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Intra-amniální zánět u spontánního předčasného porodu se zachovalým vakem blan – klinické a experimentální aspekty

Intra-amniotic inflammation in women with preterm labor with intact membranes – clinical and experimental aspects

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DECLARATION

I declare hereby that this thesis is my own original work and that I indicated by references all used information sources. I also agree with depositing my thesis in the Medical Library of the Charles University in Prague, Faculty of Medicine in Hradec Králové and with making use of it for study and educational purpose provided that anyone who will use it for his/her publication or lectures is obliged to refer to or cite my work properly.

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ABBREVIATIONS

BMI	Body mass index
BPD	Bronchopulmonary dysplasia
CFU	Colony forming units
CRP	C-reactive protein
DAMP	Damage-associated molecular pattern
dpc	Day post conception
ELISA	Enzyme-linked immunoassay
EOS	Early-onset sepsis
FcgammaBP	IgGFc-binding protein
fFN	Fetal fibronectin
FIRS	Fetal inflammatory response syndrome
HMGB	High mobility group box
IAI	Intra-amniotic inflammation
IL	Interleukin
IQR	Interquartile range
IVH	Intraventricular hemorrhage
LOS	Late-onset sepsis
LPS	Lipopolysaccharide
MCP-1	Monocyte chemotactic protein-1
MIAC	Microbial invasion of the amniotic cavity
NF-κB	Nuclear factor kappa B
NICU	Neonatal intensive care unit
PAMG-1	Placental alpha-macroglobulin-1
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
PCT	Procalcitonin
PIGFBP-1	Phosphorylated insulin-like growth factor binding protein-1
PPROM	Preterm prelabor rupture of membranes
PTL	Preterm labor with intact membranes
RCO	Receiver operating characteristic
RDS	Respiratory distress syndrome,
ROP	Retinopathy of prematurity
PRR	Pattern recognition receptors
TLR	Toll like receptor
TNF	Tumor necrosis factor
WBC	White blood cell count
WHO	World Health Organization

SUMMARY

Preterm labor with intact membranes (PTL) is responsible for approximately 40% of all preterm deliveries. PTL is frequently complicated by intra-amniotic inflammation (IAI), characterized by the elevation of inflammatory mediators in the amniotic fluid. Based on the presence or absence of microbial invasion of the amniotic cavity (MIAC), two different clinical phenotypes of IAI are distinguished: i) intra-amniotic infection, when microorganisms are present in the amniotic fluid, and ii) sterile IAI, when there are no microorganisms in the amniotic fluid. The clinical severity of both phenotypes of IAI is underlined by their association with adverse neonatal outcomes.

In addition to the presence or absence of MIAC, there are also differences between the phenotypes of IAI in terms of their intra-amniotic inflammatory status characteristics. The clinical part of this thesis has addressed these differences in women with PTL. The first specific aim of this clinical study was to determine the concentration of interleukin (IL)-6 in the cervical fluid of women with PTL complicated by intra-amniotic infection and sterile IAI. The second specific aim was to determine the concentration of IgGFc-binding protein (FcgammaBP) in the amniotic and cervical fluids of women with PTL complicated by intra-amniotic infection and sterile IAI.

Both specific aims of the clinical part of this thesis were investigated in the same study population, consisting of 79 women with PTL. The presence of both phenotypes of IAI was associated with a higher concentration of IL-6 in the cervical fluid than the absence of IAI. However, there were no differences in the concentration of IL-6 in the cervical fluid between the phenotypes of IAI. The concentration of FcgammaBP in amniotic fluid was elevated in the presence of both phenotypes of intra-amniotic inflammation, being more pronounced in the presence of intra-amniotic infection. The concentration of FcgammaBP in the cervical fluid was not altered by the presence of either phenotype of IAI.

Animal models of IAI represent a unique tool in the research of preterm delivery, enabling the study of aspects that cannot be evaluated in human clinical studies. Therefore, the objective of this thesis was to develop a rat model of IAI established by ultrasound-guided intra-amniotic administration of an inflammatory agent. The first specific aim of the experimental part of this thesis was to perform a systematic review of literature on methods of intra-amniotic administration of infectious and/or inflammatory agents to develop a rodent model of inflammation-driven preterm delivery. The second specific aim was to assess the effect of

ultrasound-guided intra-amniotic administration of lipopolysaccharide (LPS) on the concentration of IL-6 in the amniotic fluid of rats. The third specific aim was to define a detailed protocol for ultrasound-guided intra-amniotic administration of an agent in a rat.

A systematic review of the literature revealed that intra-amniotic administration of triggering agents was used to model intra-amniotic infection/inflammation in rodents. Intra-amniotic administration under ultrasound guidance has been described in mice, but not in rats. Our experiments performed on seven rat dams showed that ultrasound-guided intra-amniotic administration of an agent was feasible in rats. Administration of 10 μg of *Escherichia coli* LPS serotype O55:B5 per gestational sac resulted in the development of IAI and did not induce labor or fetal mortality. The processes of ultrasound-guided intra-amniotic administration of an agent in a rat were summarized as a protocol to offer detailed guidelines supporting the feasibility and reproducibility of this technique for future research.

SOUHRN

Předčasný porod se zachovalým vakem blan (PTL) představuje přibližně 40 % všech předčasných porodů. PTL je často komplikován intraamniálním zánětem (IAI), který je charakterizován zvýšenou koncentrací zánětlivých mediátorů v plodové vodě. Na základě přítomnosti mikrobiální invaze do amniální dutiny (MIAC) se rozlišují dva klinické fenotypy IAI: i) intraamniální infekce, kdy jsou v plodové vodě přítomny mikroorganismy, a ii) sterilní IAI bez přítomnosti mikrobů v plodové vodě. Oba fenotypy IAI jsou spojeny s horšími neonatálními výsledky, což podtrhuje jejich klinickou závažnost.

Oba fenotypy IAI vykazují kromě přítomnosti MIAC další odlišnosti v charakteristikách intraamniálního zánětu. Těmito rozdíly se zabývá klinická část disertace. Prvním specifickým cílem klinické části bylo stanovení koncentrací interleukinu (IL) - 6 v cervikální tekutině žen s PTL komplikovaným intraamniální infekcí a sterilním IAI. Druhým specifickým cílem bylo stanovení IgGFc-binding proteinu (FcgammaBP) v amniální a cervikální tekutině žen s PTL komplikovaným intraamniální infekcí a sterilním IAI.

Oba specifické cíle klinické části této disertace byly zkoumány na stejné kohortě 79 žen s PTL. Přítomnost obou fenotypů IAI byla spojena se zvýšenými hladinami IL-6 v cervikální tekutině. Mezi jednotlivými fenotypy IAI však v koncentracích IL-6 v cervikální tekutině nebyly rozdíly. Koncentrace FcgammaBP v plodové vodě byla zvýšena u obou fenotypů IAI, výrazněji v případě intraamniální infekce. Hladiny FcgammaBP v cervikální tekutině nebyly ovlivněny přítomností žádného z fenotypů IAI.

Animální modely IAI představují jedinečný nástroj k výzkumu předčasného porodu. Umožňují studovat aspekty předčasného porodu, které nelze vyhodnotit v klinických studiích u lidí. Experimentálním cílem této práce bylo vyvinout model IAI u potkana pomocí intraamniální aplikace induktorů zánětu pod ultrazvukovou kontrolou.

Prvním specifickým cílem experimentální části bylo vypracovat systematický přehled literatury zaměřený na metody intraamniální aplikaci induktorů infekce a zánětu za účelem vytvoření zánětem indukovaného modelu předčasného porodu u hlodavců. Druhým specifickým cílem bylo posoudit účinek ultrazvukem navigované intraamniální aplikace lipopolysacharidu (LPS) na hladinu IL-6 v plodové vodě u potkanů. A třetím specifickým cílem bylo vytvořit protokol pro ultrazvukem navigované intraamniální podání induktorů zánětu u potkana.

Systematický přehled literatury ukázal, že se intraamniální podávání induktorů k modelování intraamniální infekce či zánětu u hlodavců používá. Ultrazvukem navigované intraamniální podání bylo popsáno pouze u myši, ale ne u potkana. Naše studie prováděná na sedmi samicích ukázala, že intraamniální aplikace navigovaná ultrazvukem je proveditelná u potkana. Podání 10 μg *E. coli* LPS sérotypu O55: B5 intraamniálně vedlo k rozvoji IAI a nebylo spojeno s předčasným porodem a ani s vyšší úmrtností plodů. Ultrazvukem navigovaná intraamniální aplikace induktorů zánětu u potkana byla podrobně popsána v protokolu, který podporuje proveditelnost a reprodukovatelnost této techniky pro budoucí výzkum.

1. INTRODUCTION

1.1 PRETERM DELIVERY

Preterm delivery is defined by the World Health Organization (WHO) as delivery occurring before 37 weeks of gestation [1,2]. It is a heterogeneous entity resulting from various maternal and/or fetal disorders and is a leading cause of infant morbidity and mortality [3].

The precise prevalence of preterm delivery worldwide is unknown because of the lack of data on preterm delivery in many countries [4,5]. However, based on available sources from more than 180 countries, the estimated global rate of preterm delivery is approximately 11%, with almost 15 million babies born preterm annually [3,6]. The rate varies widely between countries, with the highest rates reported in Africa, South Asia [6,7]. In European countries and other developed countries, the rate of preterm delivery is between 6% and 9% [8,9]. This heterogeneity can be explained by differences in race/ethnicity, female education, poverty level, increasing maternal age, or the number of smoking individuals [10].

It is estimated, that more than 1 million children under 5 years of age die due to preterm delivery and associated complications per year [11]. In addition, those who survive are at a greater risk of a range of short- and long-term morbidities. In general, 75% of all perinatal deaths and more than 50% of all postnatal morbidities are related to preterm delivery [6].

Adverse neonatal outcomes are inversely related to gestational age at delivery [12-14]. With respect to the fact that gestational age is a crucial determiner of neonatal outcome, preterm delivery is stratified into the following categories:

i) Extremely preterm delivery occurs before 28 weeks of gestation and accounts for 5% of all preterm deliveries.
ii) Very preterm delivery occurs between 28 and 31 weeks of gestation and accounts for 10% of all preterm deliveries.
iii) Moderate preterm delivery occurs between 32 and 34 weeks of gestation and accounts for 10%–15% of all preterm deliveries.
iv) Late preterm delivery occurs between 34 and 36 weeks of gestation and accounts for 60%–75% of all preterm deliveries [12].

One-third of preterm deliveries represent iatrogenic preterm delivery, when the delivery is medically indicated due to maternal and/or fetal complications, such as preeclampsia and fetal

growth restriction [15-17]. The remaining two-thirds of preterm deliveries represent spontaneous preterm deliveries [15,17,18]. Spontaneous preterm delivery and labor at term involve three basic clinical events—increased uterine contractility, cervical dilatation, and rupture of the fetal membranes [19]. All these events constitute the "common pathway" of labor, which is physiologically activated during labor at term. In the case of spontaneous preterm delivery, pathological processes activate one or more components of the common pathway [15,17,19].

Based on the evolving clinical presentation, two basic phenotypes of spontaneous preterm delivery can be distinguished:

i) Preterm labor with intact membranes (PTL) is defined as regular uterine contractions accompanied by cervical ripening and accounts for 40%–45% of all preterm deliveries.
ii) Preterm prelabor rupture of membranes (PPROM) is characterized by the spontaneous rupture of fetal membranes prior to the onset of uterine contractions and accounts for 30%–35% of all preterm deliveries [20].

1.2 PRETERM LABOR WITH INTACT MEMBRANES

1.2.1 Definition of preterm labor with intact membranes

PTL is defined as regular uterine contractions accompanied by cervical ripening before the gestational age of 37 weeks [15,21,22].

1.2.2 Pathophysiology of preterm labor with intact membranes

Increased uterine contractility and cervical ripening are the dominant clinical events in PTL [15]. The transformation of the quiescent myometrium into a contractile state is associated with the shift between anti-inflammatory and pro-inflammatory pathways and increased synthesis of contraction-associated proteins, such as oxytocin and prostaglandin receptors and the gap junction protein connexin 43 [19]. Cervical ripening is mediated by changes in the structure of extracellular matrix proteins. The loss of collagen cross-linking and an increase in glycosaminoglycans decrease the tensile strength of the cervix and enable cervical dilatation [19,23].

However, the underlying causes and processes of these pathways are not completely understood. Based on the current knowledge, PTL is a heterogeneous entity attributable to multiple pathological conditions and processes [17,19,24].

The main pathogenic processes leading to PTL include the following: i) infection and exaggerated inflammatory response, ii) decidual hemorrhage and vascular disease, iii) disruption of maternal-fetal tolerance, iv) uterine overdistention, and v) premature activation of the maternal or fetal hypothalamic-pituitary-adrenal axis related to stress [15,19].

1.2.2.1 *Infection and exaggerated inflammatory response*

Inflammation is a highly coordinated process that protects organisms from various infectious and non-infectious stimuli [25]. When properly controlled, inflammation is beneficial; however, when dysregulated, it is harmful [26]. Increased production of chemokines, cytokines (such as interleukin [IL]-1b, IL-6, and tumor necrosis factor-alpha [TNF-α]), prostaglandins, and proteases (such as extracellular matrix metalloproteinases) [19,27-29] is a powerful activator of the common pathway of parturition [28,29].

Infection and inflammation are the leading causes of PTL, with a prevalence of approximately 30%–40% [30,31], and will be discussed in detail in Section 1.3.

1.2.2.2 *Decidual hemorrhage and vascular disease*

Vaginal bleeding from decidual hemorrhage in the first and second trimesters is associated with an increased risk of PTL [32,33]. Thrombin generated during decidual hemorrhage stimulates myometrial contractility [34]. The generation of thrombin also appears to initiate functional progesterone withdrawal by inhibiting the expression of progesterone receptors in decidual cells [35].

The failure of physiological transformation of the spiral arteries in the myometrial and decidual segments of the placental bed is a frequent finding in cases of PTL [36]. However, it is unclear why some women with these vascular lesions develop preeclampsia and other women develop PTL [19]. Placental lesions, alone or in combination with other pathological processes, such as placental ischemia associated with fetal stress and intrauterine infection, may lead to the development of PTL [36].

1.2.2.3 *Disruption of maternal-fetal tolerance*

The fetus can be regarded as the most successful semi-allograft. Therefore, maternal immune tolerance of the fetus is essential for successful pregnancy [37,38]. A subset of women with PTL experience maternal anti-fetal cellular and antibody-mediated rejection similar to graft-versus-host disease and alloimmune reactions [39]. Maternal anti-fetal rejection is associated with maternal T-cell infiltration of the chorioamniotic membranes and chorionic plate and increased concentration of C-X-C motif chemokine ligands 9 and 10 in the amniotic fluid [40,41]. In addition, the systemic inflammatory response in fetuses born to mothers with evidence of maternal anti-fetal rejection has been described [42].

1.2.2.4 *Uterine overdistention*

Uterine overdistention due to multiple gestation, polyhydramnios, and other causes is a well-described risk factor for PTL [19]. The mechanisms by which uterine stretch can promote the development of PTL are complex. Extensive stretching of the myometrium induces production of inflammatory cytokines (such as IL-1β, TNF-α, IL-8, and IL-6) and prostaglandins in both

non-primate animal models and human maternal tissue explants [43]. Uterine overdistention also induces the formation of gap junctions [44], upregulation of oxytocin receptors [45], and production of myosin light chain kinase [46], which are critical events preceding uterine contractions and cervical dilation.

1.2.2.5 *Premature activation of the maternal or fetal hypothalamic-pituitary-adrenal axis related to stress*

Maternal stress has been identified as a risk factor for PTL [47-49]. The risk of PTL increases with the severity of stressful stimuli, ranging from heavy workload to anxiety and depression [50]. Both maternal and fetal stress signals may activate the hypothalamic-pituitary-adrenal axis, triggering a cascade of endocrinological, immunological, and vascular reactions [51]. Stress signals increase the production of maternal and fetal cortisol, which in turn can stimulate production of corticotropin-releasing hormone in the placenta [51]. When it is released into the maternal and fetal circulation, it enhances prostaglandin production by the amnion, chorion, and decidua [52]. The increase in prostaglandin levels ultimately results in parturition via increased expression of genital tract proteases (such as matrix metalloproteinases) and enhanced myometrial contractility [53].

1.2.3 Outcomes of children born preterm

PTL is responsible for 40%–45% of all preterm deliveries and makes a significant contribution to neonatal and childhood morbidity and mortality. Adverse consequences are inversely related to gestational age at delivery [12-14]. Thus, extremely preterm infants have the highest mortality rate, and surviving infants have the greatest risk of severe impairment. Nevertheless, the outcome is also modified by other factors, such as the underlying pathology that causes preterm delivery [54]. Complications of preterm infants are divided into short-term complications, which occur during the neonatal period, and long-term sequelae, which occur among patients who survive.

1.2.3.1 *Short-term complications*

Short-term complications arise from the anatomical and functional immaturity of neonates born preterm. The most common short-term complications include hypothermia, respiratory distress

syndrome (RDS), bronchopulmonary dysplasia (BPD), intraventricular hemorrhage (IVH), retinopathy of prematurity (ROP), sepsis, and necrotizing enterocolitis [13,55].

Because of their relatively large body surface and inability to produce enough heat, preterm infants have a high risk of hypothermia. Rapid heat loss occurs immediately after birth in the delivery room and on admission to the neonatal intensive care unit (NICU) [56]. In preterm infants, hypothermia is associated with an increased risk of mortality, IVH, respiratory morbidities, and metabolic disorders such as hypoglycemia and acidosis [56-58]. Specific preventive measures (such as placement into a plastic bag or body wrap) are needed to avoid heat loss during transfer to the NICU and into the incubator [59,60].

RDS is a condition of pulmonary insufficiency caused by a deficit of alveolar surfactant along with structural immaturity of the lungs [61]. It represents frequent short-term complications, with an incidence of over 90% among extremely preterm infants. The incidence of RDS decreases with the increasing gestational age at delivery, but it occurs in a significant number of late preterm infants [62,63].

BPD is a late respiratory complication that commonly occurs in preterm infants. It is defined as oxygen requirement at 28 postnatal days or 36 weeks of postmenstrual age [64,65]. The etiology of BPD is multifactorial and involves disruption of lung development and injury due to mechanical ventilation, oxygen toxicity, and infection [66-68]. Currently, BPD is the leading cause of chronic lung disease in infants and contributes to pulmonary abnormalities in adulthood [69].

IVH occurs most frequently in infants born before 32 weeks of gestation. The bleeding originates from the germinal matrix, an abundantly vascularized layer in the subependymal and subventricular regions of the brain [70]. The risk of bleeding results from the structural fragility of the germinal matrix and cerebral blood flow instability in preterm infants [71,72]. The severity of hemorrhage is based on the extent of bleeding. IVH can be clinically silent, saltatory, or catastrophic [73].

ROP is a common cause of potentially preventable childhood blindness [55]. It is a process of abnormal neovascularization in the retina of preterm infants due to hyperoxia and retinal ischemia causing visual impairment [55,74]. The incidence and severity of ROP increase with decreasing gestational age and birth weight and are rarely identified after 32 weeks of gestational age [75]. The incidence of visual impairment due to ROP has decreased

considerably since the 1990s as a result of improved screening for ROP and advances in treatment, including laser photocoagulation [76].

Neonatal sepsis is a major cause of neonatal mortality and morbidity in preterm infants [77-79]. It is classified as early-onset sepsis (EOS) that occurs within the first 72 hours of life, and late-onset sepsis (LOS) that occurs at a later time point. EOS is caused by vertical transmission of infection in utero or during vaginal delivery from bacteria colonizing the maternal lower genital tract [80]. LOS can be acquired either by maternal vertical transmission, resulting in initial neonatal colonization that evolves into later infection, or by horizontal transmission from environmental sources. EOS and LOS occur in approximately 2% and 10%–40% of infants with very low birth weight, respectively [62,79,81-83]. In preterm infants, the spectrum of symptoms of neonatal sepsis ranges from nonspecific subtle findings to fulminant septic shock with cardiovascular dysfunction, RDS, and multiorgan failure [84,85].

Taken together, the presence of short-term complications increases the risk of death and the likelihood of long-term complications among survivors [55].

1.2.3.2 *Long-term complications*

The long-term sequelae in preterm infants include abnormal neurodevelopmental outcomes, pulmonary dysfunction, visual impairment, and chronic disease in adulthood [14,86,87].

Long-term neurodevelopmental disability is a major cause of morbidity among survivors. It includes impaired cognitive skills, behavioral and psychological problems, loss of vision and hearing, motor deficits, and cerebral palsy [88,89].

Moreover, preterm adults appear to have increased insulin resistance [90,91] and blood pressure [92,93] and a decreased reproductive rate [94] compared to full-term adults. Preterm birth is associated with an increased risk of death in adulthood due to cardiovascular disease, diabetes, and chronic lung disease [77,95].

Collectively, long-term disabilities of preterm survivors commonly result in functional limitations and require additional healthcare and educational services [96]. Longitudinal data have shown that preterm delivery is associated with a lower likelihood of completing university education and a lower net salary in a stepwise manner [97,98]. Reducing the occurrence of preterm delivery can considerably reduce the burden on the society worldwide [97-99].

1.2.4 Prediction of delivery among women with symptoms of preterm labor with intact membranes

Premature uterine contractions, the main symptom of PTL, are a frequent cause of hospital admission. This creates a clinical dilemma because only one-third of women admitted to the hospital with premature uterine contractions will deliver within the following 7 days and some of them will not deliver until term [100-102]. Nevertheless, prediction of delivery within 7 days is extremely important because it allows the application of appropriate measures such as hospital admission, in utero transfer, and antenatal management, primarily including induction of fetal lung maturation [103]. Moreover, proper identification of women with a low risk of preterm delivery avoids unnecessary interventions.

Cervical length measured by transvaginal sonography in women with uterine activity has been shown to be a strong predictor of preterm delivery [102,104]. A short cervix measuring 15 mm or less in symptomatic women is associated with a high risk of delivery within 7 days [102,104]. In contrast, a long cervix measuring 30 mm or more has a high negative predictive value [105].

The unsatisfactory predictive value of cervical length interval between 15 and 30 mm can be improved by incorporating biomarkers, such as fetal fibronectin (fFN), phosphorylated insulin-like growth factor binding protein-1 (PIGFBP-1), and placental alpha-macroglobulin-1 in the cervical/vaginal fluid [103,106-108]. The advantage of qualitative fFN and PIGFBP-1 tests is their high negative predictive value [109,110].

1.3 INTRA-AMNIOTIC INFLAMMATORY AND INFECTION-RELATED COMPLICATIONS IN PRETERM LABOR WITH INTACT MEMBRANES

1.3.1 Microbial invasion of the amniotic cavity

Under normal circumstances, the amniotic fluid is sterile [111]. The presence of microorganisms in the amniotic fluid is considered a pathological finding, referred to as microbial invasion of the amniotic cavity (MIAC) [112]. Microorganisms can enter the amniotic cavity by i) ascension from the vagina and the cervix, ii) hematogenous spread through the placenta, iii) retrograde dissemination from the peritoneal cavity through the fallopian tubes, and iv) iatrogenic inoculation during invasive intrauterine procedures [15].

The most common pathway is the ascending route of the lower genital tract. This theory is supported by the fact that the microorganisms frequently found in the amniotic cavity are normally present in the lower genital tract [113], and that these microorganisms are capable of crossing intact membranes [114]. Moreover, women with vaginal dysmicrobia in the form of bacterial vaginosis are at an increased risk of PTL [115,116]. According to the ascendent theory, microorganisms from the lower genital tract pass through the endocervical canal, invade the decidua and chorioamnion, cross intact membranes, and enter the amniotic cavity [117]. Prior to MIAC, this pathway is considered to have localized decidual infection close to the uterine cervix or widespread propagation of bacteria within the chorioamnionic membranes [117-119].

The frequency of MIAC in women with PTL ranges from 16% to 40% depending on the gestational age at sampling, population, and detection techniques. Low gestational age is typically associated with a higher frequency of MIAC [31,113,117,120,121].

The most common bacteria found in the amniotic fluid of women with PTL are genital mycoplasmas, specifically *Ureaplasma* spp. and *Mycoplasma hominis*. Other bacteria such as *Fusobacterium* spp., *Sneatia* spp., *Streptococcus* spp., *Gardnerella vaginalis*, *Haemophilus influenzae*, *and Escherichia coli*, can also be involved in MIAC in PTL pregnancies. A substantial portion of PTL findings are polymicrobial [112,122].

1.3.2 Intra-amniotic inflammation

Intra-amniotic inflammation (IAI) is characterized by the elevation of many inflammatory cytokines, chemokines, antimicrobial peptides, and lipids in the amniotic fluid. Based on the presence or absence of MIAC, two different clinical phenotypes of IAI are distinguished.

i. Intra-amniotic infection, when microorganisms are present in the amniotic fluid.

ii. Sterile IAI, when the amniotic fluid is free of microorganisms.

1.3.2.1 *Intra-amniotic infection*

Intra-amniotic infection complicates approximately 11% of PTL pregnancies. The microorganisms present in the amniotic fluid activate inflammatory host responses that involve the production of cytokines and chemokines [19]. The effect is dependent on the type of microorganism and the bacterial load [123-125].

Microbes activate the innate immune system through the engagement of pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are essential PRRs that can recognize specific components of microorganisms called pathogen-associated molecular patterns (PAMPs) [126]. To date, 10 TLRs (TLR-1–10) have been identified in humans [127]. Individual types of TLRs recognize specific microbial components. TLR-2 and TLR-4 are crucial for the recognition of the most frequent microorganisms involved in MIAC. TLR-2 detects products of gram-positive bacteria and genital mycoplasmas [126,128,129], while TLR-4 is an essential receptor for recognition of lipopolysaccharide (LPS), a bacterial endotoxin of gram-negative bacteria [126,130]. TLRs are expressed in dendritic cells, monocytes/macrophages, and in a wide range of epithelial cells, including the cells of the decidua and fetal membranes [130,131]. Ligation of TLRs leads to the downstream molecular chain of events that results in the activation of nuclear factor kappa B (NF-κB), a transcription factor that regulates many pro-inflammatory and labor-associated genes [132,133]. NF-κB directly binds to the promoters of genes that cause uterine contractility and genes encoding pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-8 [133]. It has been demonstrated that both IL-1β and TNF-α stimulate NF-κB activity, causing a positive feedback loop, resulting in amplification of cytokine production in the amniotic fluid [134,135].

The presence of intra-amniotic infection in PTL is associated with a shorter latency period and a lower gestational age at delivery than the absence of IAI in PTL [30,136].

Moreover, in 30% of cases of intra-amniotic infection, the bacteria can be identified even in the fetal circulation [125,137]. This condition might trigger systemic inflammation in the fetal compartment, which is called fetal inflammatory response syndrome (FIRS) [138]. Fetuses with FIRS exhibit a higher rate of severe neonatal morbidity, such as neonatal sepsis, pneumonia, IVH, and RDS [138]. Moreover, fetal microbial invasion and FIRS can progress to multiple organ dysfunction, septic shock, and possibly death in the absence of timely delivery [111]. In addition, adverse long-term neurodevelopmental outcomes and higher rates of cerebral palsy were observed in children born to women with PTL complicated by intra-amniotic infection [139-141].

1.3.2.2 *Sterile intra-amniotic inflammation*

Sterile IAI complicates 26% of pregnancies with PTL. Thus, sterile IAI is more common than intra-amniotic infection [142]. Despite intensive research, the development of sterile IAI in PTL is not completely clear [142,143]. In the absence of bacteria in the amniotic fluid, the following conditions might lead to sterile IAI: i) damage of fetal membranes, leading to the release of endogenous molecules (damage-associated molecular patterns [DAMPs]), called alarmins, into the amniotic fluid, resulting in a subsequent inflammatory response through the PRR system [142-147]; ii) microbial colonization or infection of the choriodecidual space, which stimulates the fetal membranes to produce inflammatory mediators that are released from the fetal membranes into the amniotic fluid [148,149]; or iii) a combination of these two processes.

Alarmins released after tissue damage may represent an initiating step in the development of sterile IAI. High mobility group box-1 (HMGB-1), a prototypical alarmin [150] that is released in response to different kinds of cellular stress, was found to be significantly higher in women with PTL complicated by sterile IAI [142]. Moreover, a higher concentration of HMGB-1 in the amniotic fluid was found to be associated with a shorter amniocentesis-to-delivery interval [142]. Other alarmins, such as protein S100B and heat shock proteins, are increasingly expressed in the amniotic fluid of women with PTL [144,151]. This increase in alarmins could be the earliest event in the cascade leading to sterile IAI and pro-labor response [152].

The theory of choriodecidual space infection as a source of sterile IAI is supported by several pieces of evidence. Andrews et al. cultivated chorioamniotic membranes collected from women with PTL who delivered via cesarean section. In this study, the presence of microbes in the chorioamnion was associated with increased levels of IL-6 in the amniotic fluid, despite the

amniotic fluid culture revealing negative findings [153]. Similar results were reported by Jacobsson et al. [148]. The detection of microbes in membranes and MIAC determination was based on a combination of specific polymerase chain reaction (PCR) for genital mycoplasmas and cultivation. The amniotic fluid IL-6 and IL-8 levels were elevated in PTL patients with bacteria in the chorioamniotic membranes without MIAC [148]

The sterile IAI can be characterized by a milder intra-amniotic inflammatory response, measured by concentrations of amniotic fluid IL-6 than intra-amniotic infection [142]. Surprisingly, sterile IAI has a similar rate of adverse pregnancy and neonatal outcomes to intra-amniotic infection [142]. These facts highlight the clinical seriousness of sterile IAI in PTL pregnancies.

1.3.3 Diagnostic approaches

Intra-amniotic infection and sterile IAI complicating PTL are subclinical in most cases without any specific clinical presentation [154,155]. With the respect to their significance, it is crucial to precisely diagnose these intra-amniotic inflammatory complications.

1.3.3.1 *Assessment of amniotic fluid*

The "gold standard" for the diagnosis of intra-amniotic infection and sterile IAI is based on the examination of the amniotic fluid collected by transabdominal amniocentesis [156,157]. It enables the assessment of intra-amniotic levels of multiple mediators and is the only approach for MIAC detection.

1.3.3.1.1 Diagnostic determination of microbial invasion of the amniotic cavity

Cultivation of the amniotic fluid was considered the "gold standard" for the diagnosis of MIAC for decades. However, many microbes involved in MIAC are difficult or impossible to cultivate. Therefore, the adoption of non-cultivation, PCR-based techniques to diagnose MIAC is necessary [113,122,158]. These methods include specific PCR focusing on targeted bacteria and non-specific PCR focusing on the detection of the 16S rRNA gene.

Specific PCR is frequently used to diagnose genital mycoplasmas [122]. This technique also enables quantification of the microbial load in the amniotic fluid [124].

Other bacteria in the amniotic fluid can be identified by non-specific PCR, focusing on the 16S rRNA gene. This method detects conserved regions, which are ubiquitous in all bacteria [122]. Sequencing of the amplified variable region and sequence comparison to a reference sequence from a database enables the identification of specific microbes [122].

1.3.3.1.2. *Diagnosis of intra-amniotic inflammation*

Several markers, including white blood cell count (WBC) and levels of glucose, lactate, cytokines, chemokines, antimicrobial peptides, and lipids in the amniotic fluid, have been proposed to be useful in the identification of IAI [159-165]. Currently, the evaluation of IL-6 levels in the amniotic fluid is considered the gold standard for the diagnosis of IAI. It has been shown that IL-6 is superior to other standard amniotic fluid markers, such as WBC and glucose, but not inferior to modern proteomic markers [166].

A IL-6 level of 2600 pg/mL in the amniotic fluid, when measured by enzyme-linked immunoassay (ELISA), has been broadly accepted as a cut-off value for IAI [31,167,168]. However, the use of ELISA in clinical medicine is very limited because it takes hours to obtain results, and the results are not rapidly available for clinical management.

Therefore, there is an urgent need to introduce point-of-care tests for IL-6 assessment in the amniotic fluid in clinical practice. A point-of-care test using a lateral flow-based immunoassay offers results within 20 min [169]. For this test performed on a Milenia Quickline IL-6 analyzer (Milenia Biotec, Bad Nauheim, Germany), a cut-off value of 745 pg/mL for IAI was proposed by Romero et al. [169]. Another alternative is an automated electrochemiluminescence immunoassay method developed for use in large-volume clinical biochemistry laboratories and measuring IL-6 concentrations in body fluids in less than 20 min. The cut-off value of 3000 pg/mL for IAI using this method with the immune-analyzer Cobas e602, which is a part of the Cobas 8000 platform (Roche Diagnostics, Basel, Switzerland), was determined by our team [170].

1.3.3.2 *Assessment of other body fluids*

Despite the fact that amniocentesis in women with PTL is associated with a low complication rate [171,172], some clinicians might be reluctant to broadly apply amniocentesis in PTL management due to the invasive nature of this procedure. As amniocentesis requires expertise, the accessibility of this procedure can also be limited. Furthermore, this procedure may cause

a certain amount of discomfort in some women. Thus, all these aspects support the introduction of non-invasive testing as an alternative strategy that enables an easy, available, and rapid diagnosis. The potential directions of non-invasive testing for intra-amniotic inflammatory complications include the evaluation of maternal blood, vaginal fluid, and cervical fluid.

Several studies have reported increased maternal blood IL-6, C-reactive protein (CRP), and procalcitonin levels in association with intra-amniotic inflammatory complications in women with PTL [173,174]. However, intra-amniotic inflammatory complications elicit a much weaker inflammatory response in the maternal plasma than in the amniotic fluid of women with PTL. As they are non-specific, the inflammatory biomarkers in the maternal plasma cannot be considered an exclusive reflection of the inflammatory status of the amniotic cavity and, therefore, have limited clinical value [173-175].

The diagnostic indices of the vaginal fluid in the detection of intra-amniotic inflammatory complications of PTL have been investigated in several studies [176-178]. The most frequently assessed biomarker was IL-6; however, the findings were contradictory [179]. The concentration of cytokines in the vaginal fluid might be determined by the local vaginal microbiome rather than by the intra-amniotic environment. Therefore, neither vaginal fluid component serves as an appropriate source of potential biomarkers.

The cervical fluid, collected from the cervix of women with PTL, is a unique mixture of cervical and uterine origin. Given the close anatomical proximity between the cervix and fetal membranes, the composition of cervical fluid might reflect the microbial and inflammatory status of intra-amniotic and choriodecidual space [180]. Several cervical fluid biomarkers, including IL-6, IL-8, IL-18, IL-17, and monocyte chemotactic protein-1 (MCP-1), have been reported to be elevated in women with PTL associated with intra-amniotic inflammatory complications [181-184]. The presence of MIAC was related to the elevation of IL-6 [182,185], IL-8 [182,183,186], IL-18 [184], and MCP-1 [187] in the cervical fluid, whereas IAI was associated with elevated IL-6 [182], and IL-8 [182] in the cervical fluid.

1.3.4 Antibiotic use in preterm labor with intact membranes

Given the frequency and importance of intra-amniotic infection/inflammation, antibiotics is a logical treatment method for women with PTL. Several studies, performed approximately 20 years ago, have tested the effectiveness and safety of antibiotics; however, they failed to show any beneficial effects of antibiotic treatment in an unselected cohort of women with PTL [188-

192]. Moreover, a 7-year follow-up study showed that prescription of erythromycin for women with PTL was associated with an increase in functional impairment among their children [193]. The failure of antibiotic treatment in PTL, including unfavorable outcomes in children, could reflect the vast heterogeneity in its etiology and pathophysiology. Thus, it is unlikely that one strategy would be suitable for the treatment of all women with PTL [194].

To address this issue, tailored management seems to be an appropriate approach. Recent evidence has shown that in carefully selected patients with PTL and proven intra-amniotic infection/inflammation, broad-spectrum antibiotic treatment can successfully eradicate infection and inflammation and prolong pregnancy [195,196]. These data highlight the urgent need to better understand the processes underlying intra-amniotic inflammatory complications. The reliable detection of these complications in women with PTL, including the proper differentiation between intra-amniotic infection and sterile IAI, is an essential prerequisite for this goal.

1.3.5 Significance of intra-amniotic infection and sterile intra-amniotic inflammation in preterm labor with intact membranes

The main difference between the phenotypes of IAI is the presence or absence of MIAC. Nevertheless, distinct characteristics of the inflammatory response are also expressed between these phenotypes. Levels of amniotic fluid IL-6 are significantly higher in intra-amniotic infections than in sterile IAI [142]. In addition, women with intra-amniotic infection had higher concentrations of several inflammatory-related proteins than those with sterile IAI [197]. These data demonstrate a much stronger inflammatory response in the amniotic fluid of women with intra-amniotic infection than in that of women with sterile IAI. With respect to the fact that the confirmation/exclusion of MIAC in common clinical practice requires more than 24 hours, additional markers enabling early differentiation between intra-amniotic infection and sterile IAI might be of value.

These observations provide evidence that the inflammatory response, characterized by elevated WBC and levels of glucose and IL-6 in the amniotic fluid, differs between intra-amniotic infection and sterile IAI in PTL [142,197]. However, there is a lack of knowledge on whether the concentrations of other inflammatory mediators in amniotic and other body fluids vary between women with PTL and presence of intra-amniotic infection and those with PTL and sterile IAI.

Therefore, the clinical part of this thesis has focused on the differences in concentrations of two thoroughly selected inflammatory mediators in the amniotic fluid and the cervical fluid between women with PTL and intra-amniotic infection and those with PTL and sterile IAI.

1.4. ANIMAL MODELS OF PRETERM DELIVERY

There is a substantial lack of knowledge in the field of preterm delivery. However, many aspects cannot be addressed in human studies. Therefore, animal models play an essential role in preterm delivery [198]. Currently, there is no ideal animal model for simulating all pathways of human preterm delivery. Thus, various animal species have been used in research, all of them with specific ability to mimic processes in clinical cases of human preterm delivery and with important differences in terms of reproductive biology [199].

1.3.6 Reproductive biology of animal models of preterm delivery

1.3.6.1 *Anatomical background*

There are important anatomical differences between animal models and humans. The rodent uterus is a bicornuate with multiple fetuses arranged in a "beads-on-a-string" pattern. The placentas of rodents and humans are both anatomically classified as discoid and hemochorial. However, the placental histological structure and fetal-maternal interface differ between rodents and humans [200,201]. The major difference is the presence of the labyrinth zone in rodent placentas (an alternative to the villous architecture of the human placenta), which functions as a maternofetal exchange layer [200,201].

The uterine cavity of sheep is similar to that of humans. However, the shape of the sheep uterus is bicornuate, and it allows the maintenance of one or two fetuses per gestation [202]. The sheep placenta is cotyledonary with numerous attachment sites of the fetal cotyledons and maternal caruncles, unlike the large discoid placenta that occurs in humans. However, the human placenta is structurally divided into cotyledons too, and the villous tree of the sheep cotyledon is similar to that of the human placenta [203].

The reproductive biology of non-human primates is the most similar to that of humans in terms of uterine anatomy and litter size [204]. Non-human primates represent a near-ideal choice to study preterm delivery; however, ethical reasons and high cost limit the wider use of this animal model [198].

1.3.6.2 *Gestational period*

The difference in gestational period between species are extensive and significantly modify the utilization of animal models. The gestational period of rodents is very short, with mice delivering at 19–20 days post-conception (dpc) and rats delivering at 21–22 dpc [198]. This brief gestational period is challenging for researchers because all procedures must be carefully planned. In contrast, it shortens the study period and enables fast acquisition of results.

The gestation period in sheep is 145–147 days [198]. This fact enables longitudinal sampling and analysis of the effects of chronic fetal exposure to inflammatory and infectious agents, drugs, and surgical interventions [203].

Among non-human primates, rhesus monkeys are the most frequently used species in preterm delivery research [205,206]. The gestational period of rhesus monkeys is 160–174 days [207].

1.3.6.3 *Mechanisms of parturition*

Unlike the situation in humans, systemic withdrawal of progesterone precedes labor in many animal species. In lower mammalian species (such as mouse, rat, and rabbit), term parturition occurs after involution of the corpus luteum and a subsequent fall in circulating progesterone [208]. In mice, progesterone receptor antagonists (mifepristone) reliably cause preterm delivery within 24 hours [209]. A similar phenomenon occurs in the rat model, where preterm delivery is initiated after administration of mifepristone [210] or after ovariectomy and estrogen supplementation [211].

In sheep, fetal cortisol production increases at the end of gestation. Increased fetal cortisol levels modify steroidogenesis in the sheep placenta, resulting in increased estrogen concentrations and decreased progesterone output. This leads to parturition [212]. In a sheep model, administration of cortisol or glucocorticoids induces preterm delivery [213]. In contrast, systemic progesterone withdrawal does not seem essential for the occurrence of parturition in non-human primates [198].

1.3.7 Preterm delivery animal models associated with inflammation and infection

Investigators have focused on creating models of infection or inflammation-induced preterm delivery to mimic processes frequently observed in human preterm delivery. Numerous

infectious and inflammatory agents have been used to create models for different animals, including the following:

i) Inactivated and live microorganisms*: E. coli*, *Ureaplasma parvum,* and *Fusobacterium* spp. [214-217]

ii) PAMPs: LPS and lipoteichoic acid, a constituent of the cell wall of gram-positive bacteria [218-221]

iii) DAMPs: HMGB-1 [222]

iv) Cytokines and immune proteins: IL-1 and TNF-α [223]

The animal species used to model preterm delivery associated with inflammation/infection are rat, mouse, rabbit, sheep, and rhesus monkey. Currently, rodents (rats and mice) are the most frequently used animals because they are easy to house and treat. In addition, they are relatively inexpensive [198].

1.3.7.1 *Routes of administration to create animal model of preterm delivery associated with inflammation and infection*

Animal models of preterm delivery associated with inflammation and infection can be classified as systemic or localized [198].

1.3.7.1.1 Systemic administration

The systemic model is induced by intraperitoneal or intravenous administration of triggering agents. Because intraperitoneal or intravenous administration is relatively easy to accomplish in most species, systemic models are widely used to model preterm delivery associated with inflammation and infection. Intraperitoneal administration of LPS to mice causes systemic activation of the immune system, with a dramatic increase in maternal serum cytokines and a high rate of preterm delivery among animals. A large proportion of fetal deaths have been observed in this model, with some pups consequently reabsorbed [218,224].

Systemic illnesses (such as pyelonephritis or sepsis) can cause preterm delivery in humans, but this form of preterm delivery is rare. Most women with preterm delivery do not present with generalized symptoms (such as fever) and do not have a significant increase in WBC and CRP levels [175,225]. Therefore, systemic administration might not provide sufficient insight into

the pathways that occur in most women with preterm delivery associated with inflammation and infection.

1.3.7.1.2 Localized administration

Localized models, which include intrauterine, intra-cervical, and intra-amniotic administration of agents, are a better representation of the regional nature of human preterm delivery associated with inflammation and infection [198].

Intrauterine administration involves direct administration of a triggering agent into the choriodecidual space between the gestational sacs. This approach is frequently used in rodents. It induces a high rate of preterm delivery, similar to that induced by intraperitoneal administration, in this subgroup of animals. In addition, the pup mortality rates are comparable between both approaches [226]. In rhesus monkeys, the administration of *Streptococcus* group B into catheters placed in the choriodecidual space caused an increase in uterine contractility and preterm delivery [227]. However, in sheep, LPS administered into the choriodecidual space of the uterus did not cause an increase in uterine contractility and preterm delivery [228]. The rodent and non-human primate models of intrauterine infection/inflammation might mimic processes observed clinically in cases of immune responses occurring in the choriodecidual space of women with preterm delivery [198].

Intra-cervical administration may be comparable to the ascendent theory of the development of intra-amniotic infection in women, when microorganisms from the lower genital tract enter the chorioamnion and amniotic cavity. Endoscope-assisted intra-cervical administration of LPS and *E. coli* in mice successfully induced preterm delivery associated with infection and inflammation [229]. A similar approach failed to induce preterm delivery in rats [230]. Intra-cervical administration is difficult to achieve because the cervix in rodents is small. A trained operator is required to ensure that the inflammatory agent is not misplaced intraperitoneally [198].

The intra-amniotic administration of triggering agents constitutes a direct method of IAI induction. This represents a clinically relevant issue as both forms of IAI are frequently observed in human cases of preterm delivery [142,143]. Given the specific anatomy of the rodent uterus with multiple small gestational sacs, intra-amniotic administration may be technically challenging. In contrast, the intra-amniotic administration route is commonly used in sheep [228,231] and Rhesus monkey models [206,227].

Developing an animal model of IAI is an issue addressed in this thesis. In our view, intra-amniotic administration of a triggering agent is an optimal route for the induction of precisely defined IAI. Rodents are the most available, easy to house, and treat animal models [198]. As a low volume of the amniotic fluid in mice limits the availability of a sufficient amount for analysis [232], we consider rats as a better option to create a rodent animal model of IAI. Intra-amniotic administration can be performed via laparotomy or under ultrasound guidance [233]. However, laparotomy might represent a stress stimulus with an endocrine response that influences the function of many organs [216]. Therefore, a rat model with ultrasound-guided intra-amniotic administration of a triggering agent was chosen in this study.

