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Analysis of Immunological Biomarkers in Amniotic Fluid Predicting Fetal Inflammatory Response Syndrome and Outcomes in Preterm Neonates

DOCTORAL DISSERTATION

Medicine and Health Sciences,
Medicine (M 001)

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The dissertation was prepared between 2020 and 2024 at the Clinic of Obstetrics and Gynecology, Clinical Medicine Institute, Faculty of Medicine, Vilnius University.

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Violeta Gulbinienė

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ABBREVIATIONS

AF, amniotic fluid.
AIC, Akaike information criteria.
AUC, the area under the curve.
BPD, bronchopulmonary dysplasia.
CAI, chorioamnionitis.
CCL2, chemokine C-C motif ligand 2.
CI, confidence interval.
CMV, cytomegalovirus.
COX, cyclooxygenase.
CRP, C-reactive protein.
DEFA3, α-defensin 3.
ECM, extracellular matrix.
EGF, epidermal growth factor.
ELISA, enzyme-linked immunosorbent assay.
EOS, early-onset sepsis.
FHR, fetal heart rate.
FIRS, fetal inflammatory response syndrome.
GA, gestational age.
IAI, intra-amniotic infection and/or inflammation.
IL-10, interleukin-10.
IL-17, interleukin-17.
IL-1β, interleukin 1β.
IL-6, interleukin-6.
iNOS, inducible nitric oxide synthase.
IQR, interquartile range.
IT ratio, immature to total neutrophile ratio.
IVH, intraventricular hemorrhage.
MIAC, microbial invasion into the amniotic cavity.
MMP-8, matrix metalloproteinase-8.
MMPs, matrix metalloproteinases.
MRI, magnetic resonance imaging.
MV, mechanical ventilation.
NICU, neonatal intensive care unit.
NPV, negative predictive value.
OOB, Out-of-Bag error.
OR, odds ratio.
PAMP/PAMPs, pathogen-associated molecular pattern/patterns.

PCR, polymerase chain reaction.
PDA, patent ductus arteriosus.
PMN, polymorphonuclear neutrophils.
PPROM, preterm premature rupture of membranes.
PPV, positive predictive value.
PVL, periventricular leucomalacia.
RANTES, regulated upon activation, normal T cell expressed and presumably secreted.
RDS, respiratory distress syndrome.
ROP, retinopathy of prematurity.
RS, respiratory support.
SD, standard deviation.
SHAP, Shapley additive explanations.
sIL-6R, soluble IL-6 receptor.
sTLR-2, soluble Toll-like receptor 2.
sTLR-4, soluble Toll-like receptor 4.
sTLRs, soluble Toll-like receptors.
suPAR, soluble urokinase-type plasminogen activator receptor.
TNF-α, tumor necrosis factor-α.
uIL-6, umbilical IL-6.
VSMC, vascular smooth muscle cell.
VUHSK, Vilnius University Hospital Santaros Klinikos.
WBC, white blood cells.

CONTENTS

1. INTRODUCTION

1.1 Relevance, novelty, and clinical significance

Fetal inflammatory response syndrome (FIRS) represents a systemic fetal reaction to intra-amniotic infection and/or inflammation (IAI), characterized by an elevation in various cytokines [1,2]. Intraamniotic infection and/or inflammation stands as one of the leading causes of spontaneous preterm birth, accounting for 40-70% of all preterm births [3]. FIRS is commonly followed by preterm premature rupture of membranes (PPROM) and preterm delivery, heightening the risk of adverse neonatal outcomes [4]. This syndrome is defined as an elevation of cytokines in fetal blood [5] or identified histologically as acute funisitis [6].

FIRS leads to increased neonatal morbidity and mortality, with short-and long-term outcomes: respiratory distress syndrome (RDS), sepsis, bronchopulmonary dysplasia (BPD), intraventricular hemorrhage (IVH), periventricular leukomalacia (PVL), retinopathy of prematurity (ROP), sensorineural hearing loss and neurodevelopmental disabilities, including cerebral palsy [1,5,7–9]. Due to potential maternal and fetal risks and complications, the diagnosis of IAI often leads to immediate delivery [10,11].

Diagnosing IAI, as well as FIRS, particularly following PPROM, has posed significant challenges. Currently, FIRS diagnosis relies on biochemical and histological criteria, with histological assessment of the umbilical cord feasible only post-delivery [8]. While inflammatory biomarkers in maternal blood offer limited sensitivity and specificity, providing more maternal response information than fetal [12–15]. Analysis of biomarkers in fetal plasma obtained by cordocentesis or amniotic fluid attained by transabdominal amniocentesis presents high sensitivity and specificity [5,16–21]. However, these methods are invasive, contrasting with the safe and reliable detection of umbilical cord blood biomarkers immediately post-birth, albeit unsuitable for antenatal diagnosis [12,14,22]. These limitations complicate FIRS diagnosis in clinical practice, given the challenging and interventional diagnostic methods involved. With ongoing research aimed at validating a rapid, easy-to-perform, specific, and sensitive test method for early FIRS diagnosis, the focus is shifting towards non-invasive diagnostic possibilities to address the gap in prenatal FIRS diagnosis.

The analysis of inflammatory biomarkers in amniotic fluid has proven to be the optimal approach for diagnosing IAI and FIRS before delivery [1,17,23–25]. Numerous amniotic fluid biomarkers, such as interleukin-6 (IL-

6), matrix metalloproteinase-8 (MMP-8), tumor necrosis factor-α (TNF-α), soluble Toll-like receptor 2 (sTLR-2), α defensins (DEFA3), interleukin-10 (IL-10), and interleukin-17 (IL-17), have been extensively researched as predictive tools for IAI, FIRS, and adverse neonatal outcomes. However, most studies have focused on amniotic fluid obtained via transabdominal amniocentesis [16–21] or fetal blood samples from cordocentesis [5,26]. Only a limited number of studies have investigated non-invasively obtained amniotic fluid to predict FIRS, and even fewer have evaluated the role of biomarkers in vaginally collected amniotic fluid for predicting FIRS-associated neonatal outcomes [23,25,27–29]. To date, no research has explored the association between amniotic fluid levels of soluble urokinase-type plasminogen activator receptor (suPAR), soluble Toll-like receptor 4 (sTLR-4), epidermal growth factor (EGF), and FIRS, particularly in vaginally obtained amniotic fluid.

The selection of biomarkers for this study was based on their established roles in the pathogenesis of IAI and FIRS, as well as their ability to predict adverse neonatal outcomes. Many of these markers – such as IL-6, TNF-α, MMP-8, IL-10, IL-17 and DEFA3 – are recognized as key inflammatory mediators in IAI, making them critical for diagnosing FIRS before delivery. In addition to these well-studied biomarkers, suPAR and sTLR-4 were included due to their roles as potent inflammatory markers. suPAR is a strong predictor of systemic inflammation, a crucial factor in both IAI and FIRS progression. EGF, on the other hand, was selected for its importance in fetal and neonatal growth, development, and tissue repair. Including EGF provides valuable insight into its potential impact on neonatal outcomes in the context of FIRS.

The present study aimed to determine FIRS and assess the risk of adverse neonatal outcomes by analyzing various immunological biomarkers—including IL-6, TNF-α, MMP-8, sTLR-2, sTLR-4, RANTES, IL-10, IL-17, DEFA3, suPAR, and EGF – in non-invasively collected vaginal amniotic fluid following PPROM before 34^{+6} weeks of gestation. This non-invasive sampling technique, which is simple to perform, requires no specialized skills, and carries minimal risk of complications, holds promise for becoming part of routine clinical practice. An improved, non-invasive antenatal diagnosis of FIRS could assist in better evaluating prenatal risk and reducing adverse perinatal outcomes.

1.2 Aim and objectives

This thesis aims to evaluate the significance of immunological biomarkers in non-invasively collected amniotic fluid concerning fetal inflammatory response syndrome and outcomes of preterm neonates.

Objectives:

1. To evaluate the relationship between immunological biomarkers concentrations in amniotic fluid and fetal inflammatory response syndrome.
2. To determine the association between amniotic fluid immunological biomarkers, predictive of inflammatory response, and the umbilical cord blood biomarker IL-6.
3. To assess the relationship between immunological biomarkers levels in amniotic fluid and outcomes of preterm neonates.
4. To identify the risk of adverse outcomes in preterm neonates by evaluating threshold concentrations of immunological biomarkers in amniotic fluid.
5. To improve recommendations for preterm neonatal care based on FIRS diagnosis and neonatal outcome prognosis, by assessing changes in immunological biomarkers concentrations in amniotic fluid.

The hypothesis of the thesis is: immunological biomarkers in non-invasively collected amniotic fluid predict fetal inflammatory response syndrome and preterm neonatal outcomes.

1.3 Defended statements

1. The concentrations of immunological biomarkers – TNF-α, IL-6, MMP-8, sTLR-2, sTLR-4, RANTES, IL-10, IL-17, DEFA3, suPAR, and EGF – in non-invasively collected amniotic fluid are associated with FIRS.
2. The levels of amniotic fluid immunological biomarkers, predictive of FIRS, correlate with the levels of umbilical cord blood biomarker IL-6.
3. The immunological biomarkers in non-invasively collected amniotic fluid are associated with outcomes of preterm neonates, including RDS, the need for surfactant, duration and type of respiratory support, BPD, sepsis, early hypotension, PDA, IVH, ROP, and death.

4. The threshold concentrations of biomarkers – TNF-α, IL-6, MMP-8, sTLR-2, sTLR-4, RANTES, IL-10, IL-17, DEFA3, suPAR, and EGF – predict increased odds of FIRS and adverse outcomes in preterm neonates.
5. The analysis of immunological biomarkers of non-invasively collected amniotic fluid could improve antenatal diagnosis of FIRS and help stratify the prognosis of neonatal outcomes.

2. LITERATURE REVIEW

The literature review was conducted by searching for relevant articles in the Cochrane, Medline, and Science Direct databases. The search utilized the following keywords: "FIRS", "Fetal Inflammatory Response Syndrome", "Intraamniotic inflammation and infection", "Amniotic fluid", "Cervicovaginal fluid", "Vaginal amniotic fluid", "PPROM", "Rupture of membranes", "Preterm delivery", "Preterm birth", "Biomarker", "IL-6", "TNF-α", "IL-10", "IL-17", "RANTES", "TLR-2", "TLR-4", "suPAR", "defensins", "DEFA3", "EGF", "MMP-8", "Neonatal outcomes", "Neonatal morbidity", "neonatal death", "neonatal mortality", "RDS", "Respiratory outcomes", “Bronchopulmonary dysplasia", "Neonatal sepsis", "Early onset sepsis", "Hypotension", "Patent ductus arteriosus", "Intraventricular hemorrhage" and "Retinopathy of prematurity." The review included retrospective, prospective, cohort, case-control, meta-analysis, and literature review studies, as long as they were published in English.

2.1 Definition of FIRS

FIRS refers to the systemic inflammatory reaction of the fetus caused by the activation of the innate immune system, which is mediated by the release of cytokines and chemokines, resulting in multiorgan dysfunction, neonatal mortality, and morbidity [30]. FIRS is diagnosed by an increased level of umbilical cord blood acute phase reactants, mostly IL-6 with a level of >11 pg/mL, and/or identified histologically as acute funisitis. Originally, FIRS was determined by IL-6 levels in fetal plasma obtained by transabdominal cordocentesis [5]. Over time and after extensive research, it was established that umbilical cord blood IL-6 levels correspond with fetal plasma levels. Although the most commonly used cut-off value for diagnosing FIRS is an umbilical cord blood IL-6 level greater than 11 pg/mL [1,2,5,31–33], there is ongoing debate about the optimal cut-off value, with some suggesting IL-6 levels greater than 100 pg/mL [25].

FIRS represents the fetal response to the intraamniotic infection and/or inflammation. IAI is categorized into maternal and fetal inflammatory responses [34]. Histological chorioamnionitis represents maternal inflammatory response and is defined as the presence of neutrophils in the chorioamnion membrane or chorionic plate. It is a histological counterpart of maternal IAI.

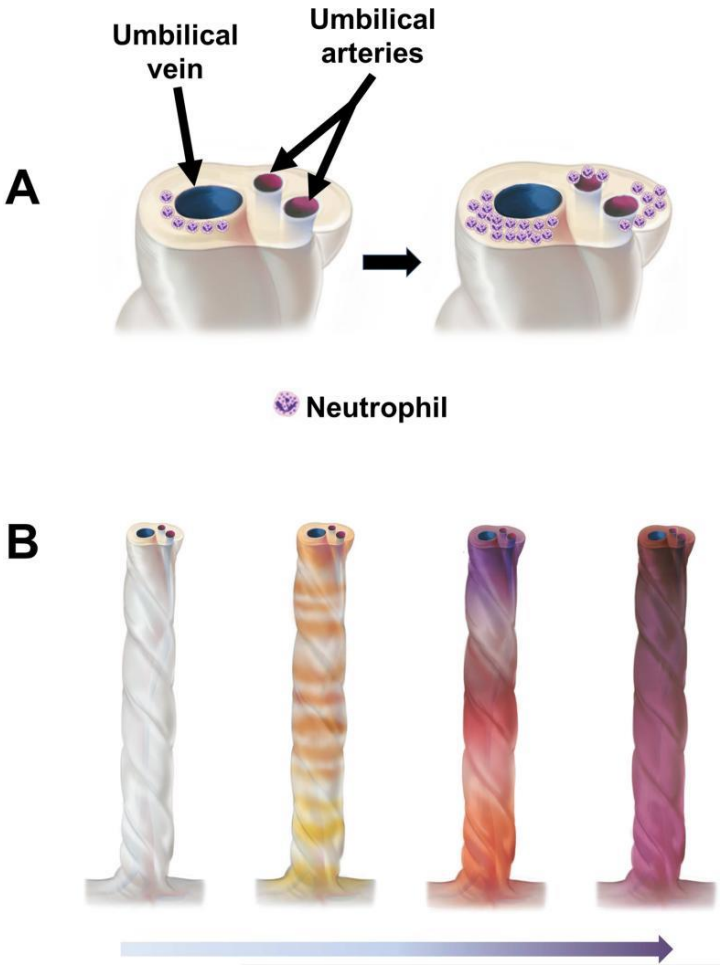


Figure 1. Inflammatory progress in the umbilical cord. A. umbilical phlebitis (blue vessel), followed by umbilical arteritis (red vessels). B. Inflammation progresses from multifocal (yellow orange) to diffuse (purple) lesions in the umbilical cord. Adopted with permission from Kim et al., 2015 [2].

Funisitis is the histological counterpart of FIRS defined by the infiltration of neutrophils into the umbilical cord (umbilical vein, umbilical arteries, and Wharton's jelly) (Figure 1) [2]. Inflammation of the umbilical cord begins with phlebitis (infiltration in the vein) and can progress to arteritis (infiltration in arteries). Neutrophil infiltration into Wharton's jelly is common in funisitis. Initially, the inflammation is multifocal; the affected areas merge over time, resulting in widespread funisitis in the umbilical cord. This infiltration starts from the umbilical vein due to higher expression of IL-8 messenger RNA in the vein compared to the arteries, making the umbilical vein more susceptible to a proinflammatory response than the umbilical arteries [35].

2.2 Etiopathogenesis of FIRS

The fetus can deploy an inflammatory response when exposed to bacteria, bacterial products (e.g., endotoxins), viruses (such as herpes simplex virus, cytomegalovirus (CMV), rubella virus), fungi (Candida), and protozoa (*Toxoplasma gondii, Plasmodium* species), as well as non-infectious stimuli (known as danger signals or alarmins, such as stress and cell death) [1]. The rate of microbial invasion into the amniotic cavity (MIAC) is reported to be 38%, while sterile intraamniotic inflammation is associated with 40-60% of cases of histological chorioamnionitis [2]. Although studies report that 30% of cases of MIAC have positive fetal blood cultures [5,36], the prevalence of microbial invasion into the fetus remains uncertain and is likely higher than reported. Several factors may contribute to this discrepancy, including intermittent bacteremia in the neonatal period, limitations in culture techniques for certain microorganisms like genital mycoplasmas, the lack of routine culture testing for these organisms in neonatal intensive care units (NICU), and maternal treatment with antibiotics [2]. Nonetheless, both inflammation with MIAC and without it are linked to adverse neonatal outcomes [8].

The risk factors for IAI encompass a broad range of lifestyle choices, medical procedures, infections, health conditions, and labor-related factors [3]. Lifestyle factors such as alcohol use and cigarette smoking can increase susceptibility to infection. Certain medical procedures, including amnioinfusion, epidural anesthesia, and multiple vaginal examinations, particularly with ruptured membranes, also elevate the risk. Pre-existing infections like bacterial vaginosis, Group B streptococcus colonization, and sexually transmitted infections further increase the likelihood of IAI. Additionally, health conditions such as obesity, and an immunocompromised

state predispose individuals to this infection. Labor-related factors, including prolonged labor, prolonged second stage of labor, meconium-stained amniotic fluid, nulliparity, prolonged membrane rupture, and twin pregnancies with preterm labor and intact membranes, also play significant roles in developing IAI [3].

Microorganisms are considered to enter the amniotic cavity through several routes: ascending from the lower genital tract, hematogenous or transabdominal [37]. A retrograde seeding route from the fallopian tubes to the peritoneum, known as transfallopian, has been proposed, but the evidence supporting this pathway is limited.

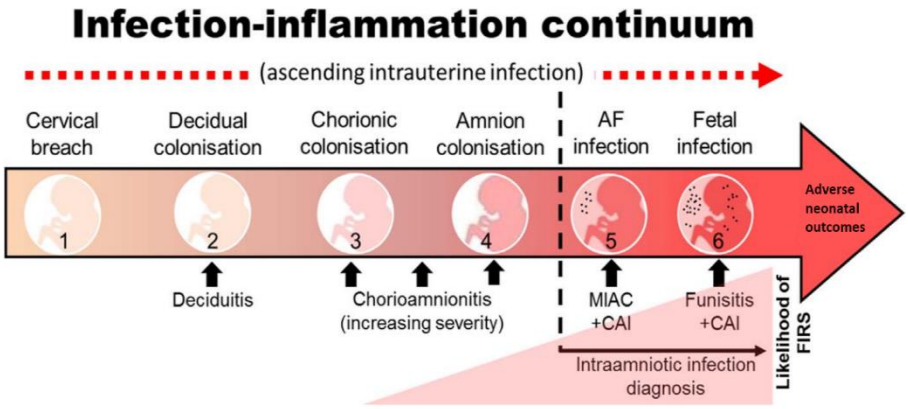


Figure 2. The progression of ascending intraamniotic infection: microorganisms from the vagina/cervix access the decidua (the pregnant endometrium), then proceed through the chorion and amnion to the amniotic cavity (dotted line), leading to intraamniotic infection. Eventually, the infection may invade the fetus. Abbreviations: CAI, chorioamnionitis; FIRS, fetal inflammatory response syndrome, AF, amniotic fluid, MIAC, microbial invasion of the amniotic cavity. Modified with permission from Keelan JA, 2018 [37].

The most common pathway for intraamniotic infection appears to be ascending from the lower genital tract (Figure 2). Microbes most frequently detected in the amniotic cavity include genital mycoplasmas, including *Ureaplasma* species, *Gardnerella vaginalis, Fusobacterium* species, multiple species of *Streptococci*, including *Streptococcus agalactiae, Lactobacillus* species, *Bacteroides* species, *Escherichia coli*, *Sneathia, Prevotella* species, *Peptostreptococcus* species and etc. Fungi, such as *Candida albicans*, can also

be found [38–40]. Group B streptococci can create "clouds" of bacteria in the absence of a proper maternal inflammatory response, particularly in cases of chronic carriage [41].

Hematogenous transmission can occur through blood-borne maternal infections caused by *Listeria monocytogenes*, *Treponema pallidum*, *Yersinia pestis*, CMV, *Plasmodium* species, dental pathogens etc. [2,37,41,42].

The transabdominal route is associated with the accidental introduction of microorganisms during procedures such as amniocentesis, percutaneous umbilical cord blood sampling, fetoscopy, or other invasive techniques [2].

The invasion of microorganisms into the amniotic cavity can advance to fetal invasion. Bacteria enter the fetus through the respiratory tract, gastrointestinal tract, skin, ears, and, depending on the gestational age (GA), even the eyes. Once microorganisms breach the fetal mucosa, they stimulate pattern recognition receptors such as Toll-like receptors, initiating transcription factors, and prompting a localized inflammatory response within an organ, which later becomes systemic as inflammatory mediators enter circulation [1,8]. The immune response to stimuli (infection or tissue injury) activates pro- and anti-inflammatory responses. The early pro-inflammatory phase has been attributed predominantly to the activity of the innate immune system, while the anti-inflammatory response has been attributed to a dysregulated adaptive immune system. As the disorder progresses, the anti-inflammatory phase becomes predominant, and patients recovering from primary infection are more susceptible to secondary ones.

In addition, Lee et al. described chronic inflammatory lesions of the placenta associated with a unique fetal inflammatory response, defined by elevated CXCL10 rather than IL-6, proposing maternal anti-fetal rejection. Hence, FIRS Type II was suggested, indicating that FIRS can occur without intra-amniotic infection or inflammation [43].

2.3 Diagnosis of FIRS

The diagnosis of FIRS can be divided into antenatal and postnatal. The antenatal methods include the assessment of fetal parameters and analysis of inflammatory biomarkers in fetal blood or amniotic fluid. Additionally, it can be inferred indirectly through clinical and biochemical signs of maternal inflammation. Postnatally, FIRS is confirmed histologically or biochemically by evaluating inflammatory changes in the umbilical cord blood.

2.3.1 Clinical diagnosis of FIRS

As IAI's counterpart, the diagnosis of FIRS may be primarily based on clinical presentation antenatally. One of the most commonly used are Gibbs's criteria: maternal fever ≥ 37.8 or 38.0°C and at least 2 of the following signs: maternal tachycardia (> 100 beats per minute), fetal tachycardia (> 160 beats per minute), foul-smelling purulent discharge or amniotic fluid, uterine tenderness (soreness), leukocytosis (> 15000/mm^3) [44]. In 2015, the term "Triple I", meaning "Intrauterine Infection, Inflammation, or both", was suggested to replace chorioamnionitis by an expert panel at the National Institute of Child Health and Human Development [45]. Under this suggestion, Triple I diagnostic criteria were defined, as follows: maternal fever (≥ 39.0°C or in the range of 38.0-39.0°C retaking temperature in 30 minutes for confirmation) with one or more of the following: fetal tachycardia (> 160 beats per minute for ≥ 10 minutes), maternal white blood cell (WBC) count > 15000/mm^3 in the absence of corticosteroids, purulent fluid from the cervical (confirmed visually on speculum examination to be coming from the cervical canal) and biochemical or microbiologic amniotic fluid results consistent with microbial invasion of the amniotic cavity (amniocentesis-proven infection through a positive Gram stain, low glucose or positive amniotic fluid culture, placental pathology revealing diagnostic features of infection).

