathogenesis is increased obstructions in maternal blood flow during pre-eclampsia, due to which dNK cells cannot interact with trophoblast cells and with other decidual cells, thus are restrained in promoting an adequate trophoblast invasion, causing dysfunction in spiral artery remodeling in PE (Fraser et al., 2012). However, inconsistent results are found over the varied role of dNK cells in PE giving the explanation of geographical indications, that even the environmental factors have an impact in modulating the immune system (Valenzuela et al., 2012; Shashar et al., 2020; Steinthorsdottir et al., 2020).

Gestational Diabetes Mellitus

Affecting 15% of pregnant mothers in developing countries GDM is a metabolic disorder which if left untreated may result in PTB due to hyperglycemia (Salomon et al., 2016). Hyperglycemia in GDM is associated with increased inflammation which occurs due to activation of inflammasomes in trophoblasts. The potent reason behind this activation of the inflammasome is excessive glucose

which induces NLRP3 resulting in the generation of pro-inflammatory cytokine storms mainly IL-1β and IL-18 (**Figure 2C**) (Han et al., 2015; Corrêa-Silva et al., 2018). Excessive neutrophilia, high glycaemic levels, and increased homeostatic model assessment of insulin resistance are associated with GDM diagnosis as early as in the first trimester (**Figure 2C**) (Sun et al., 2020). The increased numbers of neutrophils are intended to be more reliable than leukocyte numbers i.e., the neutrophil to leukocyte ratio is used as an inflammatory marker for diagnosis of GDM in the second trimester. In addition, during the third trimester of pregnancy for GDM prediction a serum delta neutrophil index representing increased neutrophil numbers and inflammation is adopted (Sahin Uysal et al., 2020). The innate immune system contributes to increased inflammation in GDM via inflammatory signals secreting monocytes (Chandra et al., 2012). Monocyte/MØ activation has been proposed to be an early predictor of GDM in as early as 14–16 weeks of gestation. A hemoglobin-haptoglobin scavenger receptor CD163 (sCD163) is scraped out of MØ as an activation marker of these cells and this shedding is significantly increased in GDM women thus, the increased circulatory levels of CD163 from the placenta as well as from adipose tissue are reflective of GDM (**Figure 2C**) (Dige et al., 2014). Another study reveals elevated levels of CD163 + cells, IL-6, TNF-α, and TLR2 are associated with a pro-inflammatory milieu in GDM patients when compared to healthy pregnancies (Ueland et al., 2019; Bari et al., 2014). Another MØ secretory signal, a pro-inflammatory cytokine known as MØ migration inhibitory factor (MIF) which stimulates TH1 cells, induces IL-17 release, and increases TLR-4 expression on MØ is used for GDM prediction (**Figure 2C**) (Yilmaz et al., 2012). Moreover, GDM susceptibility has also been determined by specific genotypes associated with MIF (Aslani et al., 2011; Zhan et al., 2015). Decreased Treg numbers are associated with GDM prognosis, as shown in multiple studies where subsets of suppressive Tregs, $CD4^+CD127LOW+/CD25$ + Tregs and CD45RA Tregs were evaluated during GDM pregnancies and represented a decline of anti-inflammatory function of Tregs as early as in the first trimester of GDM pregnancy (Schober et al., 2014). In addition, $CD4^+$ CD25 and $CD4^+CD25$ + FOXP3 cells numbers were decreased whereas, TNF-α, a pro-inflammatory cytokine expression by Tregs ($CD4^+CD25$ + FOXP3+CD127-) were found to be significantly upregulated in women with GDM pregnancies compared to women with healthy pregnancies (Schober et al., 2014). Aggravated circulatory $CD4^+$ and $CD8^+$ T cells responses in GDM pregnancy contribute to GDM pro-inflammatory milieu with significantly higher expression of CD69 (T cell activation marker) in insulin-untreated cases and higher expression of HLA-DR in insulin-treated cases (Lobo et al., 2018). Thus, the above-mentioned studies project towards an extensive pro-inflammatory build-up in GDM patients. In addition, increased levels of circulating Th-17 cells, a higher Th17: Treg cells ratio, and Th1: Treg ratios have been associated with GDM pregnancies compared to uncomplicated pregnancies (Sheu et al., 2018; Zhao et al., 2020). Thus, in order to predict a pregnancy complication only studying Th1/Th2 imbalance is insufficient however, a more comprehensive understanding can be attained by taking the Th1/Th2/Th17/Treg paradigm into consideration.

Preterm Birth

PTB is defined globally as any live birth that occurs before 37 weeks of gestation or less than 259 days. According to the world health organization (WHO), an estimated 15 million infants are born prematurely every year. One-fifth of those 15 million prematurely born infants across the world are, born in India PTB is stratified as spontaneous PTB with an intact membrane (sPTB-IM), induced PTB, preterm premature rupture of membrane (pPROM), and caregiver induced PTB. Among the PTB populations, the prevalence of sPTB is 40–45%, induced is 30–35% and pPROM is 25–30% (Goldenberg et al., 2008). The immunological status of an idiopathic PTB is more complicated than that of PE or GDM because of the absence of pathological cues. Whereas, the infection-induced PTB and labor are more frequently studied. Neutrophils are the phagocytic cells that reach predominantly at the infection site or site of injury thereafter recruiting other effector immune cells. Several rodent studies have reported that depletion of neutrophils prior to LPS administration could not delay the preterm labor however, it did help in reducing the IL-1 beta levels at the feto-maternal interface (Arenas-Hernandez et al., 2019; Gomez-Lopez et al., 2016) implicating an indirect role of neutrophils in creating an inflammatory milieu underlying PTB or pPROM. Histological evidence of PTB placentae has shown a more prominent invasion of $CD8^+$ Tc cells indicating chorioamnionitis as similarly observed in cases of pPROM and fetal death (**Figure 2D**) (Galaz et al., 2020). Flow cytometric analysis of these cases revealed an influx of effector memory T cells, secreting high levels of perforins and granzymes at the feto-maternal interface in preterm labor (Arenas-Hernandez et al., 2019). The chorioamnionitis membranes in preterm placentae are infiltrated by the increased number of Th17 subtypes that release IL-17 at the maternal-fetal interface and also in amniotic fluid indicating a chronic inflammatory status (**Figure 2D**) (Ostojic et al., 2003; Wu et al., 2014; Lombardelli et al., 2016; Pinget et al., 2016). At the feto-maternal interface, the elevated expression of Th1 and Th17 related genes with declined FOXP3 expressions were associated with unexplained recurrent pregnancy loss and spontaneous abortion patients (Lee et al., 2011; Wu et al., 2016; Zhu et al., 2017). Invariant NK cells (iNKTs) are the bridges between innate and adaptive immunity, where they provide an intense immune activation by upregulating the signalling pathways responsible for Th1 and Th2 cytokine release (Miller et al., 2018). Studies have reported increased expression of iNKT in the first and third trimester of pregnancy thus, implying their roles during term labor (Wang et al., 2002; Boyson et al., 2002). Preterm murine studies have revealed an inverse relation of iNKT and Tregs

at the feto-maternal interface (Gomez-Lopez et al., 2017). The expansion of iNKT cells was accompanied by increased Th17 and decreased Treg expression. Thus, inhibiting iNKT cells activation reduced the immune responses at feto-maternal interface, thus delaying preterm labour in mice (St Louis et al., 2016). Moreover, in humans increased expression of iNKT cells at the decidua were revealed in a transcriptomic analysis and immunophenotyping of lymphocytes in placentae of preterm cases when compared to control terms (St Louis et al., 2016). Given that iNKT cells are present at the murine maternal–fetal interface throughout pregnancy, other than the innate immune cells contributing to infection induced PTB, the adaptive immune cells also have important roles in PTB (Gomez-Lopez et al., 2017; St Louis et al., 2016). Exhausted and senescent T-cells are present at the maternal-fetal interface and help in regulating inflammation throughout gestation in a normal pregnancy. Chronic/repetitive antigen exposure on T cells can result in their functional loss which is identified by the expression of exhaustion markers such as TIM-3, PD-1, CTLA-4, and LAG-3. Whereas, T cell senescence is characterized by vanished proliferative ability along with the absence of these inhibitory markers and presence of senescent markers (increased CD57, KLRG-1 and decreased CD27 and CD28 (Wherry and Kurachi, 2015). In humans, CD4+T cells exhibiting effector memory phenotype showed upregulated expression of inhibitory marker PD-1 at the second trimester during normal pregnancy (Meggyes et al., 2020). During infectious preterm pregnancy, a decline in senescent CD4+/CD8+ T cell numbers and exhausted $CD4^+$ T cell numbers have been reported at the feto-maternal interface (Slutsky et al., 2019). The existence of T cell subsets in the above-mentioned effector memory phenotypes concludes a pro-inflammatory milieu responsible for preterm labour leading to PTB (**Figure 2D**). Moreover, blocking the inhibitory markers using antibodies to PD-1, TIM-3 has been associated with increased rates of fetal loss and thus emphasizing the fact that balanced cellular exhaustion and senescence are required for the execution of a healthy pregnancy (Wang et al., 2015b). This was further supported by the observation that CD8+PD-1+TIM-3+ T cells were impaired in decidual tissues from women with miscarriage (Wang et al., 2015b; Slutsky et al., 2019). Another aspect contributing to the pregnancy complications as explained in PE and GDM also exists in PTB i.e., decrease in Tregs numbers. Immunophenotyping performed on the lymphocytes isolated from women undergoing preterm labor revealed that chorioamnionitis accompanied preterm labouring women at the time of delivery had significantly lower numbers of Tregs as compared to term labouring women (Xiong et al., 2010). Studies have revealed the existence of reduced Tregs at the feto-maternal interface in women with idiopathic preterm birth. In a mice model of endotoxin (LPS) induced PTB the depletion of Tregs in the third week of mice pregnancy resulted in PTB. The endotoxin-induced PTB was reversible by adoptive transfer of depleted Tregs from allogeneic mice, implying the importance of Tregs in delivering a full-term pregnancy (Gomez-Lopez et al., 2020). Moreover, human cellular studies are accompanied by cytokine studies, which represented a decrease in the levels of IL-10 an anti-inflammatory cytokine with each approaching trimester in PTB. Serum levels of IL-10 and IL-10 receptors in endometrial biopsy of women with preterm labor were also found to be lower when compared to women with normal labor (Pereira et al., 2016). However, the trigger behind the perturbed immune responses in idiopathic PTB still remains unclear and requires thorough investigations.

Pla-Xosomes: Connecting Link Between Immune Clock and Pregnancy Complications

Ongoing research for identification of the one triggering factor responsible for bringing about perturbations of the immune system that lead to such pregnancy complications and adverse pregnancy outcomes is still unknown. However, of the multiple studies underway that are being investigated for identification of this trigger, one such investigation involves the study of extracellular vesicles also known as exosomes (EVs). Discovered almost 40 years ago in 1989 (Trams et al., 1981; Pan and Johnstone, 1983; Harding et al., 2013), the extracellular vesicles named exosomes were characterized later as lipid-bilayered-intraluminal microvesicles (ILVs) (30–150 nm) yielded by invagination of multivesicular bodies (MVBs) derived from endosomes during stress response or for cell-to-cell communication (Harding et al., 1984). Exosomes being the most biological residual structures with the least cytotoxicity are loaded with cargo in the form of RNAs (miRNAs) (Menon et al., 2019), proteins (cytokines) (Pillay et al., 2020), hormones (estrogen, progesterone (Fitzgerald et al., 2018), cDNAs, and metabolites making them chief molecules of cell-cell communication (Kurian and Modi, 2019). Since exosomes act as power shots of clues/factors for regulating the proximal and distal cellular responses, they are being studied to unravel the trail leading to the trigger of immune dysregulation in pregnancy complications. The involvement of exosomes in facilitating feto-maternal cross-talk during a successful pregnancy through reported literature on the cargo investigated at regular stages of gestation has led to a deeper understanding of these power shots as physiological modifiers through their action on the immune system of the pregnant mother. Exosomes act as messengers between the fetal and maternal tissues during pregnancy, delivering their payload to target cells towards making an incremental functional impact. They also have crucial roles e.g., in embryo implantation (Kurian and Modi, 2019), accelerating the glucose metabolism (James-Allan et al., 2020), and acting as a mediator for executing immune responses bring about either activation, suppression, or tolerance (Mincheva-Nilsson and Baranov, 2014a). In early pregnancy, exosomes produced by the placental cells (pla-xosomes) induce endothelial cells and vascular smooth muscle cells to promote angiogenesis (Salomon et al., 2014b). Apart from maintaining the conducive environment for the healthy growth of the developing fetus, the inflammatory signals required to initiate parturition at the last trimester of pregnancy are also provided by exosomes (Sheller-Miller et al., 2018).

Exosomes Facilitate a Fetal Sustaining Environment During a Healthy Pregnancy

Exosomes from trophoblast cell lines have been reported to trigger the recruitment and differentiation of immune cells specifically monocytes. Placenta-derived exosomes (Pla-xosomes) concentration increases with each progressive gestation of a healthy pregnancy (Salomon et al., 2014a). Pla-xosomes can cause phenotypic changes in monocytes i.e., phagocytic classical monocytes (CD14++ $CD16^+$) are transformed into intermediate monocytes (CD14 + $CD16^+$) with enhanced migratory capabilities, and pro-inflammatory factors like IL-1beta, IL-6, serpin1, GM-CSF, M-CSF, and TNF-α are secreted (Al-ofi et al., 2012; Tagliani et al., 2011). These responses are essential to function in an optimal manner so as to provide regulated angiogenesis and invasion of trophoblast cells. Along with pregnancy, M1 polarization to M2 occurs to contribute to an anti-inflammatory phase for fetal survival (**Figure 5B**). This transition is caused by the presence of an immune checkpoint inhibitory molecule known as PDL-1 on the pla-xosomes (Petroff et al., 2003; Enninga et al., 2018). Effector responses of T cells have to be reduced in order to aid the successful growth of the fetus. Multiple mechanisms such as inhibition of T cell proliferation, T cells apoptosis, T regulatory expansion, and reduction of Tc cells occur so as to shield effector T cell responses (**Figure 5B**). The immune cells have been reported to express the FAS and TRAIL receptors. Interestingly pla-xosomes isolated from the placenta or that from blood biopsies express apoptotic molecules like FAS ligand and TRAIL, thus inducing apoptosis in Jurkat cells and activating PBMCs via their receptors in a dose-dependent manner (Stenqvist et al., 2013). In addition, pla-xosomes from maternal blood downregulate the expression of CD3 and JAK3 inhibiting T cell activation ($CD4^+$ and $CD8^+$) (Sabapatha et al., 2006). MHC class I chain-related (MIC) and UL-16 binding protein (ULBP) expression on pla-xosomes downregulates expression of NKG2D receptor on $CD8^+$ T cells thus inhibiting their cytotoxic responses (**Figure 5B**) (Hedlund et al., 2009). Syncytin-2 an endogenous retroviral protein is expressed on pla-xosomes and has been reported to reduce Th1 cytokine secretion using PBMCs invitro culture causing immunosuppression (**Figure 5B**) (Lokossou et al., 2020). Although, pla-xosomes inhibit lymphocyte proliferation and induce regulatory/memory T cells differentiation in a similar manner the tumor-derived exosomes manipulate the immune cells by inhibiting immune cell attacks (Mikami et al., 2020; Yu et al., 2020). The induction of Tregs is crucial for the sustenance of the fetus during the second trimester of the pregnancy. EVs from BeWo cells showed expression of a 10 KDa heat shock protein which initiated the helper T-cell differentiation to Treg cells (Kovács et al., 2019). As described above the exosomes are potential mediators of cell-cell communication during a healthy pregnancy. The immune perturbations in pregnancy complications alter the cargo of exosomes and their numbers, which have been associated with perturbed pregnancies like pre-eclampsia, GDB, and PTB.

Pla-Xosomes in Adverse Pregnancy Outcomes

Preeclampsia

Compared to a healthy pregnancy, the placental EVs from PE patients remain in circulation for longer. Pla-xosomes levels in pre-eclamptic pregnancies in the third trimester have been reported to be elevated in comparison to healthy control (Pillay et al., 2016). Exosomal cargo has been described as biomarkers for pre-eclampsia. In the C19MC miRNAs, a set of placental unique miRNAs (miR- 517-5p, miR-520a-5p, and miR-525-5p) measured in the first trimester were reported as a biomarker panel (AUC: region underneath the curve 0.719) for predicting the PE prognosis (**Figure 3D**) (Hromadnikova et al., 2019). Proteomic studies on pre-eclamptic maternal plasma-derived exosomes have revealed higher expression of peptidase inhibitor (PAI)-1, S100 calcium-binding protein (S100b), TGF-β, VEGFR1, and natriuretic peptide B(BNP) (Tan et al., 2017; Tan et al., 2014) compared to their healthy counterparts. Increase in sFLT-1 (soluble fms-like tyrosine kinase-1) and sENG (soluble endoglobin), the causative agents of PE are found to have upregulated expression in PE exosomes compared to controls (**Figure 3D**) (Chang et al., 2018). Providing the indications of PE pathology, a reduction of immune-suppressive markers like PD-L1 and syncytin 1 or 2 (regulates M1 polarization, T reg cell differentiation, and inhibits T cell activation respectively) on exosomal membranes have been reported in preeclamptic patients (Levine et al., 2020). RNA sequencing has revealed elevated enrichment of mir-210 in preeclamptic patients that downregulates potassium channel modulatory factor 1 and thus inhibits trophoblast invasion (Luo et al., 2014). In pregnant mice, exosomes derived from the plasma of PE patients can induce PE-like phenotypes in the mother as well as the fetus (Sheller-Miller et al., 2019). PE STBs derived-EVs induces the production of superoxide by neutrophils which have been thought to surge the neutrophil extracellular traps (NETs) formation and showed more interaction with monocytes, MØ, thus increasing the pathological inflammation (Gupta et al., 2006). Pla-xosomes carry the destined cargo to prepare the mother by modulating the physiological, structural, and immunological status towards the healthy development of the fetus.

Gestational Diabetes Mellitus

In humans, the PLAP content per exosome (PLAP ratio) is used to define the existence of placental exosomes in total exosomes. In GDM, this ratio has been found to be lower in comparison to normal pregnancy irrespective of the higher number of total and placental exosomes implying that there are alterations in the number of exosomes released by the placentae, increased non-placental exosomes secretion, or convergence of both (Salomon et al., 2016). Exosomes from the plasma of GDM patients also cause glucose intolerance, decreased glucose-induced insulin secretion, and poor insulin responsiveness (James-Allan et al., 2020). Exosomal miRNAs are extensively studied for the prediction of GDM in humans eg. miR-125a-3p, miR-99b-5p, miR-197-3p, miR-22-3p, and miR-224-5p are consistently

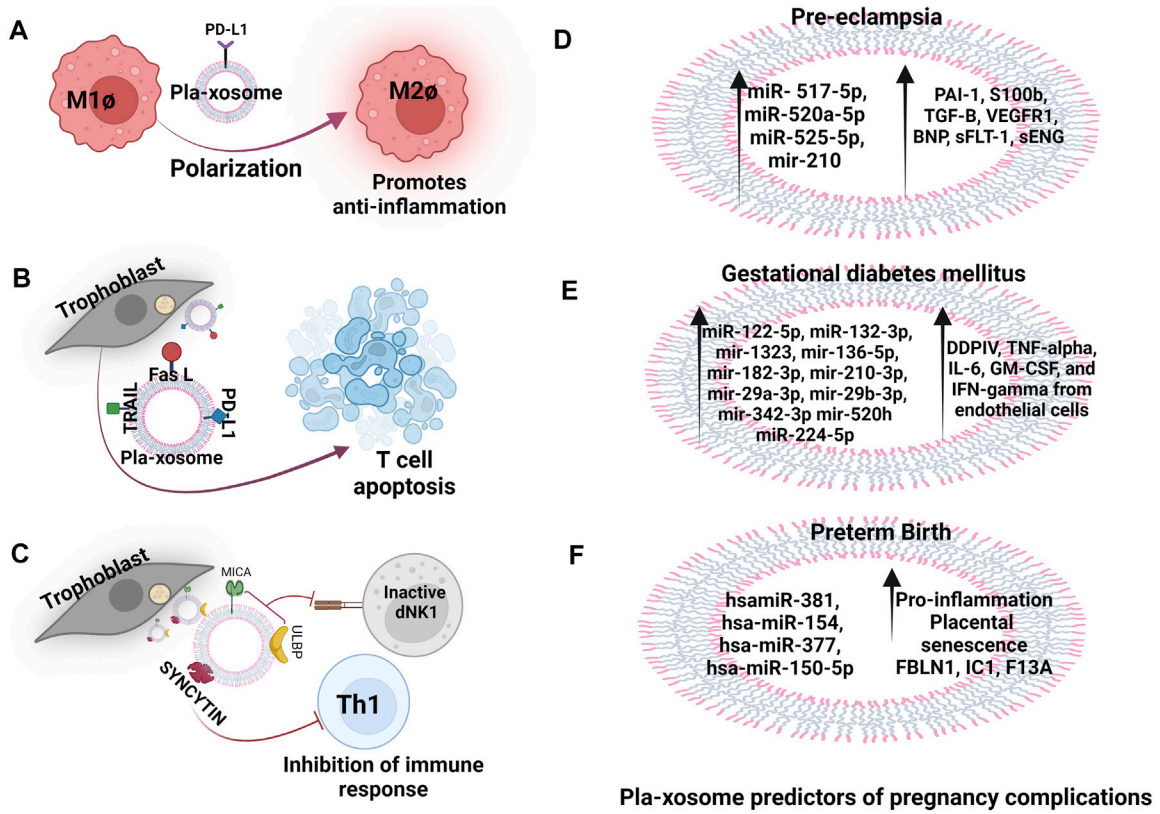


FIGURE 3 | Pla-xosomes in heathy, complicated pregnancies and adverse outcomes. **(A)** Pla-xosome (PD-L1+) causes M1Ø to M2Ø polarization to increase anti-inflammation **(B)** Pla-xosomes (Fas L + TRAIL + PD-L1+) cause T cell apoptosis for regulated immunosuppression **(C)** Pla-xosomes (ULBP + MICA + Syncytin+) cause inhibition of innate and adaptive immune response to support restructuring of materno-fetal interface **(D)** Increased expression of pla-xosomal miR- 517-5p, miR-520a-5p, miR-525-5p, and miR- 210 and the proteins PAI-1, S100b, TGF-B, VEGFR1, BNP, sFLT-1, sENG as predictors **(E)** miR-122-5p, miR-132-3p, mir-1323, mir-136-5p, mir-182-3p, mir-210-3p, mir-29a-3p, mir-29b-3p, mir-342-3p and mir-520h along with proteins like DDPIV, GLP-1, tumor-necrosis factor-alpha (TNF-α), IL-6, GM-CSF, and IFN-gamma in GDM reported to be predictors of the disease **(F)** hsamiR-381, hsa-miR-154, hsa-miR-377, and hsa-miR-150-5p and proteins FBLN1, IC1, F13A are associated with preterm labour that causes increased placental senescence and inflammation.

detected in higher concentrations in the placenta, skeletal muscles, placental and total exosomes representing their metabolic involvement (Nair et al., 2018). In addition, miR-122-5p, miR-132-3p, mir-1323, mir-136-5p, mir-182-3p, mir-210-3p, mir-29a-3p, mir-29b-3p, mir-342-3p, and mir-520h have significantly higher expression in GDM cases than in controls and have been reported to be involved in trophoblast proliferation, differentiation and insulin regulation and glucose transport in pregnant women (**Figure 3E**) (Gillet et al., 2019). A urine exosomal study in GDM patients in the third trimester of pregnancy revealed that miR-516-5p, miR-517-3p, miR-518-5p, miR-222-3p, and miR-16-5p are present in lower levels compared to a healthy pregnancy (Herrera-Van Oostdam et al., 2020). Increased level of exosomal dipeptidyl peptidase IV (DDPIV) is associated with GDM pathogenesis and a mice study showed that inhibitors of DDPIV inhibit glucose homeostasis by cleaving glucagon-like peptide 1. This could be used to treat type 2 diabetes (**Figure 3E**) (Kandzija et al., 2019). Thus, not only exosomes can serve as predictors for pathological pregnancy like GDM but can also be used as target molecules for the assessment of given therapeutics. Hyperglycaemic condition induces exosomes release in GDM pregnancy and interestingly these exosomes promote the release of pro-inflammatory cytokines like TNF-α, IL-6, GM-CSF, and IFN-γ from endothelial cells, thus contributing to the pathological inflammation in GDM (Salomon et al., 2016).

Preterm Birth

Studies on placental-derived exosomes in PTB are less and limited. Exosomes have been reported to carry miRNAs involved in the regulation of trophoblast invasion, proliferation and angiogenesis as potential biomarkers for predicting PTB such as hsamiR-381, hsa-miR-154, hsa-miR-377, and hsa-miR-150-5p (**Figure 3F**) (Menon et al., 2019; Cook et al., 2019). A set of proteins (FBLN1, IC1, F13A etc.) from plasma exosomes collected at 10–12 weeks of gestation are reported to be associated with the diagnosis of moderate PTB with the area under the receiver operating characteristic curve of 0.74 (**Figure 3F**) (McElrath et al., 2019). A comprehensive analysis of miRNA profiles of maternal plasma-derived exosomes differs at term and preterm and the miRNA's target genes are associated with TGF-β signaling, p53, and

glucocorticoid receptor signalling (Menon et al., 2019). A comprehensive proteomic profiling of PTB plasma-derived placental exosomal cargo has further verified that the alterations in protein compositions are also associated with inflammatory and metabolic signals. Interestingly, the placental senescence that occurs due to the encounter of oxidative and mitochondrial stress is reported to be influenced by these inflammatory signals (**Figure 3F**) (Cook et al., 2019). Studies performed on amniotic fluid-derived exosomes from preterm patients have confirmed these results (Dixon et al., 2018). A study in mice and cows demonstrated that *in-vitro* btamiR-499 found in pla-xosomes isolated from early pregnancy collected plasma, inhibited the activation of NF-κB via Lin28B/let-7 axis (lin 28B is an RNA Binding Protein and let7 is its targeted a miRNA) in bovine endometrial epithelial cells, suggesting that placental exosomes have a vital role in regulating uterine inflammatory balance determining a threshold for the onset of labor (Zhao et al., 2018b). *In-vivo* studies on mice have revealed labor-triggering properties of exosomes isolated from plasma of CD-1 mice from late gestation (E18) (Sheller-Miller et al., 2019). It emphasizes the importance of exosomal signals in the early termination of pregnancy.

Similarities in the Development of the Placenta and Cancer

As pregnancy disorders involve the failure of feto-maternal cells to function normally, cancer begins with the failure of cells to reproduce and differentiate in a regulated manner. The development of the placenta and fetal-placental communication during pregnancy mimics a regulated form of cancer. Cancer manipulates the immune system for its survival in a similar manner as the placenta does for fetal survival. The cross-talk between cancer cells and immune cells is mediated via tumor exosomes (TEVs) (He et al., 2021). Interestingly, the cargo of TEVs also resembles similar to pla-xosomes indicating initiation of some similar pathways e.g., angiogenesis, T cell suppression, and expansion of anti-inflammatory responses during the growth spurt, later we will be exploring these aspects in detail. Expression of factors such as angiopoietins and members of the VEGF family occurs in placental and cancer development to aid in angiogenesis (Shore et al., 1997; Charnock-Jones et al., 2004). Therefore, a similarity can be drawn between the cellular invasion of EVT and cancer cells as early events in both the cases. Both of these cell types use the epithelial-to-mesenchymal transition to promote movement across the endometrium (during placental development) or normal (cancerous growth) tissue (Yang and Weinberg, 2008).

Just like tumor cells are found in the systemic circulation, intact trophoblasts are also known to circulate in maternal peripheral blood during the early first trimester of pregnancy. Irrespective of HLA disparity these fetal-derived cells can embed in the maternal system establishing long-term microchimerism that persists for decades after parturition as a change accepted by the maternal immune system (Evans et al., 1999). Apart from the similar mechanism of development, the process for evading host immune response in cancer and trophoblast is also similar. Total or selective loss of HLA class I molecules is a frequently reported mechanism in various human tumors to escape recognition and destruction by cytotoxic T lymphocytes cells (Garcia-Lora et al., 2003). Trogocytosis (i.e., rapid cell-to-cell contacts that are dependent upon membrane transfer) is the primary mechanism by which HLAG + suppressive NK cells are generated within a tumor microenvironment (Caumartin et al., 2007). This mechanism is similar to HLA variants protection of trophoblasts in pregnancy where the trophoblast escape NK cell attack by inducing killer inhibitory receptors on NK cells reference from above (**Figure 4A**). Cancer cells also present the HLA class II antigen in the absence of the CD80/CD86 universe-stimulating molecules, this frequent representation of cancer cell antigens drives T-cell anergy thus, imparting cancer tolerance (Byrne and Halliday, 2003). Immune tolerance against cancer cells may also be the result of the knockout of lymphocyte lines that respond against autoantigens called tumour-associated antigens (TAA). These TAAs are abnormally expressed or overexpressed on malignant cells and is present in dissolved form in the circulation (Ko et al., 2003). Whereas, in the fetus, a combination of maternal and paternal antigens could contribute in chronic stimulation of T-cells thereby disrupting their effector functions. To ensure clearance from the immune system tumours are able to destroy immunocompetent T cells through a FasR/FasL-dependent mechanism causing T-cell apoptosis (Byrne and Halliday, 2003). A similar mechanism is executed by trophoblast cells for inducing T cell apoptosis. The tumor itself is resistant to Fas-mediated lysis by activated lymphocytes presumably because tumor cells overexpress BCL2 in the cytoplasm (Mese et al., 2000). Expressions of BCL2 have also been shown along the gestations in trophoblast cells however, contradicting studies revealed that expression of BCL2 is higher in the first and second trimester whereas, it has lower expressions in the third trimester of pregnancy emphasizing on the notion of pregnancy mirroring a regulated form of cancer which is a spatio-temporal need of the mother and the developing fetus (Soni et al., 2010). Just like fetal signals drive naïve T-cell differentiation into T regs, the tumor-specific antigens cause expansion of Treg cells in cancer implicating an impaired antitumor immunity, suppressed T cell proliferation, and increased tumor blood vessel density. This dampens the antitumor immune responses to promote angiogenesis (Beyer and Schultze, 2006). Immuno-regulatory mechanisms protect the fetus from the NK cell attack in the decidua. It was shown, Prostaglandin E2 (PGE2) (**Figure 4B**) which is derived from and localized in decidua aids in protecting the fetus by hindering the production of IL2 and the IL2 receptors on NK and T cells (Munn et al., 1998). This mechanism of host immune protection is hijacked by cancerous cells (Park et al., 2018). During pregnancy, membrane-bound and soluble molecules like LAG-3, Tim-3, PD-1, CTLA-4, and TIGIT are found which influence the Treg cell functions by decreasing the effectiveness of pro-inflammatory T cells (Zhang and Sun, 2020). Signals from cancer cells induce the expression of inhibitory receptor PD-1 on effector T cells setting them in a resting stage

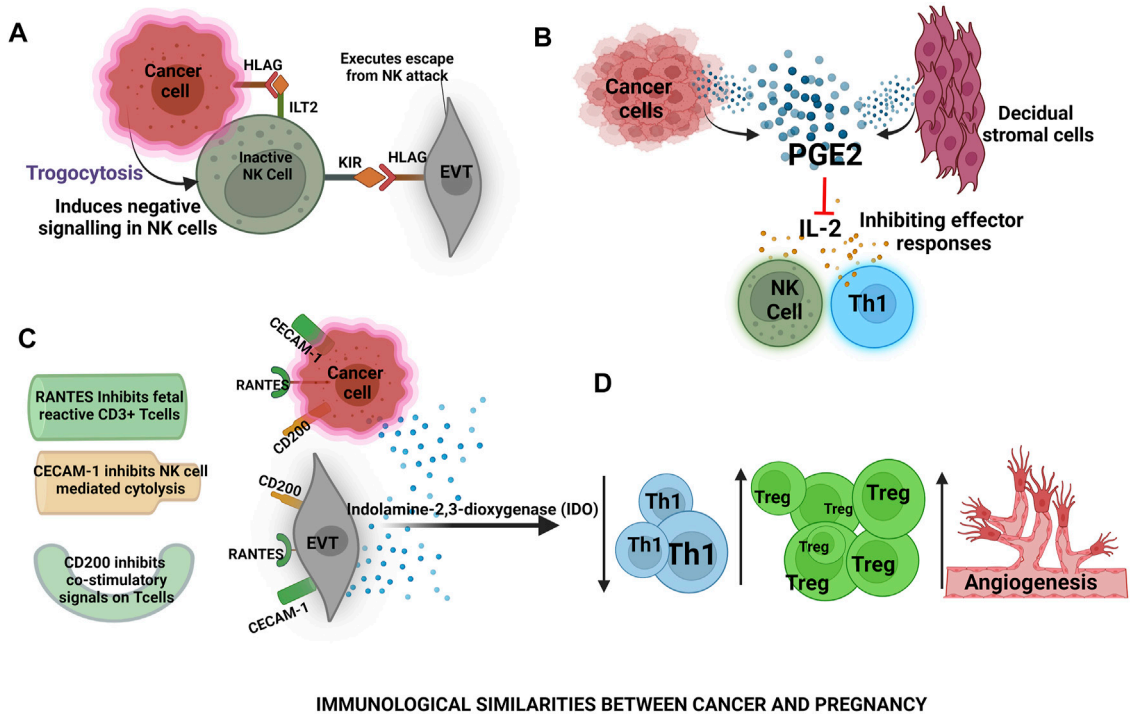


FIGURE 4 | Immunological similarities between cancer and pregnancy- **(A)** In cancer, HLA-G causes suppression of NK cells activity and in placental development trophoblasts expressing the human leukocyte antigen G-5 (HLAG) induce killer inhibitory receptors on NK cells **(B)** Cancer cells and decidual stromal cells release prostaglandins that inhibit IL-2 production thus, masking pro-inflammatory responses by NK cells and T helper 1 cells **(C)** RANTES, CECAM-1, CD200 are the common surface molecules among cancer cells and extravillous trophoblasts (EVTs) that inhibit pro-inflammatory cellular responses **(D)** Cancer cells and EVTs secrete indolamine-2,3-dioxygenase (IDO) which is toxic to Th1 cells thereby ameliorating the Th1 expression and increasing regulatory T (Treg) cell responses to promote angiogenesis for the fetus and cancer survival.

also known as T cell exhaustion. During the last decade PD-1, PD-L1 and CTLA-4 inhibitors have been used and were successful in aborting the solid tumours by setting the immune cells in their attacking state (Homet Moreno and Ribas, 2015; Robert, 2020). CD200 (OX-2) (**Figure 4C**) and carcinoembryonic antigen-related cell adhesion molecules (CEACAM-1), the cell surface tolerance signals exist commonly between trophoblasts and cancer cells (Clark et al., 2003; Gray-Owen and Blumberg, 2006). *In-vitro*, trophoblasts expressing CD200 can inhibit the generation of $CD8^+$ T cells called cytotoxic lymphocytes (CTLs) and shift the balance of cytokines towards TH2 (Clark et al., 2003). CD200 in TME of melanomas, ovarian cancers, and renal cancers suppresses Th1 cytokines *in-vitro* (Moreaux et al., 2006). Inhibition of NK-mediated cytolysis also occurs by CEACAM-1 (CD66a), expressed on trophoblasts, whereas, CEACAM-1 in tumor cells diminishes expression of NKG2D receptors on NK cells, thus suppressing NK cytolysis implying another common link between cancer and pregnancy (Gray-Owen and Blumberg, 2006). A chemokine produced by trophoblasts known as RANTES is known to induce apoptosis of fetal-reactive $CD3^+$ cells and the same chemokine is shown to be secreted by tumor-infiltrating lymphocytes following their apoptosis creating a mechanism for immune response evasion (Fraccaroli et al., 2009). Importantly, Indoleamine 2,3-dioxygenase (IDO) (**Figure 4D**) a tryptophan degrading enzyme is required for maintaining the tolerogenic state at the feto-maternal interface as well as in tumor microenvironment (TME) (Munn and Mellor, 2016). This enzyme converts tryptophan to kyneuirine, an effector T cell toxic compound inhibiting their proliferation and causing T cell apoptosis (Hwu et al., 2000). In a study performed on mouse models the action of enzyme IDO, when expressed at the interface of fetus and mother by MØ and trophoblast cells, was shown to be required for the protection of the semi-allogenic fetus. Moreover, the inhibition of IDO turned out cynical and lead to the death of the semi-allogeneic fetus (Munn et al., 1998). Whereas, IDO in TME, positively regulates the activity of Treg cells and this property has been used for the advantage of immunotherapy with IDO inhibitors (Yentz and Smith, 2018). In women with normal pregnancies, soluble CD30, a member of the tumor necrosis superfamily of receptors and a marker of TH2 polarization, is increased, while it is reduced in women with PE and intrauterine growth retardation (**Figure 4D**) (Kusanovic et al., 2007). Microarray analysis of placentae from pre-eclamptic pregnancies revealed changes in gene expression pathways including angiogenesis, immune defense responses as well as apoptosis, and cell survival which is also associated with cancer (Louwen et al., 2012).