2. OBJECTIVES OF THE THESIS

The objectives of this thesis were divided into two basic components. The first part, based on clinical studies in pregnant women with PTL, addresses the differences between the two phenotypes of IAI. The second part, which was experimental, focuses on the establishment of an animal model of IAI as a unique and irreplaceable tool in the research of preterm delivery.

2.1 CLINICAL OBJECTIVES

The clinical objective of this thesis was to determine the levels of selected inflammatory mediators in the amniotic fluid and the cervical fluid of women with PTL with respect to the presence of both phenotypes of IAI—intra-amniotic infection and sterile IAI.

There were two specific aims of the clinical part:

I-A. To determine the concentration of IL-6 in the cervical fluid of women with PTL complicated by intra-amniotic infection and sterile IAI

I-B. To determine the concentration of IgGFc-binding protein (FcgammaBP) in the amniotic fluid and the cervical fluid of women with PTL complicated by intra-amniotic infection and sterile IAI

2.2 EXPERIMENTAL OBJECTIVES

The experimental objective of this thesis was to develop a rat model of IAI established by ultrasound-guided intra-amniotic administration of an inflammatory agent.

There were three specific aims of the experimental part:

II-A. To perform a systematic review of available methods of intra-amniotic administration of infectious and/or inflammatory agents to create a rodent model of inflammation-driven preterm delivery

II-B. To assess the effect of ultrasound-guided intra-amniotic administration of LPS on the concentration of IL-6 in the amniotic fluid in rats

II-C. To develop a step-by-step protocol for ultrasound-guided intra-amniotic administration of an agent in a rat to support the reproducibility and feasibility of this approach

3. SET OF PATIENTS, METHODS AND STATISTICAL ANALYSIS

3.1 CLINICAL OBJECTIVES

The specific aims of the clinical part of the thesis were derived from the same cohort of patients. Most of the methodology was identical for both aims, and the statistical analysis was based on the same approach. Therefore, the clinical objectives have been described together, with an emphasis on items specific to a particular aim.

3.1.1 Set of patients

This retrospective cohort included pregnant women who were admitted to the Department of Obstetrics and Gynecology at the University Hospital Hradec Kralove in the Czech Republic between March 2017 and May 2020.

The inclusion criteria were as follows: 1) singleton pregnancy, 2) maternal age ≥ 18 years, 3) gestational age between 22+0 and 36+6 weeks, 4) PTL, and 5) the performance of transabdominal amniocentesis at the time of admission to determine IAI.

The exclusion criteria were as follows: 1) pregnancy-related and other medical complications such as fetal growth restriction, gestational or pre-gestational diabetes, gestational or chronic hypertension, and preeclampsia; 2) structural or chromosomal fetal abnormalities; 3) signs of fetal hypoxia; and 4) significant vaginal bleeding.

Gestational age was determined by first-trimester fetal biometry. PTL was diagnosed as the presence of regular uterine contractions (at least two contractions every 10 minutes) and cervical length, measured using transvaginal ultrasound, shorter than 15 mm or within the 15–30 mm range with a positive PartoSure test (Parsagen Diagnostics Inc., Boston, MA, USA) result [106].

3.1.2 Methods

3.1.2.1 *Sample collection*

Paired cervical fluid and amniotic fluid samples were collected at the time of admission from all women included in this study prior to the administration of antibiotics, tocolytics, and/or

corticosteroids. Each cervical fluid sample was obtained by placing a Dacron polyester swab in the cervical canal for 20 seconds to achieve saturation. Once collected, the polyester swab was inserted into a polypropylene tube containing 1.5 mL of phosphate-buffered saline; the tube was then shaken for 20 min. On removal of the polyester swab, the tube was centrifuged at 300 × *g* for 15 min at room temperature. The supernatant was divided into aliquots and stored at −80°C until further analysis.

Ultrasonography-guided transabdominal amniocentesis was performed after cervical fluid sampling. Approximately 2–3 mL of the amniotic fluid was aspirated, and the amniotic fluid was immediately divided into polypropylene tubes. The amniotic fluid samples were used for the following: i) the assessment of amniotic fluid IL-6; ii) PCR analysis of *Ureaplasma* spp., *Mycoplasma hominis*, and *Chlamydia trachomatis*; iii) sequencing of the 16S rRNA gene; iv) aerobic and anaerobic cultivation; v) stored at −80°C until further analysis.

3.1.2.2 *Sample assessment*

3.1.2.2.1 Assessment of interleukin-6 in the amniotic fluid

The concentration of IL-6 in the amniotic fluid (fresh samples) was assessed using an automated electrochemiluminescence immunoassay method. IL-6 concentrations were measured using an immuno-analyzer Cobas e602, which is part of the Cobas 8000 platform (Roche Diagnostics, Basel, Switzerland) [234]. The basic measuring 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. The coefficients of variation for the inter-assay and intra-assay precisions were <10%.

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

DNA was isolated from the amniotic fluid using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Real-time PCR was conducted on a Rotor-Gene 6000 instrument (Qiagen) using the commercial AmpliSens® *C. trachomatis*/*Ureaplasma*/*M. hominis*-FRT kit (Federal State Institution of Science, Central Research Institute of Epidemiology, Moscow, Russia) to detect the DNA from *Ureaplasma* spp., *M. hominis*, and *C. trachomatis* in the same PCR tube (multiplex format). We included a

PCR run for beta-actin, a housekeeping gene that served as the control, to examine the presence of PCR inhibitors.

3.1.2.2.3 Non-cultivation detection of other bacteria in the amniotic fluid

Bacterial DNA was identified by PCR targeting the 16S rRNA gene with the following primers: 5'-CCAGACTCCTACGGGAGGCAG-3' (V3 region) and 5'-ACATTTCACAACAC-GAGCTGACGA-3' (V6 region) [235,236]. Each reaction contained 3 μL of target DNA, 500 nM forward and reverse primers, and Q5 High-Fidelity DNA polymerase (NEB, Ipswich, MA, USA) in a total volume of 25 μL. Amplification was performed using a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The products were visualized on an agarose gel. Positive reactions yielded 950-bp products that were subsequently analyzed by sequencing. The 16S rDNA PCR products were purified and subjected to sequencing using the abovementioned primers and the BigDye Terminator kit v.3.1 (Thermo Fisher Scientific). The bacteria were then identified via searches for the obtained sequences using BLAST® and SepsiTest™ BLAST.

3.1.2.2.4 Aerobic and anaerobic cultures of amniotic fluid

The amniotic fluid samples were cultured on Columbia agar with sheep's blood, *Gardnerella vaginalis* selective medium, MacConkey agar, a *Neisseria*-selective medium (modified Thayer-Martin medium), Sabouraud agar, or Schaedler anaerobe agar. The plates were cultured for 6 days and checked daily. The species were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using MALDI Biotyper software (Bruker Daltonics, Bremen, Germany).

3.1.2.2.5 Assessment of interleukin-6 in the cervical fluid (specific aim I-A)

The concentration of IL-6 in the cervical fluid (samples with one freezing/thawing cycle) was assessed using the automated electrochemiluminescence immunoassay method. IL-6 concentrations were measured using immuno-analyzer Cobas e602, which is part of the Cobas 8000 platform (Roche Diagnostics, Basel, Switzerland) [234]. The basic measuring 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. The coefficients of variation for inter-assay and intra-assay precisions were both <10%.

3.1.2.2.6 Assessment of FcgammaBP in the amniotic fluid and the cervical fluid (Specific aim I-B)

The concentrations of FcgammaBP were assessed in the amniotic fluid and the cervical fluid (samples with one freezing/thawing cycle) using an ELISA, the Human FCGBP ELISA Kit (LifeSpan BioSciences, Inc., Seattle, WA, USA), according to the manufacturer's instructions. The amniotic fluid and cervical fluid samples were diluted 10-fold and 50-fold, respectively. The sensitivity of the kit was 0.117 ng/mL. The absorbance values were read at 450 nm using a Multiskan RC ELISA reader (Thermo Fisher Scientific, Waltham, MA, USA).

3.1.3 Clinical definitions

3.1.3.1 *Microbial invasion of the amniotic cavity*

MIAC was determined based on a positive PCR result for *Ureaplasma* spp., *M. hominis*, *C. trachomatis*, or a combination of these species or positivity for the 16S rRNA gene, findings of aerobic/anaerobic culture of the amniotic fluid, or a combination of these parameters.

3.1.3.2 *Intra-amniotic inflammation*

IAI was defined as an IL-6 concentration of ≥3000 pg/mL in the amniotic fluid [237].

3.1.3.3 *Intra-amniotic infection*

Intra-amniotic infection was defined by both MIAC and IAI.

3.1.3.4 *Sterile intra-amniotic inflammation*

Sterile IAI was defined as the presence of IAI without concomitant MIAC.

3.1.3.5 *Negative amniotic fluid*

Negative amniotic fluid was defined as the absence of MIAC and IAI.

3.1.4 Statistical analyses

The normality of the data was tested using the Anderson-Darling test.

The women's demographic and clinical characteristics were compared using a nonparametric Mann-Whitney *U* test for continuous variables and Fisher's exact test for categorical variables and are presented as median values (interquartile range [IQR]) and as number (%), respectively.

Because the concentrations of all studied inflammatory mediators were not normally distributed, the nonparametric Kruskal-Wallis and Mann-Whitney *U* tests were used for the analyses, as appropriate, and the results are presented as median values (IQR). Spearman partial correlation was performed to adjust the results for gestational age at sampling. Spearman correlation was used to assess the relationship between the concentrations of the evaluated inflammatory mediators in the amniotic fluid or the cervical fluid and gestational age at sampling. Receiver operating characteristic (ROC) curves were constructed to assess the predictive values of selected inflammatory mediators in the amniotic fluid and the cervical fluid for the presence of intra-amniotic infection.

All *p*-values were obtained using two-tailed tests. Differences were considered significant at $p < 0.05$. All statistical analyses were performed using the IBM SPSS Statistics for Mac OS version 27.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 9 for Mac OS X (GraphPad Software, San Diego, CA, USA).

3.2 EXPERIMENTAL OBJECTIVES

3.2.1 Specific aim II-A

3.2.1.1 *Search strategy*

This systematic review was conducted in accordance with PRISMA guidelines [238]. We searched for studies that employ intra-amniotic administration of infectious or inflammatory agents to establish a rodent model of inflammation-driven preterm delivery. The search was conducted in two electronic databases (PubMed and Scopus) on February 2, 2019. The following search terms and synonyms were used, adapted according to each database: (animal OR animals OR rat OR rats OR mouse OR mice OR hamster OR hamsters OR rodent OR rodents) AND (preterm birth OR preterm delivery OR preterm labor OR preterm labour) AND (intra-amniotic OR intraamniotic OR intraamniale OR ultrasound-guided OR IA OR injection OR application OR administration). Search for unpublished studies was not performed. All identified citations were downloaded into the Rayyan web app tool for systematic reviews [239], and duplicates were excluded. Using the Rayyan app, the titles and abstracts of unique citations were screened independently by two reviewers, and potentially relevant studies were selected for full-text reading, and those that fulfilled the selection criteria were included in the review.

3.2.1.2 *Selection criteria for studies*

Type of studies: Studies that used a rodent model of inflammation-driven preterm delivery initiated by intra-amniotic administration of infectious or inflammatory agents were considered eligible. Primary experimental case-control studies were included.

Type of outcomes: preterm delivery, intra-amniotic infection, sterile IAI, intra-uterine (histological chorioamnionitis) inflammation, and inflammatory complications.

3.2.1.3 *Exclusion criteria for studies*

Studies were excluded if any of the following applied: 1) human or in vitro studies, 2) animal models other than rodents, 3) models of preterm delivery other than inflammation-driven

preterm delivery, 4) routes of administration of infectious or inflammatory agents other than administration into the gestational sac, and 6) rodent models to study other conditions or diseases without relation to preterm delivery.

3.2.1.4 *Data extraction*

The following data were extracted from each article included in this review: author, year of publication, study methodology, information about the study animals and their type (number, species, strain), information about the timing of intervention, description of technique of intra-amniotic administration, information about the infectious or inflammatory agent used, and outcomes of the study.

3.2.1.5 *Quality assessment*

For quality assessment of identified studies, the checklist of the CAMARADES group was adjusted [240]. The criteria of quality assessment were as follows: (1) provided description of used animals (species, strain, and origin of animal), (2) calculation of sample size (3), description of randomized allocation to the treatment or control group; (4) description of the intervention (technique for intra-amniotic administration, number of gestational sacs injected), and (5) thorough description of infectious or inflammatory agent. One point was assigned per item, and the final quality score was the sum of particular items.

3.2.2 Specific aim II-B

3.2.2.1 *Experimental animals*

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 Czech Ministry of Education Youth and Sports (No. 41058/2016-MZE-17214). The study was approved by the animal welfare body of the Faculty of Medicine in Hradec Králové and the Institutional Review Board of the University Hospital Hradec Králové.

Pregnant Wistar rats were purchased from Velaz laboratory (Prague, Czech Republic) and bred in the animal care facility of the Faculty of Medicine in Hradec Králové. All animals were kept

in an environment with a steady temperature of 22°C ± 2°C and relative air humidity of 50% ± 10% under a circadian cycle (light:dark = 12:12 h). The animals were fed by a classical pellet diet with water available ad libitum. The day one of gestation was defined as the morning of the vaginal plug.

3.2.2.2 *Methods*

3.2.2.2.1 Ultrasound-guided intra-amniotic administration

On embryonic day 18, pregnant Wistar rats were anesthetized by inhalation of 5% isoflurane with 2 L/min of oxygen in the induction chamber. The animals were positioned and fixed on a heating pad of Vevo Imaging Station (FUJIFILM VisualSonics Inc., Toronto, ON, Canada). Anesthesia was maintained with 1.5%–2% isoflurane and 2 L/min of oxygen. Body temperature was maintained at 37°C ± 1°C and measured using a rectal probe (FUJIFILM VisualSonics Inc., Toronto, ON, Canada). The heart and respiratory rates were monitored using electrodes embedded in the heating pad. The fur was removed using depilatory cream. An ultrasound transducer MX250S (15-30 MHz) of a Vevo 3100 ultrasound machine (FUJIFILM VisualSonics Inc., Toronto, ON, Canada) was placed in the transducer holder (FUJIFILM VisualSonics Inc., Toronto, ON, Canada). Mapping of the number and localization of pups was performed and recorded. The ultrasound transducer was replaced by the MX400 transducer (20–46 MHz) and intra-amniotic administration of 10 µg of *E. coli* LPS (serotype O55:B5, Sigma-Aldrich, Prague, Czech Republic) in 100 µL of phosphate-buffered saline (PBS) was performed using a 27 G × 40 mm needle (B. Braun Melsungen, Germany) with a Omnican® 50 syringe (B. Braun, Melsungen, Germany) stabilized in a holder. Controls were injected with 100 µL PBS alone. Each accessible gestational sac was injected and its localization was recorded. Following the procedure, the animals were kept under a heat lamp for recovery and then returned to their cages.

3.2.2.2.2 Sample collection

On embryonic day 19 and 24 hours after ultrasound-guided intra-amniotic administration, the animals were anesthetized and prepared for the ultrasound examination in the same way as for intra-amniotic administration. Using the ultrasound transducer MC250 (15–30 MHz), the position of the gestational sac and the vitality of the pups were assessed. The uterine horns were then exposed using a midline abdominal incision. Prior to any manipulation of the uterine horns,

the injected sacs were identified with respect to localization recorded a day before during the administration. After identification, both uterine horns were removed from the abdominal cavity. Using a sterile 30 G × 13 mm needle (B. Braun Melsungen, Germany), the amniotic fluid was harvested from all sacs and stored in polypropylene tubes at -70°C until analysis. After the procedure, the animals were sacrificed by exsanguination under anesthesia.

3.2.2.2.3 Sample assessment

The 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, USA), 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. Absorbance was measured at 450 nm using a Multiskan RC ELISA reader (Thermo Fisher Scientific, Waltham, MA, USA).

3.2.2.3 *Statistical analyses*

The normality of the data was tested using the Anderson-Darling test. Because the IL-6 levels in the amniotic fluid were not normally distributed, non-parametric Mann-Whitney *U* tests were used for the analyses, as appropriate. All *p*-values were obtained using two-tailed tests.

All statistical analyses were performed using GraphPad Prism version 9 for Mac OS X (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $p < 0.05$.

3.2.3 Specific aim II-C

3.2.3.1 *Protocol development*

All steps of the entire process of ultrasound-guided intra-amniotic administration of an agent performed on the experimental animals used in Specific aim II-B were recorded and summarized in the protocol.

4. RESULTS

4.1 CLINICAL OBJECTIVES

4.1.1 Clinical characteristics of study population

A total of 79 women with singleton pregnancies with PTL were included in the study. IAI was found in 42% (33/79) of the women; intra-amniotic infection and sterile IAI were observed in 15% (12/79) and 27% (21/79) of the women, respectively. Demographic and clinical data are shown in Table 1.

MIAC was revealed only in women with IAI. Polymicrobial findings in the amniotic fluid were identified in three women (*Klebsiella pneumoniae* + *Streptococcus anginosus,* n = 1; *Gardnerella vaginalis* + *Lactobacillus plantus*, n = 1; and *Ureaplasma* spp. + *Mycoplasma hominis*, n = 1). The remaining nine women had only one microorganism in the amniotic fluid (*Ureaplasma* spp., n = 4; *Haemophilus influenzae*, n = 1; *Lachnoanaerobaculum* species, n = 1; *Lactococcus lactis*, n = 1; *Sneathia sanguinegens*, n = 1; and non-identifiable bacteria via sequencing, n = 1).

Table 1.

Demographic and clinical characteristics of women with spontaneous preterm labor with intact membranes with intra-amniotic infection, with sterile intra-amniotic inflammation, and negative amniotic fluid.

Characteristic	Intra-amniotic infection (n=12)	Sterile intra-amniotic inflammation (n=21)	Negative amniotic fluid (n=46)	*p*-value[1]	*p*-value[2]	*p*-value[3]
Maternal age [years, median (IQR)]	27 (24-28)	26 (22-29)	28 (24-31)	0.56	0.58	0.21
Primiparous [number (%)]	7 (58%)	17 (81%)	30 (65%)	0.23	0.74	0.26
Body mass index [kg/m^2, median (IQR)]	27.5 (23.1-30.6)	23.8 (22.4-25.4)	26.0 (23.7-28.2)	0.22	0.77	**0.03**
Smoking [number (%)]	1 (8%)	4 (19%)	3 (7%)	0.63	1.00	0.19
Gestational age at admission [weeks, median (IQR)]	27+6 (24+6-31+2)	26+6 (24+2-31+1)	31+2 (29+1-32+5)	0.78	**0.03**	**0.006**
Gestational age at delivery [weeks, median (IQR)]	29+0 (27+1-33+3)	27+5 (25+1-32+4)	34+0 (32+0-37+2)	0.49	**0.0006**	**<0.0001**
Interval from amniocentesis to delivery [days, median (IQR)]	2 (0-15)	2 (0-5)	15 (1-47)	0.81	**0.03**	**0.005**
Delivery within 7 days from amniocentesis [number (%)]	9 (75%)	18 (86%)	20 (43%)	0.64	0.10	**0.001**
Amniotic fluid IL-6 levels at admission [pg/mL, median (IQR)]	43,431 (23,597-50,000)	9,464 (4,279-50,000)	848 (286-1,683)	**0.02**	**<0.0001**	**<0.0001**
CRP levels at admission [mg/L, median (IQR)]	42.0 (7.5-75.08)	9.2 (5.5-18.0)	4.6 (2.0-9.8)	0.05	**0.0002**	**0.003**
WBC count at admission [x10^9 L, median (IQR)]	16.3 (14.1-19.5)	16.2 (12.3-19.36)	13.31 (10.3-15.6)	0.70	**0.01**	**0.02**
Administration of corticosteroids [number (%)]	9 (75%)	20 (95%)	42 (91%)	0.13	0.15	1.00
Spontaneous vaginal delivery [number (%)]	9 (75%)	18 (86%)	37 (80%)	0.64	0.70	0.74
Cesarean delivery [number (%)]	3 (25%)	3 (14%)	8 (17%)	0.64	0.68	1.00
Forceps delivery [number (%)]	0 (0%)	0 (0%)	1 (2%)	-	1.00	1.00
Birth weight of the newborn [grams, median (IQR)]	1230 (936-1958)	1180 (720-2045)	2060 (1803-2845)	0.73	**0.0007**	**<0.0001**
Apgar score <7; 5 minutes [number (%)]	3 (25%)	5 (24%)	2 (4%)	1.00	0.05	**0.03**
Apgar score <7; 10 minutes [number (%)]	2 (17%)	3 (14%)	0 (0%)	1.00	**0.04**	**0.03**

Abbreviations:
CRP: C-reactive protein
IQR: interquartile range
WBC: white blood cells

Continuous variables were compared using a nonparametric Mann-Whitney *U* test. Categorical variables were compared using the Fisher's exact test. Continuous variables are presented as median (IQR) and categorical as number (%).
Statistically significant results are marked in bold.

p-value[1] - comparison between women with intra-amniotic infection and sterile intra-amniotic inflammation
p-value[2] - comparison between women with intra-amniotic infection and negative amniotic fluid
p-value[3] - comparison between women with sterile intra-amniotic inflammation and negative amniotic fluid

4.1.2 Specific aim I-A

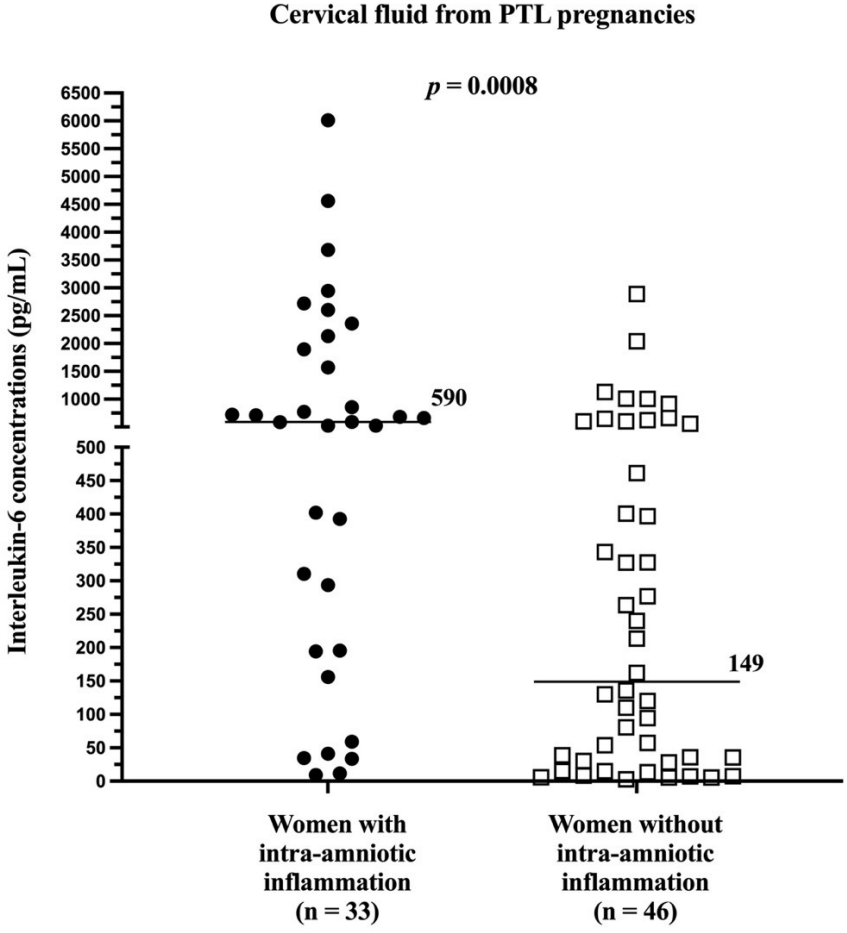
4.1.2.1 *Concentrations of IL-6 in the cervical fluid according to the presence of intra-amniotic inflammation*

Concentrations of IL-6 were measurable in all samples of cervical fluid. A positive correlation was found between the concentrations of IL-6 cervical and amniotic fluids (rho = 0.46; $p < 0.0001$).

Women with IAI had higher concentrations of IL-6 in the cervical fluid than those without IAI in the crude analysis (median, 590 pg/mL; IQR, 195 - 2014 vs. without: median 149 pg/mL, IQR 30 – 569; $p = 0.0008$; Figure 1) as well as after adjustment for gestational age at sampling ($p = 0.004$).

Figure 1.

Cervical fluid interleukin-6 concentrations based on the presence or absence of intra-amniotic inflammation.



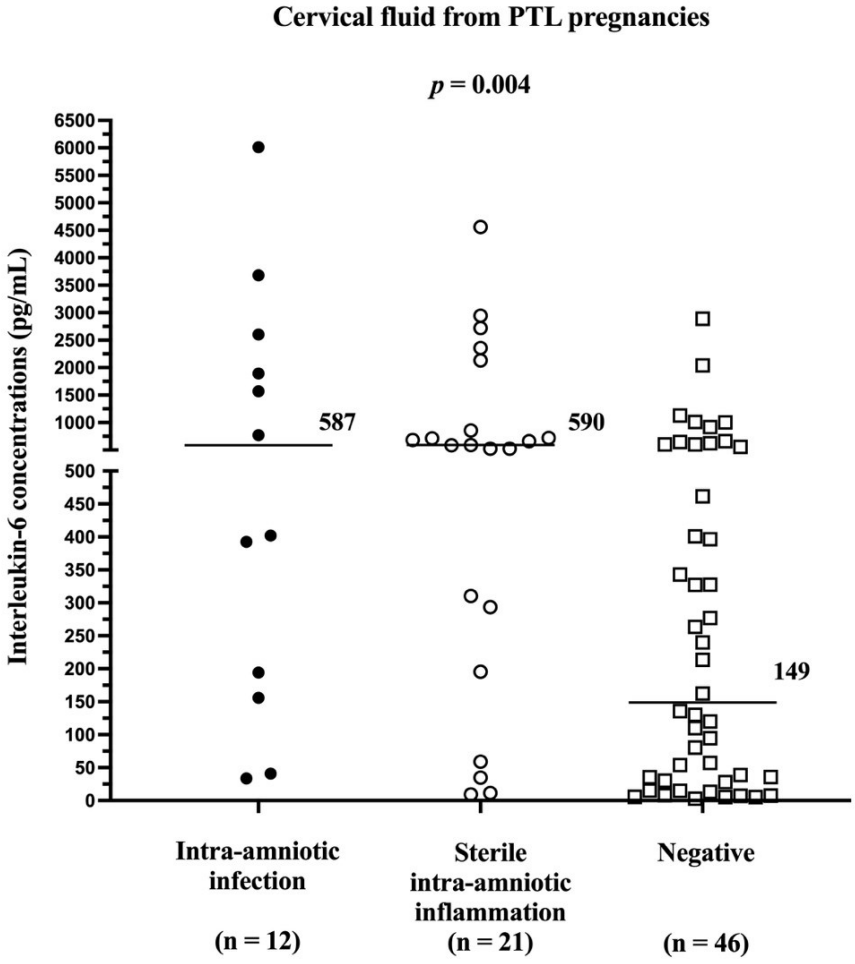
Abbreviations: PTL, preterm labor with intact membranes

4.1.2.2 *Concentrations of IL-6 in the cervical fluid according to the presence of intra-amniotic infection and sterile IAI*

Differences in the concentrations of IL-6 in the cervical fluid were found among the subgroups of women with PTL in crude analysis (intra-amniotic infection: median, 587 pg/mL; IQR, 166 – 2427; sterile IAI: median 590 pg/mL; IQR, 245 – 1495; with negative amniotic fluid, 149 pg/mL; IQR, 30 – 569; $p = 0.004$; Figure 2), as well as after the adjustment for gestational age at sampling ($p = 0.002$).

Figure 2.

Cervical fluid interleukin-6 concentrations among the subgroup of women with intra-amniotic infection, sterile intra-amniotic inflammation, and with negative amniotic fluid.



Abbreviations: PTL, preterm labor with intact membranes

Women with intra-amniotic infection and sterile IAI had higher concentrations of IL-6 than those with negative amniotic fluid (Table 2). No differences in the concentrations of IL-6 in the cervical fluid were found between women with intra-amniotic infection and sterile IAI (Table 2).

Table 2.

Cervical fluid IL-6 – the comparisons among the subgroups of the women with intra-amniotic infection, with sterile intra-amniotic inflammation, and negative amniotic fluid.

	Intra-amniotic infection	**Sterile intra-amniotic inflammation**	**Negative**
Intra-amniotic infection	x	$p = 0.81$ adj. $p = 0.38$	**$p = 0.01$** **adj. $p = 0.004$**
Sterile intra-amniotic inflammation	$p = 0.81$ adj. $p = 0.38$	x	**$p = 0.005$** **adj. $p = 0.003$**
Negative	**$p = 0.01$** **adj. $p = 0.004$**	**$p = 0.005$** **adj. $p = 0.003$**	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 age at sampling (a Spearman partial correlation)

Statistically significant results are marked in bold.

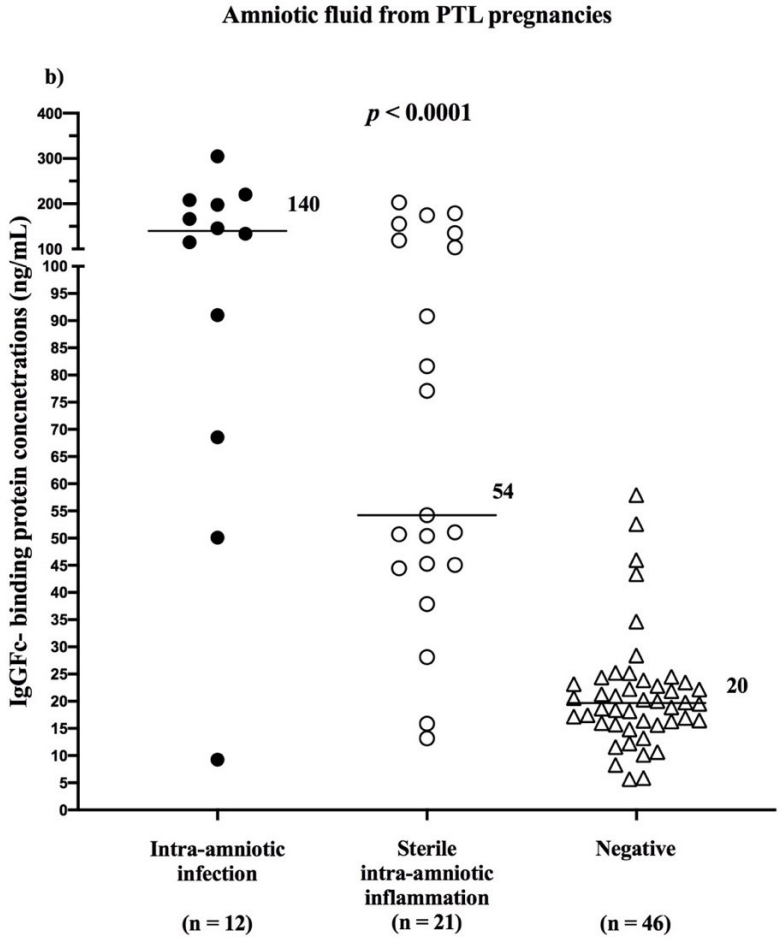
4.1.3 Specific aim I-B

4.1.3.1 *Concentrations of FcgammaBP in the amniotic fluid based on the phenotype of IAI*

Differences in the concentrations of FcgammaBP were identified among the subgroups of women with intra-amniotic infection, sterile IAI, and negative amniotic fluid (infection: median 139.7 ng/mL, IQR 74.2-205.3; sterile: median 54.2 ng/mL, IQR: 44.8-127.0; negative: median 19.7 ng/mL, IQR: 15.9-23.6; Figure 3) in the crude analysis and after the adjustment for gestational age at sampling (both $p < 0.0001$).

Figure 3.

Amniotic fluid IgGFc-binding protein concentrations in the subgroups of the women with PTL



Abbreviations: PTL, preterm labor with intact membranes

Women with intra-amniotic infection had higher amniotic fluid FcgammaBP concentrations than did women with sterile IAI and with negative amniotic fluid (Table 3). Women with sterile IAI had higher amniotic fluid FcgammaBP concentrations than those with negative amniotic fluid (Table 3).

Table 3.

IgGFc-binding protein in amniotic fluid from preterm labor with intact membranes: the comparisons among the subgroups of the women with intra-amniotic infection, sterile intra-amniotic inflammation, and negative amniotic fluid.

	Intra-amniotic infection	**Sterile intra-amniotic inflammation**	**Negative**
Intra-amniotic infection	x	**$p = 0.04$** **adj. $p = 0.02$**	**$p < 0.0001$** **adj. $p < 0.0001$**
Sterile intra-amniotic inflammation	**$p = 0.04$** **adj. $p = 0.02$**	x	**$p < 0.0001$** **adj. $p < 0.0001$**
Negative	**$p < 0.0001$** **adj. $p < 0.0001$**	**$p < 0.0001$** **adj. $p < 0.0001$**	x

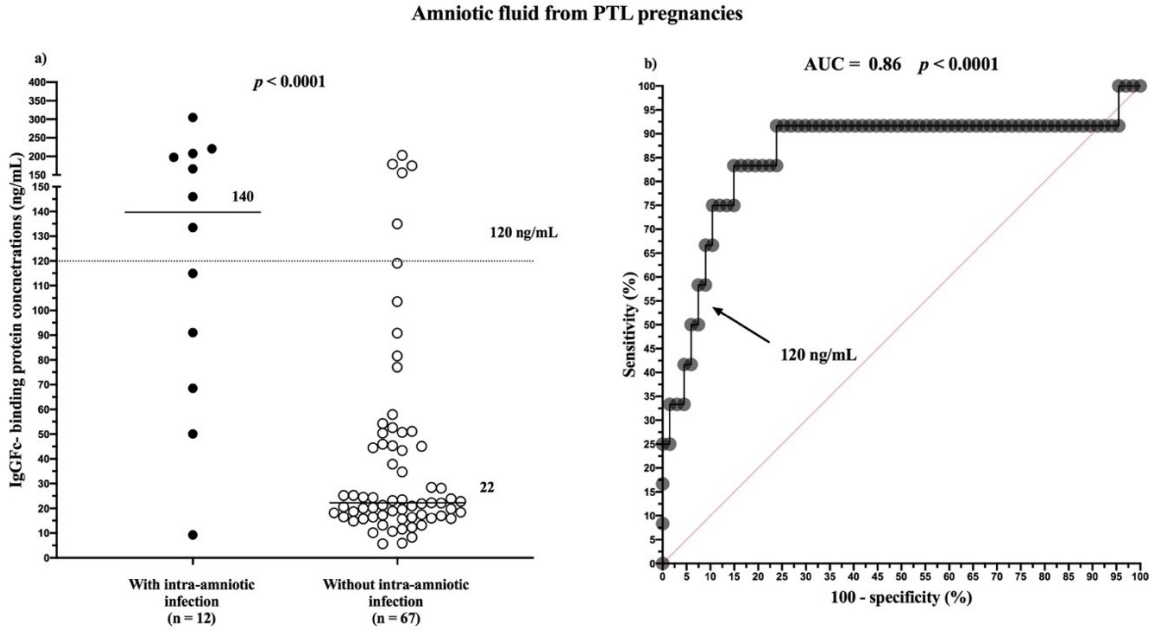
p-value: a comparison between two subgroups (a nonparametric Mann-Whitney *U* test)
adj. *p*-value: a comparison between two subgroups after the adjustment for gestational age at sampling (a Spearman partial correlation)

Statistically significant results are marked in bold.

Women with intra-amniotic infection had higher concentrations of amniotic fluid FcgammaBP than those without intra-amniotic infection (with infection: median IQR 139.7 ng/mL, IQR 74.2-205.3 vs. without infection: median 22.2 ng/mL, IQR 16.5-46.0; Figure 4a). The amniotic fluid FcgammaBP cutoff value of 120 ng/mL was found to be optimal in the prediction of intra-amniotic infection with sensitivity 58% (7/12; 95% confidence interval 32-81), specificity of 93% (62/67; 95% confidence interval 84-97), positive predictive value 58% (7/12; 95% confidence interval 32-81), negative predictive value 89% (62/67; 95% confidence interval 81-93), area under the receiver operating characteristic curve of 86% ($p < 0.0001$; Figure 4b), positive likelihood ratio of 7.8 (95% confidence interval 3.0-20.6), negative likelihood ratio of 0.45 (95% confidence interval 0.25-0.88), and odds ratio of 17 (95% confidence interval 3-64).

Figure 4.

Amniotic fluid IgGFc-binding protein concentrations based on the presence of intra-amniotic infection in women with PTL (a) and receiver operating characteristic curves for amniotic fluid IgGFc-binding protein in women with PTL with intra-amniotic infection (b). The best cutoff value is marked.



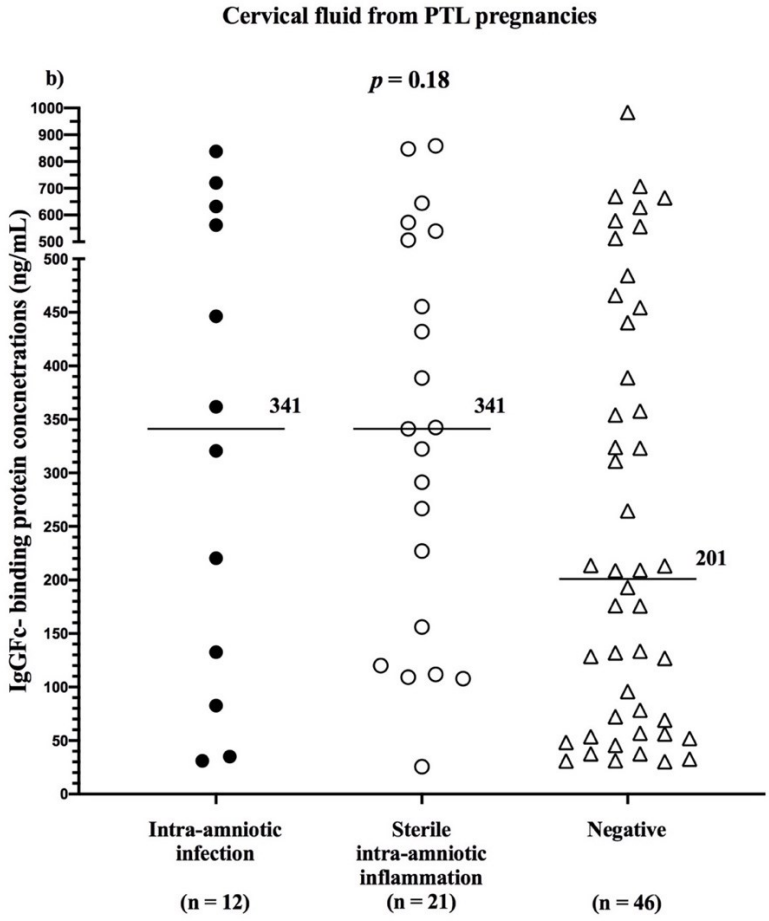
Abbreviations: PTL, preterm labor with intact membranes; AUC, Area under the receiver operating characteristic curve

4.1.3.2 *Concentration of FcgammaBP in cervical fluid based on the phenotype of intra-amniotic inflammation*

A weak positive correlation was observed between the concentrations of FcgammaBP in amniotic and cervical fluids (rho = 0.25*; p* = 0.02). No difference in cervical fluid FcgammaBP concentrations was found among the subgroups (intra-amniotic infection: median 341.1 ng/mL, IQR 95.2-614.8; sterile IAI: median 341.2 ng/mL, IQR 138.1-523.4; and negative amniotic fluid: median 200.9 ng/mL, IQR 56,7-443.8; $p = 0.18$; Figure 5). There was no difference in cervical fluid FcgammaBP concentrations between women with and without intra-amniotic infection (with infection: median 341.1 ng/mL, IQR 95.2-614.8 vs. without infection: median 227.0 ng/mL, IQR 95.7-455.4; $p = 0.45$).

Figure 5.

Cervical fluid IgGFc-binding protein concentrations in the subgroups of the women with PTL



Abbreviations: PTL, preterm labor with intact membranes

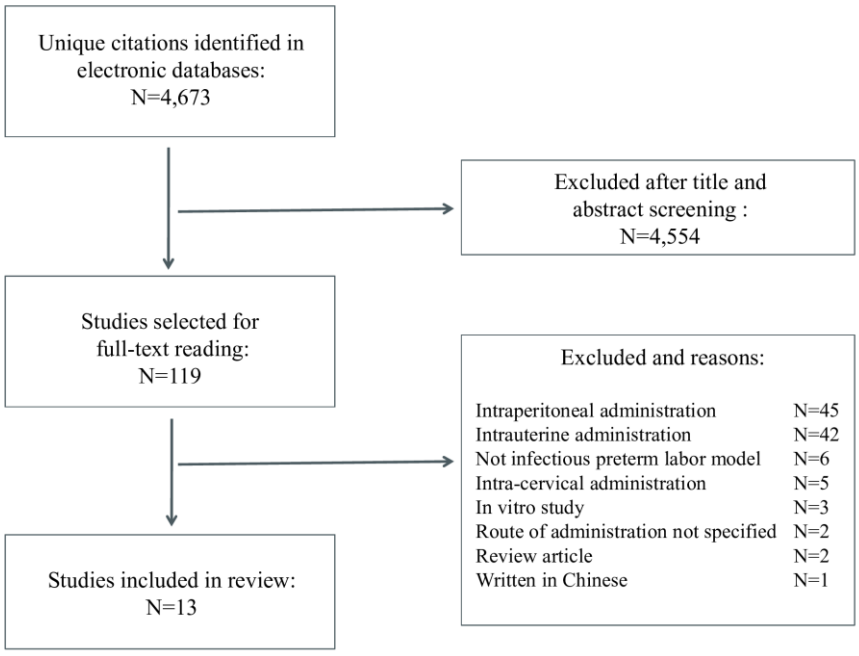
4.2 EXPERIMENTAL OBJECTIVES

4.2.1 Specific aim II-A

4.2.1.1 *Identification, selection, and inclusion of studies*

The process of identification, selection, and inclusion of studies is shown in Figure 6. The initial search in both databases identified a total of 4673 unduplicated articles, 4554 of which were excluded after screening titles and abstracts, and 119 potentially relevant citations were selected for full-text reading. The high number of citations for full-text reading was selected due to the fact that the description of used animal model was not given in many abstracts. A total of 106 studies were excluded due to reasons shown in Figure 6; finally, 13 studies that fulfilled our selection criteria were included in the review [215,222,241-251].

Figure 6.
Flowchart of the process of identification and selection of studies



The main characteristics of the studies are listed in Table 4. All 13 studies were published between 2004 and 2019; 38% (5/13) of the studies were published in 2018. Sprague–Dawley rats were used in 38% (5/13) of the studies [242,243,245,246,249]. Moreover, 62% (8/13) used different strains of mice [215,222,241,244,247,248,250,251]. The most common mouse strain was C57BL/6 (6/13) [222,244,247,248,250,251]. IRC mice [241] and CD-1 mice [215] were used in one study each. No other rodent animals than rats and mice were used in the included articles. Two distinct ways of administration of infectious or inflammatory agents into the gestational sacs were used. Five mice studies used transabdominal ultrasound-guided intra-amniotic administration of the agent [222,247,248,250,251]. Laparotomy with visualization of the uterine horns followed by direct puncture of the gestational sacs was the second identified route of administration of agents. Laparotomy was used in three mice studies [215,241,244] and in all studies with rats [242,243,245,246,249].

Infectious or inflammatory agents used in the studies were classified as follows: (1) live microorganisms, *Ureaplasma parvum;* (2) bacterial products, extracellular membrane vesicles; (3) PAMPs, LPS; and (4) DAMPs, HMGB-1, S100-B, and surfactant protein A .

Ureaplasma parvum was the only live microorganism used in the included studies. Intra-amniotic administration of 5000 colony forming units (CFU) of *Ureaplasma parvum* to mice did not lead to preterm delivery [215].

Extracellular membrane vesicles from group B *Streptococcu*s strain A909 were used in one study. Extracellular membrane vesicles injected at a dose of 5 and 10 μg per sac in mice caused preterm delivery in 55% and 68% of cases, respectively [244].

LPS was the most common triggering agent. LPS serotype O55:B55 was used in one study [245]. while serotype O111:B4 was utilized in seven studies [242,243,246-250]. In mice, intra-amniotic administration of 100 ng of LPS O111:B4 per sac on 16.5 day-post-conception (dpc) caused preterm delivery in 80%–88% of the animals [247,248,250]. Mice delivered approximately 20 hours (median) after the procedure [248,250]. Lower dosage of 50 ng of LPS (O111:B4) did not cause any preterm delivery [248]. In rats, intra-amniotic LPS O111:B4 at a dose of 4 μg and 10 μg per sac did not cause preterm delivery [242,243,246]. In addition, LPS O55:B55 at a dose of 10 μg per sac did not cause any preterm delivery [245]. The administration was used for modeling encephalopathy of prematurity and neonatal lung injury [242,243,246].

The fourth group of the triggering agents consisted of DAMPs that induced sterile IAI. In one study, HMGB-1 protein at a dose of 9 ng per sac caused preterm delivery in 57% of cases [222].

Similarly, protein S100B at a dose of 60 ng per sac caused preterm delivery in 50% of the cases [251]. The administration of 3 μg of surfactant protein A per sac in 15 dpc caused sterile IAI and preterm delivery in 82% of cases [241].