However, diagnosing IAI based on these criteria is challenging. These symptoms define clinical chorioamnionitis, which represents the clinical manifestation of a maternal systemic inflammatory response and is present in only 10% to 20% of all patients with proven intra-amniotic infection [8]. Maternal fever is a nonspecific and common symptom that should be evaluated and differentiated from other possible causes. In addition to maternal fever, the most frequent criteria of clinical chorioamnionitis are maternal and fetal tachycardia (91% and 76%, respectively), followed by maternal leukocytosis (72%). The main sign of fetal distress, and the only fetal symptom in these criteria, is fetal tachycardia, which can be detected via ultrasound or fetal monitoring.

The accuracy of clinical criteria in detecting histological placental lesions is about 50%. Biomarkers in maternal blood offer limited sensitivity and specificity, providing more information about the maternal response than the fetal response. [12–15]. Furthermore, intrauterine infection may be clinically silent before the onset of PPROM or preterm birth [46]. A recent review

comparing the diagnostic criteria for IAI found that both the Gibbs and Triple I criteria exhibit similar diagnostic characteristics. However, neither set of criteria is particularly sensitive for predicting intra-amniotic infection in women experiencing preterm labor and/or PPROM [47].

Another clinical method to predict fetal inflammation or infection is through fetal monitoring (fetal heart rate (FHR) patterns), fetal biophysical profile, which includes a nonstress test, fetal movements, fetal breathing movements, fetal tone, and amniotic fluid volume as indirect signs of fetal stress due to infection [48–50]. In women with PPROM or preterm labor with intact membranes, significant category II or III FHR patterns, such as fetal tachycardia, increased FHR baseline, decreased variability, and severe variable decelerations, suggest intrauterine infection and/or inflammation. These FHR patterns, particularly when accompanied by normal umbilical cord gases, should prompt clinicians to consider the likelihood of fetal infection and/or inflammation [48]. Additionally, a low biophysical profile score (≤ 7) has proven to be a good predictor of impending fetal infection in patients with premature rupture of membranes [49]. These methods are easy, non-invasive, and commonly used in everyday clinical practice. However, they are nonspecific, as an impaired fetal condition and heart rate can result from various factors, such as fetal infection and/or inflammation, anemia, congenital heart disease and arrhythmias, and central nervous system injury due to hypoxia-ischemia [48]. Additionally, while monitoring fetal heart rate has a high negative predictive value, its positive predictive value is comparatively low [50].

The specialists agree that biomarkers that objectively assess the risk of fetal/neonatal inflammation and infection must be discovered, validated, and implemented into the clinical workflow [45].

2.3.2 Pathophysiological (biochemical and microbiological) diagnosis

Experts suggest that the diagnosis of IAI should be based on the underlying pathological processes, which include microbial invasion and the inflammatory response. Attempts to assess intrauterine processes indirectly by evaluating inflammatory biomarkers in maternal blood have not proven very informative. The best way to assess intra-amniotic processes is by directly analyzing amniotic fluid or fetal blood. Amniotic fluid can be collected invasively through amniocentesis or non-invasively by collecting vaginal/cervical fluid after PPROM. Fetal blood can be obtained antenatally via cordocentesis or postnatally by collecting umbilical cord blood

immediately after birth. Invasive procedures, like amniocentesis and cordocentesis, remain the gold standard for antenatal IAI diagnosis.

The pathophysiological analysis of amniotic fluid and fetal blood includes:

1. Microbiological Assessment: This can be done by Gram stain, cultures, or molecular microbiologic techniques. Amniotic fluid should be obtained by amniocentesis [2,39]. Fetal blood can be microbiologically assessed by cordocentesis or from the umbilical cord after delivery.
2. Cytological Analysis: A WBC count ≥ 50 cells/mm³ in amniotic fluid indicates intra-amniotic inflammation [8].
3. Glucose Level Analysis: Assessing glucose levels in amniotic fluid.
4. Biochemical Analysis: Evaluating inflammatory biomarkers in amniotic fluid or fetal blood. Amniotic fluid samples can be collected by amniocentesis or non-invasively as vaginal amniotic fluid. Fetal blood can be obtained after cordocentesis or from the umbilical cord after delivery.

Standard microbiological techniques (cultivation of microorganisms in the laboratory) have limitations, particularly in obtaining an informative sample, as amniotic fluid requires amniocentesis, and is time-consuming. However, Polymerase Chain Reaction (PCR) analysis of amniotic fluid, when available, accelerates the diagnostic process and is highly sensitive and more accurate [39]. The primary disadvantage of PCR analysis is that PCR can only identify the presence or absence of a known pathogen. Another limitation is that the PCR primers can bind non-specifically to similar sequences, that are not completely identical to target DNA. Moreover, depending on the test range or possible selections of pathogens, there is an increased risk of false negative results [39]. However, relying solely on microbiological analysis, the diagnosis of IAI can be made in less than half of the cases, as MIAC occurs in up to 38% of histological chorioamnionitis cases [2]. Furthermore, diagnosing fetal infection is even more challenging, with only 30% of MIAC cases having positive fetal blood cultures [5,36]. Lately, relevant microbial cell-free cfDNA detection in maternal plasma and umbilical cord plasma has been reported in patients with clinical and/or histological chorioamnionitis [51]. Previous studies have suggested that detecting microbes in umbilical cord or placental blood using culture and molecular methods is a safe and reliable approach for evaluating early-onset sepsis, provided that a specific

collection method is used to minimize contamination [40,52]. Using placental or umbilical blood can benefit the neonate by delaying the onset of anemia of prematurity, reducing the need for blood transfusions and associated complications, and minimizing the number of painful laboratory procedures [52,53].

White blood cells in amniotic fluid are easily quantified using standard automatic cell counters or manual cell counters. It is also biologically relevant since the majority of neutrophils in amniotic fluid come from the fetus and are therefore a reflection of the inflammatory response to infection in the fetus. However, WBC count in amniotic fluid is difficult to interpret when it is contaminated by blood (a 'bloody tap') and it is a less accurate indicator of inflammation and infection [54].

In preterm gestations with PPROM, lower amniotic fluid glucose concentration may be used to assess the prospect of intraamniotic inflammation; however, this concentration decreases as gestation progresses [55,56]. The optimal concentration of amniotic fluid glucose for the identification of intra-amniotic inflammation in PPROM pregnancies was 0.56 mmol/L and could be used as additional criteria with very good specificity [56].

The analysis of inflammatory biomarkers in amniotic fluid or fetal plasma indicates pathological inflammatory processes occurring during IAI and FIRS. This evaluation assesses various cytokines and enzymes activated in the inflammatory cascade upon contact with a pathogen in the amniotic cavity. These highly sensitive and specific diagnostic methods are considered the gold standard for diagnosing IAI and FIRS antenatally [2,8].

Various inflammatory markers, including cytokines such as interleukins (IL-1α, IL-1β, IL-6, IL-10, IL-17), TNF-α, and chemokines (IL-8, RANTES (CCL5), CCL2-3-4-8), as well as antimicrobial peptides (defensins DEFA1-3), sTLR-2, sTLR-4), and extracellular matrix-degrading enzymes like MMP-8, have been implicated in the pathogenesis of intra-amniotic inflammation and infection (Figure 3) [2,16,31,57–63]. Inflammatory agents do not act alone but typically trigger a cascade of processes that stimulate each other's production. The induced intra-amniotic inflammatory reaction triggers a wide range of cytokines and chemokines. Cytokines act as messengers for immune cells, while chemokines (or chemotactic cytokines) stimulate the migration of inflammatory cells, particularly white blood cells. Stimulated cells produce more cytokines and defensins, which are capable of killing microbes or inhibiting their growth, and release extracellular matrix-degrading enzymes

and proteins that induce myometrial contractility, cervical ripening, and rupture of membranes [1,2,31].

Extensive research has investigated whether various inflammatory factors released during inflammation can be used to diagnose intraamniotic infection and FIRS or to predict neonatal outcomes. Amniotic fluid concentrations of MMP-8 and IL-6 are highly effective for detecting inflammation, with rapid bedside assays available for both markers [17,55,64]. The diagnosis of IAI is often based on IL-6 levels greater than 2.6 ng/mL or MMP-8 concentrations greater than 23 ng/mL in amniotic fluid obtained via amniocentesis [8].

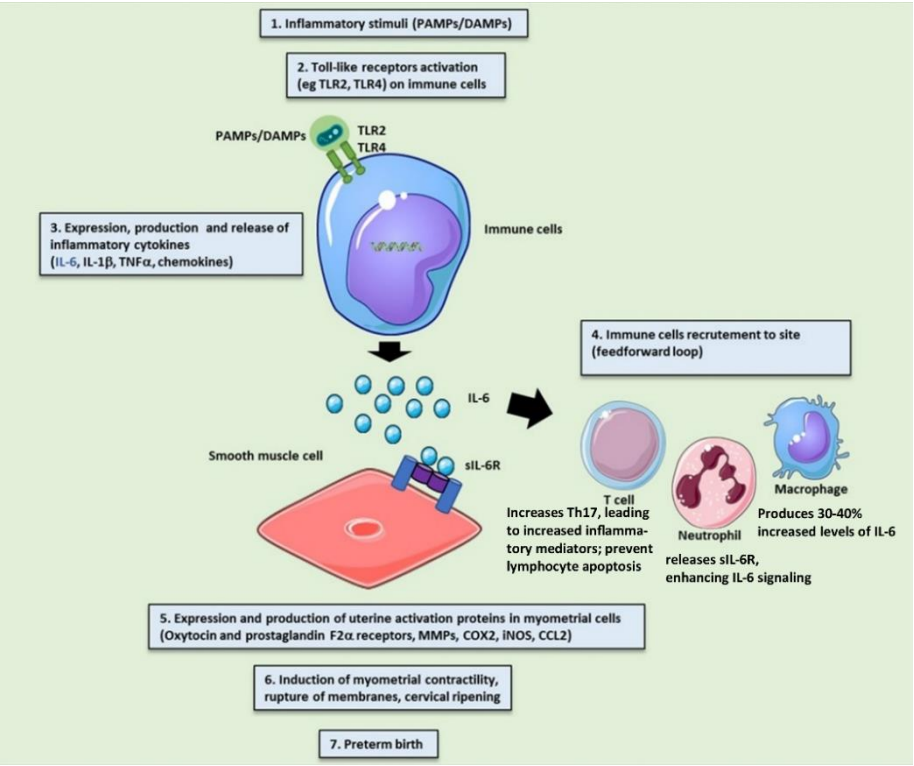


Figure 3. The role of inflammatory biomarkers in intraamniotic infection and/or inflammation and FIRS, leading to preterm birth. Abbreviations: IL-6, interleukin 6, sIL-6R, soluble IL6 receptor, PAMPs, pathogen-associated molecular patterns; TLR2, Toll-like receptor 2, TLR 4, Toll-like receptor 4, IL-1β, interleukin 1β, TNF-α, tumor necrosis factor α, MMPs, matrix metalloproteinases, COX, cyclooxygenase, iNOS; Inducible Nitric Oxide Synthase, CCL2, Chemokine C-C motif ligand 2. Adapted with permission according to E. Prairie et al., 2021 [57].

The diagnosis of FIRS is based on fetal blood IL-6 levels, with a cutoff of 11 pg/mL [5]. Additionally, non-invasive methods, such as transcervical amniotic fluid collectors, syringes, etc., have been introduced to assess inflammation in patients with ruptured membranes [23–25,65]. Furthermore, some studies concluded that the prediction of intraamniotic infection using non-invasively collected cervical fluid proteins was as accurate as using proteins from amniotic fluid or a combination of both sources [23,63]. Although further studies are needed to evaluate its diagnostic utility [24,65].

The various inflammatory factors contributing to intraamniotic inflammation, FIRS, and neonatal outcomes are presented below.

2.3.2.1 Interleukin-6

Interleukin-6 (IL-6) – is a prompt and transient cytokine with a pleiotropic effect on acute phase inflammation, immune response, and hematopoiesis. In the initial stages of both infectious and non-infectious inflammation, IL-6 is generated by monocytes and macrophages following TLRs activation [31]. In addition, T-cells, B-cells, fibroblasts, keratinocytes, endothelial cells, mesangial cells, adipocytes, and some tumor cells synthesize this cytokine. IL-6 expression plays a central role in intraamniotic infection and/or inflammation and FIRS, as well as, in PPROM and preterm birth (Figure 3). During intraamniotic inflammation, IL-6 recruits immune cells (neutrophils, macrophages, and T lymphocytes) to the site. Then neutrophils release sIL-6R (soluble receptor of IL-6), enhancing IL-6 signaling, activated macrophages produce up to 30 to 40 % increased levels of IL-6, and T lymphocyte ratio favors Th17 cells, leading to increased pro-inflammatory mediators. IL-6 directly participates in lymphocyte migration and prevents lymphocyte apoptosis, ensuring a sustained influx of lymphocytes at the infection site [57].

FIRS is defined based on increased fetal or umbilical cord blood plasma levels of IL-6 > 11 pg/mL[5]. Whereas, the diagnosis of IAI might be based on increased amniotic fluid IL-6 levels > 2.6 ng/mL [8]. Furthermore, the elevated concentrations of fetal blood and amniotic fluid IL-6 may identify neonates at risk for adverse neonatal outcomes, such as early-onset sepsis, white matter injury, periventricular leukomalacia, and cerebral palsy, BPD [31,66–72]. Increased concentrations of umbilical blood IL-6 serve as independent marker for predicting severe ROP and the need for laser treatment, with combined models accurately predicting ROP progression [68].

2.3.2.2 Tumor necrosis factor-α

Tumor necrosis factor-α (TNF-α) is a cytokine that plays a key role in the onset of the inflammatory cascade and mediating septic shock and death [26]. It is a multifunctional diverse factor that induces cell survival and death, activates and suppresses angiogenesis, recruits and regulates immune cells, and assists in constructing the extracellular matrix (ECM). TNF-α is produced by immune cells like macrophages and lymphocytes, endothelial and epithelial cells [73]. This cytokine demonstrates a dose-related effect on cells, causing induction of the processes at low levels and suppression or destruction at high levels [74]. During inflammation and/or infection in the amniotic cavity, TNF-α is found in high concentrations in amniotic fluid [75,76] and suppresses the growth of amnion cells, stimulates the prostaglandin synthesis, and induces the release of MMPs, thus triggering PPROM and preterm delivery [58]. TNF-α and IL-6 in cord blood were reported to be associated with brain injuries in preterm infants exposed to severe chorioamnionitis [77]. Increased TNF-α and IL-6 expression in brain cells was observed in association with PVL [67]. Increased levels of IL-6 and TNF-α in umbilical cord blood were identified to predict grade 3 and more IVH during early adaptation, as well as CNS damage during later adaptation in preterm infants [33]. Elevated amniotic fluid TNF-α (≥ 41 pg/ml) was proven to be an independent predictor of early-onset neonatal sepsis in patients with preterm labor and intact membranes with an odds ratio of 12.9 [20]. In addition, TNF-α plays an important role in persistent inflammation in the premature lungs, contributing to the development of BPD [78]. The levels of TNF-α and IL-6 in amniotic fluid were found to be higher in mothers of infants with BPD [72]. High levels (10-fold or more) of TNF-α seem to contribute to hypotension in septic shock through various mechanisms. TNF-α lowers blood pressure in septic shock by reducing cardiac contractility, increasing endothelial permeability, enhancing NO production, causing resistance to catecholamines, boosting sodium excretion, and decreasing the presence of vasopressin receptors and aquaporin 2 [73]. Moreover, patent ductus arteriosus (PDA) has been linked to preterm labor and intrauterine inflammation [79], and TNF-α may have a potential pathological or clinical role in PDA [80]. Studies on TNF-α levels in relation to ROP development in preterm infants have yielded inconsistent results, potentially due to variations in sample collection times and locations [81]. Some studies report increased TNF-α concentrations in plasma 24h after birth or in the umbilical cord blood in association with ROP

[33,82]. Others found low TNF-α levels in the amniotic fluid retrieved during cesarean delivery correlating with the development of ROP in preterm infants [83]. Furthermore, there was no difference found in umbilical cord blood or plasma TNF-α levels comparing ROP and control groups in other studies [84,85].

2.3.2.3 Matrix metalloproteinase 8

Matrix metalloproteinase-8 (MMP-8) (also referred to as neutrophil collagenase and collagenase 2), is an ECM-degrading enzyme that belongs to the MMPs family and is released from activated neutrophils [86]. MMP-8 has a significant role at the site of inflammation, initiating the breakdown of ECM, mediating tissue remodeling, angiogenesis, and wound healing [16,86]. During intraamniotic bacterial invasion, this enzyme is responsible for the degradation of ECM of the chorioamnion, resulting in premature rupture of membranes, preterm birth, and is found in high levels in the amniotic fluid as well [16]. Since the neutrophils found in the amniotic fluid, which secrete MMP-8, are of fetal origin, amniotic fluid MMP-8 is recognized as a marker of funisitis and IAI, with a cut-off greater than 23 ng/mL in amniotic fluid obtained via amniocentesis [17,19,55,64]. Other authors report a higher amniotic fluid MMP-8 cut-off value of 41.5 ng/mL for the diagnosis of MIAC [87]. Vaginal fluid MMP-8 concentration was associated with adverse neurological outcomes (IVH, PVL and hypoxic ischemic encephalopathy) after PPROM [29]. Furthermore, increased levels of MMP-8 in amniotic fluid are linked to the development of cerebral palsy at the age of 3 years [21].

2.3.2.4 Soluble urokinase plasminogen activator receptor

Soluble urokinase plasminogen activator receptor (suPAR) is a nonspecific marker of immune activation and systemic inflammation in various infectious, autoimmune, and malignant diseases [88–90]. The plasminogen activator system, consisting of urokinase-like plasminogen activator (uPA), its receptor (uPAR), and an inhibitor, participates in the processes of cell adhesion, migration, and invasion, which are essential for inflammation [91]. In a membrane-bound form, uPAR is expressed on immune cells (activated T cells, neutrophils, macrophages, etc.) and other cells (trophoblast cells, fetal membrane cells, etc.) [88,91,92]. Cleaved uPAR becomes soluble urokinase plasminogen activator receptor (suPAR) and is found in various body fluids, including blood, urine, saliva, and cerebrospinal fluid [88,93,94]. Previous

studies demonstrated that uPA/uPAR activity in local fibrinolysis and proteolysis contributes to processes of reproduction: ovulation, implantation and placentation, tissue remodeling, and angiogenesis [92]. The plasminogen activator system is thought to be involved in the development of pregnancy and its inflammatory complications (preeclampsia) [92,95,96]. SuPAR levels in umbilical blood serum are predictive of bronchopulmonary dysplasia in preterm neonates [97], while neonatal blood suPAR was reported to be used as a prognostic factor for early and late neonatal sepsis [98,99].

2.3.2.5 Soluble Toll-like receptors

Soluble Toll-like receptors (sTLRs) are glycoproteins found on cell membranes that identify microbial membrane components, so-called pathogen associated molecular patterns (PAMP), like unmethylated double-stranded DNA, single-stranded RNA, lipoproteins, lipopolysaccharides, and flagellin. When activated by these components, sTLRs trigger the release of inflammatory cytokines cascade (IL-1, IL-6, TNF-α, DEFA3). In humans, there are 10 functional sTLRs known as sTLR1–10. TLRs are present in innate immune cells like dendritic cells (DCs) and macrophages, as well as in non-immune cells such as fibroblasts and epithelial cells [100]. Histologic chorioamnionitis, irrespective of membrane status (intact or ruptured), in preterm delivery is linked to elevated expression of TLR-2 and TLR-4 in the chorioamniotic membranes [59]. FIRS-associated dermatitis, known as the early stage of FIRS, is also associated with increased expression of TLR-2 and TLR-4 in the fetal epidermis [60]. Likewise, cerebral white matter injury, is thought to stem from TLR4 activation in the fetus [101]. TLRs, including soluble TLR-2 and TLR-4, play a pivotal role in identifying pathogens linked to MIAC [102,103]. Soluble TLR-2, known for its versatility, forms heterodimers with TLR-1 and TLR-6 to recognize a broad spectrum of microbial components from gram-positive bacteria, genital *Mycoplasmas*, *Ureaplasma* species, and fungi. On the other hand, soluble TLR-4 is essential in responding to lipopolysaccharides of gram-negative bacteria, such as *E. coli*, *Prevotella*, *Leptotrichia, Sneathia*, and other gram-negative species [4,104], aiding in the identification of other microorganisms associated with MIAC [103,105].

2.3.2.6 Human α-defensin 3

Defensins, including Human α-defensin 3 (DEFA3), are a group of antimicrobial peptides crucial for host defense against a wide range of pathogens, including gram-negative and gram-positive bacteria, fungi, and viruses. They are primarily found in high levels in neutrophil granules, particularly DEFA1–3, and are also present in lower levels in the epithelia lining mucosal surfaces, such as those of the small intestine (Paneth cells), respiratory tract, urinary tract, female reproductive tract (from the vagina to the oviduct), placenta, fetal membranes, and amniotic fluid. The primary role of α-defensins is to eliminate microbes that have been phagocytosed by cells during their transport to phagolysosomes. Additionally, DEFA exhibits anti-inflammatory properties, regulates autoimmunity, demonstrates immunomodulatory and chemotactic activities, preserves mucosal barrier function, maintains gut microbiota balance, and regulates organ development and cell death [106,107].

Increased levels of α-defensins in amniotic fluid have been linked to MIAC, preterm birth, PPROM, and intra-amniotic inflammation [62,108]. Elevated α-defensin levels in vaginal secretions after PPROM are also associated with adverse neonatal outcomes [109]. Lucovnik et al. reported that increased levels of α-defensin in vaginal secretions after PPROM were associated with histological chorioamnionitis, FIRS, and predicted neonatal mortality or neurological impairment at one year of age. The optimal cut-off value for FIRS was >2.1 ng/mL with a sensitivity of 100% and specificity of 53%; the cut-off for infant death or neurological impairment was >9.4 ng/mL with a sensitivity of 29% and specificity of 100% [109].