Cancer Escaping the Immune System: Unraveling the Trail of Cancer-Derived Exosomes (CEV_S)

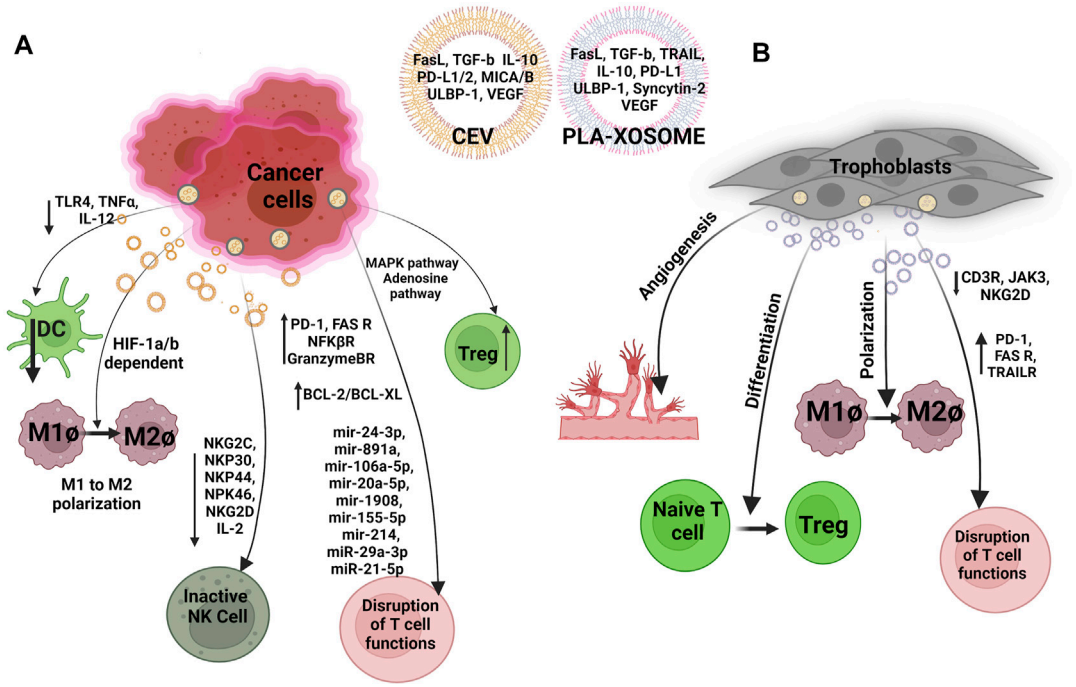
Pregnancy and cancer connect with each other at another aspect that is immunomodulation via exosomes. Studies have demonstrated the presence of similar signalling molecules (RNAs and/or proteins) encapsulated inside cancer-derived and placental-derived exosomes. Rigorous studies carried out in the field of cancer provide the initial understanding of the mechanistic pitfalls that may lead to pregnancy complications and adverse outcomes. The manipulation of host immune cells by cancer derived-exosomes to strengthen a tolerogenic milieu for the progression of cancer has been very well studied. This well-trodden path in the field of cancer biology can be tested using appropriate animal models and subsequent clinical trials to restore the lost tolerance and recreation of the anti-inflammatory milieu for the betterment of pregnancy complications. Therefore, it would be interesting to track the trail of cross-talk of cancer- and host immune cells via exosomes.

CEVs Modulate Innate Immune Cells

CEVs deviate the conventional pathway of the expansion of the myeloid and bone marrow precursor cells that are committed towards stimulatory DC into their suppressor phenotypes thus, altering the cancer antigen presentation via DCs and augmenting the tolerogenic niche (Ning et al., 2018; Tung et al., 2018). The miRNA-212 in pancreatic CEVs upon its internalization in DCs, downregulates the expression of transcription factor RFXAP (Regulatory factor X associated protein) which simultaneously demeanours the expression of MHC-II on DCs affecting the antigen presentation via these DCs (Ding et al., 2015). Moreover, CEVs interfere with the expression of co-stimulatory molecules like MHC-II, CD80, CD86 on DCs and increase the expression of co-inhibitory receptors on DCs like PD-1. Thus, affecting the maturation and migration process of DCs and converting the existing DCs into suppressive phenotypes (Ludwig et al., 2018). Another *in vivo* study on pancreatic cancer reported that in DCs, CEVs affect their proliferation and expansion by down-regulating TLR4, downstream TNF-α, and IL-12 cytokines via miR-203 (**Figure 5A**) (Zhou et al., 2014). CEVs also modulate MØ, since mutation acquired abilities of cancer cells enable them to hijack M1 and re-engineer them into M2. The existence of M2 polarized state in malignant cancer forte, expressing functional Arg1, VEGF, and CD163, CD23, CD204, along with cytokines like IL-10, TGF-β, TGF- α, and chemokines including CCL16, CCL17, and CCL22, confirms a congenial M2 state (Cheng et al., 2019). Increased cancer growth creates a hypoxic environment, which results in the release of CEVs that polarizes M1 into M2 in a HIF-1α and HIF-1β dependent manner (**Figure 5A**) (Hood et al., 2011). Thus, CEVs manipulate M1 to exhibit M2 anti-inflammatory phenotype to help aid angiogenesis for fulfilling the oxygen demand of growing cancer. Interestingly, ovarian CEVs carrying miRNAs like mir-222-3p, have been shown to disrupt Treg/Th17 immune balance. They have been implicated in inducing M2 polarization via STAT-3 signal-dependent pathway thereby, increasing Treg and M2 expansion. Besides a decrease in the Th-17 cell population has been observed contributing to the anti-inflammatory cancer microenvironment (Ying et al., 2016). CEVs also have been reported to inhibit caspases involved in apoptosis and transfer a functional receptor tyrosine kinase initiating the monocyte MAPK pathway (Song et al., 2016). Thus, these altered MØ can then encourage angiogenesis and metastasis required for cancer progression. Another important innate immune subset, NK cells contain switches in the form of activating as well as inhibitory receptors. Apoptosis of cancer cells in prostate cancer and acute leukemia is prevented by CEVs internalization in NK cells, which inhibits the expression of NK activating receptors like NKG2C, NKP30, NKP44, NPK46, and NKG2D (**Figure 5A**) (Garcia-Iglesias et al., 2009). CEVs have also been shown to target the TGF-β pathway, TGF-β which exists as TGF-latency associated peptide (LAP) in CEVs when bound to integrin a6βV is activated and induces Smad phosphorylation subsequently reducing NKG2D expression thus preventing NK cell cytotoxicity (Szczepanski et al., 2011). In a mice model, CEVs treatment affected the generation of NK cells and also impaired their responses. CEVs encapsulate the stress-inducible NKG2D ligands, MHC-class I related protein chain A/B (MICA/B) and Ul-16 binding protein-1 (ULBP-1) and -2 that acts as a decoy, by down-regulating the NKG2D-mediated cytotoxicity of NK cells in T- and B-cell leukemia/lymphoma (Clayton and Tabi, 2005; Mincheva-Nilsson and Baranov, 2014b). In addition, CEVs suppressed the cyclinD3 expression and inactivate the JAK3 pathway by inhibiting IL-2 stimulation via NK cells thereby, breaking one connective link in innate and adaptive immunity by preventing T cell interaction with NK cells. Murine mammary carcinoma exosomes promote tumor growth by suppression of NK cell function (Liu et al., 2006). However, as disconnecting a single link cannot produce desirable results, thus CEVs interact with adaptive immune cells too.

CEVs Modulate Adaptive Immune Cells

CEVs express CD39 (NTP-Dase) and CD73, which work together to convert extracellular ATP to immunosuppressive adenosine and 5 AMP phosphate (Clayton et al., 2011; Muller et al., 2016). Extracellular adenosine production is high, which adversely affects T cells around cancerous tissues, allowing it to evade immune responses. In addition, the presence of CEVs carrying miR-24-3p, miR-891a, miR-106a-5p, miR-20a-5p, and miR-1908 inhibits T-cell activity in nasopharyngeal cancer (**Figure 5A**) (Bell and Taylor, 2017; Ye et al., 2014). Interestingly, co-culturing CEVs with T cells resulted in elevated expression of BAX (proapoptotic marker) and decline in expression of BCL-2/BCL-XL (anti-apoptotic markers) indicating cancer mediated T cell suppression (**Figure 5A**). FasL in CEVs causes the apoptosis of FasR + T cells by initiating FasL/FasR signalling (Alzahrani et al., 2018). According to an analysis of EVs recovered from the serum of patients with head and neck cancer and melanoma, cell death ligands such as Fas on $CD8^+$ cytotoxic T lymphocytes (CTLs), were particularly sensitive to CEVs. They affected signal transduction and proliferation of $CD8^+$ CTLs thus, affecting cytotoxic responses on cancer cells



Role of exosomes in immunosuppression aiding cancer and placental development

FIGURE 5 | Targets of cancer-derived exosomes (CEVs) and placenta derived exosomes (Pla-xosomes)- **(A)** CEVs contains mir-203 which downregulates expression of toll-like receptor 4 (TLR4), downstream tumor necrosis factor-alpha (TNF-α) and interleukin-12 (IL-12) cytokines responsible for DCs proliferation and expansion, CEVs causes M1Ø to M2Ø polarization in a HIF-1a and HIF-1b dependent manner to promote immune suppression, CEVs internalization in NK cells, inhibits the expression of NK activating receptors like NKG2C, NKP30, NKP44, NPK46, and NKG2D to escape NK cell cytotoxicity, CEVs increases FasL/FasR signalling, PD-L1/PD-1 signalling and BCL2 (anti-apoptotic protein) expressions to evade apoptosis of cancer cells. CEVs carrying miR-29a-3p and miR-21-5p, miRNA 155-5p, miRNA-214, miR-24-3p, miR-891a, miR-106a-5p, miR-20a-5p, and miR-1908 inhibits T-cell activity, CEVs also cause T reg expansion thus aiding cancer development **(B)** Pla-xosomes promote angiogenesis via VEGF, help in Treg expansion, M2Ø polarization, causes upregulated expression of PD-L1, Fas, TRAIL and downregulates the expression of CD3 receptor, JAKR and NKG2D. All of these are essential to promote an effective immune microenvironment in the mother.

(Maybruck et al., 2017). Peritoneal tissue from patients with metastatic ovarian cancer had higher Treg levels than Th17 cells suggesting a requirement of more suppressed TME for metastasis (Zhou et al., 2018). It was found that exosomes play a unique role in this imbalance. Favoring T reg functions, exosomes originating from TAMs transfer miR-29a-3p and miR-21-5p to helper T cells and inhibit intracellular STAT3 signalling which decreases pro-inflammatory cytokine secretion from $CD4^+$ T cells (**Figure 5A**). This disturbs the Tregs/Th17 balance creating an immunosuppressive environment for ovarian cancer progression (Zhou et al., 2018). In addition, there have been recent reports of CEVs containing PD-L1, which inhibit the immune system by targeting multiple pathways, thus aiding cancer growth (**Figure 5A**) (Mrizak et al., 2015). The transfer of PD-L1 via CEV from $PD\text{-}L1_{high}$ cancer cells to $PD\text{-}L1_{low}$ cancer cells elevated the PD-L1 release which further inhibited the T cell response by initiating PD-L1/PD-1 signalling. The membrane-bound PD-L1 carried by exosomes suppresses anti-cancer immune responses both locally in the TME and systemically. PD-L1+ exosomes produced by a breast cancer cell line inhibited co-stimulatory molecule (CD3/CD28) -induced ERK phosphorylation and NFκB activation of T-cells *in vitro*. Exosomal PD-L1 harbors active defense function to suppress T cell killing of breast cancer cells and promote tumor growth (Yang et al., 2018). The experiment carried out *in-vivo* revealed suppression of granzyme B activity of T cells found in the TME, thus reducing cytotoxic T-cell activity (Vignard et al., 2020). In another study, exosomes isolated from head and HNSCC patients' plasma inhibited the activatory receptor CD69 expression on human activated $CD8^+$ T cells, and the PD-L1 levels on exosomes correlated with their T-cell inhibitory activity. Murine CEVs carrying PD-L1 were immunosuppressive, and blocking of PD-L1 activity with neutralizing mAbs restored the immune competence of T cells and inhibited tumor growth (Theodoraki et al., 2018). CEVs caused the expansion of Tregs. Tregs are one of the most important subsets of T-cells required for sustaining the development and growth of biological entities. Secretion of anti-inflammatory cytokines like IL-10, TGFβ-1, and CTLA4 promotes the suppressive phenotype of Treg which is immensely exploited by cancer cells. Researchers have confirmed the transformation and proliferation of $CD4^+CD25^+$ T-cells into $CD4^+CD25$ + Foxp3+ Tregs *in-vivo* upon administration of CEVs via MAPK pathway and adenosine pathway (**Figure 5A**) (Mrizak et al., 2015) miRNA-155-5p and

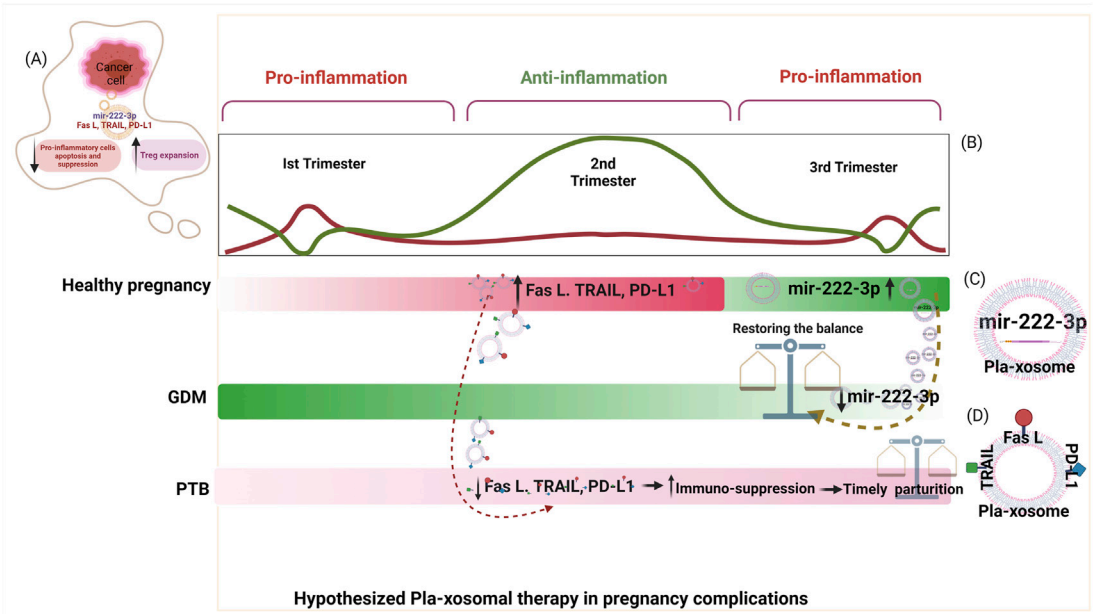


FIGURE 6 | Hypothesized pla-xosomal therapy for pregnancy complications: Taking lessons from cancer **(A)** Cancer cells derived exosomes contain mir-222-3p, PD-L1, Fas L, and TRAIL that cause suppression of pro-inflammatory cells and expansion of anti-inflammatory cells (Tregs) **(B)** Balanced pro-and anti-inflammatory milieu in a healthy pregnancy with increased expression of PD-L1, Fas L, TRAIL in second Trimester and mir-222-3p in third Trimester **(C)** Pla-xosome containing mir-223: postulated therapy for GDM **(D)** Pla-xosome containing PD-L1, Fas, TRAIL: postulated therapy for PTB. Dotted lines represent the donor and recipient gestational windows of plax-osomes intervention.

miRNA-214 in CEV inhibited the precursor T-cell differentiation into Th1/Th17 phenotypes and reduces the PTEN-tumor suppressor homolog in T cells respectively, therefore increasing anti-inflammation and decreasing pro-inflammation parallelly (**Figure 5A**) (Yao et al., 2012; Sharma et al., 2015; Sun et al., 2019). *In-vitro*, CEV's surface markers CD39 + $CD73^{+}$ (NTPDases) bind to the T cell surface adenosine receptor 2 (A2AR) and send out a signal via cAMP. This upregulates the T cells to generate adenosine and prime Tregs thereby inducing their effector responses (Clayton et al., 2011). The elevated content of CD39/CD73 in CEVs reflected the presence of advanced-stage disease in HNSCC patients. These studies give strong evidence of impaired host immune response directed via CEVs (Allard et al., 2017). Interestingly, analyzing T cell-derived exosomes from cancer patient's plasma for clues of the immune status in CEVs-reprogrammed T cells has recently become possible. Chimeric antigen receptor (CAR+) exosomes derived from CAR-T cells administered in cancer patients are enriched in immunosuppressive proteins and consistently inhibit functions of other T cells, thus their internalization causes intracellular changes in T cells (Fu et al., 2019).

Therapeutic Potential of Exosomes in Pregnancy Complications

The role of exosomes in cancer diagnosis and immune therapy has been extensively studied. As mentioned previously, cancer cells release PD-L1+ exosomes that interact with T cell's surface PD-1 initiating intracellular suppressive signalling. In the advanced stages of cancer expression levels of soluble PD-L1 are increased that can be detected in circulation thus, cancer-derived exosomal PD-L1 can serve as cancer predicting biomarker (**Figure 6A**) (Shimada et al., 2021). Even for cancer therapy, the immune checkpoints are known targets for inhibitory antibodies. Moreover, the use of human umbilical cord blood mesenchymal stem cells-derived exosomal mir-503-3p has been reported to abort endometrial cancer and target biological functions of endometrial cancer cells by downregulating mesoderm-specific transcript (Pan et al., 2022). However, the use of exosomes for providing therapies in pregnancy complications is a big challenge because of the need for a balanced treatment at a particular time, simultaneously protecting the fetus from any harm. Irrespective of the challenges, multiple trials for creating therapeutics in restoring the balance of healthy pregnancy processes in pregnancy complications have been attempted. For e.g., in a mouse model study, exosomes from human umbilical cord mesenchymal stem cell-derived (HUMSC) exosomes have been reported to improve endometrial injury by stimulating endometrial regeneration via PTEN/AKT signalling pathway. This further increases the expression of BCL-2 (anti-apoptotic protein) via AKT activation and decreases the expression of activated caspase-3 facilitating cell proliferation thus promoting endometrial regeneration (Wang et al., 2020b). Another study demonstrated that the administration of HUCMSC exosomes results in upregulation of mir-18b-3p, which targets leptin to reduce pro-inflammatory factors and prevent cellular apoptosis in the PE rat placenta (Huang et al., 2021). Interestingly in the mouse model of PE, the therapeutic effects of

HUCMSCs derived EVs have been reported where administration of HUCMSC-exos during pregnancy prevented soluble Fms-like Tyrosine kinase (sFLT-1) induce preeclamptic complications. sFLT is a negative regulator of VEGF thus aiding angiogenesis, HUMSCs-exos input resulted in decreased sFLT levels thereby, ultimately improving the fetal and placental weight. The exosomes have engineered to encapsulate IκBa that inhibit pro-inflammatory cytokine transcription factor NFkB in feto-maternal uterine tissues thus, delaying LPS-induced PTB (Sheller-Miller et al., 2021). Administration of mesenchymal stromal cell-derived extracellular vesicles alters inflammatory mediators' expression in the preeclamptic intrauterine compartment, thus normalizing the formation of fetal lung branches and their morphogenetic gene expressions (Taglauer et al., 2021).

Taking Lessons From CEVs

Due to the uncanny resemblance of the underlying biological processes of pregnancy with cancer, the signal carrying exosomal cargo in both are also close to similar. The immunosuppressive entities harbored in the exosomes e.g., PD-L1, VEGF, MICA, ULBP-1, HLA variants, Fas L, TRAIL, IL-10 etc. target similar immune cell subsets like Th1, Tregs, DCs and NK cells thus, promoting the anti-inflammatory niche required for the fetus and cancer development post its implantation and establishment respectively (**Figure 6B**). Interestingly, ovarian-cancer-derived exosomes contain mir-222-3p that is shown to increase Tregs thus, promoting anti-inflammation required for cancer survival (Stenqvist et al., 2013). Whereas, in GDM patients the expression of placental derived exosomal mir-222-3p significantly decreases by the third trimester and affects the metabolic processes like steroid hormone biosynthesis and tryptophan metabolism triggering insulin resistance and inflammation in GDM (Herrera-Van Oostdam et al., 2020). However, as a healthy pregnancy progresses, elevated levels of mir-222-3p have been observed, implying that the increased expression of this miRNA is a requirement for an uncomplicated pregnancy (Herrera-Van Oostdam et al., 2020). Since the mir-222-3p is enriched within placental exosomes, these exosomes could be used in a spatio-temporal manner to ameliorate pregnancy complications like GDM as a therapeutic agent (**Figure 6C**). Similarly, the apoptosis-inducing ligands like FasL, TRAIL, and immune exhaustion markers like PD-L1 are enriched on pla-xosomes and their isolation would be more appropriate from the second trimester of a pregnancy where an anti-inflammatory milieu is a necessity for fetal development (**Figure 6D**) (Stenqvist et al., 2013). These pla-xosomes may be used as therapeutics for treatment in pregnancy complications like PTB where inflammatory responses are high causing early parturition. However, isolation and delivery of these power shots should be carried out in a timely manner i.e., pla-xosmes isolated from the second trimester of a healthy pregnancy need to be administered to a high-risk mother diagnosed for preterm delivery so as to decrease the inflammation and lengthen the gestational age *in utero*. However, for such a successful execution of this hypothesized therapy, the identification of early predictive markers for adverse pregnancies is an obligation and clinical trials are vital.

AUTHOR CONTRIBUTIONS

HD contributed to the planning, literature search, writing, and diagrammatic representations. RK contributed in refining some sections of the review. AM was responsible for assisting in the literature search. SB supported the idea and provided inputs. PK contributed to the conceptualization, planning, supervision, implications, and final editing.

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Pregnancy-Related Stress Among Pregnant Women Receiving Tocolytic and Non-Tocolytic Treatments Where Both Used Complementary Medicine

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Objective: This study aimed to compare the pregnancy stress among pregnant women in receiving tocolytic and non-tocolytic treatments where both used complementary medicine.

Methods: A cross-sectional survey was conducted among 35 pregnant women receiving tocolytic treatment and 35 receiving non-tocolytic treatment, where both used complementary medicine in a medical center in central Taiwan. A basic information questionnaire that contained demographic variables and types of complementary medicine used and the Pregnancy Stress Rating Scale were used for the analysis.

Results: The types of complementary medicines were surveyed using the multiple-choice questionnaire. Natural products (77.5%) were most commonly used by pregnant women receiving tocolytic treatment, followed by alternative medicine (13.75%), manipulative and body-based practices (5%), and mind and body medicine (3.75%). In pregnant women who were receiving non-tocolytic treatment, natural products (59.1%) were most commonly used, followed by manipulative and body-based practices (16.4%), alternative medicine (15.4%), mind and body medicine (7.3%), and energy therapy (1.8%). According to the analysis of covariance test results, while both used complementary medicine in groups, pregnant women receiving tocolytic treatment were less stressed than those who were receiving non-tocolytic treatment (Pregnancy Stress Rating Scale score, $p = 0.038$), especially in dimension 2 (stress caused by infant care and changes in family relationships) ($p = 0.015$) and dimension 5 (stress caused by changes in physical appearance and function) ($p = 0.008$), which showed statistically significant differences ($p < 0.05$). Linear regression analysis results showed that the gestational age significantly associated with pregnancy stress (Pregnancy Stress Rating Scale score, $p = 0.029$; dimension 2, $p = 0.016$; and dimension 5, $p = 0.001$).

Conclusion: Among both who used complementary medicine, pregnancy stress was significantly lower in pregnant women who were receiving tocolytic treatment than in those who were receiving non-tocolytic treatment. This finding can be used as a reference for future pregnant women's health studies.

Keywords: tocolysis, tocolytic, pregnant women, pregnancy stress, complementary medicine

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1 INTRODUCTION

Physiological, psychological, social, or fetal factors may be detrimental to prenatal health, possibly leading to miscarriages, premature birth, or stillbirths (Hu, 2006; Dhillon et al., 2017). Thus, pregnant women may experience pregnancy stress; to reduce or eliminate such stress, they resort to using complementary medicine. Complementary medicine, such as religion, music, or acupuncture can reduce anxiety, unease, and uncertainty in tocolytic (a category of drugs used to delay the labor process) pregnant women and increase the therapeutic effectiveness of tocolysis (Tsai and Lee, 2012). Given these findings, complementary medicine and pregnancy stress are worthy to be investigated.

Complementary medicine has diverse types but is generally divided into five major categories: alternative medicine, natural products, mind and body medicine, energy therapy, and manipulative and body-based practices. Alternative medicines such as traditional Chinese medicine and naturopathy are slow-acting but can improve chronic diseases (Chiang, 2014; Pallivalappila et al., 2014; Mitchell, 2016). A 2008 survey involving obstetricians from the American Medical Association considered biofeedback, chiropractic care, acupuncture, and meditation as highly effective complementary medicine treatments (Babbar et al., 2017). Natural products emphasize that the human body itself has natural healing capabilities and that natural substances, such as natural herbs, high doses of vitamins, minerals, dietary fiber, probiotics, Lingzhi mushroom, shark cartilage, and cod liver oil, can be used to prevent diseases (Holst et al., 2009). In mind and body medicine, psychological stability can be improved using certain techniques, such as imagery, meditation, music therapy, yoga, prayer, biofeedback, journal therapy, art therapy, sitting still, humor, Tai Chi, and psychotherapy. Energy therapies, such as healing touch, therapeutic touch, Reiki, Qigong, and magnet therapy, refer to using energy from certain substances to stimulate the human body to achieve health. Last, manipulative and body-based practices are based on body manipulation and body movements, and these include chiropractic medicine, various massage techniques, body movements (e.g., osteopathy), rolfing, light therapy, hydrotherapy, and chromotherapy (Chen et al., 2010; Birdee et al., 2014; Guardino et al., 2014).

Currently, many pregnant women receiving tocolytic treatment use complementary medicine at suitable times to alleviate prenatal psychological stress and reduce stress, anxiety, labor pain, and depression, especially during labor. Hence, complementary medicine provides therapeutic effects during the entire pregnancy period (Deligiannidis and Freeman, 2014). Some types of complementary medicines are also specifically effective in mental disorders (Barcelona de Mendoza et al., 2016; Smith et al., 2019). However, pregnancy stress varies from individual to individual. The severity and type of stress experienced at the same pregnancy stage still varies because women differ in individual factors and life events. Stress occurs when pregnant women feel that they lose control or unable to cope (Yang and Chung, 2009). Rubin believes that maternal roles develop during pregnancy; one role is ensuring a healthy fetomaternal state until delivery (Rubin, 1976).

Frequently, pregnant women receiving tocolytic treatment possess factors that threaten maternal and fetal health, leading to difficulty in adapting to maternal roles (Rubin, 1976). This event causes pregnancy stress, which worsens if the pregnancy outcomes are uncertain; hence, pregnant women need more social support to adapt to pregnancy, particularly from their husbands and other family members. To alleviate such stress and uncertainty, using complementary medicine with pregnant women can be taught to use research-validated relaxation techniques, such as music, breathing techniques, and art therapy (Fang et al., 2011).

In Taiwan, most studies on complementary medicine treatment during pregnancy focused on the effectiveness of a certain type of complementary medicine, such as mind and body medicine (Mitchell, 2016). In addition, pregnancy stress in pregnant women receiving tocolytic treatment who use complementary medicine remains unreported. Therefore, this study aimed to compare pregnancy stress between pregnant women receiving tocolytic treatment and those who were receiving non-tocolytic treatment, where both used complementary medicine, to provide a reference for future studies by clinical staff.

2 MATERIALS AND METHODS

2.1 Study Design

This study is a cross-sectional survey that used convenience sampling from January 2019 to April 2019. After collecting data using structured questionnaires, we compared pregnancy stress among pregnant women receiving tocolytic treatment and those who were receiving non-tocolytic treatment, where both used complementary medicine. In this study, pregnant women receiving tocolytic treatment were defined as those within 20 and 37 gestational weeks undergoing tocolysis because of the risk of placenta previa, premature labor, or premature rupture of membrane. Conversely, pregnant women who were receiving non-tocolytic treatment were defined as those within 20–40 gestational weeks who were not prone to premature birth or had high-risk pregnancy and did not undergo tocolysis.

2.2 Study Site and Subjects

We recruited pregnant women receiving tocolytic treatment and those who were receiving non-tocolytic treatment admitted to the delivery room, obstetric ward, and obstetric outpatient unit of Taichung Veterans General Hospital in central Taiwan. The inclusion criteria were women with confirmed pregnancy; with and without tocolysis; who could listen, speak, read, and write Chinese and communicate in Chinese or Taiwanese Hokkien; and who were willing to participate in this study. The exclusion criteria were a history of mental illness, impaired consciousness, or communication disorder.

Pregnant women who use the following as prescribed by the medical center as kind of tocolytics and doses (drug treatments are from the lowest to the highest dose, and the dose was adjusted according to the maternal fetus condition) were recruited in receiving the tocolytic treatment group. 1) Drug name:

TABLE 1 | Analysis of demographic variables (n = 70).