Table 4.

Main characteristics of studies of rodent inflammation-driven preterm delivery model initiated by intra-amniotic administration

Study	Year	Animal model	Total number of animals (n)	Agent for preterm delivery induction	Time of induction (days post coitum)	Procedure of administration	Preterm delivery rate[a]	Interval between procedure and PTD (hours)	Neonatal mortality rate at birth or shortly after birth (%)	Comment
Condon et al., [241]	2004	ICR mice	n = 17	3 µg surfactant protein A per sac	15 dpc	laparotomy injection into all sacs in the right uterine horn	14/17 (82.4%)	6-24 h	no data	PTD of the fetuses from the injected (right) uterine horn, reabsorption of the fetuses from the left horn
Normann et al., [215]	2009	CD-1 mice	n = 15	*Ureaplasma parvum* - 5000 CFU per sac	13.5 dpc	laparotomy injection into all amniotic sacs	0/15 (0%)	no PTD	no effect on postnatal survival	*Ureaplasma parvum* caused intra-amniotic infection and a mild fetal lung inflammation
Jantzie et al., [242]	2015	Sprague–Dawley rats	no data	4 µg of LPS per sac (LPS O111:B4)	18 dpc	laparotomy injection into all sacs + clamping of uterine arteries for 60 min	0%	no PTD	approx. 40%	animal model of encephalopathy of prematurity
Maxwell et al., [243]	2015	Sprague–Dawley rats	n = 9	4 µg of LPS per sac (LPS O111:B4)	18 dpc	laparotomy injection into all sacs + clamping of uterine arteries for 60 min	no data animals sacrificed at 19 dpc or 21 dpc	no PTD animals sacrificed at 19 dpc or 21 dpc	15% at 19 dpc and 45% at 21 dpc	animal model of chorioamnionitis and neonatal brain injury
Gomez-Lopez et al. [222]	2016	C57BL/6 mice	n = 7	9 ng HMGB1 per sac	14.5 dpc	ultrasound-guided intraamniotic application of all sacs	4/7 (57%)	100.8 ± 7.2 h[b]	15% (in 1 week 61%)	
Surve et al., [244]	2016	C57BL6 mice	no data	extracellular membrane vesicles from GBS strain A909 – 5 µg and 10 µg per sac (two groups)	14.5 dpc	laparotomy injection into all sacs	55% (for 5 µg) 68% (for 10 µg)	delivered within 48 h	36% (for 5 µg) 29% (for 10 µg)	
Cookson et al., [245]	2018	Sprague–Dawley rats	n = 6	10 µg of LPS per sac (LPS O55:B55)	20 dpc	laparotomy number of injected sacs was not specified	0%	no PTD (delivered by caesarean section)	no data	animal model of chorioamnionitis
Dedja et al., [246]	2018	Sprague–Dawley rats	n = 4	10 µg of LPS per sac (LPS O111:B4)	20 dpc	laparotomy injection into all sacs	0%	no PTD	32%	animal model of chorioamnionitis and neonatal lung injury
Garcia-Flores et al., [247]	2018	C57BL/6 mice	n = 8	100 ng of LPS per sac (LPS O111:B4)	16.5 dpc	ultrasound-guided intra-amniotic injection into all sacs	7/8 (88%)	no data	exceeded 85% (in 1 week 100%)	
Gomez-Lopez et al., [248]	2018	C57BL/6 mice	n = 3+10	50 ng and 100 ng of LPS per sac (O111:B4) (two groups)	16.5 dpc	ultrasound-guided intra-amniotic injection into all sacs	3/0 (0%) (for 50 ng) 8/10 (80%) (for 100 ng)	no PTD (for 50 ng) 20.4 h[c] (for 100 ng)	no data	
Jantzie et al., [249]	2018	Sprague–Dawley rats	no data	4 µg of LPS per sac (LPS O111:B4)	18 dpc	laparotomy injection of all sacs + clamping of uterine arteries for 60 min	0%	no PTD	no data	animal model of encephalopathy of prematurity
Faro et al., [250]	2019	C57BL/6 mice	n = 10	100 ng of LPS per sac (LPS O111:B4)	16.5 dpc	ultrasound-guided intraamniotic application of all sacs	8/10 (80%)	20 h[c]	90,70%	
Gomez-Lopez et al., [251]	2019	C57BL/6 mice	n = 10	60 ng of S100B per sac	16.5 dpc	ultrasound-guided intra-amniotic injection into all sacs	5/10 (50%)	approx. 24 h[c]	60%	

[a] number of animals delivered preterm / number of used animals; (%) percentage of animals delivered preterm

[b] mean ± standard deviation

[c] median

Abbreviations: CFU, colony-forming units; dpc, days post coitum; GBS, group B Streptococcus; HMGB-1, high mobility group box-1; LPS, lipopolysaccharide; S100B – S100 calcium binding protein B

4.2.1.2 *Quality assessment*

The quality assessment of the included studies is listed in Table 5. The median number of study quality checklist items scored was 3 of 5. The animal model used in the study was described in all articles, including the rodent strain and its origin. Only one study (8%) described the sample size calculation. Randomized allocation to treatment group was reported 2 of 13 studies (16%). Of included studies, all except one (92%) described in detail the intervention technique. Proper characterization of triggering agent was described in all included studies.

Table 5.

Report of the study quality score

	Detailed description of the used animal model	Sample size calculation	Random allocation to treatment or control group	Detailed description of the used intervention	Detailed description of triggering agent
Condon et al., Proc Natl Acad Sci USA., 2004 [241]	yes	no	no	yes	yes
Normann et al., Pediatr Res., 2009 [215]	yes	no	no	yes	yes
Jantzie et al., J Vis Exp., 2015 [242]	yes	no	no	yes	yes
Maxwell et al., Placenta, 2015 [243]	yes	no	no	yes	yes
Gomez-Lopez et al., Am J Reprod Immunol., 2016 [222]	yes	no	no	yes	yes
Surve et al., plos Pathog., 2016 [244]	yes	no	no	yes	yes
Cookson et al., Am J Perinatol., 2018 [245]	yes	no	no	no	yes
Dedja et al., Exp Lung Res., 2018 [246]	yes	no	yes	yes	yes
Garcia-Flores et al.,Front Immunol., 2018 [247]	yes	no	no	yes	yes
Gomez-Lopez et al., J Matern Fetal Neonatal Med.,2018 [248]	yes	no	no	yes	yes
Jantzie et al., Front Neurol., 2018 [249]	yes	yes	yes	yes	yes
Faro et al., Biol Reprod., 2019 [250]	yes	no	no	yes	yes
Gomez-Lopez et al., Biol Reprod., 2019 [251]	yes	no	no	yes	yes

4.2.2 Specific aim II-B

4.2.2.1 *Animal characteristics*

In total, four rats were administered LPS and three rats were administered PBS. In total, 19 gestational sacs were injected in the LPS group and 17 gestational sacs were injected in the PBS group.

Twenty-four hours after administration, all animals remained alive and had not delivered. All fetuses, except one, were alive. The dead fetus was from an injected gestational sac in the LPS subgroup. The amniotic fluid was not sampled from this dead fetus.

From the rats administered LPS, a sufficient volume of the amniotic fluid was obtained from 16 gestational sacs with LPS and from 33 without LPS for the analysis. From the rats administered PBS, sampling was successful from nine gestational sacs with PBS and 32 sacs without PBS.

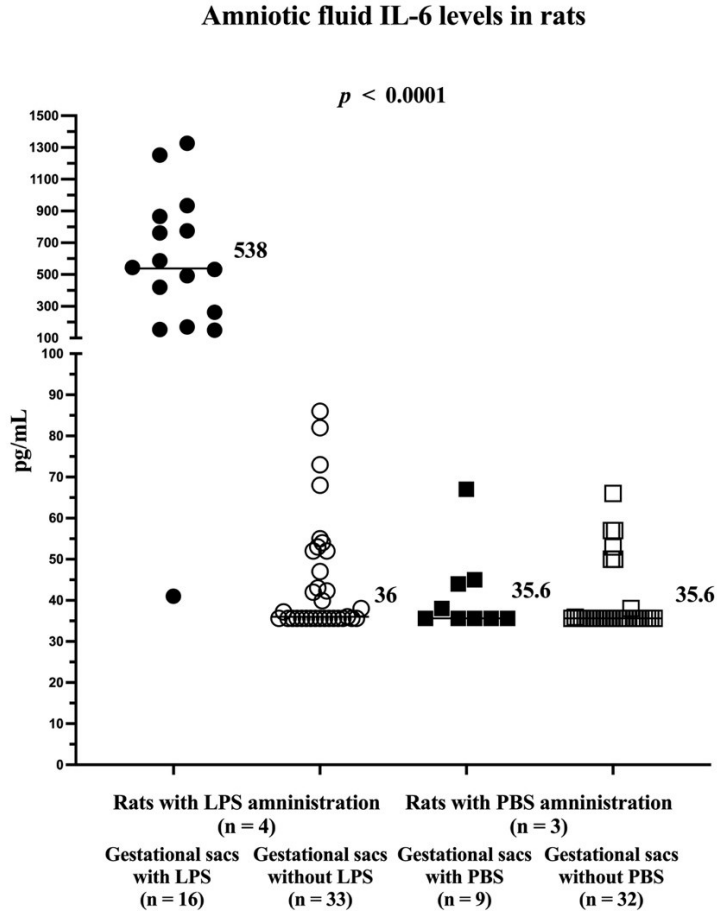
4.2.2.2 *Concentration of IL-6 in the amniotic fluid after intra-amniotic LPS administration*

Differences in the concentration of IL-6 in the amniotic fluid were found among the subgroups of gestational sacs (with LPS: median 538 pg/mL; IQR 192.6–843.2 pg/mL; without LPS: median 36 pg/mL, IQR 35.6–52 pg/mL; with PBS: median 35.6 pg/mL, IQR 35.6–44.5 pg/mL; without PBS: median 35.6 pg/mL, IQR 35.6–35.8 pg/mL; $p \leq 0.0001$; Figure 8).

The concentration of IL-6 in the amniotic fluid from gestational sacs with LPS were higher than that in the amniotic fluid from gestational sacs with PBS and those without LPS and without PBS (Table 5). No differences in the concentration of IL-6 in the amniotic fluid were identified between gestational sacs with PBS and those gestational sacs without LPS and without PBS (Table 5). The concentration of IL-6 in the amniotic fluid from gestational sacs without LPS from rats administered LPS was higher than that in the amniotic fluid from gestational sacs without PBS from rats administered PBS (Table 5).

Figure 8.

Concentration of IL-6 in the amniotic fluid of rats after intra-amniotic administration of either lipopolysaccharide or phosphate-buffered saline



Abbreviations: LPS, lipopolysaccharide; PBS, Phosphate-buffered saline

Table 5.

Comparisons of interleukin-6 concentrations in the amniotic fluid from gestational sacs of rats after administration of either lipopolysaccharide or phosphate-buffered saline

		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	***p* < 0.0001**	***p* < 0.0001**	***p* < 0.0001**
	Gestational sac without LPS	***p* < 0.0001**	x	$p = 0.63$	***p* = 0.04**
Administration of PBS	Gestational sac with PBS	***p* < 0.0001**	$p = 0.63$	x	$p = 0.36$
	Gestational sac without PBS	***p* < 0.0001**	***p* = 0.04**	$p = 0.36$	x

p-value: a comparison between two subgroups (a nonparametric Mann-Whitney *U* test).
Statistically significant results are marked in bold.
Abbreviations: LPS, lipopolysaccharide; PBS, Phosphate-buffered saline

4.2.3 Specific aim II-C

The protocol with photographs is included in section Appendices (Appendix A)

4.2.3.1 *Preparation*

The bottle warmer was turned on in advance to warm up the ultrasound gel. The temperature of the warmer was set to 37°C. The oxygen tank was checked to determine if there was enough oxygen to perform the procedure. The level of isoflurane in the anesthesia vaporizer was checked. If the level was low, isoflurane was refilled before starting the procedure. The connection of the hose of the anesthesia system to the induction chamber and Vevo Imagine Station was checked. The hose switch was opened to allow anesthetic gases to flow into the induction chamber. The hose switch that led to the imagining station was closed. The heating of the Vevo Imagine Station heating pad was turned on. The temperature of the heating pad was set to 40°C. The infrared lamp was turned on and placed above the heating pad to maintain a stable body temperature during anesthesia. Five pieces of tape, approximately 10 cm in length, were prepared and used to attach the animal and rectal probe to the heating pad during the procedure. Hair removal cream and a few pieces of cotton swabs and gauze pads were prepared and used to remove fur. The preparation took approximately 10 minutes.

4.2.3.2 *Setting Up the Vevo ultrasound machine*

A Vevo 3100 ultrasound machine was turned on. The MX250S transducer (15–30 MHz) was connected to an ultrasound machine. Different transducers were used during different stages of the procedure. The pre-set to "mouse (large) abdominal" was chosen. The study name for data storage in ultrasound machine was automatically set to "series 1" The series number was changed for every animal to maintain records. The setup of the ultrasound machine required 5 minutes.

4.2.3.3 *Anesthesia*

The oxygen tank was turned on, and the flow rate was set to 2 L/min. Isoflurane was initiated at a concentration of 5%. The rat was removed from the cage and placed in an induction chamber. The behavior of the animals was assessed. After anesthesia was initiated, the isoflurane concentration was lowered to 1.5%. The hose switch connecting the anesthesia

vaporizer and nasal mask on the heating pad at the ultrasound station was open. The second hose switch connecting the anesthesia vaporizer and induction chamber was closed. The rat was carefully removed from the induction chamber and moved to the heating pad of the imaging station. The animal was placed in the prone position on the heating pad, and the rat's snout was placed on the heating pad to the nasal mask. The animal was placed in this position for a few minutes to stabilize the anesthesia. Breathing frequency and movement were carefully monitored. The isoflurane concentration was adjusted as necessary. After stabilization, the animal was placed in the supine position (with the face and torso facing up). The snout of the rat was always connected to the nasal mask on the heating pad. The body temperature, heart rate, and breathing frequency were monitored throughout the procedure. Anesthetic induction took 5 minutes.

4.2.3.4 *Fixation of the mouse and insertion of the rectal probe*

The electrode gel was applied between the rat's paws and the electrodes of the heating pad. The rat's paws were fixed to the electrodes of the heating pad using previously prepared tapes. A drop of the lubricating gel was placed on the rectal probe. The probe was inserted into the rectum of the rat to measure body temperature. The rectal probe was fixed to the heating pad using a previously prepared tape. This part of the procedure took 5 minutes.

4.2.3.5 *Fur Removal*

Depilatory cream was applied to the rat's abdomen. The cream was spread to cover the entire area of the abdomen using a cotton swab. The cream was allowed to stand for 3 min. The fur and cream were removed using pieces of wet gauze. The abdominal fur had to be removed completely prior to the procedure. Fur removal took 10 minutes.

4.2.3.6 *General ultrasound scan with mapping of the number and the position of the pups*

The ultrasound gel was removed from the bottle heater and shaken to move the gel toward the nozzle to avoid creating bubbles, which can distort the images. The lower part of the rat's abdomen was covered with the ultrasound gel, and a 1-cm thick layer was created. The transducer was placed in a transverse orientation on the abdomen. To avoid shaking, the wrist of the hand with the transducer was fixed. The maternal bladder was identified as an

anechogenic round structure located at the midline of the lower abdomen. The urinary bladder was used as a midline reference point for localizing and mapping gestational sacs with pups. First, the right part of the abdomen of the animal was scanned from the bladder to the thorax. Then, the left part of the abdomen of the animal was scanned from the bladder to the thorax. The positions of the pups and placentas were recorded. This initial scan took approximately 10 minutes.

4.2.3.7 *Ultrasound-guided intra-amniotic administration*

The MX250S transducer was replaced by an MX400 transducer (20–46 MHz). The MX400 transducer, offering higher axial resolution, enables more precise needle navigation during the procedure. The MX400 transducer was placed in the transducer holder of the Vevo Imagine Station. A sufficient ultrasound gel layer of at least 1-cm thickness was restored below the transducer. The syringe with the substance was placed in the syringe holder of the Vevo Imagine Station. The target gestational sac was visualized on the right side of the ultrasound image. The syringe holder was moved toward the animal body surface inside the gel layer with a 27 G × 40 mm needle perpendicular to the skin until the needle tip could be visualized on the left side of the ultrasound image. When the tip of the needle was visible on the ultrasound image, puncture was performed. The thick rat skin made the passage of the needle difficult. Therefore, the puncture had to be slow to allow the tip of the needle to spontaneously slide through the skin tissue and membranes of the gestational sac. The agent was injected when the tip of the needle was inside the gestational sac. Successful application was confirmed by visualization of the fluid jet. The needle was then slowly pulled out. The procedure was repeated to inject all accessible gestational sacs in the animal. A new sharp needle was used for each new administration to ensure the successful passage of the needle through the skin. Intra-amniotic administration required approximately 30 minutes.

4.2.3.8 *End of the procedure*

The entire layer of the ultrasound gel was removed from the animal's abdomen with gauze pads. The abdomen was completely dried before the animal was moved into the cage. The rectal probe was removed, and the paws were released. The isoflurane vaporizer was turned off. The animal was placed in a cage, where it was kept under a heat lamp and watched until recovery

from anesthesia. The ultrasound machine and equipment were turned off. All instruments were cleaned. This part of the procedure took 10 minutes.

5. DISCUSSION

5.1 CLINICAL OBJECTIVES

5.1.1 Specific aim I-A

The principal findings of the specific aim I-A, evaluated in women with PTL, were as follows: i) intra-amniotic infection and sterile IAI were found in 15% and 27% of the women, respectively; ii) cervical fluid IL-6 concentration was positively correlated with amniotic fluid IL-6 concentration; iii) women with intra-amniotic infection and sterile IAI had a higher concentration of IL-6 in the cervical fluid than those without IAI; and iv) no differences in the concentration of IL-6 in the cervical fluid were found between women with intra-amniotic infection and those with sterile IAI.

The presence of sterile IAI has been described so far in pregnancies complicated by preterm delivery [142,143,222,251-259], PPROM [143,145,260-263], and a short cervix [264]. In pregnancies with PTL, the rate of sterile IAI is approximately two-fold more frequent than the rate of intra-amniotic infection [142]. However, the frequency of both phenotypes of IAI in PTL decreases with advancing gestational age [142]. The results of this study regarding the frequency of both clinical phenotypes of IAI are in line with the results of a pioneering study by Dr. Romero on sterile IAI in PTL, in which sterile IAI and intra-amniotic infection were found in 26% and 11% of women, respectively [142].

In this study, the concentration of IL-6 was measured in cervical fluid samples obtained from women with PTL. Previous studies have reported a positive correlation between the concentration of IL-6 in the cervical fluid and the amniotic fluid [148,182,185,265]. In addition, there is evidence that an increased concentration of IL-6 in the cervical fluid in women with PTL is related to the following: i) a higher risk of preterm delivery [182,265-270]; ii) increased concentration of IL-6 in the amniotic fluid [182,185]; iii) a higher risk of IAI [182,185]; iv) microbial invasion of the chorioamniotic membranes [148]; v) MIAC [182,185]; vi) the presence of histological [185]; and clinical [271] chorioamnionitis.

In this study, we confirmed the results reported in previous studies [182,185] that in PTL pregnancies, IAI is associated with higher cervical fluid concentrations of IL-6 than in those without IAI. To extend the knowledge of this field, women with PTL were further divided into

three subgroups: intra-amniotic infection, sterile IAI, and without IAI. As expected, women with both clinical phenotypes of IAI had higher concentrations of IL-6 in the cervical fluid than those without IAI. However, no difference in the cervical fluid IL-6 concentration was found between women with intra-amniotic infection and sterile IAI. These observations show that an inflammatory and/or infectious environment in the cervical compartment is present in both clinical phenotypes of IAI. Given the tight anatomical proximity between the cervix and fetal membranes, we hypothesize that the composition of the cervical fluid might reflect the microbial and inflammatory status of the choriodecidual space. This hypothesis is driven by the fact that the presence of bacteria in the chorioamnion is associated with an elevation of IL-6 concentration in the cervical fluid [148]. In addition, the presence of microorganisms in the chorioamnion is also related to higher concentrations of IL-6 in the amniotic fluid, irrespective of the presence or absence of microorganisms in the amniotic fluid [148,153]. These facts collectively suggest that presence of microorganisms in the chorioamniotic membranes closely related to the elevation of the concentration of IL-6 in both cervical and amniotic fluids.

Therefore, the elevation of IL-6 concentration in the cervical fluid in women with PTL with sterile IAI can be explained by the possible presence of microorganisms in the chorioamnion and/or inflammation in the choriodecidual space. This observation supports the hypothesis that these conditions represent one of the mechanisms playing pivotal roles, apart or in combination with the release of alarmins from necrotic cells or cells undergoing cellular stress, on the development of a sterile intra-amniotic environment in women with PTL.

This study has several strengths. First, the presence of MIAC was assessed using a combination of culture and non-culture methods (specific PCR for *Ureaplasma* spp., *M. hominis*, and *C. trachomatis* and non-specific evaluation of the 16S rRNA gene). This comprehensive approach allowed us to precisely identify a subset of women with sterile IAI. Second, paired amniotic and cervical fluid samples were obtained at the time of admission before the administration of corticosteroids, tocolytics, and antibiotics.

This study also has limitations. First, number of women with intra-amniotic infection and sterile IAI in this study (n = 12 and n = 21, respectively) was relatively small. Second, the concentration of IL-6 in the amniotic fluid was assessed in fresh samples; however, the concentration of IL-6 in the cervical fluid was measured in the samples that underwent one freezing/thawing cycle. Third, the cervical fluid microbiota and microbiome were not assessed in this study.

5.1.2 Specific aim I-B

The principal findings of the specific aim I-B, evaluated in women with PTL, were as follows: i) FcgammaBP was identified as a constituent of amniotic and cervical fluids, ii) the concentration of FcgammaBP in amniotic fluid was elevated in the presence of both phenotypes of IAI, being higher in the presence of intra-amniotic infection, iii) FcgammaBP in the amniotic fluid might be a marker of intra-amniotic infection in women with PTL, and iv) the concentration of FcgammaBP in the cervical fluid was not altered by the presence of either phenotype of IAI.

FcgammaBP is a relatively unknown protein, with limited reports in relation to conditions such as bowel inflammatory disease, autoimmune disease, or thyroid gland tumors [272-274], however, it also represents one of the proteins identified in the amniotic fluid using proteomics [275-278]. FcgammaBP was discovered more than 30 years ago as a specific site for the fragment of crystallizable (Fc) region of the immunoglobulin (Ig) G antibody in the small intestinal and colonic epithelia [279]. This specific site differed from previously recognized receptors in the Fc region of IgG [279]. The specific site for the Fc region of IgG was later termed FcgammaBP and identified as a protein primarily localized in the mucosal granules of the small intestinal and colonic epithelia that are secreted into the intestinal lumen Based on the current knowledge, FcgammaBP is considered a protein that provides immunological protection to the intestinal tissue and facilitates the interaction between the intestinal mucus and potentially harmful stimuli (such as microorganisms and alarmins) with the ultimate goal of protecting the mucosal surface [272,279,280]. However, its exact biological function has yet to be fully elucidated.

The production of FcgammaBP has been described to occur in the intestinal epithelial cells, placenta, and thyroid tissue [272,274]. However, its expression has not been observed in the brain, heart, kidney, liver, lung, and skeletal muscles [272]. Interestingly, the ability to produce FcgammaBP was confirmed only in humans and monkeys, it has not been confirmed in mice, rats, rabbits, dogs, bovines, and porcines [272].

FcgammaBP has been found in low concentrations in human serum from healthy individuals [273]. However, its serum concentrations were elevated in the presence of autoimmune diseases such as Crohn's disease, ulcerative colitis, rheumatoid arthritis, systemic lupus erythematosus, and progressive systemic sclerosis [273]. The presence of FcgammaBP has been further proven in the amniotic fluid, urine, saliva, and cerebrospinal fluid [275,278]. Liu et al. found the presence of FcgammaBP in the amniotic fluid in the second trimester of uncomplicated

pregnancies [275]. In addition, FcgammaBP was shown to be among the most abundant (35/1624) proteins found in the amniotic fluid [275]. Our group described the presence of FcgammaBP in the amniotic fluid in pregnancies complicated by PPROM and PTL [276,277]. The finding of this study that FcgammaBP is a constituent of the amniotic fluid in PTL pregnancies is in line with the abovementioned findings.

Previously, the concentration of FcgammaBP in the amniotic fluid was shown to be higher in women with PPROM with MIAC and acute histological chorioamnionitis than in those without these complications [276]. Interestingly, no differences in the amniotic fluid concentration of FcgammaBP between the presence and absence of the abovementioned complications were identified in women with PTL, where amniotic fluid was obtained from the forewaters at the end of the first stage of labor [277].

In this study, we found an elevated amniotic fluid concentration of FcgammaBP in the presence of both phenotypes of IAI. Interestingly, the concentrations of FcgammaBP in amniotic fluid were higher in the presence of intra-amniotic infection than in the presence of sterile IAI. The results from this study show that both infectious and non-infectious stimuli might trigger the production of FcgammaBP.

In this study, the concentration of FcgammaBP was measured in paired amniotic and cervical fluid samples. Interestingly, the FcgammaBP concentrations were higher in the cervical fluid samples than in the amniotic fluid samples, despite the fact that cervical fluid samples obtained with a swab were diluted in 1.5 mL of the buffer. These observations suggest that epithelial cells and/or immune cells in the endocervical canal are able to produce FcgammaBP. This finding supports the key role of the cervix during pregnancy, which is immunological protection against the ascension of microorganisms from the vagina and the cervix toward the upper genital tract [281-284].

Cervical fluid sampling can be clinically relevant given the non-invasive nature of this procedure. However, only a weak positive correlation between the concentration of FcgammaBP in the amniotic fluid and the cervical fluid was found in PTL. Due to intact membranes in pregnancies with PTL, the protein composition of a cervical fluid sample may reflect the situation in the cervical compartment rather than that in the intra-amniotic cavity. The study shows that FcgammaBP in the cervical fluid is not a useful marker for the diagnosis of intra-amniotic complications in women with PTL.

Confirmation of intra-amniotic infection is a challenge for clinicians. The necessity to rule in or rule out the presence of microorganisms in amniotic fluid makes the diagnosis of intra-amniotic infection time-consuming and more expensive when the techniques used to identify either non-culturable or difficult-to-culture microorganisms are employed. Therefore, from a clinical point of view, there is an urgent need to discover a single marker of intra-amniotic infection that has reliable sensitivity and specificity. In this study, FcgammaBP in the amniotic fluid was identified as a potential marker of intra-amniotic infection in PTL pregnancies.

The strength of this study is the relatively large cohort of paired amniotic and cervical fluid samples. Second, fluid samples were collected from a well-defined phenotype of PTL. Finally, a thorough assessment of MIAC using a combination of culture and non-culture methods provided an opportunity to precisely distinguish the subsets of women with intra-amniotic infection and sterile IAI.

This study also has some limitations that are worth mentioning. For example, there was a small number of women with intra-amniotic infection (n = 12). To confirm whether the concentration of FcgammaBP in amniotic fluid is a reliable marker of intra-amniotic infection, the results need to be replicated in independent cohorts. Next, despite the FcgammaBP expression in the placenta that was described [272], the questions of which part of the placenta is a source of FcgammaBP and whether fetal membranes produce FcgammaBP still remain unanswered. A body of evidence has shown that intestinal epithelial cells produce FcgammaBP [272,273], but no data are available on whether amniotic epithelial cells can produce FcgammaBP. Given the importance of the amniotic epithelium as a barrier against the ascension of microorganisms into the amniotic cavity [285-287], some similarities between intestinal and amniotic epithelial cells might be identifiable such as, 1) to serve as mechanical barriers [285-289]; 2) to have spatially expressed toll-like receptors [290,291]; and 3) to indicate that the expression of toll-like receptors changes when inflammation is present [131,290,291]. Therefore, we hypothesize that the amniotic epithelium might be involved in FcgammaBP production.

5.2 EXPERIMENTAL OBJECTIVES

5.2.1 Specific aim II-A

The key findings of specific aim II-A were as follows: 1) intra-amniotic administration of agents to model intra-amniotic inflammation/infection associated with preterm delivery has been used since 2004; 2) the published approaches to administer triggering agents into the gestational sacs include open surgery with direct puncture and transabdominal ultrasound-guided administration; 3) four kinds of triggering agents were used: i) live microorganisms, ii) bacterial products, iii) PAMPs, and iv) DAMPs; 4) LPS was the most commonly used triggering agent; 5) *Ureaplasma parvum* was the only live microorganism used; and 6) HMGB-1, S100B, and surfactant protein A were DAMPs used to model sterile IAI.

The rodent model of inflammatory preterm delivery initiated by intraperitoneal or intrauterine administration of endotoxins is used in research for decades [199]. However, the intra-amniotic administration of triggering agents started to be used only recently. Our search (even though there was no time restriction) identified only the studies published after 2004, with majority of studies published in 2018. The possible explanations are the following: i) a change of researchers' view on the importance of intra-amniotic inflammatory response in the research of intra-amniotic inflammation/infection associated with preterm delivery, and ii) better availability of high-frequency ultrasound devices that made the intra-amniotic administration of triggering agents under ultrasound guidance possible.

The majority of the included studies used mini-laparotomy to establish access to the pregnant uterus. During the procedure, a rodent was anesthetized and a short (usually 1-3 cm) midline incision was made in the lower abdomen. The rodent uterus is bicornuate, and the sacs with fetuses are arranged in a "beads-on-a-string" pattern. One or both horns were exposed, and the individual fetal sacs were directly punctured usually using a 31 G needle. After the procedure, the abdominal wall was closed using sutures. Laparotomy is an invasive procedure and has some drawbacks. Pain along with surgical stress can result in a major endocrine response influencing function of many organs [292]. Laparotomy itself can cause preterm delivery among sham controls and therefore can confound the results [216].

High-frequency ultrasound devices have recently become available for small laboratory animal imaging. This high-resolution technology enables reliable guidance of the needle passage through an intact abdominal wall. Rinaldi et al. presented the method of transabdominal

ultrasound-guided puncture in the uterine cavity of mice [293]. Of the included studies, only one research group took advantage of a high-frequency ultrasound device to guide the transabdominal administration into gestational sacs of mice [222,247,248,250,251]. During the procedure, anesthetized animals were positioned on a pad. The ultrasound probe and syringe with an agent were stabilized by a mechanical holder. After fur removal, gestational sacs were transabdominally punctured by 30 G needle under ultrasound guidance. This approach is less invasive than direct puncture of gestational sacs from mini-laparotomy. However, the ultrasound-guided approach requires both high-quality ultrasound device and a well-trained operator.

The use of transabdominal ultrasound-guided administration has been reported only in mice but not in rats. In our experience, thicker rat skin impedes the needle passage through their abdominal wall. On the contrary, Serriere et al. showed that transabdominal ultrasound-guided aspiration of amniotic fluid was possible even in rats [294]. Therefore, rats can be used for modeling of intra-amniotic inflammation/infection associated with preterm delivery with caution.

For animal modeling, intra-amniotic infection can be triggered by live microorganisms, their components, or PAMPs. PAMPs are diverse sets of microbial molecules that share a number of different recognizable biochemical features that trigger an immune responses [295]. Except for one study, the included LPS studies used LPS serotype O111:B4. Intra-amniotic administration of LPS O111:B4 to C57BL/6 mice caused preterm delivery in 80%-88% of the cases. The remaining mice delivered at term [247,248,250]. In studies with intraperitoneal and intrauterine administrations, almost all animals delivered preterm [226,296]. It is likely that the intensity of IAI triggered by the intra-amniotic administration of LPS was not strong enough to cause preterm delivery in all animals. However, the exposed pups suffered from severe mortality regardless of preterm or term delivery. The advantage of the intra-amniotic administration of LPS is the absence of signs of systematic involvement and changes in body temperature in pregnant mice [248]. This scenario mimics a clinical situation in pregnant women.

The intra-amniotic administration of LPS to Sprague–Dawley rats did not cause preterm delivery among animals in five included studies regardless of the dose or serotype of LPS used. This might suggest that Sprague–Dawley rats were not as sensitive to LPS as C57BL/6 mice. On the contrary, intra-amniotic administration of LPS in Sprague–Dawley rats caused histologic chorioamnionitis [242,243,245,246,249].

Genital mycoplasmas are the most frequent microorganisms diagnosed in the amniotic cavity of women with preterm delivery [124,297]. Therefore, their use to model intra-amniotic infection is more clinically relevant than LPS-based studies. In addition to their effect on triggering intra-amniotic infection, ureaplasmas can play a role in the development of bronchopulmonary dysplasia in preterm newborns [298,299]. Interestingly, in a study by Norman et al., intra-amniotic administration of live *Ureaplasma parvum* did not cause preterm delivery in the CD-1 mouse model [215]. The absence of preterm delivery induction by *Ureaplasma parvum* in this study can be the consequence of bypassing the process of ascending infection due to direct intra-amniotic administration. Other possible explanation is that the serovar of *Ureaplasma parvum* used in this study lacked the capacity to induce preterm delivery. However, exposed pups suffered from mild postnatal inflammation and worsened oxygen-induced lung injury, which demonstrate the significance of intra-amniotic infection [215].

Three kinds of DAMPs were used for the induction of sterile IAI in the included studies; namely, HMGB-1, S100B, and surfactant protein A [222,241,251]. HMGB-1 and S100B were elevated in the amniotic fluid of women with sterile IAI [142,144,147]. In C57BL/6 mice, intra-amniotic administration of HMGB-1 and S100B caused a similar rate of preterm delivery (approximately 50%). Interestingly, intraperitoneal administration of HMGB-1 to C57BL/6 mice did not cause preterm delivery [222]. These findings provide evidence that DAMPs can induce preterm delivery associated with sterile IAI and probably mimic similar situation among humans.

The main limitation of this systematic review is its focus on rodent models, although other animals (such as sheep) have been used for intra-amniotic administration of triggering agents to simulate intra-amniotic inflammation/infection associated with preterm delivery [199]. The intra-amniotic administration of triggering agents to rodents is relatively new compared with other well-established animal models. An additional limitation is that we only used two databases for the initial search and excluded the articles published in other languages than English. Finally, there is heterogeneity of the outcomes of included studies as well as in the used strains of mice and rats.

5.2.2 Specific aim II-B

The principal findings of the specific aim II-B were as follows: i) ultrasound-guided intra-amniotic administration of an agent was feasible in rats; ii) ultrasound-guided intra-amniotic administration of 10 μg of *E. coli* LPS serotype O55:B5 induced a marked elevation in the concentration of IL-6 in the amniotic fluid of rats; iii); the concentration of IL-6 in the amniotic fluid was elevated in gestational sacs treated with LPS; iv) intra-amniotic administration of 10 μg of *E. coli* LPS serotype O55:B5 did not induce labor within 24 hours; and v) after LPS administration, 95% of fetuses remained alive.

Animal models represent a unique tool in the research of preterm delivery, enabling us to improve our understanding of the underlying mechanisms associated with intra-amniotic inflammatory and infection-related complications. Here, we have reported the development of a novel, minimally invasive rat model of IAI based on ultrasound-guided LPS administration.

Ultrasound-guided injections of triggering agents have only been recently used to develop a model of inflammation/infection associated with preterm delivery in small laboratory animals [233,293]. This approach has become possible because of the high-frequency ultrasound devices specifically designed for use in small laboratory animals. Owing to their minimal invasiveness, ultrasound-guided injection is advantageous over the classical open surgery approach [233]. Ultrasound-guided intra-amniotic administration of triggering agents to create a model of IAI in mice is currently used by one research group [222,248,250,251]. Serriere *et al.* showed that transabdominal ultrasound-guided aspiration of the amniotic fluid was also possible in rats [294]. However, to the best of our knowledge, our study is the first to use ultrasound-guided intra-amniotic administration of a triggering agent to develop a model of IAI in rats.

LPS, a component of the cell wall of gram-negative bacteria, has been used to model infection and inflammation associated with preterm delivery in animal models for decades [204]. Gayle *et al.* demonstrated a 12-fold and 5-fold increase in IL-6 levels in the amniotic fluid at 6 and 12 hours, respectively, after systemic administration of LPS to rats [300]. In our study, the intra-amniotic injection of 10 μg of *E. coli* LPS serotype O55:B5 per gestational sac under ultrasound guidance triggered a marked elevation in IL-6 levels in the amniotic fluid. Twenty-four hours after administration, the concentration of IL-6 in the amniotic fluid in the gestational sacs with LPS were 15-fold higher than that in the amniotic fluid in the gestational sacs without LPS. Elevated IL-6 levels were observed only in the gestational sacs with LPS. However, the

amniotic fluid from non-injected gestational sacs in rats administered LPS, had slightly higher IL-6 concentrations than that from non-injected gestational sacs in rats administered PBS. This phenomenon could be explained by a potential weak leak of LPS from the injected gestational sacs into the choriodecidual space surrounding non-injected gestational sacs. We can only hypothesize that the presence of LPS leak in the choriodecidual space from injected gestational sacs might have triggered a weak inflammatory response with the release of IL-6 in the amniotic fluid into non-injected sacs.

There is evidence that intra-amniotic administration of LPS 0.1 μg per gestational sac in mice causes a high frequency of preterm delivery (80%) [247,248,250]. In our study, dams were not delivered within 24 h after intra-amniotic administration of LPS despite the development of IAI. This is in line with other rat studies, in which intra-amniotic administration of LPS via small laparotomy did not induce preterm delivery [245,246]. Moreover, intra-amniotic administration of LPS to mice is associated with a high fetal mortality rate, which is not seen in rat studies [245,248-250]. In our study, only 1 of the 19 fetuses from gestational sacs injected with LPS died 24 hours after administration. This intrauterine death could be attributed to IAI induced by LPS; however, needle injury during the intra-amniotic puncture might have also been a possible mechanism. The important attribute of this model is that intra-amniotic administration of LPS did not induce labor and 95% of fetuses remained alive in the inflammatory environment. Due to its endurance and stability, it may represent a more valuable model for research of intra-amniotic inflammatory complications in maternal and fetal compartments than in a similar mouse model.

The assessment of IL-6 concentrations in the amniotic fluid in an animal model represents an approach relevant to human clinical practice. Therefore, we focused on the evaluation of this body fluid in our rat model. To the best of our knowledge, this is the first study to investigate IL-6 levels in the amniotic fluid of rats after intra-amniotic administration of LPS. The possible reason for the absence of other studies could be the fact that the acquisition of a sufficient volume of amniotic fluid for analysis could be a challenge after intra-amniotic injection. In our study, the amniotic fluid volume was reduced in the injected gestational sacs, which impaired amniotic fluid harvesting. The reduction of amniotic fluid volume could have been associated with the development of IAI; however, based on the concurrent occurrence of this event in gestational sacs administered PBS, the leakage of the amniotic fluid through the traumatized membranes was a more probable mechanism.

The strength of this study is that it is a minimally invasive method of intra-amniotic administration of LPS. Nevertheless, this study also has limitations. First, an sufficient amount of the amniotic fluid for analysis could not be obtained from all gestational sacs due to the reduction in its volume. Second, the inflammatory responses to LPS in the maternal, placental, and fetal compartments were not examined. Third, only one dose of LPS was administered.

5.2.3 Specific aim II-C

In specific aim II-C, the protocol of ultrasound-guided intra-amniotic administration of an agent to create a rat animal model of IAI was summarized.

The value of research can be enhanced by applying a consistent methodology that supports reproducibility. The course of actions can be summarized step by step into protocols, which enable us to proceed with the research without technical issues. This is especially important when animal models are involved, where a consistent methodology facilitates adherence to ethical standards [301].

The protocol provides complete and consistent instructions on how to develop a rat animal model of IAI by ultrasound-guided intra-amniotic administration of an agent. The ultrasound-guided mini-invasive approach minimizes trauma and stress in animals. The protocol describes this technique in detail and focuses on specific technical aspects of gestational sac puncture and the use of high-resolution ultrasound guidance. In our study, we used LPS administration; however, other agents such as different bacteria and their products or DAMPs can be used in the same way to trigger different phenotypes of IAI in rats. Therefore, this protocol can be a supportive and helpful basis for the establishment of other rat models of IAI.

6. CONCLUSION

6.1 CLINICAL OBJECTIVES

In women with PTL, the presence of both phenotypes of IAI, intra-amniotic infection and sterile IAI, was associated with an elevated concentration of IL-6 in the cervical fluid. However, no differences in the concentration of IL-6 in the cervical fluid were found between the two conditions.

In women with PTL, the concentrations of FcgammaBP in amniotic fluid were elevated in the presence of both phenotypes of IAI, being higher in the presence of intra-amniotic infection. Therefore, FcgammaBP in the amniotic fluid can be considered a potential marker of intra-amniotic infection in women with PTL. The concentration of FcgammaBP of the cervical fluid was not altered by the presence of either phenotype of IAI.

6.2 EXPERIMENTAL OBJECTIVES

A systematic review of the literature demonstrated that intra-amniotic administration of triggering agents is used to model intra-amniotic infection/inflammation in rodents. Intra-amniotic administration under ultrasound guidance has been described in mice, but not in rats.

Our experiments showed that ultrasound-guided intra-amniotic administration of an agent was feasible in rats. The administration of 10 µg of *E. coli* LPS serotype O55:B5 per gestational sac resulted in the development of IAI and did not induce labor or fetal mortality.

Finally, a step-by-step protocol for ultrasound-guided intra-amniotic administration of an agent in a rat to support the reproducibility and feasibility of this approach was developed.

7. LITERATURE REVIEW

1. Dbstet A. WHO: recommended definitions, terminology and format for statistical tables related to the perinatal period and use of a new certificate for cause of perinatal deaths. *Acta Obstetricia et Gynecologica Scandinavica.* 1977;56(3):247-53.
2. Vogel JP, Chawanpaiboon S, Moller A-B, et al. The global epidemiology of preterm birth. *Best Practice & Research Clinical Obstetrics & Gynaecology.* 2018 2018/10/01/;52:3-12.
3. Blencowe H, Cousens S, Oestergaard MZ, et al. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. *The Lancet*. 2012 2012/06/09/;379(9832):2162-2172.
4. Walani SR. Global burden of preterm birth. *International Journal of Gynecology & Obstetrics*. 2020;150(1):31-33.
5. Lumley J. Defining the problem: the epidemiology of preterm birth. *International Journal of Obstetrics & Gynaecology*. 2003;110(s20):3-7.
6. Chawanpaiboon S, Vogel JP, Moller A-B, et al. Global, regional, and national estimates of levels of preterm birth in 2014: a systematic review and modelling analysis. *The Lancet Global Health.* 2019 2019/01/01/;7(1):e37-e46.
7. Fantom NJ, Serajuddin U. The World Bank's classification of countries by income. *The World Bank*; 2016.
8. Martin JA, Hamilton BE, Osterman MJ, et al. Births: final data for 2015. 2017.
9. Morken N-H, Vogel I, Kallen K, et al. Reference population for international comparisons and time trend surveillance of preterm delivery proportions in three countries. *BMC Women's Health.* 2008 2008/09/25;8(1):16.
10. Cobo T, Kacerovsky M, Jacobsson B. Risk factors for spontaneous preterm delivery. *International Journal of Gynecology & Obstetrics*. 2020;150(1):17-23.
11. Blencowe H, Cousens S, Chou D, et al. Chapter 2: 15 million preterm births: Priorities for action based on national, regional and global estimates. Born Too Soon: The Global Action Report on Preterm Birth New York: March of Dimes, PMNCH, Save the Children, *World Health Organization*. 2012.
12. Frey HA, Klebanoff MA. The epidemiology, etiology, and costs of preterm birth. *Seminars in Fetal and Neonatal Medicine.* 2016 2016/04/01/;21(2):68-73.
13. Purisch SE, Gyamfi-Bannerman C. Epidemiology of preterm birth. *Seminars in Perinatology.* 2017 2017/11/01/;41(7):387-391.
14. Saigal S, Doyle LW. An overview of mortality and sequelae of preterm birth from infancy to adulthood. *The Lancet.* 2008 Jan 19;371(9608):261-9.
15. Goldenberg RL, Culhane JF, Iams JD, et al. Epidemiology and causes of preterm birth. *The Lancet*. 2008 2008/01/05/;371(9606):75-84.

16. Souza RT, Cecatti JG, Passini Jr R, et al. The burden of provider-initiated preterm birth and associated factors: evidence from the brazilian multicenter study on preterm birth (EMIP). *PLoS One.* 2016;11(2):e0148244.

17. Kramer MS, Papageorghiou A, Culhane J, et al. Challenges in defining and classifying the preterm birth syndrome. *American Journal of Obstetrics and Gynecology.* 2012 2012/02/01/;206(2):108-112.

18. Savitz DA, Blackmore CA, Thorp JM. Epidemiologic characteristics of preterm delivery: etiologic heterogeneity. *American Journal of Obstetrics and Gynecology.* 1991;164(2):467-471.

19. Romero R, Dey SK, Fisher SJ. Preterm labor: One syndrome, many causes. *Science.* 2014;345(6198):760-765.

20. Gilman-Sachs A, Dambaeva S, Salazar Garcia MD, et al. Inflammation induced preterm labor and birth. *Journal of Reproductive Immunology*. 2018 2018/09/01/;129:53-58.