2.3.2.7 Interleukin-10

Inreleukin-10 (IL-10), a pregnancy-compatible anti-inflammatory cytokine, plays a crucial role in maintaining immune tolerance [110]. It is secreted by both immune (dendritic cells, macrophages, mast cells, natural killer cells, eosinophils, neutrophils, T and B cells) and non-immune cells in an autocrine and paracrine manner. IL-10 mainly targets various leukocytes and primarily suppresses excessive inflammatory responses by reducing pro-inflammatory cytokines such as IL-1, IL-6, IL-12, and TNF-α. In addition, it induces heme oxygenase-1 and inhibits antigen presentation by blocking major histocompatibility complex class II expression [111,112]. IL-10 inhibits

cyclo-oxygenase-2 expression and reduces prostaglandin E2 release in cultured placental explants from preterm labor deliveries, but not in term labor samples [110].

Dysregulation of IL-10 is associated with adverse pregnancy outcomes such as preterm birth and miscarriage [111], as well as neonatal outcomes, with IL-10 showing a protective effect against the development of BPD [113]. Preterm delivery accompanied by intra-amniotic infection/inflammation has been reported to be associated with elevated concentrations of IL-10 in the amniotic fluid [114]. Furthermore, increased concentrations of IL-10 were observed in the umbilical cord blood of neonates born with FIRS [115].

2.3.2.8 Interleukin-17

Interleukin-17 (IL-17) is a proinflammatory cytokine produced by various types of immune cells, including T cells (especially Th17), as well as other innate cells like macrophages, dendritic cells, natural killer cells, natural killer T cells, lymphoid tissue inducer cells, and γδ-T cells, along with epithelial cells. When it synergizes with potent mediators of proinflammatory immune responses, such as IL-6, IL-8, and TNF-α, IL-17 demonstrates significant proinflammatory effects, while exhibiting limited biological activity when acting alone [116]. There is a family of six IL-17 cytokines (IL-17A-F), that demonstrate diverse biological functions. Under homeostatic conditions, IL-17 stimulates the production of neutrophils, exerts a potent chemotactic effect on neutrophils and monocytes, induces the production of mucus and antimicrobial peptides, maintains epithelial integrity through the secretion of claudins, and triggers the production of inflammatory chemokines/cytokines. Intensive research has been dedicated to this cytokine due to its pivotal role in the pathogenesis of various inflammatory and autoimmune diseases such as psoriasis, rheumatoid arthritis, asthma, Crohn's disease, and multiple sclerosis [117].

Recently, IL-17A has emerged as a crucial initiator of FIRS and a potential contributor to chronic conditions resulting from fetal exposure to inflammatory or infectious agents [116,118,119]. Increased amniotic fluid IL-17 levels were found to be associated with histological chorioamnionitis and preterm birth [119]. Jacobsson et al. demonstrated that elevated concentrations of cervical fluid IL-17, but not in amniotic fluid, significantly predicted MIAC [63]. In addition, preterm neonates exposed to histological chorioamnionitis in utero exhibit elevated levels of IL-17A-producing Th17 lymphocytes and IL17+ Treg cells in cord blood, potentially disrupting normal fetal immune

development and predisposing to chronic diseases later in life [120]. Elevated IL-17 levels compromise intestinal integrity, heightening the risk of bacteremia, organ damage, and sepsis [116]. Activation of IL-17 pathways during fetal development due to IAI can exacerbate fetal inflammatory responses, leading to increased neonatal morbidities and mortality associated with conditions like sepsis, BPD, PDA, and NEC [116,121]. IL-17A-mediated FIRS can also trigger adverse neurologic conditions in neonates, such as IVH, ROP [122], and PVL, potentially resulting in cerebral white matter injury and subsequent neurodevelopmental impairment like cerebral palsy [66,123,124].

2.3.2.9 RANTES

RANTES (CCL5, regulated on activation, normal T cell expressed and secreted), a potent and multifaceted chemokine of the CC group, possesses the proinflammatory ability to recruit monocytes, lymphocytes, basophils, and eosinophils to the inflammation site. Following TLRs activation, this chemokine is produced by various cells, including T cells, macrophages, endothelial cells, and gestational tissue [125,126]. RANTES is implicated in the processes of human childbirth and in modulating the host's reaction to intrauterine infection. MIAC was linked to a notable rise in median amniotic fluid RANTES levels during preterm labor. Remarkably, RANTES concentration in amniotic fluid decreased with advancing gestational age [126]. Jacobsson et al. reported that the highest predictive values for MIAC were achieved by amniotic fluid markers IL-18, IL-1β, IL-6, and RANTES. [63]. Furthermore, CCL5 has been linked to the pathogenesis of ROP in the neonatal period. Sood et al. reported, that RANTES levels in the neonatal blood were lower with increasing ROP severity.[122]

2.3.2.10 Epidermal growth factor

Epidermal growth factor (EGF) is a small mitogenic polypeptide that belongs to the family of human EGF-related growth factors [13]. EGF is found in most body fluids, including amniotic fluid [14,15]. The amniotic fluid has high levels of EGF, which is essential to fetal growth and development [16]. EGF acts as a promoter of epithelial cell growth and has an important role in airway branching, stimulating the growth of the epithelial tubules, and in differentiating lung cells during embryonic, fetal, and postnatal lung development [12,17]. Moreover, EGF is involved in lung surfactant synthesis

by accelerating the maturation of alveolar type II cells [18–20] and producing surfactant protein A [21,22]. Throughout gestation, amniotic fluid EGF levels increase [14]. In animal models, intraamniotic EGF injections decrease the severity and duration of respiratory disease in preterm newborns [22]. Conversely, modified expression of growth factors, such as EGF, has been reported in pathological lung conditions such as bronchopulmonary dysplasia, bronchial asthma, and pulmonary fibrosis [12,13]. Currie et al. indicated decreased levels of EGF in the bronchoalveolar lavage fluid after birth in preterm newborns who developed BPD [13]. Moreover, Shobokshi [34] previously reported elevated EGF concentrations in cases of premature rupture of membranes (PROM) with intraamniotic infection.

EGF and its receptor (EGFR) are linked to PDA, with EGF inducing PDA contraction. New treatment options are being explored through the EGFR transactivation pathway, aiming to either close PDA in preterm neonates or, conversely, keep it open in congenital heart diseases [127]. EGF protects against intestinal injury and is important in the pathogenesis of necrotizing enterocolitis (NEC) in preterm infants. In animal studies, oral administration of EGF at physiologically relevant doses significantly decreased the incidence of NEC [128].

Furthermore, EGF enhances myelination, astrogliosis, and neurologic recovery in animal models of white matter injury. EGF has been identified as a factor associated with the efficacy of intranasal mesenchymal stem cell therapy in enhancing myelination following encephalopathy in premature infants [129]. Vinukonda's study revealed that recombinant EGF treatment might improve the neurological outcome of premature infants with IVH [130].

2.3.3 Fetal and placental imaging in diagnosis of FIRS

Recent studies suggest that imaging techniques such as ultrasound and magnetic resonance imaging (MRI) may be important for the non-invasive antenatal diagnosis of IAI [131–135]. Imaging the fetal immune system, particularly MRI of the thymus and spleen and the placenta, can provide valuable insights for the prenatal diagnosis of FIRS. Ultrasound is a cost-effective and widely available method for assessing fetal organs and blood flow, though its image quality can be affected by fetal position, anhydramnios, and maternal body habitus [135]. Ultrasound can detect functional and anatomical changes associated with FIRS, including a decrease in thymus volume, pulsatile splenic venous wave patterns, increased adrenal gland volume, and various modifications in heart Doppler ultrasound (higher E/A

ratio, higher velocity time integral, increased Tei index, increased left and right ventricular compliance, decreased right ventricular systolic function, and longitudinal myocardial dyskinesia) [131–136]. One of the most studied ultrasound indicators is the reduction in fetal thymus size. Fetal thymic involution, a diagnostic marker of FIRS, has been shown to occur before changes in maternal inflammatory markers, such as C-reactive protein (CRP) and erythrocyte sedimentation rate [136].

While ultrasound is largely limited to providing structural information, MRI can complement it with functional insights into the metabolic activities of the fetal immune system and placenta. Fetal MRI, though more expensive and less widely accessible, offers significant advantages such as superior image quality regardless of fetal position or anhydramnios, and advanced techniques for correcting fetal motion [135]. Research on fetal MRI in the context of IAI has primarily focused on evaluating thymus size. Recent studies have also explored placental MRI for diagnosing chorioamnionitis, revealing distinctive changes in the placenta of affected women compared to those with low-risk pregnancies [135,137]. These changes correspond to histopathological findings, such as increased neutrophils in the chorion and thrombi in the villous tree. While placental ultrasound is limited to identifying anatomical issues, functional MRI of the placenta shows promise in evaluating high-risk pregnancies, although further studies are needed to determine its predictive value for adverse outcomes [135].

2.3.4 Histological FIRS diagnosis

The histological changes of IAI, including histological chorioamnionitis and funisitis, are determined by examining the placenta, fetal membranes, and umbilical cord both macroscopically and microscopically after delivery. The histological pattern of FIRS is funisitis, defined as fetal neutrophil infiltration within the umbilical vessels, with or without extension into Wharton's jelly. Histological chorioamnionitis, which is defined as the presence of neutrophils in the chorioamnion membrane or chorionic plate, is a histological counterpart of maternal IAI.

The severity of IAI can be described evaluating placenta reaction patterns according to guidelines from the Amniotic Fluid Infection Nosology Committee of the Perinatal Section of the Society for Pediatric Pathology (Table 1) [34] and the Amsterdam Placental Workshop Group Consensus (Table 2) [138]. Based on neutrophil infiltration into anatomical regions, "stage" describes the disease's progress, while "grade" indicates the intensity

of acute inflammation [34]. The severity and intensity of funisitis are linked to IL-6 levels in fetal plasma and amniotic fluid [139]. Although histological examination of the placenta, fetal membranes, and umbilical cord is one of the most accurate methods for diagnosing intra-amniotic inflammation, its main disadvantage is that it provides a retrospective diagnosis after birth and is not suitable for antenatal diagnosis of FIRS.

Table 1. Nomenclature and definitions of placenta reaction patterns related to intraamniotic infection.

Diagnostic categories	Suggested diagnostic terminology	Definitions
Maternal inflammatory response		
Stage 1 – Early	Acute subchorionitis or chorionitis	PMN in subchorionic fibrin and/or membrane trophoblast.
2 – Intermediate	Acute chorioamnionitis	Diffuse-patchy PMN in fibrous chorion and/or amnion.
3 – Advanced	Necrotizing chorioamnionitis	PMN karyorrhexis, amniocyte necrosis, and/or amnion basement membrane thickening/ hypereosinophilia
Grade 1 – Mild–moderate required	No special terminology	Not severe as defined below.
2 – Severe	Severe acute chorioamnionitis or with subchorionic microabscesses	Confluent PMN (≥10 x 20 cells in extent) between chorion and decidua; ≥ 3 isolated foci or continuous band.
Other	Chronic (or subacute) chorioamnionitis	Subamnionic mononuclear cell infiltrate with occasional PMN (meconium and hemosiderin-laden macrophages excluded)
Fetal inflammatory response		
Stage 1 – Early	With chorionic vasculitis or umbilical phlebitis	Intramural PMN-chorionic vessels and/or umbilical vein

Diagnostic categories	Suggested diagnostic terminology	Definitions
2 – Intermediate	With umbilical vasculitis (one or two arteries ±vein) or umbilical panvasculitis (all vessels)	Intramural PMN-umbilical artery or arteries (± umbilical vein)
3 – Advanced	With (subacute) necrotizing funisitis or with concentric umbilical perivasculitis.	PMN ± associated debris in concentric bands-rings-halos around one or more umbilical vessels.
Grade 1 – Mild–Moderate	No special terminology required.	Not severe as defined below.
2 – Severe	With a severe fetal inflammatory response or with intense chorionic (umbilical) vasculitis	Near confluent intramural PMN-chorionic and/or umbilical vessels with attenuation/degeneration of VSMC
Other	With associated fetal vessel thrombi	Recent thrombosis associated with intramural PMN
Other specific features	Peripheral funisitis	Focal aggregates of PMN at the umbilical cord surface
	Acute villitis	PMN in villous stroma (or between trophoblast and stroma)
	Acute intervillositis with intervillous abscesses.	Patchy-diffuse PMN in intervillous space.
	Decidual plasma cells	Unequivocal plasma cells in decidua basalis or capsularis.

Cited from R.W. Redline 2003[34]. Abbreviations: PMN, polymorphonuclear neutrophils; VSMC, vascular smooth muscle cell.

Table 2. Staging and Grading of the Maternal and Fetal Inflammatory Responses in Ascending Intrauterine Infection.

Maternal Inflammatory Response Stage	
Stage 1 - acute subchorionitis or chorionitis. Stage 2 – acute chorioamnionitis: PMNS extend into fibrous chorion and/or amnion. Stage 3 – necrotizing chorioamnionitis: karyorrhexis of PMNs, amniocyte necrosis, and/or amnion basement membrane hypereosinophilia;	Grade 1—not severe as defined. Grade 2 – severe: confluent PMNs or with subchorionic microabscesses.
Fetal Inflammatory Response Stage	
Stage 1 – chorionic vasculitis or umbilical phlebitis. Stage 2 – involvement of the umbilical vein and one or more umbilical arteries. Stage 3 – necrotizing funisitis.	Grade 1 – not severe as defined. Grade 2 – severe: near-confluent intramural PMNs with attenuation of VSMC.

Cited from T. Y. Khong 2016 [138]. Abbreviations: PMN, polymorphonuclear neutrophils; VSMC, vascular smooth muscle cell.

2.3.5 The neonatal outcomes of FIRS

The presence of FIRS is linked to the following onset of spontaneous preterm delivery in cases with PPROM [36], a shorter interval to delivery, higher neonatal morbidity after adjustment for gestational age at birth [5], increased risk of early-onset sepsis and multiorgan involvement in utero or the immediate neonatal period [1,8,140]. Multisystemic involvement includes the hematopoietic system, thymus, spleen, lungs, heart, adrenal glands, kidneys, skin, brain, gastrointestinal tract, thyroid, liver, eyes, ears [1,2,5,8] (Figure 4 and Table 3).

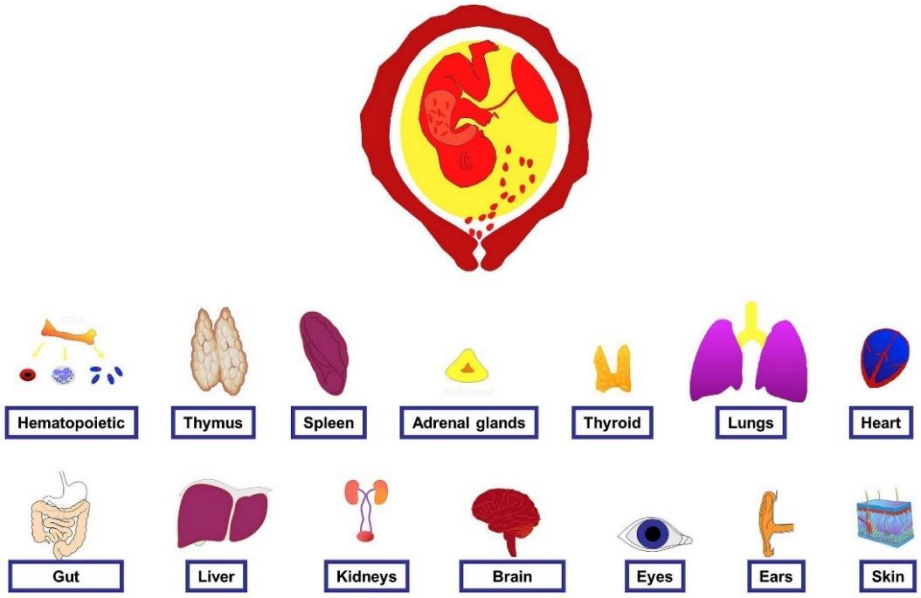


Figure 4. Multiorgan involvement associated with FIRS. Adapted from Jung. E et al. (2020)[1].

Table 3. Multiorgan involvement associated with FIRS

Organ	Outcome	References
Hematopoietic system	Neutrophilia > 95th percentile; Monocytosis; Higher nucleated RBC count.	[141,142]
Thymus	Involution of thymus.	[131,143,144]
Spleen	Splenic depletion; Changes in the fetal splenic circulation (pulsatile flow pattern in the fetal splenic vein).	[132,133,145]
Adrenal glands	Increase in cortisol; Increased ratio of fetal plasma cortisol and dehydroepiandrosterone sulfate; Changes in fetal programming.	[146,147]
Thyroid	Transient hypothyroxinemia	[148]
Lungs	Increased rate of RDS vs. decreased rate of RDS Increased rate of BPD	[7,66,78,113,149–151]
Heart	Fetal heart rate disturbances; Systolic and diastolic ventricular dysfunction (increased left and right ventricular compliance, decreased systolic contractile function of the right ventricle, and longitudinal myocardial dyskinesia);	[79,80,116,121,152–154]

Organ	Outcome	References
	Hemodynamic abnormalities (low systolic, mean, and diastolic blood pressure, lower systemic vascular resistance); Persistent patent ductus arteriosus.	
Gastroin-testinal tract	Increased risk of necrotizing enterocolitis due to impaired gut barrier and immune dysfunction)	[155,156]
Liver	Increase in fetal hepatic hematopoiesis and myelopoiesis; Fetal hepatic inflammation and impaired lipid metabolism (increase in total cholesterol, high-density lipoprotein, low-density lipoprotein and triglycerides in umbilical cord blood)	[157–159]
Kidneys	Oligohydramnios and altered fetal urine production; Higher serum blood urea nitrogen levels; Renal injury: reduction in nephron numbers, low glomerular density, altered renal biochemical profile (in animal models)	[160–164]
Brain	Fetal/neonatal neuroinflammation; Brain injury (PVL, IVH, neuronal injury) Neurodevelopmental disorders (cerebral palsy, mental illness)	[7,21,29,31, 66,67,77,123 ,124]
Eyes	Retinopathy of prematurity; Cerebrovisual impairment.	[33,68,81,83 ,122,165]
Ears	Sensorineural hearing loss; Microbial invasion or inflammation of the middle ear	[166–168]
Skin	Fetal dermatitis	[60]

3. METHODS

3.1 Study design and setting

The entire analysis of this thesis is based on data from the "Non-Invasive Immunological Analysis of Amniotic Fluid in Preterm Birth" study, funded by the Lithuanian Research Council under grant no. S-MIP-19-57. This prospective cohort study, initiated by Vilnius University Faculty of Medicine (principal investigator prof. Diana Ramašauskaitė) was performed in Vilnius University Hospital Santaros Klinikos (VUH SK) in 2017 – 2022. Immunological assays of amniotic fluid samples were performed at the State Research Institute, Centre for Innovative Medicine. The study was approved by the Vilnius Regional Biomedical Research Ethics Committee (2017-07-04 No. 158200-17-931-434 and additional approval in 2019-09-19 No.2019-LP-40, Supplement 1 and 2). Authorization from the State Data Protection Inspectorate for the processing of personal data has also been obtained (2018-03-09, No 2R-1765 (2.6-1.)) (Supplement 3).

The study prospectively enrolled singleton pregnant women admitted to Vilnius University Hospital Santaros Klinikos due to preterm premature rupture of membranes at 22^{+0}-34^{+6} weeks of gestation. After delivery, preterm newborns were also enrolled. All participants provided informed written consent before enrollment. Both parents provided written consent due to newborn enrollment.

The inclusion criteria were:

- Maternal age ≥ 18 years,
- Singleton pregnancy,
- Duration of pregnancy 22^{+0}-34^{+6} weeks,
- Diagnosed PPROM.

The exclusion criteria were:

- Multiple gestations,
- Vaginal bleeding,
- Placenta previa,
- Fetal malformations,
- Non-reassuring fetal status.

3.2 Collected maternal data

After enrollment maternal data collection was performed: age, number of previous pregnancies and deliveries, comorbidities, date of the last menstrual cycle, time of PPROM, pregnancy-related conditions, GBS test result, laboratory data (C-reactive protein (CRP), WBC), administered treatment (antibiotics, corticosteroids, tocolytics). After delivery additional maternal data was collected: mode of delivery, induction, latency period, and gestational age at birth.

Gestational age was based on the first day of the last menstrual period if the menstrual cycle was regular. If the menstrual cycle was irregular or the difference in the ultrasound scan at week 11^{+0} – 13^{+6} was more than 5 days, GA was determined from the ultrasound scan. Premature rupture of membranes was diagnosed with a sterile speculum during vaginal examination verifying amniotic fluid pooling in the back of the vagina or leaking from the cervix. In uncertain cases, rupture of membranes was confirmed by the presence of the placental alpha microglobulin-1 protein in the cervicovaginal fluid (AmniSure®, QIAGEN, Germantown, MD, USA). The latency period was defined as the time from preterm premature rupture of the membranes to delivery.

Following the obstetric guidelines approved by the Lithuanian Society of Obstetricians and Gynaecologists (2019) and management protocols approved by the VUH SK (2017) [169,170], women with PPROM up to 34 weeks of gestation were on expectant management. They received antibiotics, one course of prenatal corticosteroids, and, if necessary, tocolytics during the lung maturation course. Antibiotic therapy included intravenous ampicillin (2 g every 6 hours) and erythromycin (250 mg every 6 hours) for 48 hours followed by oral amoxicillin (500 mg every 8 hours) and erythromycin (250 mg every 6 hours) for five days. Dexamethasone was injected intramuscularly in two 12-mg doses every 12 hours for fetal lung maturation. After fetal lung maturation, labor started spontaneously or was induced. The indications for labor induction were intrauterine infection, bleeding, or non-reassuring fetal status. Participation in the study did not change routine clinical practice.

Clinical chorioamnionitis was diagnosed according to Gibb's criteria: maternal fever ≥ 38 °C and at least 2 of the following signs: maternal tachycardia (> 100 beats per minute), fetal tachycardia (> 160 beats per minute), foul-smelling purulent discharge or amniotic fluid, uterine tenderness (soreness), leukocytosis (> 15 x 10^9/l) and CRP > 40 mg/l [44].

3.3 Amniotic fluid sampling and immunological analysis

Free leaking amniotic fluid was collected vaginally with a sterile speculum into the sterile centrifuge tube every second day. The last sample obtained within 48 hours before labor was included in further analysis. We chose a sampling period of less than 48 hours to maintain a significant timing relationship between the results of amniotic fluid tests and histological findings of the placenta and umbilical cord at birth. Mucous, bloody samples with less volume were considered invalid and excluded from the study. To minimize contamination and attain clear specimens, samples were centrifuged at 3000 rpm for 5 min at 4°C and stored at −80°C.

A single investigator at the State Research Institute performed immunological assays of stored amniotic fluid samples. The amniotic fluid samples were thawed and analyzed in panels. Dilution and preparation of the samples for analysis were carried out according to the manufacturers' recommendations for commercial kits. Diluents were provided by the manufacturers. All samples were assayed in duplicate. The optical density was measured using a spectrophotometer (BioTek Instruments, Winooski, VT, USA). The concentrations of each biomarker were calculated according to standard curves using a dedicated computer program to evaluate immunoassay results: Gen5 Microplate Data Collection & Analysis Software (BioTek Instruments, Winooski, VT, USA).