Variable	Pregnant women receiving tocolytic treatment(n = 35) n (%)	Pregnant women receiving non-tocolytic treatment (n = 35) n (%)	p
Age			1.000
20–34 years	23 (65.7)	23 (65.7)	
35–45 years	12 (34.3)	12 (34.3)	
Occupation			0.271
Bureaucrat	2 (5.7)	6 (17.1)	
Farmer	0 (0)	1 (2.9)	
Artisan	5 (14.3)	4 (11.4)	
Merchant	12 (34.3)	6 (17.1)	
Homemaker	15 (42.9)	18 (51.4)	
Service industry	1 (2.9)	0 (0)	
Marital status			0.314
Married	34 (97.1)	35 (100)	
Unmarried	1 (2.9)	0 (0)	
Education level			1.000
Below senior high school	6 (17.1)	6 (17.1)	
Senior high school and above	29 (82.9)	29 (82.9)	
Religious beliefs			0.811
Present	17 (48.6)	16 (45.7)	
Absent	18 (51.4)	19 (54.3)	
Gravidity			0.803
Primigravida	22 (62.9)	23 (65.7)	
Multigravida	13 (37.1)	12 (34.3)	
Tocolysis experience (women with a history of tocolysis in previous pregnancy)			0.615
Yes	13 (37.1)	11 (31.4)	
No	22 (62.9)	24 (68.6)	

nifedipine/5 gm, it is advised to take one to two tablets orally, once every 4 or 6 hours or to use when necessary (PRN, pro re nata/as necessary). 2) Drug name: Yutopar (atosiban) 250 mg/amp, 5 amp, normal saline (N/S) (225 ml) is added and maintained at 3–21 ml per hour. 3) Drug name: $MgSO_4$ (magnesium sulfate) maintained at 1–2 g per hour. 4) Drug name: atosiban two vials normal saline (N/S) (90 ml) were added and maintained at 2–12 ml per hour. Pregnant women who did not receive tocolytic treatment were recruited in the non-tocolytic treatment group.

2.3 Rights and Ethical Considerations of the Study Subjects

This study was reviewed and approved by the Institutional Review Board of the hospital (IRB NO: CE18194A). Data collection officially began after fulfilling the administrative procedures and obtaining written informed consent from all study participants.

2.4 Study Tools

This study employed structured questionnaires to collect patient data. The study tools included a basic information questionnaire and the Pregnancy Stress Rating Scale (Chen, 2015), which are briefly summarized as follows:

2.4.1 Basic Information Questionnaire

This questionnaire was created by ourselves, and it includes demographic variables such as last menstrual period (LMP) to understand the gestational age, age, occupation, marital status, education level, religious beliefs, gravidity, and tocolysis experience (e.g., women with a history of tocolysis in previous pregnancy), as well as a survey on the types of complementary medicine used.

2.4.2 Pregnancy Stress Rating Scale

We used the Pregnancy Stress Rating Scale (Chen, 2015) to evaluate pregnancy stress. It contains five dimensions as follows: dimension 1 (stress caused by ensuring mother and child health and safety, with nine questions); dimension 2 (stress caused by infant care and changes in family relationships, with nine questions); dimension 3 (stress caused by acknowledging maternal role, with eight questions), dimension 4 (stress caused by seeking social support, with four questions), and dimension 5 (stress caused by changes in physical appearance and function, with six questions). Each question was answered using a 5-point Likert scale (never, one point; occasionally, two points; sometimes, three points; often, four points; and always, five points). The higher the score, the greater will be the pregnancy stress. The validity and reliability tests of the scale (Chen, 2015) obtained $\alpha = 0.92$ and can jointly achieve 52.17% of the total variance; thus, the scale is fairly valid and reliable.

2.5 Data Analysis

All statistical data were analyzed using the Statistical Package for the Social Sciences version 22.0. The demographic variables and usage distribution for complementary medicine were

TABLE 2 | Analysis of the types of complementary medicine used by pregnant women receiving tocolytic and non-tocolytic treatment (n = 70).

Variable (multiple-choice)	(*n*)	Pregnant women receiving tocolytic treatment *n* (%)	(*n*)	Pregnant women receiving non-tocolytic treatment *n* (%)	*p*
Alternative medicine		11 (13.75)		17 (15.4)	0.069
Traditional Chinese medicine	6		12		
Acupuncture	2		1		
Moxibustion	1		0		
Chinese herbal medicine	1		3		
Tuina	1		0		
Tai chi	0		0		
Qigong	0		1		
Natural products		62 (77.5)		65 (59.1)	0.088
Natural herbs	2		0		
Vitamins	24		28		
Minerals	4		5		
Dietary fiber	6		9		
Probiotics	18		13		
Cod liver oil	8		10		
Mind and body medicine		3 (3.75)		8 (7.3)	0.101
Meditation	0		3		
Yoga	2		4		
Hypnosis	1		0		
Progressive relaxation and guided imagery	0		1		
Energy therapy		0 (0)		2 (1.8)	0.151
Artificial magnet	0		1		
Natural magnet	0		1		
Manipulative and body-based practices		4 (5)		18 (16.4)	0.162
Osteopathy	0		1		
Massage	4		17		

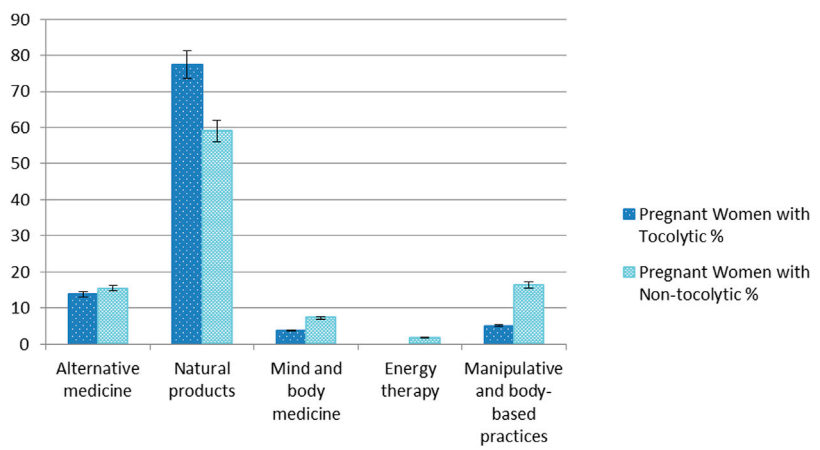

FIGURE 1 | Types of complementary medicine used by pregnant women receiving tocolytic and non-tocolytic treatment.

analyzed by descriptive statistics. Proportions were compared by the χ^2 test. For inferential statistics, the means between groups were compared by the analysis of covariance (ANCOVA) test. Furthermore, the predictive relationship between variables was evaluated by regression analysis. To estimate the sample size, we employed power analysis (Polit and Hungler, 1999), with >0.8 as the statistical power. When α (significance level) was set at 0.05, the power was 0.8, and the effect size was 0.4; thus, 35 subjects were required for each group. In consideration of the timeliness of subject enrollment and sample loss, 70 subjects were enrolled in all groups. The questionnaire was personally sent to the participants, and after the completion of all questions, no data were missed. The reliability of this research was assessed using Cronbach's α to assess the internal consistency. Cronbach's α was 0.92. For all statistical tests, $p < 0.05$ indicated significance.

TABLE 3 | Comparison of pregnancy stress among pregnant women receiving tocolytic and non-tocolytic treatment where both used complementary medicine (n = 70).

Pregnancy Stress Rating Scale	Pregnant women receiving tocolytic treatment (n = 35) (mean ± SD)	Pregnant women receiving non-tocolytic treatment (n = 35) (mean ± SD)	F	p
Pregnancy Stress Rating Scale score	81.74 ± 17.20	92.17 ± 21.61	3.43	0.038[a]
Dimension 1: stress caused by ensuring mother and child health and safety	23.71 ± 6.52	25.00 ± 8.38	1.81	0.171
Dimension 2: stress caused by infant care and changes in family relationships	16.91 ± 4.49	20.40 ± 6.00	4.48	0.015[a]
Dimension 3: stress caused by acknowledging the maternal role	20.14 ± 4.52	21.31 ± 4.33	1.32	0.294
Dimension 4: stress caused by seeking social support	6.57 ± 2.48	7.11 ± 2.96	1.51	0.227
Dimension 5: stress caused by changes in physical appearance and function	14.40 ± 4.76	18.34 ± 6.14	5.15	0.008[a]

[a]p < .05.
SD, standard deviation.

TABLE 4 | Gestational age and pregnancy stress (n = 70).

Pregnancy Stress Rating Scale	Gestational age			
	β	R^2	p	95% Confidence interval
Pregnancy Stress Rating Scale score	0.261	0.068	0.029[a]	0.086–1.52
Dimension 1: stress caused by ensuring mother and child health and safety	0.127	0.016	0.296	–0.130–0.422
Dimension 2: stress caused by infant care and changes in family relationships	0.286	0.082	0.016[a]	0.046–0.442
Dimension 3: stress caused by acknowledging the maternal role	0.101	0.010	0.406	–0.096–0.233
Dimension 4: stress caused by seeking social support	–0.003	0.000	0.978	–0.103–0.100
Dimension 5: stress caused by changes in physical appearance and function	0.392	0.154	0.001[a]	0.151–0.549

[a]p < .05.

3 RESULTS

3.1 Distribution of the Basic Information of Pregnant Women Receiving Tocolytic and Non-tocolytic Treatments

Tocolysis usually occurs in the second trimester. In this study, the mean gestational age of pregnant women receiving tocolytic treatment and those who were receiving non-tocolytic treatment was 27.94 and 38.54 weeks, respectively. Most of the pregnant women were aged 20–34 years, homemaker, married, had a senior high school education or above, absent of religious beliefs, primigravida, and never had a history of tocolysis in previous pregnancy in both the groups. According to the χ^2 test results, the demographic variables were not significantly different between the two groups (**Table 1**).

3.2 Analysis of the Types of Complementary Medicine Used by Pregnant Women Receiving Tocolytic and Non-tocolytic Treatments

The types of complementary medicine used during pregnancy were surveyed using the multiple-choice questionnaire, and the results were calculated according to the number of subjects. Natural products (n = 62, 77.5%) were most commonly used by pregnant women receiving tocolytic treatment, followed by alternative medicine (n = 11, 13.75%), manipulative and body-based practices (n = 4, 5%), and mind and body medicine (n = 3, 3.75%). In pregnant women who were receiving non-tocolytic treatment, natural products (n = 65, 59.1%) were most commonly used, followed by manipulative and body-based practices (n = 18, 16.4%), alternative medicine (n = 17, 15.4%), mind and body medicine (n = 8, 7.3%), and energy therapy (n = 2, 1.8%). The natural products used by these pregnant women mainly consisted of supplements, such as various vitamins, probiotics, and cod liver oil; meanwhile, alternative medicine, manipulative and body-based practices, and mind and body medicine were mainly traditional Chinese medicine, massage, and yoga, respectively. According to the χ2 test results, the types of complementary medicine used during pregnancy were not significantly different between the two groups (**Table 2**; **Figure 1**).

3.3 Comparison of Pregnancy Stress Among Pregnant Women Receiving Tocolytic and Non-tocolytic Treatments Where Both Used Complementary Medicine

The groups were compared by the ANCOVA test to determine the pregnancy stress between pregnant women receiving tocolytic and non-tocolytic treatments where both used complementary medicine. The influence of covariates (i.e. gestational age) was also examined on the pregnancy stress has been adjusted for gestational age as a confounder. The Pregnancy Stress Rating Scale score was significantly (p = 0.038) higher in pregnant women who were receiving non-tocolytic treatment (Mean ± SD: 92.17 ± 21.61) than in their tocolytic (Mean ± SD: 81.74 ± 17.20) counterparts where both used complementary medicine. Dimension 2 (stress caused by infant care and changes in family relationships) between the pregnant women with the tocolytic

group (Mean ± SD: 16.91 ± 4.49) and the non-tocolytic group (Mean ± SD: 20.40 ± 6.00) showed statistically significant differences (p = 0.015). Dimension 5 (stress caused by changes in physical appearance and function) between the pregnant women with the tocolytic (Mean ± SD: 14.40 ± 4.76) and non-tocolytic groups (Mean ± SD: 18.34 ± 6.14) showed statistically significant differences (p = 0.008). Whereas other dimensions, revealed no significant differences (**Table 3)**. Both the groups used complementary medicine, and pregnancy stress was significantly lower in pregnant women who were receiving tocolytic treatment than those who were receiving non-tocolytic treatment.

3.4 Gestational Age and Pregnancy Stress

Linear regression analysis results revealed that the gestational age significantly associated pregnancy stress according to the Pregnancy Stress Rating Scale score (p = 0.029), especially in dimension 2 [stress caused by infant care and changes in family relationships (p = 0.016)] and dimension 5 [stress caused by changes in physical appearance and function (p = 0.001)]. Regarding its predictive ability, gestational age obtained 6.8% for the overall Pregnancy Stress Rating Scale score, 8.2% for dimension 2, and 15.4% (R2) for dimension 5. Thus, dimensions 2 and 5 explained the gestational age associated with stress in those categories (**Table 4**).

4 DISCUSSION/CONCLUSION

The distribution of basic information between the pregnant women receiving tocolytic treatment and those who were receiving non-tocolytic treatment in this study was similar to that in a previous research involving pregnant women in Taiwan (Holst et al., 2009); most of the pregnant women aged 20–34 years, married, had a senior high school education or above, and primigravida. The present study then discussed and compared in the usage of complementary medicine and pregnancy stress between pregnant women receiving tocolytic treatment and those who were receiving non-tocolytic treatment. The findings can serve as future care guidelines and research directions. The insights into whether gestational age, preterm birth risk, and uncertainty can increase pregnancy stress should also be understood in detail. Pregnant women require sufficient time to rest, with a more accurate understanding of current complementary medicine usage during pregnancy through interviews, and environmental interference factors can be reduced.

This study analyzed the types of complementary medicine commonly used by pregnant women (Chiang, 2014; Pallivalappila et al., 2014; Mitchell, 2016) and found five major categories: alternative medicine, natural products, mind and body medicine, energy therapy, and manipulative and body-based practices. The complementary medicine types were reported between the groups of pregnant women receiving tocolytic treatment and those who were receiving non-tocolytic treatment. Both groups most frequently used natural products, the second most commonly used approach was alternative medicine in pregnant women receiving tocolytic treatment, and manipulative and body-based practices in those who were receiving non-tocolytic treatment. The study results clearly demonstrated those pregnancy characteristics among pregnant women, the choice and application of complementary medicine. As Sheraton et al. (2018) reported that women are increasingly using complementary and alternative therapies during pregnancy. This view is worthy of further investigation.

Regarding the use of alternative medicine during pregnancy, traditional Chinese medicine was most commonly used, followed by acupuncture, Chinese herbal medicine, and Tuina, similar to a study in New Zealand (Zheng et al., 2018). Both groups mostly used vitamins, probiotics, and cod liver oil as natural products, similar to previous studies (Johnson et al., 2016; Gilmartin et al., 2018; Zheng et al., 2018). Moreover, yoga and hypnosis were mainly used as mind and body medicine, consistent with previous studies (Chen et al., 2013; Gavin et al., 2020). In this study, pregnant women receiving tocolytic treatment did not use energy therapy, and no literature study has reported the use of energy therapy during pregnancy. In pregnant women who were receiving non-tocolytic treatment, massage was mostly used as a manipulative and body-based practice, similar to previous studies (Hung and Chiang, 2017; Fogarty et al., 2019).

The Pregnancy Stress Rating Scale score, dimension 2 (stress caused by infant care and changes in family relationships), and dimension 5 (stress caused by changes in physical appearance and function) were significantly different between the two patient groups. Although both groups used complementary medicine, the pregnant women receiving tocolytic treatment showed less pregnancy stress than those who were receiving non-tocolytic treatment. Dimension 2, which focuses on stress caused by infant care and changes in family relationships, was significantly lower in pregnant women receiving tocolytic treatment than those who were receiving non-tocolytic treatment. Family relationship changes require important family support to achieve the entire process of adaptation. Therefore, the acceptance of others during pregnancy is very important to the psychology of pregnancy, such as pregnant women receiving tocolytic treatment who just focus on a fetus, hope to ensure the safety of themselves and their fetus (Chen, 2015; Chang et al., 2016).

Dimension 5, which refers to stress caused by changes in physical appearance and function, showed the same finding. The pregnancy stress can be caused by changes in body appearance and function becomes more obvious as the pressure increases during pregnancy (Chen, 2015). Therefore, we hypothesized that as the gestational age advances in pregnant women, their physical appearance changes more significantly. This view is consistent with a previous study (Chang et al., 2016), which reported that stress caused by changes in physical appearance and function appears in early pregnancy and gradually peaks at late pregnancy.

However, the Pregnancy Stress Rating Scale scores for dimension 1 (stress caused by ensuring mother and child health and safety), dimension 3 (stress caused by acknowledging maternal role), and dimension 4 (stress caused by seeking social support) were not significantly different between the two groups. These dimensions are critical for both pregnant women receiving tocolytic and those who were receiving non-

tocolytic treatment, consistent with Rubin's view that maternal tasks develop during pregnancy. Examples for such tasks include ensuring that the mother and fetus can successfully undergo pregnancy and delivery and adapt to maternal roles (Rubin, 1976). This result is consistent with a previous study (Fang et al., 2011). Thus, pregnant women require more social support to adapt to pregnancy. Generally, pregnant women receiving tocolytic treatment have factors that are detrimental to maternal and fetal health, resulting in difficulty in overcoming pregnancy-related stress.

Mental health should be considered during pregnancy (Gong et al., 2020; Martínez-Borba et al., 2020; Rodríguez-Muñoz et al., 2020). The present study revealed pregnancy stress. However, convenience sampling limits the generalizability of the findings. We collected data from only one medical center in central Taiwan. Therefore, generalizing the results to other pregnant women receiving tocolytic and those who were receiving non-tocolytic treatment should be undertaken with caution.

This study provides the following recommendations for future research directions, education, and clinical practice. First, regarding future research, healthcare professionals should thoroughly examine community homes to explore the correlation between pregnant women receiving tocolytic treatment who use complementary medicine and their pregnancy stress. They must ensure that they understand the types of complementary medicine and their effects in such people. Regarding educational recommendations, healthcare professionals should develop knowledge and skills related to complementary medicine to improve their care ability for pregnant women receiving tocolytic treatment. In clinical settings, they can explore and understand how to provide comprehensive care for pregnant women receiving tocolytic treatment and to know the pregnancy stress of pregnant women.

In conclusion, although both pregnant women receiving tocolytic treatment and those who were receiving non-tocolytic treatment used complementary medicine, pregnancy stress was significantly lower in the former than in the latter. This finding can be used as a reference for future studies on pregnant women's health. All findings in this study support the use of complementary medicine at suitable times if pregnant women agree that complementary medicine is beneficial during pregnancy.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the regional ethics committee of the Institutional Review Board of Taichung Veterans General Hospital, Taichung, Taiwan (IRB NO: CE18194A). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

C-YH: conceptualization, formal analysis, resources, writing—original draft preparation, methodology, software, data curation, writing—review and editing, visualization, supervision, and project administration. C-YH, C-LC, L-YT, and J-MT: validation. C-LC: investigation. All authors contributed to the manuscript and approved the submitted version.

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Fetal DNA Causes Sex-Specific Inflammation From Human Fetal Membranes

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Inflammation is central to the mechanisms of parturition, but the lack of understanding of how it is controlled in normal parturition hampers our ability to understand how it may diverge resulting in preterm birth. Cell-free fetal DNA is found in the amniotic fluid, and it is thought to be able to activate inflammation as a danger-associated molecular pattern. Although its levels increases with gestational age, its effect has not been studied on the human fetal membranes. Thus, the aim of this study was to determine if the fetal DNA can trigger inflammation in the human fetal membranes and, thus, potentially contribute to the inflammatory load. Isolated human amniotic epithelial cells and fetal membrane explants were treated apically with fetal DNA causing the translocation of NF-KB into the nucleus of cells and throughout the cells of the explant layers with time. Fetal membrane explants were treated apically with either small or larger fragments of fetal DNA. IL-6, TNFα, and GM-CSF secretion was measured by ELISA, and pro-MMP2 and pro-MMP9 activity was measured by zymography from apical and basal media. Increased apical IL-6 secretion and basal pro-MMP2 activity was seen with small fragments of fetal DNA. When the data were disaggregated based on fetal sex, males had significant increases in IL-6 secretion and basal increased activity in pro-MMP2 and 9, whereas females had significantly increased basal secretion of TNFα. This was caused by the smaller fragments of fetal DNA, whereas the larger fragments did not cause any significant increases. Male fetal DNA had significantly lower percentages of methylation than females. Thus, when the cytokine and pro-MMP activity data were correlated with methylation percentage, IL-6 secretion significantly correlated negatively, whereas GM-CSF secretion positively correlated. These data support the role of fetal DNA as an inflammatory stimulus in the FM, as measured by increased NF-κB translocation, cytokine secretion, and increased pro-MMP activity. However, the data also suggested that the responses are different from FM tissues of male and female fetuses, and both the fragment size and methylation status of the fetal DNA can influence the magnitude and type of molecule secreted.

Keywords: fetal membranes, fetal DNA, inflammation, fetal sex, cytokine, methylation

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INTRODUCTION

Normal pregnancy concludes *via* the integration of several complex parturition pathways that coalesce in the human myometrium, cervix, and fetal membranes (FMs). However, these pathways and the specifics of their interactions are not fully understood. This knowledge gap not only hampers our ability to understand normal parturition mechanisms but also presents a significant barrier to our recognition of how they diverge into those pregnancies that terminate in preterm birth. What is currently clear is that regardless of the gestational age at the onset of parturition, inflammation is critical for its initiation (Rice, 2001). In normal pregnancies, this inflammation leads to changes in several maternal and fetal tissues but specifically results in FM weakening through the proinflammatory mediators that promote the production of cytokines (Kumar et al., 2006), apoptosis (Kumar et al., 2016), and increased matrix metalloproteinase (MMP) activity (Kumar et al., 2018). Indeed, the treatment of human FM, with interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα), and granulocyte-macrophage colony-stimulating factor (GM-CSF), has been shown to directly weaken human FM (Kobayashi et al., 2010; Kumar et al., 2016).

The primary initiator of inflammation has not been established, but stretch (Kendal-Wright, 2007) and other amassing cell stressors have been implicated (Menon et al., 2016a; Sheller et al., 2016). Growing evidence also suggests that endogenous inflammatory mediators such as danger-associated molecular patterns (DAMPs) may contribute to this inflammation and its consequences (Menon et al., 2016b; Padron et al., 2020). Oxidative stress and hypoxia in the placenta cause the production of several DAMPs, including; uric acid, high mobility group box1 (HMGB1), S100 calcium-binding protein A (S100A), S100 calcium binding protein alpha-12 (S100A12), heat shock protein (HSP) 70 kD, and cell-free fetal DNA (cffDNA) (Baker et al., 2021).

Although little is known about the effects of cffDNA on the human FM, fetal DNA is readily found in the amniotic fluid (AF) where it is at much higher concentrations than in maternal plasma (Bianchi et al., 2001). The results from several studies show that there is no correlation between the amount of DNA in the AF and the maternal plasma, indicating that this source of fetal DNA is physiologically independent of the cffDNA circulating in the maternal vascular system (Hui and Bianchi, 2011). This is because the primary source of the nucleic acids in the AF is from the fetus (Larrabee et al., 2005) rather than from the placental trophoblast. However, they are both of fetal origin. Interestingly, the fetal DNA levels are high in the AF of premature preterm rupture of the membrane pregnancies with those who have a microbial-associated intrauterine infection (IAI) having the highest levels (Kacerovsky et al., 2018). On average, the size of the AF DNA is <200 bp, and it is thought to be more fragmented than that found in the maternal circulation (Burnham et al., 2020) but the methylation status can vary. This form of AF DNA is understood because its transcriptome characterization can be used to detect fetal genetic abnormalities. It is a useful, but an invasive diagnostic tool. Not only is it affected in normal pregnancies by gestational age, fetal maturity, and fetal sex, but also by pathologic states such as maternal obesity and various genetic syndromes (Park et al., 2021). The pregnancy complications of preeclampsia (Jung et al., 2019), intrauterine growth restriction (Cho et al., 2018) and preterm birth (Bhatti et al., 2021) have also been shown to be detectable through the AF transcriptome.

cffDNA released from the apoptotic and necrotic cells of placental origin into the maternal circulation has been studied more extensively (Kazemi et al., 2021). These DNA fragments are <313 bp (Sin et al., 2020) in length and present within the maternal circulation as early as 35 days gestation. They have been shown to increase throughout the pregnancy, peaking at term and then dropping after birth (Liu et al., 2007). Indeed, this source of cffDNA is also used for prenatal diagnostic detection of fetal chromosomal abnormalities (Wang et al., 2021) as it is less invasive than sampling from the AF. Increased circulating levels of cffDNA have also been associated with adverse pregnancy outcomes and pathologies such as; preeclampsia (Martin et al., 2014; Kwak et al., 2020), gestational diabetes (Hopkins et al., 2020), and preterm birth (Gomez-Lopez et al., 2020). cffDNA from the trophoblast is also known to increase by sterile inflammation, *via* HMGB1 (Yaganeh Kazemi et al., 2021), but its ability to generate inflammation itself is contentious. Some studies have clearly shown that it is able to interact with toll-like receptor 9 (TLR9) (Goldfarb et al., 2018) and that immune cells respond to it by producing inflammatory cytokines (Yeganeh Kazemi et al., 2021). However, others have not been able to confirm this (van Boeckel et al., 2020).

Like placental cffDNA, it is thought that fetal DNA in the AF could activate TLR9. Although much of the evidence for this comes from the work showing that mouse FM release cffDNA that is hypomethylated, can increase IL-6 through TLR9 *in vitro* (Sawyer et al., 2018). Interestingly, TLR9 is highly expressed in the cells of the human amnion (Sato et al., 2016). Thus, it is possible that in humans hypomethylated regions of fetal DNA bind to TLR9 expressed in the amnion to elicit an inflammatory signal that may contribute to the initiation of parturition. Therefore, the aim of this study was to determine if fetal DNA, similar to that which would be obtained from AF, can trigger inflammation in the human FM and thus potentially contribute to the inflammatory load central to the normal mechanisms of parturition.

MATERIALS AND METHODS

Tissue Collection and Amnion Cell Culture

Term (≥38 week's gestation) fetal membranes were collected from singleton, repeat Cesarean sections at Kapi'olani Medical Center for Women and Children (Honolulu, HI, United States) with approval from the Institutional Review Board (Hawaii Pacific Health). The reflected fetal membranes were isolated 1 inch from the placenta and washed in sterile phosphate-buffered saline (PBS) within 30 min of collection to remove blood. The primary amnion epithelial cells (AEC) were isolated as previously

described (Kendal-Wright et al., 2010). Briefly, the primary amnion was stripped from the choriodecidua fetal membrane layers, and the amnion epithelial cells were subjected to four consecutive trypsin (0.2%) digestions (Gibco, Waltham, MA, United States) for 30 min at 37°C at 150 rpm. AECs were cultured in DMEM/F12 media (Gibco, Waltham, MA, United States) containing 10% fetal bovine serum (FBS, Gibco, Waltham, MA, United States), penicillin (100 U/mL), streptomycin (100 µg/ml) and incubated at 37°C in 95% air/5% CO_2. AECs were utilized without passage upon reaching 90% confluence.

Primary Explant System and Fetal DNA Treatment

The full thickness amnion choriodecidua fetal membrane integrity was assessed to ensure all layers were intact with no separation from other layers. The membranes were rinsed in sterile PBS and then cut into 2.5 cm^2 × 2.5 cm^2 pieces and placed onto sterilized Transwell frames (Corning Inc., Corning, NY, United States) without synthetic membrane. The two-compartment system was created by placing an elastic latex dental band around the tissue (Astern et al., 2013). The amnion side of the fetal membrane tissue faced apical to the Transwell insert creating the inner, upper well, while the decidua layer faced outward creating a completely separate, outer, lower well. Each mounted fetal membrane was placed in a single well within a 12-well tissue culture plate with DMEM/F12 medium on both sides of the membranes to allow equilibration for 24 h. The culture medium was removed and the apical side (upper well) of the fetal membrane compartment was treated with 100 ng/ml cffDNA in 500 µl DMEM/F12 media and the basal side (lower well) was supplemented with only 1.5 ml DMEM/F12 media for 24 h 1000 ng/ml of lipopolysaccharide (LPS, Sigma Aldrich, St. Louis, MO, United States) was also used to treat the apical well of the fetal membrane compartment system. The condition medium from the top and bottom wells were collected and centrifuged for 10 mins, 10,000 x g at 4°C to remove cell debris. The fetal membrane explant was removed from the Transwell apparatus and placed in 10% formalin (Epredia, Kalamazoo, MI, United States) for 24 h and then stored in sterile PBS until further analysis.

DNA Isolation From Amnion Epithelial Cells, Sonication, and Verification

DNA isolation was performed using the QIAamp DNA Mini isolation kit (Qiagen, Hilden, Germany) from isolated primary AECs following the manufacturer's instructions. DNA was quantified using a NanoDrop (Thermo Fisher Scientific, Waltham, MA, United States). To create smaller fragments of cffDNA that would be more similar to those seen in AF, it was sonicated after dilution to 10 ng/µl in AE buffer (Qiagen, Hilden, Germany) for 4 min within an ultra sonicating water bath (PS-20A, Vevor, Shanghai, China). cffDNA fragment size of the whole (non-sonicated) and sonicated samples were verified on a 2% agarose gel with ethidium bromide (Invitrogen, Waltham, MA, United States) and visualized under UV transillumination using a Chemidoc (Bio-Rad, Hercules, CA, United States).

Immunocytochemistry

Primary isolated AECs (100,000 cells/well) were seeded into 4-well chamber slides and grown to 80% confluency. The AECs were treated with 0, 1, 10, 100, and 1,000 ng/ml 4 min sonicated cffDNA or 1,000 ng/ml of lipopolysaccharide (LPS). After treatment, the AECs were fixed with 4% paraformaldehyde (PFA) (Sigma Aldrich, St. Louis, MO, United States) in 1X PBS for 15 mins, followed by two washes of 1X PBS. Non-specific binding was blocked with 5% bovine serum albumin (BSA) in PBS for 1 h and subsequent incubation with 1:500 rabbit polyclonal anti-NF-kκB p65 antibody (06-418, Sigma Aldrich, St. Louis, MO, United States) incubation in 1% BSA in PBS for 1 h at room temperature. Secondary antibody Alexa flur-488 anti-rabbit at 1:2000 (Life Technologies, Waltham, MA, United States) incubation were performed for 1 h at room temperature. The cells were then washed with PBS and counterstained with DAPI at 1:5000 (Calbiochem, Billerica, MA, United States) for 5 min. The slides were mounted with ClearMount with Tris buffer (Electron Microscopy Sciences, Hatfield, PA, United States) before imaging with confocal microscopy (Nikon C1 Plus Ti Eclipse epi-fluorescence). The effects of treatment with cffDNA and LPS were for 30 mins and were measured as percent NF-κB p65 nuclear translocation, and quantified using immunofluorescent imaging.

Immunohistochemistry

The fetal membrane tissues that were subjected to 30 mins, 1 h, and 2 h of control (no DNA), whole (non-sonicated), and sonicated cffDNA treatment were placed in 10% formalin for 24 h before being stored in sterile PBS. The fetal membranes were embedded in paraffin and cut tissue sections (5 µM) mounted on charged microscope slides. Immunohistochemistry was performed according to the Vectastain ABC immunoperoxidase staining manufacturer's protocol (n = 8) using 1:100 rabbit polyclonal NF-κB p65 primary antibody (06-418, Sigma Aldrich, St. Louis, MO, United States) and the 3,3′-Diaminobenzidine (DAB) peroxidase HRP substrate (Vector Laboratories, Burlingham, CA, United States). The sections were then re-dehydrated and mounted with Permount (Fisher Chemicals, Pittsburg, PA, United States).

IL-6, TNF-α, and GM-CSF ELISA

All cytokine secretion was assessed from condition media samples collected from the top and bottom well of the Transwell explant system. The Human IL-6, TNF-α, and GM-CSF Quantikine ELISA Kits (R&D Systems, Minneapolis, MN, United States), were all used following the manufacturer's protocol. The secreted protein levels were normalized to total protein concentrations for each sample, measured by protein assay compatible with conditioned media (Pierce, Waltham, MA, United States).

Zymography

The condition media collected from the top and bottom wells of the fetal membrane explant system were assessed for pro-matrix metalloproteinase-2/9 (pro-MMP2, pro-MMP9) activity. The condition media (5 μg) samples were mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) sample loading buffer. The samples were run on a 10% SDS PAGE gel containing 1 mg/ml gelatin and separated by electrophoresis. The resolving gel was incubated in an assay buffer (40 mM Tris, 0.2 M NaCl, 10 mM CaCl2, 0.1 μM zinc chloride, pH 8.8) overnight at 37°C in a shaking incubator. After incubation, the transfer gels were exposed to a staining buffer (methanol, acetic acid, dH2O, and Coomassie blue) at room temperature while shaking for 45 min. They were then rinsed with dH2O until excess staining solution was removed. The gels were incubated with destaining solution (methanol, acetic acid, and dH2O) until the bands could clearly be seen. The clear bands on the gel indicated the area of enzyme activity. The clear bands on the gel were quantified using ImageJ (Rasband, 2018).

DNA Sex Determination

Polymerase chain reaction (PCR) based on sex determination was performed to identify the presence of sex region y gene (SRY) for males or alanine aminotransferase-1 gene (ALT1) for females. The sequences of primers for SRY were 5′-CATGAACGCATT CATCGTGTGGTC-3′, 5′-CTGCGGGAAGCAAACTGCAAT TCT T-3′ and 5′-CCCTGATGAAGAACTTGTATCTC-3', and 5′-GAAATTACACACATAGGTGGCACT-3′ for ATL1 (Settin et al., 2008). Each PCR reaction comprised 100 ng DNA, 1X PCR buffer minus Mg, 0.2 mM dNTP mixture, 1.5 nM MgCl2, 0.5 μM SRY and ALT1 primers (FWD and REV), and 2.5 units Taq DNA Polymerase (5 U/μl) (Invitrogen, Waltham MA, United States). All PCR reactions were performed in a thermal cycler (PTC-225, Peltier Thermal Cycler, MJ Research, NH, United States) at 94°C (3 min) for initial DNA denaturation, followed by 35 cycles of 94°C (15 s) for DNA denaturation, 55°C (30 s) for primer annealing and 72°C (90 s) for primer extension, with a final extension of the cycle at 72°C (10 min). The amplified PCR products were separated on a 2.5% agarose gel with ethidium bromide and imaged under UV transillumination. The product size of SRY was 254 bp for the Y chromosome and ALT1 was 300 bp for the X chromosome. Two product bands at 254 bp and 300 bp identified male samples and one band at 300 bp female samples.