21. Menon R. Spontaneous preterm birth, a clinical dilemma: Etiologic, pathophysiologic and genetic heterogeneities and racial disparity. *Acta Obstetricia et Gynecologica Scandinavica.* 2008 2008/01/01;87(6):590-600.

22. Berghella V, Saccone G. Cervical assessment by ultrasound for preventing preterm delivery. *Cochrane Database of Systematic Reviews*. 2019 (9).

23. Mahendroo M. Cervical remodeling in term and preterm birth: insights from an animal model. *Reproduction*. 2012;143(4):429.

24. Manuck TA, Esplin MS, Biggio J, et al. The phenotype of spontaneous preterm birth: application of a clinical phenotyping tool. *American Journal of Obstetrics and Gynecology*. 2015;212(4):487.e1-487.e11.

25. Ramos GC. Inflammation as an Animal Development Phenomenon. *Clinical and Developmental Immunology*. 2012 2011/10/19;2012:983203.

26. Medzhitov R. Inflammation 2010: new adventures of an old flame. *Cell*. 2010 Mar 19;140(6):771-6.

27. Norman JE, Bollapragada S, Yuan M, et al. Inflammatory pathways in the mechanism of parturition. *BMC Pregnancy and Childbirth*. 2007 2007/06/01;7(1):S7.

28. Tosto V, Giardina I, Tsibizova V, et al. Preterm Birth, From the Biological Knowledges to the Prevention: An Overview. *Maternal-Fetal Medicine*. 2020;2(3):162-171.

29. Romero R, Espinoza J, Gonçalves LF, et al. Inflammation in preterm and term labour and delivery. *Seminars in Fetal and Neonatal Medicine*. 2006 2006/10/01/;11(5):317-326.

30. Romero R, Miranda J, Chaiworapongsa T, et al. Prevalence and clinical significance of sterile intra-amniotic inflammation in patients with preterm labor and intact membranes. *American journal of reproductive immunology*. 2014;72(5):458-474.

31. Yoon BH, Romero R, Moon JB, et al. Clinical significance of intra-amniotic inflammation in patients with preterm labor and intact membranes. *American Journal of Obstetrics and Gynecology.* 2001 Nov;185(5):1130-6.

32. Yang J, Hartmann KE, Savitz DA, et al. Vaginal Bleeding during Pregnancy and Preterm Birth. *American Journal of Epidemiology*. 2004;160(2):118-125.

33. Salafia CM, López-Zeno J, Sherer DM, et al. Histologic evidence of old intrauterine bleeding is more frequent in prematurity. *American Journal of Obstetrics and Gynecology*. 1995 1995/10/01/;173(4):1065-1070.

34. Elovitz MA, Saunders T, Ascher-Landsberg J, et al. Effects of thrombin on myometrial contractions in vitro and in vivo. *American Journal of Obstetrics and Gynecology*. 2000 Oct;183(4):799-804.

35. Lockwood CJ, Kayisli UA, Stocco C, et al. Abruption-induced preterm delivery is associated with thrombin-mediated functional progesterone withdrawal in decidual cells. *American Journal of Pathology*. 2012;181(6):2138-2148.

36. Kim YM, Bujold E, Chaiworapongsa T, et al. Failure of physiologic transformation of the spiral arteries in patients with preterm labor and intact membranes. *American Journal of Obstetrics and Gynecology*. 2003 2003/10/01/;189(4):1063-1069.

37. von Rango U. Fetal tolerance in human pregnancy--a crucial balance between acceptance and limitation of trophoblast invasion. *Immunology Letters*. 2008 Jan 15;115(1):21-32.

38. Guleria I, Sayegh MH. Maternal acceptance of the fetus: true human tolerance. *Journal of Immunology*. 2007 Mar 15;178(6):3345-51.

39. Lee J, Romero R, Xu Y, et al. A Signature of Maternal Anti-Fetal Rejection in Spontaneous Preterm Birth: Chronic Chorioamnionitis, Anti-Human Leukocyte Antigen Antibodies, and C4d. *PLoS One*. 2011;6(2):e16806.

40. Kim CJ, Romero R, Kusanovic JP, et al. The frequency, clinical significance, and pathological features of chronic chorioamnionitis: a lesion associated with spontaneous preterm birth. *Modern Pathology*. 2010;23(7):1000-1011.

41. Maymon E, Romero R, Bhatti G, et al. Chronic inflammatory lesions of the placenta are associated with an up-regulation of amniotic fluid CXCR3: A marker of allograft rejection. *Journal of Perinatal Medicine*. 2018;46(2):123-137.

42. Lee J, Romero R, Chaiworapongsa T, et al. Characterization of the Fetal Blood Transcriptome and Proteome in Maternal Anti-Fetal Rejection: Evidence of a Distinct and Novel Type of Human Fetal Systemic Inflammatory Response. *American Journal of Reproductive Immunology*. 2013;70(4):265-284.

43. Adams Waldorf KM, Singh N, Mohan AR, et al. Uterine overdistention induces preterm labor mediated by inflammation: observations in pregnant women and nonhuman primates. *American Journal of Obstetrics and Gynecology*. 2015 Dec;213(6):830.e1-830.e19.

44. Ou CW, Orsino A, Lye SJ. Expression of connexin-43 and connexin-26 in the rat myometrium during pregnancy and labor is differentially regulated by mechanical and hormonal signals. *Endocrinology*. 1997 Dec;138(12):5398-407.

45. Terzidou V, Sooranna SR, Kim LU, et al. Mechanical stretch up-regulates the human oxytocin receptor in primary human uterine myocytes. *The Journal of Clinical Endocrinology and Metabolism*. 2005 Jan;90(1):237-46.

46. Shim SS, Romero R, Hong JS, et al. Clinical significance of intra-amniotic inflammation in patients with preterm premature rupture of membranes. *American Journal of Obstetrics and Gynecology*. 2004 Oct;191(4):1339-45.

47. Vollrath ME, Sengpiel V, Landolt MA, et al. Is maternal trait anxiety a risk factor for late preterm and early term deliveries? *BMC Pregnancy and Childbirth*. 2016 2016/09/29;16(1):286.

48. Li D, Liu L, Odouli R. Presence of depressive symptoms during early pregnancy and the risk of preterm delivery: a prospective cohort study. *Human Reproduction.* 2009 Jan;24(1):146-53.

49. Lima SAM, El Dib RP, Rodrigues MRK, et al. Is the risk of low birth weight or preterm labor greater when maternal stress is experienced during pregnancy? A systematic review and meta-analysis of cohort studies. *PLoS One*. 2018;13(7):e0200594.

50. Dole N, Savitz DA, Hertz-Picciotto I, et al. Maternal Stress and Preterm Birth. *American Journal of Epidemiology.* 2003;157(1):14-24.

51. Petraglia F, Imperatore A, Challis JR. Neuroendocrine mechanisms in pregnancy and parturition. *Endocrine Reviews.* 2010 Dec;31(6):783-816.

52. Jones SA, Challis JR. Effects of corticotropin-releasing hormone and adrenocorticotropin on prostaglandin output by human placenta and fetal membranes. *Gynecologic and Obstetric Investigation.* 1990;29(3):165-8.

53. Gibb W. The role of prostaglandins in human parturition. *Annals of Medicine*. 1998 Jun;30(3):235-41.

54. Basso O, Wilcox A. Mortality risk among preterm babies: immaturity vs. underlying pathology. *Epidemiology*. 2010;21(4):521.

55. Ward RM, Beachy JC. Neonatal complications following preterm birth. *BJOG: An International Journal of Obstetrics and Gynaecology*. 2003 2003/04/01/;110:8-16.

56. Laptook AR, Bell EF, Shankaran S, et al. Admission temperature and associated mortality and morbidity among moderately and extremely preterm infants. *The Journal of Pediatrics.* 2018;192:53-59. e2.

57. de Almeida MF, Guinsburg R, Sancho GA, et al. Hypothermia and early neonatal mortality in preterm infants. *The Journal of Pediatrics.* 2014 Feb;164(2):271-5.e1.

58. Ting JY, Synnes AR, Lee SK, et al. Association of admission temperature and death or adverse neurodevelopmental outcomes in extremely low-gestational age neonates. *Journal of Perinatology*. 2018;38(7):844-849.

59. Hu XJ, Wang L, Zheng RY, et al. Using polyethylene plastic bag to prevent moderate hypothermia during transport in very low birth weight infants: a randomized trial. *Journal of Perinatology.* 2018 Apr;38(4):332-336.

60. Reilly MC, Vohra S, Rac VE, et al. Randomized trial of occlusive wrap for heat loss prevention in preterm infants. *Journal of Perinatology.* 2015 Feb;166(2):262-8.e2.

61. Sweet DG, Carnielli V, Greisen G, et al. European Consensus Guidelines on the Management of Neonatal Respiratory Distress Syndrome in Preterm Infants - 2013 Update. *Neonatology*. 2013;103(4):353-368.

62. Stoll BJ, Hansen NI, Bell EF, et al. Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. *Pediatrics*. 2010;126(3):443-456.

63. Smolarova S, Kocvarova L, Matasova K, et al. Impact of Updated European Consensus Guidelines on the Management of Neonatal Respiratory Distress Syndrome on Clinical

Outcome of Preterm Infants. In: Pokorski M, editor. *Respiratory Infections*. 2015. p. 61-66.

64. Sinkin RA, Cox C, Phelps DL. Predicting risk for bronchopulmonary dysplasia: selection criteria for clinical trials. *Pediatrics*. 1990;86(5):728-736.

65. Kraybill EN, Runyan DK, Bose CL, et al. Risk factors for chronic lung disease in infants with birth weights of 751 to 1000 grams. *The Journal of Pediatrics*. 1989;115(1):115-120.

66. Laughon MM, Langer JC, Bose CL, et al. Prediction of bronchopulmonary dysplasia by postnatal age in extremely premature infants. *American Journal of Respiratory and Critical Care Medicine*. 2011;183(12):1715-1722.

67. Wai KC, Kohn MA, Ballard RA, et al. Early cumulative supplemental oxygen predicts bronchopulmonary dysplasia in high risk extremely low gestational age newborns. *The Journal of Pediatrics*. 2016;177:97-102. e2.

68. Lahra MM, Beeby PJ, Jeffery HE. Intrauterine inflammation, neonatal sepsis, and chronic lung disease: a 13-year hospital cohort study. *Pediatrics*. 2009;123(5):1314-1319.

69. Northway Jr WH, Moss RB, Carlisle KB, et al. Late pulmonary sequelae of bronchopulmonary dysplasia. *New England Journal of Medicine*. 1990;323(26):1793-1799.

70. Perlman JM, editor The relationship between systemic hemodynamic perturbations and periventricular-intraventricular hemorrhage—a historical perspective. *Seminars in Pediatric Neurology*; 2009: Elsevier.

71. Tortora D, Severino M, Malova M, et al. Differences in subependymal vein anatomy may predispose preterm infants to GMH‐IVH. *Archives of Disease in Childhood-Fetal and Neonatal Edition*. 2018;103(1):F59-F65.

72. Noori S, Seri I, editors. Hemodynamic antecedents of peri/intraventricular hemorrhage in very preterm neonates. *Seminars in Fetal and Neonatal Medicine*; 2015: Elsevier.

73. Inder TE, Perlman JM, Volpe JJ. Preterm intraventricular hemorrhage/posthemorrhagic hydrocephalus. *Volpe's Neurology of the Newborn: Elsevier*. 2018. p. 637-698. e21.

74. Wheatley C, Dickinson J, Mackey D, et al. Retinopathy of prematurity: recent advances in our understanding. *British Journal of Ophthalmology*. 2002;86(6):696-700.

75. Hussain N, Clive J, Bhandari V. Current incidence of retinopathy of prematurity, 1989‐1997. *Pediatrics*. 1999;104(3):e26-e26.

76. Tan Z, Chong C, Darlow B, et al. Visual impairment due to retinopathy of prematurity (ROP) in New Zealand: a 22-year review. *British Journal of Ophthalmology*. 2015;99(6):801-806.

77. Lawn JE, Wilczynska-Ketende K, Cousens SN. Estimating the causes of 4 million neonatal deaths in the year 2000. *International Journal of Epidemiology*. 2006 Jun;35(3):706-18.

78. Stoll BJ, Hansen N. Infections in VLBW infants: studies from the NICHD Neonatal Research Network. *Seminars in Perinatology*. 2003 Aug;27(4):293-301.

79. Stoll BJ, Hansen N, Fanaroff AA, et al. Changes in pathogens causing early-onset sepsis in very-low-birth-weight infants. *New England Journal of Medicine*. 2002 Jul 25;347(4):240-7.

80. Puopolo KM, Benitz WE, Zaoutis TE, et al. Management of neonates born at≥ 35 0/7 weeks' gestation with suspected or proven early-onset bacterial sepsis. *Pediatrics*. 2018;142(6).

81. Stoll BJ, Hansen N, Fanaroff AA, et al. Late-onset sepsis in very low birth weight neonates: the experience of the NICHD Neonatal Research Network. *Pediatrics.* 2002 Aug;110(2 Pt 1):285-91.

82. Stoll BJ, Hansen NI, Bell EF, et al. Trends in Care Practices, Morbidity, and Mortality of Extremely Preterm Neonates, 1993-2012. *Jama.* 2015 Sep 8;314(10):1039-51.

83. Stoll BJ, Hansen NI, Sánchez PJ, et al. Early onset neonatal sepsis: the burden of group B Streptococcal and E. coli disease continues. *Pediatrics*. 2011 May;127(5):817-26.

84. Bekhof J, Reitsma JB, Kok JH, et al. Clinical signs to identify late-onset sepsis in preterm infants. *European Journal of Pediatrics.* 2013 Apr;172(4):501-8.

85. Griffin MP, Lake DE, O'Shea TM, et al. Heart rate characteristics and clinical signs in neonatal sepsis. *Pediatric Research.* 2007 Feb;61(2):222-7.

86. Dehaene I, Scheire E, Steen J, et al. Obstetrical characteristics and neonatal outcome according to aetiology of preterm birth: a cohort study. *Archives of Gynecology and Obstetrics.* 2020 2020/10/01;302(4):861-871.

87. Repka MX. Ophthalmological problems of the premature infant. *Developmental Disabilities Research Reviews.* 2002;8(4):249-257.

88. Stark A, Carlo W, Tyson J, et al. National Institute of Child Health and Human Development Neonatal Research Network Adverse effects of early dexamethasone in extremely-low-birth-weight infants. National Institute of Child Health and Human Development Neonatal Research Network. *New England Journal of Medicine*. 2001;344(2):95-101.

89. Vohr BR, Wright LL, Dusick AM, et al. Neurodevelopmental and functional outcomes of extremely low birth weight infants in the National Institute of Child Health and Human Development Neonatal Research Network, 1993‐1994. *Pediatrics*. 2000;105(6):1216-1226.

90. Hovi P, Andersson S, Eriksson JG, et al. Glucose regulation in young adults with very low birth weight. *New England Journal of Medicine*. 2007 May 17;356(20):2053-63.

91. Morrison KM, Ramsingh L, Gunn E, et al. Cardiometabolic Health in Adults Born Premature With Extremely Low Birth Weight. *Pediatrics*. 2016 Oct;138(4).

92. Rotteveel J, van Weissenbruch MM, Twisk JW, et al. Infant and childhood growth patterns, insulin sensitivity, and blood pressure in prematurely born young adults. *Pediatrics*. 2008 Aug;122(2):313-21.

93. Odberg MD, Sommerfelt K, Markestad T, et al. Growth and somatic health until adulthood of low birthweight children. *Archives of Disease in Childhood-Fetal and Neonatal Edition*. 2010 May;95(3):F201-5.

94. Swamy GK, Ostbye T, Skjaerven R. Association of preterm birth with long-term survival, reproduction, and next-generation preterm birth. *Jama*. 2008 Mar 26;299(12):1429-36.

95. Crump C. Preterm birth and mortality in adulthood: a systematic review. *Journal of Perinatology.* 2020 Jun;40(6):833-843.

96. Holsti A, Adamsson M, Hägglöf B, et al. Chronic conditions and health care needs of adolescents born at 23 to 25 weeks' gestation. *Pediatrics.* 2017;139(2).

97. Lindström K, Winbladh B, Haglund B, et al. Preterm infants as young adults: a Swedish national cohort study. *Pediatrics*. 2007 Jul;120(1):70-7.

98. Moster D, Lie RT, Markestad T. Long-term medical and social consequences of preterm birth. *New England Journal of Medicine.* 2008 Jul 17;359(3):262-73.

99. Mangham LJ, Petrou S, Doyle LW, et al. The cost of preterm birth throughout childhood in England and Wales. *Pediatrics*. 2009 Feb;123(2):e312-27.

100. Cobo T, Aldecoa V, Figueras F, et al. Development and validation of a multivariable prediction model of spontaneous preterm delivery and microbial invasion of the amniotic cavity in women with preterm labor. *American Journal of Obstetrics and Gynecology*. 2020 2020/09/01/;223(3):421.e1-421.e14.

101. McPheeters ML, Miller WC, Hartmann KE, et al. The epidemiology of threatened preterm labor: A prospective cohort study. *American Journal of Obstetrics and Gynecology*. 2005 2005/04/01/;192(4):1325-1329.

102. Sotiriadis A, Papatheodorou S, Kavvadias A, et al. Transvaginal cervical length measurement for prediction of preterm birth in women with threatened preterm labor: a meta-analysis. *Ultrasound in Obstetrics and Gynecology*. 2010;35(1):54-64.

103. Suff N, Story L, Shennan A. The prediction of preterm delivery: What is new? *Seminars in Fetal and Neonatal Medicine.* 2019 2019/02/01/;24(1):27-32.

104. National Collaborating Centre for Ws, Children's H. National Institute for Health and Care Excellence: Clinical Guidelines. Preterm Labour and Birth. London: National Institute for Health and Care Excellence (UK) Copyright © 2015 National Collaborating Centre for Women's and Children's Health.; 2015.

105. Berghella V, Palacio M, Ness A, et al. Cervical length screening for prevention of preterm birth in singleton pregnancy with threatened preterm labor: systematic review and meta-analysis of randomized controlled trials using individual patient-level data. *Ultrasound in Obstetrics and Gynecology*. 2017;49(3):322-329.

106. van Baaren G-J, Vis JY, Wilms FF, et al. Predictive Value of Cervical Length Measurement and Fibronectin Testing in Threatened Preterm Labor. *Obstetrics & Gynecology*. 2014;123(6):1185-1192.

107. Radan A-P, Aleksandra Polowy J, Heverhagen A, et al. Cervico-vaginal placental α-macroglobulin-1 combined with cervical length for the prediction of preterm birth in women with threatened preterm labor. *Acta Obstetricia et Gynecologica Scandinavica*. 2020;99(3):357-363.

108. Bolotskikh V, Borisova V. Combined value of placental alpha microglobulin-1 detection and cervical length via transvaginal ultrasound in the diagnosis of preterm labor in symptomatic patients. *Journal of Obstetrics and Gynaecology Research*. 2017;43(8):1263-1269.

109. Abbott DS, Radford SK, Seed PT, et al. Evaluation of a quantitative fetal fibronectin test for spontaneous preterm birth in symptomatic women. *American Journal of Obstetrics and Gynecology.* 2013;208(2):122. e1-122. e6.

110. Kumari A, Saini V, Jain P, et al. Prediction of delivery in women with threatening preterm labour using phosphorylated insulin-like growth factor binding protein-1 and cervical

length using transvaginal ultrasound. *Journal of Clinical and Diagnostic Research*. 2017;11(9):QC01.

111. Romero R, Gotsch F, Pineles B, et al. Inflammation in pregnancy: its roles in reproductive physiology, obstetrical complications, and fetal injury. *Nutrition Reviews*. 2007;65(suppl_3):S194-S202.
112. Jacobsson B, Mattsby-Baltzer I, Andersch B, et al. Microbial invasion and cytokine response in amniotic fluid in a Swedish population of women in preterm labor. *Acta Obstetricia et Gynecologica Scandinavica.* 2003 2003/01/01;82(2):120-128.
113. Romero R, Miranda J, Chaiworapongsa T, et al. A Novel Molecular Microbiologic Technique for the Rapid Diagnosis of Microbial Invasion of the Amniotic Cavity and Intra-Amniotic Infection in Preterm Labor with Intact Membranes. *American Journal of Reproductive Immunology.* 2014;71(4):330-358.
114. Galask RP, Varner MW, Petzold CR, et al. Bacterial attachment to the chorioamniotic membranes. *American Journal of Obstetrics and Gynecology*. 1984;148(7):915-928.
115. Chaim W, Mazor M, Leiberman JR. The relationship between bacterial vaginosis and preterm birth. A review. *Archives of Gynecology and Obstetrics*. 1997 1997/01/01;259(2):51-58.
116. Romero R, Chaiworapongsa T, Kuivaniemi H, et al. Bacterial vaginosis, the inflammatory response and the risk of preterm birth: a role for genetic epidemiology in the prevention of preterm birth. *American Journal of Obstetrics and Gynecology*.. 2004;190(6):1509-1519.
117. Goldenberg RL, Hauth JC, Andrews WW. Intrauterine Infection and Preterm Delivery. *New England Journal of Medicine*. 2000;342(20):1500-1507.
118. Goldenberg RL, Andrews WW, Hauth JC. Choriodecidual infection and preterm birth. *Nutrition reviews*. 2002;60(suppl_5):S19-S25.
119. Kim MJ, Romero R, Gervasi MT, et al. Widespread microbial invasion of the chorioamniotic membranes is a consequence and not a cause of intra-amniotic infection. *Laboratory Investigation.* 2009 2009/08/01;89(8):924-936.
120. Gardella C, Riley DE, Hitti J, et al. Identification and sequencing of bacterial rDNAs in culture-negative amniotic fluid from women in premature labor. *American Journal of Perinatology.* 2004;21(06):319-323.
121. Cobo T, Vives I, Rodríguez-Trujillo A, et al. Impact of microbial invasion of amniotic cavity and the type of microorganisms on short-term neonatal outcome in women with preterm labor and intact membranes. *Acta Obstetricia et Gynecologica Scandinavica*. 2017;96(5):570-579.
122. DiGiulio DB. Diversity of microbes in amniotic fluid. *Seminars in Fetal and Neonatal Medicine*. 2012 Feb;17(1):2-11.
123. Menon R, Peltier MR, Eckardt J, et al. Diversity in cytokine response to bacteria associated with preterm birth by fetal membranes. *American Journal of Obstetrics and Gynecology.* 2009;201(3):306. e1-306. e6.
124. Kacerovsky M, Pliskova L, Bolehovska R, et al. The impact of the microbial load of genital mycoplasmas and gestational age on the intensity of intraamniotic inflammation. *American Journal of Obstetrics and Gynecology*. 2012 2012/04/01/;206(4):342.e1-342.e8.

125. Carroll SG, Ville Y, Greenough A, et al. Preterm prelabour amniorrhexis: intrauterine infection and interval between membrane rupture and delivery. *Archives of Disease in Childhood - Fetal and Neonatal Edition*. 1995;72(1):F43-F46.

126. Takeda K, Akira S. Toll-like receptors in innate immunity. *International Immunology*. 2005;17(1):1-14.

127. Rindsjö E, Holmlund U, Sverremark-Ekström E, et al. Toll-like receptor-2 expression in normal and pathologic human placenta. *Human Pathology*. 2007;38(3):468-473.

128. Schwandner R, Dziarski R, Wesche H, et al. Peptidoglycan-and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *Journal of Biological Chemistry*. 1999;274(25):17406-17409.

129. Yoshimura A, Lien E, Ingalls RR, et al. Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *The Journal of Immunology*. 1999;163(1):1-5.

130. Kim YM, Romero R, Chaiworapongsa T, et al. Toll-like receptor-2 and-4 in the chorioamniotic membranes in spontaneous labor at term and in preterm parturition that are associated with chorioamnionitis. *American Journal of Obstetrics and Gynecology*.. 2004;191(4):1346-1355.

131. Hayati AR, Mohamed AE, Tan GC. An immunohistochemical study of Toll-like receptors 2 and 4 in placenta with and without infection. *The Malaysian Journal of Pathology.* 2010 Jun;32(1):13-9.

132. Green ES, Arck PC, editors. Pathogenesis of preterm birth: bidirectional inflammation in mother and fetus. *Seminars in Immunopathology*; 2020: Springer.

133. Lindstrom TM, Bennett PR. The role of nuclear factor kappa B in human labour. *Reproduction*. 2005;130(5):569-581.

134. Lappas M, Permezel M, Georgiou HM, et al. Nuclear Factor Kappa B Regulation of Proinflammatory Cytokines in Human Gestational Tissues In Vitro1. *Biology of Reproduction.* 2002;67(2):668-673.

135. Belt AR, Baldassare JJ, Molnár M, et al. The nuclear transcription factor NF-kappaB mediates interleukin-1beta-induced expression of cyclooxygenase-2 in human myometrial cells. *American Journal of Obstetrics and Gynecology*. 1999 Aug;181(2):359-66.

136. Combs CA, Gravett M, Garite TJ, et al. Amniotic fluid infection, inflammation, and colonization in preterm labor with intact membranes. *American Journal of Obstetrics and Gynecology*. 2014 2014/02/01/;210(2):125.e1-125.e15.

137. Gomez R, Ghezzi F, Römern R, et al. Two thirds of human fetuses with microbial invasion of the amniotic cavity have a detectable systemic cytokine response before birth. *Acta Diabetologica Latina*. 1997;176(1 PART II).

138. Gomez R, Romero R, Ghezzi F, et al. The fetal inflammatory response syndrome. *American Journal of Obstetrics and Gynecology*. 1998 1998/07/01/;179(1):194-202.

139. Thorell A, Hallingström M, Hagberg H, et al. Microbial invasion of the amniotic cavity is associated with impaired cognitive and motor function at school age in preterm children. *Pediatric Research*. 2020 2020/04/01;87(5):924-931.

140. Rodríguez-Trujillo A, Ríos J, Ángeles MA, et al. Influence of perinatal inflammation on the neurodevelopmental outcome of premature infants. *The Journal of Maternal-Fetal & Neonatal Medicine*. 2019 Apr;32(7):1069-1077.

141. Yoon BH, Romero R, Park JS, et al. Fetal exposure to an intra-amniotic inflammation and the development of cerebral palsy at the age of three years. *American Journal of Obstetrics and Gynecology*. 2000;182(3):675-681.

142. Romero R, Miranda J, Chaiworapongsa T, et al. Prevalence and Clinical Significance of Sterile Intra-amniotic Inflammation in Patients with Preterm Labor and Intact Membranes. *American Journal of Reproductive Immunology*. 2014;72(5):458-474.

143. Romero R, Miranda J, Chaemsaithong P, et al. Sterile and microbial-associated intra-amniotic inflammation in preterm prelabor rupture of membranes. *The Journal of Maternal-Fetal & Neonatal Medicine*. 2015;28(12):1394-1409.

144. Friel LA, Romero R, Edwin S, et al. The calcium binding protein, S100B, is increased in the amniotic fluid of women with intra-amniotic infection/inflammation and preterm labor with intact or ruptured membranes. *Journal of Perinatal Medicine*. 2007;35(5):385-393.

145. Musilova I, Kutová R, Pliskova L, et al. Intraamniotic inflammation in women with preterm prelabor rupture of membranes. *PLoS One*. 2015;10(7):e0133929.

146. Bredeson S, Papaconstantinou J, Deford JH, et al. HMGB1 promotes a p38MAPK associated non-infectious inflammatory response pathway in human fetal membranes. *PLoS One*. 2014;9(12):e113799.

147. Romero R, Chaiworapongsa T, Alpay Savasan Z, et al. Damage-associated molecular patterns (DAMPs) in preterm labor with intact membranes and preterm PROM: a study of the alarmin HMGB1. *The Journal of Maternal-Fetal & Neonatal Medicine*. 2011;24(12):1444-1455.

148. Jacobsson B, Mattsby-Baltzer I, Hagberg H. Interleukin-6 and interleukin-8 in cervical and amniotic fluid: relationship to microbial invasion of the chorioamniotic membranes. *BJOG: An International Journal of Obstetrics and Gynaecology*. 2005 Jun;112(6):719-24.

149. Andrews WW, Hauth JC, Goldenberg RL, et al. Amniotic fluid interleukin-6: correlation with upper genital tract microbial colonization and gestational age in women delivered after spontaneous labor versus indicated delivery. *American Journal of Obstetrics and Gynecology*. 1995 Aug;173(2):606-12.

150. Saito Reis CA, Padron JG, Norman Ing ND, et al. High-mobility group box 1 is a driver of inflammation throughout pregnancy. *American Journal of Reproductive Immunology*. 2021 Jan;85(1):e13328.

151. Chaiworapongsa T, Erez O, Kusanovic JP, et al. Amniotic fluid heat shock protein 70 concentration in histologic chorioamnionitis, term and preterm parturition. *The Journal of Maternal-Fetal & Neonatal Medicine*. 2008 2008/01/01;21(7):449-461.

152. Nadeau-Vallée M, Obari D, Palacios J, et al. Sterile inflammation and pregnancy complications: a review. *Reproduction*. 2016 01 Dec. 2016;152(6):R277-R292.

153. Andrews WW, Hauth JC, Goldenberg RL, et al. Amniotic fluid interleukin-6: Correlation with upper genital tract microbial colonization and gestational age in women delivered after spontaneous labor versus indicated delivery. *American Journal of Obstetrics and Gynecology*. 1995 1995/08/01/;173(2):606-612.

154. Wei S-Q, Fraser W, Luo Z-C. Inflammatory cytokines and spontaneous preterm birth in asymptomatic women: a systematic review. *Obstetrics and Gynecology*. 2010;116(2):393-401.

155. Gravett MG, Hummel D, Eschenbach DA, et al. Preterm labor associated with subclinical amniotic fluid infection and with bacterial vaginosis. *Obstetrics and Gynecology*. 1986;67(2):229-237.

156. Armer TL, Duff P. Intraamniotic infection in patients with intact membranes and preterm labor. *Obstetrical & Gynecological Survey*. 1991;46(9):589-593.

157. Bobitt JR, Hayslip CC, Damato JD. Amniotic fluid infection as determined by transabdominal amniocentesis in patients with intact membranes in premature labor. *American Journal of Obstetrics and Gynecology*. 1981 1981/08/15/;140(8):947-952.

158. DiGiulio DB, Romero R, Amogan HP, et al. Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. *PloS One.* 2008;3(8):e3056.

159. Romero R, Jimenez C, Lohda AK, et al. Amniotic fluid glucose concentration: a rapid and simple method for the detection of intraamniotic infection in preterm labor. *American Journal of Obstetrics and Gynecology* 1990 Sep;163(3):968-74.

160. Buhimschi IA, Nayeri UA, Laky CA, et al. Advances in medical diagnosis of intra-amniotic infection. *Expert Opinion on Medical Diagnostics*. 2013;7(1):5-16.

161. Buhimschi IA, Christner R, Buhimschi CS. Proteomic biomarker analysis of amniotic fluid for identification of intra-amniotic inflammation. *BJOG: An International Journal of Obstetrics & Gynaecology.* 2005;112(2):173-181.

162. Buhimschi CS, Bhandari V, Hamar BD, et al. Proteomic profiling of the amniotic fluid to detect inflammation, infection, and neonatal sepsis. *PLoS Medicine*. 2007;4(1):e18.

163. Chaemsaithong P, Romero R, Docheva N, et al. Comparison of rapid MMP-8 and interleukin-6 point-of-care tests to identify intra-amniotic inflammation/infection and impending preterm delivery in patients with preterm labor and intact membranes. *The Journal of Maternal-Fetal & Neonatal Medicine.* 2018 2018/01/17;31(2):228-244.

164. Hussey MJ, Levy ES, Pombar X, et al. Evaluating rapid diagnostic tests of intra-amniotic infection: Gram stain, amniotic fluid glucose level, and amniotic fluid to serum glucose level ratio. *American Journal of Obstetrics and Gynecology*. 1998;179(3):650-656.

165. Kirshon B, Rosenfeld B, Mari G, et al. Amniotic fluid glucose and intraamniotic infection. *American Journal of Obstetrics and Gynecology*. 1991;164(3):818-820.

166. Romero R, Kadar N, Miranda J, et al. The diagnostic performance of the Mass Restricted (MR) score in the identification of microbial invasion of the amniotic cavity or intra-amniotic inflammation is not superior to amniotic fluid interleukin-6. *The Journal of Maternal-Fetal & Neonatal Medicine.* 2014 2014/05/01;27(8):757-769.

167. Lee J, Oh KJ, Yang HJ, et al. The importance of intra-amniotic inflammation in the subsequent development of atypical chronic lung disease. *The Journal of Maternal-Fetal & Neonatal Medicine.* 2009 2009/01/01;22(10):917-923.

168. Gomez R, Ghezzi F, Romero R, et al. Premature Labor and Intra-Amniotic Infection: Clinical Aspects and Role of the Cytokines in Diagnosis and Pathophysiology. *Clinics in Perinatology*. 1995 1995/06/01/;22(2):281-342.

169. Chaemsaithong P, Romero R, Korzeniewski SJ, et al. A rapid interleukin-6 bedside test for the identification of intra-amniotic inflammation in preterm labor with intact membranes. *The Journal of Maternal-Fetal & Neonatal Medicine.* 2016;29(3):349-359.

170. Musilova I, Andrys C, Holeckova M, et al. Interleukin-6 measured using the automated electrochemiluminescence immunoassay method for the identification of intra-amniotic inflammation in preterm prelabor rupture of membranes. *The Journal of Maternal-Fetal & Neonatal Medicine.* 2020 2020/06/02;33(11):1919-1926.

171. Gordon MC, Narula K, O'Shaughnessy R, et al. Complications of third-trimester amniocentesis using continuous ultrasound guidance. *Obstetrics & Gynecology*. 2002;99(2):255-259.

172. Stark CM, Smith RS, LaGrandeur RM, et al. Need for urgent delivery after third-trimester amniocentesis. *Obstetrics & Gynecology*. 2000;95(1):48-50.

173. Park H, Park KH, Kim YM, et al. Plasma inflammatory and immune proteins as predictors of intra-amniotic infection and spontaneous preterm delivery in women with preterm labor: a retrospective study. *BMC pregnancy and childbirth*. 2018;18(1):146-146.

174. Cobo T, Tsiartas P, Kacerovsky M, et al. Maternal inflammatory response to microbial invasion of the amniotic cavity: analyses of multiple proteins in the maternal serum. *Acta Obstetricia et Gynecologica Scandinavica.* 2013;92(1):61-68.

175. Dulay AT, Buhimschi IA, Zhao G, et al. Compartmentalization of acute phase reactants Interleukin-6, C-Reactive Protein and Procalcitonin as biomarkers of intra-amniotic infection and chorioamnionitis. *Cytokine.* 2015 Dec;76(2):236-243.

176. Hitti J, Hillier SL, Agnew KJ, et al. Vaginal indicators of amniotic fluid infection in preterm labor. *Obstetrics & Gynecology*. 2001;97(2):211-219.

177. Park JW, Park KH, Lee SY. Noninvasive prediction of intra-amniotic infection and/or inflammation in women with preterm labor: various cytokines in cervicovaginal fluid. *Reproductive Sciences*. 2013;20(3):262-268.

178. Combs CA, Garite TJ, Lapidus JA, et al. Detection of microbial invasion of the amniotic cavity by analysis of cervicovaginal proteins in women with preterm labor and intact membranes. *American Journal of Obstetrics and Gynecology*. 2015;212(4):482. e1-482. e12.

179. Cobo T, Kacerovsky M, Jacobsson B. Noninvasive sampling of the intrauterine environment in women with preterm labor and intact membranes. *Fetal Diagnosis and Therapy.* 2018;43:241-249.

180. Adnane M, Meade KG, O'Farrelly C. Cervico-vaginal mucus (CVM) - an accessible source of immunologically informative biomolecules. *Veterinary Research Communications.* 2018 Dec;42(4):255-263.

181. Holst R-M, Hagberg H, Wennerholm U-B, et al. Prediction of microbial invasion of the amniotic cavity in women with preterm labour: analysis of multiple proteins in amniotic and cervical fluids. *BJOG: An International Journal of Obstetrics & Gynaecology*. 2011;118(2):240-249.

182. Holst R-M, Mattsby-Baltzer I, Wennerholm U-B, et al. Interleukin-6 and interleukin-8 in cervical fluid in a population of Swedish women in preterm labor: relationship to microbial invasion of the amniotic fluid, intra-amniotic inflammation, and preterm delivery. *Acta Obstetricia et Gynecologica Scandinavica*. 2005;84(6):551-557.

183. Rizzo G, Capponi A, Vlachopoulou A, et al. Ultrasonographic assessment of the uterine cervix and interleukin-8 concentrations in cervical secretions predict intrauterine infection in patients with preterm labor and intact membranes. *Ultrasound in Obstetrics & Gynecology.* 1998;12(2):86-92.

184. Jacobsson B, Holst R-M, Mattsby-Baltzer I, et al. Interleukin-18 in cervical mucus and amniotic fluid: relationship to microbial invasion of the amniotic fluid, intra-amniotic inflammation and preterm delivery. *BJOG: An International Journal of Obstetrics & Gynaecology.* 2003;110(6):598-603.

185. Rizzo G, Capponi A, Rinaldo D, et al. Interleukin-6 concentrations in cervical secretions identify microbial invasion of the amniotic cavity in patients with preterm labor and intact membranes. *American Journal of Obstetrics and Gynecology* 1996 Oct;175(4 Pt 1):812-7.

186. Rizzo G, Capponi A, Arduini D, et al. The prognostic value of interleukin-8 and fetal fibronectin concentrations in cervical secretions in patients with preterm labor. *American Journal of Obstetrics and Gynecology.* 1997;176(1):S6.

187. Jacobsson B, Holst R-M, Wennerholm U-B, et al. Monocyte chemotactic protein-1 in cervical and amniotic fluid: relationship to microbial invasion of the amniotic cavity, intra-amniotic inflammation, and preterm delivery. *American Journal of Obstetrics and Gynecology.* 2003 2003/10/01/;189(4):1161-1167.

188. Romero R, Sibai B, Caritis S, et al. Antibiotic treatment of preterm labor with intact membranes: A multicenter, randomized, double-blinded, placebo-controlled trial. *American Journal of Obstetrics and Gynecology.* 1993 1993/10/01/;169(4):764-774.

189. Kenyon SL, Taylor DJ, Tarnow-Mordi W. Broad-spectrum antibiotics for spontaneous preterm labour: the ORACLE II randomised trial. *The Lancet*. 2001 2001/03/31/;357(9261):989-994.

190. Cox SM, Bohman VR, Sherman ML, et al. Randomized investigation of antimicrobials for the prevention of preterm birth. *American Journal of Obstetrics and Gynecology*. 1996 1996/01/01/;174(1, Part 1):206-210.

191. Gordon M, Samuels P, Shubert P, et al. A randomized, prospective study of adjunctive ceftizoxime in preterm labor. *American Journal of Obstetrics and Gynecology*. 1995 1995/05/01/;172(5):1546-1552.

192. Keuchkerian SE, Sosa CG, Fernandez A, et al. Effect of amoxicillin sulbactam in threatened preterm labour with intact membranes: a randomised controlled trial. *European Journal of Obstetrics & Gynecology and Reproductive Biology*. 2005 2005/03/01/;119(1):21-26.

193. Kenyon S, Pike K, Jones DR, et al. Childhood outcomes after prescription of antibiotics to pregnant women with spontaneous preterm labour: 7-year follow-up of the ORACLE II trial. *The Lancet*. 2008 2008/10/11/;372(9646):1319-1327.

194. Boyle AK, Rinaldi SF, Norman JE, et al. Preterm birth: Inflammation, fetal injury and treatment strategies. *Journal of Reproductive Immunology*. 2017 2017/02/01/;119:62-66.

195. Yoon BH, Romero R, Park JY, et al. Antibiotic administration can eradicate intra-amniotic infection or intra-amniotic inflammation in a subset of patients with preterm labor and intact membranes. *American Journal of Obstetrics and Gynecology*. 2019 2019/08/01/;221(2):142.e1-142.e22.

196. Oh KJ, Romero R, Park JY, et al. Evidence that antibiotic administration is effective in the treatment of a subset of patients with intra-amniotic infection/inflammation presenting with cervical insufficiency. *American Journal of Obstetrics and Gynecology*. 2019 Aug;221(2):140.e1-140.e18.

197. Romero R, Grivel J-C, Tarca AL, et al. Evidence of perturbations of the cytokine network in preterm labor. *American Journal of Obstetrics and Gynecology*. 2015 2015/12/01/;213(6):836.e1-836.e18.

198. Elovitz MA, Mrinalini C. Animal models of preterm birth. *Trends in Endocrinology & Metabolism*. 2004;15(10):479-487.

199. Nielsen BW, Bonney EA, Pearce BD, et al. A cross-species analysis of animal models for the investigation of preterm birth mechanisms. *Reproductive Sciences*. 2016;23(4):482-491.

200. Dilworth MR, Sibley CP. Review: Transport across the placenta of mice and women. *Placenta*. 2013 2013/03/01/;34:S34-S39.

201. Furukawa S, Tsuji N, Sugiyama A. Morphology and physiology of rat placenta for toxicological evaluation. *Journal of Toxicologic Pathology*. 2019;32(1):1-17.

202. Bezold KY, Karjalainen MK, Hallman M, et al. The genomics of preterm birth: from animal models to human studies. *Genome Medicine*. 2013 2013/04/29;5(4):34.

203. Barry JS, Anthony RV. The pregnant sheep as a model for human pregnancy. *Theriogenology*. 2008 2008/01/01/;69(1):55-67.

204. Kemp MW, Saito M, Newnham JP, et al. Preterm birth, infection, and inflammation advances from the study of animal models. *Reproductive Sciences*. 2010 Jul;17(7):619-28.

205. Waldorf KMA, Persing D, Novy MJ, et al. Pretreatment With Toll-like Receptor 4 Antagonist Inhibits Lipopolysaccharide-Induced Preterm Uterine Contractility, Cytokines, and Prostaglandins in Rhesus Monkeys. *Reproductive Sciences*. 2008 2008/02/01;15(2):121-127.

206. Gravett MG, Witkin SS, Haluska GJ, et al. An experimental model for intraamniotic infection and preterm labor in rhesus monkeys. *American Journal of Obstetrics and Gynecology*. 1994 Dec;171(6):1660-7.

207. Adams Waldorf K, Rubens C, Gravett M. Use of nonhuman primate models to investigate mechanisms of infection-associated preterm birth. *BJOG: An International Journal of Obstetrics & Gynaecology*. 2011;118(2):136-144.

208. Ratajczak CK, Muglia LJ. Insights Into Parturition Biology From Genetically Altered Mice. *Pediatric Research*. 2008 2008/12/01;64(6):581-589.

209. Dudley DJ, Branch DW, Edwin SS, et al. Induction of preterm birth in mice by RU486. *Biology of Reproduction*. 1996 Nov;55(5):992-5.

210. Garfield RE, Gasc JM, Baulieu EE. Effects of the antiprogesterone RU 486 on preterm birth in the rat. *American Journal of Obstetrics and Gynecology*. 1987 1987/11/01/;157(5):1281-1285.

211. Garfield RE, Puri CP, Csapo AI. Endocrine, structural, and functional changes in the uterus during premature labor. *American Journal of Obstetrics and Gynecology*. 1982 1982/01/01/;142(1):21-27.

212. Challis JRG, Matthews SG, Gibb W, et al. Endocrine and paracrine regulation of birth at term and preterm. *Endocrine Reviews.* 2000 Oct;21(5):514-50.

213. Liggins GC. Premature delivery of foetal lambs infused with glucocorticoids. *Journal of Endocrinology.* 1969 Dec;45(4):515-23.

214. Han YW, Redline RW, Li M, et al. Fusobacterium nucleatum induces premature and term stillbirths in pregnant mice: implication of oral bacteria in preterm birth. *Infection and Immunity*. 2004 Apr;72(4):2272-9.

215. Normann E, Lacaze-Masmonteil T, Eaton F, et al. A novel mouse model of Ureaplasma-induced perinatal inflammation: effects on lung and brain injury. *Pediatric Research*. 2009;65(4):430-436.

216. Hirsch E, Saotome I, Hirsh D. A model of intrauterine infection and preterm delivery in mice. *American Journal of Obstetrics and Gynecology*. 1995 May;172(5):1598-603.

217. Davies JK, Shikes RH, Sze CI, et al. Histologic inflammation in the maternal and fetal compartments in a rabbit model of acute intra-amniotic infection. *American Journal of Obstetrics and Gynecology*. 2000 Nov;183(5):1088-93.

218. Fidel PL, Jr., Romero R, Wolf N, et al. Systemic and local cytokine profiles in endotoxin-induced preterm parturition in mice. *American Journal of Obstetrics and Gynecology*. 1994 May;170(5 Pt 1):1467-75.

219. Masaoka N, Watanabe M, Nakajima Y. The effects of sivelestat sodium hydrate on uterine contraction and the concentration of maternal and fetal blood cytokines in a sheep model of intra-amniotic infection induced by lipopolysaccharide. *The Journal of Maternal-Fetal & Neonatal Medicine.* Med. 2011 Aug;24(8):1013-8.