The levels of IL-6 were measured using an enzyme-linked immunosorbent assay (ELISA) with a commercial kit “Human IL-6 ELISA kit” (Bender MedSystems GmbH, Vienna, Austria). The kit’s sensitivity (minimum detectable dose) was < 1 pg/ml, and the assay range was 10.2–400 pg/mL. The samples were used undiluted, but if the measured optical density of the sample exceeded the optical density of the highest standard, the samples were diluted 1:10, 1:100, or 1:1000, as appropriate, and retested.

TNF-α concentrations were determined using a commercially available “Human TNFα ELISA Kit” (Life Technologies, Waltham, USA). The assay range was 15.6–1000 pg/mL with a minimum detectable dose of < 2 pg/mL. The samples were non-diluted. If the measured optical density exceeded the optical density of the highest standard, the samples were diluted 1:5 and reanalyzed accordingly.

MMP-8 concentrations were determined using the commercial kit “Human Total MMP-8, Quantikine ELISA” (R&D Systems, Minneapolis, USA). The assay range was 0.156–10 ng/mL, and the kit’s sensitivity was 0.013 ng/mL.

Specimens for MMP-8 analysis were diluted to 1:10. If the sample's measured optical density surpassed the optical density of the highest standard, the samples were diluted to 1:100 or 1:1000, as appropriate, and tested repeatedly.

The levels of sTLR-2 were determined using the commercial kit "Enzyme-linked Immunosorbent Assay Kit for Toll Like Receptor 2 (TLR2)" (Cloud-clone Corp., Katy, USA), and sTLR-4 levels were determined using the commercial kit "Enzyme-linked Immunosorbent Assay Kit For Toll Like Receptor 4 (TLR4)" (Cloud-clone Corp., Katy, USA). Both assay ranges were 0.312–20 ng/mL for both receptors, with sensitivities of 0.112 ng/mL and 0.118 ng/mL for TLR-2 and sTLR-4, respectively. In both cases, undiluted samples were used.

The concentration of DEFA3 was determined using the commercial kit "Human Defensin Alpha 3, Neutrophil Specific (DEFA3) ELISA Kit" (Abbexa Ltd, Cambridge, UK). The assay range was 0.47–30 ng/mL, and the sensitivity was < 0.21 ng/mL. Samples were diluted to 1:10.

SuPAR concentrations were determined using the commercial kit "Human uPAR Immunoassay, Quantikine ELISA" (R&D Systems, Minneapolis, USA). The assay range was 62.5–4000 pg/mL and the kit's sensitivity was < 33 pg/mL. The samples were diluted to 1:5. If the measured concentrations of analytes exceeded the highest point on the standard curve, dilutions of 1:10 or 1:100 were accomplished, and re-tested.

IL-10 concentrations were determined using the commercial kit "Human IL-10 ELISA^{PRO} kit" (Mabtech, Stockholm, Sweden). The standard range was 3.16–1000 pg/mL, and the minimum detectable dose was 1 pg/mL. The IL-10 samples were diluted 1:2 as specified in the kit methodology and those samples above the limits of the highest standard were diluted 1:10.

The levels of EGF were determined using the commercial kit "Human EGF ELISA kit" (Bender MedSystems, Vienna, Austria). The samples diluted 1:5 were used to determine EGF concentrations. If the measured concentrations of analytes exceeded the highest point on the standard curve, dilutions 1:10 or 1:100 were performed. The analytical sensitivity of human EGF was <1 pg/mL. The assay range was 3.9 – 250 pg/mL.

Immunological AF assays of IL-17 were performed using a „LEGEND MAX Human IL-17A ELISA Kit"(BioLegend, San Diego, USA). A four-parameter logistic (4-PL) standard curve was calculated and plotted between 250 and 3.9 pg/ml, the sensitivity of the kit was 0.8 pg/mL. The samples for IL-17 analysis were used undiluted.

The levels of RANTES were determined by "Human CCL5/RANTES DuoSet ELISA" (R&D Systems, Minneapolis, USA). A standard curve was calculated and plotted in the 15.6–1.000 pg/mL range and RANTES samples were diluted as specified in the kit methodology.

The concentrations of amniotic fluid biomarkers (IL-6, MMP-8, TNF-α, sTLR-2, sTLR-4, suPAR, DEFA3, IL-10, IL-17, RANTES, and EGF) were concealed from investigators and clinical personnel.

3.4 Collected neonatal data

After birth, newborns were also registered for the study. Neonatal evaluations were conducted after birth, and follow-up assessments were performed before discharge from the hospital. Neonatal data including birth weight, Apgar scores, the necessity for neonatal resuscitation, and laboratory data such as umbilical cord arterial blood pH, umbilical cord blood IL-6, WBC, immature to total (I/T) neutrophil ratio in the blood 6-24 hours after birth, CRP, blood culture, and chest radiography were collected. The umbilical cord IL-6 concentration was determined in umbilical cord serum by automated chemiluminescent enzyme immunoassay using a kit (DPC, Los Angeles, CA, USA). The following outcomes were included: RDS, the need for surfactant, duration (in days) and type of respiratory support (mechanical ventilation, non-invasive respiratory support, or none), BPD, early-onset sepsis, early hypotension, NICU duration, PDA and PDA treatment (medications, surgery, or none), IVH with grading, ROP, ROP treatment, and death.

According to national RDS management guidelines, adapted from European Consensus guidelines [171], RDS was diagnosed based on a combination of respiratory symptoms observed in a preterm newborn requiring respiratory support after delivery, along with findings on chest radiography. These findings typically include low lung volumes with a 'reticulogranular' appearance and air bronchograms [172]. The severity of RDS was determined according to chest radiography findings: no RDS – no typical findings, mild RDS (Grade 1) – a diffuse, linear granular pattern; moderate RDS (Grade 2) – bilateral widespread more prominent air bronchograms, severe RDS (Grade 3 and 4) – the opaque lungs and/or alveolar shadowing obscuring the cardiac border [172]. As per European guidelines, surfactant therapy was administered to all neonates ≤ 26 weeks GA prophylactically and to infants > 26 weeks GA based on clinical evaluations of breathing and an inspired oxygen requirement of > 0.3 – 0.35 (very early in the clinical course) [171]. Bronchopulmonary dysplasia was diagnosed based

on the need for oxygen supplementation or respiratory support at 28 days postnatal age, 36 weeks postmenstrual age, or until discharge to home.

Early hypotension was defined as systolic and/or mean blood pressure falling below the third percentile for gestational age during the transitional period, necessitating vasoactive medications [173,174].

Early-onset sepsis (EOS) was defined as clinical and/or laboratory evidence of sepsis with a positive or negative blood culture occurring within 72 hours after birth according to the hospital's EOS protocol [175,176].

Intraventricular hemorrhage was diagnosed upon detection of intraventricular bleeding via cranial ultrasound. The severity of IVH was determined using adapted Papile and Volpe classifications: Grade 1 – subependymal hemorrhage limited to germinal matrix; Grade II – IVH without ventricular dilatation, with blood occupying < 50% of the ventricle; Grade III - IVH with ventricular dilatation and blood occupying > 50% of the ventricle. Grade IV – IVH with parenchymal extension (hemorrhagic venous infarction in the periventricular white matter). Grade III and IV were categorized as severe IVH [177,178].

Patent ductus arteriosus was defined as a hemodynamically significant PDA persisting beyond 72 hours of age, as per the hospital's protocol. The criteria for diagnosing significant PDA were a ductal diameter exceeding 1.5 mm, a left atrium-to-aorta ratio surpassing 1.4 in the parasternal long axis view, a pulmonary artery-to-aorta ratio greater than 1.2, along with a left ventricular and left atrium enlargement, and holodiastolic flow reversal in the descending aorta [179,180].

Retinopathy of prematurity diagnosis was based on the presence of retinal vascular and capillary proliferation within specific zones and stages as outlined in The International Classification of Retinopathy of Prematurity [181] and the hospital's protocol. Severe ROP was identified for cases necessitating surgical intervention. The screening was conducted by a designated hospital ophthalmologist.

The prevalence of individual neonatal outcome parameters was rather low. Therefore, we used a substituted composite outcome of mortality and severe morbidity as in other neonatal studies [11,25]. Major neonatal outcomes were diagnosed if one or more of the following were present: severe RDS, requirement for mechanical ventilation, death, EOS, early hypotension, severe IVH, BPD, and severe ROP.

According to current hospital guidelines [170] for preterm birth, the placentas of all women underwent histological examination after delivery. A single investigator at the State Pathology Centre conducted histological examinations of the placentas. Inflammatory lesions in the placenta, fetal membranes, and umbilical cord were assessed following the guidelines outlined by the Amsterdam Placental Workshop Group Consensus and the Society for Paediatric Pathology Diagnostic criteria for intraamniotic infection (Table 1 and Table 2). Histological chorioamnionitis was defined by the infiltration of neutrophils into the choriodecidua and amnion. Funisitis was identified by neutrophilic infiltration in the umbilical vascular wall or Wharton's jell [34,138]. FIRS was defined according to the umbilical cord blood IL-6 levels > 11 pg/mL and/or histological funisitis [5,6,32].

3.5 Study methodology

The study was divided into three parts (Figure 5).

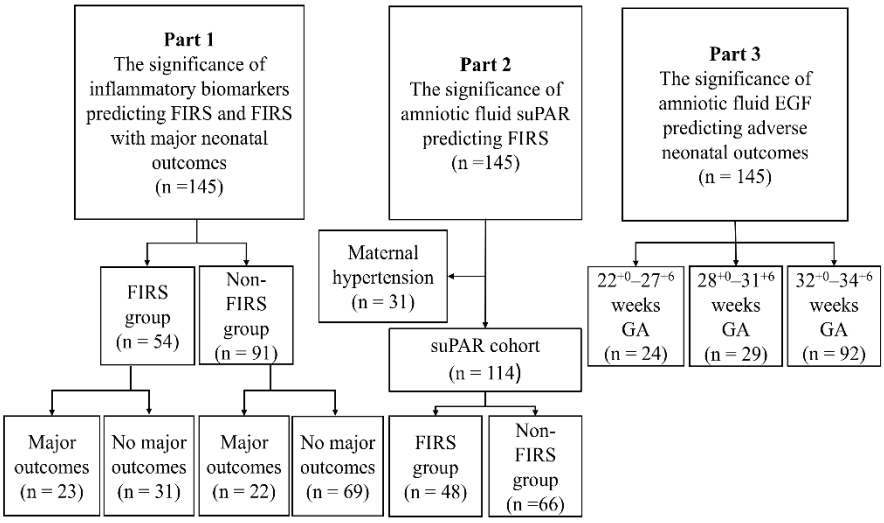


Figure 5. Scheme of thesis parts.

3.5.1 Part 1: Immunological biomarkers predicting FIRS and FIRS with the major neonatal outcome.

In the first part of the study, we aimed to evaluate the significance of inflammatory biomarkers in predicting FIRS and FIRS with major adverse neonatal outcomes. The study population consisted of singleton pregnant women with PPROM at 22^{+0}-34^{+6} weeks of gestation who were admitted to

VUH SK between 2017 and 2020, as well as their newborns, based on the inclusion and exclusion criteria of the study. After enrollment, 34 patients were excluded due to inadequate specimens (contaminated with mucus, blood, and lower volume), and six cases were excluded due to severe congenital anomalies that were undiagnosed antenatally. Figure 6 presents the patient flow diagram, showing 145 participants in the final analysis. The study population was then divided into the FIRS group (n=54) and the non-FIRS group (n=91) based on FIRS diagnosis.

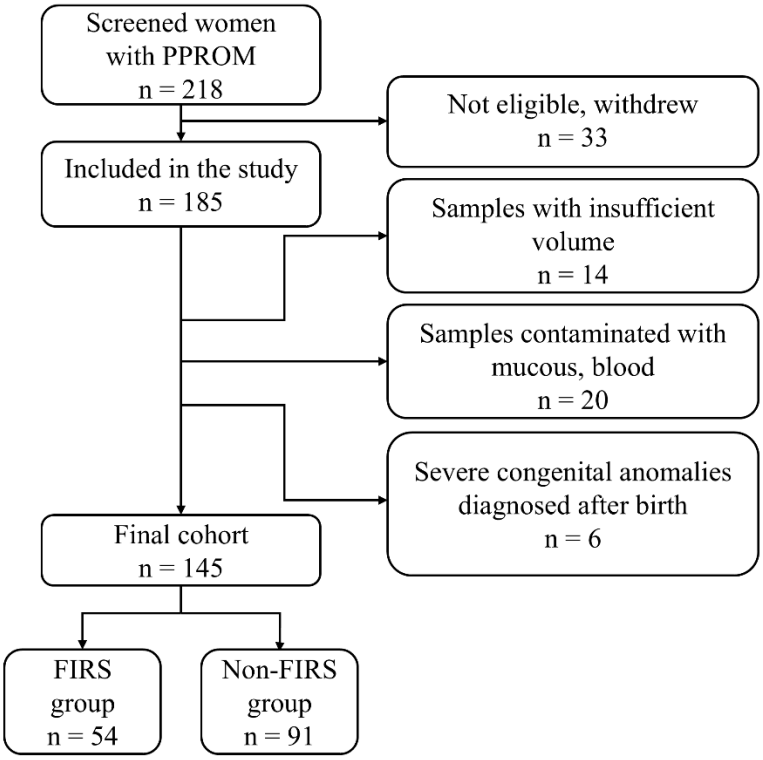


Figure 6. The patient flow diagram for Part 1.

For the analysis of FIRS with major outcomes, each group was further assessed based on the presence or absence of major neonatal outcomes, a composite variable that included neonatal mortality and severe morbidity. Major neonatal outcomes were diagnosed if one or more of the following were present: severe RDS, requirement for mechanical ventilation, death, EOS, early hypotension, severe IVH, BPD, and severe ROP.

3.5.2 Part 2: SUPAR cohort

In the second part of the study, we aimed to evaluate the significance of suPAR in predicting FIRS and FIRS with major adverse neonatal outcomes. Due to suPAR association with hypertensive diseases, we retrospectively excluded all cases with maternal hypertensive disorders for the assessment of suPAR in vaginally collected amniotic fluid. Figure 7 shows the patient flow diagram with 114 participants in the final analysis. The study cohort of 114 women with PPROM before 34^{+6} weeks of gestation and their newborns was divided into the FIRS group (n=48) and the non-FIRS group (n=66).

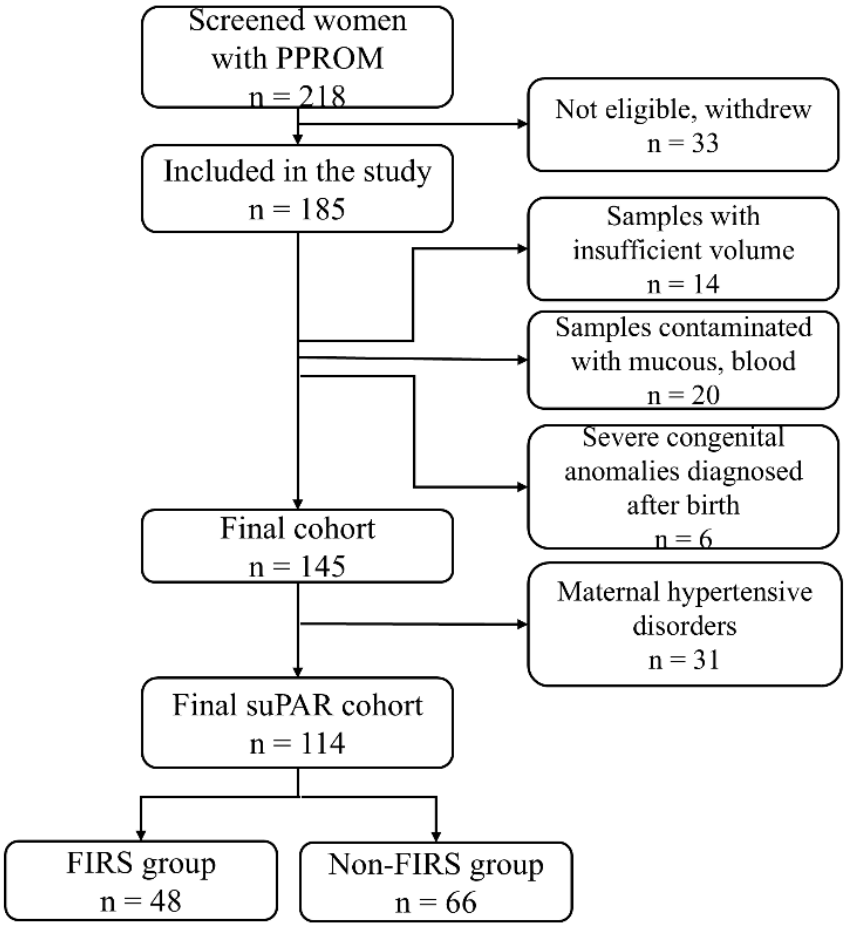


Figure 7. The patient flow diagram for Part 2.

3.5.3 Part 3: Amniotic fluid EGF in predicting adverse neonatal outcomes

In the third part of the study, we aimed to evaluate the significance of EGF in predicting adverse neonatal outcomes. The study population of 145 women and their neonates was categorized into three gestational age groups based on the WHO classification [182]: extremely preterm infants (22^{+0}–27^{+6} weeks GA), very preterm infants (28^{+0}–31^{+6} weeks GA), and moderate to late preterm infants (32^{+0}–34^{+6} weeks GA). Figure 8 presents the flow chart of Part 3.

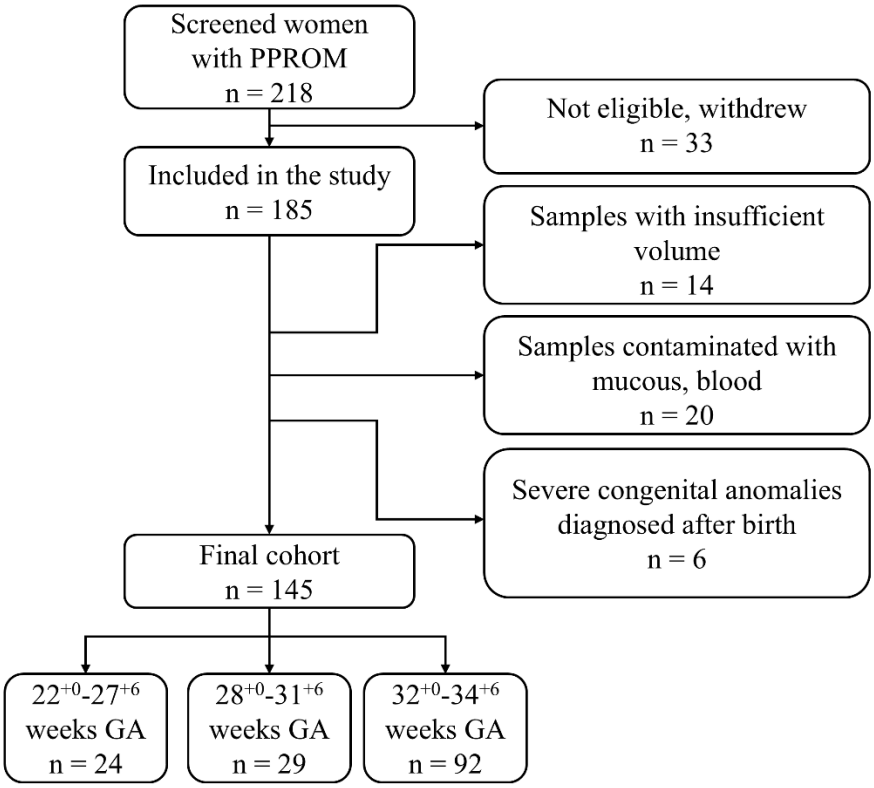


Figure 8. The patient flow diagram for Part 3.

3.6 Study organization

The PhD student was one of the study investigators, responsible for organizing the study process, preparing the protocol and documentation for study permissions, managing participant enrollment, collecting maternal and neonatal data, conducting statistical analyses, and writing publications.

3.7 Calculation of the sample size

The G*Power software (version 3.1.9.4) was used to determine the sample size. The significance level of the criterion was set at $\alpha = 0.05$. The power of the criterion was considered to be at least 0.8 ($0.8 < \beta < 0.9$). The sample size chosen was appropriate.

3.8 Statistical analysis

Statistical analysis was conducted using R software version R–4.0.5 (R Core Team, 2021). The distribution of continuous data was ensured by comparing means and medians, visually assessing histograms, and considering skewness parameters. Continuous data are presented as means with standard deviation (SD) or medians with interquartile range (IQR), as appropriate. Non-normally distributed variables were log-transformed for further analysis. Categorical variables are presented as frequencies and percentages.

Parametric (two-sample Student t-test) or non-parametric (Mann-Whitney-Wilcoxon rank sum test) tests were employed, as appropriate, to compare continuous variables between two independent groups. Continuous variables among multiple independent groups were assessed using either analysis of variance (ANOVA) or the Kruskal-Wallis test, depending on the data distribution. Spearman correlation analysis assessed relationships between biomarkers and maternal and neonatal parameters in a correlation matrix. In the matrix, each rectangle indicates the correlation between two variables. Blue represents positive correlations, and red signifies negative correlations, with color intensity reflecting the strength of correlation coefficients. The numbers in the rectangles indicate the correlation coefficients of each interaction and the numbers in the complementing correlation matrix represent p values. The legend on the right displays correlation coefficients alongside their respective colors.

The receiver operator curves (ROC) were plotted and the areas under the curve (AUC) were calculated to assess the prediction performance and diagnostic characteristics of all biomarkers in non-invasively obtained amniotic fluid for FIRS. The DeLong test was used to compare the area under different models' curves (AUC). The Youden index determined the best cut-off values.

Using univariate logistic regression analysis, we estimated biomarkers as predictors. We determined the odds ratio (OR) with a 95% confidence interval

(CI) for the outcomes, as follows: FIRS, FIRS with major outcomes, respiratory outcomes, PDA, ROP, and early hypotension. Variables demonstrating a p value < 0.05 in the univariate regression analysis were chosen for inclusion in the multivariable model construction. Stepwise multivariable (forward-backward) logistic regression for FIRS and FIRS with major outcomes with forced gestational age was constructed with model selection based on the Akaike Information Criteria (AIC). The multiple regression analysis was applied to estimate the reliability of biomarkers predicting FIRS and FIRS with major outcomes, controlling for GA. Estimated coefficients, p values, and odds ratios with confidence intervals were reported. The univariate and multivariate logistic regression was also performed using optimal cut-off values of biomarkers.