Methylation Status Quantification

The percent DNA methylation status of cffDNA samples was calculated and quantified using MethylFlash Global DNA Methylation (5-mC) ELISA Easy Kit (colorimetric) according to the manufacturer's protocol (Epigentek, Farmingdale, NY, United States).

Statistical Analysis

All statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, United States). The data throughout the figures are expressed as ± standard error of the mean (SEM). All statistical comparisons between the groups were identified by using one-way ANOVA analysis followed by Bonferroni's multiple comparison tests or by paired t-tests, appropriately. The correlation statistics were performed using Pearson's correlation coefficient calculation. The differences $*p < 0.05$ and $**p < 0.01$ were considered statistically significant.

RESULTS

Sonication Breaks Cell-Free Fetal DNA Into Smaller Fragments but Does Not Decrease the Percentage of Methylation of the DNA

In order to study the potential of fetal DNA to stimulate inflammation in the human FM, we first sought to obtain DNA fragments more consistent with the sizes of DNA fragments found in AF. The non-sonicated whole DNA (w.cffDNA) freshly isolated DNA from AEC contained much larger >1000 bp fragments (**Figure 1A**). While the DNA that was sonicated for 4 min (4 m.s cffDNA) broke into fragments that ranged from 100 to 1000 bp. As it has also been shown *in utero* that DNA fragments released from cells *via* apoptosis or necrosis are often hypomethylated (Gordevičius et al., 2020), we assessed the methylation status of the cffDNA. The methylation ranged from 0.1% to 0.4% (**Figure 1B**) but this did not decrease after 4 min of sonication as no statistical difference in the percentages of methylation was seen when the DNA was sheared into smaller fragments (variation between the samples before and after sonication ± 5%–10%, which was within the range of assay variation for technical repeat samples).

Fetal DNA Causes the Nuclear Translocation of the NFκB p65 Subunit in Both Isolated Human Amnion Epithelial Cells and in Cells Throughout the Layers of Fetal Membrane Explants

Following the isolation and characterization of fetal DNA, we treated isolated primary human AECs with 4 m.s cffDNA to determine if it would cause the translocation of the inflammatory transcription factor, NFκB. After immunocytochemistry to visualize the NFκB p65 subunit (**Figure 2A**), the nuclear location was clearly seen at low levels in untreated cells (22.91%). However, after only 30 min of treatment with 4 m.s cffDNA, quantitation of the NFκB p65 subunit nuclear translocation (**Figures 2A,B**) after treatment with the higher concentrations of cffDNA (100 and 1,000 ng/ml) was 29.16% and 43.75% respectively (**Figure 2B**). This response was similar to that seen with 100 ng/ml of LPS (34.37%).

As we detected an increase in the nuclear translocation of NFκB p65 within individual isolated AECs after treatment with 4 m.s cffDNA, we treated human FM explants held in transwell holders with 100 ng/ml of 4 m.s cffDNA just to the apical side of the explant. This was because we wanted to determine specifically if the apical treatment of the FM explant with the 4 m.s cffDNA, would be able to propagate a signal from the amnion surface of the FM to the underlying cells over time. This strategy was

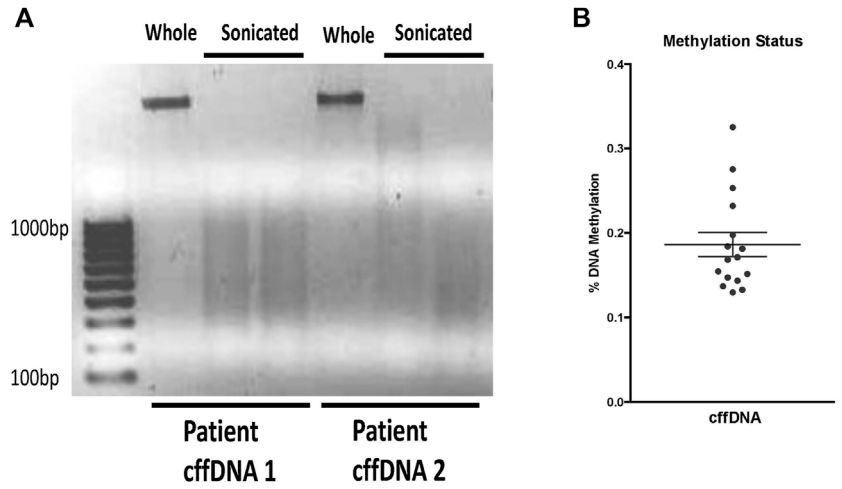


FIGURE 1 | Characterization of fetal DNA fragments isolated from AECs. **(A)** Ethidium bromide stained gel of DNA either; whole—the cffDNA upon immediate isolation from primary human amnion epithelial cells or, sonicated—cffDNA isolated from primary human amnion epithelial cells but sonicated for 4 min. Two different patient samples of fetal DNA are shown as examples. **(B)** Quantification of methylation percentage of DNA from the individual patients' collected FM samples. Each dot indicates separate patients' DNA $n = 16$.

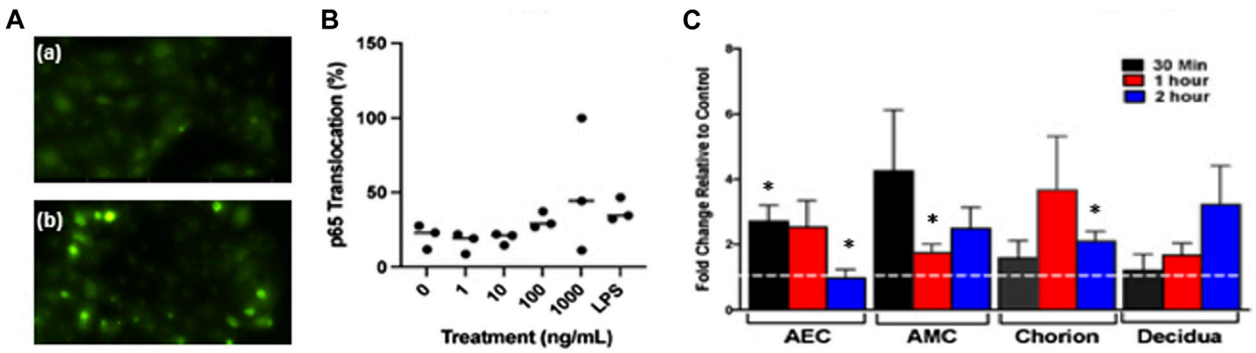


FIGURE 2 | Fetal DNA causes the translocation of the NF-κB subunit p65. **(A)** Immunocytochemistry of the nuclear translocation of p65 **(a)** not treated with cffDNA and **(b)** treated with 100 ng/ml of 4 m.s. cffDNA. Green—p65. **(B)** Quantitation of the percentage of p65 translocation in AECs treated with 1, 10, 100, and 1,000 ng/ml 4 m.s. cffDNA or LPS 1000 ng/ml for 30 min $n = 3$ AEC from different patients' isolated cells. **(C)** Fetal membrane explants treated only apically with 100 ng/ml 4 m.s. cffDNA for 30 mins, 1, and 2 h. The dotted white line represents untreated control, AEC—amnion epithelial cells. AMC—amnion mesenchymal cells. The data displayed as fold change from no treatment control. ($n = 3$ different patients' fetal membrane explants). *$p = < 0.05$.

conceived as the fetal DNA source we were seeking to study was that which would originate in the AF. An increase in the translocation of nuclear NFκB p65 (compared to no treatment control) observed after only 30 min of apical treatment of the FM explant was statistically significant in the AEC (171% $p = 0.023$) (**Figure 2C**). This translocation was sustained over 1 h (153.23% $p = 0.05$) compared to no treatment control but after 2 h had returned to untreated explant control levels. Although the 4 m.s cffDNA was only applied to the AEC (the apical side of the FM explant), translocation was also seen in the amnion mesenchymal cells (AMC) after 30 min (324.98%). This was reduced after 1 h (74.88% $p = 0.041$) but remained higher than baseline for the remainder of the 2 h tested (149.06% $p = 0.07$) (**Figure 2C**). In addition, the cells of the chorion under the AMC also showed translocation of NFκB p65 compared to the untreated explants but this appeared to take 1 h to reach levels higher than that seen at baseline (267.73%). This then began to return toward untreated levels after 2 h but due to low interpatient variation was a statistically significant difference (110.24% $p = 0.02$ compared to untreated control). Finally, p65 nuclear translocation at levels higher than the untreated explants was also seen in the cells of the decidua (under the chorion) but this only became apparent after 2 h and this increase did not reach significance (222.06%) of treatment (**Figure 2C**). Thus, a significant increase in NF-κB p65 nuclear translocation was seen in all of the layers of the FM except the decidua, (although this may have occurred if the FM were treated for longer times), appearing to take longer for the translocation to peak the further away from the treated surface the cells were in the FM explant model (**Figure 2C**).

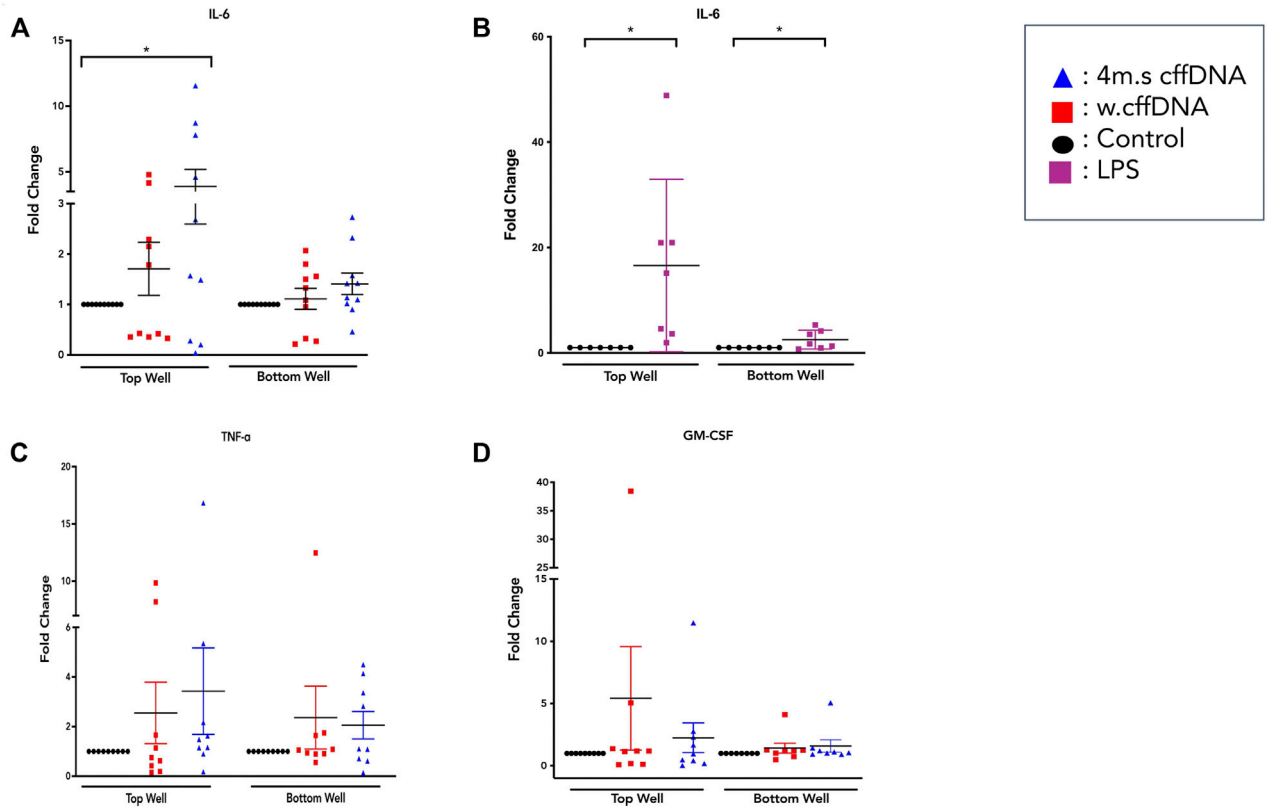


FIGURE 3 | Cytokine secretion from fetal membrane explants after treatment with w.cffDNA or 4 m.s cffDNA. Fold change secretion, compared to no treatment control, of cytokines into the apical (top) well or basal (bottom) well of the transwell fetal membrane explant after treatment with (red) w.cffDNA or (blue) 4 m.s. cffDNA at 100 ng/m. **(A)** IL-6, **(B)** IL-6 after treatment with LPS (100 ng/ml), **(C)** TNF-α, and **(D)** GM-CSF. n = 7–10 patients. *p = < 0.05.

Effect of Fetal DNA on the Secretion of Proinflammatory Cytokines and Matrix Metalloproteinases From Fetal Membrane Explants

As we were able to see an increase in the translocation of NF-κB p65 to more nuclei in cells from the amnion and cells throughout our FM explant model after treatment with cffDNA, we tested if this stimulus was also able to increase the secretion of cytokines that are important for FM weakening at the end of gestation. However, first we measured the levels of activity of lactate dehydrogenase from the media from the apical and basal wells of our explant, over the total 48 h time taken for our experiment. This was performed to test the health of the tissue from collection to the end of treatment with fetal DNA. No changes in the level of lactate dehydrogenase were seen over the 48 h, or due to the addition of fetal DNA (data not shown). Despite the FM explant being treated only on the apical side of the explant with fetal DNA for 24 h, IL-6 secretion was increased into both apical and basal compartments with both 4 m.s.cffDNA (fold change 3.05 apical, 1.39 basal, respectively) and w. cffDNA (fold change 1.70 apical, 1.13 basal, respectively) (**Figure 3A**). However, the increase in secretion only reached statistical significance in the apical wells treated with 4 m.s cffDNA (p = 0.038) compared to the untreated control (**Figure 3A**). Although the NF-κB p65 translocation response seen in the cells of the amnion was similar for both the cffDNA and LPS, the IL-6 secretion following the treatment with LPS treatment caused a much higher increase in IL-6 secretion than that seen with cffDNA (fold change, apical 16.55, basal 2.52, p = 0.02 and p = 0.04, respectively) (**Figure 3B**). It was interesting to note that the LPS only caused a small increase in IL-6 secretion into the basal compared to the apical media compartment. A non-significant increase in TNF-α secretion level was seen after treatment with both 4 m.s (fold change apical 3.43, basal 2.05) and w. cffDNA (fold change apical 2.55, basal 2.36) into the apical and basal media (**Figure 3C**). Non-significant increases in GM-CSF secretion (fold change apical 2.25, basal 1.59) were also observed (**Figure 3D**).

Because our data showed that the fetal DNA was able to increase the activation of NF-κB and increase the secretion of some of its downstream cytokines, we also wanted to determine whether it was able to increase the levels of the enzymes that are crucial to the degradation of the extracellular matrix. Our MMP activity assay data showed that some patients' explants did show non-significant increases in activity of pro-MMP9 (**Figure 4A,B**), especially into the apical well. The results for pro-MMP2 (**Figure 4C**) showed

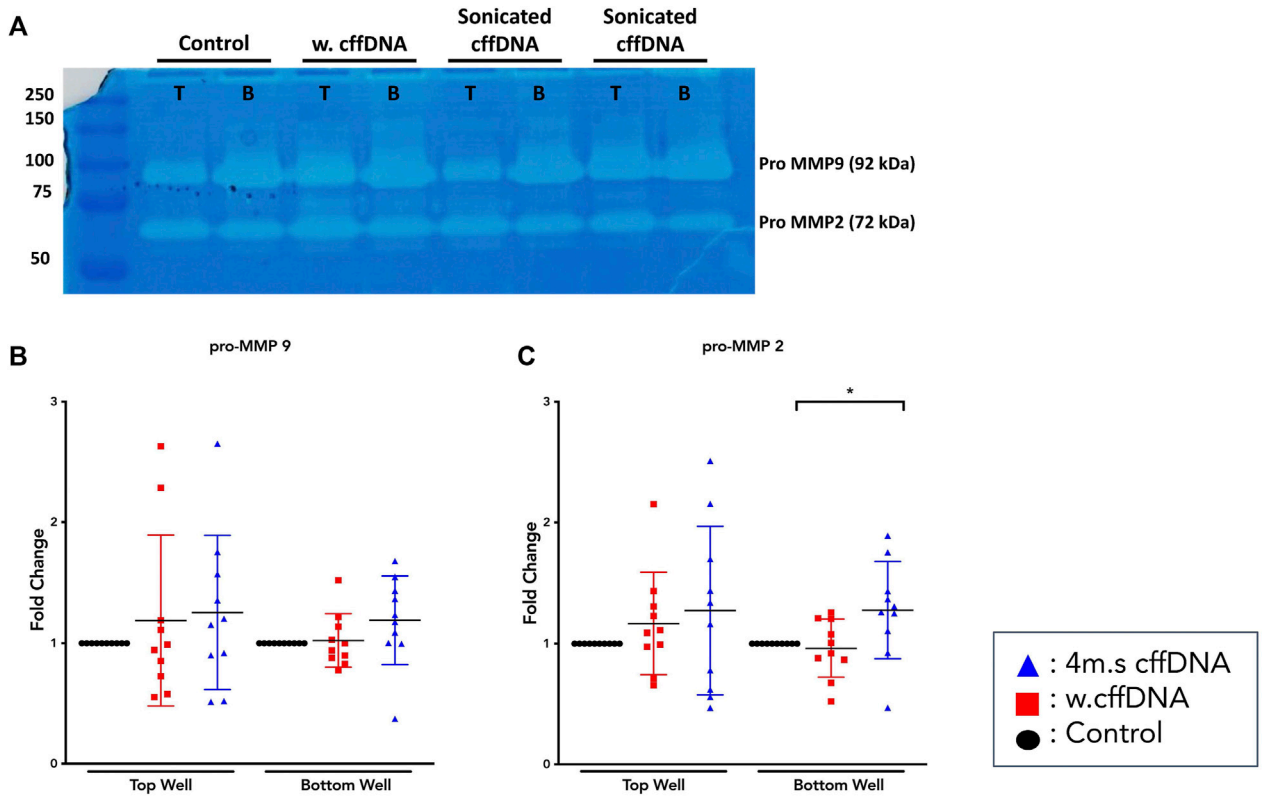


FIGURE 4 | Matrix metalloproteinase activity from fetal membrane explants after treatment with w.cffDNA or 4 m.s. cffDNA. The fold change activity, compared to no treatment control, of MMPs from the apical (top) well or basal (bottom) well of the transwell fetal membrane explant after treatment with (red) w.cffDNA or (blue) 4 m.s cffDNA at 100 ng/ml. **(A)** Example zymography gel. T = apical, B = basal, **(B)** Pro-MMP9, and **(C)** pro-MMP2. *n* = 10 patients. *p = < 0.05.

a more consistent increase in activity after all fetal DNA treatment conditions, reaching significance after apical 4 m.s cffDNA treatment (fold change 1.27 p = 0.04) of the enzyme into the basal media compartment.

The Effect of Fetal Sex on the Fetal Membrane Explant Responses to cffDNA

The data from the treated FM explants showed large interpatient variation throughout the measurement of cytokine secretion and MMP activity. Therefore, as it is known that fetal sex can alter the inflammatory response (Mitchell et al., 2017; Velten et al., 2018; Allard et al., 2019) we determined the sex of each fetus for the tissues we used for this series of experiments. The males were identified by the dual expression of SRY and ALT1 expression, whereas the females only expressed the X-linked SRY gene (**Figure 5A**). As the response to the cffDNA that had been sonicated for 4 min appeared to be the more robust for several of our targets of interest, we first analyzed this data by separating the male and female responses (**Figures 5B–F**) and then repeated this analysis strategy for the w. cffDNA (**Figures 6A–E**), to uncover any sex-specific patterns of response.

Overall, it was clear that the FM explants from pregnancies with a male fetus had a larger cytokine response than those with females to 4 m.s cffDNA; secreting more IL-6 into both the apical (fold change 5.38, p = 0.041) and basal (fold change 1.28, p = 0.011) wells (**Figure 5B**). Similar to IL-6, the males also appeared to have more of an increase in GM-CSF secretion into both apical (fold change 3.27) and basal (fold change 2.16) wells but this did not reach significance (**Figure 5D**). When the data was reviewed based on sex for the pro-MMP activity, both activities of pro-MMP9 and pro-MMP2 were statistically significantly higher in males (fold change pro-MMP9 1.28, pro-MMP2 1.37; p = 0.02 and p = 0.03) from the basal wells (**Figures 5E,F**). Interestingly, this was not the case for TNF-α, as although some of the males clearly increased their secretion of this cytokine, especially apically, only the FM explants from female fetuses significantly (fold change 3.15; p = 0.04) increased their TNF-α secretion into the basal well (**Figure 5C**). When the data was then analyzed from the FM explants treated with w. cffDNA, no significant differences were seen for males or females, for any of the cytokines secreted, or for the activity levels of the pro-MMPs (**Figure 6**). However, several of the targets were (non-significantly) increased from the male FM explants, particularly those measured from the apical wells. Thus, collectively this analysis of the data separated by fetal sex (**Figures 5**, **6**) illustrated that the male and female FM tissues responded differently.

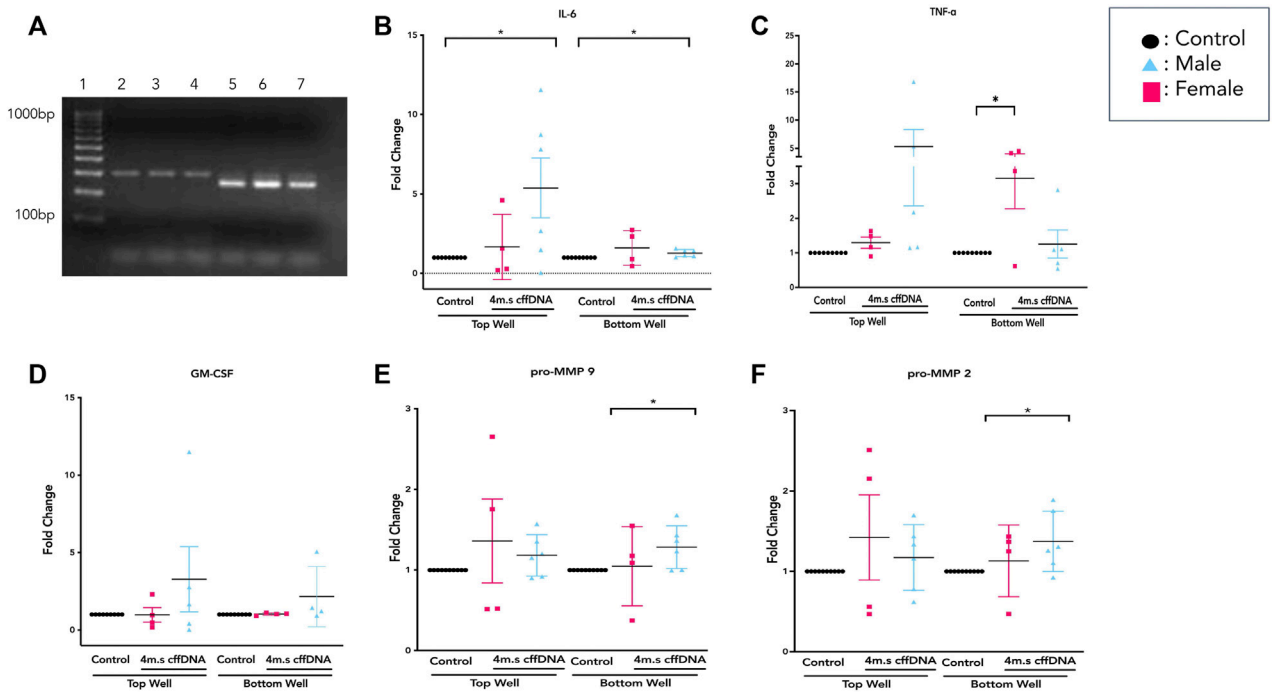


FIGURE 5 | Sex-specific response of FM explants to 4 m.s cffDNA. **(A)** Example of PCR product gel shows that in lanes 2–4 the expression of SRY indicates a female fetus, whereas the expression of both SRY and ALT1 in lanes 5–7 indicated a male fetus. The fold change secretion, compared to no treatment control, of cytokines into the apical (top) well or basal (bottom) well of the transwell fetal membrane explant after treatment with 4 m.s cffDNA at 100 ng/ml. **(B)** IL-6, **(C)** TNF-alpha, and **(D)** GM-CSF **(E)** pro-MMP9 **(F)** pro-MMP2. Black—untreated control. Blue- male fetus, and pink—female fetus. n = 7–10 patients. *p = < 0.05 **p = < 0.001.

The Effect of the Level of Methylation on the Fetal Membrane Explant Response to cffDNA

The initial characterization of the sonicated and non-sonicated fetal DNA (**Figure 1**) showed that there was some variation in the percentages of methylation from different patients. Thus, we also sought to evaluate the influence of the percentage of methylation on our treatment response to fetal DNA. We wanted to determine if this would affect the FM response, but also to see if any difference would, in part, explain the variation between the male and female responses.

When the percentage of methylation for all of the male fetal DNA was compared to the female fetal DNA, they had a significantly lower (p = 0.040) percentage of methylation (0.16%, 0.24% respectively) (**Figure 7A**). Next, we took our cytokine secretion and our pro-MMP activity data and for each sample correlated the response with the percentage of methylation. We looked at a number of correlations with the percentage methylation; the specific target of interest in the apical or the basal well irrespective of fetal DNA type, the target in the apical or basal also split by fetal DNA type (w.cffDNA or 4 m.s. cffDNA), the target in the apical or basal divided into male and female and also the cffDNA type. A complete record of all correlations performed, regardless of statistical significance, is documented in **Supplementary Data Table S1**. When we correlated the IL-6 secretion into the apical well, regardless of cffDNA type or fetal sex (**Figure 7B**) we found a significant negative correlation with the percentage of methylation (p = 0.019). In addition, we found that there was a strong negative correlation for IL-6 secreted into the apical well (**Figure 7B**) with w. cffDNA (p = 0.09) and that when this was analyzed for just the male fetus FM with 4 m.s cffDNA (**Figure 7C**) it was statistically significant (p = 0.004). Thus, when the percentage of methylation is lower, more IL-6 was secreted into the apical well, particularly from the male FM when treated with 4 m.s cffDNA (**Figures 7B,C**).

Interestingly, the other cytokine that correlated with the percentage of methylation was GM-CSF (**Figure 7D**). When the data were combined for both types of cffDNA and both fetal sexes (**Figure 7E**), a positive correlation was seen for GM-CSF in the basal well (p = 0.00012). In addition, when the cffDNA type was separated, both the 4 m.s (**Figure 7E**) and w. cffDNA (**Figure 7E**) treatments caused GM-CSF secretion into the basal well that positively correlated with the percentage of methylation (p = 0.018 and p = 0.004 respectively). Finally, when the data was separated into the two sexes and the cffDNA type, a strong correlation was seen for the female FM explant (**Figure 7E**) secretion of GM-CSF into the basal well with w.cffDNA (p = 0.08). Thus, when the percentage of methylation is higher, more GM-CSF is secreted into the basal well, which may be stronger than the female FM treated with w.cffDNA (**Figure 7E**).

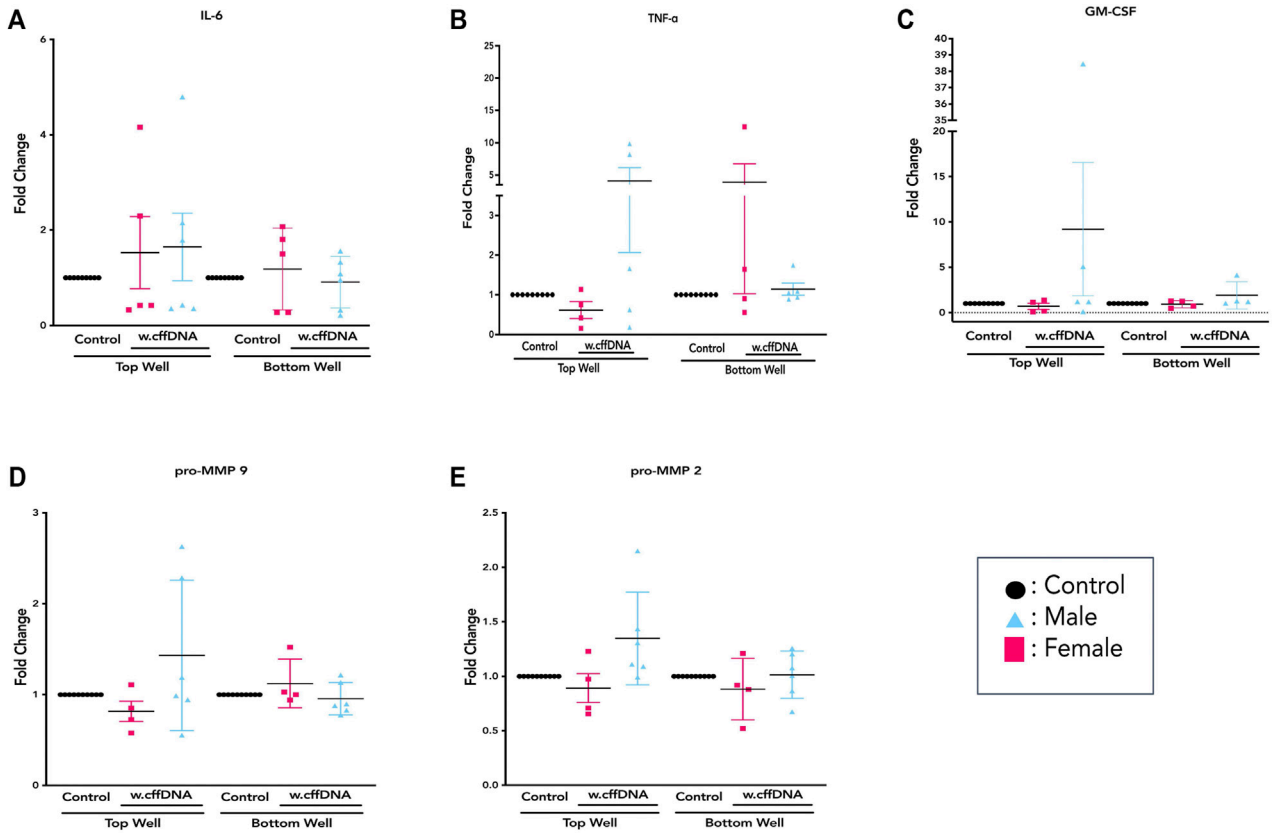
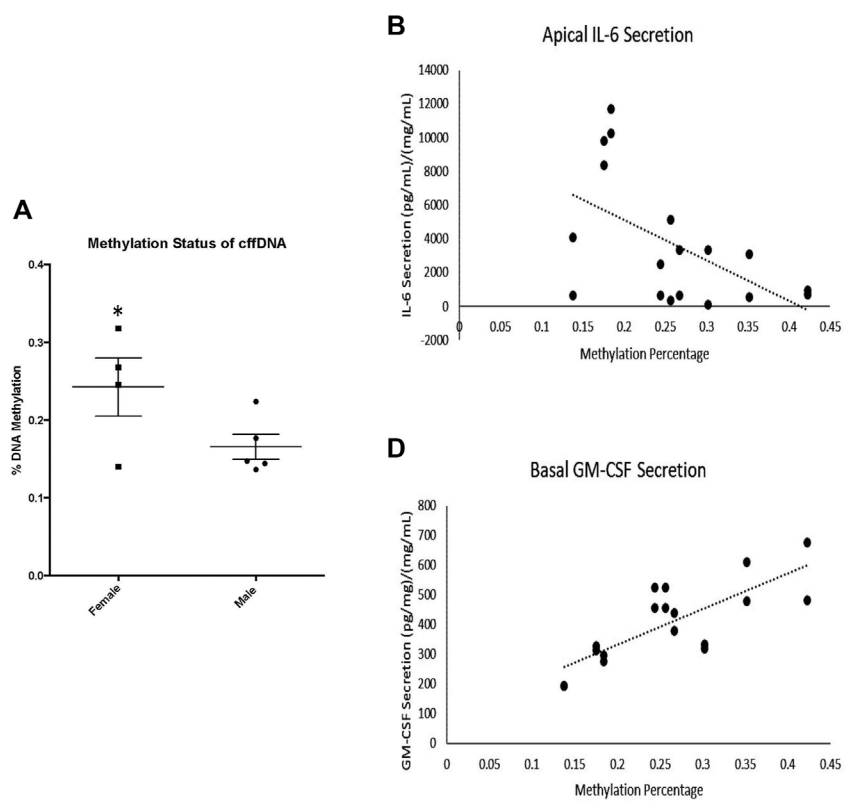


FIGURE 6 | Sex-specific response of FM explants to w.cffDNA. The fold change secretion, compared to no treatment control, of cytokines into the apical (top) well or basal (bottom) well of the transwell fetal membrane explant after treatment with w.cffDNA at 100 ng/ml. **(A)** IL-6, **(B)** TNF-alpha, and **(C)** GM-CSF **(D)** pro-MMP9 **(E)** pro-MMP2. Black—untreated control. Blue- male fetus, and pink—female fetus. n = 7–10 patients. $^{*}p$ = < 0.05 $^{**}p$ = < 0.001.