220. Kajikawa S, Kaga N, Futamura Y, et al. Lipoteichoic acid induces preterm delivery in mice. *Journal of Pharmacological and Toxicological Methods.* 1998 Apr;39(3):147-54.

221. Ilievski V, Hirsch E. Synergy between viral and bacterial toll-like receptors leads to amplification of inflammatory responses and preterm labor in the mouse. *Biology of Reproduction*. 2010;83(5):767-773.

222. Gomez-Lopez N, Romero R, Plazyo O, et al. Intra-amniotic administration of HMGB1 induces spontaneous preterm labor and birth. *American Journal of Reproductive Immunology.* 2016;75(1):3-7.

223. Romero R, Mazor M, Tartakovsky B. Systemic administration of interleukin-1 induces preterm parturition in mice. *American Journal of Obstetrics and Gynecology*. 1991 Oct;165(4 Pt 1):969-71.

224. Salminen A, Paananen R, Vuolteenaho R, et al. Maternal Endotoxin-Induced Preterm Birth in Mice: Fetal Responses in Toll-Like Receptors, Collectins, and Cytokines. *Pediatric Research*. 2008 2008/03/01;63(3):280-286.

225. Wei SQ, Fraser W, Luo ZC. Inflammatory cytokines and spontaneous preterm birth in asymptomatic women: a systematic review. *Obstetrics & Gynecology.* 2010 Aug;116(2 Pt 1):393-401.

226. Elovitz MA, Wang Z, Chien EK, et al. A new model for inflammation-induced preterm birth: the role of platelet-activating factor and Toll-like receptor-4. *American Journal of Pathology*. 2003 Nov;163(5):2103-11.

227. Gravett MG, Haluska GJ, Cook MJ, et al. Fetal and maternal endocrine responses to experimental intrauterine infection in rhesus monkeys. *American Journal of Obstetrics and Gynecology*. 1996 1996/06/01/;174(6):1725-1733.

228. Grigsby PL, Hirst JJ, Scheerlinck J-P, et al. Fetal Responses to Maternal and Intra-Amniotic Lipopolysaccharide Administration in Sheep1. *Biology of Reproduction*. 2003;68(5):1695-1702.

229. Reznikov LL, Fantuzzi G, Selzman CH, et al. Utilization of endoscopic inoculation in a mouse model of intrauterine infection-induced preterm birth: role of interleukin 1beta. *Biology of Reproduction*. 1999 May;60(5):1231-8.

230. Hirsch E, Filipovich Y, Romero R. Failure of E. coli bacteria to induce preterm delivery in the rat. *Journal of Negative Results in BioMedicine*. 2009 2009/01/04;8(1):1.

231. Kramer BW, Kramer S, Ikegami M, et al. Injury, inflammation, and remodeling in fetal sheep lung after intra-amniotic endotoxin. *American Journal of Physiology-Lung Cellular and Molecular Physiology*. 2002;283(2):L452-L459.

232. Cheung CY, Brace RA. Amniotic Fluid Volume and Composition in Mouse Pregnancy. *The Journal of the Society for Gynecologic Investigation.* 2005 2005/12/01;12(8):558-562.

233. Stranik J, Kacerovsky M, Vescicik P, et al. A rodent model of intra-amniotic inflammation/infection, induced by the administration of inflammatory agent in a gestational sac, associated with preterm delivery: a systematic review. *The Journal of Maternal-Fetal & Neonatal Medicine.* 2020:1-9.

234. Fraunberger P, Pfeiffer M, Cremer P, et al. Validation of an Automated Enzyme Immunoassay for Interleukin-6 for Routine Clinical Use. *Clinical Chemistry and Laboratory Medicine*. 1998 13 Oct. 1998;36(10):797-801.

235. Fouhy F, Deane J, Rea MC, et al. The Effects of Freezing on Faecal Microbiota as Determined Using MiSeq Sequencing and Culture-Based Investigations. *PLoS One*. 2015;10(3):e0119355.

236. Greisen K, Loeffelholz M, Purohit A, et al. PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. *Journal of Clinical Microbiology*. 1994;32(2):335-351.

237. Musilova I, Andrys C, Holeckova M, et al. Interleukin-6 measured using the automated electrochemiluminescence immunoassay method for the identification of intra-amniotic inflammation in preterm prelabor rupture of membranes. *The Journal of Maternal-Fetal & Neonatal Medicine* 2020 Jun;33(11):1919-1926.

238. Moher D, Liberati A, Tetzlaff J, et al. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. *PLoS Medicine*. 2009;6(7):e1000097.

239. Ouzzani M, Hammady H, Fedorowicz Z, et al. Rayyan−a web and mobile app for systematic reviews. *Systematic Reviews*. 2016 2016/12/05;5(1):210.

240. Macleod MR, O'Collins T, Howells DW, et al. Pooling of animal experimental data reveals influence of study design and publication bias. *Stroke*. 2004 May;35(5):1203-8.

241. Condon JC, Jeyasuria P, Faust JM, et al. Surfactant protein secreted by the maturing mouse fetal lung acts as a hormone that signals the initiation of parturition. *Proceedings of the National Academy of Sciences.* 2004;101(14):4978-4983.

242. Jantzie LL, Winer JL, Maxwell JR, et al. Modeling encephalopathy of prematurity using prenatal hypoxia-ischemia with intra-amniotic lipopolysaccharide in rats. *Journal of Visualized Experiments*. 2015 (105):e53196.

243. Maxwell JR, Denson JL, Joste NE, et al. Combined in utero hypoxia-ischemia and lipopolysaccharide administration in rats induces chorioamnionitis and a fetal inflammatory response syndrome. *Placenta*. 2015;36(12):1378-1384.

244. Surve MV, Anil A, Kamath KG, et al. Membrane vesicles of group B Streptococcus disrupt feto-maternal barrier leading to preterm birth. *PLoS pathogens*. 2016;12(9):e1005816.

245. Cookson MW, Ryan SL, Seedorf GJ, et al. Antenatal vitamin D preserves placental vascular and fetal growth in experimental chorioamnionitis due to intra-amniotic endotoxin exposure. *American Journal of Perinatology*. 2018;35(13):1260-1270.

246. Dedja A, Gucciardi A, Giordano G, et al. Lipopolysaccharide-induced chorioamnionitis and postnatal lung injury: The beneficial effects of L-citrulline in newborn rats. *Experimental Lung Research*. 2018;44(4-5):226-240.

247. Garcia-Flores V, Romero R, Miller D, et al. Inflammation-induced adverse pregnancy and neonatal outcomes can be improved by the immunomodulatory peptide exendin-4. *Frontiers in Immunology*. 2018;9:1291.

248. Gomez-Lopez N, Romero R, Arenas-Hernandez M, et al. Intra-amniotic administration of lipopolysaccharide induces spontaneous preterm labor and birth in the absence of a body temperature change. *The Journal of Maternal-Fetal & Neonatal Medicine*. 2018;31(4):439-446.

249. Jantzie LL, Oppong AY, Conteh FS, et al. Repetitive neonatal erythropoietin and melatonin combinatorial treatment provides sustained repair of functional deficits in a rat model of cerebral palsy. *Frontiers in Neurology*. 2018;9:233.

250. Faro J, Romero R, Schwenkel G, et al. Intra-amniotic inflammation induces preterm birth by activating the NLRP3 inflammasome. *Biology of Reproduction*. 2019;100(5):1290-1305.

251. Gomez-Lopez N, Romero R, Garcia-Flores V, et al. Inhibition of the NLRP3 inflammasome can prevent sterile intra-amniotic inflammation, preterm labor/birth, and adverse neonatal outcomes. *Biology of Reproduction*. 2019;100(5):1306-1318.

252. Bhatti G, Romero R, Rice GE, et al. Compartmentalized profiling of amniotic fluid cytokines in women with preterm labor. *PLoS One*. 2020;15(1):e0227881.

253. Romero R, Grivel J-C, Tarca AL, et al. Evidence of perturbations of the cytokine network in preterm labor. *American Journal of Obstetrics and Gynecology*. 2015;213(6):836. e1-836. e18.

254. Gomez-Lopez N, Romero R, Galaz J, et al. Cellular immune responses in amniotic fluid of women with preterm labor and intra-amniotic infection or intra-amniotic inflammation. *American Journal of Reproductive Immunology*. 2019;82(5):e13171.

255. Gomez-Lopez N, Romero R, Tarca AL, et al. Gasdermin D: Evidence of pyroptosis in spontaneous preterm labor with sterile intra-amniotic inflammation or intra-amniotic infection. *American Journal of Reproductive Immunology*. 2019;82(6):e13184.

256. Gomez-Lopez N, Romero R, Panaitescu B, et al. Inflammasome activation during spontaneous preterm labor with intra-amniotic infection or sterile intra-amniotic inflammation. *American Journal of Reproductive Immunology*. 2018;80(5):e13049.

257. Motomura K, Romero R, Garcia-Flores V, et al. The alarmin interleukin-1α causes preterm birth through the NLRP3 inflammasome. *Molecular Human Reproduction*. 2020;26(9):712-726.

258. Peiris HN, Romero R, Vaswani K, et al. Preterm labor is characterized by a high abundance of amniotic fluid prostaglandins in patients with intra-amniotic infection or sterile intra-amniotic inflammation. *The Journal of Maternal-Fetal & Neonatal Medicine*. 2019:1-16.

259. Plazyo O, Romero R, Unkel R, et al. HMGB1 induces an inflammatory response in the chorioamniotic membranes that is partially mediated by the inflammasome. *Biology of Reproduction*. 2016;95(6):130, 1-14.

260. Kacerovsky M, Holeckova M, Stepan M, et al. Amniotic fluid glucose level in PPROM pregnancies: a glance at the old friend. *The Journal of Maternal-Fetal & Neonatal Medicine.* 2020:1-13.

261. Kacerovsky M, Romero R, Stepan M, et al. Antibiotic administration reduces the rate of intraamniotic inflammation in preterm prelabor rupture of the membranes. *American Journal of Obstetrics and Gynecology.* 2020;223(1):114. e1-114. e20.

262. Spacek R, Musilova I, Andrys C, et al. Extracellular granzyme A in amniotic fluid is elevated in the presence of sterile intra-amniotic inflammation in preterm prelabor rupture of membranes. *The Journal of Maternal-Fetal & Neonatal Medicine*. 2020:1-10.

263. Theis KR, Romero R, Motomura K, et al. Microbial burden and inflammasome activation in amniotic fluid of patients with preterm prelabor rupture of membranes. *Journal of Perinatal Medicine.* 2020;48(2):115-131.

264. Romero R, Miranda J, Chaiworapongsa T, et al. Sterile intra-amniotic inflammation in asymptomatic patients with a sonographic short cervix: prevalence and clinical significance. *The Journal of Maternal-Fetal & Neonatal Medicine*. 2015;28(11):1343-1359.

265. Holst RM, Hagberg H, Wennerholm UB, et al. Prediction of spontaneous preterm delivery in women with preterm labor: analysis of multiple proteins in amniotic and cervical fluids. *Obstetrics & Gynecology.* 2009 Aug;114(2 Pt 1):268-77.

266. Inglis SR, Jeremias J, Kuno K, et al. Detection of tumor necrosis factor-α, interleukin-6, and fetal fibronectin in the lower genital tract during pregnancy: relation to outcome. *American Journal of Obstetrics and Gynecology*. 1994;171(1):5-10.

267. Goepfert AR, Goldenberg RL, Andrews WW, et al. The Preterm Prediction Study: association between cervical interleukin 6 concentration and spontaneous preterm birth. *American Journal of Obstetrics and Gynecology*. 2001;184(3):483-488.

268. Kurkinen-Räty M, Ruokonen A, Vuopala S, et al. Combination of cervical interleukin-6 and-8, phosphorylated insulin-like growth factor-binding protein-1 and transvaginal cervical ultrasonography in assessment of the risk of preterm birth. *British Journal of Obstetrics and Gynaecology*. 2001;108(8):875-881.

269. Lange M, Chen FK, Wessel J, et al. Elevation of interleukin-6 levels in cervical secretions as a predictor of preterm delivery. *Acta Obstetricia et Gynecologica Scandinavica*. 2003;82(4):326-329.

270. Lockwood CJ, Wein R, Lapinski R, et al. Increased interleukin-6 concentrations in cervical secretions are associated with preterm delivery. *American Journal of Obstetrics and Gynecology*. 1994;171(4):1097-1102.

271. Brik M, Aguar M, Valiente A, et al. Cervical IL-6 and pIGFBP-1 and the prediction of neonatal outcome in symptomatic preterm labour. *The Journal of Maternal-Fetal & Neonatal Medicine.* 2014;27(12):1241-1247.

272. Harada N, Iijima S, Kobayashi K, et al. Human IgGFc binding protein (FcgammaBP) in colonic epithelial cells exhibits mucin-like structure. *Journal of Biological Chemistry*. 1997 Jun 13;272(24):15232-41.

273. Kobayashi K, Yagasaki M, Harada N, et al. Detection of Fcgamma binding protein antigen in human sera and its relation with autoimmune diseases. *Immunology Letters.* 2001 Dec 3;79(3):229-35.

274. O'Donovan N, Fischer A, Abdo EM, et al. Differential expression of IgG Fc binding protein (FcgammaBP) in human normal thyroid tissue, thyroid adenomas and thyroid carcinomas. *Journal of Endocrinology.* 2002 Sep;174(3):517-24.

275. Liu X, Song Y, Guo Z, et al. A comprehensive profile and inter-individual variations analysis of the human normal amniotic fluid proteome. *Journal of Proteomics*. 2019 Feb 10;192:1-9.

276. Tambor V, Kacerovsky M, Andrys C, et al. Amniotic fluid cathelicidin in PPROM pregnancies: from proteomic discovery to assessing its potential in inflammatory complications diagnosis. *PLoS One*. 2012;7(7):e41164.

277. Tambor V, Vajrychova M, Kacerovsky M, et al. Potential Peripartum Markers of Infectious-Inflammatory Complications in Spontaneous Preterm Birth. *BioMed Research International.* 2015;2015:343501.

278. Zhao M, Yang Y, Guo Z, et al. A Comparative Proteomics Analysis of Five Body Fluids: Plasma, Urine, Cerebrospinal Fluid, Amniotic Fluid, and Saliva. *Proteomics Clinical Applications.* 2018 Nov;12(6):e1800008.

279. Kobayashi K, Blaser M, Brown W. Identification of a unique IgG Fc binding site in human intestinal epithelium. *The Journal of Immunology*. 1989;143(8):2567-2574.

280. Kobayashi K, Hamada Y, Blaser MJ, et al. The molecular configuration and ultrastructural locations of an IgG Fc binding site in human colonic epithelium. *The Journal of Immunology.* 1991;146(1):68-74.

281. Frew L, Makieva S, McKinlay AT, et al. Human cathelicidin production by the cervix. *PLoS One*. 2014;9(8):e103434.

282. Hansen LK, Becher N, Bastholm S, et al. The cervical mucus plug inhibits, but does not block, the passage of ascending bacteria from the vagina during pregnancy. *Acta Obstetricia et Gynecologica Scandinavica*. 2014 Jan;93(1):102-8.

283. Hein M, Valore EV, Helmig RB, et al. Antimicrobial factors in the cervical mucus plug. *American Journal of Obstetrics and Gynecology*. 2002 Jul;187(1):137-44.

284. Racicot K, Cardenas I, Wünsche V, et al. Viral infection of the pregnant cervix predisposes to ascending bacterial infection. *Journal of Immunology.* 2013 Jul 15;191(2):934-41.

285. Kjaergaard N, Hein M, Hyttel L, et al. Antibacterial properties of human amnion and chorion in vitro. *European Journal of Obstetrics & Gynecology and Reproductive Biology*. 2001 Feb;94(2):224-9.

286. Kjaergaard N, Helmig RB, Schønheyder HC, et al. Chorioamniotic membranes constitute a competent barrier to group b streptococcus in vitro. *European Journal of Obstetrics & Gynecology and Reproductive Biology*. 1999 Apr;83(2):165-9.

287. Winram SB, Jonas M, Chi E, et al. Characterization of group B streptococcal invasion of human chorion and amnion epithelial cells In vitro. *Infection and Immunity*. 1998 Oct;66(10):4932-41.

288. Azzarelli B, Lafuze J. Amniotic basement membrane: a barrier to neutrophil invasion. *American Journal of Obstetrics and Gynecology*. 1987 May;156(5):1130-6.

289. Turley E, Tretiak M, Tanguay K. Effect of glycosaminoglycans and enzymes on the integrity of human placental amnion as a barrier to cell invasion. *Journal of the National Cancer Institute.* 1987 May;78(5):787-95.

290. Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nature Reviews Immunology.* 2010 Feb;10(2):131-44.

291. Kim YM, Romero R, Chaiworapongsa T, et al. Toll-like receptor-2 and -4 in the chorioamniotic membranes in spontaneous labor at term and in preterm parturition that are associated with chorioamnionitis. *American Journal of Obstetrics and Gynecology*. 2004 Oct;191(4):1346-55.

292. Richardson CA, Flecknell PA. Anaesthesia and post-operative analgesia following experimental surgery in laboratory rodents: are we making progress? *Alternatives to Laboratory Animals.* 2005;33(2):119-127.

293. Rinaldi SF, Makieva S, Frew L, et al. Ultrasound-guided intrauterine injection of lipopolysaccharide as a novel model of preterm birth in the mouse. *American Journal of Pathology*. 2015;185(5):1201-1206.

294. Serriere S, Nadal-Desbarats L, Seguin F, et al. Ultrasound-guided collection of amniotic fluid in pregnant rats. *Journal of the American Association for Laboratory Animal Science.* 2006;45(4):49-53.

295. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. *Journal of Leukocyte Biology.* 2007;81(1):1-5.

296. Lee PR, Kim S-R, Jung B-K, et al. Therapeutic effect of cyclo-oxygenase inhibitors with different isoform selectivity in lipopolysaccharide-induced preterm birth in mice. *American Journal of Obstetrics and Gynecology*. 2003;189(1):261-266.

297. Yoon BH, Romero R, Lim J-H, et al. The clinical significance of detecting Ureaplasma urealyticum by the polymerase chain reaction in the amniotic fluid of patients with preterm labor. *American Journal of Obstetrics and Gynecology*. 2003;189(4):919-924.

298. Colaizy TT, Morris CD, Lapidus J, et al. Detection of ureaplasma DNA in endotracheal samples is associated with bronchopulmonary dysplasia after adjustment for multiple risk factors. *Pediatric Research*. 2007;61(5):578-583.

299. Schelonka RL, Katz B, Waites KB, et al. Critical appraisal of the role of Ureaplasma in the development of bronchopulmonary dysplasia with metaanalytic techniques. *The Pediatric Infectious Disease Journal.* 2005;24(12):1033-1039.

300. Gayle DA, Beloosesky R, Desai M, et al. Maternal LPS induces cytokines in the amniotic fluid and corticotropin releasing hormone in the fetal rat brain. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*. 2004;286(6):R1024-R1029.

301. Russell WMS, Burch RL. The principles of humane experimental technique. *Methuen*. 1959.

8. APPENDICES

8.1 APPENDIX A: STEP-BY-STEP PROTOCOL FOR ULTRASOUND-GUIDED INTRA-AMNIOTIC ADMINISTRATION OF AN AGENT IN A RAT

1. Preparation

- The bottle warmer was turned on in advance to warm up the ultrasound gel. The temperature of the warmer was set to 37°C.
- The oxygen tank was checked to determine if there was enough oxygen to perform the procedure.
- The level of isoflurane in the anesthesia vaporizer (Figures 1.1 and 1.2) was checked. If the level was low, isoflurane was refilled before starting the procedure.
- The connection of the hose of the anesthesia system to the induction chamber and Vevo Imagine Station was checked. The hose switch (Figure 1.3) was opened to allow anesthetic gases to flow into the induction chamber. The hose switch that led to the imagining station was closed.
- The heating of the Vevo Imagine Station heating pad (Figure 1.4 and 1.5) was turned on. The temperature of the heating pad was set to 40°C (Figure 1.6).
- The infrared lamp was turned on and placed above the heating pad to maintain a stable body temperature during anesthesia.
- Five pieces of tape, approximately 10 cm in length, were prepared and used to attach the animal and rectal probe to the heating pad during the procedure.
- Hair removal cream and a few pieces of cotton swabs and gauze pads were prepared and used to remove fur.

Figure 1.1

Vevo Anesthesia System.

Figure 1.2

Isoflurane vaporizer of Vevo Anesthesia System.

Figure 1.3

Hoses with switches connecting anesthesia system to the induction chamber and imagine station.

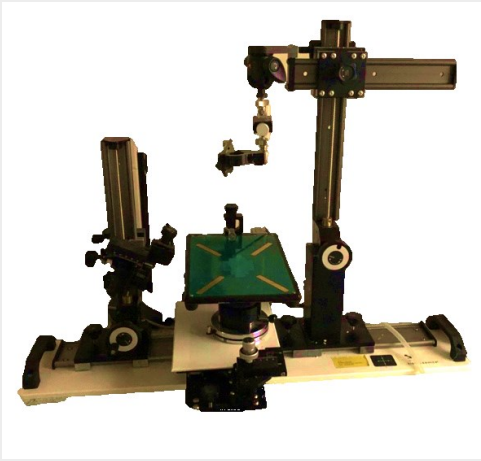


Figure 1.4

Vevo Imagine Station.

Figure 1.5

Vevo Imagine Station heating pad.

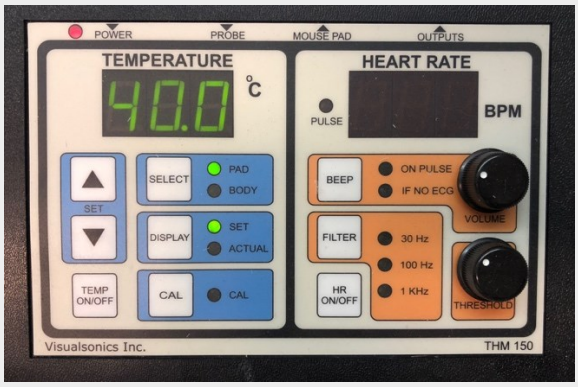


Figure 1.6

Temperature setting of the Vevo Imagine Station heating pad.

2. Setting Up the Vevo ultrasound machine

- A Vevo 3100 ultrasound machine (Figure 2.1) was turned on.
- The MX250S transducer (15–30 MHz) was connected to an ultrasound machine (Figure 2.2). Different transducers were used during different stages of the procedure.
- The pre-set to "mouse (large) abdominal" was chosen (Figure 2.3).
- The study name for data storage in ultrasound machine was automatically set to "series 1" The series number was changed for every animal to maintain records.

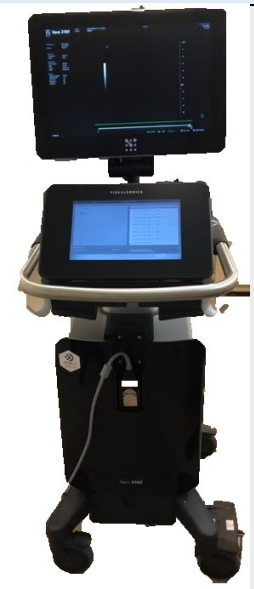


Figure 2.1

Vevo 3100 ultrasound machine.

Figure 2.2

Connection of the transducer to the ultrasound machine.

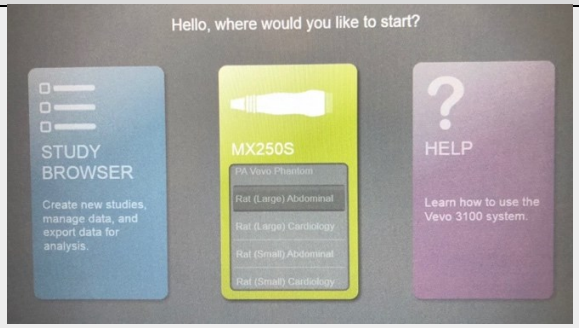


Figure 2.3

Transducer pre-set window of the ultrasound machine.

3. Anesthesia

- The oxygen tank was turned on, and the flow rate was set to 2 L/min (Figure 3.1). Isoflurane was initiated at a concentration of 5%.
- The rat was removed from the cage and placed in an induction chamber (Figure 3.2). The behavior of the animals was assessed. After the animal was introduced into anesthesia, the isoflurane concentration was lowered to 1.5%.
- The hose switch connecting the anesthesia vaporizer and nasal mask on the heating pad at the ultrasound station was open. The second hose switch connecting the anesthesia vaporizer and induction chamber was closed.
- The rat was carefully removed from the induction chamber and moved to the heating pad of the imaging station.
- The animal was placed in the prone position on the heating pad (Figure 3.3), and the rat's snout was placed on the heating pad to the nasal mask. The animal was placed in this position for a few minutes to stabilize the anesthesia. Breathing frequency and movement were carefully monitored. The isoflurane concentration was adjusted as necessary.
- After stabilization, the animal was placed in the supine position (with the face and torso facing up [Figure 3.4]). The snout of the rat was always connected to the nasal mask on the heating pad.
- The body temperature, heart rate, and breathing frequency were monitored throughout the procedure.

Figure 3.1

Setting of an oxygen flow of anesthesia system.

Figure 3.2

Animal placed in an induction chamber.

Figure 3.3

Animal placed in the prone position on the heating pad.

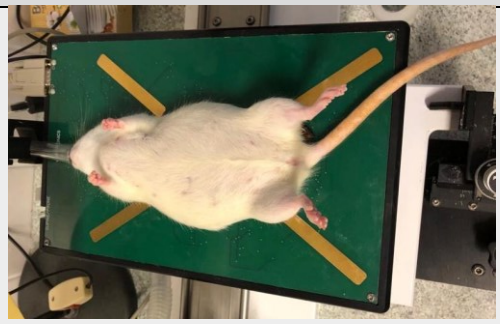


Figure 3.4

Animal placed in the supine position on the heating pad.

4. Fixation of the mouse and insertion of the rectal probe

- The electrode gel was applied between the rat's paws and the electrodes of the heating pad.
- The rat's paws were fixed to the electrodes of the heating pad using previously prepared tapes Figures 4.1 and 4.2).
- A drop of the lubricating gel was placed on the rectal probe (Figure 4.3). The probe was inserted into the rectum of the rat to measure body temperature. The rectal probe was fixed to the heating pad using a previously prepared tape.

Figure 4.1

The rat's paw fixed to the electrode of the heating pad.

Figure 4.1

Rat fixed to the heating pad.

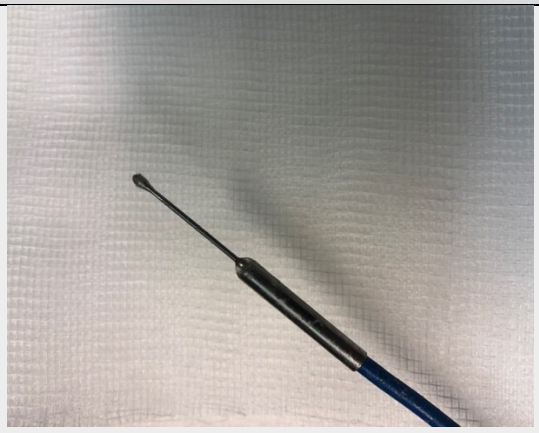


Figure 4.1

The rectal probe for measurement of animal body temperature during anesthesia.

5. Fur Removal

- Depilatory cream was applied to the rat's abdomen (Figure 5.1).
- The cream was spread to cover the entire area of the abdomen using a cotton swab (Figure 5.2). The cream was allowed to stand for 3 min.
- The fur and cream were removed using pieces of wet gauze. The abdominal fur had to be removed completely prior to the procedure (Figure 5.3).

Figure 5.1

Animal in the supine position on the heating pad. The depilatory cream is applied to the animal's abdomen.

Figure 5.2

Animal in the supine position on the heating pad. The depilatory cream is spread to cover the entire area of the abdomen.

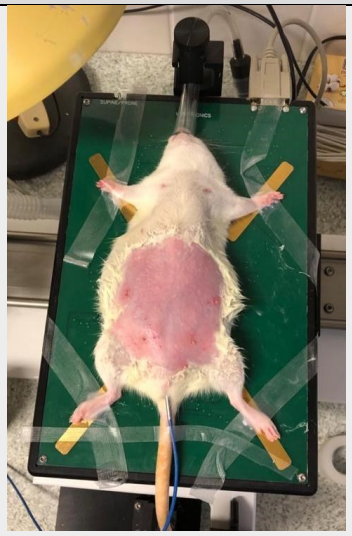


Figure 5.3

Animal in the supine position on the heating pad with completely removed abdominal fur.

6. General ultrasound scan with mapping of the number and the position of the pups

- The ultrasound gel was removed from the bottle heater and shaken to move the gel toward the nozzle to avoid creating bubbles, which can distort the images.
- The lower part of the rat's abdomen was covered with the ultrasound gel, and a 1-cm thick layer was created (Figure 6.1).
- The transducer was placed in a transverse orientation on the abdomen. To avoid shaking, the wrist of the hand with the transducer was fixed (Figure 6.2).
- The maternal bladder was identified as an anechogenic round structure located at the midline of the lower abdomen. The urinary bladder was used as a midline reference point for localizing and mapping gestational sacs with pups (Figures 6.3 and 6.4).
- First, the right part of the abdomen of the animal was scanned from the bladder to the thorax.
- Then, the left part of the abdomen of the animal was scanned from the bladder to the thorax. The positions of the pups and placentas were recorded.

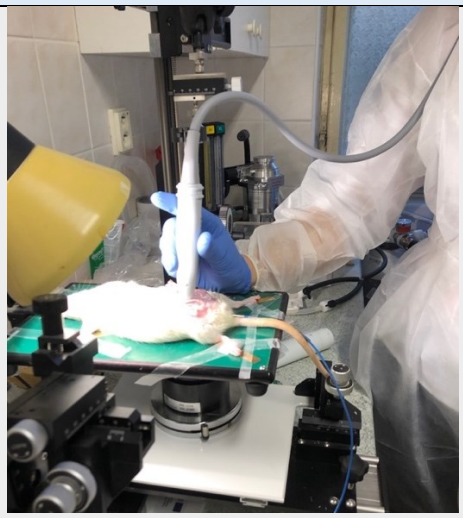


Figure 6.1

Technique used for mapping the number and localization of gestational sacs. The wrist of the hand with the transducer is fixed to avoid shaking.

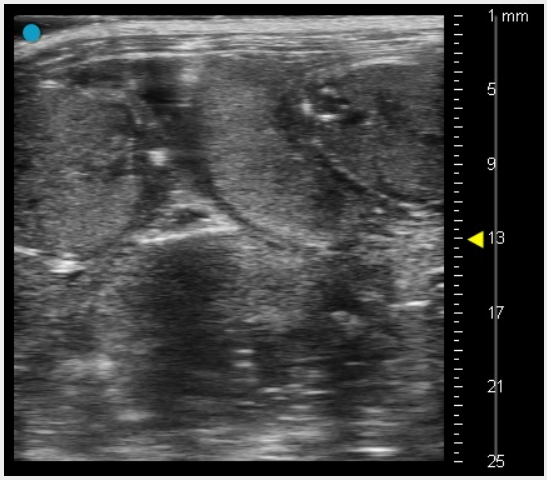


Figure 6.2

Ultrasound image of several gestational sacs with placentas and pups.

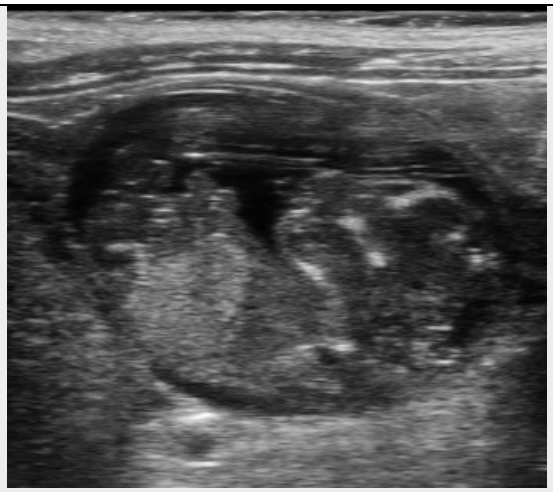


Figure 6.3

Detailed ultrasound image of rat embryo.

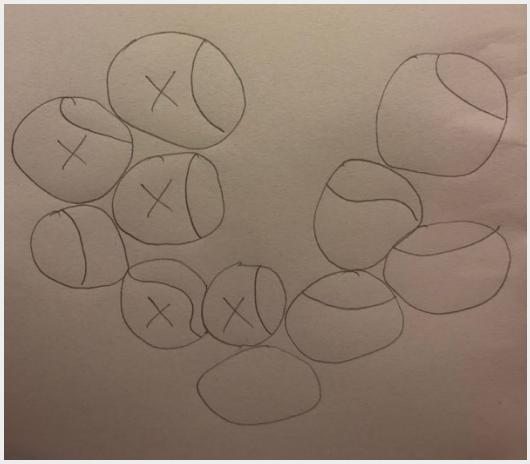


Figure 6.4

Example of drawn record showing the location of each gestational sac with the placenta. Injected sacs are marked (x).

7. Ultrasound-guided intra-amniotic administration

- The MX250S transducer was replaced by an MX400 transducer (20–46 MHz). The MX400 transducer, offering higher axial resolution, enables more precise needle navigation during the procedure.
- The MX400 transducer was placed in the transducer holder of the Vevo Imagine Station (Figure 7.1).
- A sufficient ultrasound gel layer of at least 1-cm thickness was restored below the transducer.
- The syringe with the substance was placed in the syringe holder of the Vevo Imagine Station (Figure 7.2).
- The target gestational sac was visualized on the right side of the ultrasound image (Figure 7.3). The syringe holder was moved toward the animal body surface inside the gel layer with a 27 G × 40 mm needle perpendicular to the skin until the needle tip could be visualized on the left side of the ultrasound image.
- When the tip of the needle was visible on the ultrasound image, puncture was performed. The thick rat skin made the passage of the needle difficult. Therefore, the puncture had to be slow to allow the tip of the needle to spontaneously slide through the skin tissue and membranes of the gestational sac.
- The agent was injected when the tip of the needle was inside the gestational sac (Figure 7.4). Successful application was confirmed by visualization of the fluid jet.
- The needle was then slowly pulled out.
- The procedure was repeated to inject all accessible gestational sacs in the animal. A new sharp needle was used for each new administration to ensure the successful passage of the needle through the skin.

Figure 7.1

Ultrasound transducer placed in the transducer holder of the Vevo Imagine Station.

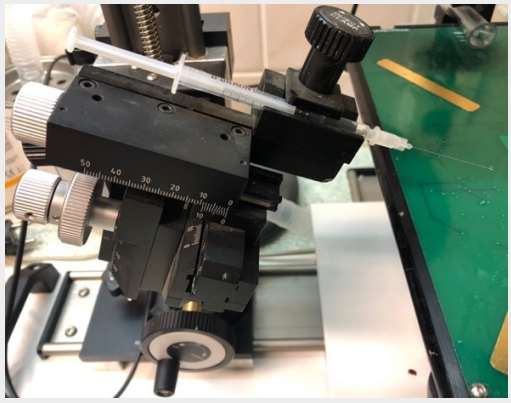


Figure 7.2

Syringe placed in the syringe holder of the Vevo Imagine Station.

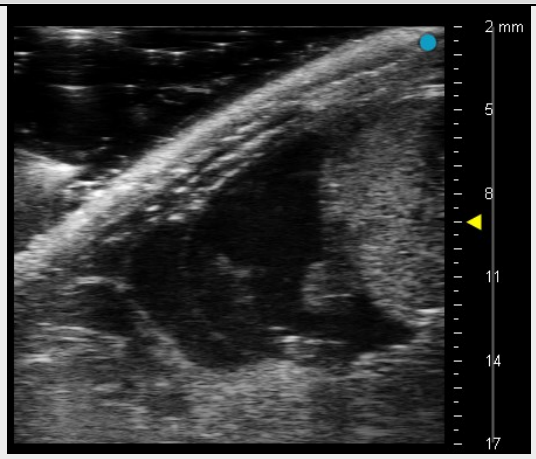


Figure 7.3

Ultrasound image. The tip of the needle is visible on the left part of the ultrasound image in the proximity of animal skin.

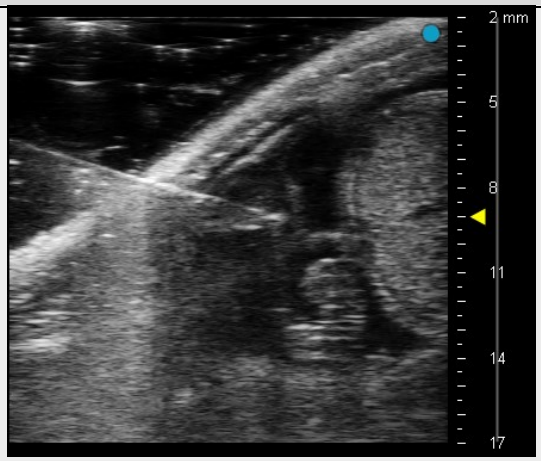


Figure 7.4

Ultrasound image. The tip of the needle is inside the gestational sac.

8. End of the procedure

- The entire layer of the ultrasound gel was removed from the animal's abdomen with gauze pads. The abdomen was completely dried before the animal was moved into the cage.
- The rectal probe was removed, and the paws were released.
- The isoflurane vaporizer was turned off.
- The animal was placed in a cage, where it was kept under a heat lamp and watched until recovery from anesthesia.
- The ultrasound machine and equipment were turned off. All instruments were cleaned.

8.2 APPENDIX B: PUBLICATIONS RELATED TO DISSERTATION THESIS

8.2.1 Specific aim I-A

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ORIGINAL ARTICLE

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Intra-amniotic infection and sterile intra-amniotic inflammation are associated with elevated concentrations of cervical fluid interleukin-6 in women with spontaneous preterm labor with intact membranes

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ABSTRACT

Objective: To determine the concentration of interleukin-6 (IL-6) in the cervical fluid in women with spontaneous preterm labor with intact fetal membranes (PTL) complicated by intra-amniotic infection (the presence of both microbial invasion of the amniotic cavity and intra-amniotic inflammation), or sterile intra-amniotic inflammation (the presence of intra-amniotic inflammation alone).

Methods: Eighty women with singleton pregnancies complicated by PTL between gestational ages 22 + 0 and 34 + 6 weeks were included in this retrospective cohort study. Samples of amniotic and cervical fluids were collected at the time of admission. Amniotic fluid samples were obtained via transabdominal amniocentesis, and cervical fluid was obtained using a Dacron polyester swab. Microbial invasion of the amniotic cavity was diagnosed based on the combination of culture and molecular biology methods. The concentration of IL-6 in the amniotic and cervical fluids were measured using an automated electrochemiluminescence immunoassay method. Intra-amniotic inflammation was defined as an amniotic fluid IL-6 concentration ≥3000 pg/mL.

Results: The presence of intra-amniotic infection and sterile inflammation was identified in 15% (12/80) and 26% (21/80) of the women, respectively. Women with intra-amniotic infection (median: 587 pg/mL; $p = .01$) and with sterile intra-amniotic inflammation (median: 590 pg/mL; $p = .005$) had higher concentrations of IL-6 in the cervical fluid than those without intra-amniotic inflammation (intra-amniotic infection: median 587 pg/mL vs. without inflammation, median: 136 pg/mL; $p = .01$; sterile intra-amniotic inflammation, median: 590 pg/mL vs. without inflammation, $p = .005$). No differences were found in the concentrations of IL-6 in the cervical fluid between women with intra-amniotic infection and sterile intra-amniotic inflammation ($p = .81$).

Conclusion: In pregnancies with PTL, both forms of intra-amniotic inflammation are associated with elevated concentrations of IL-6 in the cervical fluid.

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KEYWORDS
Microbial invasion of the amniotic cavity; cytokine; intra-amniotic inflammation; noninvasive sample; preterm delivery

Introduction

Spontaneous preterm labor with intact fetal membranes (PTL) is responsible for approximately one-third of all preterm deliveries [1–3]. PTL is considered one of the major obstetric syndromes owing to: (i) having multiple etiologies, (ii) a long preclinical phase, (iii) fetal involvement, and (iv) a complex interaction between the environment and the maternal/fetal genome [4–8]. Collectively, PTL represents a pregnancy complication with serious medical and socio-economic impacts [9,10].

A recent view of PTL clearly shows that the majority of cases are complicated by the presence of intra-amniotic inflammation, a condition characterized by

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the elevation of pro- and anti-inflammatory mediators in the amniotic fluid [3,11–13]. Intra-amniotic inflammation has two different clinical phenotypes: (i) intra-amniotic infection, when microorganisms are present in the amniotic fluid; or (ii) sterile intra-amniotic inflammation, when the amniotic fluid is free of microorganisms [14–16].

Intra-amniotic infection and sterile intra-amniotic inflammation complicate approximately 11% and 26% of pregnancies with PTL, respectively [15]. The latter form of intra-amniotic inflammation can be characterized by a milder intra-amniotic inflammatory response, compared with intra-amniotic infection, by measuring the concentration of interleukin (IL)-6 and white blood cell count in the amniotic fluid [15]. Regardless, sterile intra-amniotic inflammation has similar rates of acute placental inflammatory lesions and adverse neonatal outcomes to those of intra-amniotic infection [15]. These facts highlight the clinical seriousness of sterile intra-amniotic inflammation in pregnancies with PTL.

Despite intensive research on sterile intra-amniotic inflammation in PTL [12,13,15,17–24], its development has yet to be fully revealed. The following conditions might lead to this intra-amniotic complication: (i) damage of the fetal membranes, leading to the release of endogenous molecules, called alarmins, into the amniotic fluid, resulting in a subsequent inflammatory response through the system of pattern recognition receptors [15,25–29]; (ii) microbial colonization/infection of the choriodecidual space, which stimulates the fetal membranes to produce inflammatory mediators that are released from the fetal membranes into the amniotic fluid [30,31], or (iii) a combination of these two processes.

There is solid evidence that intra-amniotic inflammation is associated with a higher cervical inflammatory response, measured by the concentration of IL-6 in the cervical fluid of PTL women [32,33]. Nevertheless, there is a shortage of information about cervical fluid IL-6 concentrations related to both clinical phenotypes of intra-amniotic inflammation.

Therefore, the main aim of this study was to determine the concentrations of interleukin-6 in the cervical fluid of women with PTL complicated by intra-amniotic infection or sterile intra-amniotic inflammation.

Material and methods

This was a retrospective study of pregnant women admitted to the Department of Obstetrics and Gynecology of the University Hospital Hradec Kralove, in Czech Republic, between March 2017 and May 2020, who met the following criteria: (i) maternal age $\geq$ 18 years; (ii) singleton gestation between gestational ages 22 + 0 and 34 + 6 weeks; and (iii) preterm labor with intact fetal membranes; (iv) transabdominal amniocentesis was performed for the evaluation of inflammatory and microbial status of the amniotic cavity. The exclusion criteria were as follows: (i) pregnancy-related and other medical complications (e.g. fetal growth restriction, gestational or pregestational diabetes, gestational hypertension, and preeclampsia); (ii) congenital or chromosomal fetal abnormalities; (iii) signs of fetal hypoxia; (iv) significant vaginal bleeding; and (v) rupture of the fetal membranes with leakage of amniotic fluid.

Gestational age was determined based on first-trimester fetal biometry. PTL was defined as the presence of regular uterine contractions (at least two every 10 min), along with the cervical length, measured using transvaginal ultrasound, shorter than 15 mm or within the 15–30 mm range with a positive PartoSure test (Parsagen Diagnostics Inc., Boston, MA) [34]. Transabdominal amniocentesis and cervical fluid sampling were performed at the time of admission, before administration of corticosteroids, antibiotics, or tocolytics.

All women received a course of corticosteroids (betamethasone 14 mg intramuscularly, 24 h apart) and tocolytic therapy with either intravenous atosiban (those with a gestational age $\leq$28 weeks) or nifedipine, administered orally, for 48 h. Women with proven intra-amniotic inflammation received treatment with intravenous clarithromycin for seven days, unless delivery occurred earlier. Women positive for group B Streptococcus (GBS) in the vaginal-rectal swab or with an unknown GBS status received intravenous benzylpenicillin (clindamycin, in case of penicillin allergy) during an active labor. Once the final result of microbial invasion from the amniotic cavity via culture or molecular biology methods was known, the attending clinician made individualized treatments to determine the optimal antibiotic therapy. Those who delivered prior to gestational age 32 + 0 weeks received a neuroprotection treatment with magnesium sulfate during labor.

This study was approved by the Institutional Review Board Committee (June 2015; No 201408 I96L). All participants in the study were Caucasian. Informed consent was obtained from all participants.

Amniotic fluid sampling

Ultrasonography-guided transabdominal amniocentesis was performed, and approximately 5 mL of amniotic

fluid was aspirated. Amniotic fluid was dispensed into polypropylene tubes. The first tube was sent for the assessment of IL-6 level. The second tube was transported to the microbiology laboratories for aerobic/anaerobic cultivation of amniotic fluid. The third tube was sent to the molecular biology laboratory for polymerase chain reaction (PCR) testing for *Ureaplasma* spp., *Mycoplasma hominis*, *Chlamydia trachomatis*, and 16S rRNA gene.