Random Forest analysis was conducted to predict FIRS, FIRS with major outcomes, and various major outcomes separately, and to rank the importance of predictors. The test classification accuracy of Random Forest models with sensitivity and specificity was determined. A Shapley Additive Explanations (SHAP) summary plot was generated to visualize the impact of individual features on the Random Forest model's predictions. The x-axis demonstrates Shapley values (impact on the model output and direction). On the y-axis, the features (predictors) are listed in descending order of their importance in the Random Forest model. SHAP feature importance represents how a feature influences the prediction of a single row relative to the other features in that row and to the average outcome in the dataset. The colors represent the feature values of predictors: higher values are in red-orange, and lower values are in dark blue.

It can be noted that Random Forest often outperforms logistic regression in predictive accuracy, especially with complex, non-linear relationships, due to its ensemble learning approach. SHAP summary plot effectively identifies the strength and direction of association between FIRS or adverse neonatal outcomes and its major predictors. However, logistic regression remains advantageous for interpretability and understanding the influence of individual predictors in linear relationships.

The two-sided p value of <.05 was considered significant.

4. RESULTS

4.1 Part 1: Immunological biomarkers predicting FIRS and FIRS with major neonatal outcomes

4.1.1 Study population characteristics

The study population was divided into the FIRS group (n=54) and the non-FIRS group (n=91) based on FIRS diagnosis. Table 4 shows the demographic and clinical characteristics of the study population. Most maternal factors did not differ between groups, except for a higher frequency of diagnosed hypertensive disorders in the non-FIRS group. As expected, the FIRS group exhibited a higher rate of spontaneous delivery. Both clinical and histological chorioamnionitis were more prevalent in the FIRS group. Newborns in the FIRS group had smaller birth weight and lower gestational age. They were more likely to have lower Apgar scores of less than seven after the 1st and 5th minute and a higher need for resuscitation after birth compared to those without FIRS. The FIRS group also presented a higher incidence of respiratory issues, notably severe RDS, and an elevated need for respiratory support, particularly mechanical ventilation. Furthermore, there was an increased incidence of EOS and ROP in the FIRS group than in the non-FIRS group. However, no significant differences were observed between the groups regarding median umbilical cord arterial pH and neonatal death rates, early hypotension, PDA, BPD, and IVH.

Table 4. Demographic and clinical characteristics of the study population.

Characteristics	FIRS group (n=54)	Non-FIRS group (n=91)	P value
Maternal characteristics			
Maternal age, years, mean ± SD	31.22 ±5.5	31.26 ±5.7	0.966
Latency period, hours, median, IQR	64 (16–200)	75 (34–216)	0.408
Hypertensive disorders, n, %	6 (11)	25 (28)	0.020
Gestational diabetes, n, %	12 (22)	22 (24)	0.761
Group B streptococcus test positive, n, %	7(13)	10 (11)	0.444
Primigravida, n, %	20 (37)	30 (33)	0.618
Primiparous, n, %	23 (43)	45 (50)	0.424
Antenatal glucocorticoids, n, %	47 (87)	78 (86)	0.823

Characteristics	FIRS group (n=54)	Non-FIRS group (n=91)	P value
Tocolytics, n, %	23 (43)	41 (48)	0.501
Mode of delivery, n, % vaginal delivery cesarean section	 46 (85) 8 (15)	 68 (75) 23 (25)	0.079
Induction of delivery or cesarean section n, % Spontaneous delivery n, %	22 (41) 32 (59)	56 (61) 35 (39)	0.015
Clinical chorioamnionitis, n, %	6 (11)	1 (1)	0.011
Histological chorioamnionitis, n, %	38 (70)	16 (18)	<0.001
Funisitis, n, %	21 (39)	0	<0.001
Neonatal characteristics			
Gestational age at birth, weeks, median, IQR	31.5 (28–33)	33.0 (31–34)	0.003
Birth weight, grams, mean ± SD	1675 ± 640	1938 ± 613	0.020
Apgar scores <7 at 1 min., n, %	14 (26)	8 (9)	0.005
Apgar scores <7 at 5 min., n, %	6 (11)	1 (1)	0.007
Umbilical cord arterial pH, mean ± SD	7.34 ± 0.09	7.32 ± 0.09	0.331
The need for resuscitation after birth, n, %	14 (26)	8 (9)	0.008
Major outcome, n, %	22 (42)	22 (24)	0.029
Respiratory distress n, %	51 (94)	71 (78)	0.009
Severe RDS, n, %	12 (22)	16 (18)	0.049
Respiratory support, n, % None Mechanical ventilation Non-invasive respiratory therapy	 3 (6) 12 (22) 39 (72)	 20 (22) 13 (14) 58 (64)	0.025
The need for surfactant, n, %	16 (30)	25 (28)	0.812
Neonatal death, n, %	2 (4)	0	0.193
Sepsis, n, %	8 (15)	7 (8)	0.173
Early-onset sepsis, n, %	7 (13)	2 (2)	0.009
Early hypotension, n, %	10 (19)	8 (9)	0.086
Bronchopulmonary dysplasia, n, %	9 (17)	8 (9)	0.142
Patent ductus arteriosus, n %	9 (17)	10 (11)	0.305
Retinopathy of prematurity, n, %	16 (30)	9 (10)	0.003
Treated retinopathy of prematurity, n, %	3 (6)	2 (2)	0.358
Intraventricular hemorrhage, n, % None 1-2 grade 3-4 grade	 34 (63) 15 (28) 5 (9)	 64 (70) 23 (25) 4 (4)	0.437

4.1.2 Biomarkers' characteristics in amniotic fluid and umbilical cord blood

The summary characteristics of all biomarkers in amniotic fluid obtained vaginally, as well as the characteristics of IL-6 in umbilical cord blood, are presented in Table 5.

Table 5. Biomarkers' characteristics in amniotic fluid and umbilical cord blood. Amniotic fluid biomarker variables that were not normally distributed were log- or squared transformed.

	Mean	SD	Median	Q1	Q3	Min	Max
MMP-8 (ng/mL)	701.89	1311.96	147.18	25.95	710.27	0.03	8190.84
TNF-α (pg/mL)	506.40	1977.61	20.29	7.94	108.08	3.26	13342.63
IL-6 (pg/mL)	25686.97	63119.54	2600.91	564.09	15898.74	20.59	433001.59
IL-10 (pg/mL)	74.95	161.54	10.71	0.00	59.33	0.00	882.32
IL-17 (pg/mL)	5.71	18.46	0.648	0.23	2.93	0.00	162.29
sTLR-2 (ng/mL)	0.38	0.50	0.20	0.06	0.43	0.002	2.39
sTLR-4 (ng/mL)	0.44	0.46	0.30	0.10	0.56	0.00	2.50
uIL-6 (pg/mL)	547.52	3845.53	8.21	3.79	38.83	0.00	40000.00
EGF (pg/mL)	71708.3	523092.41	81.15	41.07	133.38	3.56	4897359.0
RANTES (pg/mL)	948.35	1418.70	397.59	114.03	1115.96	0.00	8427.27
DEFA3 (ng/mL)	24.95	16.30	21.18	14.833	31.153	3.566	132.865
Log-TNF-α	3.72	1.99	3.01	2.07	4.68	1.18	9.50
Log-IL-6	7.93	2.20	7.86	6.34	9.67	3.02	12.98
Log-IL-10	2.53	2.04	2.46	0.00	4.10	0.00	6.78
Log-IL-17	0.95	1.06	0.50	0.21	1.37	0.00	5.10
Log-MMP-8	4.88	2.14	4.99	3.29	6.57	0.03	9.01
Log-RANTES	5.78	1.71	5.99	4.75	5.99	0.00	9.04
Log-TLR2	0.28	0.29	0.19	0.06	0.36	0.001	1.22
Sqrt-TLR4	1.19	0.17	1.14	1.05	1.25	1	1.87
Log-EGF	4.59	2.20	4.40	3.72	4.89	1.27	15.40
Log-DEFA3	3.03	0.63	3.05	2.70	3.44	1.27	4.89

4.1.3 Correlation analysis

Spearman correlation analysis assessed the correlation between biomarkers and their relationship with maternal and neonatal parameters, as depicted in Figure 9. The figure illustrates a correlation matrix, a tabular representation showing correlation coefficients among variables. Figure 10 complements Figure 9, showing the corresponding p values of each interaction between variables. Several important interactions are highlighted below.

Most of the immunological biomarkers in the vaginally collected amniotic fluid, umbilical cord blood, maternal and neonatal blood show different degrees of correlation with each other. For instance, amniotic fluid biomarkers, such as TNF-α, IL-10, IL-6, IL-17 and MMP-8 demonstrate moderate to strong positive correlations with each other, indicating potential relationships between these inflammatory markers and coordinated immune responses in inflammatory conditions.

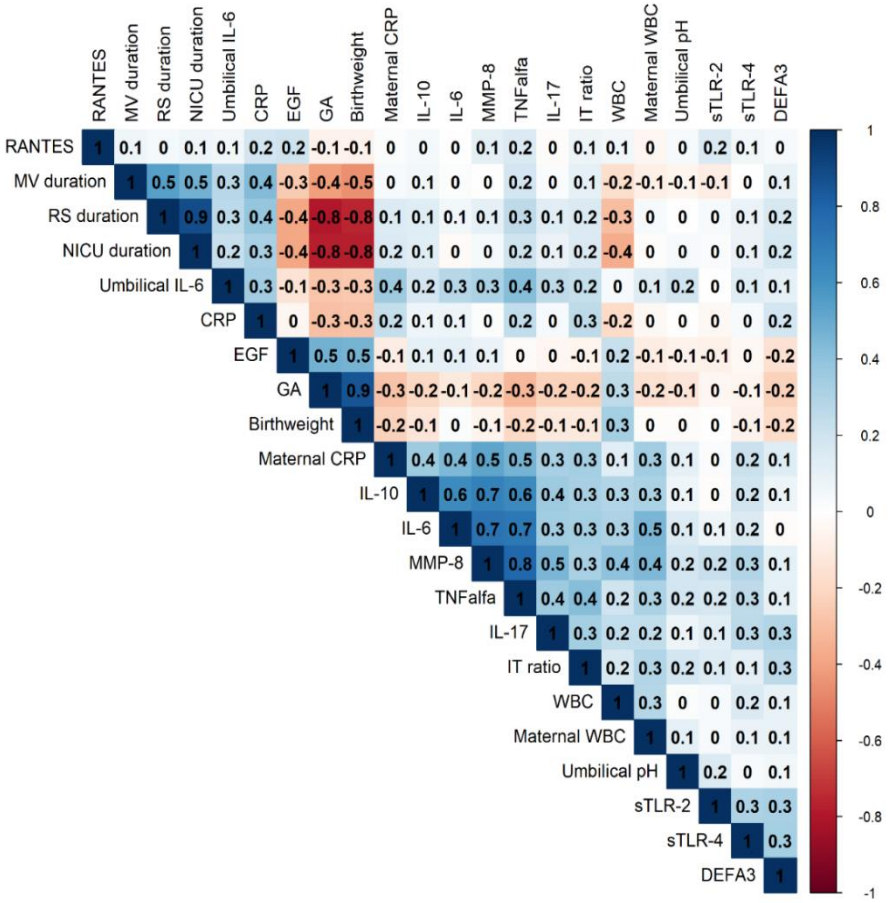


Figure 9. Spearman correlation analysis assessing relationships between biomarkers and maternal and neonatal parameters in a correlation matrix. In the matrix, each rectangle indicates the correlation between two variables. Blue represents positive correlations, and red signifies negative correlations, with color intensity reflecting the strength of correlation coefficients. The numbers in the rectangles indicate the correlation coefficients of each interaction. The legend on the right displays correlation coefficients alongside their respective colors.

Maternal blood biomarker CRP displays a positive moderate correlation with amniotic fluid cytokines, whereas maternal WBC and neonatal blood markers, such as CRP, I/T ratio, and WBC, showed weak correlations with each other and with cytokines. DEFA3, sTLR-2, sTLR-4, and RANTES demonstrate mostly weak or insignificant correlations with other variables.

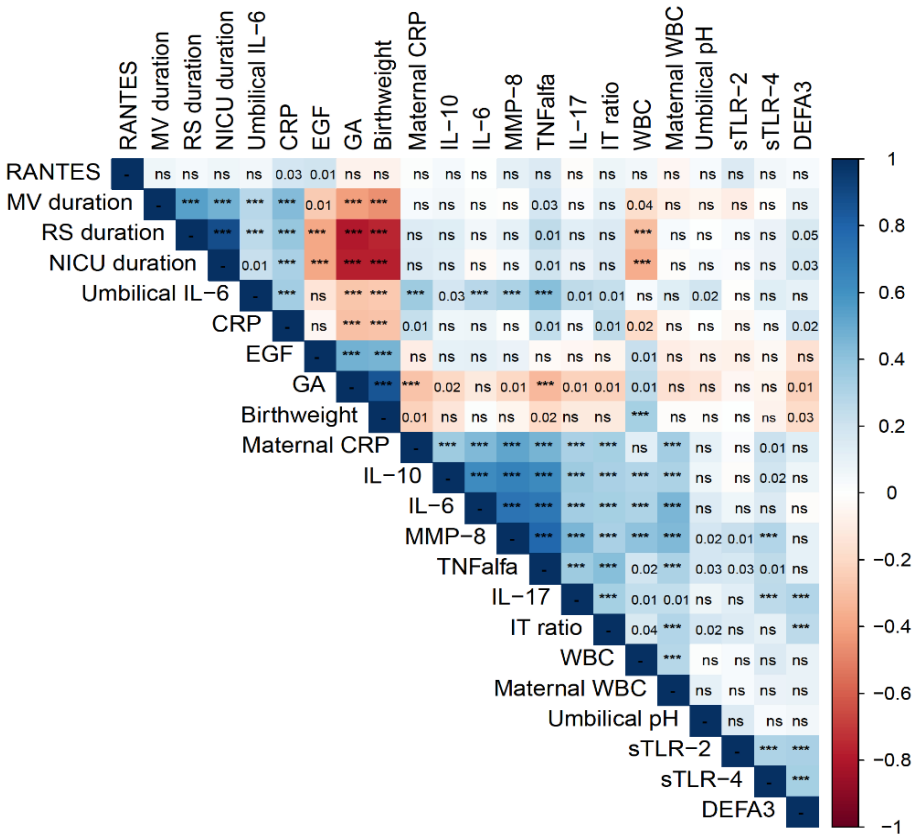


Figure 10. Spearman correlation analysis between biomarkers and maternal and neonatal parameters presenting p values of each variable interaction. P values less than 0.001 are marked as three asterisks (***); not significant results $p > 0.05$ are marked as ns.

Neonatal growth and respiratory health parameters form a significant cluster of correlations. Birthweight and gestational age demonstrate strong positive correlations, aligning with established fetal growth patterns. Measures related to respiratory morbidities, such as respiratory support (RS) duration, and mechanical ventilation (MV) duration, and measures, representing the general well-being of preterm neonates, such as treatment duration in NICU, demonstrate strong to moderate positive correlations, suggesting a shared pathophysiological basis and potential co-occurrence in affected neonates. As expected, respiratory parameters and NICU duration showed a strong negative association with GA and birth weight, implying that preterm infants with lower GA and birthweight tend to have longer respiratory

support, mechanical ventilation, and NICU stay. Surprisingly, in this cluster, EGF has appeared, revealing a moderate negative correlation with respiratory parameters and a positive moderate correlation with GA and birthweight.

4.1.4 The association of amniotic fluid biomarkers levels with FIRS

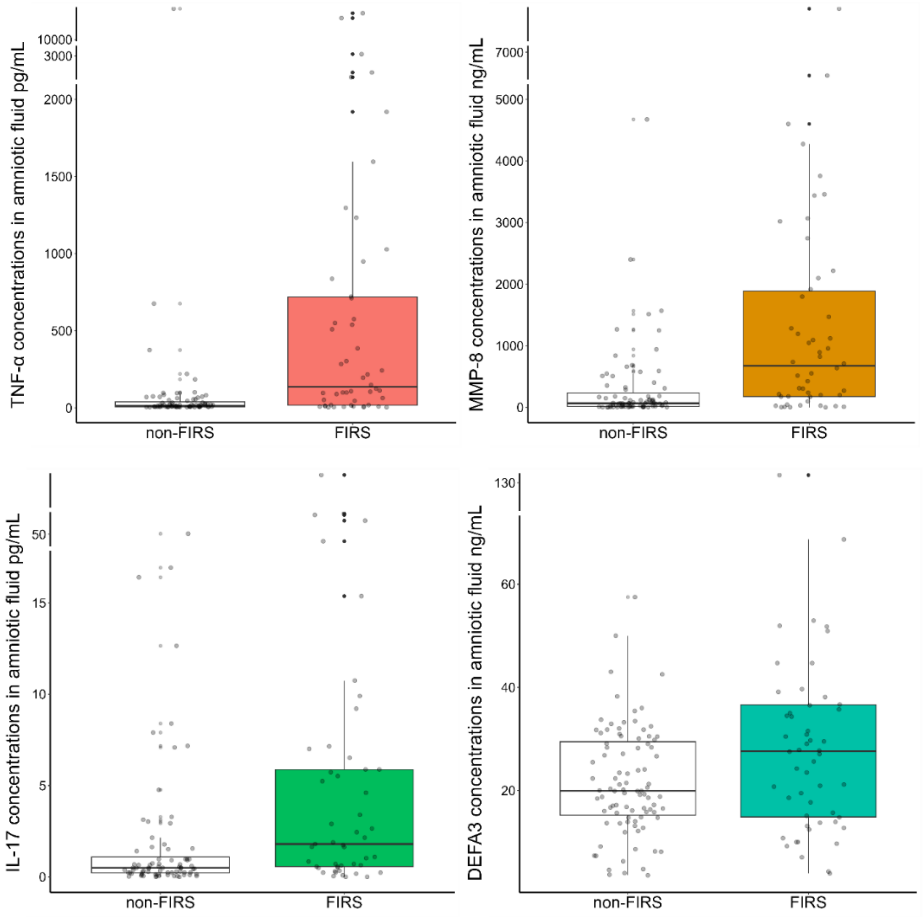
4.1.4.1 The comparison of biomarkers medians between groups

We compared the median levels of biomarkers in vaginal amniotic fluid in the FIRS group and the non-FIRS group (Table 6, Figure 11 and Figure 12). The results are presented using the Wilcoxon test, including medians and IQR.

Table 6. The concentrations (median and IQR) of biomarkers in vaginal amniotic fluid according to the presence and absence of FIRS. Amniotic fluid biomarker variables that were not normally distributed were log- or squared transformed (mean and SD).

Biomarker	FIRS group		Non-FIRS group		P value
	Median	IQR	Median	IQR	
MMP-8 (ng/mL)	673.30	175.02-1886.61	65.57	15.69-34.54	<0.001
TNF-α (pg/mL)	136.43	17.65-18.59	14.35	6.71-9.58	<0.001
IL-6 (pg/mL)	4079.09	2761.82-97294.34	1425.45	437.53-3301.46	<0.001
IL-10 (pg/mL)	59.43	5.21-170.35	6.26	0-21.24	<0.001
IL-17 (pg/mL)	1.80	0.56-5.88	0.50	0.21-1.09	<0.001
sTLR-2 (ng/mL)	0.22	0.06-0.64	0.197	0.06-0.39	0.472
sTLR-4 (ng/mL)	0.29	0.15-0.56	0.30	0.08-0.56	0.453
uIL-6 (pg/mL)	52.70	18.15-184.25	4.16	2.97-5.87	<0.001
EGF (pg/mL)	80.95	37.70-126.61	84.13	42.67-147.86	0.436
RANTES (pg/mL)	335.45	160.75-935.30	476.32	108.28-1223.25	0.501
DEFA3 (ng/mL)	27.63	14.87-36.65	19.94	15.23-29.47	0.025
	Mean	SD	Mean	SD	P value
Log-TNF-α	4.95	2.20	2.95	1.37	<0.001
Log-MMP-8	5.93	2.14	4.20	1.86	<0.001
Log-IL-6	9.15	2.38	7.21	1.72	<0.001
Log-IL-10	3.53	2.17	1.92	1.71	<0.001
Log-IL-17	1.38	1.26	0.67	0.79	<0.001
Log-EGF	4.35	1.10	4.73	2.13	0.159
Log-TLR2	0.32	0.34	0.25	0.25	0.352
Sqrt-TLR4	1.19	0.16	1.18	0.18	0.563
Log-RANTES	5.71	1.40	5.82	1.87	0.693
Log-DEFA3	3.19	0.70	2.94	0.58	0.032

In vaginal amniotic fluid, the median concentrations of MMP-8, TNF-α, IL-6, IL-10, IL-17, and DEFA3 were higher in the FIRS group than in the non-FIRS group (Figure 11); the difference was statistically significant ($P < 0.001$ for MMP-8, TNF-α, IL-6, IL-10, IL-17; and $P = 0.025$ for DEFA3). The levels of other markers, such as sTLR-2, sTLR-4, EGF, and RANTES, were not significantly different in these groups (Figure 12).



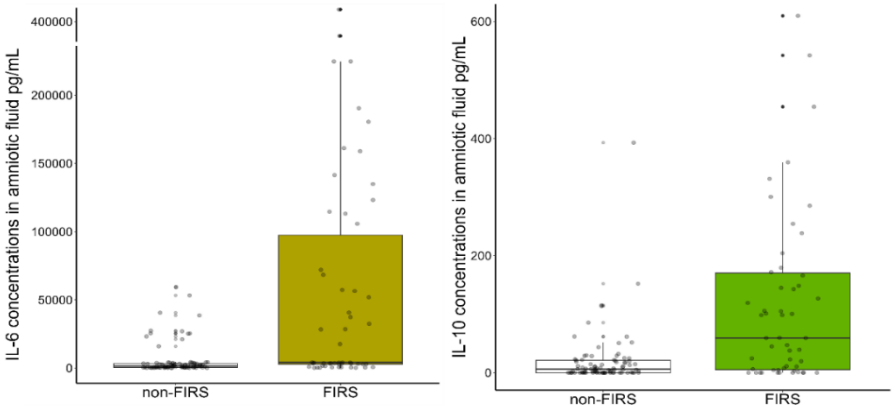


Figure 11. Comparison of the levels of immunological biomarkers, that differed significantly, in vaginal amniotic fluid between FIRS and non-FIRS groups.