DISCUSSION

It is known that cffDNA can stimulate inflammation in several tissues and cell types (Goldfarb et al., 2018; Kazemi et al., 2021; Yeganeh Kazemi et al., 2021) but its effects have not yet been studied on the human FM. In addition to this, it is also known that the sex of the fetus can influence both the magnitude and the resultant cytokine profile of the inflammatory response, depending on the specific circumstances (Burns et al., 2015; Mitchell et al., 2017). Therefore, as the AF's concentration of fetal DNA increases with gestational age (Park et al., 2021), we sought to understand its effect(s) on the human FM. Together our data support the role of fetal DNA as an inflammatory stimulus in the FM, as measured by increased NF-κB translocation and cytokine secretion. However, our data also suggest that this response is different in FM tissues from male and female fetus pregnancies and that both the fragment size and methylation status of the fetal DNA could influence the magnitude and type of molecule secreted.

The proinflammatory transcription factor NF-κB has been shown to be important to drive inflammatory cascades at the end of pregnancies in the myometrium, cervix, and FM (Gomez-Chavez et al., 2021). Thus, it is considered central to what understand about the progression of normal parturition. Interestingly, the NF-κB dimer can be activated by many different receptors signaling cascades that result in the phosphorylation of its inhibitory protein IκB (Schiedereit, 2006), this activated dimer then causes the transcription of a whole cadre of cytokines (Lui et al., 2017) that are key for parturition including; IL-1β, IL-6, IL-8, TNFα, and IFN-γ (Burns et al., 2015). The consequence of this is that NF-κB is able to influence a wide range of cellular pathways, in addition to inflammation, including cell survival, proliferation, and angiogenesis (Lui et al., 2017). Our data clearly shows the propagation of the translocation of its p65 subunit to the nucleus in cells through the FM with time (**Figures 2**, **8**). This illustrates the potential capacity of stimulating factors in the AF to activate this transcription factor, leading to wide-ranging downstream consequences. Interestingly, this means that factors in the AF could also readily lead to changes below the AEC, affecting the ability of AMC to regulate the ECM in the amnion and hence its strength. They could also influence the behavior of the chorion and decidua in a variety of ways. Our data in the layers of the FM also demonstrated the known trafficking behavior of this transcription factor; as it quickly translocates to the nucleus when its inhibitory protein (IκB) is phosphorylated, and then out again, as this inhibitor is *de novo* synthesized. This results in pulsatile waves of activation and deactivation of this



C

Conditions correlated with methylation	R^2	p-Value
Apical IL-6 Secretion (w.cffDNA)	0.35	0.09
Apical Male IL-6 Secretion (4m.s cffDNA)	0.95	0.004

E

Conditions correlated with methylation	R^2	p-Value
Basal GM-CSF Secretion (w.cffDNA)	0.76	0.004
Female Basal GM-CSF Secretion (w.cffDNA)	0.84	0.08
Basal GM-CSF Secretion (4m.s cffDNA)	0.63	0.018

FIGURE 7 | Fetal membrane response to fetal DNA relative to methylation percentage. **(A)** Percentage methylation of male and female fetal DNA. The correlation of cytokine secretion from FM correlated with percentage methylation of fetal DNA used for apical treatment. **(B)** Apical secretion of IL-6 regardless of fetal DNA fragment size. **(C)** Table illustrating the correlation between the percentage of methylation and the apical secretion of IL-6 with w.cffDNA; the apical secretion of IL-6 from the male explants with 4 m.s cffDNA. **(D)** Basal GM-CSF secretion regardless of fetal DNA size is used for treatment. **(E)** Table illustrating the correlation between the percentage of methylation and the basal secretion of GM-CSF of FM treated with w. cffDNA; the basal GM-CSF secretion of FM treated with 4 m.s. cffDNA; the basal secretion of GM-CSF from the female explants treated with w.cffDNA.

transcription factor (Klinke et al., 2008). This propagation of signaling through the tissue is also supported by the detection of increased levels of the cytokines, IL-6 and TNFα and both pro-MMP 2 and pro-MMP 9, in the basal compartment explant media (**Figure 8**), despite this side of the membrane not being treated.

Although *ex vivo* FM explants lack the immune cells that are crucial for a full understanding of how this tissue behaves at the end of pregnancy, they still enable the study of the resident cell types and their complex interactions. As there is currently no reliable test to determine the proximity to labor, experiments with these tissues often exhibit large interpatient variation. This can also come from inaccuracies in the estimation of gestational age and the normal variation in the immune responses between individuals (Klein and Flanagan, 2016). This leads to experiments with FM tissues that may seem to have little response to treatment due to factors like increasing levels of cellular senescence or apoptosis with age (Reti et al., 2007; Menon et al., 2019). Thus, without a legitimate way to dismiss these results, they should be included in data analysis. Newly developed models like the fetal membrane organ on a chip (Richardson et al., 2020), coupled with *ex vivo* FM explants, and may help us to further understand some nuances of cell-to-cell interactions in this tissue.

Despite the variation in our FM responses, we saw many samples were clearly very responsive, and the disaggregation of the data based on fetal sex was illuminating. We saw that male and female FM both led to the increased secretion of cytokines and pro-MMPs but that this response varied between the sexes. The others have shown that males have a more robust inflammatory response but other studies have also shown that females, in other circumstances, have a more robust response than males (Burns et al., 2015; Mitchell et al., 2017). This might be explained by the variety of the targets measured between the studies (Makinson et al., 2017; Wegner et al., 2017; Allard et al., 2019; Na et al., 2021; Goldstein et al., 2021). We saw that the species of fetal DNA also caused differential responses between the sexes (**Figure 8**). Overall, the male FM seemed to have a larger response to the male fetal DNA, especially when it was sonicated into smaller fragments. We also saw that the male fetal DNA was generally more hypomethylated (**Figure 7A**). This supports other reports that have demonstrated that in different cell and tissue types that the X chromosome in females was hypermethylated compared to the males (Bianca-Miotto et al., 2016; Garcia-Calzón

A

Methylation Correlation		
Condition	**Target**	**P-value**
Apical, 4m.s cffDNA	Male IL-6	0.004
Apical, w.cffDNA	All IL-6	0.092
Basal, 4m.s cffDNA	All GM-CSF	0.018
Basal, w.cffDNA	All GM-CSF	0.004
Basal, w.cffDNA	Female GM-CSF	0.084
Protein Secretion		
Condition	**Target**	**P-value**
Apical, 4m.s cffDNA	Male IL-6	0.041
Basal, 4m.s cffDNA	Male IL-6	0.011
Basal, 4m.s cffDNA	Male MMP9	0.02
Basal, 4m.s cffDNA	Male MMP2	0.03
Basal, 4m.s cffDNA	Female TNF-a	0.04

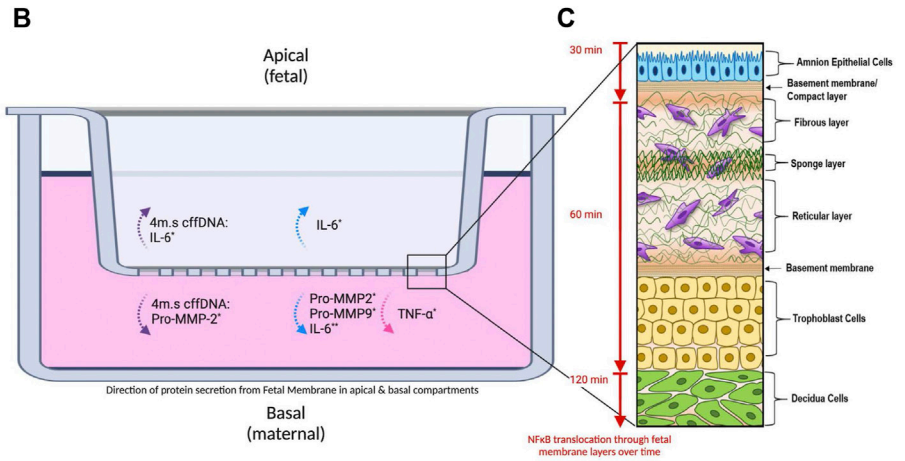


FIGURE 8 | Summary of the effect of fetal DNA on fetal membrane explants from male and female fetus pregnancies. **(A)** Table of treatment conditions, secretion locations with DNA species causing a response. **(B)** Transwell system with fetal membrane explant. The secretion into the apical media compartment of IL-6, and pro-MMP-2, into the basal media compartment of IL-6, TNF-α, pro-MMP2, and pro-MMP9. The dotted purple arrow indicates secretion regardless of fetal sex. The dotted blue arrows indicated secretion by male fetus FM. The dotted pink arrows indicated secretion by female fetus FM. n = 7–10 patients. $^{*}p$ = < 0.05 $^{**}p$ = < 0.001. **(C)** Diagram of fetal membranes (modified from Richardson et al., 2020) with attached decidua, illustrating the wave of NF-κB p65 subunit translocation to the nucleus over time (amnion→chorion→decidua) in response to fetal DNA treatment (apical).

et al., 2018). However, the cause for this is not understood. Although the females in our study generally secreted lower levels of cytokines than the males, they did have a significant increase in basal TNF-α secretion, a small increase in the pro-MMPs, and an increase in GM-CSF that correlated well with the higher levels of methylation of female fetal DNA (**Figure 8**). Thus, both males and females did increase their section of proinflammatory cytokines and pro-MMPs, although their responses were different. An increased IL-6 response by the males might precipitate a strong inflammatory response driven by JAK/STAT and MAPK signaling (Rose-John, 2021), whereas the increased TNF-α response in females may more likely lead to further NF-κB activation. Thus, although both cytokines are themselves increased through NF-κB pathway activation, they may result in different pathway activation and consequences. Indeed, it has been shown in other tissues that TLR activation rapidly results in TNF release which is then followed by IL-6, and that blocking TNF decreases IL-6 levels (Ghezzi et al., 2000). Therefore, future work should focus on understanding the differential responses and how these may change and influence specific pathological processes during pregnancy.

Our results are composite responses influenced by both sex-specific FM tissues, coupled with sex-specific fetal DNA from the same pregnancy. This is different from the studies that aimed to understand the role of cffDNA in maternal circulation because these studies always measure the female response to either male or female cffDNA, while our tissue and DNA are sex-matched. However, a limitation of our study is that although we are using FM and fetal DNA from the same pregnancy, we did not isolate cffDNA directly from the AF. Thus, there may be differences between our DNA and AF DNA that are not based on fragment size or methylation status, and that have not been fully characterized to date. cffDNA is thought to be liberated *via* the processes of apoptosis and necrosis, and only recently have studies begun to sequence circulating fetal versus maternal cffDNA to further understand its specific characteristics (Enninga 2022).

Originally it was thought that our not sonicated "whole" DNA would be unlikely to elicit an FM response and may serve as a good additional negative control in our experiments. However, we did see a response, but this differed from that seen with the sonicated, smaller fragments of fetal DNA. It is thought that *in vivo* small hypomethylated fragments of DNA interact with intracellular TLR9 (Scharfe-Nugent et al., 2012) after it enters the cell *via* a variety of mechanisms (van Boeckel et al., 2018). As we saw an FM response with this w.cffDNA, it may be that this larger DNA could be interacting with receptors such as RAGE or STING that are known to be able to bind larger DNA fragments (van Boeckel et al., 2018). Indeed, it is known that the STING receptor can also work with HMGB1 to augment its function (Lee et al., 2021). Our results demonstrating a positive correlation between the percentage of methylation and GM-CSF and the negative correlation between methylation and IL-6 are intriguing. However, they also support the premise that different forms of DNA, whether based on size, or in this instance methylation status, are able to elicit different responses. This further emphasizes the importance of the study of the interaction of these different species with potential receptors to improve our understanding of fetal DNA as a signaling molecule in pregnancy.

Collectively, our data also highlight the need to improve our understanding of the influence of having a male or female fetus in normal pregnancy and also those with negative outcomes. They also suggest that the fetal DNA that builds up in the AF with gestational age may signal to the cells of the FM and potentially the underlying maternal tissues. Indeed, it may be able to contribute to the inflammatory load that builds to initiate

parturition. Currently, it is not understood how the different fetal DNA fragment sizes or methylation level contributes to this, but it may be through differential receptor interaction and activation. It is also clear that although this DNA is able to activate inflammation in the FM it may only contribute to it once the functional progesterone block is removed from NF-κB (Gomez-Chavez et al., 2021). Thus, in conclusion, our data suggested that fetal DNA can be proinflammatory but the magnitude of response and the resultant downstream signaling molecules is dependent on the sex of the fetus and the specific characteristics of the fetal DNA.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This study used human fetal membrane tissue collected at repeat cesarean section. The protocol used was reviewed and approved by the IRB committee that oversees research at Kapiolani Hospital (Hawaii Pacific Health), the facility where the tissues were collected, and also Chaminade University of Honolulu's IRB committee, the site where experimentation took place. No identified information about the patients was ever collected, and therefore, the protocol was approved under both committees' exempted expedited procedures.

AUTHOR CONTRIBUTIONS

CR, collected the fetal membrane tissues and treated them with the DNA and performed the IL-6 ELISA. PN and CK performed the TNF and GM-CSF ELISA and processed and analyzed all the data and helped draw the figures and write the manuscript. JP isolated the amnion epithelial cells and performed the NF-KB translocation experiment in the primary cells. CK-W was involved in all aspects of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.901726/full#supplementary-material

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GLOSSARY

DAB 3,3′-Diaminobenzidine
DAPI 4′,6-Diamidino-2-phenylindole
ALT-1 Alanine aminotransferase-1
BSA Albumin standard
AEC Amniotic epithelial cells
AF Amniotic fluid
AMC Amniotic mesenchymal cells
ANOVA Analysis of variance
BP Base pair
BCA Bicinchoninic acid
CaCl2 Calcium chloride
cffDNA Cell free fetal DNA
DAMPs Danger-associated molecular patterns
DMEM/F12 Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
ELISA Enzyme-linked immunoassay
ECM Extracellular matrix
FM Fetal membranes
FBS Fetal bovine serum
4 m.s. cffDNA Four minute sonicated cell-free fetal DNA
GM-CSF Granulocyte macrophage colony-stimulating factor
HSP Heat shock proteins
HMGB1 High mobility group box protein 1
HRP Horseradish peroxidase
IHC Immunohistochemistry
IFNα Interferon-alpha
IL-1β Interleukin-1 beta
il-6 Interleukin-6
IAI Intrauterine infection
JAK Janus Kinase
LPS Lipopolysaccharide
MAPK Mitogen-activated protein kinase
MMP Matrix metalloproteinase
NF-κB Nuclear factor-kappa beta
PFA Paraformaldehyde
PBS Phosphate buffered saline
PCR Polymerase chain reaction
RAGE Receptor for advanced glycation end products
S100A12 S100 calcium-binding protein alpha-12
S100A S100 calcium-binding protein alpha
SRY Sex region Y
NaCl Sodium chloride
SDS Sodium dodecyl sulfate
SEM Standard error of the mean
STAT Signal transducer and activator of transcription
STING Stimulator of interferon genes
TLR9 Toll-like receptor 9
TNFα Tumor necrosis factor alpha
w.cffDNA Whole cell-free DNA.

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SYSTEMATIC REVIEW
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Motherwort Injection for Preventing Uterine Hemorrhage in Women With Induced Abortion: A Systematic Review and Meta-Analysis of Randomized Evidence

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Objective: Motherwort injection (MI) is a modern patented injection extracted from motherwort (Leonurus japonicus Hoult). Empirical studies and systematic reviews have shown the benefits of motherwort injection for preventing postpartum hemorrhage after vaginal delivery and cesarean section. This study was conducted to explore the efficacy and safety of motherwort injection for women with the prevention of post-abortion uterine hemorrhage.

Methods: A comprehensive literature search was conducted to identify RCTs regarding the effect of the use of motherwort injection in women after abortion. Data from trials were pooled by meta-analysis and a random-effects model was used to calculate the summarized relative risks (RRs) and their 95% confidence intervals (CIs). The grading of recommendations assessment, development, and evaluation (GRADE) methodology was used to access the quality of the evidence.

Results: Nine trials with a total of 1,675 participants were identified. Overall, motherwort injection combined with oxytocin compared to oxytocin had a significantly lower blood loss within 2 hours (MD = –50.00, 95% CI –62.92 to –37.08, very low quality); lower blood loss within 24 h (MD = –50.00, 95% CI –62.92 to –37.08, very low quality); however, there was no significant difference between motherwort injection and oxytocin (24 h: MD: 0.72, 95% CI –7.76 to 9.20; 48 h: MD: –0.01, 95% CI –11.35 to 11.33; 72 h: MD: –1.12, 95% CI –14.39 to 12.15, very low quality). Compared with oxytocin or no intervention, both motherwort injection and motherwort injection combined with oxytocin had a significantly decreased duration of blood loss (MI vs. O: MD –2.59, 95% CI –4.59 to –0.60, very low quality; MI + O vs. O: MD –2.62, 95% CI -3.02 to –2.22, very low quality; MI + O vs. No intervention: MD: –1.80, 95% CI –2.28 to –1.33, low quality). Seven of nine included trials reported adverse event outcomes. Three cases were found in the motherwort injection group, and five induced abortion syndromes were found in the motherwort injection plus oxytocin group. 29 adverse events were reported in the oxytocin group instead. The recovery time of normal menstruation after abortion was significantly earlier in the group using motherwort injection compared with oxytocin (MDs –3.77, 95% CI –6.29 to –1.25, very low quality), and the endometrial thickness in the motherwort injection group was

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significantly different from that in the oxytocin group (MD: 2.24, 95% CI 1.58 to 2.90, very low quality).

Conclusion: The results of this meta-analysis indicate prophylactic use of motherwort injection may reduce the risk of uterine hemorrhage in women after abortion, and more high-quality research is needed to confirm the efficacy and safety of motherwort injection in preventing uterine hemorrhage after abortion.

Systematic Review Registration: https://www.crd.york.ac.uk/PROSPERO/display_record.php?RecordID=274153, identifier CRD42021274153

Keywords: induced abortion, motherwort injection, Oxytocin, uterine hemorrhage, meta-analysis, systematic review, randomized controlled trials

1 INTRODUCTION

An estimated 205 million pregnancies occur each year worldwide, with 20% being terminated by induced abortion. Medication and surgery are both highly effective methods for induced abortion and are determined by the gestational age of the embryo or tutus. Abortion is one of the safest procedures when properly done, but unsafe abortion is associated with significant morbidity (Organization., 2021). Hemorrhage is the common consequence of misled management of abortion care and risks including retained tissues, uterine injury, uterine atony, and vaginal laceration are related to uterine bleeding after an abortion (Kerns and Steinauer, 2013; Perriera et al., 2017). The administration of uterotonic agents, such as oxytocin, misoprostol, and methylergonovine, is recommended to prevent hemorrhage after induced abortion if the uterus is atonic (Evensen et al., 2017; Bienstock et al., 2021; Steinauer and Patil, 2021). However, excessive use of oxytocin and misoprostol may cause high fever, shaking, chills, vomiting, hypertension, and other complications of toxicity (Widmer et al., 2010; Cleland et al., 2013).

Motherwort injection (MI) is a modern patented injection made from aqueous extracts of motherwort (Leonurus japonicus Hoult), which is a traditional Chinese herb used by thousands for gynecological conditions in China (Lulin et al., 2019). Pharmacological studies have shown that the active ingredients of motherwort injection (i.e. alkaloid, leonurine) could significantly facilitate hemostatic outcomes by promoting uterine contraction and blocking the uterine spiral vessels (Jian et al., 2005; Ojewole, 2005; Xiaoju, 2009; Huizhen et al., 2021). Moreover, motherwort injection works on the lower uterus without the receptor saturation effect, which reduces the risk of adverse events caused by the excessive use of uterotonic agents (Wenjing, 2022). Therefore, the prophylactic use of motherwort injection with or without oxytocin has been widely applied in Chinese tertiary hospitals to prevent postpartum and postabortion hemorrhage since 2005.

Empirical studies (Jianhua et al., 2009; Wei et al., 2016) and systematic reviews (Wenwen et al., 2018; Jiajie et al., 2019) have illustrated the benefits of motherwort injection for preventing PPH (postpartum hemorrhage) after vaginal delivery and cesarean section. Meanwhile, some trials have been published to explore the effect of motherwort injection on women after induced abortion and the findings were inconsistent. Therefore, we conducted a systematic review of randomized trials to determine the efficacy and safety of motherwort injection compared to oxytocin in women with induced abortion.

2 MATERIALS AND METHOD

This systematic review has been registered in the PROSPERO database (CRD42021274153) and reported according to The Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement guidelines (Page et al., 2021) (**Supplementary Material S1**).

2.1 Eligibility Criteria

Randomized control trials were eligible if they met the following criteria: 1) Participants: pregnant women anticipating an induced abortion; 2) Intervention: motherwort injection given by any route of administration and dose used alone or in combination with oxytocin; 3) Control: no intervention or oxytocin alone; 4) Outcomes measures: duration of uterine hemorrhage, the volume of blood loss, adverse events, the recovery time of normal menstruation, and endometrial thickness. We excluded trials reporting blood loss as a categorical variable due to a lack of classification criteria.

2.2 Data Source and Search Strategy

Relevant studies were identified from PubMed, EMbase, Cochrane Central Register of Controlled Trials (CENTRAL), Chinese National Knowledge Infrastructure Database (CNKI) and WanFang database inception to December 2021, updated to May 2022. An information expert was consulted to optimize our search strategies (**Supplementary Material S2**). ClinicalTrial.gov and Chinese Clinical Trial Registry were searched to identify unpublished studies, and the reference lists of included trials were searched for additional eligible studies. The search strategy was based on Mesh terms and their variants. No restriction in language was applied.

We also contacted a content expert and industry representatives and searched conference abstracts for additional information.

2.3 Data Selection and Data Extraction

Two reviewers (Xue XY and Tang XT) used predefined, pilot-tested forms to screen studies for eligibility, independently

screened titles/abstracts, and full text of potentially eligible articles. They independently assessed the risk of bias, quality of evidence and extracted data. If necessary, discrepancies were resolved through discussion. We collected information regarding study characteristics (sample size, publication year, author name, affiliation, and multicenter study), participants' characteristics (age, gestational week, and risk factors), interventions (dosage, timing, injection site, and duration of treatment), and outcomes (duration of hemorrhage, blood loss, adverse events, recovery time of normal menstruation, and endometrial thickness).

2.4 Risk of Bias Assessment

We assessed the risk of bias using the revised Cochrane Risk of Bias tool (Higgins et al., 2011; Akl et al., 2012) in our published study. The items included randomization sequence generation, allocation concealment, blinding of patients and personnel, or outcome assessors, infrequent missing outcome data, selective outcome reporting, and funding resources. The risk of bias for each item will be classified into low risk, high risk, and unclear. The risk of bias for each item will be classified into low risk, high risk, and unclear. The options for an overall risk of bias are the same as for individual items and are based on the following criteria: 1) trials were judged to be at low risk of bias if all items were assessed as low risk; 2) to be at high risk in at least one item assessed as high risk, or multiple items were assessed as an unclear risk; 3) to be an unclear risk in at least one item assessed as unclear but not to be at high risk for any item (Higgins et al., 2022).

2.5 Data Analysis and Rating Quality of Evidence

The data were pooled using a random-effects model for potential heterogeneity among studies when two or more studies assessed the same outcome. Heterogeneity among studies was assessed by Cochran's Q test and the I^2 statistic. We expressed dichotomous data as risk ratio (RR) with 95% confidence intervals (CIs) and continuous data as mean differences (MDs) with 95% CIs. If the trial was comparing three groups, we separately analyzed the data in terms of their interventions. The intervention arm was included twice in the analysis; however, this was related to only one trial, and this double inclusion would not influence the outcomes. Subgroup analyses were performed based on the type of administration (immediate administration versus consecutive administration) and risk for hemorrhage after abortion (high risk vs. moderate risk vs. low risk) when applicable. We summarized the adverse event data from all included studies and qualitatively described the data for rare data. Publication bias was assessed using Egger test plots when ten or more studies were available (Higgins JPT et al., 2022). RevMan 5.4 software was used for meta-analysis. We used the grading of recommendations assessment, development, and evaluation (GRADE) methodology to assess the quality of the evidence (Guyatt et al., 2008).

3 RESULTS

3.1 Search Results

Seven databases were screened yielding a total of 1823 studies. After removing duplicates and title and abstract screening, 48 studies were selected for a full-text review. Of 48 potentially relevant studies, 37 were excluded (e.g., studies were not properly randomized, or did not report relevant outcomes, etc.,) and two were abstracts without outcome measures. Finally, nine studies involving 1,675 women were included in the systematic review. The selection process is listed in **Figure 1**.

3.2 Study Characteristics

One multicenter RCT was identified and the remaining RCTs were single centers. All studies were reported in Chinese except one (Wanting et al., 2020) in English. These studies were all conducted in China between 2006 and 2019, from 60 to 366, and the characteristics of included studies are shown in **Table 1**. The mean age of pregnant women was 26.43 (SD:6.72), the mean pregnancy time was 2.31 (SD:1.10), and the mean gestation was 54.18 (SD:10.62) days. Two studies (Juan, 2016; Yun and Dengyu, 2016) evaluated motherwort injection combined with oxytocin versus oxytocin alone, four studies (Jiaogui, 2010; Li and Lixia, 2012; Yingjie and Junling, 2016; Yunyan, 2019) compared motherwort injection with oxytocin, and two studies (Yimei et al., 2008; Wanting et al., 2020) compared motherwort injection to no intervention. There was one trial (Yanxia, 2012) with multiple arms (MI vs. O vs. None).

3.3 Risk of Bias Within Studies

Among these nine trials, two (Yingjie and Junling, 2016; Wanting et al., 2020) adequately generated random sequences by random number table or computer; none of them clearly stated how to conceal the random sequence and blind the participants, health care providers, or outcome assessors; none of them reported selective outcomes, and one trial reported the funding resource. **Table 2** contains detailed results of the assessment. Overall, each of the included studies assessed the risk of bias to be unclear.

3.4 Outcome measures

3.4.1 Blood Loss

3.4.1.1Motherwort Injection vs. Oxytocin

Only one RCT (Li and Lixia, 2012) involving 87 participants reported blood loss within 24 h, 48 h, and 72 h. The data from this trial showed no significant difference between motherwort injection and oxytocin in all three assessments (24 h: MD: 0.72, 95% CI −7.76 to 9.20; 48 h: MD: −0.01, 95% CI −11.35 to 11.33; 72 h: MD: −1.12, 95% CI −14.39 to 12.15, very low quality).

3.4.1.2 Motherwort Injection Plus Oxytocin vs. Oxytocin

One trial (n = 230) (Juan, 2016) compared motherwort injection to oxytocin reporting blood loss within 2 hours and 24 h. There was a significant decrease in blood loss in the combined group compared to oxytocin alone (2 h: MD: −50.00, 95% CI −62.92 to −37.08; 24 h: MD: −50.00, 95% CI −62.92 to -37.08, very low quality).

3.5 Duration of Blood Loss (days)

3.4.1 Motherwort Injection vs. Oxytocin

There was statistically significant heterogeneity among two trials (n = 240, I^2 = 97%) (Jiaogui, 2010; Yunyan, 2019), and pooled

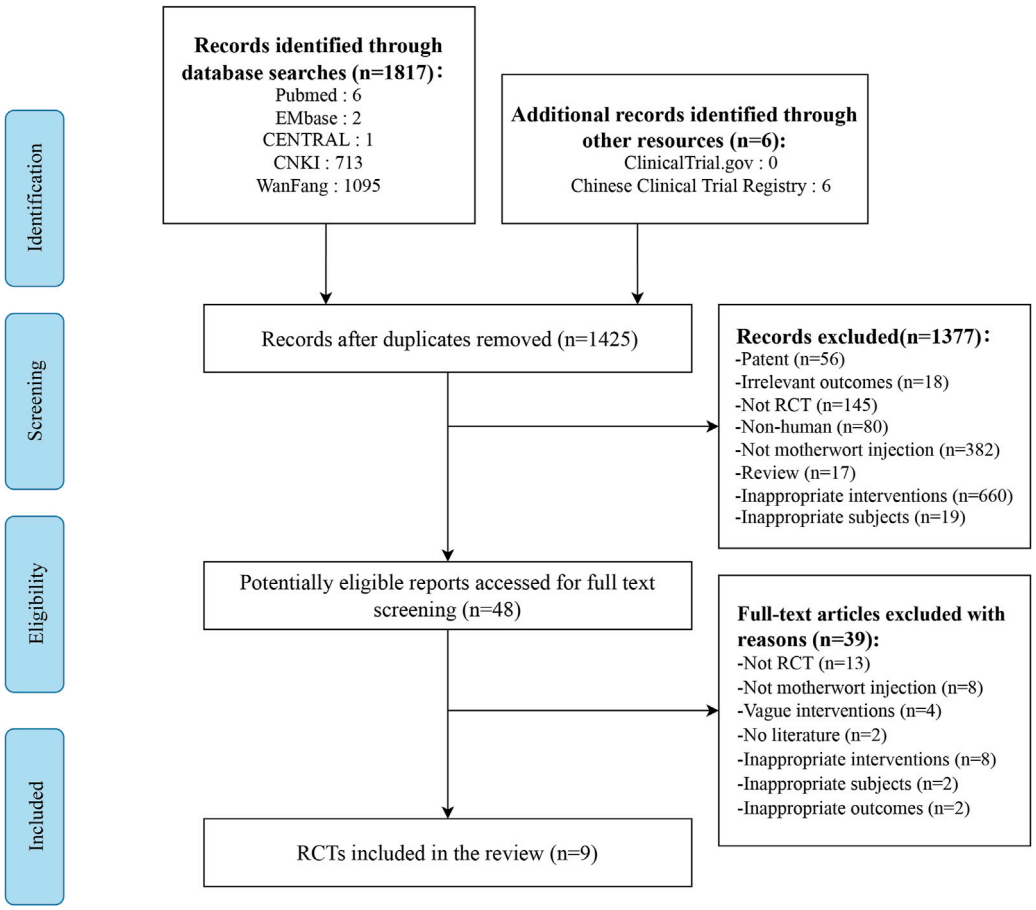

FIGURE 1 | Screening flow chart.

data demonstrated that motherwort injection significantly decreased the duration of blood loss compared to oxytocin (MD −2.59, 95% CI −4.59 to −0.60, very low quality) (**Figure 2**).

3.5.2 Motherwort Injection Plus Oxytocin vs. Oxytocin

We also observed a significantly decreased duration of blood loss in the combination administration group in one trial (MD -2.62, 95% CI −3.02 to −2.22, very low quality) (Yun and Dengyu, 2016) (**Figure 2**).

3.5.3 Motherwort Injection vs. no Intervention

Two trials (n = 516) (Yimei et al., 2008; Wanting et al., 2020) collected data for the duration of blood loss, and there was a significant reduction in days after motherwort injection (MD: −1.80, 95% CI −2.28 to −1.33, low quality) (**Figure 2**).

3.6 Adverse Events

Seven of nine included trials commented on adverse event outcomes, and three of them (Jiaogui, 2010; Yanxia, 2012; Juan, 2016) stated none had occurred. The remaining four trials (Yingjie and Junling, 2016; Yun and Dengyu, 2016; Yunyan, 2019; Wanting et al., 2020) reported adverse events, three cases (mild erythema, nausea and vomiting) (1.2%, 3/248) were found in the motherwort injection group, and five induced abortion syndromes were found in the motherwort injection plus oxytocin group (5.5%, 5/90). 29 adverse events (e.g., induced abortion syndromes, nausea, vomiting, infection, etc.) were reported in the oxytocin group instead (19.3%, 29/150) (**Table 3**).

3.7 Recovery Time of Normal Menstruation

3.7.1 Motherwort Injection vs. Oxytocin (days)

Only one trial (n = 60) (Yunyan, 2019) comparing motherwort injection with oxytocin discussed the recovery time of normal menstruation. The data showed significantly earlier recovery of normal menstruation in the motherwort injection group than those in the oxytocin group (MDs −3.77, 95% CI −6.29 to −1.25, very low quality).

3.8 Endometrial Thickness

3.8.1 Motherwort Injection vs. Oxytocin (mm)

Only one RCT (Yunyan, 2019) involving 60 participants reported endometrial thickness after abortion. The data from this trial showed a significant difference in endometrial thickness after abortion between the two groups (MD: 2.24, 95% CI 1.58 to 2.90, very low quality).

TABLE 1 | Characteristics of included studies.