Cervical fluid sampling

Cervical fluid samples were collected using a Dacron polyester swab placed in the cervical canal for 20 s to achieve saturation. Upon collection, the Dacron polyester swab was inserted into a polypropylene tube containing 1.5 mL of phosphate-buffered saline. The tube was shaken for 20 min, the Dacron swab was removed, and the tube was centrifuged for 15 min at 300 × *g*. The supernatant and pellets were aliquoted and stored at −80 °C until further analysis.

Assessment of interleukin-6

The concentration of IL-6 in the amniotic fluid (fresh samples) and cervical fluid (samples with one freezing/thawing cycle) were assessed using the automated electrochemiluminescence immunoassay method. IL-6 concentrations were measured using immuno-analyzer Cobas e602, which is part of the Cobas 8000 platform (Roche Diagnostics, Basel, Switzerland) [35]. The basic measuring range was 1.5–5000 pg/mL, which could be extended to 50,000 pg/mL with a 10-fold dilution of the sample. The coefficients of variation for inter-assay and intra-assay precisions were <10%.

Aerobic and anaerobic culture of amniotic fluid

The amniotic fluid samples were cultured in Columbia agar with sheep's blood, *Gardnerella vaginalis* selective medium, MacConkey agar, *Neisseria*-selective medium (modified Thayer-Martin medium), Sabouraud agar, and Schaedler anaerobe agar. The plates were cultured for 6 days and checked daily. The species were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using MALDI Biotyper software (Bruker Daltonics, Bremen, Germany).

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

DNA was isolated from the amniotic fluid using the QIAamp DNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions (using the protocol for isolating the bacterial DNA from biological fluids). Real-time PCR was performed using the Rotor-Gene 6000 instrument (Qiagen, Hilden, Germany) using the commercial AmpliSens® *C. trachomatis/Ureaplasma/M. hominis*-FRT kit (Federal State Institution of Science, Central Research Institute of Epidemiology, Moscow, Russia) to detect the DNA from *Ureaplasma* species, *M. hominis*, and *C. trachomatis* in a common PCR tube. As a control, we included a PCR run for beta-actin, a housekeeping gene, to exclude the presence of PCR inhibitors.

Detection of other bacteria in the amniotic fluid

Bacterial DNA was identified using PCR, targeting the 16S rRNA gene using the following primers: 5′-CCAGACTCCTACGGGAGGCAG-3′ (V3 region) and 5′-ACATTTCACAACACGAGCTGACGA-3′ (V6 region). Each reaction contained 3 µL of the target DNA, 500 nM forward and reverse primers, and Q5 High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) in a total volume of 25 µL. Amplification was performed using a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The products were visualized on an agarose gel. Positive reactions yielded products of 950 base pairs, which were subsequently analyzed using sequencing. The 16S PCR products were cleaned and used for sequencing PCR using the above-mentioned primers and the BigDye Terminator kit, v3.1 (Thermo Fisher Scientific). The bacteria were then typed using the sequences obtained from BLAST® and SepsiTest™ BLAST.

Clinical definitions

Microbial invasion of the amniotic cavity was determined based on a positive PCR analysis of *Ureaplasma* species, *M. hominis*, *C. trachomatis*, or a combination of these species, or positivity for the 16S rRNA gene, aerobic/anaerobic cultivation of the amniotic fluid, or a combination of these parameters. Intra-amniotic inflammation was defined as amniotic fluid IL-6 concentrations ≥ 3000 pg/mL [36]. Intra-amniotic infection was defined as the presence of both microbial invasion of the amniotic cavity and intra-amniotic inflammation. Sterile intra-amniotic inflammation was

defined as the presence of intra-amniotic inflammation without the concomitant presence of microbial invasion of the amniotic cavity.

Statistical analyses

The women's demographic and clinical characteristics were compared using a nonparametric Mann–Whitney *U* test for continuous variables and Fisher's exact test for categorical variables and are presented as median values (interquartile range [IQR]) and as number (%), respectively. The normality of the data was tested using the Anderson–Darling test. Because the cervical fluid IL-6 levels were not normally distributed, nonparametric Kruskal–Wallis and Mann–Whitney *U* tests were used for the analyses, as appropriate. Spearman partial correlation was performed to adjust the results for gestational age at sampling. Spearman correlation was used to assess the association between the concentrations of IL-6 in the amniotic and cervical fluids. Differences were considered significant at a $p < .05$. All *p* values were obtained from two-tailed tests, and all statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 19.0 for Mac OS X (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 8 for Mac OS X (GraphPad Software, San Diego, CA, USA).

Results

A total of 80 women with singleton pregnancies with PTL were included in the study. Intra-amniotic inflammation was found in 41% (33/80) of the women; intra-amniotic infection and sterile intra-amniotic inflammation were observed in 15% (12/80) and 26% (21/80) of the women, respectively. Demographic and clinical data are shown in Table 1.

Microbial invasion of the amniotic cavity was revealed only in women with intra-amniotic inflammation. Polymicrobial findings in the amniotic fluid were identified in three women (*Klebsiella pneumoniae* + *Streptococcus anginosus*, $n = 1$; *Gardnerella vaginalis* + *Lactobacillus plantus*, $n = 1$; and *Ureaplasma* species + *Mycoplasma hominis*, $n = 1$). The remaining nine women had only one microorganism in the amniotic fluid (*Ureaplasma* species, $n = 4$; *Haemophilus influenzae*, $n = 1$; *Lachnoanaerobaculum* species, $n = 1$; *Lactococcus lactis*, $n = 1$; *Sneathia sanguinegens*, $n = 1$; and nonidentifiable bacteria via sequencing, $n = 1$).

Concentrations of IL-6 were measurable in all samples of cervical fluid. A positive correlation was found between the concentrations of IL-6 cervical and amniotic fluids (rho = 0.47; $p < .0001$).

Concentrations of IL-6 in the cervical fluid according to the presence of intra-amniotic inflammation

Women with intra-amniotic inflammation had higher concentrations of IL-6 in the cervical fluid than those without intra-amniotic inflammation in the crude analysis (median, 590 pg/mL; IQR, 195–2014 vs. without: median 136 pg/mL, IQR 30–558; $p = .0007$; Figure 1) as well as after adjustment for gestational age at sampling ($p = .003$).

Concentrations of IL-6 in the cervical fluid according to the presence of intra-amniotic infection and sterile intra-amniotic inflammation

Differences in the concentrations of IL-6 in the cervical fluid were found among the subgroups of women with PTL in crude analysis (intra-amniotic infection: median, 587 pg/mL; IQR, 166–2427; sterile intra-amniotic inflammation: median 590 pg/mL; IQR, 245–1495; without intra-amniotic inflammation: median, 136 pg/mL; IQR, 30–558; $p = .004$; Figure 2), as well as after the adjustment for gestational age at sampling ($p = .001$).

Women with intra-amniotic infection and sterile intra-amniotic inflammation had higher concentrations of IL-6 than those without intra-amniotic inflammation (Table 2). No differences in the concentrations of IL-6 in the cervical fluid were found between women with intra-amniotic infection and sterile intra-amniotic inflammation (Table 2).

Discussion

The principal findings of this study, carried out on women with PTL, are the following: (i) intra-amniotic infection and sterile intra-amniotic inflammation were found in 15% and 26% of the women, respectively; (ii) cervical fluid IL-6 concentration was positively correlated with amniotic fluid IL-6 concentration; (iii) women with intra-amniotic infection and sterile intra-amniotic inflammation had higher concentrations of IL-6 in the cervical fluid than those without intra-amniotic inflammation; and (iv) no differences in the concentration of IL-6 in cervical fluid were found between those with intra-amniotic infection and sterile intra-amniotic inflammation.

defined as the presence of intra-amniotic inflammation without the concomitant presence of microbial invasion of the amniotic cavity.

Statistical analyses

The women's demographic and clinical characteristics were compared using a nonparametric Mann–Whitney *U* test for continuous variables and Fisher's exact test for categorical variables and are presented as median values (interquartile range [IQR]) and as number (%), respectively. The normality of the data was tested using the Anderson–Darling test. Because the cervical fluid IL-6 levels were not normally distributed, nonparametric Kruskal–Wallis and Mann–Whitney *U* tests were used for the analyses, as appropriate. Spearman partial correlation was performed to adjust the results for gestational age at sampling. Spearman correlation was used to assess the association between the concentrations of IL-6 in the amniotic and cervical fluids. Differences were considered significant at a $p < .05$. All *p* values were obtained from two-tailed tests, and all statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 19.0 for Mac OS X (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 8 for Mac OS X (GraphPad Software, San Diego, CA, USA).

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Differences in the concentrations of IL-6 in the cervical fluid were found among the subgroups of women with PTL in crude analysis (intra-amniotic infection: median, 587 pg/mL; IQR, 166–2427; sterile intra-amniotic inflammation: median 590 pg/mL; IQR, 245–1495; without intra-amniotic inflammation: median, 136 pg/mL; IQR, 30–558; $p = .004$; Figure 2), as well as after the adjustment for gestational age at sampling ($p = .001$).

Women with intra-amniotic infection and sterile intra-amniotic inflammation had higher concentrations of IL-6 than those without intra-amniotic inflammation (Table 2). No differences in the concentrations of IL-6 in the cervical fluid were found between women with intra-amniotic infection and sterile intra-amniotic inflammation (Table 2).

Discussion

The principal findings of this study, carried out on women with PTL, are the following: (i) intra-amniotic infection and sterile intra-amniotic inflammation were found in 15% and 26% of the women, respectively; (ii) cervical fluid IL-6 concentration was positively correlated with amniotic fluid IL-6 concentration; (iii) women with intra-amniotic infection and sterile intra-amniotic inflammation had higher concentrations of IL-6 in the cervical fluid than those without intra-amniotic inflammation; and (iv) no differences in the concentration of IL-6 in cervical fluid were found between those with intra-amniotic infection and sterile intra-amniotic inflammation.

Table 1. Demographic and clinical characteristics of women with spontaneous preterm labor with intact membranes with intra-amniotic infection, with sterile intra-amniotic inflammation, and without intra-amniotic inflammation.

Characteristic	Intra-amniotic infection ($n = 12$)	Sterile intra-amniotic inflammation ($n = 21$)	Absence of intra-amniotic inflammation ($n = 47$)	p value[a]	p value[b]	p value[c]
Maternal age [years, median (IQR)]	27 (25–28)	26 (22–28)	28 (24–32)	.56	.53	.17
Primiparous [number (%)]	7 (58%)	17 (81%)	30 (64%)	.12	.75	.14
Pre-pregnancy body mass index [kg/m^2, median (IQR)]	27.5 (23.3–29.7)	23.8 (22.7–25.0)	26.0 (23.9–28.2)	.22	.81	**.03**
Smoking [number (%)]	1 (8%)	4 (19%)	3 (6%)	.63	1.00	.19
Gestational age at admission [weeks, median (IQR)]	27 + 6 (26 + 3–30 + 2)	26 + 6 (24 + 2–30 + 6)	31 + 2 (29 + 3–32 + 4)	.78	**.04**	**.006**
Gestational age at delivery [weeks, median (IQR)]	29 + 0 (27 + 2–32 + 5)	27 + 5 (25 + 1–31 + 4)	34 + 1 (32 + 2–37 + 2)	.49	**.0004**	**<.0001**
Interval from amniocentesis to delivery [days, median (IQR)]	2 (0–8)	2 (0–5)	18 (2–45)	.81	**.02**	**.004**
Delivery within 7 days from amniocentesis [number (%)]	9 (75%)	18 (86%)	20 (43%)	.64	.06	**.001**
Amniotic fluid IL-6 levels at admission [pg/mL, median (IQR)]	43,431 (23,597–50,000)	9,464 (4,279–50,000)	847 (286–1,651)	**.02**	**<.0001**	**<.0001**
CRP levels at admission [mg/L, median (IQR)]	42.0 (12.7–67.8)	9.2 (6.4–17.0)	4.6 (2.1–9.7)	.05	**.0002**	**.003**
WBC count at admission [$\times 10^9$ L, median (IQR)]	16.3 (14.3–18.5)	16.2 (12.7–18.2)	13.0 (10.2–15.5)	.70	**.009**	**.01**
Administration of corticosteroids [number (%)]	9 (75%)	20 (95%)	43 (91%)	.13	.14	1.00
Clinical chorioamnionitis [number (%)]	1 (8%)	0 (0%)	0 (0%)	.36	.20	–
Vaginal delivery [number (%)]	9 (75%)	18 (86%)	39 (83%)	.64	.70	.74
Cesarean delivery [number (%)]	3 (25%)	3 (14%)	8 (17%)	.64	.68	1.00
Birth weight of the newborn [grams, median (IQR)]	1230 (979–1913)	1180 (740–1980)	2060 (1830–2810)	.73	**.0007**	**<.0001**
Apgar score < 7; 5 min [number (%)]	3 (25%)	5 (24%)	2 (4%)	1.00	.05	**.03**
Apgar score < 7; 10 min [number (%)]	2 (17%)	3 (14%)	0 (0%)	1.00	**.04**	**.03**

CRP: C-reactive protein; IL: interleukin; IQR: interquartile range; WBC: white blood cells.
Continuous variables were compared using a nonparametric Mann–Whitney *U* test. Categorical variables were compared using the Fisher's exact test.
Continuous variables are presented as median (IQR) and categorical as number (%).
Statistically significant results are marked in bold.
[a]Comparison between women with intra-amniotic infection and sterile intra-amniotic inflammation.
[b]Comparison between women with intra-amniotic infection and without intra-amniotic inflammation.
[c]Comparison between women with sterile intra-amniotic inflammation and without intra-amniotic inflammation.

The presence of sterile intra-amniotic inflammation has been described so far in pregnancies complicated by PTL [12–15,17–24], preterm prelabor rupture of the membranes [15,27,37–40], and a short cervix [16]. In pregnancies with PTL, the rate of sterile intra-amniotic inflammation is approximately two-fold more frequent than the rate of intra-amniotic infection [14]. However, the frequency of both forms of intra-amniotic inflammation in PTL diminishes with an advancing gestational age [14]. The results of this study regarding the frequency of both clinical phenotypes of intra-amniotic inflammation are in line with the results from pioneering Dr. Romero's study on sterile intra-amniotic inflammation in PTL, in which sterile intra-amniotic inflammation and intra-amniotic infection were revealed in 26% and 11% of the women, respectively [14].

In this study, IL-6 concentrations were measured in the cervical fluid samples obtained from women with PTL. Previous studies have reported a positive correlation between IL-6 concentration in the cervical and amniotic fluids [30,32,33,41]. In addition, there is evidence that an increased concentration of IL-6 in the cervical fluid in women with PTL is related to: (i) a higher risk of preterm delivery [33,41–46]; (ii) an elevation of amniotic fluid IL-6 concentration [32,33]; (iii) a higher risk of intra-amniotic inflammation [33]; (iv) microbial invasion of the chorioamniotic membranes [30]; (v) microbial invasion of the amniotic fluid [32,33]; and (vi) the presence of histological [32] and clinical [47] chorioamnionitis.

In this study, we confirmed the results from previous studies [32,33] that in PTL pregnancies, intra-amniotic inflammation is associated with higher cervical

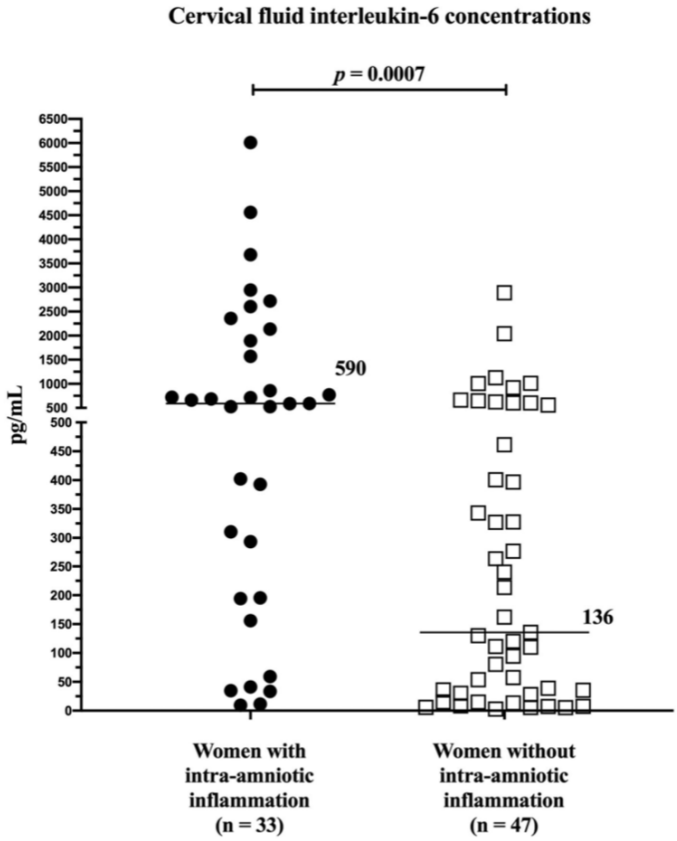

Figure 1. Cervical fluid interleukin-6 concentrations based on the presence or absence of intra-amniotic inflammation.

fluid concentrations of IL-6 than in those without intra-amniotic inflammation. To extend the knowledge of this field, women with PTL were further divided into three subgroups: intra-amniotic infection, sterile intra-amniotic inflammation, and without intra-amniotic inflammation. As expected, women with both clinical phenotypes of intra-amniotic inflammation had higher concentrations of IL-6 in the cervical fluid than those without intra-amniotic inflammation. However, no difference in the cervical fluid IL-6 concentration was found between women with intra-amniotic infection and sterile intra-amniotic inflammation. These observations show that an inflammatory and/or infectious environment in the cervical compartment is present in both clinical phenotypes of intra-amniotic inflammation. Given the tight anatomical proximity between the cervix and fetal membranes, we hypothesize that the composition of the cervical fluid might reflect the microbial and inflammatory status of the choriodecidual space. This hypothesis is driven by the fact that the presence of bacteria in the chorioamnion is associated with an elevation of IL-6 concentration in the cervical fluid [30]. In addition, the presence of microorganisms in the chorioamnion is also related to higher concentrations of IL-6 in the amniotic fluid, irrespective of the presence or absence of microorganisms in the amniotic fluid [30,31]. Taken together, the presence of microorganisms in chorioamniotic membranes is closely related to the elevation of the concentration of IL-6 in both cervical and amniotic fluids.

Therefore, the elevation of IL-6 concentration in the cervical fluid in women with PTL with sterile intra-amniotic inflammation can be explained as the result

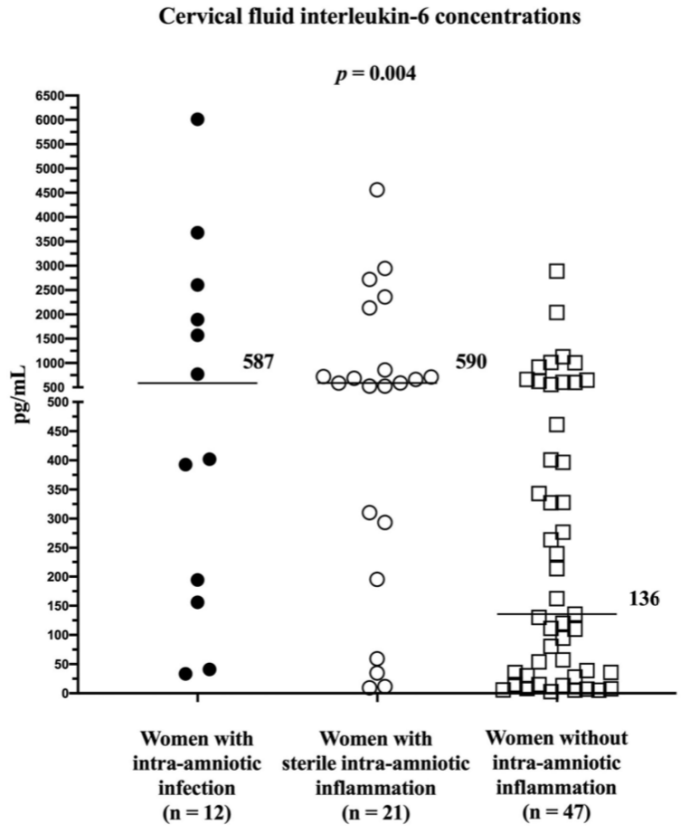

Figure 2. Cervical fluid interleukin-6 concentrations among the subgroup of women with intra-amniotic infection, sterile intra-amniotic inflammation, and without intra-amniotic inflammation.

Table 2. Cervical fluid IL-6 – the comparisons among the subgroups of the women with intra-amniotic infection, with sterile intra-amniotic inflammation, and without intra-amniotic inflammation.

	With intra-amniotic infection	With Sterile intra-amniotic inflammation	Without intra-amniotic inflammation
With intra-amniotic infection	x	$p = .81$; adj. $p = .18$	**$p = .01$; adj. $p = .003$**
With sterile intra-amniotic inflammation	$p = .81$; adj. $p = .18$	x	**$p = .005$; adj. $p = .003$**
Without intra-amniotic inflammation	**$p = .01$; adj. $p = .003$**	**$p = .005$; adj. $p = .003$**	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 age at sampling (a Spearman partial correlation).
Statistically significant results are marked in bold.

of the possible presence of microorganisms in the chorioamnion and/or inflammation in the choriodecidual space. This observation supports the hypothesis that these conditions represent one of the mechanisms playing pivotal roles, apart or in combination with the release of alarmins from necrotic cells or cells undergoing cellular stress, on the development of a sterile intra-amniotic environment in women with PTL.

This study has several strengths. First, the presence of microbial invasion of the amniotic cavity was assessed using a combination of culture and non-culture methods (specific PCR for *Ureaplasma* spp., *M. hominis*, and *C. trachomatis* and nonspecific evaluation of the 16S rRNA gene). This comprehensive approach allowed us to precisely dissect a subset of women with sterile intra-amniotic inflammation. Second,

paired amniotic and cervical fluid samples were obtained at the time of admission before the administration of corticosteroids, tocolytics, and antibiotics.

This study also has limitations that are worth mentioning. First, the groups of women with intra-amniotic infection and sterile intra-amniotic inflammation in this study ($n = 12$ and $n = 21$, respectively) were relatively small. Second, the amniotic fluid concentration of IL-6 was assessed in fresh samples; however, the concentration of IL-6 in the cervical fluid was measured in the samples that underwent one freezing/thawing cycle. Third, the cervical fluid microbiota or microbiome was not assessed in this study.

In conclusion, in women with PTL, the presence of both phenotypes of intra-amniotic inflammation, sterile intra-amniotic inflammation, and intra-amniotic inflammation, was associated with elevated concentrations of IL-6 in the cervical fluid.

Disclosure statement

The authors report that they have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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References

[1] Romero R, Mazor M, Munoz H, et al. The preterm labor syndrome. Ann N Y Acad Sci. 1994;734:414–429.
[2] Muglia LJ, Katz M. The enigma of spontaneous preterm birth. N Engl J Med. 2010;362(6):529–535.
[3] Romero R, Dey SK, Fisher SJ. Preterm labor: one syndrome, many causes. Science. 2014;345(6198):760–765. 15
[4] Romero R. The child is the father of the man. Prenat Neonat Med. 1996;1:8–11.
[5] Romero R, Espinoza J, Kusanovic JP, et al. The preterm parturition syndrome. BJOG. 2006;113(Suppl.3):17–42.
[6] Romero R. Prenatal medicine: the child is the father of the man. 1996. J Matern Fetal Neonatal Med. 2009;22(8):636–639.
[7] Di Renzo GC. The great obstetrical syndromes. J Matern Fetal Neonatal Med. 2009;22(8):633–635.
[8] Brosens I, Pijnenborg R, Vercruysse L, et al. The "Great Obstetrical Syndromes" are associated with disorders of deep placentation. Am J Obstet Gynecol. 2011;204(3):193–201.
[9] Liu L, Oza S, Hogan D, et al. Global, regional, and national causes of child mortality in 2000-13, with projections to inform post-2015 priorities: an updated systematic analysis. Lancet. 2015;385(9966):430–440.
[10] Murphy SL, Mathews TJ, Martin JA, et al. Annual summary of vital statistics: 2013–2014. Pediatrics. 2017;139(6):e20163239.
[11] Yoon BH, Romero R, Moon JB, et al. Clinical significance of intra-amniotic inflammation in patients with preterm labor and intact membranes. Am J Obstet Gynecol. 2001;185(5):1130–1136.
[12] Romero R, Grivel JC, Tarca AL, et al. Evidence of perturbations of the cytokine network in preterm labor. Am J Obstet Gynecol. 2015;213(6):836 e1–836 e18.
[13] Bhatti G, Romero R, Rice GE, et al. Compartmentalized profiling of amniotic fluid cytokines in women with preterm labor. PLoS One. 2020;15(1):e0227881.
[14] Romero R, Miranda J, Chaiworapongsa T, et al. Prevalence and clinical significance of sterile intra-amniotic inflammation in patients with preterm labor and intact membranes. Am J Reprod Immunol. 2014;72(5):458–474.
[15] Romero R, Miranda J, Chaemsaithong P, et al. Sterile and microbial-associated intra-amniotic inflammation in preterm prelabor rupture of membranes. J Matern Fetal Neonatal Med. 2015;28(12):1394–1409.
[16] Romero R, Miranda J, Chaiworapongsa T, et al. Sterile intra-amniotic inflammation in asymptomatic patients with a sonographic short cervix: prevalence and clinical significance. J Matern Fetal Neonatal Med. 2015;28(11):1343–1359.
[17] Gomez-Lopez N, Romero R, Tarca AL, et al. Gasdermin D: evidence of pyroptosis in spontaneous preterm labor with sterile intra-amniotic inflammation or intra-amniotic infection. Am J Reprod Immunol. 2019;82(6):e13184.
[18] Gomez-Lopez N, Romero R, Garcia-Flores V, et al. Inhibition of the NLRP3 inflammasome can prevent sterile intra-amniotic inflammation, preterm labor/birth, and adverse neonatal outcomes†. Biol Reprod. 2019;100(5):1306–1318.
[19] Gomez-Lopez N, Romero R, Panaitescu B, et al. Inflammasome activation during spontaneous preterm labor with intra-amniotic infection or sterile intra-amniotic inflammation. Am J Reprod Immunol. 2018;80(5):e13049.
[20] Gomez-Lopez N, Romero R, Galaz J, et al. Cellular immune responses in amniotic fluid of women with preterm labor and intra-amniotic infection or intra-amniotic inflammation. Am J Reprod Immunol. 2019;82(5):e13171.
[21] Gomez-Lopez N, Romero R, Plazyo O, et al. Intra-amniotic administration of HMGB1 induces spontaneous preterm labor and birth. Am J Reprod Immunol. 2016;75(1):3–7.
[22] Peiris HN, Romero R, Vaswani K, et al. Preterm labor is characterized by a high abundance of amniotic fluid prostaglandins in patients with intra-amniotic infection or sterile intra-amniotic inflammation. J Matern Fetal Neonatal Med. 2019;29:1–16.
[23] Motomura K, Romero R, Garcia-Flores V, et al. The alarmin interleukin-1α causes preterm birth through the NLRP3 inflammasome. Mol Hum Reprod. 2020;26(9):712–726.

[24] Plazyo O, Romero R, Unkel R, et al. HMGB1 Induces an inflammatory response in the chorioamniotic membranes that is partially mediated by the inflammasome. Biol Reprod. 2016;95(6):130.
[25] Friel LA, Romero R, Edwin S, et al. The calcium binding protein, S100B, is increased in the amniotic fluid of women with intra-amniotic infection/inflammation and preterm labor with intact or ruptured membranes. J Perinat Med. 2007;35(5):385–393.
[26] Kim KW, Romero R, Park HS, et al. A rapid matrix metalloproteinase-8 bedside test for the detection of intraamniotic inflammation in women with preterm premature rupture of membranes. Am J Obstet Gynecol. 2007;197(3):292 e1–5.
[27] Musilova I, Kutova R, Pliskova L, et al. Intraamniotic inflammation in women with preterm prelabor rupture of membranes. PLoS One. 2015;10(7):e0133929.
[28] Bredeson S, Papaconstantinou J, Deford JH, et al. HMGB1 promotes a p38MAPK associated non-infectious inflammatory response pathway in human fetal membranes. PLoS One. 2014;9(12):e113799.
[29] Romero R, Chaiworapongsa T, Alpay Savasan Z, et al. Damage-associated molecular patterns (DAMPs) in preterm labor with intact membranes and preterm PROM: a study of the alarmin HMGB1. J Matern Fetal Neonatal Med. 2011;24(12):1444–1455.
[30] Jacobsson B, Mattsby-Baltzer I, Hagberg H. Interleukin-6 and interleukin-8 in cervical and amniotic fluid: relationship to microbial invasion of the chorioamniotic membranes. BJOG: Int J O&G. 2005; 112(6):719–724.
[31] Andrews WW, Hauth JC, Goldenberg RL, et al. Amniotic fluid interleukin-6: correlation with upper genital tract microbial colonization and gestational age in women delivered after spontaneous labor versus indicated delivery. Am J Obstet Gynecol. 1995; 173(2):606–612.
[32] Rizzo G, Capponi A, Rinaldo D, et al. Interleukin-6 concentrations in cervical secretions identify microbial invasion of the amniotic cavity in patients with preterm labor and intact membranes. Am J Obstet Gynecol. 1996;175(4 Pt 1):812–817.
[33] Holst RM, Mattsby-Baltzer I, Wennerholm UB, et al. Interleukin-6 and interleukin-8 in cervical fluid in a population of Swedish women in preterm labor: relationship to microbial invasion of the amniotic fluid, intra-amniotic inflammation, and preterm delivery. Acta Obstet Gynecol Scand. 2005;84(6):551–557.
[34] van Baaren GJ, Vis JY, Wilms FF, et al. Predictive value of cervical length measurement and fibronectin testing in threatened preterm labor. Obstet Gynecol. 2014;123(6):1185–1192.
[35] Fraunberger P, Pfeiffer M, Cremer P, et al. Validation of an automated enzyme immunoassay for Interleukin-6 for routine clinical use. Clin Chem Lab Med. 1998;36(10):797–801.
[36] Musilova I, Andrys C, Holeckova M, et al. Interleukin-6 measured using the automated electrochemiluminescence immunoassay method for the identification of intra-amniotic inflammation in preterm prelabor rupture of membranes. J Matern Fetal Neonatal Med. 2020;33(11):1919–1926.
[37] Kacerovsky M, Romero R, Stepan M, et al. Antibiotic administration reduces the rate of intraamniotic inflammation in preterm prelabor rupture of the membranes. Am J Obstet Gynecol. 2020;223(1):114 e1–114 e20.
[38] Kacerovsky M, Holeckova M, Stepan M, et al. Amniotic fluid glucose level in PPROM pregnancies: a glance at the old friend. J Matern Fetal Neonatal Med. 2020;24: 1–13.
[39] Spacek R, Musilova I, Andrys C, et al. Extracellular granzyme A in amniotic fluid is elevated in the presence of sterile intra-amniotic inflammation in preterm prelabor rupture of membranes. J Matern Fetal Neonatal Med. 2020;10:1–10.
[40] Theis KR, Romero R, Motomura K, et al. Microbial burden and inflammasome activation in amniotic fluid of patients with preterm prelabor rupture of membranes. J Perinat Med. 2020;48(2):115–131.
[41] Holst RM, Hagberg H, Wennerholm UB, et al. Prediction of spontaneous preterm delivery in women with preterm labor: analysis of multiple proteins in amniotic and cervical fluids. Obstet Gynecol. 2009; 114(2 Pt 1):268–277.
[42] Inglis SR, Jeremias J, Kuno K, et al. Detection of tumor necrosis factor-alpha, interleukin-6, and fetal fibronectin in the lower genital tract during pregnancy: relation to outcome. Am J Obstet Gynecol. 1994;171(1): 5–10.
[43] Lockwood CJ, Ghidini A, Wein R, et al. Increased interleukin-6 concentrations in cervical secretions are associated with preterm delivery. Am J Obstet Gynecol. 1994;171(4):1097–1102.
[44] Goepfert AR, Goldenberg RL, Andrews WW, et al. The Preterm Prediction Study: association between cervical interleukin 6 concentration and spontaneous preterm birth. National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network. Am J Obstet Gynecol. 2001;184(3):483–488.
[45] Lange M, Chen FK, Wessel J, et al. Elevation of interleukin-6 levels in cervical secretions as a predictor of preterm delivery. Acta Obstet Gynecol Scand. 2003; 82(4):326–329.
[46] Kurkinen-Raty M, Ruokonen A, Vuopala S, et al. Combination of cervical interleukin-6 and -8, phosphorylated insulin-like growth factor-binding protein-1 and transvaginal cervical ultrasonography in assessment of the risk of preterm birth. BJOG. 2001; 108(8):875–881.
[47] Brik M, Aguar M, Valiente A, et al. Cervical IL-6 and pIGFBP-1 and the prediction of neonatal outcome in symptomatic preterm labour. J Matern Fetal Neonatal Med. 2014;27(12):1241–1247.

8.2.2 Specific aim I-B

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IgGFc-binding protein in pregnancies complicated by spontaneous preterm delivery: a retrospective cohort study

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To determine the IgGFc-binding protein (FcgammaBP) concentration in amniotic and cervical fluids in preterm prelabor rupture of membranes (PPROM) and preterm labor with intact membranes (PTL) and to assess the diagnostic indices of FcgammaBP to predict intra-amniotic infection (the presence of both microbial invasion of the amniotic cavity and intra-amniotic inflammation). In this study, we included 170 and 79 women with PPROM and PTL, respectively. Paired cervical and amniotic fluid samples were obtained using a Dacron polyester swab and transabdominal amniocentesis, respectively. The FcgammaBP concentrations in the samples were assessed using an enzyme-linked immunosorbent assay. The presence of intra-amniotic infection was associated with elevated FcgammaBP concentrations in pregnancies with PPROM and PTL [PPROM—presence: 86 ng/mL vs. absence: 13 ng/mL, $p < 0.0001$, area under receiver operating characteristic curve (AUC) = 0.94; PTL—presence: 140 ng/mL vs. absence: 22 ng/mL, $p < 0.0001$, AUC = 0.86]. In cervical fluid, the concentrations of FcgammaBP were elevated in the presence of intra-amniotic infection in pregnancies with PPROM only (presence: 345 ng/mL vs. absence: 60 ng/mL, $p < 0.0001$, AUC = 0.93). FcgammaBP in amniotic fluid might be a marker of intra-amniotic infection in women with both PPROM and PTL However, in cervical fluid, it is only observed in women with PPROM.

Preterm delivery, defined as delivery before 37 weeks of gestation, is divided into two major subgroups: (i) iatrogenic preterm delivery and (ii) spontaneous preterm delivery[1,2]. The latter form of preterm delivery is more frequent and is responsible for more than two-thirds of all preterm deliveries[1,2]. Spontaneous preterm delivery can also be divided into two clinical phenotypes: (i) preterm prelabor rupture of the membranes (PPROM) and (ii) preterm labor with intact membranes (PTL)[1,2].

Some pregnancies can be complicated by the elevation of amniotic fluid concentrations of various inflammatory mediators, such as cytokines, chemokines, and antimicrobial peptides[3–11]. This condition is termed intra-amniotic inflammation and can be identified in both PPROM[12,13] and PTL[14]. Based on the presence or absence of microorganisms and/or their nucleic acids in amniotic fluid, intra-amniotic inflammation can be further divided into two clinical subtypes: (i) intra-amniotic infection and (ii) sterile intra-amniotic inflammation[12,14,15]. Clinical

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Characteristic	The presence of intra-amniotic infection (n = 19)	The absence of intra-amniotic infection (n = 151)	*p*-value
Maternal age [years, median (IQR)]	30 (24–33)	31 (27–35)	0.19
Primiparous [number (%)]	10 (53%)	90 (60%)	0.63
Pre-pregnancy body mass index [kg/m^2, median (IQR)]	22.8 (20.9–25.2)	24.0 (21.2–27.2)	0.25
Smoking [number (%)]	3 (16%)	25 (17%)	1.00
Interval between PPROM and amniocentesis [hours, median (IQR)]	3 (2–8)	4 (2–7)	0.57
Gestational age at admission [weeks, median (IQR)]	29 + 3 (27 + 0–33 + 4)	34 + 3 (32 + 3–35 + 3)	**0.0003**
Gestational age at delivery [weeks, median (IQR)]	29 + 6 (27 + 3–33 + 4)	34 + 5 (33 + 0–35 + 6)	**<0.0001**
Latency between PPROM and delivery [hours, median (IQR)]	60 (17–113)	45 (13–157)	0.70
Amniotic fluid IL-6 levels at admission [pg/nL, median (IQR)]	30,932 (9427–50,000)	667 (329–50,000)	**<0.0001**
CRP levels at admission [mg/L, median (IQR)]	19.2 (3.3–36.5)	5.2 (2.5–8.2)	**0.003**
WBC count at admission [×10^9 L, median (IQR)]	14.9 (11.2–17.6)	11.9 (9.9–14.3)	**0.03**
Administration of corticosteroids [number (%)]	15 (79%)	89 (59%)	0.13
Vaginal delivery [number (%)]	13 (68%)	108 (72%)	0.79
Birth weight [grams, median (IQR)]	1460 (1090–2220)	**2290 (1930–3620)**	**<0.0001**
Apgar score <7; 5 min [number (%)]	3 (16%)	5 (3%)	**0.05**
Apgar score <7; 10 min [number (%)]	2 (11%)	2 (1%)	0.06

Table 1. Maternal and clinical characteristics of women with preterm prelabor rupture of membranes based on the presence and absence of intra-amniotic infection. Continuous variables were compared using a nonparametric Mann–Whitney *U* test. Categorical variables were compared using the Fisher's exact test. Continuous variables are presented as median (IQR) and categorical as number (%). Statistically significant results are marked in bold. CRP: C-reactive protein; IL-6: interleukin-6; IQR: interquartile range; WBC: white blood cells.

relevance of intra-amniotic inflammation, its association with adverse short- and long-term neonatal outcomes, and optimal diagnostic markers, are still a matter of intense debate[16–28].

The broad availability and recent advances in proteomics, an unbiased technology, bring it within the scope of researchers working in the field of intra-amniotic inflammatory complications[29–38]. The ability to identify hundreds of proteins and to quantify changes in their abundance across multiple amniotic fluid samples makes proteomics very appealing, particularly in the discovery phase of the biomarker search process[29–61]. Therefore, proteomic analysis of amniotic fluid may reveal new proteins involved in the complex pathogenesis of intra-amniotic inflammation[29–40].

IgGFc-binding protein (FcgammaBP) is one of the proteins identified in amniotic fluid using proteomics[36,37,40,42]. FcgammaBP is a relatively unknown protein, with limited reports in relation to conditions such as bowel inflammatory disease, autoimmune disease, or thyroid gland tumors[62–64]. Nevertheless, FcgammaBP has been identified in amniotic fluid obtained from women with uncomplicated pregnancies[40,42] as well as from women with pregnancies complicated by PPROM[36] and PTL[37]. In pregnancies with PPROM, an elevation of FcgammaBP concentration in amniotic fluid has been observed during microbial invasion of the amniotic cavity and in acute histological chorioamnionitis[36]. However, there is a paucity of information on whether concentrations of FcgammaBP in amniotic and cervical fluid reflect the presence of intra-amniotic inflammatory complications in both the clinical phenotypes of spontaneous preterm delivery.

To fill this gap in the knowledge, we conducted this study with the following goals: (i) to quantify the FcgammaBP concentration in amniotic fluid samples from pregnant women with PPROM and PTL based on the phenotype of intra-amniotic inflammation; (ii) to quantify the FcgammaBP concentration in cervical fluid samples from pregnant women with PPROM and PTL based on the phenotype of intra-amniotic inflammation; and (iii) to assess the predictive value of FcgammaBP concentrations in amniotic and cervical fluids for intra-amniotic infection in pregnant women with PPROM and PTL.

Results

In total, 170 and 79 women with PPROM and PTL, respectively were included in the study. Among women with PPROM, the presence of intra-amniotic infection, sterile intra-amniotic inflammation, colonization, and negative amniotic fluid were observed in 11% (19/170), 5% (9/170), 10% (16/170), and 74% (126/170) of women, respectively. Among women with PTL, intra-amniotic infection, sterile intra-amniotic inflammation, and negative amniotic fluid were found in 15% (12/79), 27% (21/79), and 58% (46/79) of the women, respectively. None of the women with PTL had colonization.

The demographics of all the women in this study and clinical data of the women with PPROM and PTL, based on the presence and absence of intra-amniotic infection, are shown in Tables 1 and 2, respectively. The microorganisms identified in the amniotic fluid from women with PPROM and PTL are listed in Table 3.

Amniotic fluid FcgammaBP concentrations based on the phenotype of intra-amniotic inflammation. *PPROM pregnancies.* PPROM pregnancies with intra-amniotic infection and sterile intra-amniot-

Characteristic	The presence of intra-amniotic infection (n = 12)	The absence of intra-amniotic infection (n = 67)	*p*-value
Maternal age [years, median (IQR)]	27 (25–28)	28 (23–30)	0.86
Primiparous [number (%)]	7 (58%)	47 (70%)	0.50
Pre-pregnancy body mass index [kg/m^2, median (IQR)]	27.5 (23.1–30.6)	25.0 (23.0–27.9)	0.51
Smoking [number (%)]	1 (8%)	7 (10%)	1.00
Gestational age at admission [weeks, median (IQR)]	27 + 6 (26 + 6–31 + 2)	30 + 6 (26 + 6–32 + 3)	0.17
Gestational age at delivery [weeks, median (IQR)]	29 + 0 (27 + 1–33 + 3)	32 + 6 (29 + 0–36 + 5)	**0.04**
Interval from amniocentesis to delivery [days, median (IQR)]	2 (0–15)	5 (1–39)	0.10
Amniotic fluid IL-6 levels at admission [pg/mL, median (IQR)]	43,431 (23,597–50,000)	1495 (484–4050)	**<0.0001**
CRP levels at admission [mg/L, median (IQR)]	42.0 (7.5–75.1)	6.0 (2.4–11.4)	**0.0007**
WBC count at admission [×10^9 L, median (IQR)]	16.3 (14.1–19.5)	13.9 (10.6–16.5)	0.05
Administration of corticosteroids [number (%)]	9 (75%)	57 (85%)	0.41
Vaginal delivery [number (%)]	9 (75%)	55 (82%)	0.69
Birth weight of the newborn [grams, median (IQR)]	1230 (936–1958)	1940 (130–2690)	**0.03**
Apgar score <7; 5 min [number (%)]	3 (25%)	7 (10%)	0.17
Apgar score <7; 10 min [number (%)]	2 (17%)	3 (5%)	0.16

Table 2. Maternal and clinical characteristics of women with spontaneous preterm labor with intact membranes based on the presence and absence of intra-amniotic infection. Continuous variables were compared using a nonparametric Mann–Whitney *U* test. Categorical variables were compared using the Fisher's exact test. Continuous variables are presented as median (IQR) and categorical as number (%). Statistically significant results are marked in bold. RP: C-reactive protein; IL: interleukin; IQR: interquartile range; WBC: white blood cells.

Preterm prelabor rupture of membranes	Preterm labor with intact membranes
Ureaplasma spp. (n = 18)	*Ureaplasma* spp. (n = 4)
Ureaplasma spp. + *Mycoplasma hominis* (n = 1)	*Klebsiella pneumoniae* + *Streptococcus anginosus* (n = 1)
Ureaplasma spp. + *Gardnerella vaginalis* (n = 1)	*Gardnerella vaginalis* + *Lactobacillus plantum* (n = 1)
Ureaplasma spp. + *Streptococcus mitis* (n = 1)	*Ureaplasma* spp. + *Mycoplasma hominis* (n = 1)
Ureaplasma spp. + *Escherichia coli* (n = 1)	*Haemophilus influenzae* (n = 1)
Ureaplasma spp. + *Fusobacterium nucleatum* v	*Lachnoanaerobaculum* spp. (n = 1)
Ureaplasma spp. + *Dialister micraerophilus* + *Atopobium vaginae* (n = 1)	*Lactococcus lactis* (n = 1)
Gardnerella vaginalis + *Sneathia sanguinegens* (n = 1)	*Sneathia sanguinegens* (n = 1)
Haemophilus influenzae (n = 4)	Non-identifiable bacteria by sequencing (n = 1)
Anaerococcus tetradius (n = 1)	
Chlamydia trachomatis (n = 1)	
Lactobacillus iners (n = 1)	
Lactobacillus jensenii (n = 1)	
Mycoplasma hominis (n = 1)	
Streptococcus anginosus (n = 1)	

Table 3. The microbial species identified in the amniotic fluid of women with preterm prelabor rupture of membranes and with spontaneous preterm labor with intact membranes.

ic inflammation had higher amniotic fluid FcgammaBP concentrations than did the women with colonization and with negative amniotic fluid (infection: median 85.6 ng/mL, IQR 37.3–146.0, sterile: median 41.3 ng/mL, IQR 22.5–91.4, colonization: median 12.3 ng/mL, IQR 8.0–19.8, negative 12.2 ng/mL, IQR 9.0–17.8; $p < 0.0001$; Fig. 1a). No differences in amniotic fluid FcgammaBP concentrations were found between the women with colonization and negative amniotic fluid (Table 4).