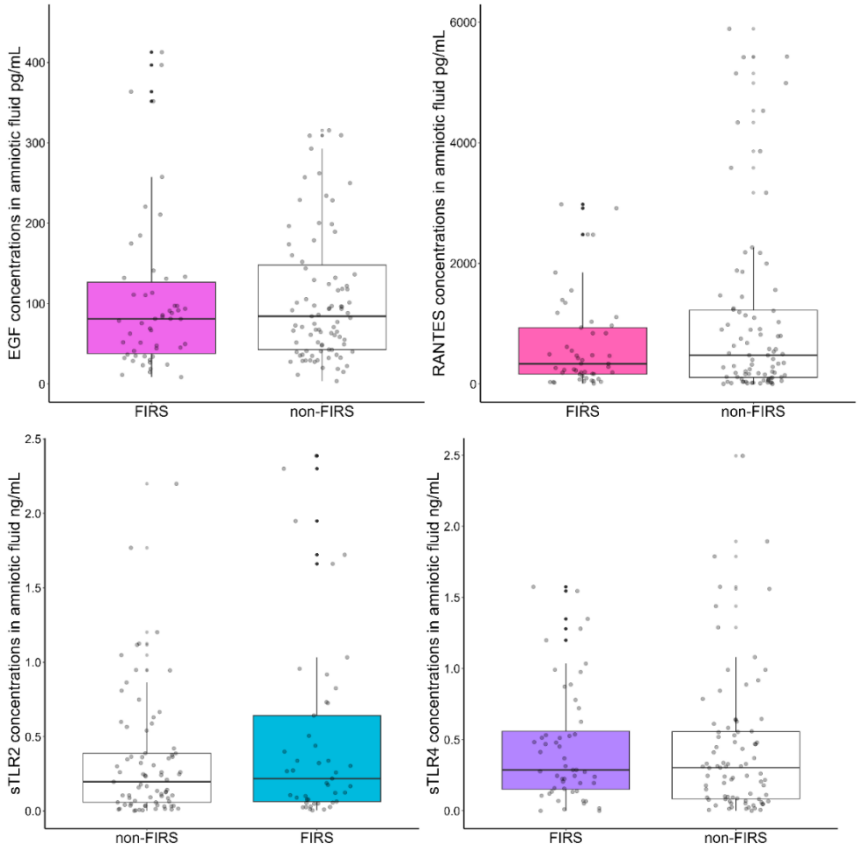


Figure 12. Comparison of the levels of immunological biomarkers, that did not differ significantly, in vaginal amniotic fluid between FIRS and non-FIRS groups.

4.1.4.2 ROC curves analysis and cut-off values for predicting FIRS

The receiver operator curves were plotted and the areas under the curve were calculated to assess the prediction performance of all biomarkers in non-invasively obtained amniotic fluid for FIRS (Figures 13 and 14, Table 7). Table 7 displays the AUCs, the cutoff values, and diagnostic characteristics of inflammatory markers in vaginal amniotic fluid identifying FIRS. TNF-α and MMP-8 demonstrated good discriminatory power (AUC of 0.77 and 0.75, respectively) in distinguishing between cases of FIRS and non-FIRS in the dataset. The specificity and sensitivity values indicate that MMP-8 can correctly identify a significant portion of true negatives and true positives, respectively. The positive predictive value (PPV), however, is relatively lower, indicating that there might be a notable number of false positives among the positive predictions. Furthermore, while the TNF-α specificity is high, indicating a low rate of false positives, the sensitivity is moderate, indicating that there is room for improvement in correctly identifying true positives. IL-6, IL-10 and IL-17 revealed moderate discriminatory capability, with AUC values of 0.74, 0.72, and 0.69, respectively, in recognizing between FIRS and non-FIRS groups. The test displays a reasonably balanced performance of IL-6 and IL-17 featuring moderate sensitivity and specificity. PPV of IL-6 and IL-17 suggests the possibility of a significant number of false positives among the positive predictions. Whereas IL-10, like TNF-α, indicates that while this cytokine has good specificity, its sensitivity is relatively lower, suggesting that it might be better at correctly identifying true negatives than true positives. DEFA3 showed weak discriminatory power (AUC of 0.61) in distinguishing between cases of FIRS and non-FIRS. The ROC analysis for sTLR-2, sTLR-4, RANTES and EGF in predicting FIRS outcomes yields AUC of 0.54, 0.46, 0.54 and 0.54, respectively, indicating limited effectiveness as predictors in distinguishing between FIRS and non-FIRS. Based on these findings, we excluded the following cytokines – sTLR-2, sTLR-4, RANTES, and EGF– from further analysis of FIRS.

Table 7. The cutoff values and diagnostic characteristics of inflammatory biomarkers in amniotic fluid identifying FIRS.

Biomarker	Cutoff value	AUC 95% CI		Sensitivity		Specificity		PPV		NPV	
				%	95% CI	%	95% CI	%	95% CI	%	95% CI
MMP-8	170.76	0.75	0.65-0.84	78	64-88	71	61-81	63	55-72	84	76-91
TNF-α	89.20	0.77	0.67–0.86	66	52-78	90	84-96	81	69-92	81	74-87
IL-6	2695.68	0.74	0.66-0.83	76	65-87	76	65-87	58	50-67	82	75-90
IL-10	35.05	0.72	0.63-0.81	61	48-74	87	80-93	73	63-85	79	74-85
IL-17	1.003	0.69	0.60-0.79	61	47-76	75	64-84	61	51-73	75	68-83
sTLR-2	0.64	0.54	0.43- 0.65	27	13-40	84	75-92	50	32-68	66	62-71
sTLR-4	1.55	0.46	0.36-0.56	98	94-100	6	1-12	40	38-41	83	50-100
EGF	91.63	0.54	0.44-0.64	63	50-76	47	36-57	41	34-49	68	59-77
RANTES	493.45	0.54	0.44-0.63	65	53-80	48	38-59	42	34-49	72	62-80
DEFA3	34.04	0.61	0.51-0.71	35	22-48	92	87-98	73	57-89	71	66-75

Abbreviations: AUC, the area under the curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

Table 8. Comparison of immunological biomarkers in vaginal amniotic fluid predicting FIRS using the DeLong test for two correlated ROC curves. Each count represents the P value of the DeLong test comparing the ROC curves of two biomarkers. The significant differences are marked in grey.

	MMP-8	TNF-α	IL-6	IL-10	IL-17	sTLR-2	sTLR-4	EGF	RANTES	DEFA3
MMP-8	-	0.507	0.751	0.194	0.022	0.009	<0.001	0.015	0.001	0.029
TNF-α	0.507	-	0.364	0.070	0.004	0.003	<0.001	0.006	<0.001	0.011
IL-6	0.751	0.364	-	0.524	0.202	0.006	<0.001	0.004	0.007	0.049
IL-10	0.194	0.070	0.524	-	0.386	0.010	<0.001	0.018	0.024	0.121
IL-17	0.022	0.004	0.202	0.386	-	0.072	0.205	0.066	0.219	0.301
sTLR-2	0.009	0.003	0.006	0.010	0.072	-	0.906	0.928	0.990	0.093
sTLR-4	<0.001	<0.001	<0.001	<0.001	0.205	0.906	-	0.304	0.440	0.101
EGF	0.015	0.006	0.004	0.018	0.066	0.928	0.304	-	0.908	0.283
RANTES	0.001	<0.001	0.007	0.024	0.219	0.990	0.440	0.908	-	0.275
DEFA3	0.029	0.011	0.049	0.121	0.301	0.093	0.101	0.283	0.275	-

The DeLong test was conducted to assess the difference in diagnostic characteristics (AUC) between two correlated ROC curves generated from each immunological biomarker in predicting FIRS. Table 8 presents the test results as p values of the DeLong tests, indicating a statistically significant difference or the lack thereof when comparing two ROC curves of biomarkers. These results suggest that there is no significant difference in the discriminatory power between MMP-8, TNF-α, IL-6, and IL-10, all of them presenting good predicting characteristics. These four biomarkers (MMP-8, TNF-α, IL-6, and IL-10) are statistically significantly different in the prediction performance than sTLR-2, sTLR-4, EGF, RANTES, DEFA3 and demonstrate higher performance in recognizing FIRS. The cytokines, like sTLR-2, sTLR-4, EGF, RANTES, and DEFA3, are not significantly different from each other in distinguishing between cases of FIRS and non-FIRS. IL-17, being a rather moderate predictor of FIRS, has weaker predicting performance than MMP-8 and TNF-α, but the same as the rest biomarkers, based on the DeLong test.

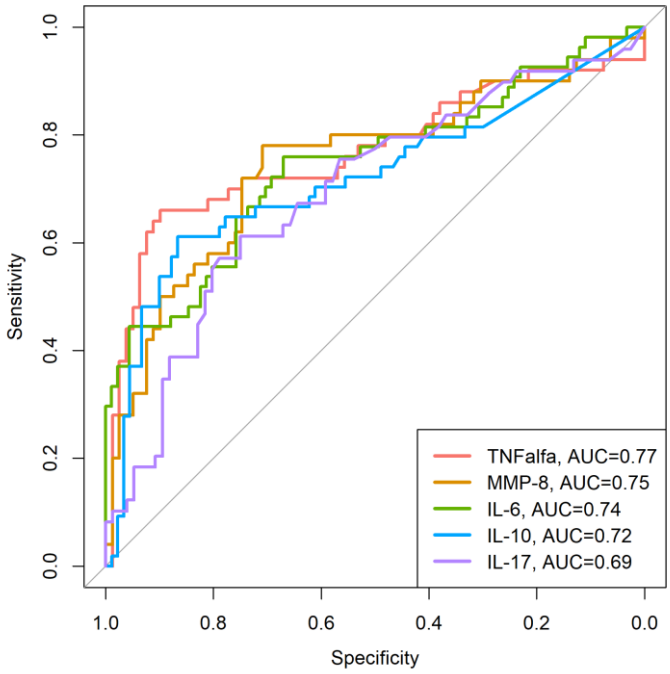


Figure 13. The ROC curves of immunological biomarkers (TNF-α, MMP-8, IL-6, IL-10, IL-10), with the highest AUCs in non-invasively obtained amniotic fluid predicting FIRS.

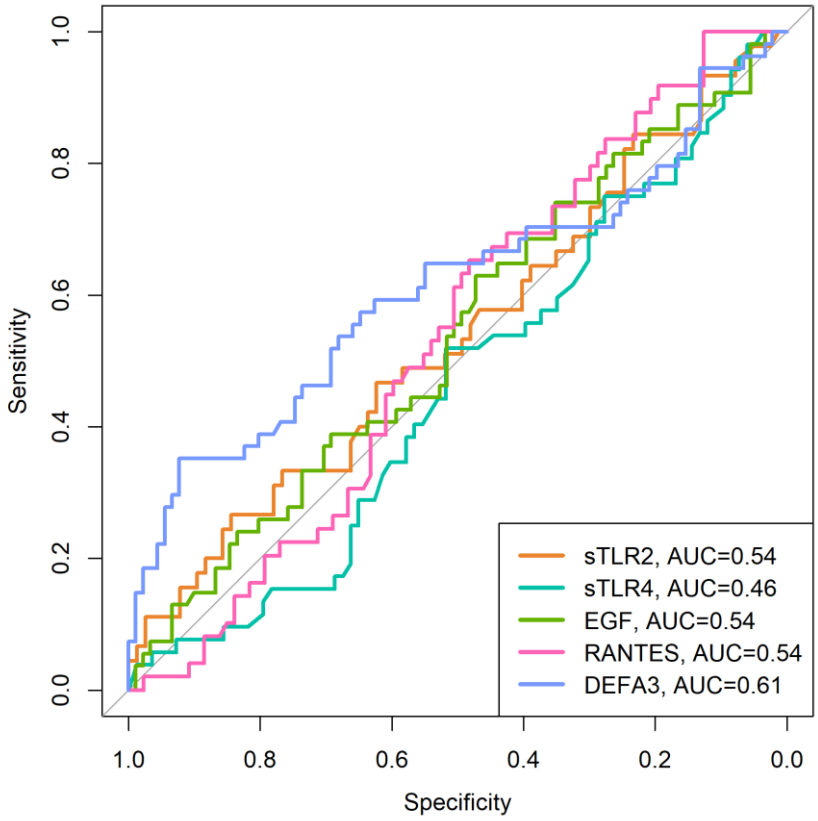


Figure 14. The ROC curves of immunological biomarkers (sTLR-2, sTLR-4, EGF, RANTES, and DEFA3), with the lower AUCs in non-invasively obtained amniotic fluid predicting FIRS.

4.1.4.3 Logistic regression analysis predicting FIRS

For a comprehensive evaluation of biomarkers as predictors and evaluating the odds of FIRS, we conducted univariate and multivariate logistic regression analyses to assess the significance of inflammatory markers in vaginal amniotic fluid predicting FIRS.

Table 9. Univariate and multivariate logistic regression of log-transformed biomarkers in vaginal amniotic fluid as predictors of FIRS. The variables, that were not normally distributed, were log-transformed and used for the analysis.

Biomarker	Univariate logistic regression				
	Estimate	P value	Odds ratio	95% CI	P value
Log-TNF-α	0.608	<0.001	1.84	1.46 - 2.38	<0.001
Log-MMP-8	0.446	<0.001	1.56	1.28 - 1.95	<0.001
Log-IL-6	0.463	<0.001	1.59	1.33 - 1.95	<0.001
Log-IL-10	0.424	<0.001	1.53	1.27 - 1.87	<0.001
Log-IL-17	0.702	<0.001	2.02	1.37 - 3.14	<0.001
Log-DEFA3	0.664	0.026	1.94	1.10 - 3.60	0.026
GA	-0.170	0.004	0.84	0.75-0.94	0.004
Birthweight	-0.001	0.0171	0.99	0.998-0.999	0.017
Hypertensive disorders (ref. yes)	-1.109	0.024	0.33	0.12 - 0.82	0.024
Spontaneous delivery (ref. yes)	0.845	0.016	2.33	1.18 - 4.68	0.016
Multivariate logistic regression N=145, AIC=158.95					
Log-TNF-α	0.635	<0.001	1.83	1.39 - 2.50	<0.001
Log-IL-17	0.072	0.793	1.01	0.61 - 1.69	0.793
Log-DEFA3	0.662	0.062	1.94	0.98 – 4.02	0.063
GA	-0.064	0.440	0.95	0.83 - 1.11	0.440
Hypertensive disorders (ref. yes)	0.429	0.546	0.76	0.23- 2.31	0.546
Spontaneous delivery (ref. yes)	0.416	0.435	1.27	0.51 – 3.11	0.435

In the univariate logistic regression analysis, log-transformed levels of TNF-α, MMP-8, IL-6, IL-10, IL-17, DEFA3, GA, hypertensive disorders, and spontaneous delivery were found to be significantly associated with FIRS (Table 9). However, considerable collinearity was observed among several variables: log-TNF-α and log-MMP-8 (rho = 0.67, p< 0.001), log-TNF-α and log-IL-6 (rho = 0.71, p < 0.001), log-MMP-8 and log-IL-6 (rho = 0.72, p<0.001), log-TNF-α and logIL-10 (rho = 0.65, p<0.001), and GA and birthweight (rho = 0.87, p<0.001). Variables with stronger effect estimates and lower p values were selected for further multivariate logistic regression analysis.

In the multivariate logistic regression analysis, we examined the association between various selected predictor variables (log-transformed levels of TNF-α, IL-17, DEFA3, GA, hypertensive disorders, and spontaneous delivery) and the occurrence of FIRS. The results are presented in Table 9. A significant positive association was observed between log-transformed TNF-α levels and the likelihood of FIRS, with an odds ratio of 1.83 (95% CI: 1.39 - 2.50, $p < 0.001$). The results suggest that log-TNF-α is a significant predictor of FIRS, with higher levels associated with increased odds of FIRS (OR of 1.83 (95% CI: 1.39 - 2.50, $p < 0.001$). While log-DEFA3 showed a marginally significant association, other variables such as log-IL-17, GA, hypertensive disorders, and mode of delivery did not demonstrate significant predictive value for FIRS.

Table 10. Stepwise (forward-backward) logistic regression of log-transformed biomarkers for predicting FIRS controlling for gestational age.

Stepwise logistic regression N=145, AIC=153.68				
Biomarker	Estimate	P value	Odds ratio	95% CI
Log-TNF-α	0.655	<0.001	1.93	1.51 - 2.53
Log-DEFA3	0.653	0.051	1.92	1.01 - 3.81
GA (forced)	-0.037	0.614	0.96	0.84 - 1.11

The stepwise (forward-backward) logistic regression analysis was performed with gestational age as a controlled variable (Table 10). A significant positive association was found between log-transformed TNF-α levels and the likelihood of FIRS. OR was 1.93, indicating that for each unit increase in log-TNF-α, the odds of FIRS increased by a factor of 1.93 (95% CI: 1.51 - 2.53). Log-transformed DEFA3 levels exhibited a marginally significant positive association with FIRS ($p = 0.051$). OR was 1.92, suggesting an increased likelihood of FIRS for higher levels of log-DEFA3 (95% CI: 1.01 - 3.81). Gestational age, included in the model as a forced variable, did not show a significant association with FIRS ($p = 0.614$, OR = 0.96, 95% CI: 0.84 - 1.11). Log-TNF-α and log-DEFA3 demonstrate independent associations with FIRS, underscoring their potential as predictive biomarkers. Including gestational age in the model did not significantly impact these associations.

Due to the dose-dependent effect of biomarkers, we tested predictive models of FIRS with optimal cut-off values in univariate and multivariate logistic regression (Table 11). The cut-off values for amniotic fluid biomarkers

were detected using ROC curves and the Youden index (refer to Table 7). In the univariate logistic regression with cut-offs, we explored the association between selected biomarkers and the probability of a specific outcome, employing pre-defined cut-off values for each biomarker. The selection of biomarkers was based on univariate logistic regression results presented in Table 9 with p values greater than 0.05, including TNF-α, MMP-8, IL-6, IL-10, IL-17, and DEFA3.

TNF-α levels >89 pg/mL were significantly associated with FIRS, with an OR of 14.92 (95% CI: 5.91 – 41.79, $p < 0.001$). Elevated MMP-8 levels >170 ng/mL significantly increased the odds of FIRS 7.62 times (95% CI: 3.37 – 18.32, $p < 0.001$). IL-6 levels > 2695 pg/mL were significantly associated with 6.41 times higher odds of having FIRS. The odds for FIRS were 10.21 times higher with IL-10 level > 35 pg/mL. IL-17 levels > 1 pg/mL were significantly associated with the outcome, with an odds ratio of 4.74 (95% CI: 2.21 - 10.48). DEFA3 levels above 34 ng/mL increased odds of FIRS, yielding an odds ratio of 6.51 (95% CI: 2.61 - 17.97).

With the same set of biomarkers and the addition of two more variables, hypertensive disorders, and spontaneous delivery, we investigated the association between biomarkers and the likelihood of FIRS while considering predefined cut-off values in a combined model of multivariable logistic regression. In this multivariate regression analysis, only two independent variables, TNF-α > 89 and DEFA3 > 34, showed a significant association with increased odds of FIRS, with odds ratio adjusted for GA of 11.50 (95% CI: 2.73 - 57.32, $p = 0.001$) and 3.42 (95% CI: 0.84 - 16.25, $p = 0.025$), respectively. Other covariates did not demonstrate a significant association in this combined model. The multivariate logistic regression analysis provides insights into the potential role of TNF-α, DEFA3, and their respective cut-offs as predictive markers for FIRS.

Table 11. Univariate and multivariate logistic regression using cut-off values of inflammatory biomarkers predicting FIRS.

Univariate logistic regression with cut-offs				
Biomarker	Estimate	P value	Odds ratio	95% CI
TNF-α >89	2.70	<0.001	14.92	5.91 – 41.79
MMP-8 >170	2.03	<0.001	7.62	3.37 – 18.32
IL-6 >2695	1.89	<0.001	6.41	3.06 - 14.16
IL-10 >35	2.32	<0.001	10.21	4.63 - 23.94
IL-17 >1	1.56	<0.001	4.74	2.21 - 10.48
DEFA3 >34	1.87	<0.001	6.51	2.61 - 17.97

Multivariate logistic regression with cut-off; N=145, AIC =143.49				
TNF-α >89	2.44	0.001	11.50	2.73 - 57.32
MMP-8 >170	1.07	0.138	2.90	0.70 - 12.22
IL-6 > 2695	-0.55	0.424	0.47	0.06 - 2.61
IL-10 >35	0.52	0.410	2.12	0.55 - 7.67
IL-17 >1	0.11	0.837	1.33	0.41 - 4.06
DEFA3 >34	1.43	0.025	3.42	0.84 - 16.25
GA	-0.002	0.970	0.96	0.80 - 1.14
Hypertensive disorders (ref. yes)	-0.10	0.885	2.09	0.39 - 12.14
Spontaneous delivery (ref. yes)	-0.50	0.385	0.80	0.20 - 2.80

Table 12. Stepwise (forward-backward) logistic regression using cut-off values of inflammatory biomarkers to predict FIRS controlling for gestational age.

Stepwise logistic regression N=145, AIC=135.44				
Biomarker	Estimate	P value	Odds ratio	95% CI
TNF-α > 89	2.297	<0.001	9.94	3.18 - 34.69
DEFA3 > 34	1.482	0.015	4.40	1.38 - 15.16
MMP-8 > 170	0.904	0.101	2.47	0.81 - 7.23
GA (forced)	-0.005	0.4392	0.99	0.85 - 1.16

Multivariable logistic regression with stepwise (forward-backward) selection confirmed independently observed associations with FIRS (Table 12). The stepwise analysis, incorporating gestational age as a forced factor, identified TNF-α > 89 and DEFA3 > 34 as robust and significant predictors of FIRS. However, despite being selected based on AIC, MMP-8 did not reveal significance as a predictive variable in this model.

4.1.4.4 Random Forest analysis predicting FIRS

Based on our previous findings, we performed Random Forest analysis to predict FIRS and to rank the importance of variables using nine predictors, as follows: TNF-α, MMP-8, IL-6, IL-17, IL-10, DEFA3, GA, hypertension disorders and spontaneous delivery. We tested a model with 300, 500, 1000, 2000, and 3000 trees, and the number of variables to be tested (mtry) was set as the square root of the total number of predictors (the square root of 9 equals 3) with nearby values from 1 to 6. We verified the model using the Out-of-Bag error (OOB). The OOB error rate was lowest when mtry was 3, and it did not improve the model with an increasing number of trees. The test classification accuracy of this random forest model was 80% with sensitivity

estimated to be 63% and specificity to be 92%. The 3 most important predictors of FIRS were TNF-α, IL-10, and IL-6.

A SHAP summary plot was generated to visualize the impact of individual features on the model's predictions (Figure 15). Figure 15 demonstrates that out of the 9 variables selected, higher values of TNF-α, IL-6, and IL-10 were found to be the most important variables in predicting FIRS. Meanwhile, MMP-8, DEFA3 and IL-17 performed moderately. GA, maternal hypertension disorders and spontaneous preterm delivery were the least important predictors of FIRS, GA being with negative direction. FIRS was less expected with higher GA.