Study	Intervention T/C	Participants T/C	Age (year) Rang/ mean	Pregnancy time Rang/ mean	Gestation (DAY) Rang/Mean	Injection site T/C	Usage T/C	Dosage T/C
Peng 2016	M + O	115	27.60 (5.30)	—	62.30 (5.80)	Intramuscular	Immediate	2 ml(M)+ 1 ml(O)
	O	115	27.60 (5.30)	—	62.30 (5.80)	Intramuscular	Immediate	1 ml(O)
He 2016	M + O	90	26.03 (6.21)	2.12 (0.35)	57.33 (9.94)	Intramuscular(M) Cervical(O)	Immediate	2 ml(M)+Unclear(O)
	O	90	25.36 (6.78)	2.03 (0.32)	56.84 (11.76)	Cervical	Immediate	Unclear
Yuan 2010	M	90	23.95 (9.91)	—	53.54 (9.11)	Intramuscular	Continuous	3 ml(M)
	O	90	24.14 (9.98)	—	53.69 (8.86)	Intramuscular	Continuous	3 ml(O)
Huang 2012b	M	129	17–39	—	—	Intramuscular	Immediate	2 ml(M)
	O	131	17–39	—	—	Intramuscular	Immediate	2 ml(O)
Ouyang 2019	M	30	28.24 (3.63)	2.08 (0.33)	42.13 (1.56)	Intramuscular	Immediate	2 ml(M)
	O	30	29.51 (3.45)	2.14 (0.43)	41.87 (1.35)	Intramuscular	Immediate	1 ml(O)
Zhang 2016	M	30	29.10 (7.10)	3.19 (0.27)	41.10 (1.70)	Intramuscular	Immediate	2 ml(M)
	O	30	27.20 (6.70)	3.06 (0.14)	42.40 (1.80)	Intramuscular	Immediate	1 ml(O)
Yuan 2012	M	43	26.58 (6.09)	2.81 (1.29)	43.88 (6.53)	Cervical + Intramuscular	Continuous	2 ml(M)
	O	44	27.59 (6.62)	3.00 (1.53)	45.86 (6.78)	Cervical + Intramuscular	Continuous	2 ml(O)
Xia 2020	M	188	26.86 (5.89)	2.24 (1.33)	—	Intramuscular	Continuous	8 ml(M)
	No Intervention	178	25.89 (5.50)	2.12 (1.26)	—	None	None	None
Huang 2012a	M	129	17–39	—	—	Intramuscular	Immediate	2 ml(M)
	No Intervention	102	17–39	—	—	None	None	None
Zhang 2008	M	100	—	—	—	Intramuscular	Immediate	1 ml(M)/2 ml(M)
	No Intervention	50	—	—	—	None	None	None

M, motherwort injection; O, oxytocin.

TABLE 2 | Risk of bias.

Study	Randomization	Concealed allocation	Blinding		Integrity of result	Selective reporting	Other bias (funding resources)
			For participants	For outcome assessment			
Peng 2016	Only mentioned	NR	NR	NR	Complete	NR	NR
Yuan 2010	Only mentioned	NR	NR	NR	Complete	NR	NR
Huang 2012	Only mentioned	NR	NR	NR	Complete	NR	NR
Ouyang 2019	Only mentioned	NR	NR	NR	Complete	NR	NR
Zhang 2016	Random number table	NR	NR	NR	Complete	NR	NR
He 2016	Only mentioned	NR	NR	NR	Complete	NR	NR
Yuan 2012	Only mentioned	NR	NR	NR	7 cases in MI group had incomplete records of bleeding, and 6 cases in the oxytocin group had incomplete records	NR	NR
Xia 2020	Random number table	NR	NR	NR	Completed in 398 patients (201 patients assigned to MI and 199 assigned to no-treatment), and 366 patients completed the follow-up assessment (188 patients assigned to LHI and 178 assigned to no-treatment)	NR	Supported by the science and technology support project of Sichuan province and the science and technology achievements transformation demonstration project of Sichuan province
Zhang 2008	Only mentioned	NR	NR	NR	Complete	NR	NR

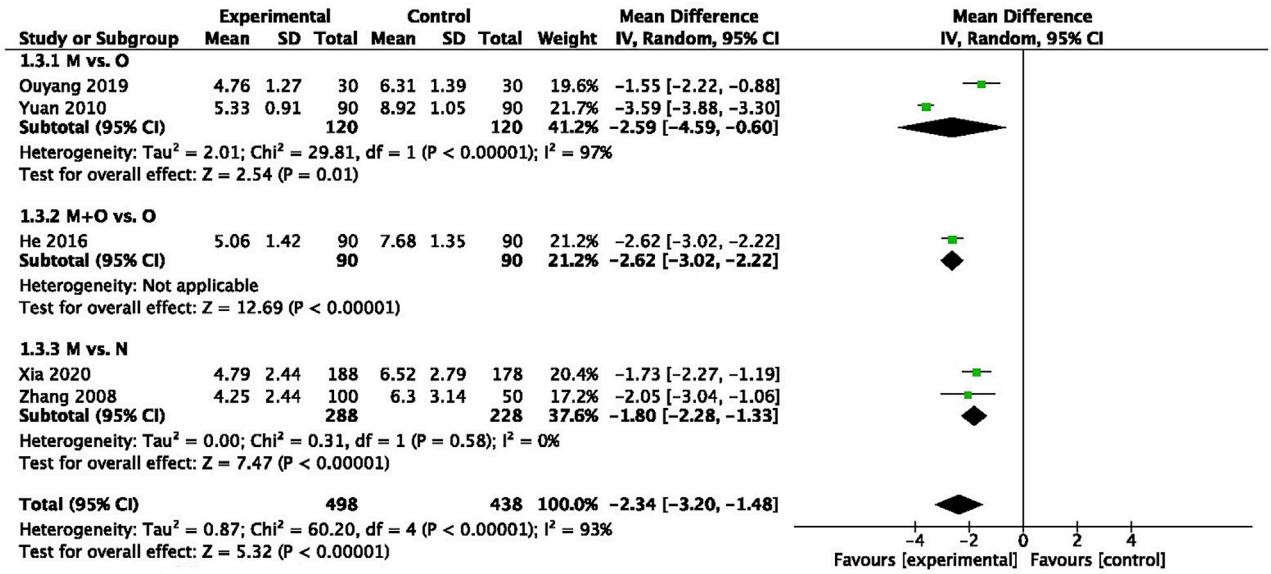

FIGURE 2 | Duration of blood loss.

TABLE 3 | Adverse events.

Study	Intervention	Participants	Adverse events
	T/C	T/C	T/C
Peng 2016	M + O	115	None
	O	115	None
He 2016	M + O	90	5 induced abortion syndromes
	O	90	19 induced abortion syndromes
Yuan 2010	M	90	None
	O	90	None
Ouyang 2019	M	30	None
	O	30	1 gastrointestinal discomfort
Zhang 2016	M	30	1 nausea, 1 vomiting
	O	30	1 allergy, 1 nausea, 3 vomiting, 1 diarrhea, 3 infections
Xia 2020	M	188	1 mild erythema
	No Intervention	178	None
Huang2012	M	129	None
	O	131	None
	No Intervention	102	None

3.9 Subgroup Analysis and Publication Bias

We failed to conduct the subgroup analysis and assess publication bias for the small number of studies included in each outcome measure.

4 DISCUSSION

This review brings all randomized evidence together to assess the effect of motherwort injection for postabortion hemorrhage. Compared with oxytocin, a significant benefit of prophylactic use of motherwort injection with or without oxytocin was reported for four outcomes: duration of blood loss, adverse events, and the recovery time of normal menstruation and endometrial thickness. In contrast, only two trials reported blood loss as a continuous variable and no significant difference was found between motherwort injection and oxytocin. Considering the low and very low methodological quality, small sample size and the observed difference between groups, this evidence supporting the use of prophylactic use of motherwort injection for women with postabortion hemorrhage must be generalized with caution.

Atony of the uterine body or fundus is a common cause of postabortion hemorrhage (Gill et al., 2022), and the risk of bleeding is associated with prior cesarean section, history of obstetrical hemorrhage, increasing maternal age, gestational age and obesity (Upadhyay et al., 2015; Kerns et al., 2019). Uterotonic agents are a priority protocol for the prevention and treatment of postabortion hemorrhage in women with

uterine atony, including methylergonovine, misoprostol and oxytocin. However, little evidence exists to recommend starting with a particular agent. Motherwort injection, approved for marketing in 1971 by the Chinese FDA, has been used for stopping bleeding and regulating menstruation for decades (Yulin et al., 2018). Modern pharmacological studies have demonstrated that the active ingredients of motherwort injection, such as leonurine and stachydrine (Yulin et al., 2018), could exhibit angiogenic activity and have an excitatory effect on the uterus, without adverse effects such as elevated blood pressure (Dan et al., 2013; Xiaofei et al., 2014; Rebonato et al., 2016; Juan et al., 2018; Liefang, 2021; Qiang et al., 2021). These active ingredients can also dilate blood vessels and protect the cardiovascular system (Chengping et al., 2019), and will not affect women's temperature and respiration (Yanfang et al., 2017). In addition, oxytocin is a polypeptide hormone with uterine contraction and has a rapid onset of action (Aiqun et al., 2007), while motherwort injection causes contractions for a longer period than oxytocin after injection into the uterine wall (half-life is 6 h) with a relatively slow onset of action. Therefore, the additional use of motherwort injection on oxytocin has been widely applied in routine clinical practice.

Our published SRs (Wenwen et al., 2018; Jiajie et al., 2019) have suggested the preferable outcomes of motherwort injection for preventing PPH (postpartum hemorrhage) after vaginal delivery and cesarean section. However, even with wide application in clinical settings, limited studies have been conducted to discuss the effect of motherwort injection on a postabortion hemorrhage. To the best of our knowledge, this is the first systematic review and meta-analysis to address the effect of motherwort injection on a postabortion hemorrhage.

4.1 Limitations

We conducted a comprehensive systematic review including all published RCTs with rigorous methods to evaluate the effect of motherwort injection for women with induced abortion. However, our study also has a few significant limitations. First, the trials included suffered from a high risk of bias, and only two trials clearly stated the method of random sequence generation. Second, we were unable to conduct a subgroup analysis to explore the source of heterogeneity for a limited number of studies. Most of the trials included in our analyses had small sample sizes and resulted in an imprecise estimation of effects with very low quality. Third, the trials we included were all conducted in China mainland and included women who were in the first termination; and no trials on the use of motherwort injection in women with intermediate or late abortions. Fourth, the most appropriate dosing schedule is still unknown for the limited evidence we found.

5 CONCLUSION

In conclusion, due to the small number of events and sample sizes and severe limitations, the current body of evidence is inadequate to establish the positive effects—including blood loss, duration of blood time, adverse events, the recovery time of normal menstruation and endometrial thickness—of motherwort injection preventing hemorrhage after abortion. Given the insufficiently high quality of these trials, future adequately powered, well-designed, and conducted trials are warranted to test the effects of the different treatments preventing hemorrhage fairly. Observational studies that carefully collect and analyze the data may also provide important insights regarding the effects of motherwort injection.

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

YJ and LY conceived and designed this study; XX and TX searched the literature and extracted data, XX synthesized data, and developed the first draft of the manuscript; YJ provided critical methodological guidance; WF provided clinical guidance. All authors critically revised the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.916665/full#supplementary-material

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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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SYSTEMATIC REVIEW
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Developing a Core Outcome Set for the Evaluation of Antibiotic Use in Prelabor Rupture of Membranes: A Systematic Review and Semi-Structured Interview

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Background: Prelabor rupture of membranes (PROM) is associated with maternal and neonatal infections. Although guidelines suggest prophylactic antibiotics for pregnant women with PROM, the optimal antibiotic regimen remains controversial. Synthesizing the data from different studies is challenging due to variations in reported outcomes.

Objective: This study aimed to form the initial list of outcomes for the core outcome set (COS) that evaluates antibiotic use in PROM by identifying all existing outcomes and patients' views.

Methods: Relevant studies were identified by searching PubMed, EMBASE, Cochrane Library, Chinese National Knowledge Infrastructure, Wanfang, and VIP databases. We also screened the references of the included studies as a supplementary search. We extracted basic information from the articles and the outcomes. Two reviewers independently selected the studies, extracted the data, extracted the outcomes, and grouped them into domains. Then, semi-structured interviews based on the potential factors collected by the systematic review were conducted at West China Second Hospital of Sichuan University. Pregnant women who met the diagnostic criteria for PROM were enrolled. Participants reported their concerns about the outcomes. Two researchers identified the pregnant women's concerns.

Results: A total of 90 studies were enrolled in this systematic review. The median outcomes in the included studies was 7 (1–31), and 109 different unique outcomes were identified. Pre-term PROM (PPROM) had 97 outcomes, and term PROM (TPROM) had 70 outcomes. The classification and order of the core outcome domains of PPROM and TPROM were consistent. The physiological domain was the most common for PPROM and TPROM outcomes. Furthermore, 35.1 and 57.1% outcomes were only reported once in PPROM and TPROM studies, respectively. Thirty pregnant women

participated in the semi-structured interviews; 10 outcomes were extracted after normalized, and the outcomes were reported in the systematic review. However, studies rarely reported pregnant women's concerns.

Conclusion: There was considerable inconsistency in outcomes selection and reporting in studies about antibiotics in PROM. An initial core outcomes set for antibiotics in PROM was formed.

Keywords: core outcome sets, outcome reporting, pregnancy, prelabor rupture of membranes, systematic review, semi-structured interview

1 INTRODUCTION

Prelabor rupture of membranes (PROM) is a rupture of membranes before the onset of labor, which consists of "pre-term prelabor rupture of membranes (PPROM)" and "term prelabor rupture of membranes (TPROM)" (Siegler et al., 2020). It affects 2.3%–18.7% of pregnancies and increases the risk of intrauterine infection, neonatal sepsis, neonatal pneumonia, etc. (Kenyon et al., 2001a; Martin et al., 2005; Mercer, 2005; Smith et al., 2005; Clark and Varner, 2011; Reuter et al., 2014; Middleton et al., 2017; Zhuang et al., 2020). Although guidelines suggest that the use of prophylactic antibiotics could reduce infection morbidity and improve the outcomes for mothers and newborns, the optimal antibiotic regimen is still controversial (Yudin et al., 2009; Kenyon et al., 2013; Thomson and Royal College of Obstetricians and Gynaecologists, 2019; Chatzakis et al., 2020; Siegler et al., 2020). Despite many studies about the antibiotics regimens for PROM conducted, it is difficult to synthesize their data due to outcome variations. As a recent systematic review shows, only 70.0% (17/20) of the included studies reported the primary outcome. The risk of bias was 35.0% (7/20) and 90.0% (18/20) of the included studies, including risk in "Measurement of outcome" and "Selection of reported result," respectively (Chatzakis et al., 2020).

A core outcome set (COS), defined as an agreed standardized set of outcomes that should be measured and reported as a minimum, could improve consistency in outcome measurement and reduce outcome reporting bias. A COS would eliminate unnecessary waste in producing and reporting research findings (Williamson et al., 2012). The COS is drawing increasing attention across all health research areas and is referred to as a starting point for outcome selection in the work of some trialists, systematic reviewers, and guideline developers (COS users) (Gorst et al., 2016).

However, there is no COS for antibiotics in PROM or COS for treating or preventing infection in pregnant women. This systematic review and semi-structured interview would form the initial list of outcomes for the COS of antibiotics in PROM by identifying all existing outcomes and patients' views.

2 METHODS

This COS project is registered on the core outcome measures in effectiveness trials (COMET) database, and further details are available at https://www.comet-initiative.org/Studies/Details/1986.

2.1 Systematic Review

The part of the systematic review was performed and reported per the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines for systematic reviews (Preferred Reporting Items for Systematic Reviews and Meta-Analyses, 2009).

2.1.1 Search Strategy

We conducted an electronic search of PubMed, EMBASE, Cochrane Library, Chinese National Knowledge Infrastructure, Wanfang, and VIP Database from inception to September 2021. The search strategy was adjusted specifically for each database. It combined medical subject headings and free text terms for ("Fetal Membranes, Premature Rupture" "antibiotics" or "Prelabor rupture of membranes") and ("Anti-Infective Agents" or "antibiotics" or "Penicillins" or" Cephalosporins" or" azithromycin" or" erythromycin" or" Clindamycin"). **Supplementary Table S1** lists the search terms. Citation lists of the included studies were reviewed to identify any intervention reports missed by the search strategy.

2.1.2 Inclusion Criteria

The following studies were included: 1) Participants: pregnant women (no restriction for gestational age) met the diagnostic criteria for PROM according to the guidelines of the Chinese Medical Association, American College of Obstetricians and Gynecologists, Society of Obstetricians & Gynaecologists (SOGC), Royal College of Obstetricians and Gynaecologists (ROGC), etc. 2) Intervention: antibiotics. 3) Type of study: systematic reviews, randomized controlled trials, non-randomized controlled trials, or cohort studies. The following studies were excluded: 1) non-Chinese and non-English literature, 2) unobtainable full-texts.

2.1.3 Data Extraction

Titles and abstracts were independently screened by two reviewers to determine potential eligible studies, and full texts of potentially relevant articles were independently screened by two reviewers to assess for eligibility. Disagreements were resolved by consensus or consulted a third reviewer. Two reviewers independently extracted data from the included studies and cross-checked it. The extracted data included: 1) the basic information of the articles (the first author, published year, study design, country, etc.); 2) the characteristics of participants and interventions; 3) the outcomes reported (names, definitions, and measurements of each outcome).

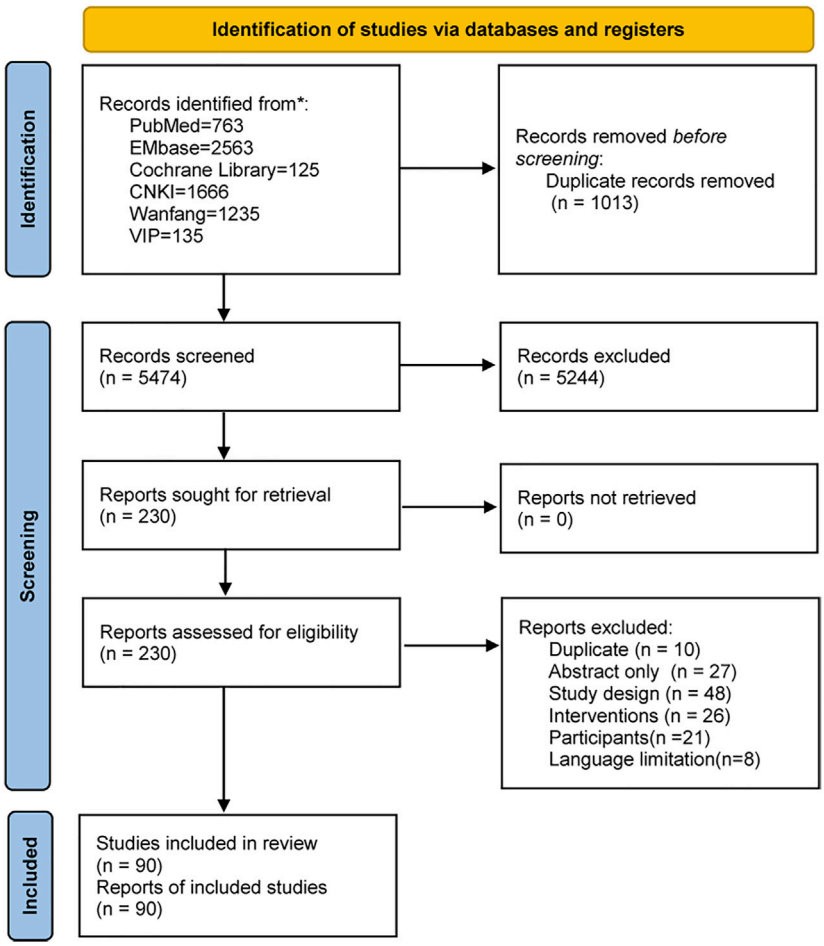

FIGURE 1 | Flow diagram of study selection.

2.1.4 Assessment of Risk of Bias

There was no assessment of the risk of bias since the purpose of this study was to identify all outcomes reported irrespective of the study quality.

2.1.5 Data Synthesis

All outcomes were extracted verbatim from studies. Variations in the same outcome reporting were revised for consistency, and the composite outcomes were split into unique outcomes by a researcher with clinical experience in obstetrics. Outcome terminologies were assigned to one of the core outcome domains according to the COMET Handbook (Williamson et al., 2017). We calculated the number of unique outcomes for each study and outcome domain, the number of reported studies for each outcome, and the median number of the reported studies for each outcome domain.

2.2 Semi-Structured Interview

According to recommendations of COS-STAndards for Development and COMET handbook (version 1.0) (Kirkham et al., 2016; Williamson et al., 2017), a list of outcomes from published clinical trials may be supplemented with semi-structured interviews with patients. Therefore, we conducted the semi-structured interview to obtain the opinions of patients on PROM treatment.

The semi-structured interview study was conducted at West China Second Hospital of Sichuan University from January to February 2022. The West China Second University Hospital, Sichuan University, provided ethical approval. The participants gave verbal consent before their interviews. The participants' socioeconomic information of participants came from the hospital information system.

2.2.1 Participants

Pregnant women in West China Second Hospital of Sichuan University, January to February 2022, who met the diagnostic criteria for PROM were enrolled. The exclusion criteria included: 1) pregnant women with serious illnesses who were not suitable to participate in the study; 2) pregnant women with communication difficulties; 3) pregnant women who refused to

Continent	Country	Before 1991 (3)	1991-2000 (16)	2001-2010 (11)	2011-2020 (51)	After 2020 (9)
Asia (49%, 44/90)	China (33)				27	6
	South Korea (4)			1	3	
	Israel (3)		1		1	1
	Japan (2)			1	1	
	India (1)				1	
	Turkey (1)				1	
America (36%, 32/90)	The United States (31)	3	12	3	11	2
	Canada (1)				1	
	Chile (1)			1		
Europe (10%, 10/90)	The United Kingdom (5)			4	1	
	Germany (1)			1		
	Finland (1)		1			
	The Greek (1)				1	
	Yugoslavia (1)				1	
Africa (4%, 4/90)	Egypt (2)				2	
	Mozambique (2)		2			

1-5
6-10
10-20
>20

FIGURE 2 | The published areas and time of the including studies.

participate. The sample size was 30 since 30 subjects could achieve data saturation reported in other studies (Keyvanara et al., 2013; Alkadhimi et al., 2020). However, if new information is generated in the final interview, the sample size of the interview will increase.

2.2.2 Procedure

The research team designed a semi-structured interview guide involving open-ended questions (Supplementary). The face-to-face semi-structured interviews took place at the patient's bedside at mutually convenient times. The researchers would explain the content and purpose of the study to the patients and interview them after obtaining their informed consent. Interviews were digitally audio-recorded using a mobile phone.

2.2.3 Analysis

All the interviews were transcribed literally by a researcher. Our systematic review developed a consensus codebook using a deduction coding process and evaluating the first 10 transcripts to identify emerging codes through an inductive coding process. Each transcript was independently coded by two researchers, and coding inconsistencies were resolved by discussion. Disagreements were resolved by consensus or a discussion in the research group. Data analysis was processed by identifying the codes to judge whether these were new outcomes and whether they should be added to the list of candidate outcomes. We would identify whether these outcomes are new and judge whether they should be added to the list of candidate outcomes.

3 RESULTS

3.1 Systematic Review

3.1.1 Study Characteristics

The search retrieved 6,487 studies. After removing duplicates and irrelevant records by screening the titles and abstracts, 230 studies were assessed for eligibility by full-text screening. Eventually, 90 studies (Chatzakis et al., 2020) were included in this systematic review (**Figure 1**). These studies were conducted in 17 countries on five continents from 1966 to 2021 (**Figure 2**). The study designs were comprised of systematic review (7/90, 7.8%) (Mercer and Arheart, 1995; Maymon et al., 1998; Kenyon et al., 2004; Cousens et al., 2010; Wojcieszek et al., 2014; Saccone and Berghella, 2015; Chatzakis et al., 2020), RCTs (32/90, 35.6%) (Brelje and Kaltreider, 1966; Amon et al., 1988; Johnston et al., 1990; McGregor et al., 1991; Kurki et al., 1992; McCaul et al., 1992; Mercer et al., 1992; Lockwood et al., 1993; Ernest and Givner, 1994; Lewis et al., 1995; Almeida et al., 1996; Grable et al., 1996; Lovett et al., 1997; Kenyon et al., 2001b; Ovalle et al., 2002; Lewis et al., 2003; Segel et al., 2003; August Fuhr et al., 2006; Kwak et al., 2013; Nabhan et al., 2014; Zhang, 2014; Kahramanoglu et al., 2016; Mai and He, 2016; Liang, 2018; Zheng, 2018; Pasquier et al., 2019; Siegel et al., 2019; Deng, 2020; Wolf et al., 2020; Chen, 2021; Cong, 2021; Zheng, 2021) and cohort studies (51/90, 56.6%) (A, 2021; Ali, 2020; Bar et al., 2020; Barišić et al., 2017; Bergström, 1991; Chang et al., 2017; Chen et al., 2020; Dotters-Katz et al., 2017; Du, 2016; Du et al., 2019; Du and Zhang, 2020; Ehsanipoor et al., 2008; Feng, 2020; Finneran et al., 2019; Finneran et al., 2017; Fitzgibbon et al., 2021; Siegel et al., 2019; Ke, 2013; Kenyon et al., 2008; Knupp et al., 2022; Kole-White et al., 2021; Lee et al., 2016; Li, 2017; Li, 2020; Lin et al., 2012; Martingano et al., 2020; Navathe et al., 2019; Pan et al., 2018;

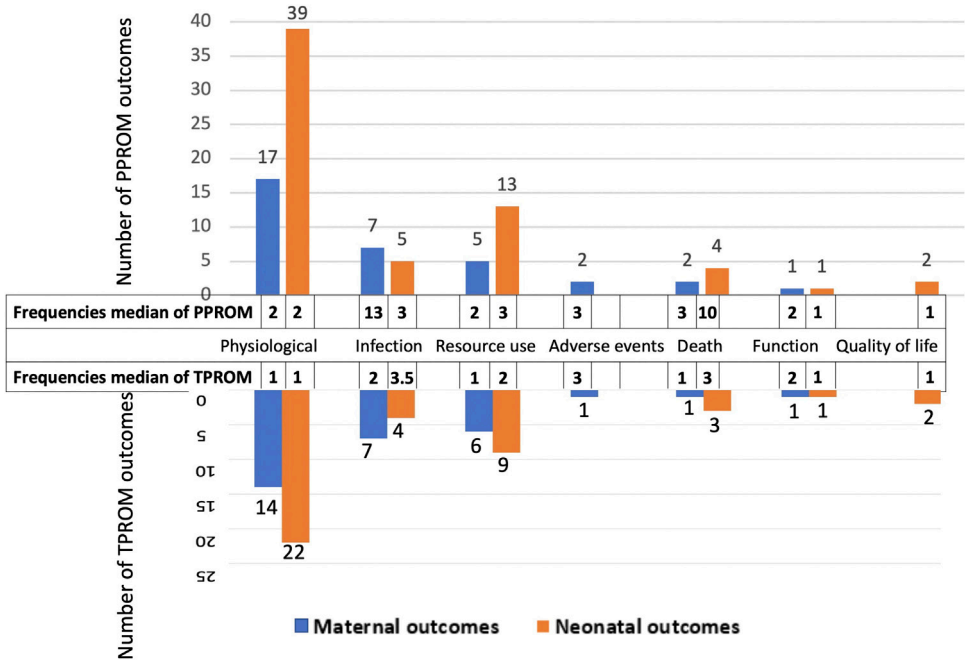

FIGURE 3 | Summary of core outcome areas.

Pawar and Reddy, 2020; Pierson et al., 2014; Edwards et al., 2020; Ryo et al., 2005; Smith et al., 2015; Song and Han, 2005; Sung et al., 2017; Tai, 2011; Tanaka et al., 2019; Kramer et al., 1996; Wu, 2018; Yeung et al., 2014; Zeng and Lin, 2020; Zhang, 2017; Zhang, 2019;Zhao, 2019a; Zhao,2019b; Zheng et al., 2016; Zhou et al., 2015; Zhou, 2020; Zou, 2021; Zheng et al., 2020). Out of the 90 studies, 78 (86.7%) studies (Ali, 2020; Almeida et al., 1996; Amon et al., 1988; Bar et al., 2000; Bergström, 1991; Chang et al., 2017; Chatzakis et al., 2020; Chen et al., 2020; Chen, 2021; Cong, 2021; Cousens et al., 2010; Lewis et al., 1995; Deng, 2020; Dotters-Katz et al., 2017; Du, 2016; Du et al., 2019; Du and Zhang, 2020; Ehsanipoor et al., 2008; Ernest and Givner, 1994; Feng, 2020; Finneran et al., 2019; Finneran et al., 2017; Fitzgibbon et al., 2021; August et al., 2006; Grable et al., 1996; Siegel et al., 2019; Johnston et al., 1990; Kahramanoglu et al., 2016; Ke, 2013; Kenyon et al., 2001; Kenyon et al., 2004; Knupp et al., 2022; Kole-White et al., 2021; Kurki et al., 1992; Kwak et al., 2013; Lee et al., 2016; Li, 2017; Li, 2020; Li ,2021; Liang, 2018; Lin et al., 2012; Lockwood et al., 1993; Lovett et al., 1997; Siegel et al., 2019; Mai et al., 2016; Martingano et al., 2020; Maymon et al., 1998; McCaul et al., 1992; McGregor et al., 1991; Mercer et al., 1992; Mercer and Arheart, 1995; Lewis et al., 2003; Nabhan et al., 2014; Navathe et al., 2019; Ovalle et al., 2002; Pan et al., 2018; Pasquier et al., 2019; Pawar and Reddy, 2020; Pierson et al., 2014; Edwards et al., 2020; Ryo et al., 2005; Saccone and Berghella, 2015; Segel et al., 2003; Smith et al., 2015; Song et al. 2005; Sung et al., 2017; Tai, 2011; Tanaka et al., 2019; Kramer et al., 1996; Wojcieszek et al., 2014; Wolf et al., 2020; Wu, 2018; Yeung et al., 2014; Zeng and Lin, 2020; Zhang, 2014; Zhang, 2017; Zhang, 2019; Zhao, 2019a; Zhao, 2019b; Zheng et al., 2016; Zheng, 2021; Zhou et al., 2015; Zhou, 2020; Zou, 2021) included PPROM women, 6 (6.7%) studies (Zheng, 2018; A, 2021; Barišić et al., 2017; Tai, 2011; Zhao, 2019a; Zheng et al., 2020) included term PROM women, 4 (4.4%) studies (Kwak et al., 2013; Nabhan et al., 2014; Wojcieszek et al., 2014; Saccone and Berghella, 2015) included both PPROM and term PROM women and 2 (2.2%) studies (Brelje and Kaltreider, 1966; Kenyon et al., 2008) did not report whether the participants were term. The study interventions/comparisons included: 1) using antibiotics vs placebo/blank control (31/90, 34.4%) (Brelje and Kaltreider, 1966; Amon et al., 1988; Johnston et al., 1990; Bergström, 1991; Kurki et al., 1992; McCaul et al., 1992; Mercer et al., 1992; Lockwood et al., 1993; Ernest and Givner, 1994; Mercer and Arheart, 1995; Almeida et al., 1996; Grable et al., 1996; Kramer et al., 1996; Maymon et al., 1998; Bar et al., 2020; Ovalle et al., 2002; Kenyon et al., 2004; Song and Han, 2005; August Fuhr et al., 2006; Cousens et al., 2010; Lin et al., 2012; Ke, 2013; Nabhan et al., 2014; Wojcieszek et al., 2014; Zhang, 2014; Saccone and Berghella, 2015; Du, 2016; Chang et al., 2017; Dotters-Katz et al., 2017; Pasquier et al., 2019; Feng, 2020); 2) different antibiotics (29/90, 32.2%) (McGregor et al., 1991; Lewis et al., 1995; Lovett et al., 1997; Edwards et al., 2020; Kenyon et al., 2001b; Ryo et al., 2005; Ehsanipoor et al., 2008; Kenyon et al., 2008; Kwak et al., 2013; Pierson et al., 2014; Yeung et al., 2014; Kahramanoglu et al., 2016; Lee et al., 2016; Zheng et al., 2016; Finneran et al., 2017; Sung et al., 2017; Wu, 2018; Zhao, 2019b; Finneran et al., 2019; Navathe et al., 2019; Siegel et al., 2019; Tanaka et al., 2019; Ali, 2020; Chatzakis et al., 2020; Martingano et al., 2020; Pawar and Reddy, 2020; Wolf et al., 2020; Fitzgibbon et al., 2021); 3) different timing of antibiotics administration (17/90, 18.9%) (Deng, 2020; Liang, 2018; Zheng, 2018; A, 2021; Barišić et al., 2017; Tai, 2011; Zhao, 2019a; Zheng et al., 2020; Chen et al., 2020; Du et al., 2019; Du and Zhang, 2020; Knupp et al., 2022; Li, 2017; Pan et al., 2018; Zeng and Lin, 2020; Zhang, 2019; Zhou et al., 2015); 4) antibiotics chosen depending on

TABLE 1 | The initial outcomes list of COS for antibiotics in PROM.