Women with intra-amniotic infection had higher amniotic fluid FcgammaBP than did those without intra-amniotic infection (with infection: median IQR 85.6 ng/mL, IQR 37.3–146.0 vs. without infection: median 12.6 ng/mL, IQR 9.1–20.3; Fig. 2a). The amniotic fluid FcgammaBP cutoff value of 60 ng/mL was optimal in the prediction of intra-amniotic infection (area under the ROC curve [AUC] = 0.94; $p < 0.0001$; Fig. 2b). The diagnostic indices of these cutoff values are in Table 5.

PTL pregnancies. Differences in the concentrations of FcgammaBP were identified among the subgroups of women with intra-amniotic infection, sterile intra-amniotic inflammation, and negative amniotic fluid (infec-

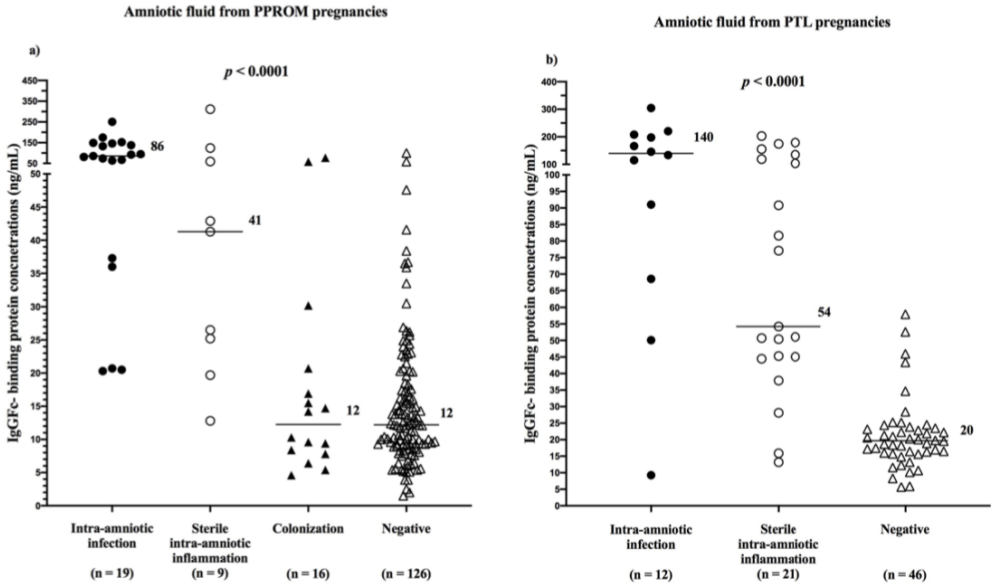

Figure 1. Amniotic fluid IgGFc-binding protein concentrations in the subgroups of the women with PPROM (**a**) and with PTL (**b**). PPROM, preterm prelabor rupture of membranes; PTL, preterm labor with intact membranes.

	Intra-amniotic infection	Sterile intra-amniotic inflammation	Colonization	Negative
Intra-amniotic infection	x	$p = 0.11$ adj. $p = 0.51$	$p < 0.0001$ **adj. p < 0.0001**	$p < 0.0001$ **adj. p < 0.0001**
Sterile intra-amniotic inflammation	$p = 0.11$ adj. $p = 0.51$	x	$p = 0.004$ **adj. p = 0.05**	$p < 0.0001$ **adj. p < 0.0001**
Colonization	$p < 0.0001$ **adj. p = 0.0001**	$p = 0.004$ **adj. p = 0.05**	x	$p = 0.98$ adj. $p = 0.22$
Negative	$p < 0.0001$ **adj. p < 0.0001**	$p < 0.0001$ **adj. p < 0.0001**	$p = 0.98$ adj. $p = 0.22$	x

Table 4. IgGFc-binding protein in amniotic fluid from preterm prelabor rupture of membranes: the comparisons among the subgroups of the women with intra-amniotic infection, sterile intra-amniotic inflammation, colonization, and negative amniotic fluid. *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 age at sampling (a Spearman partial correlation). Statistically significant results are marked in bold.

tion: median 139.7 ng/mL, IQR 74.2–205.3; sterile: median 54.2 ng/mL, IQR: 44.8–127.0; negative: median 19.7 ng/mL, IQR: 15.9–23.6; Fig. 1b) in the crude analysis and after the adjustment for gestational age at sampling (both *p*-values < 0.0001). Women with intra-amniotic infection had higher amniotic fluid FcgammaBP concentrations than did women with sterile intra-amniotic inflammation and with negative amniotic fluid (Table 6). Women with sterile intra-amniotic inflammation had higher amniotic fluid FcgammaBP concentrations than those with negative amniotic fluid (Table 6).

Women with intra-amniotic infection had higher concentrations of amniotic fluid FcgammaBP than those without intra-amniotic infection (with infection: median 139.7 ng/mL, IQR 74.2–205.3 vs. without infection: median 22.2 ng/mL, IQR 16.5–46.0; Fig. 3a). The amniotic fluid FcgammaBP cutoff value of 120 ng/mL was found to be optimal in the prediction of intra-amniotic infection (AUC = 0.86; $p < 0.0001$; Fig. 3b). The diagnostic indices of these cutoff values are in Table 5.

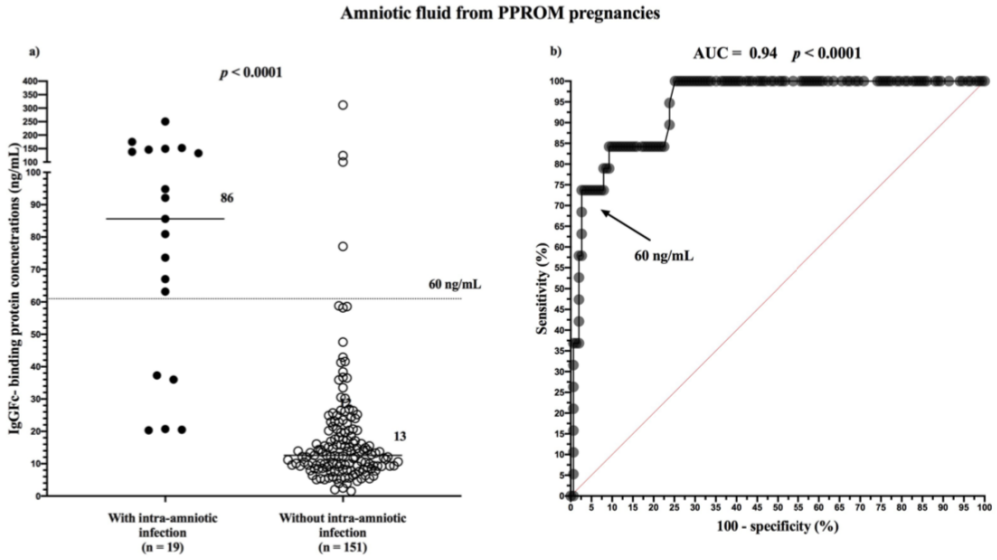

Figure 2. Amniotic fluid IgGFc-binding protein concentrations based on the presence of intra-amniotic infection in women with PPROM (**a**) and receiver operating characteristic curves for amniotic fluid IgGFc-binding protein in women with PPROM with intra-amniotic infection (**b**). PPROM, preterm prelabor rupture of membranes.

Cut-off value	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Positive likelihood ratio	Negative likelihood ratio	Odds ratio
Preterm prelabor rupture of membranes—amniotic fluid							
60 ng/mL	14/19 74% (95% CI 51–88)	147/151 97% (95% CI 93–99)	14/18 78% (95% CI 55–91)	147/152 97% (95% CI 93–99)	27.9 (95% CI 10.2–75.9)	0.27 (95% CI 0.13–0.57)	103 (95% CI 33–339)
Preterm labor with intact membranes—amniotic fluid							
120 ng/mL	7/12 58% (95% CI 32–81)	62/67 93% (95% CI 84–97)	7/12 58% (95% CI 32–81)	62/67 89% (95% CI 81–93)	7.8 (95% CI 3.0–20.6)	0.45 (95% CI 0.25–0.88)	17 (95% CI 3–64)
Preterm prelabor rupture of membranes—cervical fluid							
300 ng/mL	11/19 58% (95% CI 36–77)	145/151 96% (95% CI 92–98)	11/17 65% (95% CI 41–83)	145/153 95% (95% CI 90–97)	14.6 (95% CI 6.1–34.9)	0.44 (95% CI 0.26–74)	33 (95% CI 10–97)

Table 5. The predictive values of cut-off values of amniotic fluid IgGFcBP to identify intra-amniotic infection. CI: confidence interval.

	Intra-amniotic infection	Sterile intra-amniotic inflammation	Negative
Intra-amniotic infection	x	$p = 0.04$ **adj. p = 0.02**	$p < 0.0001$ **adj. p < 0.0001**
Sterile intra-amniotic inflammation	$p = 0.04$ **adj. p = 0.02**	x	$p < 0.0001$ **adj. p < 0.0001**
Negative	$p < 0.0001$ **adj. p < 0.0001**	$p < 0.0001$ **adj. p < 0.0001**	x

Table 6. IgGFc-binding protein in amniotic fluid from preterm labor with intact membranes: the comparisons among the subgroups of the women with intra-amniotic infection, sterile intra-amniotic inflammation, and negative amniotic fluid. *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 age at sampling (a Spearman partial correlation). Statistically significant results are marked in bold.

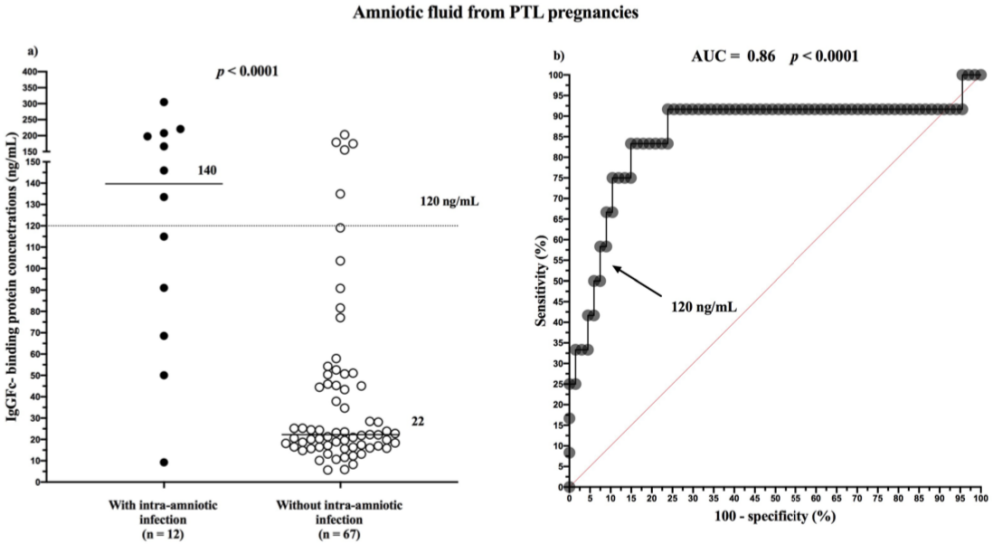

Figure 3. Amniotic fluid IgGFc-binding protein concentrations based on the presence of intra-amniotic infection in women with PTL (**a**) and receiver operating characteristic curves for amniotic fluid IgGFc-binding protein in women with PTL with intra-amniotic infection (**b**). PTL, preterm labor with intact membranes.

Concentration of FcgammaBP in cervical fluid based on the phenotype of intra-amniotic inflammation. *PPROM pregnancies.* A positive correlation was found between the concentrations of FcgammaBP in amniotic and cervical fluids ($\rho = 0.34$; $p < 0.0001$). The differences in cervical fluid FcgammaBP concentrations were revealed among the subgroups (infection: median 345.0 ng/mL, IQR 201.9–480.0; sterile: median 56.1 ng/mL, IQR 36.5–139.3; colonization: median 130.6 ng/mL, IQR 51.4–186.9; and negative: median 55.4 ng/mL, IQR 31.1–92.6; Fig. 4a) in the crude analysis as well as after the adjustment for gestational age at sampling (both $p < 0.0001$). Women with intra-amniotic infection had higher cervical fluid FcgammaBP concentrations than women with sterile intra-amniotic inflammation, colonization, and negative amniotic fluid (Table 7).

Women with intra-amniotic infection had higher cervical fluid FcgammaBP than those without intra-amniotic infection (with infection: median 345.0 ng/mL, IQR 201.9–480.0 vs. without infection: median 59.6 ng/mL, IQR 31.9–111.5; Fig. 5a). The cervical fluid FcgammaBP cutoff value of 300 ng/mL was found to be optimal in the prediction of intra-amniotic infection (AUC = 0.93; $p < 0.0001$; Fig. 5b). The diagnostic indices of these cutoff values are in Table 5.

PTL pregnancies. A weak positive correlation was observed between the concentrations of FcgammaBP in amniotic and cervical fluids ($\rho = 0.25$; $p = 0.02$). No difference in cervical fluid FcgammaBP concentrations was found among the subgroups (infection: median 341.1 ng/mL, IQR 95.2–614.8; sterile: median 341.2 ng/mL, IQR 138.1–523.4; and negative: median 200.9 ng/mL, IQR 56,7–443.8; $p = 0.18$; Fig. 4b). There was no difference in cervical fluid FcgammaBP concentrations between women with and without intra-amniotic infection (with infection: median 341.1 ng/mL, IQR 95.2–614.8 vs. without infection: median 227.0 ng/mL, IQR 95.7–455.4; $p = 0.45$).

Discussion

Principal findings of the study. (1) FcgammaBP was identified as a constituent of amniotic and cervical fluids from pregnancies complicated by PPROM and PTL; (2) the concentration of FcgammaBP in amniotic fluid was elevated in the presence of both phenotypes of intra-amniotic inflammation, being more pronounced in the presence of intra-amniotic infection in women with PTL; (3) the concentration of FcgammaBP in cervical fluid was elevated in the presence of intra-amniotic infection only in women with PPROM; (4) the FcgammaBP in amniotic fluid might be a marker of intra-amniotic infection in women with PPROM and PTL; and (5) the FcgammaBP in cervical fluid might be a non-invasive marker of intra-amniotic infection in women with PPROM.

Meaning of the study. FcgammaBP was discovered more than 30 years ago as a specific site for the fragment of crystallizable (Fc) region of the immunoglobulin (Ig) G antibody in the small intestinal and colonic epithelia[65]. This specific site differed from previously recognized receptors in the Fc region of IgG[65]. The specific

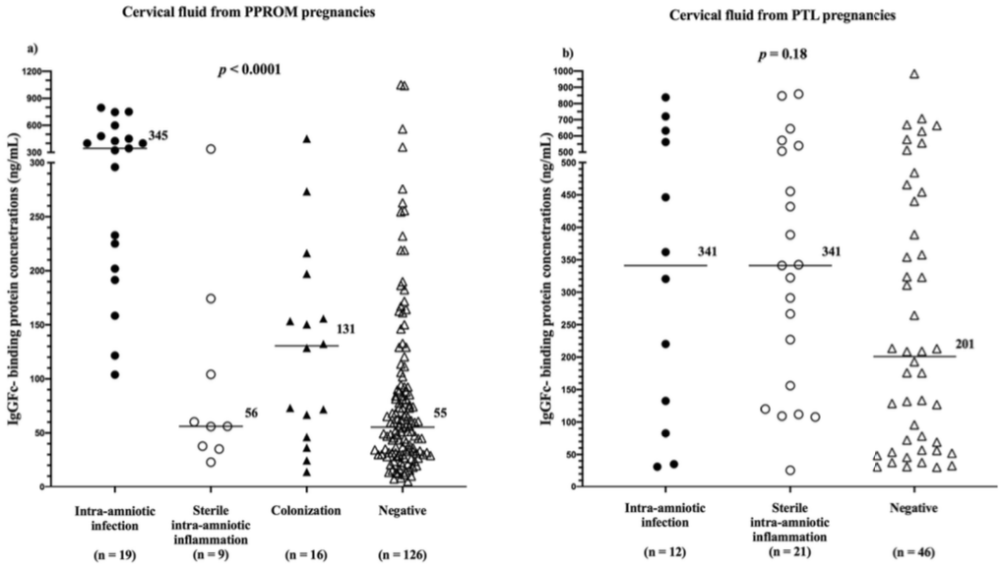

Figure 4. Cervical fluid IgGFc-binding protein concentrations in the subgroups of the women with PPROM (**a**) and with PTL (**b**). PPROM, preterm prelabor rupture of membranes; PTL, preterm labor with intact membranes.

	Intra-amniotic infection	Sterile intra-amniotic inflammation	Colonization	Negative
Intra-amniotic infection	x	$p = 0.0001$ **adj. p = 0.001**	$p < 0.0001$ **adj. p < 0.0001**	$p < 0.0001$ **adj. p < 0.0001**
Sterile intra-amniotic inflammation	$p = 0.0001$ **adj. p = 0.001**	x	$p = 0.28$ adj. $p = 0.76$	$p = 0.57$ adj. $p = 0.93$
Colonization	$p < 0.0001$ **adj. p = 0.0001**	$p = 0.28$ adj. $p = 0.76$	x	$p = 0.02$ adj. $p = 0.25$
Negative	$p < 0.0001$ **adj. p < 0.0001**	$p = 0.57$ adj. $p = 0.93$	$p = 0.02$ adj. $p = 0.25$	x

Table 7. IgGFc-binding protein in cervical fluid from preterm prelabor rupture of membranes: the comparisons among the subgroups of the women with intra-amniotic infection, sterile intra-amniotic inflammation, colonization, and negative amniotic fluid. *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 age at sampling (a Spearman partial correlation). Statistically significant results are marked in bold.

site for the Fc region of IgG was later termed FcgammaBP and identified as a protein primarily localized in the mucosal granules of the small intestinal and colonic epithelia that are secreted into the intestinal lumen[66]. Based on the current knowledge, FcgammaBP is considered to be a protein that provides immunologic protection of the intestinal tissue and facilitates the interaction between the intestinal mucus and potentially harmful stimuli (microorganisms, alarmins) with the ultimate goal of protecting the mucosal surface[62,65,66]. However, its exact biological function has yet to be fully elucidated.

The production of FcgammaBP has been described to occur in the intestinal epithelial cells, placenta, and thyroid tissue[62,63]. However, its expression has not been observed in the brain, heart, kidney, liver, lung, and skeletal muscles[62]. Interestingly, the ability to produce FcgammaBP was confirmed only in humans and monkeys, but not in mice, rats, rabbits, dogs, bovines, and porcines[62].

FcgammaBP has been found in low concentrations in human serum from healthy individuals[64]. However, its serum concentrations were elevated in the presence of autoimmune diseases such as Crohn's disease, ulcerative colitis, rheumatoid arthritis, systemic lupus erythematosus, and progressive systemic sclerosis[64]. The presence of FcgammaBP has been further proven in amniotic fluid, urine, saliva, and cerebrospinal fluid[40,42]. Liu et al. found FcgammaBP to be a constituent of amniotic fluid in the second trimester of uncomplicated pregnancies[42]. In

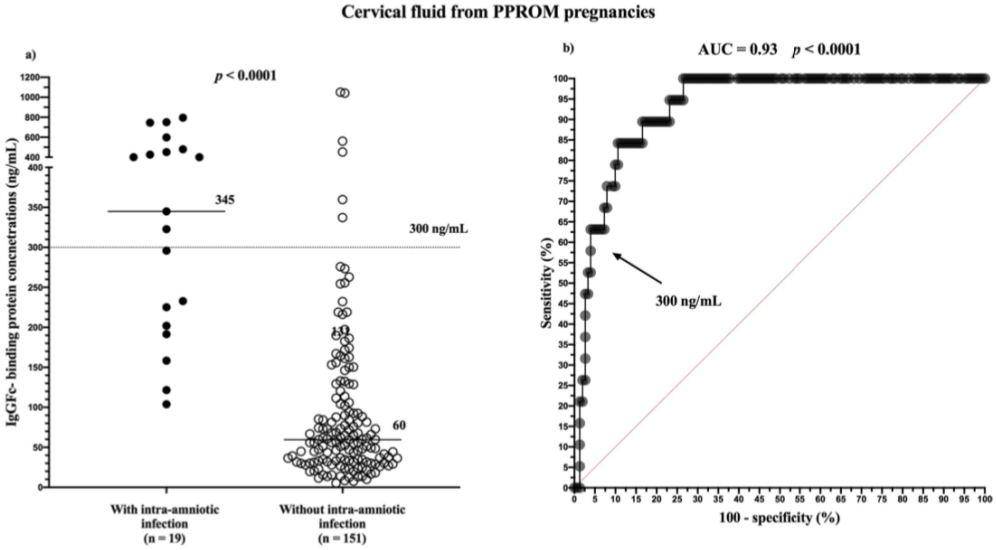

Figure 5. Cervical fluid IgGFc-binding protein concentrations based on the presence of intra-amniotic infection in women with PPROM (**a**) and receiver operating characteristic curves for cervical fluid IgGFc-binding protein in women with PPROM with intra-amniotic infection (**b**). PPROM, preterm prelabor rupture of membranes.

addition, FcgammaBP was shown to be among the most abundant (35/1624) proteins found in amniotic fluid[42]. Our group described the amniotic fluid presence of FcgammaBP in pregnancies complicated by PPROM and PTL[36,37]. The observation from this study, where we found FcgammaBP as a constituent of amniotic fluid from pregnancies with PPROM and PTL, is in line with the abovementioned findings.

Previously, concentrations of FcgammaBP in amniotic fluid have been shown to be higher in women with PPROM with microbial invasion of the amniotic cavity and acute histological chorioamnionitis than in those without these complications[36]. Interestingly, no differences in the amniotic fluid concentration of FcgammaBP between the presence and absence of the abovementioned complications were identified in women with PTL, where amniotic fluid was obtained from the forewaters at the end of the first stage of labor[37].

In this study, we found elevated amniotic fluid concentrations of FcgammaBP in the presence of both phenotypes of intra-amniotic inflammation. Interestingly, in women with PTL, the concentrations of FcgammaBP in amniotic fluid were higher in the presence of intra-amniotic infection than in the presence of sterile intra-amniotic inflammation. Collectively, the results from this study clearly show that both infectious and non-infectious stimuli might trigger the production of FcgammaBP.

In this study, the concentrations of FcgammaBP were measured in paired amniotic and cervical fluid samples obtained from women with both phenotypes of spontaneous preterm delivery. Interestingly, the FcgammaBP concentrations were higher in the cervical fluid samples than in the amniotic fluid samples, despite the fact that cervical fluid samples obtained with a swab were diluted in 1.5 mL of the buffer. These observations suggest that epithelial cells and/or immune cells in the endocervical canal are able to produce FcgammaBP. This finding supports the key role of the cervix during pregnancy, which is its immunologic protection against the ascension of microorganisms from the vagina and the cervix toward the upper genital tract[67–70].

It is obvious that the protein composition of a cervical fluid sample from pregnancies with PPROM may be substantially affected by amniotic fluid that has leaked from the amniotic cavity. Therefore, the cervical fluid samples of PPROM pregnancies may reflect such a situation in both the intra-amniotic and cervical compartments. This is a possible explanation as to why women with intra-amniotic infection had higher cervical fluid FcgammaBP concentrations than those without intra-amniotic infection in PPROM but not in PTL pregnancies. However, a weak positive correlation between amniotic and cervical fluid FcgammaBP protein concentrations was also found in PTL.

This study suggests that FcgammaBP might be a new biomarker for intra-amniotic infection in both phenotypes of spontaneous preterm delivery. This finding is clinically very relevant since confirmation of intra-amniotic infection represents a challenge for clinicians. The necessity to rule in or rule out the presence of microorganisms in amniotic fluid makes the diagnosis of intra-amniotic infection time-consuming and more expensive when the techniques used to identify either non-culturable or difficult-to-culture microorganisms are employed. Therefore, from a clinical point of view, there is an urgent need to discover a single marker of intra-amniotic infection that has reliable sensitivity and specificity. In this study, FcgammaBP in amniotic fluid was identified as a potential

marker of intra-amniotic infection in pregnancies with PPROM and PTL. Previously, Chaemsaithong et al. has described diagnostic indices of rapid matrix metalloproteinase (MMP)-8 and interleukin (IL)-6 point-of-care test (two cut-off values: 745 pg/mL and 1000 pg/mL) to identify intra-amniotic infection in pregnancies with PTL[21]. Comparing diagnostic indices among amniotic fluid FcgammaBP, MMP-8, and IL-6 to identify intra-amniotic infection in pregnancies with PTL, amniotic fluid FcgammaBP has the highest likelihood ratio [FcgammaBP (cut-off value of 120 ng/mL) 7.8, MMP-8 (cut-off value of 10 ng/mL) 3.3, IL-6 (cut-off value of 745 pg/mL) 2.6, and IL-6 (cut-off value of 1000 pg/mL) 3.0][21].

In addition, in women with PPROM, FcgammaBP in cervical fluid was also revealed as a potential marker of intra-amniotic infection. Particularly, cervical fluid FcgammaBP can be a clinically relevant marker given the non-invasive nature of cervical fluid sampling. Moreover, its diagnostic indices are better than previously published diagnostic indices of IL-6 in cervical fluid (AUC = 0.78, the positive likelihood ratio of 4.8) in PPROM pregnancies[71]. In addition, cervical fluid sampling can be safely repeated during the latency period of pregnancy complicated by PPROM. Therefore, FcgammaBP assessment in cervical fluid might be used to monitor the development of secondary intra-amniotic infection during the latency interval in women with PPROM.

Strengths and limitations of the study. The strength of this study is the relatively large cohort of paired samples of amniotic and cervical fluid. Second, the fluid samples were collected from well-defined phenotypes of spontaneous preterm delivery (PPROM and PTL). Finally, the thorough assessment of microbial invasion of the amniotic cavity, by a combination of culture and non-culture methods, provided an opportunity to precisely distinguish the subsets of women with intra-amniotic infection and sterile intra-amniotic inflammation.

This study also has some limitations that are worth mentioning. For example, there was a small number of women with intra-amniotic infection (n = 19 and n = 12). To confirm whether the concentration of FcgammaBP in amniotic fluid and cervical fluid is a reliable marker of intra-amniotic infection, the results need to be replicated in independent cohorts. Next, despite the FcgammaBP expression in the placenta that was described[62], the questions of which part of the placenta is a source of FcgammaBP and whether fetal membranes produce FcgammaBP still remain unanswered. A body of evidence has shown that intestinal epithelial cells produce FcgammaBP (97, 102, 103), but no data are available on whether amniotic epithelial cells can produce FcgammaBP. Given the importance of the amniotic epithelium as a barrier against the ascension of microorganisms into the amniotic cavity[72–74], some similarities between intestinal and amniotic epithelial cells might be identifiable such as, (1) to serve as mechanical barriers[72–76]; (2) to have spatially expressed toll-like receptors[77,78]; and (3) to indicate that the expression of toll-like receptors changes when inflammation is present[77–79]. Therefore, we hypothesize that the amniotic epithelium might be involved in FcgammaBP production.

Conclusion

The concentrations of FcgammaBP observed in amniotic and cervical fluid were elevated in women with intra-amniotic infection. Thus, after replication in an independent cohort, FcgammaBP in amniotic fluid might be a potential marker of intra-amniotic infection in pregnancies with PPROM and PTL. Moreover, FcgammaBP in cervical fluid could be a marker of intra-amniotic infection in pregnancies with PPROM.

Methods

This retrospective cohort study included pregnant women who were admitted to the Department of Obstetrics and Gynecology at the University Hospital Hradec Kralove in the Czech Republic between March 2017 and May 2020. The inclusion criteria were the following: (1) singleton pregnancy, (2) maternal age ≥ 18 years, (3) gestational age between 22 + 0 and 36 + 6 weeks, (4) PPROM or PTL, and (5) the performance of transabdominal amniocentesis at the time of admission to determine intra-amniotic inflammation. In contrast, the exclusion criteria were the following: (1) pregnancy-related and other medical complications such as fetal growth restriction, gestational or pre-gestational diabetes, gestational or chronic hypertension, and preeclampsia; (2) structural or chromosomal fetal abnormalities; (3) signs of fetal hypoxia; and (4) significant vaginal bleeding.

The gestational age was determined via the use of first-trimester fetal biometry. PPROM was diagnosed by examining the women, using a sterile speculum, for pooling of amniotic fluid in the posterior fornix of the vagina. In the case of clinical uncertainty in diagnosing PPROM, amniotic fluid leakage was confirmed by the presence of insulin-like growth factor-binding proteins (Actim PROM test; Medix Biochemica, Kauniainen, Finland) in the vaginal fluid.

PTL was diagnosed as the presence of regular uterine contractions (at least two contractions every 10 min), along with cervical length, measured using transvaginal ultrasound, shorter than 15 mm or within the 15–30 mm range with a positive PartoSure test (Parsagen Diagnostics Inc., Boston, MA)[80].

Women with PPROM were treated with antibiotics. Those with intra-amniotic inflammation received first-line treatment with intravenous clarithromycin for seven days. Unless delivery occurred earlier, the antibiotic treatment was eventually modified under the condition of microbial invasion of the amniotic cavity; the women without intra-amniotic inflammation received benzylpenicillin (clindamycin was used in women allergic to penicillin). Women with PPROM below the gestational age of 35 + 0 weeks received corticosteroids (betamethasone) to accelerate fetal lung maturation and reduce neonatal mortality and morbidity. Women with PPROM were managed expectantly, except those with intra-amniotic infection beyond the gestational age 28 + 0 weeks wherein labor was induced or an elective cesarean section was performed within 72 h of admission.

Women with PTL received a course of corticosteroids (betamethasone) and tocolytic therapy with either intravenous atosiban (for gestational age ≤ 28 weeks) or with nifedipine, which was administered orally, for 48 h. Patients with proven intra-amniotic inflammation received treatment with intravenous clarithromycin for seven days, unless delivery occurred earlier. Antibiotic treatment was eventually modified under the condition of

microbial invasion of the amniotic cavity. Women with PTL that were positive for group B Streptococcus (GBS), as determined from the vaginal-rectal swab, or with an unknown GBS status received intravenous benzylpenicillin (clindamycin, in case of penicillin allergy) during an active labor.

All participants in this study provided informed written consent prior to the collection of amniotic and cervical fluid samples. Sample collection for this research was approved by the Institutional Review Board of the University Hospital Hradec Kralove (July 2014; No. 201408 S07P). All experiments were performed in accordance with relevant guidelines and regulations. All participants were Caucasian.

Cervical and amniotic fluid sampling. Paired cervical fluid and amniotic fluid samples were collected at the time of admission from all women included in this study, prior to the administration of antibiotics, tocolytics, and/or corticosteroids. Each cervical fluid sample was obtained by placing a Dacron polyester swab in the cervical canal for 20 s to achieve saturation. Once collected, the polyester swab was inserted into a polypropylene tube containing 1.5 mL of phosphate-buffered saline; the tube was then shaken for 20 min. Upon removal of the polyester swab, the tube was centrifuged at 300×*g* for 15 min at room temperature. The supernatant was divided into aliquots and stored at − 80 °C until further analysis.

Ultrasonography-guided transabdominal amniocentesis was performed after cervical fluid sampling. Approximately 2–3 mL of amniotic fluid was aspirated, and the amniotic fluid was immediately divided among polypropylene tubes. The samples of amniotic fluid were used for (i) the assessment of amniotic fluid interleukin (IL)-6; (ii) polymerase chain reaction (PCR) analysis of *Ureaplasma* species, *Mycoplasma hominis*, and *Chlamydia trachomatis*; (iii) sequencing of the 16S rRNA gene; and (iv) aerobic and anaerobic cultivation.

Amniotic fluid IL-6 concentrations. IL-6 concentrations were assessed using the immuno-analyzer Cobas e602, a part of the Cobas 8000 platform (Roche Diagnostics, Basel, Switzerland). The measurement range was 1.5–50,000 pg/mL. The coefficient of inter- and intra-assay precision was < 10%[22].

Detection of *Ureaplasma* species, *M. hominis*, and *C. trachomatis*. DNA was isolated from amniotic fluid using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Real-time PCR was conducted on a Rotor-Gene 6000 instrument (Qiagen) using the commercial AmpliSens *C. trachomatis*/*Ureaplasma*/*M. hominis*-FRT kit (Federal State Institution of Science, Central Research Institute of Epidemiology, Moscow, Russia) to detect the DNA from *Ureaplasma* species, *M. hominis*, and *C. trachomatis* in the same PCR tube (multiplex format). We included a PCR run for beta-actin, a housekeeping gene that served as the control, to examine the presence of polymerase chain reaction inhibitors.

Detection of other bacteria in amniotic fluid. Bacterial DNA was identified by PCR targeting the 16S rRNA gene with the following primers: 5′-CCAGACTCCTACGGGAGGCAG-3′ (V3 region) and 5′-ACATTT CACAACAC-GAGCTGACGA-3′ (V6 region)[81,82]. Each reaction contained 3 µL of target DNA, 500 nM forward and reverse primers, and Q5 High-Fidelity DNA polymerase (NEB, Ipswich, MA, USA) in a total volume of 25 µL. Amplification was carried out on a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The products were visualized on an agarose gel. Positive reactions yielded 950 bp products that were subsequently analyzed by sequencing. The 16S rDNA PCR products were purified and subjected to sequencing with the above-mentioned primers and the BigDye Terminator kit v.3.1 (Thermo Fisher Scientific, Waltham, MA, USA). The bacteria were then typed via searches for the obtained sequences using BLAST and SepsiTest BLAST.

Aerobic and anaerobic cultures of amniotic fluid. The amniotic fluid samples were cultured on Columbia agar with sheep's blood, *Gardnerella vaginalis* selective medium, MacConkey agar, a *Neisseria*-selective medium (modified Thayer–Martin medium), Sabouraud agar, or Schaedler anaerobe agar. The plates were cultured for 6 days and checked daily. The species were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using MALDI Biotyper software (Bruker Daltonics, Bremen, Germany).

Clinical definitions. *Microbial invasion of the amniotic cavity* was determined based on a positive PCR analysis of *Ureaplasma* species, *M. hominis*, *C. trachomatis* or a combination of these species or positivity for the 16S rRNA gene, aerobic/anaerobic cultivation of the amniotic fluid, or a combination of these parameters. *Intra-amniotic inflammation* was defined as amniotic fluid IL-6 concentrations ≥ 3000 pg/mL[22]. *Intra-amniotic infection* was defined by both microbial invasion of the amniotic cavity and intra-amniotic inflammation. *Sterile intra-amniotic inflammation* was defined as the presence of intra-amniotic inflammation without the concomitant microbial invasion of the amniotic cavity. *Colonization* was defined as the microbial invasion of the amniotic cavity without intra-amniotic inflammation. *Negative amniotic fluid* was defined as the absence of microbial invasion of the amniotic cavity and intra-amniotic inflammation.

Quantification of FcgammaBP in amniotic and cervical fluids. The concentrations of FcgammaBP were assessed in the amniotic fluid and cervical fluid samples using an enzyme-linked immunosorbent assay (ELISA), the Human FCGBP ELISA Kit (LifeSpan BioSciences, Inc., Seattle, WA, USA), according to the manufacturer's instructions. The amniotic fluid and cervical fluid samples were diluted tenfold and 50-fold, respectively. The sensitivity of the kit was 0.117 ng/mL. The absorbance values were read at 450 nm on a Multiskan RC ELISA reader (Thermo Fisher Scientific).

Statistical analyses. The women's demographic and clinical characteristics were compared using the non-parametric Mann–Whitney *U* test for continuous variables and are presented as median values (interquartile range [IQR]). Categorical variables were compared using Fisher's exact test and are presented as a number (%). The normality of the data was tested using the Anderson–Darling test. Because the FcgammaBP concentrations in the amniotic fluid were not normally distributed, the nonparametric Kruskal–Wallis and Mann–Whitney *U* tests were performed for statistical analyses, and the results are presented as a median value (IQR). Spearman's partial correlation analysis was performed to adjust the results for gestational age at sampling. Spearman's correlation was used to assess the relationship between the concentrations of amniotic fluid FcgammaBP and cervical fluid FcgammaBP and gestational age at sampling. Receiver operating characteristic (ROC) curves were constructed to assess the predictive value of amniotic fluid and cervical fluid FcgammaBP for the presence of intra-amniotic infection. Cutoff values were determined based on the highest positive likelihood ratio. Differences were considered significant at $p < 0.05$. All p values were obtained using two-tailed tests, and all statistical analyses were performed using GraphPad Prism, version 8.1.1. for Mac OS X (GraphPad Software, San Diego, CA, USA) or the Statistical Package for Social Sciences (SPSS), version 19.0 for Mac OS X (SPSS Inc., Chicago, IL, USA).

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References

1. Goldenberg, R. L., Culhane, J. F., Iams, J. D. & Romero, R. Epidemiology and causes of preterm birth. *Lancet* **371**, 75–84. https://doi.org/10.1016/S0140-6736(08)60074-4 (2008).
2. Romero, R., Dey, S. K. & Fisher, S. J. Preterm labor: One syndrome, many causes. *Science* **345**, 760–765. https://doi.org/10.1126/science.1251816 (2014).
3. Pacora, P. *et al.* Lactoferrin in intrauterine infection, human parturition, and rupture of fetal membranes. *Am. J. Obstet. Gynecol.* **183**, 904–910. https://doi.org/10.1067/mob.2000.108882 (2000).
4. Maymon, E. *et al.* A role for the 72 kDa gelatinase (MMP-2) and its inhibitor (TIMP-2) in human parturition, premature rupture of membranes and intraamniotic infection. *J. Perinat. Med.* **29**, 308–316. https://doi.org/10.1515/JPM.2001.044 (2001).
5. Espinoza, J. *et al.* Antimicrobial peptides in amniotic fluid: Defensins, calprotectin and bacterial/permeability-increasing protein in patients with microbial invasion of the amniotic cavity, intra-amniotic inflammation, preterm labor and premature rupture of membranes. *J. Matern. Fetal Neonatal Med.* **13**, 2–21. https://doi.org/10.1080/jmf.13.1.2.21 (2003).
6. Jacobsson, B. *et al.* Microbial invasion and cytokine response in amniotic fluid in a Swedish population of women with preterm prelabor rupture of membranes. *Acta Obstet. Gynecol. Scand.* **82**, 423–431 (2003).
7. Kusanovic, J. P. *et al.* Amniotic fluid soluble human leukocyte antigen-G in term and preterm parturition, and intra-amniotic infection/inflammation. *J. Matern. Fetal Neonatal Med.* **22**, 1151–1166. https://doi.org/10.3109/14767050903019684 (2009).
8. Erez, O. *et al.* Differential expression pattern of genes encoding for anti-microbial peptides in the fetal membranes of patients with spontaneous preterm labor and intact membranes and those with preterm prelabor rupture of the membranes. *J. Matern. Fetal Neonatal Med.* **22**, 1103–1115. https://doi.org/10.3109/14767050902994796 (2009).
9. Lee, S. E., Park, I. S., Romero, R. & Yoon, B. H. Amniotic fluid prostaglandin F2 increases even in sterile amniotic fluid and is an independent predictor of impending delivery in preterm premature rupture of membranes. *J. Matern. Fetal Neonatal Med.* **22**, 880–886. https://doi.org/10.1080/14767050902994648 (2009).
10. Pacora, P. *et al.* Amniotic fluid angiopoietin-2 in term and preterm parturition, and intra-amniotic infection/inflammation. *J. Perinat. Med.* **37**, 503–511. https://doi.org/10.1515/JPM.2009.093 (2009).
11. Romero, R. *et al.* Evidence of perturbations of the cytokine network in preterm labor. *Am. J. Obstet. Gynecol.* **213**, 836.e831–836.e818. https://doi.org/10.1016/j.ajog.2015.07.037 (2015).
12. Romero, R. *et al.* Sterile and microbial-associated intra-amniotic inflammation in preterm prelabor rupture of membranes. *J. Matern. Fetal Neonatal Med.* **28**, 1394–1409. https://doi.org/10.3109/14767058.2014.958463 (2015).
13. Musilova, I. *et al.* Intraamniotic inflammation in women with preterm prelabor rupture of membranes. *PLoS ONE* **10**, e0133929. https://doi.org/10.1371/journal.pone.0133929 (2015).
14. Romero, R. *et al.* Prevalence and clinical significance of sterile intra-amniotic inflammation in patients with preterm labor and intact membranes. *Am. J. Reprod. Immunol.* **72**, 458–474. https://doi.org/10.1111/aji.12296 (2014).
15. Romero, R. *et al.* Sterile intra-amniotic inflammation in asymptomatic patients with a sonographic short cervix: Prevalence and clinical significance. *J. Matern. Fetal Neonatal Med.* **28**, 1343–1359. https://doi.org/10.3109/14767058.2014.954243 (2015).
16. Shim, S. S. *et al.* Clinical significance of intra-amniotic inflammation in patients with preterm premature rupture of membranes. *Am. J. Obstet. Gynecol.* **191**, 1339–1345. https://doi.org/10.1016/j.ajog.2004.06.085 (2004).
17. Kim, K. W. *et al.* A rapid matrix metalloproteinase-8 bedside test for the detection of intraamniotic inflammation in women with preterm premature rupture of membranes. *Am. J. Obstet. Gynecol.* **197**(292), e291-295. https://doi.org/10.1016/j.ajog.2007.06.040 (2007).
18. Kacerovsky, M. *et al.* Bedside assessment of amniotic fluid interleukin-6 in preterm prelabor rupture of membranes. *Am. J. Obstet. Gynecol.* **211**(385), e381-389. https://doi.org/10.1016/j.ajog.2014.03.069 (2014).
19. Chaemsaithong, P. *et al.* A point of care test for the determination of amniotic fluid interleukin-6 and the chemokine CXCL-10/IP-10. *J. Matern. Fetal Neonatal Med.* **28**, 1510–1519. https://doi.org/10.3109/14767058.2014.961417 (2015).
20. Chaemsaithong, P. *et al.* A point of care test for interleukin-6 in amniotic fluid in preterm prelabor rupture of membranes: A step toward the early treatment of acute intra-amniotic inflammation/infection. *J. Matern. Fetal Neonatal Med.* **29**, 360–367. https://doi.org/10.3109/14767058.2015.1006621 (2016).
21. Chaemsaithong, P. *et al.* Comparison of rapid MMP-8 and interleukin-6 point-of-care tests to identify intra-amniotic inflammation/infection and impending preterm delivery in patients with preterm labor and intact membranes(). *J. Matern. Fetal Neonatal Med.* **31**, 228–244. https://doi.org/10.1080/14767058.2017.1281904 (2018).
22. Musilova, I. *et al.* Interleukin-6 measured using the automated electrochemiluminescence immunoassay method for the identification of intra-amniotic inflammation in preterm prelabor rupture of membranes. *J. Matern. Fetal Neonatal Med.* https://doi.org/10.1080/14767058.2018.1533947 (2018).
23. Soucy-Giguere, L. *et al.* Intra-amniotic inflammation and child neurodevelopment: A systematic review protocol. *Syst. Rev.* **7**, 12. https://doi.org/10.1186/s13643-018-0683-z (2018).
24. Rodriguez-Trujillo, A. *et al.* Influence of perinatal inflammation on the neurodevelopmental outcome of premature infants. *J. Matern. Fetal Neonatal Med.* **32**, 1069–1077. https://doi.org/10.1080/14767058.2017.1399118 (2019).
25. Archabald, K. L. *et al.* Limiting the exposure of select fetuses to intrauterine infection/inflammation improves short-term neonatal outcomes in preterm premature rupture of membranes. *Fetal Diagn. Ther.* **42**, 99–110. https://doi.org/10.1159/000450997 (2017).