To determine the optimal Random Forest model with the highest accuracy and lowest OOB error, we assessed several models with different combinations of predictive variables. The Random Forest model predicting FIRS based on five variables (TNF-α, MMP-8, IL-6, IL-17, and IL-10) demonstrated superior performance metrics (Figure 16). We tested varying tree numbers (300, 500, 1000, 2000, and 3000) and mtry values (2, based on the square root of 5) ranging from 1 to 4. Model validation using the OOB error indicated that the lowest error rate occurred with mtry set at 3, and increasing the number of trees did not further improve the model. The test classification accuracy for this Random Forest model was 86%, with increased sensitivity of 81% and sustained specificity of 89%. The variable importance ranking for predicting FIRS was TNF-α, IL-10, MMP-8, IL-6, and IL-17, respectively. In summary, the Random Forest analysis revealed that TNF-α in vaginal amniotic fluid holds the most significance in predicting FIRS compared to other analyzed biomarkers.

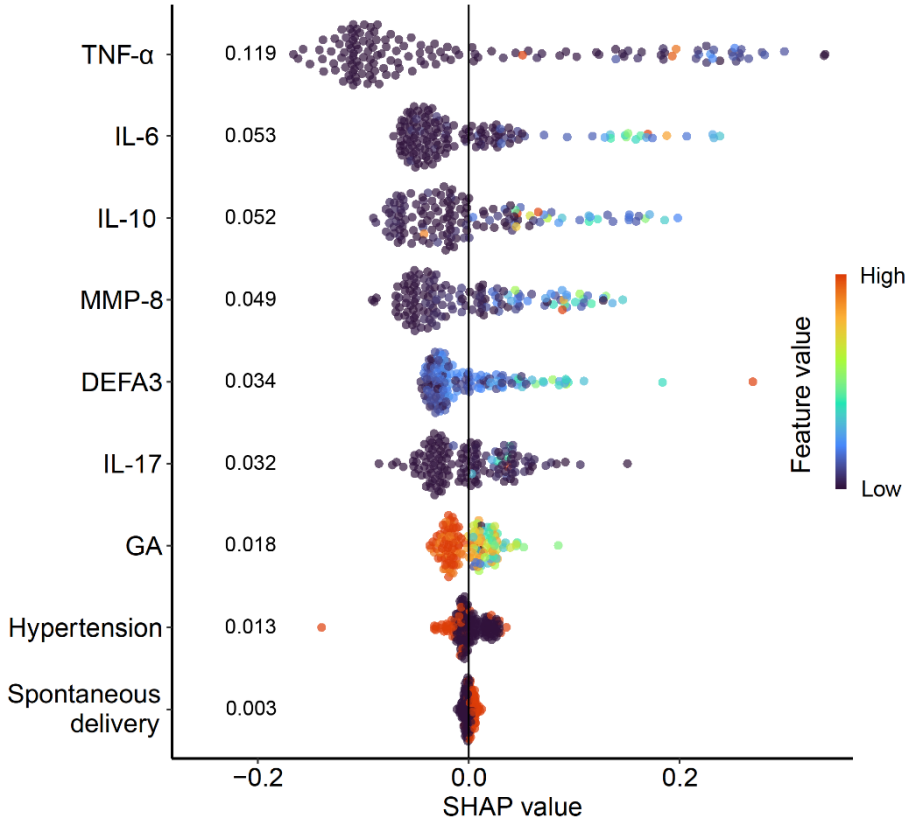


Figure 15. The importance of all significant variables in predicting FIRS in SHAP Summary Plot. The x-axis demonstrates Shapley values (impact on the model output and direction). Positive values contribute to increasing the chances of FIRS, while negative values contribute to decreasing it. On the y-axis, the predictors are listed in descending order of their importance in the Random Forest model. The colors represent the feature values of predictors: higher values are in red-orange, and lower values are in dark blue.

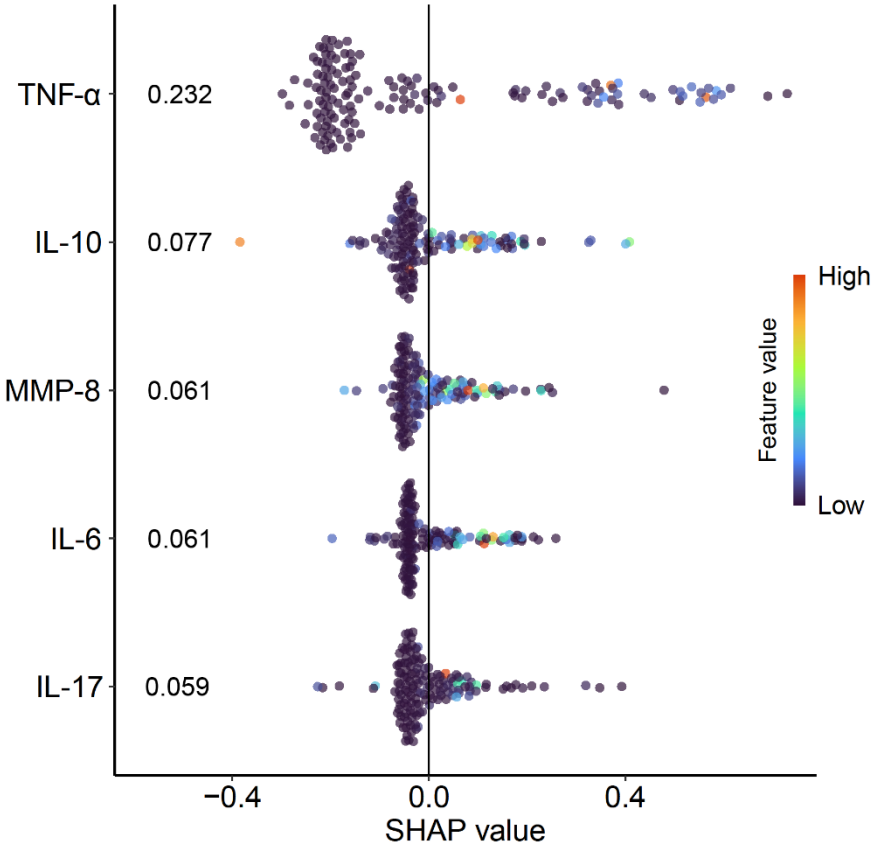


Figure 16. SHAP Summary Plot of the optimal model for the prediction of FIRS. Five predictors (TNF-α, IL-10, MMP-8, IL-6, IL-17) are listed in descending order of their importance in the optimal Random Forest model, with the numbers of SHAP feature importance of each variable.

4.1.5 The significance of inflammatory biomarkers predicting FIRS and major neonatal outcomes

4.1.5.1 The prevalence of major neonatal outcomes

We found that newborns in the FIRS group had more adverse outcomes than those in the non-FIRS group (42% vs. 24%, p = 0.02, Table 4). Figure 17 shows the predicted odds of major neonatal outcomes as a function of gestational age and the presence or absence of FIRS. Based on gestational age, the odds for major neonatal outcomes were higher in the FIRS group than in the non-FIRS group.

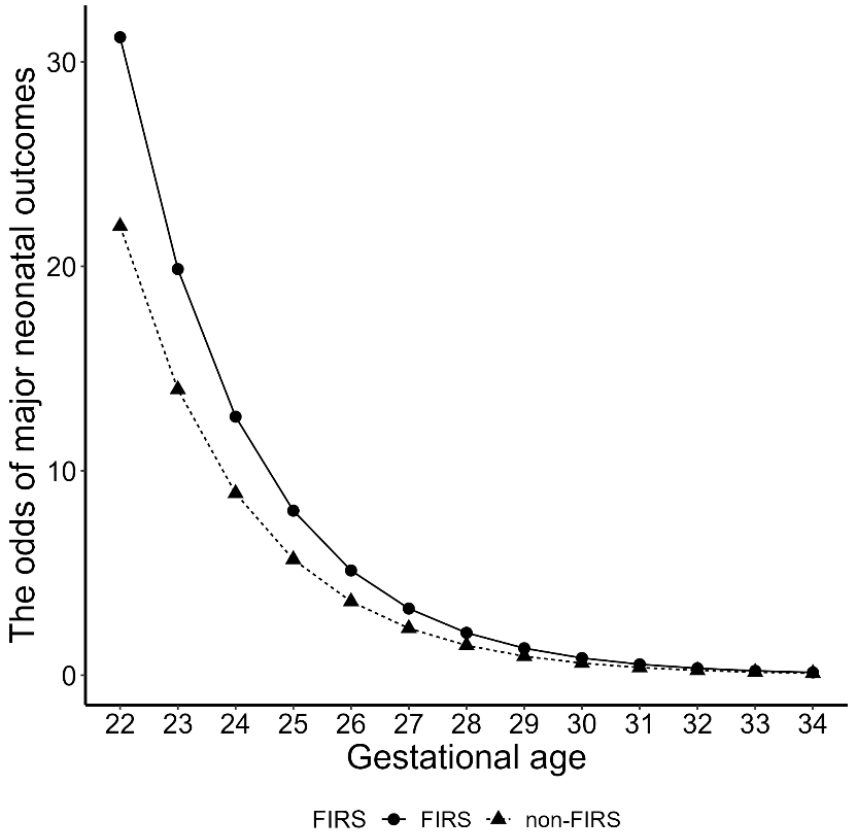


Figure 17. Plot of predicted odds of major neonatal outcomes.

In the non-FIRS group, among 91 neonates, 69 (76%) had no major outcomes, while 22 (24%) experienced major outcomes: 15% had 1-2 outcomes, 7% had 3-4 outcomes, and 2% had 5-6 outcomes. Of the 54 infants

in the FIRS group, 31 (approximately 58%) had no major outcomes, whereas 23 (approximately 42%) had major outcomes: 24% had 1-2 outcomes, 11% had 3-4 outcomes, and 7% had 5-6 outcome. However, this difference in the count of outcomes was not statistically significant ($p = 0.09$).

Neonates with GA between 22^{+0}-27^{+6} weeks had major outcomes in 50% of FIRS cases, while those with GA between 28^{+0}-31^{+6} weeks experienced major outcomes in 17% of FIRS cases. For newborns with GA between 32^{+0}-34^{+6} weeks, major outcomes occurred in 7% of FIRS cases (Table 13, $p < 0.001$).

Table 13. The relationship between GA groups and FIRS and the non-FIRS groups according to the presence and absence of major outcomes.

	GA groups	22^{+0}-27^{+6} weeks (n, %)	28^{+0}-31^{+6} weeks (n, %)	32^{+0}-34^{+6} weeks (n, %)	P value
Non-FIRS group	No major outcomes	2 (3)	9 (13)	58 (84)	<0.001
	Major outcomes	9 (41)	6(27)	7 (32)	
FIRS group	No major outcomes	1 (3)	9 (29)	21 (68)	
	Major outcomes	12 (52)	5 (22)	6 (26)	

4.1.5.2 Comparison of biomarkers levels based on the presence of FIRS and outcomes

We determined median levels of biomarkers in the FIRS and non-FIRS groups according to the presence or absence of neonatal outcomes (Table 14). The FIRS group neonates having neonatal outcomes had significantly higher levels of these biomarkers in amniotic fluid, as follows: TNF-α, MMP-8, IL-6, IL-10, IL-17, EGF, and DEFA3.

Table 14. The median concentrations of biomarkers in both the FIRS and non-FIRS groups, categorized by the presence or absence of major neonatal outcomes. The results are reported as median with IQR and mean with SD, respectively. The p value represents statistical significance determined through the Kruskal-Wallis test or ANOVA, as applicable. The variables, that were not normally distributed, were logged (squared).

	FIRS group		Non-FIRS group		
Biomarkers	Major outcomes	No outcomes	Major outcomes	No outcomes	P value
TNF-α (pg/mL)	462.16 (15.66 - 1191.02)	104.80 (17.77 - 302.91)	17.74 (6.19 - 74.30)	12.83 (6.83 - 30.46)	<0.001
MMP-8 (ng/mL)	801.82 (134.28 - 2259.07)	515.81 (177.20 - 1282.03)	16.90 (9.05 - 147.18)	76.40 (22.66 - 302.78)	<0.001
IL-6 (pg/mL)	4079.09 (1949.43 - 99180.39)	3920.91 (2808.86 - 70164.47)	871.23 (111.06 - 3751.62)	1562.55 (630.91 - 3294.89)	<0.001
IL-10 (pg/mL)	53.18 (2.30 - 198.02)	59.62 (6.28 - 146.76)	6.51 (0 - 23.88)	3.61 (0 - 20.02)	<0.001
IL-17 (pg/mL)	3.20 (0.60 - 9.38)	1.69 (0.54 - 5.31)	0.49 (0.16 - 0.91)	0.50 (0.24 - 1.30)	0.004
sTLR-2 (ng/mL)	0.27 (0.10 - 0.68)	0.19 (0.05 - 0.50)	0.24 (0.08 - 0.36)	0.17 (0.04 - 0.39)	0.664
sTLR-4 (ng/mL)	0.51 (0.23 - 0.77)	0.25 (0.12 - 0.48)	0.41 (0.09 - 0.58)	0.27 (0.09 - 0.56)	0.383
uIL-6 (pg/mL)	120.50 (25.00 - 1000.00)	33.00 (16.10 - 99.80)	4.98 (3.68 - 5.56)	3.97 (2.86 - 6.57)	<0.001
EGF (pg/mL)	48.73 (30.51 - 109.29)	88.24 (65.81 - 131.51)	55.18 (30.67 - 71.44)	96.02 (51.59 - 189.35)	0.004
RANTES (pg/mL)	395.30 (231.96-935.30)	190.32 (78.08 - 1010.85)	477.00 (75.52 - 1779.07)	413.26 (114.580 - 1138.93)	0.752
DEFA3 (ng/mL)	28.41 (19.14-37.72)	27.72 (11.76-35.85)	20.23 (16.43-31.49)	19.89 (14.72-29.05)	0.049
log-TNF-α	5.39±2.54	4.56±1.91	3.44±1.97	2.81±1.13	<0.001
log-IL-6	9.08±2.61	9.11±2.22	6.82±2.39	7.34±1.44	<0.001
log-IL-10	3.36±2.36	3.56±2.03	1.88±1.89	1.94±1.66	<0.001
log-IL-17	1.57±1.40	1.25±1.18	0.66±0.85	0.67±0.78	0.002
log-MMP-8	5.78±2.64	5.99±1.79	3.63±2.27	4.36±1.72	<0.001
log-RANTES	5.99±1.13	5.50±1.59	5.71±2.22	5.86±1.76	0.759
log-TLR2	0.35±0.34	0.31±0.35	0.23±0.27	0.26±0.21	0.535
sqrt-TLR4	1.22±0.15	1.18±0.17	1.19±0.17	1.18±0.19	0.786
log-EGF	4.12±0.94	4.54±1.19	3.82±0.93	5.02±2.32	0.024
log-DEFA3	3.37±0.58	3.07±0.56	3.05±0.79	2.91±0.55	0.029

4.1.5.3 ROC curves analysis and cut-off values for FIRS with major neonatal outcomes

The biomarkers that showed significant associations with the FIRS group, specifically those with major outcomes (Table 14), were selected for further analysis. Assessing the prediction performance of selected variables for FIRS with major outcomes, we plotted ROC curves and calculated the AUCs (Figures 18 and 19). In Table 15, inflammatory markers in vaginal amniotic fluid identifying FIRS with major outcomes are displayed with their AUC, cutoff values, and diagnostic characteristics. TNF-α demonstrated the highest discriminatory power (AUC of 0.73) in distinguishing cases with FIRS and major outcomes. Furthermore, while TNF-α demonstrates high specificity, signifying a low incidence of false positives, its moderate sensitivity suggests that there is the potential for enhancement in accurately identifying true positives. MMP-8, IL-6, IL-10, IL-17, EGF and DEFA3 displayed moderate prediction performance for FIRS with major outcomes with AUC values of 0.67, 0.67, 0.64, 0.66, 0.62, and 0.66, respectively. The specificity of these biomarkers is high, indicating a low rate of false positives, whereas the sensitivity is rather moderate. However, the PPV of all biomarkers is quite low, indicating that there might be a notable number of false positives among the positive predictions.

The outcomes of the DeLong test, displayed in Table 16 as p values, signify a statistically significant difference or absence when comparing the ROC curves of the biomarkers. These findings imply that, except for TNF-α with IL-10 or IL-17, most biomarkers show comparable discriminatory power, all demonstrating moderate predictive characteristics based on the ROC analysis in predicting FIRS with major outcomes.

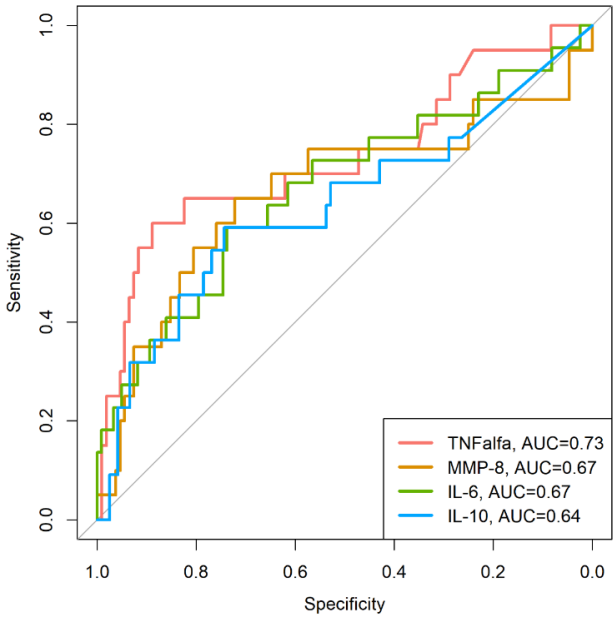


Figure 18. The ROC curves of TNF-α, MMP-8, IL-6, and IL-10 predicting FIRS with major outcomes.

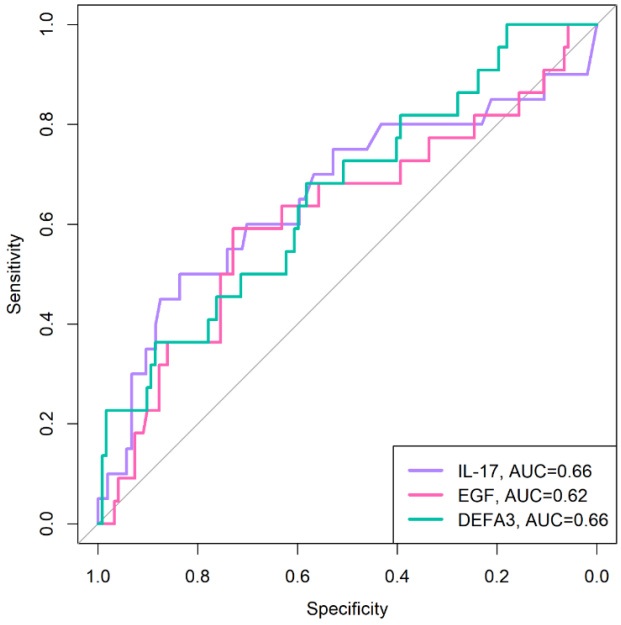


Figure 19. The ROC curves of IL-17, EGF, and DEFA3, predicting FIRS with major outcomes.

Table 15. The cutoff values and diagnostic characteristics of inflammatory markers in amniotic fluid identifying FIRS with major outcomes.

Biomarker	Cutoff value	AUC 95% CI		Sensitivity		Specificity		PPV		NPV	
				%	95% CI	%	95% CI	%	95% CI	%	95% CI
MMP-8	413.67	0.67	0.51 - 0.82	65	45 – 85	72	63 - 80	30	21 - 40	92	87 - 96
TNF-α	231.13	0.73	0.59 - 0.87	60	35 - 80	89	82 - 94	50	35 - 68	92	88 - 96
IL-6	3999.42	0.67	0.53 - 0.81	59	41 - 77	74	66 - 81	29	20 - 39	91	87 - 95
IL-10	35.05	0.64	0.49 - 0.78	59	36 - 77	74	66 - 82	30	20 - 40	91	87 - 95
IL-17	4.00	0.66	0.51 - 0.81	50	30 - 70	84	76 - 90	37	23 - 52	90	86 – 94
EGF	51.49	0.62	0.48 - 0.76	59	41 - 77	73	65 - 80	28	19 - 38	91	87 - 95
DEFA3	35.58	0.66	0.53 - 0.79	36	18 - 55	89	83 - 93	36	19 - 55	89	85 - 92

Abbreviations: AUC, the area under the curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

Table 16. Comparison of immunological biomarkers predicting FIRS with major outcomes using the DeLong test for two correlated ROC curves. Each count represents the p value of the DeLong test comparing the ROC curves of two biomarkers. The significant differences are marked in grey.

	MMP-8	TNF-α	IL-6	IL-10	IL-17	EGF	DEFA3
MMP-8	-	0.116	0.848	0.365	0.132	0.884	0.837
TNF-α	0.116	-	0.107	0.029	0.049	0.408	0.278
IL-6	0.848	0.107	-	0.549	0.651	0.603	0.883
IL-10	0.365	0.029	0.55	-	0.950	0.849	0.783
IL-17	0.132	0.049	0.651	0.950	-	0.653	0.838
EGF	0.884	0.408	0.603	0.849	0.653	-	0.665
DEFA3	0.837	0.278	0.883	0.783	0.838	0.665	-

4.1.5.4 Logistic regression analysis predicting FIRS and major neonatal outcomes

To assess the significance of inflammatory markers in vaginal amniotic fluid predicting FIRS with major outcomes, we conducted univariate logistic regression analysis (Table 17). Additionally, we estimated the odds of having FIRS with major outcomes using multivariate logistic regression with the adjustment for gestational age.

In the univariate logistic regression analysis, several biomarkers, including log-transformed levels of TNF-α, MMP-8, IL-6, IL-10, IL-17, and DEFA3 exhibit positive associations with increased odds of FIRS with major outcomes, with significant p values. Additionally, clinical factors, including GA and birthweight, are strongly associated with FIRS and major outcomes, providing a comprehensive understanding of the predictive factors. Other variables, such as hypertensive disorders, and spontaneous delivery, do not demonstrate significant associations with FIRS and major outcomes.

Due to collinearity among several variables (log-TNF-α and log-MMP-8: rho = 0.67, $p < 0.001$; log-TNF-α and log-IL-6: rho = 0.71, $p < 0.001$; log-MMP-8 and log-IL-6: rho = 0.72, $p < 0.001$; log-TNF-α and log-IL-10: rho = 0.65, $p < 0.001$; GA and birthweight: rho = 0.87, $p < 0.001$), we selected variables with stronger effect estimates and lower p values for further analysis in the multivariate logistic regression.