Outcome domain	Outcome	Number of reported studies	Definition	Participants' views
PROM (97)				
Physiological (17/97)	Latency period	41 (Johnston et al. (1990); Bergström, (1991); McGregor et al. (1991); McCaul et al. (1992); Lockwood et al. (1993); Ernest and Givner, 1994; Lewis et al. (1995); Mercer and Arheart, (1995); Almeida et al. (1996); Grable et al. (1996); Lovett et al. (1997); Maymon et al. (1998); Bar et al. (2020); Kenyon et al. (2001b); Lewis et al. (2003); Segel et al. (2003); Kenyon et al. (2004); Ryo et al. (2005); August Fuhr et al. (2006); Pierson et al. (2014); Saccone and Berghella, (2015); Smith et al. (2015); Kahramanoglu et al. (2016); Mai and He, (2016); Chang et al. (2017); Dotters-Katz et al. (2017); Finneran et al. (2017); Sung et al. (2017); Wu, (2018); Du et al. (2019); Navathe et al. (2019); Siegel et al. (2019); Zhang, (2019); Chatzakis et al. (2020); Du and Zhang, (2020); Martingano et al. (2020); Pawar and Reddy, (2020); Zeng and Lin, (2020); Fitzgibbon et al. (2021); Kole-White et al. (2021); Knupp et al. (2022))	√ (Lockwood et al. (1993); Ernest and Givner, (1994); Grable et al. (1996); Pierson et al. (2014); Smith et al. (2015); Chang et al. (2017); Dotters-Katz et al. (2017); Sung et al. (2017); Siegel et al. (2019); Fitzgibbon et al. (2021); Kole-White et al. (2021))	√
	Mode of delivery	27 Brelje and Kaltreider, (1966); Johnston et al., 1990; Lewis et al. (1995); Grable et al. (1996); Bar et al. (2020); Kenyon et al. (2001b); Ke, (2013); Lee et al. (2016); Finneran et al. (2017); Ali, (2020); Feng, (2020); Chen, (2021); Fitzgibbon et al. (2021)	—	√
	Postpartum hemorrhage	22 Lin et al. (2012); Nabhan et al. (2014); Wojcieszek et al. (2014); Zhou et al. (2015); Li, (2017); Zhang, (2017); Pan et al. (2018); Zhao, (2019b); Du et al. (2019); Chen et al. (2020); Deng, (2020); Du and Zhang, (2020); Feng, (2020); Li, (2020); Zeng and Lin, (2020); Zhou, (2020); Chen, (2021); Cong, (2021); Zheng, (2021); Zou, (2021); Knupp et al. (2022))	—	—
	Preterm delivery	6 Brelje and Kaltreider, (1966); Lee et al. (2016); Feng, (2020)	—	—
	Maternal white blood cell count	4 (Kahramanoglu et al. (2016); Liang, (2018); Wu, (2018); Fitzgibbon et al. (2021))	—	—
	Placental abruption	4 (Mercer et al. (1992); Lewis et al. (1995); Saccone and Berghella, (2015); Pawar and Reddy, (2020)	—	—
	Deep vein thrombosis	3 Dotters-Katz et al. (2017); Ali (2020); Knupp et al. (2022)	√ (Dotters-Katz et al. (2017))	—
	Maternal c-reactive protein	3 Kahramanoglu et al. (2016); Liang, (2018); Wu, (2018)	—	—
	Fever	2 Wojcieszek et al. (2014); Kahramanoglu et al. (2016)	√ Wojcieszek et al. (2014)	—
	Maternal intensive care unit admission	2 Wojcieszek et al. (2014); Fitzgibbon et al. (2021)	—	—
	Meconium-stained amniotic fluid	2 Feng, (2020); Martingano et al. (2020)	—	√
	Amniotic fluid index	2 (Lewis et al. (1995); Kahramanoglu et al. (2016))	—	√
	Cardiac arrest	1 (Wojcieszek et al. (2014))	—	—
	Cord prolapse	1 Saccone and Berghella, (2015)	—	—
	Reason for delivery	1 Finneran et al. (2017)	—	—
	Respiratory arrest	1 Wojcieszek et al. (2014)	—	—
	Trophoblastic hyperplasia	1 Ovalle et al. (2002)	—	—
Infection (7/97)	Chorioamnionitis	43 Brelje and Kaltreider, (1966); Amon et al. (1988); Johnston et al. (1990); Kurki et al. (1992); Grable et al. (1996); Bar et al. (2020); Kenyon et al. (2004); Ehsanipoor et al. (2008); Du, (2016); Lee et al. (2016); Siegel et al. (2019); Ali (2020); Chatzakis et al. (2020); Deng, (2020); Chen, (2021); Cong, (2021); Knupp et al. (2022))	√ (Amon et al. (1988); Kurki et al. (1992); Mercer et al. (1992); Grable et al. (1996); Ehsanipoor et al. (2008); Wojcieszek et al. (2014); Zheng et al. (2016); Pasquier et al. (2019); Martingano et al. (2020))	—
	Endometritis	18 Brelje and Kaltreider, (1966); Amon et al. (1988); Johnston et al. (1990); Kurki et al. (1992); Mercer et al. (1992); Ernest and Givner, (1994); Grable et al. (1996); Kramer et al. (1996); Maymon et al. (1998); Edwards et al. (2020); Segel et al. (2003); Ehsanipoor et al. (2008); Nabhan et al. (2014); Wojcieszek et al. (2014); Saccone and Berghella, (2015); Martingano et al. (2020); Knupp et al. (2022)	√ Amon et al. (1988); Mercer et al. (1992); Ernest and Givner, (1994); Grable et al. (1996); Kramer et al. (1996); Ehsanipoor et al. (2008); Wojcieszek et al. (2014); Martingano et al. (2020)	
	Puerperal infection	18 Mercer and Arheart, (1995); Zhou et al. (2015); Li, (2017); Zhang, (2017); Pan et al. (2018); Zhao, (2019b); Du et al. (2019); Ali (2020); Chen et al. (2020); Du and Zhang, (2020); Feng, (2020); Li, (2020); Zeng and Lin, (2020); Zhou, (2020); Chen, (2021); Cong, (2021); Zheng, (2021); Zou, (2021)	—	—
	Intrauterine infection	13 Ernest and Givner, (1994); Ovalle et al. (2002); Zhou et al. (2015); Du, (2016); Finneran et al. (2017); Li, (2017); Liang, (2018); Wu, (2018); Du et al. (2019); Deng, (2020); Du and Zhang, (2020); Feng, (2020); Li, (2020)	√ Ernest and Givner, (1994)	√
	Maternal sepsis	11 Johnston et al. (1990); Kurki et al. (1992); Mercer et al. (1992); Song and Han, (2005); Wojcieszek et al. (2014); Saccone and Berghella, (2015); Finneran et al. (2017); Sung et al. (2017); Siegel et al. (2019); Pawar and Reddy, (2020); Knupp et al. (2022)	√ Wojcieszek et al. (2014)	—

(Continued on following page)

TABLE 1 | (*Continued*) The initial outcomes list of COS for antibiotics in PROM.

Outcome domain	Outcome	Number of reported studies	Definition	Participants' views
	Maternal infection	3 (McCaul et al. (1992); Mai and He, (2016); Zhang, (2019)	—	—
	Wound infection	2 Wojcieszek et al. (2014); Knupp et al. (2022)	—	—
Resource use (5/97)	Length of maternal hospitalization	8 (Johnston et al. (1990); Lockwood et al. (1993); Almeida et al. (1996); Lovett et al. (1997); Kenyon et al. (2001b); Nabhan et al. (2014); Wojcieszek et al. (2014); Kahramanoglu et al. (2016))	—	—
	Steroid administration	3 (Bar et al. (2020); Kahramanoglu et al. (2016); Chang et al. (2017))	—	—
	Postpartum antibiotic administration	2 (Kenyon et al. (2001b); Wojcieszek et al. (2014))	—	—
	Tocolysis administration	1 (Kahramanoglu et al. (2016))	—	—
	Cost	1 (Finneran et al. (2019))		√
Adverse events (2/97)	Adverse drug reaction	5 (Nabhan et al. (2014); Pierson et al. (2014); Wojcieszek et al. (2014); Saccone and Berghella, 2015; Sung et al. (2017))	—	—
	Anaphylaxis	1 (Wojcieszek et al. (2014))	—	—
Death (2/97)	Maternal deaths	3 (Wojcieszek et al. (2014); Zhang, (2017); Knupp et al. (2022))	—	—
Function (1/97)	Breastfeeding	2 (Wojcieszek et al. (2014); Saccone and Berghella, (2015)	—	—
Physiological (39/97)	Birth weight	33 Johnston et al. (1990); Bergström, (1991); Ernest and Givner, (1994); Lewis et al. (1995); Almeida et al. (1996); Grable et al. (1996); Bar et al. (2020); Kenyon et al. (2001b); Kwak et al. (2013); Du, 2016; Kahramanoglu et al. (2016); Lee et al. (2016); Chang et al. (2017); Chen, (2021); Knupp et al. (2022)	—	√
	Respiratory distress syndrome	32 Johnston et al. (1990); Lewis et al. (1995); Grable et al. (1996); Bar et al. (2020); Kenyon et al. (2001b); August Fuhr et al. (2006); Ehsanipoor et al. (2008); Cousens et al. (2010); Ke, (2013); Kwak et al. (2013); Kahramanoglu et al. (2016); Lee et al. (2016); Chang et al. (2017); Finneran et al. (2017); Chatzakis et al. (2020); Knupp et al. (2022)	√ Grable et al. (1996); Ehsanipoor et al. (2008)	—
	Apgar score	30 Brelje and Kaltreider, (1966); Johnston et al. (1990); Kurki et al. (1992); Mercer et al. (1992); Lockwood et al. (1993); Grable et al. (1996); Lovett et al. (1997); Bar et al. (2020); Lewis et al. (2003); Kwak et al. (2013); Nabhan et al. (2014); Pierson et al. (2014); Wojcieszek et al. (2014); Saccone and Berghella, 2015; Zhou et al. (2015); Du, (2016); Kahramanoglu et al. (2016); Finneran et al. (2017); Li, (2017); Sung et al. (2017); Wu, (2018); Du et al. (2019); Navathe et al. (2019); Tanaka et al. (2019); Zhang, (2019); Du and Zhang, (2020); Li, (2020); Wolf et al. (2020); Zeng and Lin, (2020); Fitzgibbon et al. (2021)		
	Necrotising enterocolitis	27 (Johnston et al. (1990); Grable et al. (1996); Bar et al. (2020); Kenyon et al. (2001b); Segel et al. (2003); August Fuhr et al. (2006); Ehsanipoor et al. (2008); Cousens et al. (2010); Kwak et al. (2013); Lee et al. (2016); Chang et al. (2017); Finneran et al. (2017); Pasquier et al. (2019); Siegel et al. (2019); Chatzakis et al. (2020); Knupp et al. (2022))	√ Grable et al. (1996); Kenyon et al. (2001b); Ehsanipoor et al. (2008); Siegel et al. (2019)	
	Neonatal pneumonia	19 McGregor et al. (1991); Mercer et al. (1992); Mercer and Arheart, (1995); Ehsanipoor et al. (2008); Lin et al. (2012); Ke, (2013); Nabhan et al. (2014); Wojcieszek et al. (2014); Zhang, (2014); Smith et al. (2015); Kahramanoglu et al. (2016); Zhang, (2017); Zhao, (2019b); Feng, (2020); Zeng and Lin, (2020); Zhou, (2020); Chen, (2021); Zheng, (2021); Zou, (2021)	√ Ehsanipoor et al. (2008)	—
	Neonatal infection	15 Brelje and Kaltreider, (1966); Amon et al. (1988); Ernest and Givner, (1994); Lewis et al. (1995); Bar et al. (2020); Kenyon et al. (2004); August Fuhr et al. (2006); Saccone and Berghella, (2015); Smith et al. (2015); Du, (2016); Chang et al. (2017); Chatzakis et al. (2020); Feng, (2020); Wolf et al. (2020); Zeng and Lin, (2020)	√ Brelje and Kaltreider, (1966); Amon et al. (1988); Lewis et al. (1995); Bar et al. (2020); Smith et al. (2015)	
	Bronchopulmonary dysplasia	11 Kurki et al. (1992); Ehsanipoor et al. (2008); Kwak et al. (2013); Lee et al. (2016); Chang et al. (2017); Siegel et al. (2019); Knupp et al. (2022)	√ Ehsanipoor et al. (2008)	—
	Neonatal asphyxia	9 Ke, (2013); Zhang, (2014); Liang, (2018); Pan et al. (2018); Zhao, (2019b); Zhang, (2019); Chen et al. (2020); Du and Zhang, (2020); Feng, (2020)	—	—
	Periventricular leukomalacia	9 Lee et al. (2016); Chang et al. (2017); Siegel et al. (2019)	—	—
	Cerebral palsy	7 Kenyon et al. (2008); Lee et al. (2016); Siegel et al. (2019)	—	—
	Fetal distress	7 (Mercer et al. (1992); Lin et al. (2012); Pan et al. (2018); Zhao, 2019b; Chen et al. (2020); Du and Zhang, 2020; Feng, 2020)	—	√
	Cord arterial pH	4 (Johnston et al. (1990); Lockwood et al. (1993); Grable et al. (1996); Wolf et al. (2020))	—	—
	Neonatal icterus	4 Lin et al. (2012); Kahramanoglu et al. (2016); Pan et al. (2018); Feng, (2020)	—	—
	Retinopathy of prematurity		—	—

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TABLE 1 | (*Continued*) The initial outcomes list of COS for antibiotics in PROM.

Outcome domain	Outcome	Number of reported studies	Definition	Participants' views
		4 Song and Han, (2005); Kwak et al. (2013); Chang et al. (2017)		
	Abnormal brain sonography	3 Kenyon et al. (2004); Kwak et al. (2013); Saccone and Berghella, (2015)	√ Saccone and Berghella, (2015)	—
	Neonatal fever	2 Smith et al. (2015); Knupp et al. (2022)	—	—
	Neurological outcome	2 Kwak et al. (2013); Chang et al. (2017)	√ Chang et al. (2017)	—
	Patent ductus arteriosus	2 Lewis et al. (1995); Tanaka et al. (2019)	—	—
	Respiratory problems	2 Kenyon et al. (2008); Saccone and Berghella, (2015)	√ Kenyon et al. (2008)	—
	Seizures	2 Kenyon et al. (2008); Knupp et al. (2022)	—	—
	Small for gestational age	2 Johnston et al. (1990); McGregor et al. (1991)	—	—
	Abnormal hearing screen	1 Tanaka et al. (2019)	—	—
	Bowel disorders	1 Kenyon et al. (2008)	—	—
	Chronic lung disease	1 Kenyon et al. (2001b)	—	—
	Conjunctivitis	1 McGregor et al. (1991)	—	—
	Diabetes	1 Kenyon et al. (2008)	—	—
	Fetal placental vascular lesions	1 Ovalle et al. (2002)	—	—
	Hypoxic ischemic encephalopathy	1 Zhang, (2014)	—	—
	Neonatal group B *streptococcus* colonization	1 Yeung et al. (2014)	—	—
	Neonatal group B *streptococcus* infection	1 Yeung et al. (2014)	—	—
	Neonatal scleredema	1 Zhang, (2014)	—	—
	neonatal white cell count	1 Fitzgibbon et al. (2021)	—	—
	Patent ductus arteriosus ligated	1 Tanaka et al. (2019)	—	—
	Persistent fetal circulation	1 Grable et al. (1996)	√ Grable et al. (1996)	—
	Postnatal steroid requirement	1 Tanaka et al. (2019)	—	—
	Pulmonary hypoplasia	1 Knupp et al. (2022)	—	—
	Skeletal deformities	1 Kurki et al. (1992)	—	—
	Transient tachypnea of the newborn	1 Kahramanoglu et al. (2016)	—	—
	Weight gain	1 Johnston et al. (1990)	—	—
Resource use (13/97)	Admission to the neonatal intensive care unit	9 Lewis et al. (1995); Kenyon et al. (2001b); Lewis et al. (2003); Kwak et al. (2013); Nabhan et al. (2014); Wojcieszek et al. (2014); Saccone and Berghella, (2015); Kahramanoglu et al. (2016); Chatzakis et al. (2020)	—	—
	Duration of hospitalization of the newborns	9 McCaul et al. (1992); Mercer et al. (1992); Almeida et al. (1996); Wojcieszek et al. (2014); Saccone and Berghella, (2015); Liang, 2018; Navathe et al. (2019); Tanaka et al. (2019); Wolf et al. (2020)	—	—
	Duration of stay in the neonatal intensive care unit	7 Johnston et al. (1990); Lockwood et al. (1993); Kwak et al. (2013); Nabhan et al. (2014); Wojcieszek et al. (2014); Finneran et al. (2017); Knupp et al. (2022)	—	—
	Duration of ventilation	5 Lewis et al. (1995); Lovett et al. (1997); Kwak et al. (2013); Nabhan et al. (2014); Tanaka et al. (2019)	—	—
	Mechanical ventilation requirement	5 Kurki et al. (1992); Lovett et al. (1997); Kenyon et al. (2001b); Kwak et al. (2013); Wojcieszek et al. (2014)	—	—
	Oxygen requirement	4 Lewis et al. (1995); Lovett et al. (1997); Kenyon et al. (2001b); Pasquier et al. (2019)	—	—
	Antibiotic therapy requirement	3 Wojcieszek et al. (2014); Saccone and Berghella, 2015; Wolf et al. (2020)	—	—
	Hospital admission	3 McGregor et al. (1991); Lewis et al. (1995); Kenyon et al. (2008)	—	—
	Duration of antibiotics	2 Johnston et al. (1990); Tanaka et al. (2019)	—	—
	Duration of oxygen requirement	2 Lewis et al. (1995); Lovett et al. (1997)	—	—
	Surfactant requirement	2 Kenyon et al. (2001b); Tanaka et al. (2019)	—	—
	Internal fetal monitoring	1 Wojcieszek et al. (2014)	—	—
	Neonatal respiratory support	1 Wolf et al. (2020)	—	—
Infection (5/97)	Neonatal sepsis	35 Johnston et al. (1990); Kurki et al. (1992); Kenyon et al. (2001b); Cousens et al. (2010); Kwak et al. (2013); Kahramanoglu et al. (2016); Lee et al. (2016); Chang et al. (2017); Siegel et al. (2019); Chen et al. (2020); Knupp et al. (2022)	√ (Kramer et al. (1996); Nabhan et al. (2014); Wojcieszek et al. (2014); Saccone and Berghella, (2015); Martingano et al. (2020)	—
	Intraventricular haemorrhage	26 Johnston et al. (1990); Lewis et al. (1995); Grable et al. (1996); Bar et al. (2020); August Fuhr et al. (2006); Cousens et al. (2010); Kwak et al. (2013); Lee et al. (2016); Chang et al. (2017); Siegel et al. (2019); Chatzakis et al. (2020); Knupp et al. (2022)	√ Grable et al. (1996); Siegel et al. (2019)	—
	Funisitis	3 Lee et al. (2016)	√ Zheng et al. (2016)	—

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TABLE 1 | (*Continued*) The initial outcomes list of COS for antibiotics in PROM.

Outcome domain	Outcome	Number of reported studies	Definition	Participants' views
	Neonatal meningitis	1 Wojcieszek et al. (2014)	—	—
	Intracranial infection	1 Zeng and Lin, (2020)	—	—
Death (4/97)	Neonatal deaths	34 Brelje and Kaltreider, (1966); Johnston et al. (1990); Bergström, (1991); Kurki et al. (1992); Bar et al. (2020); Kenyon et al. (2001b); Kenyon et al. (2008); Cousens et al. (2010); Ke, (2013); Kwak et al. (2013); Kahramanoglu et al. (2016); Lee et al. (2016); Dotters-Katz et al. (2017); Finneran et al. (2017); Siegel et al. (2019); Chatzakis et al. (2020); Knupp et al. (2022)	—	√
	Perinatal death	10 McGregor et al. (1991); Kurki et al. (1992); Maymon et al. (1998); Lewis et al. (2003); Kenyon et al. (2004); Nabhan et al. (2014); Wojcieszek et al. (2014); Saccone and Berghella, 2015; Zhao, (2019b); Chatzakis et al. (2020)	√ Chatzakis et al. (2020)	—
	Stillbirth	5 Johnston et al. (1990); Bergström, (1991); Kurki et al. (1992); Mercer et al. (1992); Wojcieszek et al. (2014)	—	—
	Neonatal deaths due to infection	1 Mercer et al. (1992)	—	—
Quality of life (2/97)	Health-related quality-of-life and behavior	1 Kenyon et al. (2008)	—	√
	Developmental problems	1 Kenyon et al. (2008)	√ Kenyon et al. (2008)	
Function (1/97) TPROM (70)	Functional impairment	1 Kenyon et al. (2008)	—	—
Physiological (14/70)	Mode of delivery	5 Brelje and Kaltreider, (1966); Tai, (2011); Nabhan et al. (2014); Saccone and Berghella, (2015)	—	√
	Postpartum hemorrhage	5 Wojcieszek et al. (2014); Nabhan et al. (2014); A, (2021); Tai, (2011); Zheng et al. (2020)	√ A, (2021)	—
	Latency period	2 Saccone and Berghella, (2015); Barišić et al. (2017)	—	√
	Preterm delivery	2 Brelje and Kaltreider, (1966); Saccone and Berghella, (2015)	—	—
	Temperature	2 Zhao, (2019a); Zheng et.al. (2016); Zheng, (2018); Zheng et.al. (2020)	—	—
	Abnormalities in blood routine	1 Zhao, (2019a)	—	—
	Maternal neutrophil percentage	1 Zheng et al. (2020)	—	—
	Maternal procalcitonin	1 Zheng et al. (2020)	—	—
	Maternal white blood cell count	1 Zheng et al. (2020)	—	—
	Maternal c-reactive protein	1 Zheng et al. (2020)	—	—
	Cord prolapse	1 Saccone and Berghella, (2015)	—	—
	Fever	1 Wojcieszek et al. (2014)	√ Wojcieszek et al. (2014)	—
	Placental abruption	1 Saccone and Berghella, (2015)	—	—
	Respiratory arrest	1 (Wojcieszek et al. (2014))	—	—
Infection (7/70)	Chorioamnionitis	8 Saccone and Berghella, (2015); Wojcieszek et al. (2014); Brelje and Kaltreider, (1966); Nabhan et al. (2014); Zheng, (2018); A, (2021); Barišić et al. (2017); Zheng et al. (2020)	√ Wojcieszek et al. (2014); A, (2021)	—
	Endometritis	4 Brelje and Kaltreider, (1966); Nabhan et al. (2014); Wojcieszek et al. (2014); Saccone and Berghella, (2015)	√ Wojcieszek et al. (2014)	—
	Puerperal infection	3 A, (2021); Tai, (2011); Zheng et al. (2020)	√ A, (2021)	—
	Maternal sepsis	2 Wojcieszek et al. (2014); Saccone and Berghella, (2015)	√ Wojcieszek et al. (2014)	—
	Wound infection	2 Wojcieszek et al. (2014); Wolf et.al. (2020); Wu, (2018); Yeung et.al. (2014); Zeng and Lin, (2020); Zhang, (2014); Zhang, (2017); Zhang, (2019); Zhoa, (2019a); Zhoa, (2019b)	—	—
	Urinary tract infection	1 Zheng et al. (2020)	—	—
	Vaginitis	1 Zheng et al. (2020)	—	—
Resource use (6/70)	Length of maternal hospitalization	2 Nabhan et al. (2014); Wojcieszek et al. (2014)	—	—
	Maternal intensive care unit admission	1 Wojcieszek et al. (2014)	—	—
	Postpartum antibiotic administration	1 Wojcieszek et al. (2014)	—	—
	Anaphylaxis	1 Wojcieszek et al. (2014)	—	—
	Cardiac arrest	1 Wojcieszek et al. (2014)	—	—
Death (1/70)	Maternal deaths	1 Wojcieszek et al. (2014)	—	—
Adverse events (1/70)	Adverse drug reaction	3 Nabhan et al. (2014); Wojcieszek et al. (2014); Saccone and Berghella, (2015)	—	—
Function (1/70)	Breastfeeding	2 (Wojcieszek et al. (2014); Saccone and Berghella, (2015)	—	—
Physiological (22/70)	Apgar score	6 Saccone and Berghella, (2015); Wojcieszek et al. (2014); Brelje and Kaltreider, (1966); Kwak et al. (2013); Nabhan et al. (2014); A, (2021)	√ A, (2021)	—
	Fetal distress	3 Zheng, (2018); A, (2021); Tai, (2011)	√ A, (2021)	√
	Abnormal brain sonography	2 Kwak et al. (2013); Saccone and Berghella, (2015)	√ Saccone and Berghella, (2015)	—
	Cerebral palsy	2 Kenyon et al. (2008); Saccone and Berghella, (2015)	—	—
	Respiratory distress syndrome	2 (Kwak et al. (2013); Wojcieszek et al. (2014))	—	—
	Respiratory problems	2 Kenyon et al. (2008); Saccone and Berghella, (2015)	√ Kenyon et al. (2008)	—
	Baby gender	1 Kwak et al. (2013)	—	—

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TABLE 1 | (*Continued*) The initial outcomes list of COS for antibiotics in PROM.

Outcome domain	Outcome	Number of reported studies	Definition	Participants' views
	Birth weight	1 Kwak et al. (2013)	—	√
	Bowel disorders	1 Kenyon et al. (2008)	—	—
	Bronchopulmonary dysplasia	1 Kwak et al. (2013)	—	—
	Cord arterial pH	1 Zheng et al. (2020)	—	—
	Diabetes	1 Kenyon et al. (2008)	—	—
	Intraventricular haemorrhage	1 Kwak et al. (2013)	—	—
	Necrotising enterocolitis	1 Kwak et al. (2013)	—	—
	Neonatal asphyxia	1 Zheng et al. (2018)	—	—
	Neonatal c-reactive protein	1 Barišić et al. (2017)	—	—
	Neonatal lung injury	1 Zheng, (2018)	—	—
	Neonatal procalcitonin	1 Zheng et al. (2020)	—	—
	Neonatal white blood cell count	1 Zheng et al. (2020)	—	—
	Neurological outcome	1 Kwak et al. (2013)	—	—
	Retinopathy of prematurity	1 Kwak et al. (2013)	—	—
	Seizures	1 Kwak et al. (2013)	—	—
Resource use (9/70)	Admission to the neonatal intensive care unit	5 Kwak et al. (2013); Nabhan et al. (2014); Wojcieszek et al. (2014); Saccone and Berghella, (2015); Barišić et al. (2017))	—	—
	Antibiotic therapy requirement	3 Wojcieszek et al. (2014); Saccone and Berghella, (2015); Barišić et al. (2017)	—	—
	Duration of hospitalization of the newborns	3 Wojcieszek et al. (2014); Saccone and Berghella, (2015); Barišić et al. (2017)	—	—
	Duration of stay in the neonatal intensive care unit	3 Kwak et al. (2013); Nabhan et al. (2014); Wojcieszek et al. (2014)	—	—
	Hospital admission	2 Kenyon et al. (2008); Knupp et. al. (2022); Kole-White et.al. (2021); Kurki et.al. (1992); Kwak et al. (2013); Lee et.al. (2016)	√ Kenyon et al. (2008)	—
	Mechanical ventilation requirement	2 Kwak et al. (2013); Wojcieszek et al. (2014)	—	—
	Duration of ventilation	1 Nabhan et al. (2014)	—	—
	Duration of ventilator treatment	1 Kwak et al. (2013)	—	—
	Internal fetal monitoring	1 Wojcieszek et al. (2014)	—	—
Infection (4/70)	Neonatal sepsis	5 Saccone and Berghella, (2015); Wojcieszek et al. (2014); Kwak et al. (2013); Nabhan et al. (2014); A, (2021)	√ Saccone and Berghella, (2015); Wojcieszek et al. (2014); Nabhan et al. (2014); A, (2021)	—
	Neonatal pneumonia	4 Tai, (2011); Nabhan et al. (2014); Wojcieszek et al. (2014); Zheng, (2018)	—	—
	Neonatal infection	3 Saccone and Berghella, (2015); Brelje and Kaltreider, (1966); A, (2021)	√ Brelje and Kaltreider, (1966); A, (2021)	—
	Neonatal meningitis	1 Wojcieszek et al. (2014)	—	—
Death (3/70)	Neonatal deaths	4 Brelje and Kaltreider, (1966); Kenyon et al. (2008); Kwak et al. (2013); Wojcieszek et al. (2014)	—	√
	Perinatal death	3 Nabhan et al. (2014); Wojcieszek et al. (2014); Saccone and Berghella, (2015)	—	—
	Stillbirth	1 Wojcieszek et al. (2014)	—	—
Quality of life (2/70)	Health-related quality-of-life and behavior	1 Kenyon et al. (2008)	—	√
	Developmental problems	1 Kenyon et al. (2008)	√ Kenyon et al. (2008)	—
Function (1/70)	Functional impairment	1 Kenyon et al. (2008)	—	—

experience vs culture results (8/90, 8.9%) (Mai and He, 2016; Zhang, 2017; Zhou, 2020; Chen, 2021; Cong, 2021; Zheng, 2021; Zou, 2021); 5) different courses of antibiotics administration (4/90, 4.4%) (Lewis et al., 2003; Segel et al., 2003; Smith et al., 2015; Li, 2020); 6) different administration route (1/90, 1.1%) (Kole-White et al., 2021). The median number of the outcomes in the included studies was 7, with the range 1–31. Only 38.9% (35/90) studies (Chatzakis et al., 2020; Saccone and Berghella, 2015; Amon et al., 1988; Brelje and Kaltreider, 1966; Lewis et al., 1995; Ernest and Givner, 1994; Grable et al., 1996; Siegel et al., 2019; Kahramanoglu et al., 2016; Kenyon et al., 2001b; Kurki et al., 1992; Kwak et al., 2013; Lockwood et al., 1993; Mercer et al., 1992; Nabhan et al., 2014; Pasquier et al., 2019; Segel et al., 2003; A, 2021; Zhao, 2019a; Zheng et al., 2020; Kenyon et al., 2008; Bar et al., 2020; Chang et al., 2017; Dotters-Katz et al., 2017; Kramer et al., 1996; Ehsanipoor et al., 2008; Fitzgibbon et al., 2021; Martingano et al., 2020; Pierson et al., 2014; Sung et al., 2017; Zheng et al., 2016; Knupp et al., 2022; Smith et al., 2015; Kole-White et al., 2021) defined study outcomes and 3.3% (3/90) studies (Kenyon et al., 2008; Kwak et al., 2013; Chang et al., 2017) explained how to measure the outcomes. 16.7% (15/90) of studies used composite outcomes (Lockwood et al., 1993; Kenyon et al., 2001b; Segel et al., 2003; Kenyon et al., 2008; Kwak et al., 2013; Wojcieszek et al., 2014; Smith et al., 2015; Kahramanoglu et al., 2016; Zheng et al., 2016; Chang et al., 2017; Zhao, 2019a; Pasquier et al., 2019; Siegel et al., 2019; Zheng et al., 2020; Knupp et al., 2022). **Supplementary Table S2** shows the study characteristics.

3.1.2 Outcomes Reported in the Studies

Extraction of each verbatim outcome domain from each study, a total of 784 verbatim outcomes were identified. After merging outcomes with similar definitions and removing duplicates, we had 109 unique outcomes. Of those, 76.1% (83/109) of outcomes were not clearly

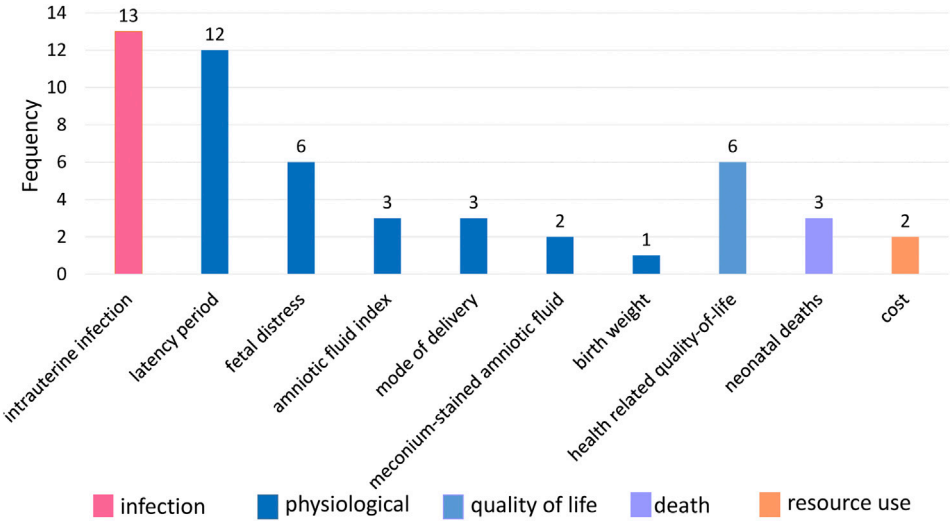

FIGURE 4 | The frequencies of outcomes extracted in the semi-structured interview.

defined and often had different definitions for the same term. For example, the definition of "latency period" was provided in 11 studies (Lockwood et al., 1993; Ernest and Givner, 1994; Grable et al., 1996; Pierson et al., 2014; Smith et al., 2015; Chang et al., 2017; Dotters-Katz et al., 2017; Sung et al., 2017; Siegel et al., 2019; Fitzgibbon et al., 2021; Kole-White et al., 2021); however, some studies meant "time from the first dose of antibiotics to delivery" (Pierson et al., 2014; Sung et al., 2017; Kole-White et al., 2021) and other studies meant "from the day of rupture of membranes to the date of delivery" (Lockwood et al., 1993; Ernest and Givner, 1994; Grable et al., 1996; Smith et al., 2015; Chang et al., 2017; Dotters-Katz et al., 2017; Siegel et al., 2019; Fitzgibbon et al., 2021).

Since the antibiotics strategy dramatically differs between PPROM and TPROM, we analyzed these subsets of pregnancy complications separately. Outcomes were categorized according to the populations in the studies reporting these outcomes, with PPROM having more outcomes than TPROM, 97 and 70, respectively.

The 97 outcomes for PPROM were grouped into maternal outcomes and neonatal outcomes. Maternal outcomes involved 33 outcomes categorized into six core domains (physiological, infection, resource use, death, adverse events, and function, from most to least). Neonatal outcomes involved 64 outcomes categorized into six core domains (physiological, resource use, infection, death, quality of life, and function, from most to least) (**Figure 3**). The physiological domain was the most common for maternal and neonatal outcomes, with the 51.5% (17/33) and 60.9% (39/64) outcomes falling into it, respectively.

Table 1 presents outcomes for PPROM with the number of reported studies (reported frequencies). **Figure 3** ranks the outcome domains by median reported frequencies from high to low. The rank for maternal outcome domains were infection, death, adverse events, physiological, function and resource use, and for neonatal domains were death, infection, resource use, physiological, quality of life, and function. Across all maternal outcomes, the top three most frequently reported outcomes were chorioamnionitis, pregnancy latency period, and mode of delivery, reported in 47.8% (43/90), 45.6% (41/90), and 30.0% (27/90), respectively of the including studies. The top three most frequently reported outcomes for newborns were neonatal sepsis, neonatal deaths, and birth weight, reported in 38.9% (35/90), 37.8% (34/90), and 36.7% (33/90) of the included studies. Nevertheless, 35.1% of outcomes (34/97, eight maternal and 26 neonatal outcomes) were reported only once in the related studies.