26. Rodriguez-Trujillo, A. *et al.* Gestational age is more important for short-term neonatal outcome than microbial invasion of the amniotic cavity or intra-amniotic inflammation in preterm prelabor rupture of membranes. *Acta Obstet. Gynecol. Scand.* **95**, 926–933. https://doi.org/10.1111/aogs.12905 (2016).
27. Lee, J. *et al.* A new anti-microbial combination prolongs the latency period, reduces acute histologic chorioamnionitis as well as funisitis, and improves neonatal outcomes in preterm PROM. *J. Matern. Fetal Neonatal Med.* **29**, 707–720. https://doi.org/10.3109/14767058.2015.1020293 (2016).
28. Cobo, T. *et al.* Intra-amniotic inflammation predicts microbial invasion of the amniotic cavity but not spontaneous preterm delivery in preterm prelabor membrane rupture. *Acta Obstet. Gynecol. Scand.* **91**, 930–935. https://doi.org/10.1111/j.1600-0412.2012.01427.x (2012).
29. Gravett, M. G. *et al.* Diagnosis of intra-amniotic infection by proteomic profiling and identification of novel biomarkers. *JAMA* **292**, 462–469. https://doi.org/10.1001/jama.292.4.462 (2004).
30. Buhimschi, I. A., Christner, R. & Buhimschi, C. S. Proteomic biomarker analysis of amniotic fluid for identification of intra-amniotic inflammation. *BJOG* **112**, 173–181. https://doi.org/10.1111/j.1471-0528.2004.00340.x (2005).
31. Klein, L. L. *et al.* Detection of intra-amniotic infection in a rabbit model by proteomics-based amniotic fluid analysis. *Am. J. Obstet. Gynecol.* **193**, 1302–1306. https://doi.org/10.1016/j.ajog.2005.06.017 (2005).
32. Ruetschi, U. *et al.* Proteomic analysis using protein chips to detect biomarkers in cervical and amniotic fluid in women with intra-amniotic inflammation. *J. Proteome Res.* **4**, 2236–2242. https://doi.org/10.1021/pr050139e (2005).
33. Gravett, M. G. *et al.* Proteomic analysis of cervical-vaginal fluid: Identification of novel biomarkers for detection of intra-amniotic infection. *J. Proteome Res.* **6**, 89–96. https://doi.org/10.1021/pr060149v (2007).
34. Romero, R. *et al.* Proteomic analysis of amniotic fluid to identify women with preterm labor and intra-amniotic inflammation/infection: The use of a novel computational method to analyze mass spectrometric profiling. *J. Matern. Fetal Neonatal Med.* **21**, 367–388. https://doi.org/10.1080/14767050802045848 (2008).
35. Romero, R. *et al.* Isobaric labeling and tandem mass spectrometry: A novel approach for profiling and quantifying proteins differentially expressed in amniotic fluid in preterm labor with and without intra-amniotic infection/inflammation. *J. Matern. Fetal Neonatal Med.* **23**, 261–280. https://doi.org/10.3109/14767050903067386 (2010).
36. Tambor, V. *et al.* Amniotic fluid cathelicidin in PPROM pregnancies: From proteomic discovery to assessing its potential in inflammatory complications diagnosis. *PLoS ONE* **7**, e41164. https://doi.org/10.1371/journal.pone.0041164 (2012).
37. Tambor, V. *et al.* Potential peripartum markers of infectious-inflammatory complications in spontaneous preterm birth. *Biomed. Res. Int.* **2015**, 343501. https://doi.org/10.1155/2015/343501 (2015).
38. Cobo, T. *et al.* Cervical alpha-actinin-4 is upregulated in women with threatened preterm labor and microbial invasion of the amniotic cavity. *Fetal Diagn. Ther.* **44**, 36–43. https://doi.org/10.1159/000478259 (2018).
39. Govia, R. N. M. *et al.* Amniotic fluid proteomic signatures of cervical insufficiency and their association with length of latency. *Am. J. Reprod. Immunol.* **80**, e13030. https://doi.org/10.1111/aji.13030 (2018).
40. Zhao, M. *et al.* A comparative proteomics analysis of five body fluids: Plasma, urine, cerebrospinal fluid, amniotic fluid, and saliva. *Proteomics Clin. Appl.* **12**, e1800008. https://doi.org/10.1002/prca.201800008 (2018).
41. Wang, Y. *et al.* Placental protein 14 as a potential biomarker for diagnosis of preterm premature rupture of membranes. *Mol. Med. Rep.* **18**, 113–122. https://doi.org/10.3892/mmr.2018.8967 (2018).
42. Liu, X., Song, Y., Guo, Z., Sun, W. & Liu, J. A comprehensive profile and inter-individual variations analysis of the human normal amniotic fluid proteome. *J. Proteomics* **192**, 1–9. https://doi.org/10.1016/j.jprot.2018.04.023 (2019).
43. Hallingstrom, M. *et al.* Proteomic analysis of early mid-trimester amniotic fluid does not predict spontaneous preterm delivery. *PLoS ONE* **11**, e0155164. https://doi.org/10.1371/journal.pone.0155164 (2016).
44. An, D. *et al.* Identification of PCSK9 as a novel serum biomarker for the prenatal diagnosis of neural tube defects using iTRAQ quantitative proteomics. *Sci. Rep.* **5**, 17559. https://doi.org/10.1038/srep17559 (2015).
45. Dixon, C. L. *et al.* Amniotic fluid exosome proteomic profile exhibits unique pathways of term and preterm labor. *Endocrinology* **159**, 2229–2240. https://doi.org/10.1210/en.2018-00073 (2018).
46. Liu, Z., Yuan, Z. & Zhao, Q. SELDI-TOF-MS proteomic profiling of serum, urine, and amniotic fluid in neural tube defects. *PLoS ONE* **9**, e103276. https://doi.org/10.1371/journal.pone.0103276 (2014).
47. Fotopoulou, C. *et al.* Proteomic analysis of midtrimester amniotic fluid to identify novel biomarkers for preterm delivery. *J. Matern. Fetal Neonatal Med.* **25**, 2488–2493. https://doi.org/10.3109/14767058.2012.712565 (2012).
48. Oh, K. J. *et al.* Proteomic biomarkers in second trimester amniotic fluid that identify women who are destined to develop preeclampsia. *Reprod. Sci.* **19**, 694–703. https://doi.org/10.1177/1933719112438441 (2012).
49. Tsangaris, G. T. *et al.* Application of proteomics for the identification of biomarkers in amniotic fluid: Are we ready to provide a reliable prediction?. *EPMA J.* **2**, 149–155. https://doi.org/10.1007/s13167-011-0083-0 (2011).
50. Kolialexi, A., Tounta, G., Mavrou, A. & Tsangaris, G. T. Proteomic analysis of amniotic fluid for the diagnosis of fetal aneuploidies. *Expert Rev. Proteomics* **8**, 175–185. https://doi.org/10.1586/epr.10.112 (2011).
51. Park, J. *et al.* Comparative proteomic analysis of human amniotic fluid supernatants with Down syndrome using mass spectrometry. *J. Microbiol. Biotechnol.* **20**, 959–967 (2010).
52. Lee, J. *et al.* Identification and characterization of proteins in amniotic fluid that are differentially expressed before and after antenatal corticosteroid administration. *Am. J. Obstet. Gynecol.* **202**, 388.e381–388.e388. https://doi.org/10.1016/j.ajog.2010.01.056 (2010).
53. Park, J. S. *et al.* Identification of proteomic biomarkers of preeclampsia in amniotic fluid using SELDI-TOF mass spectrometry. *Reprod. Sci.* **15**, 457–468. https://doi.org/10.1177/1933719108316909 (2008).
54. Mavrou, A. *et al.* Proteomic analysis of amniotic fluid in pregnancies with Turner syndrome fetuses. *J. Proteome Res.* **7**, 1862–1866. https://doi.org/10.1021/pr700588u (2008).
55. Kolialexi, A., Mavrou, A. & Tsangaris, G. T. Proteomic analysis of human reproductive fluids. *Proteomics Clin. Appl.* **1**, 853–860. https://doi.org/10.1002/prca.200700040 (2007).
56. Michaels, J. E. *et al.* Comprehensive proteomic analysis of the human amniotic fluid proteome: Gestational age-dependent changes. *J. Proteome Res.* **6**, 1277–1285. https://doi.org/10.1021/pr060543t (2007).
57. Queloz, P. A. *et al.* Proteomic analyses of amniotic fluid: Potential applications in health and diseases. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **850**, 336–342. https://doi.org/10.1016/j.jchromb.2006.12.006 (2007).
58. Tsangaris, G. T. *et al.* The normal human amniotic fluid supernatant proteome. *In Vivo* **20**, 479–490 (2006).
59. Michel, P. E. *et al.* Proteome analysis of human plasma and amniotic fluid by Off-Gel isoelectric focusing followed by nano-LC-MS/MS. *Electrophoresis* **27**, 1169–1181. https://doi.org/10.1002/elps.200500680 (2006).
60. Park, S. J. *et al.* Proteome analysis of human amnion and amniotic fluid by two-dimensional electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Proteomics* **6**, 349–363. https://doi.org/10.1002/pmic.200500084 (2006).
61. Bujold, E. *et al.* Proteomic profiling of amniotic fluid in preterm labor using two-dimensional liquid separation and mass spectrometry. *J. Matern. Fetal Neonatal Med.* **21**, 697–713. https://doi.org/10.1080/14767050802053289 (2008).
62. Harada, N. *et al.* Human IgGFc binding protein (FcgammaBP) in colonic epithelial cells exhibits mucin-like structure. *J. Biol. Chem.* **272**, 15232–15241. https://doi.org/10.1074/jbc.272.24.15232 (1997).

63. O'Donovan, N. *et al*. Differential expression of IgG Fc binding protein (FcgammaBP) in human normal thyroid tissue, thyroid adenomas and thyroid carcinomas. *J. Endocrinol.* **174**, 517–524 (2002).
64. Kobayashi, K. *et al.* Detection of Fcgamma binding protein antigen in human sera and its relation with autoimmune diseases. *Immunol. Lett.* **79**, 229–235 (2001).
65. Kobayashi, K., Blaser, M. J. & Brown, W. R. Identification of a unique IgG Fc binding site in human intestinal epithelium. *J. Immunol.* **143**, 2567–2574 (1989).
66. Kobayashi, K., Hamada, Y., Blaser, M. J. & Brown, W. R. The molecular configuration and ultrastructural locations of an IgG Fc binding site in human colonic epithelium. *J. Immunol.* **146**, 68–74 (1991).
67. Racicot, K. *et al.* Viral infection of the pregnant cervix predisposes to ascending bacterial infection. *J. Immunol.* **191**, 934–941. https://doi.org/10.4049/jimmunol.1300661 (2013).
68. Hein, M., Valore, E. V., Helmig, R. B., Uldbjerg, N. & Ganz, T. Antimicrobial factors in the cervical mucus plug. *Am. J. Obstet. Gynecol.* **187**, 137–144 (2002).
69. Hansen, L. K. *et al*. The cervical mucus plug inhibits, but does not block, the passage of ascending bacteria from the vagina during pregnancy. *Acta Obstet. Gynecol. Scand.* **93**, 102–108. https://doi.org/10.1111/aogs.12296 (2014).
70. Frew, L. *et al.* Human cathelicidin production by the cervix. *PLoS ONE* **9**, e103434. https://doi.org/10.1371/journal.pone.0103434 (2014).
71. Musilova, I. *et al.* Cervical fluid interleukin 6 and intra-amniotic complications of preterm prelabor rupture of membranes. *J. Matern. Fetal Neonatal Med.* **31**, 827–836. https://doi.org/10.1080/14767058.2017.1297792 (2018).
72. Winram, S. B., Jonas, M., Chi, E. & Rubens, C. E. Characterization of group B streptococcal invasion of human chorion and amnion epithelial cells in vitro. *Infect. Immun.* **66**, 4932–4941 (1998).
73. Kjaergaard, N. *et al.* Antibacterial properties of human amnion and chorion in vitro. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **94**, 224–229 (2001).
74. Kjaergaard, N. *et al.* Chorioamniotic membranes constitute a competent barrier to group b streptococcus in vitro. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **83**, 165–169 (1999).
75. Azzarelli, B. & Lafuze, J. Amniotic basement membrane: A barrier to neutrophil invasion. *Am. J. Obstet. Gynecol.* **156**, 1130–1136 (1987).
76. Turley, E., Tretiak, M. & Tanguay, K. Effect of glycosaminoglycans and enzymes on the integrity of human placental amnion as a barrier to cell invasion. *J. Natl. Cancer Inst.* **78**, 787–795 (1987).
77. Kim, Y. M. *et al.* Toll-like receptor-2 and -4 in the chorioamniotic membranes in spontaneous labor at term and in preterm parturition that are associated with chorioamnionitis. *Am. J. Obstet. Gynecol.* **191**, 1346–1355. https://doi.org/10.1016/j.ajog.2004.07.009 (2004).
78. Abreu, M. T. Toll-like receptor signalling in the intestinal epithelium: How bacterial recognition shapes intestinal function. *Nat. Rev. Immunol.* **10**, 131–144. https://doi.org/10.1038/nri2707 (2010).
79. Hayati, A. R., Mohamed, A. E. & Tan, G. C. An immunohistochemical study of Toll-like receptors 2 and 4 in placenta with and without infection. *Malays. J. Pathol.* **32**, 13–19 (2010).
80. van Baaren, G. J. *et al.* Predictive value of cervical length measurement and fibronectin testing in threatened preterm labor. *Obstet. Gynecol.* **123**, 1185–1192. https://doi.org/10.1097/aog.0000000000000229 (2014).
81. Fouhy, F. *et al*. The effects of freezing on faecal microbiota as determined using MiSeq sequencing and culture-based investigations. *PLoS ONE* **10**, e0119355. https://doi.org/10.1371/journal.pone.0119355 (2015).
82. Greisen, K., Loeffelholz, M., Purohit, A. & Leong, D. PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. *J. Clin. Microbiol.* **32**, 335–351 (1994).

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Competing interests

The authors declare no competing interests.

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8.2.3 Specific aim II-A

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REVIEW ARTICLE

A rodent model of intra-amniotic inflammation/infection, induced by the administration of inflammatory agent in a gestational sac, associated with preterm delivery: a systematic review

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ABSTRACT

Background: Rodents are the most commonly used animals in the study of spontaneous preterm delivery (PTD). Intra-amniotic inflammation/infection is a frequent and important cause of PTD. Intraperitoneal and intrauterine administrations of inflammatory agents are traditional methods to establish a rodent model of PTD associated with inflammation and infection. The intra-amniotic administration of inflammatory or infectious triggering agents to rodents can be useful to study not only intra-amniotic inflammatory response but also PTD associated with intra-amniotic inflammation/infection.

Objective: This systematic review aimed mainly to assess and analyze all described methods of intra-amniotic administration of infectious and/or inflammatory agents to create a rodent model of intra-amniotic inflammation associated with PTD.

Methods: A literature search through two electronic databases from their earliest entries to February 2019 was performed. The selection criteria were as follows: (1) rodents as model animals, (2) a model of intra-amniotic inflammation/infection associated with PTD, and (3) intra-amniotic administration of triggering agents. Data extraction included specification of the study (author and year of publication), characteristics of study animals (species, strain, and number of animals), characteristics of intervention (timing and used technique), substance used for induction of intra-amniotic inflammation/infection, and outcome assessment.

Results: The search identified a total of 4673 articles, of which 118 were selected for full-text reading, but only 13 studies were included in the review. Intra-amniotic administration was used only in the articles that were published beyond 2004. Two different approaches were identified: (1) open surgery with direct puncture of the amniotic sacs and (2) transabdominal ultrasound-guided puncture of the gestational sacs. Live microorganisms (*Ureaplasma parvum*), bacterial products (extracellular membrane vesicles), and pathogen-associated (lipopolysaccharide) and damage-associated molecular patterns (high mobility group box-1, S100B, and surfactant protein A) were used to simulate intra-amniotic inflammation/infection. Differences in the effect on intra-amniotic inflammation/infection associated with PTD in the mouse model were identified among triggering agents. Intra-amniotic application of lipopolysaccharide in the rat model caused intra-amniotic inflammation, but it did not lead to PTD.

Conclusion: The intra-amniotic administration of the triggering agents can be used to study intra-amniotic inflammatory response and intra-amniotic inflammation/infection in the rodents model.

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Mouse; rat; pathogen-associated molecular pattern; damage-associated molecular pattern; lipopolysaccharide; sterile inflammation

Introduction

Spontaneous preterm delivery (PTD) is the leading cause of perinatal morbidity and mortality [1]. Given the immaturity of multiple organ systems, children born prematurely are at risk of short- and long-term complications [2]. The PTD rate has not declined in the past few decades, despite the effort in prevention and treatment [3]. This failure to reduce the PTD rate is likely due to the knowledge gaps in our understanding of PTD [3]. Even if many risk factors of PTD are identified, the underlying biological mechanisms and pathophysiology pathways remain elusive [4]. The lack

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Supplemental data for this article can be accessed here.

of knowledge presents a major challenge that needs to be addressed by further research in order to improve the incidence and outcomes of PTD.

Animal models play an essential role in PTD research, because human studies have, for understandable reasons, many limitations [5]. Currently, there is no one ideal animal model for simulation of all pathways of human PTD. Multiple mechanisms including intra-amniotic inflammation/infection, uteroplacental ischemia or hemorrhage, uterine overdistension, stress, or other immunologically mediated processes can initiate PTD in humans [1]. Thus, various animal species have been used in research, all of them with specific ability to mimic processes in clinical cases of human PTD and with important differences in terms of reproductive biology [6].

Currently, rodents are the most frequently used animals to model PTD (i.e. mice and rats) because they are easy to house and treat. In addition, they are relatively inexpensive [6]. Rodents may be also useful animals to model PTD associated with inflammation or infection, which is the most common subset of PTD and responsible for at least 25–40% of PTD cases [1]. A traditional way to create a rodent model of PTD associated with inflammation or infection is by intraperitoneal or intrauterine administration of inflammatory or infectious agents [5]. Their intra-amniotic administration represents the less common way [5]. However, the direct intra-amniotic administration of triggering agents induces intra-amniotic inflammatory response leading to the development of either sterile intra-amniotic inflammation or intra-amniotic infection. This represents a clinically relevant issue as both forms of intra-amniotic inflammation are frequently revealed in both phenotypes of PTD – spontaneous preterm delivery with intact membranes and preterm prelabor rupture of the membranes [7–9].

Therefore, this systematic review aimed to assess and analyze all methods of intra-amniotic administration of inflammatory and infectious agents to create a rodent model of intra-amniotic inflammation/infection associated with PTD.

Material and methods

Search strategy

This systematic review was conducted in accordance with PRISMA guidelines (supporting information S1) [10]. We searched for studies that employ intra-amniotic administration of infectious or inflammatory agents to establish a rodent model of intra-amniotic inflammation/infection associated with PTD. The search was conducted in two electronic databases (PubMed and Scopus) from their earliest entries to February 2019. The following search terms and synonyms were used, adapted according to each database: (animal OR animals OR rat OR rats OR mouse OR mice OR hamster OR hamsters OR rodent OR rodents) AND (preterm birth OR preterm delivery OR preterm labor OR preterm labor) AND (intra-amniotic OR intra-amniotic OR intra-amniale OR ultrasound-guided OR ia OR injection OR application OR administration). Search for unpublished studies was not performed. All identified citations were downloaded into the Rayyan web application tool for systematic reviews [11], and duplicates were excluded. Using the Rayyan application, the titles and abstracts of unique citations were screened independently by two reviewers (JS, BJ), potentially relevant studies were selected for full-text reading, and those that fulfilled the selection criteria were included in the review.

Selection criteria for studies

Type of studies: Studies that used a rodent model of intra-amniotic inflammation/infection associated with PTD which was initiated by intra-amniotic administration of inflammatory or infectious agents were considered eligible. Primary experimental case–control studies were included.

Type of outcomes: PTD, intra-amniotic infection, sterile intra-amniotic inflammation, intra-uterine inflammation (histological chorioamnionitis), and inflammatory complications.

The selection was restricted to studies published in English.

Exclusion criteria for studies

Studies were excluded if any of the following applied: (1) human or *in vitro* studies, (2) animal models other than rodents, (3) models of PTD other than those associated with inflammation or infection, (4) routes of administration of infectious or inflammatory agents other than administration into the amniotic sac (intraperitoneal, intrauterine, and intra-cervical administration), and (5) rodent models to study other conditions or diseases without relation to PTD.

Data extraction

The following data were extracted from each article included in this review: author, year of publication,

study methodology, information about the study animals and their type (number, species, strain), information about the timing of intervention, description of technique of intra-amniotic administration, information about the infectious or inflammatory agent used, and outcomes of the study.

Quality assessment

For quality assessment of identified studies, the checklist of the CAMARADES group was adjusted [12]. The criteria of quality assessment were as follows: (1) provided description of used animals (species, strain, and origin of animal), (2) calculation of sample size (3), description of randomized allocation to the treatment or control group; (4) description of the intervention (technique for intra-amniotic application, number of amniotic sacs injected), and (5) thorough description of infectious or inflammatory agent. One point was assigned per item, and the final quality score was the sum of particular items.

Results

The process of identification, selection, and inclusion of studies is shown in Figure 1. The initial search in both databases identified a total of 4673 unduplicated articles, 4555 of which were excluded after screening titles and abstracts, and 118 potentially relevant citations were selected for full-text reading. The high number of citations for full-text reading was selected due to the fact that the description of used animal model was not given in many abstracts. A total of 105 studies were excluded due to reasons shown in Figure 1; finally, 13 studies that fulfilled our selection criteria were included in the review) [13–25].

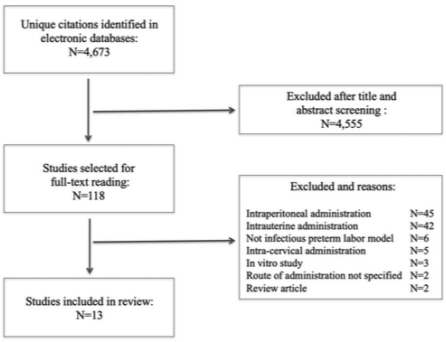

Figure 1. Flowchart of the process of identification and selection of studies.

The main characteristics of the studies are listed in Table 1. All 13 studies were published between 2004 and 2019; 38% (5/13) of the studies were published in 2018. Sprague–Dawley rats were used in 38% (5/13) of the studies [15,16,19,20,23]. Moreover, 62% (8/13) used different strains of mice [13,14,17,18,21,22,24,25]. The most common mouse strain was C57BL/6 (6/13) [17,18,21,22,24,25]. IRC mice [13] and CD-1 mice [14] were used in one study each. No other rodent animals than rats and mice were used in the included articles.

Two distinct ways of administration of infectious or inflammatory agents into the amniotic sacs were used. Five mice studies used transabdominal ultrasound-guided intra-amniotic administration of the agent [17,21,22,24,25]. Laparotomy with visualization of the uterine horns followed by direct puncture of the amniotic sacs was the second identified route of administration of agents. Laparotomy was used in three mice studies [13,14,18] and in all studies with rats [15,16,19,20,23].

Infectious or inflammatory agents used in the studies were classified as follows: (1) live microorganisms, *Ureaplasma parvum;* (2) bacterial products, extracellular membrane vesicles; (3) pathogen-associated molecular patterns (PAMPs), lipopolysaccharide (LPS); and (4) damage-associated molecular patterns (DAMPs), high mobility group box-1 (HMGB-1), S100-B, and surfactant protein A .

U. parvum was the only live microorganism used in the included studies. Intra-amniotic application of 5000 colony forming units (CFU) of *U. parvum* to mice did not lead to PTD in CD-1 mice [14].

Extracellular membrane vesicles from group B *Streptococcus* strain A909 were used in one study. Extracellular membrane vesicles injected at a dose of 5 and 10 μg per sac in C57BL/6 mice caused preterm delivery in 55% and 68% of cases, respectively [18].

LPS was the most common triggering agent. LPS serotype O55:B55 was used in one study [19], while serotype O111:B4 was utilized in seven studies [15,16,20–24]. In C57BL/6 mice, intra-amniotic application of 100 ng of LPS O111:B4 per sac on 16.5 day-post-conception (dpc) caused PTD in 80%–88% of the animals [21,22,24]. Mice delivered approximately 20 h (median) after the procedure [22,24]. Lower dosage of 50 ng of LPS (O111:B4) did not cause any preterm delivery [22]. In Sprague–Dawley rats, intra-amniotic LPS O111:B4 at a dose of 4 μg and 10 μg per sac did not cause PTD [15,16,20,23]. In addition, LPS O55:B55 at a dose of 10 μg per sac did not cause any PTD [19]. The application was used for modeling

Table 1. Main characteristics of studies of rodent intra-amniotic inflammation/infection model associated with preterm delivery initiated by intra-amniotic application.

Study	Year	Animal model	Total number of animals (n)	Agent for intra-amniotic inflammation/ infection induction	Time of induction (days post coitum)	Procedure of administration	Preterm delivery rate[a]	Interval between procedure and PTD (hours)	Neonatal mortality rate at birth or shortly after birth (%)	Comment
Condon et al. [13]	2004	ICR mice	$n = 17$	3 μg surfactant protein A per sac	15 dpc	Laparotomy injection into all sacs in the right uterine horn	14/17 (82.4%)	6–24 h	no data	PTD of the fetuses from the injected (right) uterine horn, reabsorption of the fetuses from the left horn
Normann et al. [14]	2009	CD-1 mice	$n = 15$	*Ureaplasma parvum* - 5000 CFU per sac	13.5 dpc	Laparotomy injection into all amniotic sacs	0/15 (0%)	no PTD	no effect on postnatal survival	*UP* caused intra-amniotic infection and a mild fetal lung inflammation
Jantzie et al. [15]	2015	Sprague–Dawley rats	no data	4 μg of LPS per sac (LPS O111:B4)	18 dpc	laparotomy injection into all sacs + clamping of uterine arteries for 60 min	0%	no PTD	approx. 40%	animal model of encephalopathy of prematurity
Maxwell et al. [16]	2015	Sprague–Dawley rats	$n = 9$	4 μg of LPS per sac (LPS O111:B4)	18 dpc	Laparotomy injection into all sacs + clamping of uterine arteries for 60 min	no data animals sacrificed at 19 dpc or 21 dpc	no PTD animals sacrificed at 19 dpc or 21 dpc	15% at 19 dpc and 45% at 21 dpc	animal model of chorioamnionitis and neonatal brain injury
Gomez-Lopez et al. [17]	2016	C57BL/6 mice	$n = 7$	9 ng HMGB1 per sac	14.5 dpc	Ultrasound-guided intraamniotic application of all sacs	4/7 (57%)	100.8 ± 7.2 h[b]	15% (in 1 week 61%)	
Surve et al. [18]	2016	C57BL6 mice	no data	extracellular membrane vesicles from GBS strain A909 – 5 μg and 10 μg per sac (two groups)	14.5 dpc	Laparotomy injection into all sacs	55% (for 5 μg) 68% (for 10 μg)	delivered within 48 h	36% (for 5 μg) 29% (for 10 μg)	
Cookson et al. [19]	2018	Sprague–Dawley rats	$n = 6$	10 μg of LPS per sac (LPS O55:B55)	20 dpc	Laparotomy number of injected sacs was not specified	0%	no PTD (delivered by cesarean section)	no data	animal model of chorioamnionitis
Dedja et al. [20]	2018	Sprague–Dawley rats	$n = 4$	10 μg of LPS per sac (LPS O111:B4)	20 dpc	Laparotomy injection into all sacs	0%	no PTD	32%	animal model of chorioamnionitis and neonatal lung injury
Garcia-Flores et al. [21]	2018	C57BL/6 mice	$n = 8$	100 ng of LPS per sac (LPS O111:B4)	16.5 dpc	Ultrasound-guided intra-amniotic injection into all sacs	7/8 (88%)	no data	exceeded 85% (in 1 week 100%)	
Gomez-Lopez et al. [22]	2018	C57BL/6 mice	$n = 3 + 10$	50 ng and 100 ng of LPS per sac (O111:B4) (two groups)	16.5 dpc	Ultrasound-guided intra-amniotic injection into all sacs	3/0 (0%) (for 50 ng) 8/10 (80%) (for 100 ng)	no PTD (for 50 ng) 20.4 h[c] (for 100 ng)	no data	
Jantzie et al. [23]	2018	Sprague–Dawley rats	no data	4 μg of LPS per sac (LPS O111:B4)	18 dpc	Laparotomy injection of all sacs + clamping of uterine arteries for 60 min	0%	no PTD	no data	animal model of encephalopathy of prematurity

(*continued*)

Table 1. Continued.

Study	Year	Animal model	Total number of animals (n)	Agent for intra-amniotic inflammation/ infection induction	Time of induction (days post coitum)	Procedure of administration	Preterm delivery rate[a]	Interval between procedure and PTD (hours)	Neonatal mortality rate at birth or shortly after birth (%)	Comment
Faro et al. [24]	2019	C57BL/6 mice	$n = 10$	100 ng of LPS per sac (LPS O111:B4)	16.5 dpc	Ultrasound-guided intraamniotic application of all sacs	8/10 (80%)	20 h[c]	90,70%	
Gomez-Lopez et al. [25]	2019	C57BL/6 mice	$n = 10$	60 ng of S100B per sac	16.5 dpc	Ultrasound-guided intra-amniotic injection into all sacs	5/10 (50%)	approx. 24 h[c]	60%	

[a]Number of animals delivered preterm / number of used animals; (%) percentage of animals delivered preterm.
[b]Mean ± standard deviation.
[c]Median.
CFU: colony-forming units; dpc: days post coitum; GBS: group B Streptococcus; HMGB-1: high mobility group box-1; LPS: lipopolysaccharide; PTD: preterm delivery; S100B – S100 calcium-binding protein B; UP: *Ureaplasma parvum*.

encephalopathy of prematurity and neonatal lung injury [15,16,23].

The fourth group of the triggering agents consisted of DAMPs that induced sterile intra-amniotic inflammation. In one study, HMGB1 protein at a dose of 9 ng per sac caused PTD in 57% of cases of C57BL/6 mice [17]. Similarly, protein S100B at a dose of 60 ng per sac caused PTD in 50% of the cases of C57BL/6 mice [25]. The application of 3 μg of surfactant protein A per sac in 15 dpc caused sterile intra-amniotic inflammation and PTD in 82% of cases of IRC mice [13].

Quality assessment

The quality assessment of the included studies is listed in Supporting Information S2. The median number of study quality checklist items scored was 3 of 5. The animal model used in the study was described in all articles, including the rodent strain and its origin. Only one study (8%) described the sample size calculation. Randomized allocation to treatment group was reported 2 of 13 studies (16%). Of included studies, all except one (92%) described in detail the intervention technique. Proper characterization of triggering agent was described in all included studies.

Discussion

Rodent animal models are essential components of current research on the field of PTD. Herein, we analyzed evidence regarding the intra-amniotic administration of triggering agents in rodents to model intra-amniotic inflammation/infection associated with PTD.

The key findings were as follows: (1) intra-amniotic administration of agents to model intra-amniotic inflammation/infection associated with PTD has been used since 2004; (2) the published approaches to administer triggering agents into the amniotic sacs include open surgery with direct puncture and transabdominal ultrasound-guided administration; (3) four kinds of triggering agents were used: i) live microorganisms, ii) bacterial products, iii) PAMPs, and iv) DAMPs; (4) LPS was the most commonly used triggering agent; (5) *U. parvum* was the only live microorganism used; and (6) HMGB1, S100B, and surfactant protein A were DAMPs used to model sterile intra-amniotic inflammation.

The rodent model of inflammatory PTD initiated by intraperitoneal or intrauterine administration of endotoxins is used in research for decades [6]. However, the intra-amniotic administration of triggering agents

started to be used only recently. Our search (even though there was no time restriction) identified only the studies published after 2004, with majority of studies published in 2018. The possible explanations are the following: (i) a change of researchers' view on the importance of intra-amniotic inflammatory response in the research of intra-amniotic inflammation/infection associated with PTD, and (ii) better availability of high-frequency ultrasound devices that made the intra-amniotic administration of triggering agents under ultrasound guidance possible.

The majority of the included studies used minilaparotomy to establish access to the pregnant uterus. During the procedure, a rodent was anesthetized and a short (usually 1–3 cm) midline incision was made in the lower abdomen. The rodent uterus is bicornuate, and the sacs with fetuses are arranged in a "beads-on-a-string" pattern. One or both horns were exposed, and the individual fetal sacs were directly punctured usually using a 31 G needle. After the procedure, the abdominal wall was closed using sutures. Laparotomy is an invasive procedure and has some drawbacks. Pain along with surgical stress can result in a major endocrine response influencing function of many organs [26]. Laparotomy itself can cause PTD among sham controls and therefore can confound the results [27].

High-frequency ultrasound devices have recently become available for small laboratory animal imaging. This high-resolution technology enables reliable guidance of the needle passage through an intact abdominal wall. Rinaldi et al. presented the method of transabdominal ultrasound-guided puncture in the uterine cavity of mice [28]. Of the included studies, only one research group took advantage of a high-frequency ultrasound device to guide the transabdominal administration into amniotic sacs of mice [17,21,22,24,25]. During the procedure, anesthetized animals were positioned on a pad. The ultrasound probe and syringe with an agent were stabilized by a mechanical holder. After fur removal, amniotic sacs were transabdominally punctured by 30 G needle under ultrasound guidance. This approach is less invasive than direct puncture of amniotic sacs from minilaparotomy. However, the ultrasound-guided approach requires both high-quality ultrasound device and a well-trained operator.

The use of transabdominal ultrasound-guided administration has been reported only in mice but not in rats. In our experience, thicker rat skin impedes the needle passage through their abdominal wall. On the contrary, Serriere et al. showed that transabdominal ultrasound-guided aspiration of amniotic fluid was possible even in rats [29]. Therefore, rats can be used for modeling of intra-amniotic inflammation/infection associated with PTD with caution.

In humans, intra-amniotic inflammation has two subtypes: (i) intra-amniotic infection (an elevation of inflammatory mediators in amniotic fluid along with the presence of microorganisms in amniotic fluid), and (ii) sterile intra-amniotic inflammation (an elevation of inflammatory mediators with the absence of microorganisms in amniotic fluid) [7,9]. For animal modeling, intra-amniotic infection can be triggered by live microorganisms, their components, or PAMPs. PAMPs are diverse sets of microbial molecules that share a number of different recognizable biochemical features that trigger an immune response [30]. LPS, a component of the cell wall of gram-negative bacteria, is the most frequently used PAMP to induce inflammatory delivery among animal models. LPS has different serotypes that activate distinct inflammatory pathways causing various maternal and neonatal responses [31]. Except for one study, the included LPS studies used LPS serotype O111:B4. Intra-amniotic administration of LPS O111:B4 to C57BL/6 mice caused PTD in 80%-88% of the cases. The remaining mice delivered at term [21,22,24]. In studies with intraperitoneal and intrauterine administrations, almost all animals delivered preterm [32,33]. It is likely that the intensity of intra-amniotic inflammation triggered by the intra-amniotic administration of LPS was not strong enough to cause PTD in all animals. However, the exposed pups suffered from severe mortality regardless of preterm or term delivery. The advantage of the intra-amniotic administration of LPS is the absence of signs of systematic involvement and changes in body temperature in pregnant mice [22]. This scenario mimics a clinical situation in pregnant women.

The intra-amniotic administration of LPS to Sprague–Dawley rats did not cause PTD among animals in five included studies regardless of the dose or serotype of LPS used. This might suggest that Sprague–Dawley rats were not as sensitive to LPS as C57BL/6 mice. On the contrary, intra-amniotic administration of LPS in Sprague–Dawley rats caused histologic chorioamnionitis [15,16,19,20,23].

Genital mycoplasmas are the most frequent microorganisms diagnosed in the amniotic cavity of women with PTD [34,35]. Therefore, their use to model intra-amniotic infection is more clinically relevant than LPS-based studies. In addition to their effect on triggering intra-amniotic infection, *Ureaplasma* spp. can play a role in the development of bronchopulmonary

dysplasia in preterm newborns [36,37]. Interestingly, in a study by Norman et al., intra-amniotic administration of live *U. parvum* did not cause PTD in the CD-1 mouse model [14]. The absence of PTD induction by *U. parvum* in this study can be the consequence of bypassing the process of ascending infection due to direct intra-amniotic administration. Other possible explanation is that the serovar of *U. parvum* used in this study lacked the capacity to induce PTD. However, exposed pups suffered from mild postnatal inflammation and worsened oxygen-induced lung injury, which demonstrate the significance of intra-amniotic infection [14].

Intra-amniotic inflammation without detectable microorganisms in the amniotic fluid is called sterile intra-amniotic inflammation, and its rate in women with PTD exceeds that of intra-amniotic infection [7]. In the absence of bacterial products, DAMPs are considered to trigger the inflammatory process [30]. These molecules are usually endogenous constituents of the cell and are released from the cell in response to cellular injury [38]. The initial moment of sterile intra-amniotic inflammation is unknown, and the process can be started by tissue ischemia, senescence, or oxidative stress [39,40]. Three kinds of DAMPs were used in the included studies; namely, HMGB1, S100B, and surfactant protein A [13,17,25]. HMGB1 and S100B were elevated in the amniotic fluid of women with sterile intra-amniotic inflammation [7,41,42]. In C57BL/6 mice, intra-amniotic administration of HMGB1 and S100B caused a similar rate of PTD (approximately 50%). Interestingly, intraperitoneal application of HMGB1 to C57BL/6 mice did not cause PTD [17]. These findings provide evidence that DAMPs can induce PTD associated with sterile intra-amniotic inflammation and probably mimic similar situation among humans.

The main limitation of this systematic review is its focus on rodent models, although other animals (e.g. sheep) have been used for intra-amniotic administration of triggering agents to simulate intra-amniotic inflammation/infection associated with PTD [6]. The intra-amniotic administration of triggering agents to rodents is relatively new compared with other well-established animal models. An additional limitation is that we only used two databases for the initial search and excluded the articles published in other languages than English. Finally, there is heterogeneity of the outcomes of included studies as well as in the used strains of mice and rats.

In conclusion, the intra-amniotic administration of the triggering agents can be used to study intra-amniotic inflammatory response and intra-amniotic inflammation/infection in the rodents model.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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References

[1] Goldenberg RL, Culhane JF, Iams JD, et al. Epidemiology and causes of preterm birth. The Lancet. 2008;371(9606):75–84.
[2] Rubens CE, Sadovsky Y, Muglia L, et al. Prevention of preterm birth: harnessing science to address the global epidemic. Sci Transl Med. 2014;6(262):262sr5–262sr5.
[3] Menon R, Williams SM, Lamont RF. Research to achieve a reduction in the global rate of preterm birth needs attention: preface to the special issue by the preterm Birth International Collaborative (PREBIC). Placenta. 2019;(79):1–2.
[4] Yamey G, Horvath H, Schmidt L, et al. Reducing the global burden of Preterm Birth through knowledge transfer and exchange: a research agenda for engaging effectively with policymakers. Reprod Health. 2016;13(1):26.
[5] Elovitz MA, Mrinalini C. Animal models of preterm birth. Trends Endocrinol Metab. 2004;15(10):479–487.
[6] Nielsen BW, Bonney EA, Pearce BD, et al. Preterm Birth International C. A cross-species analysis of animal models for the investigation of preterm birth mechanisms. Reprod Sci. 2016;23(4):482–491.
[7] Romero R, Miranda J, Chaiworapongsa T, et al. Prevalence and clinical significance of sterile intra-amniotic inflammation in patients with preterm labor and intact membranes. Am J Reprod Immunol. 2014;72(5):458–474.
[8] Musilova I, Kutova R, Pliskova L, et al. Intraamniotic inflammation in women with preterm prelabor rupture of membranes. PLoS One. 2015;10(7):e0133929.
[9] Romero R, Miranda J, Chaemsaithong P, et al. Sterile and microbial-associated intra-amniotic inflammation in preterm prelabor rupture of membranes. J Matern Fetal Neonatal Med. 2015;28(12):1394–1409.
[10] Moher D, Liberati A, Tetzlaff J, et al. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. PLoS Med. 2009;6(7):e1000097.
[11] Ouzzani M, Hammady H, Fedorowicz Z, et al. Rayyan—a web and mobile app for systematic reviews. Syst Rev. 2016;5(1):210.
[12] Macleod MR, O'Collins T, Howells DW, et al. Pooling of animal experimental data reveals influence of

study design and publication bias. Stroke. 2004;35(5):1203–1208.
[13] Condon JC, Jeyasuria P, Faust JM, et al. Surfactant protein secreted by the maturing mouse fetal lung acts as a hormone that signals the initiation of parturition. Proc Natl Acad Sci USA. 2004;101(14):4978–4783.
[14] Normann E, Lacaze-Masmonteil T, Eaton F, et al. A novel mouse model of ureaplasma-induced perinatal inflammation: effects on lung and brain injury. Pediatr Res. 2009;65(4):430–436.
[15] Jantzie LL, Winer JL, Maxwell JR, et al. Modeling encephalopathy of prematurity using prenatal hypoxia-ischemia with intra-amniotic lipopolysaccharide in rats. J Vis Exp. 2015;(105):e53196.
[16] Maxwell JR, Denson JL, Joste NE, et al. Combined in utero hypoxia-ischemia and lipopolysaccharide administration in rats induces chorioamnionitis and a fetal inflammatory response syndrome. Placenta. 2015;36(12):1378–1384.
[17] Gomez-Lopez N, Romero R, Plazyo O, et al. Intra-amniotic administration of HMGB1 induces spontaneous preterm labor and birth. Am J Reprod Immunol. 2016;75(1):3–7.
[18] Surve MV, Anil A, Kamath KG, et al. Membrane vesicles of group B Streptococcus disrupt feto-maternal barrier leading to preterm birth. PLoS Pathog. 2016;12(9):e1005816.
[19] Cookson MW, Ryan SL, Seedorf GJ, et al. Antenatal vitamin D preserves placental vascular and fetal growth in experimental chorioamnionitis due to intra-amniotic endotoxin exposure. Amer J Perinatol. 2018;35(13):1260–1270.
[20] Dedja A, Gucciardi A, Giordano G, et al. Lipopolysaccharide-induced chorioamnionitis and postnatal lung injury: The beneficial effects of L-citrulline in newborn rats. Exp Lung Res. 2018;44(4-5):226–240.
[21] Garcia-Flores V, Romero R, Miller D, et al. Inflammation-induced adverse pregnancy and neonatal outcomes can be improved by the immunomodulatory peptide exendin-4. Front Immunol. 2018;9:1291.
[22] Gomez-Lopez N, Romero R, Arenas-Hernandez M, et al. Intra-amniotic administration of lipopolysaccharide induces spontaneous preterm labor and birth in the absence of a body temperature change. J Matern Fetal Neonatal Med. 2018;31(4):439–446.
[23] Jantzie LL, Oppong AY, Conteh FS, et al. Repetitive neonatal erythropoietin and melatonin combinatorial treatment provides sustained repair of functional deficits in a rat model of cerebral palsy. Front Neurol. 2018;9:233.
[24] Faro J, Romero R, Schwenkel G, et al. Inflammation-induced intra-amniotic inflammation induces preterm birth by activating the NLRP3 inflammasome. Biol Reprod. 2019;100(5):1290–1305.
[25] Gomez-Lopez N, Romero R, Garcia-Flores V, et al. Inhibition of the NLRP3 inflammasome can prevent sterile intra-amniotic inflammation, preterm labor/birth, and adverse neonatal outcomes. Biol Reprod. 2019;100(5):1306–1318.
[26] Richardson CA, Flecknell PA. Anaesthesia and post-operative analgesia following experimental surgery in laboratory rodents: are we making progress. Anesth Analg. 2005;33(2):119–127.
[27] Hirsch E, Saotome I, Hirsch D. A model of intrauterine infection and preterm delivery in mice. Am J Obstet Gynecol. 1995;172(5):1598–1603.
[28] Rinaldi SF, Makieva S, Frew L, Wade J, et al. Ultrasound-guided intrauterine injection of lipopolysaccharide as a novel model of preterm birth in the mouse. Am J Pathol. 2015;185(5):1201–1206.
[29] Serriere S, Nadal-Desbarats L, Seguin F, et al. Ultrasound-guided collection of amniotic fluid in pregnant rats. J Am Assoc Lab Anim Sci. 2006;45(4):49–53.
[30] Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol. 2007;81(1):1–5.
[31] Migale R, Herbert BR, Lee YS, et al. Specific lipopolysaccharide serotypes induce differential maternal and neonatal inflammatory responses in a murine model of preterm labor. Am J Pathol. 2015;185(9):2390–2401.
[32] Elovitz MA, Wang Z, Chien EK, et al. New model for inflammation-induced preterm birth: the role of platelet-activating factor and Toll-like receptor-4. Am J Pathol. 2003;163(5):2103–2111.
[33] Lee PR, Kim SR, Jung BK, et al. Therapeutic effect of cyclo-oxygenase inhibitors with different isoform selectivity in lipopolysaccharide-induced preterm birth in mice. Am J Obstet Gynecol. 2003;189(1):261–266.
[34] Yoon BH, Romero R, Lim J-H, et al. The clinical significance of detecting Ureaplasma urealyticum by the polymerase chain reaction in the amniotic fluid of patients with preterm labor. Am J Obstet Gynecol. 2003;189(4):919–924.
[35] Kacerovsky M, Musilova I, Khatibi A, et al. Intraamniotic inflammatory response to bacteria: analysis of multiple amniotic fluid proteins in women with preterm prelabor rupture of membranes. J Matern Fetal Neonat Med. 2012;25(10):2014–2019.
[36] Colaizy TT, Morris CD, Lapidus J, et al. Detection of ureaplasma DNA in endotracheal samples is associated with bronchopulmonary dysplasia after adjustment for multiple risk factors. Pediatr Res. 2007;61(5, Part 1):578–583.
[37] Schelonka RL, Katz B, Waites KB, et al. Critical appraisal of the role of Ureaplasma in the development of bronchopulmonary dysplasia with metaanalytic techniques. Pediatr Infect Dis J. 2005;24(12):1033–1039.
[38] Klune JR, Dhupar R, Cardinal J, et al. HMGB1: endogenous danger signaling. Mol Med. 2008;14(7–8):476–484.
[39] Piccinini AM, Midwood KS. DAMPening inflammation by modulating TLR signaling. Mediators Inflamm. 2010;2010:1–21.
[40] Sheller-Miller S, Urrabaz-Garza R, Saade G, et al. Damage-associated molecular pattern markers HMGB1 and cell-free fetal telomere fragments in oxidative-stressed amnion epithelial cell-derived exosomes. J Reprod Immunol. 2017;123:3–11.

[41] Friel Lara A, Romero R, Edwin S, et al. The calcium binding protein, S100B, is increased in the amniotic fluid of women with intra-amniotic infection/inflammation and preterm labor with intact or ruptured membranes. J Perinat Med. 2007;35(5):385–393.

[42] Romero R, Chaiworapongsa T, Alpay Savasan Z, et al. Damage-associated molecular patterns (DAMPs) in preterm labor with intact membranes and preterm PROM: a study of the alarmin HMGB1. J Matern Fetal Neonat Med. 2011;24(12):1444–1455.