The 70 outcomes for TPROM were divided into maternal outcomes and neonatal outcomes. Maternal outcomes included 29 outcomes and were classified into six core domains, while neonatal outcomes included 41 outcomes classified into six core domains. Besides, the order of domains is the same as for PPROM (**Figure 3**). The physiological domain was the most common for both maternal and neonatal outcomes, with the 48.3% (14/29) and 53.7% (22/41) outcomes belonging to it, respectively.

Table 1presents the outcomes for TPROM and the number of reported studies. The rank for maternal outcome domains by reported frequencies were adverse events, infection, function, death, physiological, and resource use, and for neonatal domains were infection, death, resource use, physiological, and quality of life (**Figure 3**). The top three most frequently reported maternal outcomes were chorioamnionitis, postpartum hemorrhage, and mode of delivery, reported in 8.9% (8/90), 5.6% (5/90), and 5.6% (5/90), respectively of the included studies. And the top three most frequently reported neonatal outcomes were Apgar score, neonatal sepsis, and admission to the neonatal intensive care unit, reported in 6.7% (6/90), 5.6% (5/90), and 5.6% (5/90) of the including studies. Nevertheless, 57.1% of outcomes (40/70, 16 maternal and 24 neonatal outcomes) were reported only once in the related studies.

3.2 Semi-structured Interview

From January 2022 to February 2022, 30 pregnant women took part in the interviews. Their socioeconomic information is in **Supplementary Table S3**. Two researchers extracted 10

outcomes after normalization, and no new outcomes were obtained (**Figure 4**). The most frequently reported outcomes by PROM pregnant women were intrauterine infection (43.3%, 13/30), followed by latency period (40.0%, 12/30), fetal distress (20.0%, 6/30), and health-related quality of life and behavior (20.0%, 6/30).

DISCUSSION

To our knowledge, this is the first study to investigate study outcomes and the concerns of pregnant women on antibiotics in PROM. Our study showed a growing number of studies about antibiotics used in PROM; however, a significant inconsistency appeared in outcomes reported in antibiotics used in pregnant women with PROM. Firstly, the current studies reported many different outcomes, some of which were only reported once. Moreover, many outcomes were not clearly defined, and different definitions were frequently found for the same term. Therefore, it might not be possible to compare, contrast or combine the results of the individual studies in a systematic review to provide higher-level evidence for clinical practice (Clarke and Williamson, 2016), which contributes to waste in research (Glasziou et al., 2014). The development of the COS for antibiotics in PROM could improve the research quality of PROM and provide a reference for research about the infection in pregnant women.

Although the classification and order of the core outcome domains of PPROM and TPROM were consistent, there were some differences between the specific outcomes of PPROM and TPROM studies due to the different clinical stages of PPROM and TPROM. For example, neonatal death was one of the most concerned outcomes of PPROM researchers. However, this outcome was seldom reported in TPROM studies because pre-term birth complications are the leading cause of death among children (World Health Organization, 2018).

The outcomes identified in the including studies could cover the outcomes concerned by pregnant women. The physiological domain contained the most outcomes. Despite this, many outcomes were reported only once in studies or by pregnant women. Both the PPROM studies' researchers and the pregnant women interviewed were very concerned about the latency period. During the latency period of PROM, the fetus would be exposed to the risk of maternofetal infection, abruptio placentae, cord prolapse, and intrauterine death (Mercer, 2003). However, a large cohort study suggested that prolonged latency duration did not worsen neonatal prognosis. Moreover, survival and survival without severe morbidity improved with increased gestational age at birth (Lorthe et al., 2017). Therefore, prolonging latency if there is no contraindication was recommended in pregnant women at 24 0/7–33 6/7 weeks of gestation (Siegler et al., 2020). Nevertheless, some pregnant women's concerns, such as health-related quality of life and behavior, were rarely reported in the studies. This kind of outcome is used to assess the effect of chronic disease management on an individual's health status and is drawing the attention of researchers and policymakers (Guyatt et al., 1993). Although PROM is not a chronic disease, the sequelae of premature infants, according to PROM, require constant attention as many pre-term children develop important behavioral and educational difficulties (Bhutta et al., 2002). Future studies could pay attention to these outcomes.

Limitation and Future Research

Firstly, our study only included articles in Chinese and English, which could have a language limitation. Besides, the semi-structured interview was conducted at a single center, which could have limitations to sample representativeness. Therefore, in the next stage of this COS research, we would conduct a Delphi survey with stakeholder groups, which were based on multicenter, to add important outcomes not identified by our current study and prioritize outcomes for the COS.

CONCLUSION

An initial list of core outcomes set for antibiotics in pregnant women with prelabor rupture of membranes is formed. We identified 109 outcomes from 90 studies and a semi-structured interview. There was considerable inconsistency in outcomes selection and reporting in current studies for antibiotics in PROM. These results provide a robust foundation for the development of a COS.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

LZh and QY contributed to the conception and design of the study. DL, LW, JL, SL, and YL conducted the systematic review including screening of abstracts and full-text and extracting the data. DL, LW, CZ, and LZe conducted the semi-structured interview including data collection and data analysis. DL, LW, and LZe performed the analyses and wrote the manuscript. All authors revised it critically for important intellectual content and gave their approval of the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.915698/full#supplementary-material

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Blood vessel assessment using computed tomography : Effects of ephedrine on uterine artery

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Background: Ephedrine increased blood pressure due to the contractile properties of resistance vessels. Excessive contraction of the uterine arteries might cause fetal distress. This study was to determine the diameter of the uterine artery of female New Zealand rabbits after the administration of different doses of ephedrine using CT.

Methods: Thirty-two rabbits were randomly divided into a control group (Group C), low dosage group (Group L), medium dosage group (Group M) and high dosage group (Group H). Normal saline and doses corresponding to the human dose of 7.5, 15 and 30 mg of ephedrine were injected respectively. The marginal ear and uterine artery diameters were measured 5, 10, 15, 30, and 45 min after injection using CT, and the hemodynamic changes were recorded.

Results: The increase in mean arterial pressure in group M ($p = 0.009$), and H ($p = 0.013$) was higher than that in group C. Compared with group C, substantial contraction of the marginal ear artery was observed at the three doses of ephedrine. There were no differences in the uterine artery diameter among groups L, M and C, However, in Group H, a significant contraction of the uterine artery compared with the other groups ($p < 0.001$) was observed.

Discussion: CT can be used to evaluate the effects of drugs on organs and blood vessels. Ephedrine can not only constrict the peripheral blood vessels but also do not affect the uterine artery at a dose of 15 mg or less. However, the dose should not exceed 30 mg, which may cause severe uterine artery depression.

KEYWORDS

computed tomography, vasopressors, pharmacodynamics, uterine artery, animal models

Introduction

Spinal anesthesia is the most common method for cesarean section (Saravanan et al., 2006; Kinsella et al., 2018). However, approximately 80% of patients develop hypotension after anesthesia. The hypotension event can affect uteroplacental circulation, and impaired uteroplacental circulation is one of the main causes of severe fetal acidosis and even fetal death (Loughrey et al., 2005; Withers et al., 2009). Previous research has shown that ephedrine is beneficial to maintain uteroplacental circulation and constrict peripheral blood vessels compared with other vasoactive agents (James et al., 1970; McGrath et al., 1994). However, the dose of ephedrine needed to reverse the symptoms of hypotension varies depending on individual sensitivity (Ali Elnabtity Amand Selim, 2018; Hassani et al., 2018). With the increase in dose, the relationship between different blood vessels and ephedrine dose has rarely been reported.

Vasoactive drugs increase blood pressure owing to the contractile properties of resistance vessels. Moreover, changes in the uterine artery may be similar to those in resistance vessels under the action of vasoactive drugs. Many methods, such as umbilical artery pH and Apgar score, are used to indirectly evaluate the effect of ephedrine on the uterine artery. Additionally, Doppler ultrasound is widely used to evaluate hemodynamics, usually by measuring the vascular flow *via* the pulsatility index (PI) or resistance index. However, as the uterine artery is located deep in the abdominal cavity and surrounded by tissue, the measurement of the uterine artery is challenging and the estimation of vessel diameter changes by Doppler ultrasound is not accurate. Thus, a more direct measurement of vessel diameter is needed to quantitatively assess the effect of ephedrine. Computed tomography (CT) has demonstrated excellent penetrating ability, it can clearly show some organs and arteries, offers great potential to explore smaller branches of blood vessels, and can be used to diagnose vascular diseases such as stenosis and occlusion. Furthermore, the three-dimensional reconstruction of CT images can be utilized to accurately measure blood vessel diameter (Mon et al., 2017; Chen et al., 2014). Therefore, in this study, we observed the changes in the uterine and peripheral artery diameters using CT imaging after the injection of different doses of ephedrine.

The primary aim of this study was to explore the feasibility of measuring the uterine and peripheral artery diameter after the administration of different doses of ephedrine using CT. The secondary aim was to determine whether the maintenance effect of ephedrine on the uterine artery changes with the increase in dose. The results of this study will provide a reference for the scientific and rational use of ephedrine in the clinic, and, ultimately, improve the safety of patients and fetuses undergoing cesarean section.

Materials and methods

Research subjects

The experimental protocol was approved by the Animal Research Ethics Committee of Wannan Medical College (Approval No. 2019-018), and all procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986. In total, Thirty-two healthy non-pregnant female New Zealand rabbits (mean weight ± SD: 3.32 ± 0.21 kg; age: 5–6 months) were used in the study. All rabbits were housed in the Laboratory Animal House at a temperature of 22 ± 1°C and humidity of 55 ± 5%, under a 12-h light/dark cycle with free access to water and chow.

Anesthesia and operation

Under local anesthesia with 1% lidocaine, the left and right marginal ear veins were pierced with an indwelling needle and firmly fixed after heparinization. Anesthesia was induced in every rabbit using sodium pentobarbital (50 mg/kg; XinYu Biotechnology, Shanghai, China) and maintained by continuous infusion of sodium pentobarbital (0.05 mg/kg/min) through an injection pump (LD-P2020 Anesthesia Pump, Lande Medical Ltd., Shanghai, China). The other ear vein was used for the injection of the contract agent (ioversol injection, Hengrui Pharmaceutical Co., Ltd. Nanjing, China). When the corneal reflex and pain reflexes disappeared, the depth of anesthesia met the requirement for surgery. To eliminate the interference of the bladder to the image, urethral catheterization was performed before the experiment. The rabbit was placed on a sterile operating table, the rabbit's abdomen was sterilized, and surgical towels were spread over the abdomen. A midline laparotomy was performed, and the incision continued to the upper edge of the pubic bone. The intestinal tract, both cervices, and the uterus were observable, as was the scattered vascular structure of the uterus. Then, the uterus was separated from the intestines with a piece of sterile gauze to achieve a clear and undisturbed vascular image. To obtain the hemodynamic changes during the drug cycle, mean arterial pressure was measured using a non-invasive blood pressure measuring device for animals (NIBP220A, Ranzhe Instrument Equipment Co., Ltd., Shanghai, China). The measurement was performed in the middle of the right forelimb radius.

Angiography imaging

The 32 female rabbits were randomly divided into four groups using a free online randomization tool (http://www.randomizer.org): the control group (Group C), equivalent human dose of 7.5 mg ephedrine (Group L), 15 mg (Group

M), and 30 mg (Group H). The purpose of the design of this drug gradient was to observe the difference within the therapeutic dose. The low dose of ephedrine (0.332 mg·kg^{-1}) used in this study was converted from a human equivalent dose based on body surface area using the following formula from the US Food and Drug Administration: assuming a pregnant woman weight of 70 kg, the pregnant woman equivalent dose was 7.5 mg × 70 kg-1 (0.107 mg·kg-1) = 0.107 × 3.1 = a rabbit dose of 0.332 mg·kg-1; the conversion coefficient 3.1 was used to account for the difference in body surface area between a rabbit and a human (Chen et al., 2014; Wang et al., 2018). The doses used in groups M and H were also calculated in this way.

The contrast agents were injected into the ear veins with a dual chamber power injector (Leibs Industrial Co., ltd., Shanghai, China) at a dosage of 1.5 ml/kg, and rate of 1.5 ml/s. The head to the base of the thigh was quickly scanned using a Definition Flash 128 row dual source X-ray computed tomography machine (SOMATOM Definition Flash; Siemens Medical Solutions, Erlangen, Germany) and recorded (scan parameters: tube voltage 80 kV; tube current 310 mAs; detector width 80 mm; pitch 0.922; lamination thickness 5 mm; interval between layers 5 mm). Rabbits in groups L, M and H were then injected with the respective doses of ephedrine, and Group C was administered normal saline. Based on the blood concentration metabolism of ephedrine, CT scans were performed 5, 10, 15, 30, and 45 min after the administration of ephedrine (Persky et al., 2014). For CT scans, 741 slices were collected. The interval between every two slices was a fixed value of 0.06 mm. The total acquisition time was 39 s.

The original pictures collected from the dynamic CT were transferred to the imaging workstation. A professional processing software (Vitrea Fx6.2.3, Vital Images, Minnetonka, MN, United States) was used to analyze the data on the workstation and calculate the diameters of the uterine and marginal ear arteries (hereinafter referred to as a peripheral artery), and mark them with arrows. The rabbit marginal ear artery (representing the terminal microcirculation) is an important peripheral blood pressure regulator (Harvey and Knowles TandMurison, 2012; Ramos-Alves et al., 2012).

Each measurement was repeated twice, and the average value was recorded. All measurements were performed by a senior professional imaging doctor, who was not informed of the medication status of the rabbits. Addition, to ensure measurement accuracy, the parameters of all machines, concentration of the contrast agent, position of the measurement, and position of the rabbit were fixed. The continuous injection of anesthetics aided the latter two requirements. All rabbits were euthanized by the intravenous injection of a lethal dose of sodium pentobarbital (150 mg/kg) after image acquisition and then when breathing and heartbeat stopped, they were placed in medical waste bags and disposed of by professionals in the central laboratory.

Statistical analysis

In this study, thirty-two complete datasets were collected and used as the total sample size. The primary outcome measure was the difference in the rate of change of the uterine and peripheral artery diameters among different groups. Quantitative data are presented as mean ± standard deviation (SD). A repeated-measures analysis of variance model was used to examine group differences in measurements over time. The Greenhouse-Geisser procedure was used after checking for variance-covariance matrix sphericity assumptions. Intergroup comparisons of vessel diameter at different time points were analyzed using one-way ANOVA on ranks, using Tukey's *post-hoc* test for multiple comparisons. The effects of ephedrine infusion on hemodynamic variables was also analyzed using this method. OriginPro 2017 (OriginLab, Northampton, MA, United States) was used to draw graphics. All statistical analyses were performed using SSPS 18.0 (IBM Corporation, Armonk, NY, United States) $p < 0.05$ was considered statistically significant.

Results

All 32 rabbits provided data for image collection and measurement. The maximum intensity projection and low-density images clearly show the shape of the blood vessels of each organ and that the uterine artery is filled with contrast agent.

The effect of ephedrine on the uterine artery in the different groups is shown in Figure 1; a representative example of a dilated and a contracted uterine artery. Figure 1B shows the diameter of the initial uterine artery without medication. Observe the slight dilation of the uterine artery diameter 5 min after the equivalent 15 mg ephedrine was administered (Figure 1C). This diameter expands to a maximum after 30 min (Figure 1D). Dose of 7.5 mg and 15 mg ephedrine caused an expansion of 5% and 7%, respectively, in the uterine artery diameter after 5min compared to the initial diameter, after which a slow increase was observed, reaching a peak after 45 min with 20% and 34% expansion. After giving the equivalent 30 mg of ephedrine, the typical manifestation of the sequential contraction of the uterine artery over time is shown in Figures 1F–H. The diameter of the uterine artery contracted by 27% in 10 min in this group, and then a gradual recovery was observed.

The changes in the marginal ear arteries under the action of ephedrine are shown in Figure 2. Figure 2B is the initial blood vessel image, but after 7.5 mg of ephedrine was administered, the blood vessel contracted substantially, which made the angiography insufficient to fill the blood vessel, and the blood vessel appeared as a shadow and therefore could not be measured (Figure 2C). The same phenomenon was observed in treatment groups. Mean arterial pressure (MAP) is determined by cardiac output and peripheral resistance. Repeated measurement

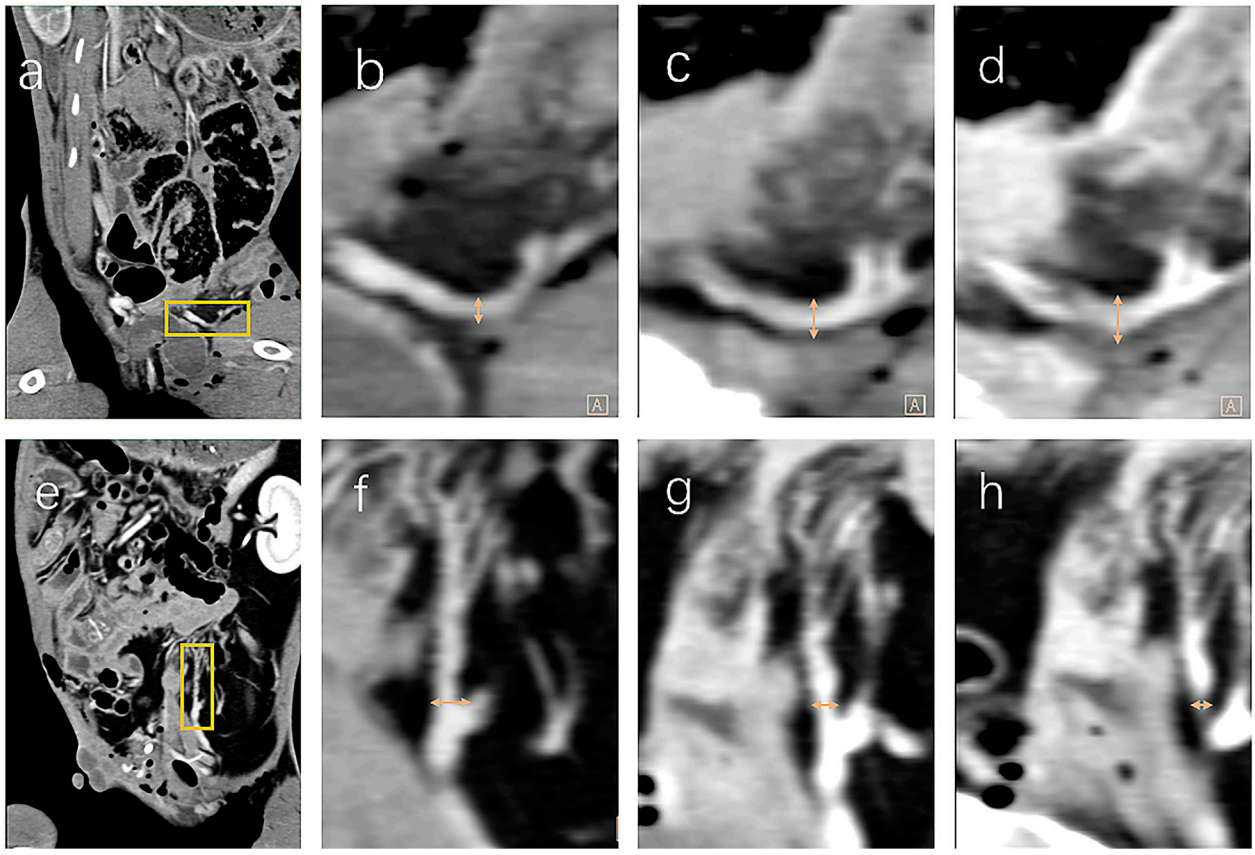

FIGURE 1
For each row (Figures 1A–H), the figures are from the same rabbits. The computed tomography sagittal plane shows the abdominal cavity of the rabbit and the uterus is marked with a yellow square (Figure 1A). After magnification, the initial distance of the uterine artery (Figure 1B), the blood vessels dilate after 15 min (Figure 1C), until the dilation is obvious at 30 min (Figure 1D). Sagittal plane scan of another rabbit with the uterus marked by the yellow square (Figure 1E). Initial distance of the uterine artery (Figure 1F), vasoconstriction at 15 min after high dose ephedrine (Figure 1G), and final severe contraction at 30 min (Figure 1H). High-density shadows in surrounding organs due to penetration of contrast media, but the blood vessels are severely stenotic, or even transiently interrupted (Figure 1H). The diameter of the uterine artery is marked with a yellow arrow.

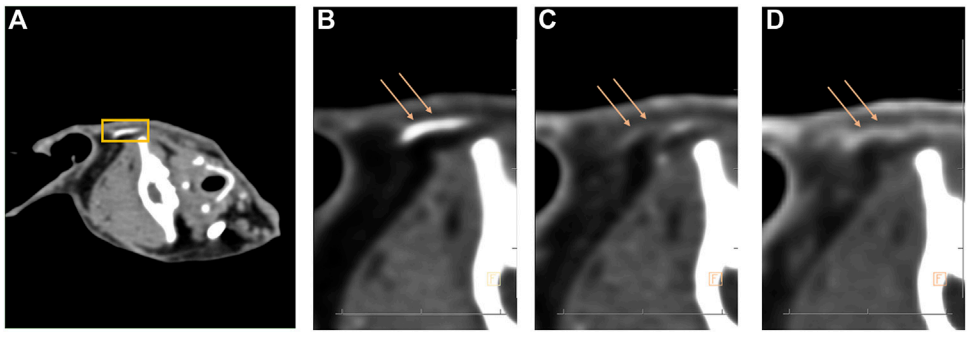

FIGURE 2
Computed tomography scans the sagittal plane of the entire skull and the marginal ear artery was marked with a yellow square (Figure 2A). The auricular artery is filled with blood vessels (Figure 2B), and the blood vessels are significantly constricted after administration of ephedrine (Figure 2C). The diameter of the marginal auricular artery partially recovered within 30 min (Figure 2D). The loss of visibility of blood vessels due to narrowing of blood vessels and insufficient filling of contrast agent (Figure 2C).

TABLE 1 Hemodynamic parameters of rabbits between groups (mean arterial pressure, MAP, mm Hg, data are expressed as means ± SD, n = 30 for each group).

	T_0	T_5	T_{10}	T_{15}	T_{30}	T_{45}
Group C	85.32 ± 6.75	88.65 ± 4.88	85.85 ± 5.95	88.14 ± 3.93	89.72 ± 5.75	84.69 ± 3.97
Group L	90.25 ± 3.85	92.62 ± 3.59[a]	95.28 ± 3.49[a,b]	97.72 ± 3.45[a,b]	94.49 ± 6.45	97.49 ± 5.58
Group M	89.14 ± 4.75	111.83 ± 6.95[a,b]	113.39 ± 5.89[a,b]	117.63 ± 4.52[a,b]	109.85 ± 4.39[a,b]	102.45 ± 7.58[b]
Group H	91.33 ± 2.18	115.41 ± 3.28[a,b]	135.15 ± 3.35[a,b]	141.49 ± 6.38[a,b]	138.28 ± 5.49[a,b]	137.49 ± 3.75[a,b]

[a]$p < 0.05$ compared with the T_0.
[b]$p < 0.05$ compared with group c.

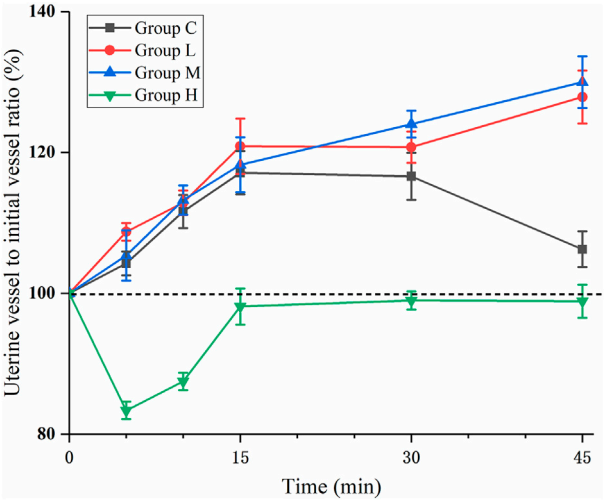

FIGURE 3
Percentage uterine artery change over time. The value is calculated as the ratio of the diameter of the uterine artery to the initial diameter at each time point. In Group H, the value decreased rapidly and reached its nadir after 5 min ($p < 0.05$ compared with C, L, and M).

analysis showed that the MAP of groups M and H had substantial changes at different time points. The difference in systolic blood pressure is most obvious between the groups at 10 min, and with the increase in dose, the blood pressure also increased (Table 1).

The diameter of the uterine artery was significantly different among groups and over time within each group ($p < 0.05$). Figure 3 shows the uterine artery diameter to initial diameter ratio over time after ephedrine injection. In groups L and M, there was no significant difference in the uterine artery diameter compared with Group C ($p = 0.82$). In Group H, the uterine artery diameter was significantly smaller than that in the other three groups ($p < 0.001$), and the diameter continued to decline within 10 min, indicating that the blood vessels were continuously contracting. The uterine artery diameter to initial diameter ratio of each sample at each time point are presented in Figure 4. After 10 min, the ratio of more than two samples in group L was larger than that in group C. Similar results were observed in Group M. In Group H, the uterine artery diameter was lower than that in the other three groups at each time point, this phenomenon was most significant at 10 min.

Discussion

In this study, the measurement of CT images showed that all three doses of ephedrine effectively constrict peripheral blood vessels after injection. Moreover, ephedrine does not interfere with the diameter of the uterine artery, or increases the diameter in some cases, at doses of 7.5 and 15 mg. However, in the 30 mg dose group, not only the peripheral artery but also the uterine artery was significantly contracted, and the inhibitory effect lasted until the end of the experiment.

Ephedrine was isolated from an herbal medicine in 1887 and has been widely used ever since. It excites adrenergic α and β receptors directly and indirectly, *via* the promotion of the release of norepinephrine from nerve endings. Additionally, ephedrine increases the contractility of the heart, expands the coronary and intracranial arteries by stimulating β-receptors, and promotes the contraction of skin, mucous membranes, and visceral blood vessels by stimulating α-receptors to increase venous return and blood pressure (Kobayashi et al., 2003; Docherty, 2008). According to previous reports, the main blood vessels supplying the uterus run within the endometrium and smooth muscle and are surrounded by an abundance of adrenergic nerves (Bower, 1966; Brauer, 2008). Some research showed that adrenergic receptors in the uterus are in a state of inhibition at different stages of pregnancy, which suggests that neurons are affected by predisposing factors, such as hormones, body fluids, or drugs (Bulat and Kannan MSandGarfield, 1989; Klukovits et al., 2002). Therefore, we speculate different doses of ephedrine affect adrenergic nerves in different locations, resulting in opposite effects in the uterus.

Similar results were obtained by Ralston et al. using a special flow tube (Ralston and Shnider SMandDeLoorimier, 1974). They measured a decrease in uterine blood flow after the infusion of a high dose of ephedrine. However, an increase in uterine blood flow was not apparent at a normal dose of ephedrine. This subtle difference with our study in the normal dose group may have been caused by the difference in sensitivity between the experimental methods.

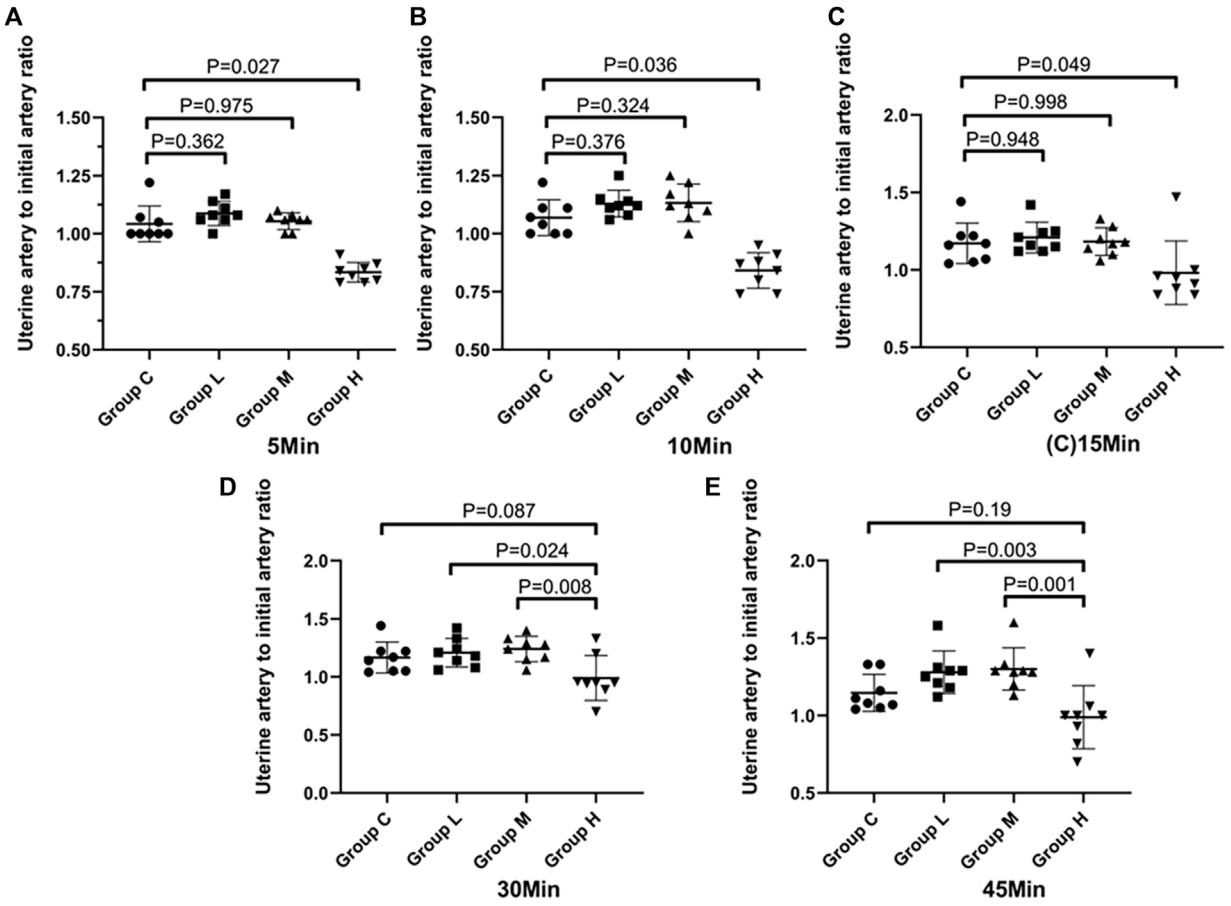

FIGURE 4
Distribution of the ratio of the diameter of the uterine artery to the initial diameter at different time points. The black line is used to link two groups with statistical significance.

The density of blood vessels and extravascular tissue is significantly different after the injection of contrast medium. CT can accurately measure subtle changes in blood vessels, which greatly improves the accuracy of measurement compared with other methods. For example Alahuhta et al. concluded that the pulsatility index (PI) does not change significantly when 5 mg of ephedrine is administered to maintain blood pressure (Alahuhta et al., 1992), whereas Ducros et al. showed that vascular resistance decreases, and flow rate increases at similar doses (Ducros et al., 2002). Although PI is a sensitive factor that reflects vascular indicators (Sun et al., 2020), cardiac contractility, blood viscosity, and the position of the Doppler probe interfere with the results, which may be the reason for the discrepancy between these studies. Therefore, the use of Doppler to evaluate the effect of ephedrine has some flaw. A recent study by Shapiro (Shapiro et al., 2020), used blood oxygen level-dependent magnetic resonance imaging (BOLD-MRI), which closely reflects oxygen delivery or extraction and has been used to accurately image the hypoxic uterus, to compare the effects of ephedrine administration on placental circulation (Wedegärtner et al., 2010). This study showed that 10–20 mg ephedrine increases the oxygen supply to the placenta, which is closely related to uterine artery dilatation. This is consistent with our conclusion that ephedrine dilates the uterine artery at this dose.

\However, there are several limitations to our study. First, the study was based on one animal models. The animal model is characterized by superficial trophoblasts on the surface of the uterine decidua, which makes the uterine artery sensitive to the sympathetic nerve (Adamson et al., 2002; Carter, 2020). In contrast, in the human womb, trophoblasts are present deep within the uterine myometrium (Brosens et al., 2002). This causes subtle changes in the uterine artery, which may change the sensitivity to vasoconstrictor drugs or sympathetic nerves (Hamzic et al., 2008; Osol GandMandala, 2009). Moreover, the

study was based on the uterine artery, and we have not discussed blood vessels, such as the placental arcuate artery and fetal umbilical cord, which are closely related to fetal health. Further studies are required to explore the effect of different doses of ephedrine in the uteroplacental circulation of pregnant women, and the differences in anesthesia methods also need further research.

In summary, CT can be used to non-invasively evaluate the changes in peripheral blood vessels over a short period of time. Additionally, this study showed that the peripheral artery contracts under the action of ephedrine, whereas the common clinical dose of ephedrine has no significant effect on the diameter of the uterine artery. However, at 30 mg, ephedrine can significantly inhibit the diameter of the uterine artery.

Data availability statement

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

Ethics statement

The animal study was reviewed and was approved by the Animal Research Ethics Committee of Wannan Medical College (Approval No. 2019-018).

Author contributions

YY, CL, and WG carried out the studies, participated in collecting data, and drafted the manuscript. JL, GG, and PZ performed the statistical analysis and participated in its design. All authors read and approved the final manuscript.

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Conflict of interest

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

Publisher's note

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