In the multivariate analysis, log-transformed TNF-α remains a significant predictor of FIRS with major outcomes, showing an adjusted odds ratio of 1.48 (95% CI: 1.09 - 2.05). Log-IL-17 did not demonstrate significant associations after adjusting for other variables, while log-DEFA3 was

marginally significant. GA continues to be a significant predictor, indicating a protective effect with an adjusted odds ratio of 0.77 (95% CI: 0.65 – 0.91), meaning that higher GA is associated with a lower risk of FIRS with major outcomes, and vice versa. The results suggest that while certain biomarkers and clinical factors individually contribute to the likelihood of FIRS with major outcomes, the multivariate model highlights the importance of considering these factors collectively.

The stepwise (forward – backward) logistic regression analysis for FIRS with major outcomes (Table 18), while controlling for GA, was conducted, optimizing AIC with a final model with an AIC value of 100.98. The negative estimate for GA indicates a protective effect, suggesting that as gestational age increases, the odds of FIRS with major outcomes decrease. Log-TNF-α and log-DEFA3 exhibit positive estimates, signifying an increased likelihood of FIRS with major outcomes as the levels of these biomarkers rise. However, log-DEFA3, with a borderline p value, should be interpreted cautiously. In summary, the stepwise logistic regression model identifies gestational age, log-TNF-α, and log-DEFA3 as significant predictors of FIRS with major outcomes.

Table 17. The Univariate and multivariate logistic regression analysis predicting FIRS with major outcomes with log-transformed biomarkers. In this table, the outcome variable is FIRS with major outcomes. The variables that were not normally distributed, were log-transformed.

	Univariate logistic regression			
Input variables	Estimate	P value	Odds ratio	95% CI
Log-TNF-α	0.474	<0.001	1.61	1.27 - 2.09
Log-MMP-8	0.263	0.038	1.30	1.03 - 1.69
Log-IL-6	0.292	0.007	1.34	1.09 - 1.67
Log-IL-10	0.252	0.033	1.29	1.03 - 1.63
Log-IL-17	0.535	0.008	1.71	1.15 - 2.57
Log-EGF	-0.333	0.163	0.72	0.43 - 1.05
Log-DEFA3	1.170	0.009	3.22	1.41 - 8.25
GA	-0.319	< 0.001	0.72	0.62 – 0.84
Birthweight	-0.002	< 0.001	0.99	0.97 - 0.99
Hypertensive disorders (ref. yes)	-0.635	0.334	0.53	0.12 - 1.70
Spontaneous delivery (ref. yes)	-0.018	0.969	0.98	0.39 - 2.45
	Multivariate logistic regression N=145, AIC= 155.26			
Log-TNF-α	0.392	0.013	1.48	1.09 - 2.05
Log-IL-17	-0.100	0.720	0.90	0.51 - 1.54
Log-DEFA3	0.887	0.069	2.43	0.99 – 6.85
GA	-0.260	0.003	0.77	0.65 – 0.91

Table 18. Stepwise (forward – backward) logistic regression for FIRS with major outcomes controlling for gestational age. The variables that were not normally distributed, were log-transformed.

Stepwise logistic regression N=145, AIC=100.98				
Biomarker	Estimate	P value	Odds ratio	95% CI
GA	-0.268	0.002	0.77	0.64 - 0.90
Log-TNF-α	0.323	0.024	1.38	1.05 - 1.85
Log-DEFA3	0.857	0.057	2.36	1.03 - 6.12

In further analysis, the optimal cut-off values were used to evaluate predictive logistic regression models of FIRS with major outcomes as a consequence of the dose-dependent effect of biomarkers (Table 19). ROC curves and the Youden index were previously used to detect cut-off values for biomarkers (Table 15). In univariate logistic regression with cut-off values, TNF-α, MMP-8, IL-6, IL-10, IL-17, DEFA3, and EGF — each biomarker, when assessed individually with predefined cut-off values — demonstrated a significant association with FIRS and major outcomes, indicating their potential as predictive indicators. In multivariate logistic regression with cut-off values, TNF-α maintained its significant predictive role even after adjusting for other biomarkers and gestational age, suggesting its robustness as an independent predictor. MMP-8, IL-6, IL-10, IL-17, DEFA3 and EGF did not exhibit significant predictive value after adjusting for covariates.

In stepwise logistic regression, TNF-α with the cut-off of > 231 pg/mL emerged as a strong predictor of FIRS with major outcomes even when controlling for gestational age, reinforcing its importance in identifying the likelihood of FIRS with major outcomes. GA showed a significant protective effect, indicating that higher gestational age is associated with a lower risk of FIRS with major outcomes.

Table 19. Univariate and multivariate logistic regression using cut-off values of biomarkers predicting FIRS with major outcomes.

Univariate logistic regression with cut-off				
Biomarker	Estimate	P value	Odds ratio	95% CI
TNF-α > 231	2.485	< 0.001	12.00	4.18 - 36.78
MMP-8 > 413	1.575	0.002	4.82	1.76 - 13.27
IL-6 > 3999	1.402	0.004	4.06	1.60 - 10.73
IL-10 > 35	1.434	0.003	4.19	1.65 - 11.10
IL-17 > 4	1.633	0.002	5.12	1.84 - 14.43

Univariate logistic regression with cut-off				
Biomarker	Estimate	P value	Odds ratio	95% CI
DEFA3 >35	1.331	0.010	3.79	1.33 - 10.39
EGF <51	1.175	0.013	3.24	1.28 - 8.37
Multivariate logistic regression with cut-off N=145, AIC =103.2				
TNF-α > 231	2.221	0.013	9.21	1.82 – 65.04
MMP-8 > 413	0.4094	0.630	1.50	0.27 - 7.89
IL-6 > 3999	-0.337	0.677	0.71	0.12 – 3.13
IL-10 > 35	0.263	0.724	1.30	0.29 – 5.61
IL-17 > 4	0.411	0.603	1.51	0.31 – 7.15
DEFA3 > 35	0.450	0.510	1.57	0.39 – 5.83
EGF<51	0.832	0.245	2.30	0.57 - 9.77
GA	-0.179	0.089	0.83	0.68 – 1.02

Table 20. Stepwise (forward – backward) logistic regression for the prediction of FIRS with major outcomes using cut-off values of biomarkers and controlling for gestational age.

Stepwise logistic regression N=145, AIC=94.415				
Biomarker	Estimate	P value	Odds ratio	95% CI
TNF-α > 231	2.335	<0.001	10.33	3.38 - 33.30
GA	-0.269	0.001	0.76	0.64 - 0.89

4.1.5.5 Random Forest predicting FIRS with major neonatal outcomes

Based on findings in univariate logistic regression, Random Forest analysis was conducted to predict FIRS with major outcomes. The importance and the rating of selected variables were determined using seven predictors, as follows: TNF-α, MMP-8, IL-6, IL-17, IL-10, DEFA3, and GA. Various model interactions were tested with 300, 500, 1000, 2000, and 3000 trees. The mtry was tested from 1 to 5 with default as the square root of the total number of predictors, resulting in a value of 2 based on the seven predictors. Model validation was performed using the OOB error, revealing that the OOB error rate reached its minimum when mtry was set to 2. The subsequent increases in the number of trees did not yield improvements in model performance. Cross-validation and accuracy assessment using a separate test set demonstrated a classification accuracy of 88%, sensitivity of 33%, specificity of 97%, PPV of 67%, and NPV of 90%. The three most influential predictors of FIRS with major outcomes were identified as GA, TNF-α, and MMP-8.

To visually depict the impact of individual features on the model's predictions based on Shapley values, a SHAP summary plot was generated (see Figure 20). The plot illustrates that, among the seven selected variables, lower values of GA and elevated values of TNF-α and MMP-8 emerged as the most critical variables in predicting FIRS with major outcomes. Meanwhile, DEFA3, IL-17, IL-6, and IL-10 exhibited moderate performance.

To identify the optimal Random Forest model with the highest accuracy and lowest OBB, we systematically evaluated many models with different combinations of predictor variables. Notably, the Random Forest model developed to predict FIRS with major outcomes using three key variables (gestational age, TNF-α and DEFA3) presented promising performance. Our tests included a range of tree numbers (300, 500, 1000, 2000 and 3000) and mtry values (set at 1, representing the square root of 3), tested from 1 to 3. The model validation process using OOB error showed that the optimum configuration was achieved when mtry was 1. Increasing the number of trees did not improve the model's performance further. The improved Random Forest model had a classification accuracy of 93%, with a sensitivity of 50% and a specificity of 100%. Gestational age, TNF-α, and DEFA3 were the most important variables in predicting FIRS with major outcomes, with gestational age being the most powerful. Figure 21 displays the SHAP summary plot for the optimal Random Forest model for FIRS with major outcomes. In summary, our comprehensive Random Forest analysis showed that gestational age and TNF-α levels in the vaginal amniotic fluid are the most significant factors, outperforming other studied biomarkers, in predicting FIRS with major outcomes.

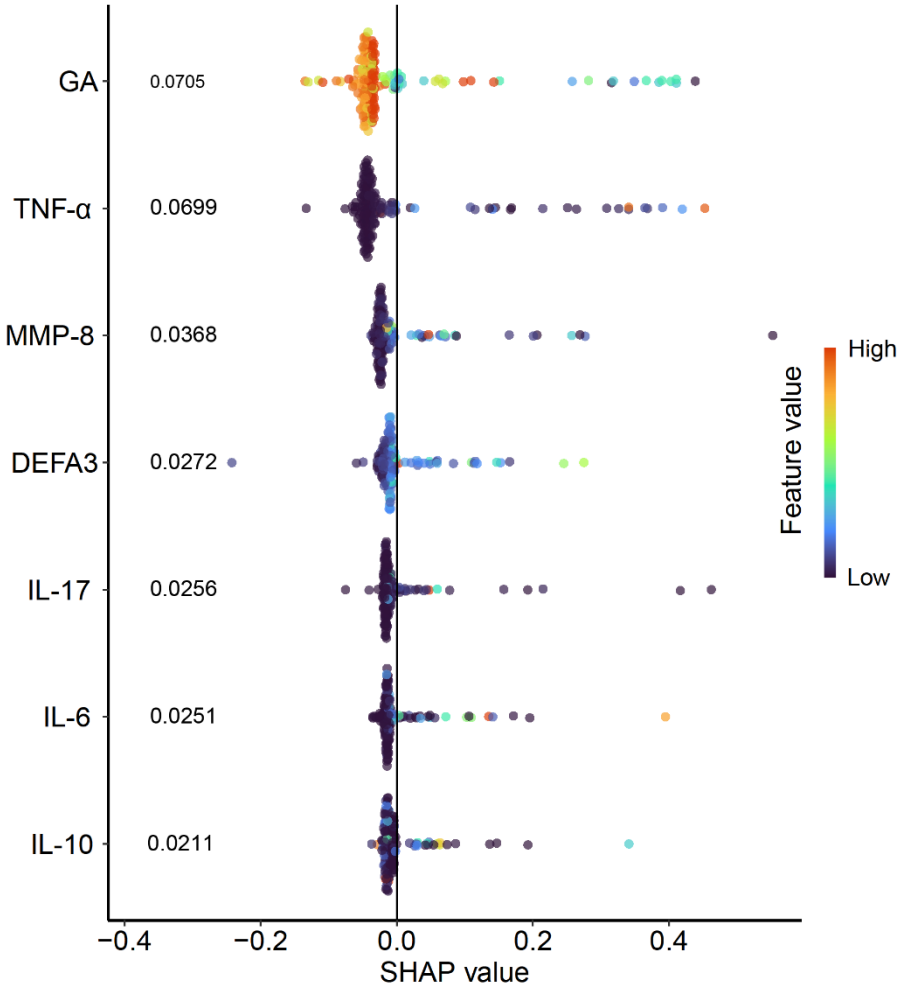


Figure 20. Variable Importance in predicting FIRS with major outcomes in SHAP Summary Plot. On the y-axis, the predictors (GA, TNF-α, MMP-8, DEFA3, IL-17, IL-6 and IL-10) are listed in descending order of their importance in the Random Forest model with the numbers of SHAP feature importance of each variable.

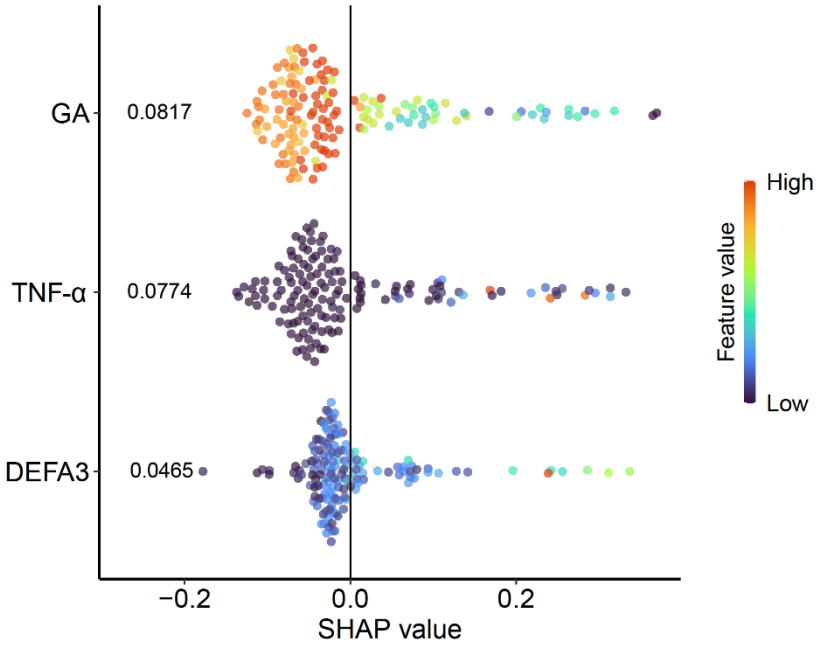


Figure 21. The optimal model for predicting FIRS with major outcomes in SHAP Summary Plot. On the y-axis, the predictors (GA, TNF-α and DEFA3) are listed in descending order of their importance in the Random Forest model, with the numbers of SHAP feature importance of each variable.

4.1.6 The association between inflammatory maternal and neonatal biomarkers and umbilical cord blood IL-6 levels

Umbilical cord blood IL-6 levels were determined in the 0–40000 pg/mL range with a median of 8.21 pg/mL (IQR, 3.79–38.83). The correlation analysis was undertaken to assess the relationship between umbilical IL-6 and amniotic fluid biomarkers and clinical parameters, yielding valuable insights into its associations and potential role in neonatal health and disease.

We observed a modest positive correlation between maternal and neonatal CRP levels, and umbilical cord blood IL-6 concentrations (refer to Figures 9 and 10). This association highlights the link between maternal, fetal, and neonatal inflammation. Conversely, the association of umbilical cord blood IL-6 with neonatal WBC, I/T ratio, and maternal WBC was weak or insignificant. Moreover, there were significant negative correlations of umbilical cord blood IL-6 levels with GA and birthweight. The levels of umbilical IL-6 were higher in neonates with low GA and birthweight,

complementing the findings that smaller infants according to GA and birthweight are more often affected by intraamniotic inflammation.

Umbilical IL-6 present moderate to weak positive correlations with other pro-inflammatory amniotic fluid cytokines, such as TNF-α, IL-6, IL-17, MMP-8, and IL-10, implicating that levels of immunological biomarkers in umbilical cord blood, as well as, in vaginally collected amniotic fluid both, correspond.

To compare the diagnostic performance of umbilical IL-6 with amniotic fluid biomarkers, we analyzed the ROC curve of umbilical IL-6 in predicting major neonatal outcomes. FIRS prediction was excluded from the analysis, as FIRS is defined by umbilical IL-6 levels. The AUC for umbilical IL-6 was 67% (95% CI: 57–80). At a cut-off of ≥ 19.3 pg/mL, the specificity was 77%, sensitivity 53%, PPV 47%, and NPV 81%. Increasing the cut-off to 100.5 pg/mL improved specificity to 92%, with a sensitivity of 34%, PPV of 61%, and NPV of 78%.

Next, we compared the diagnostic accuracy for major outcomes of umbilical IL-6 against amniotic fluid biomarkers using the Delong test. Based on AUC comparisons, umbilical IL-6 significantly outperformed the following biomarkers in predicting major neonatal outcomes ($p < 0.05$): MMP-8 ($p = 0.012$), amniotic fluid IL-6 ($p = 0.037$), and IL-10 ($p = 0.035$). However, no significant differences in AUC were observed with TNF-α ($p = 0.245$), DEFA3 ($p = 0.376$), IL-17 ($p = 0.106$), and EGF ($p = 0.886$).

A Random Forest analysis was performed to predict major neonatal outcomes, comparing the significance of amniotic fluid biomarkers with umbilical cord IL-6 and gestational age. The model included nine predictors: TNF-α, MMP-8, IL-6, IL-17, IL-10, DEFA3, umbilical IL-6, EGF, and GA. FIRS was excluded from the model because it is based on umbilical IL-6 levels, which were already included. Model evaluation across varying tree counts (from 500 to 3000) showed optimal performance with 500 trees and `mtry` value of 2. Further adjustments in tree count and `mtry` did not improve the out-of-bag (OOB) error rate. The final model achieved a classification accuracy of 84%, with a sensitivity of 54%, specificity of 97%, PPV of 86%, and NPV of 83%. Predictors were ranked by importance based on SHAP values, with GA, EGF, and TNF-α identified as the most significant contributors to neonatal outcomes. Specifically, lower GA, reduced EGF levels, and elevated TNF-α values were the strongest predictors. Amniotic fluid IL-6, umbilical IL-6, and MMP-8 showed similar moderate importance in the prediction model.

4.2 Part 2: SuPAR cohort

4.2.1 SuPAR study population characteristics

The study cohort of 114 women with PPROM before 34^{+6} weeks of gestation and their newborns was divided into the FIRS group (n=48) and the non-FIRS group (n=66). Table 21 displays the clinical characteristics of the study population.

Table 21. Demographic and clinical characteristics of the study population.

Characteristics	FIRS group (n=48)	Non-FIRS group (n=66)	P value
Maternal characteristics			
Maternal age, years, mean ± SD	31.2 ± 5.5	31.1 ± 5.7	0.932
Latency period, hours, median, IQR	93 (17-205)	106 (44-236)	0.199
Gestational diabetes, n, %	10 (21)	13 (20)	0.907
Group B streptococcus test positive, n, %	7 (24)	10 (22)	0.814
Primigravida, n, %	17 (46)	31 (40)	0.566
Primiparous, n, %	20 (39)	28 (44)	0.566
Antenatal glucocorticoids, n, %	41 (85)	55 (83)	0.764
Tocolytics, n, %	23 (48)	38 (58)	0.312
Mode of delivery, n, % vaginal delivery cesarean section	 43 (90) 5 (10)	 55 (83) 11 (17)	0.423
Induction of delivery or cesarean section n, % Spontaneous delivery n, %	17 (35) 31 (65)	33 (50) 33 (50)	0.279
Clinical chorioamnionitis, n, %	6 (13)	1 (2)	0.024
Histological chorioamnionitis, n, %	38 (79)	15 (23)	<0.001
Funisitis, n, %	21 (44)	0	<0.001
Neonatal characteristics			
GA at birth, weeks, median, IQR	31 (27-33)	33 (32-34)	0.001
Birthweight, grams, mean ± SD	1688± 669	2070± 555	0.012
Apgar scores <7 at 1 min., n, %	14 (29)	6 (9)	0.014
Apgar scores <7 at 5 min., n, %	6 (13)	1 (2)	0.037
Umbilical cord arterial pH, median, IQR	7.35 (7.29-7.42)	7.36 (7.32-7.40)	0.774

Characteristics	FIRS group (n=48)	Non-FIRS group (n=66)	P value
The need for resuscitation after birth, n, %	14 (29)	6 (9)	0.005
Major outcome, n, %	19 (40)	11 (17)	0.014
Respiratory distress, n, %	46 (96)	48 (73)	0.001
Severe RDS, n, %	10 (21)	8 (12)	0.005
Respiratory support, n, % None Mechanical ventilation Non-invasive respiratory therapy	 2 (4) 11 (23) 35 (73)	 18 (27) 6 (9) 42 (64)	0.002
The need for surfactant, n, %	15 (31)	14 (21)	0.224
Neonatal death, n, %	1 (2)	0	0.422
Sepsis, n %	8 (17)	2 (3)	0.017
Early-onset sepsis	7 (17)	1 (2)	0.007
Early hypotension	10 (21)	5 (8)	0.039
BPD, n, %	9 (19)	4 (6)	0.044
PDA, n %	8 (17)	8 (12)	0.462
ROP, n %	15 (32)	6 (9)	0.003
Treated ROP, n %	3 (6)	1(2)	0.168
IVH, n, % None 1-2 grade 3-4 grade	 29 (60) 14 (29) 5 (10)	 46 (70) 18 (27) 2 (3)	0.243

A higher prevalence of clinical and histological chorioamnionitis in the FIRS group was the only difference between the groups in the maternal factors. In the FIRS group, neonates had lower birth weight, gestational age, and Apgar scores after the 1st and 5th minute and a higher need for resuscitation after birth compared to the non-FIRS group. There was a greater incidence of respiratory problems, including severe RDS, the need for respiratory support, bronchopulmonary dysplasia, early-onset sepsis, unstable hemodynamics, and retinopathy of prematurity in the FIRS group than in the non-FIRS group. Neither group differed in umbilical cord arterial pH, neonatal mortality, PDA, the need for surfactant or IVH.

4.2.2 SuPAR characteristics in amniotic fluid

We detected suPAR in all amniotic fluid samples in the 5.36–399.98 ng/mL range with a median of 26.23 ng/mL IQR, 15.19–51.14) (Table 22).

Table 22. SuPAR characteristics in amniotic fluid

	Mean	SD	Median	Q1	Q3	Min	Max
suPAR (ng/mL)	46.07	58.99	26.23	15.19	51.14	5.36	399.98
Log-suPAR	3.36	0.91	3.27	2.27	3.93	1.68	5.99

4.2.3 Spearman correlation analysis

We performed Spearman correlation analysis to investigate the relationship between suPAR and various biomarkers, as well as maternal and neonatal parameters, as shown in Figure 22. The figure displays a correlation matrix, presenting correlation coefficients among the variables in a tabular format. Notably, suPAR displayed a strong positive correlation with MMP-8, a moderately positive correlation with TNF-α and IL-6 in vaginal amniotic fluid, as well as maternal CRP. Moreover, there was a weak positive correlation with maternal WBC, while gestational age and neonatal birthweight showed a weak negative correlation with suPAR.

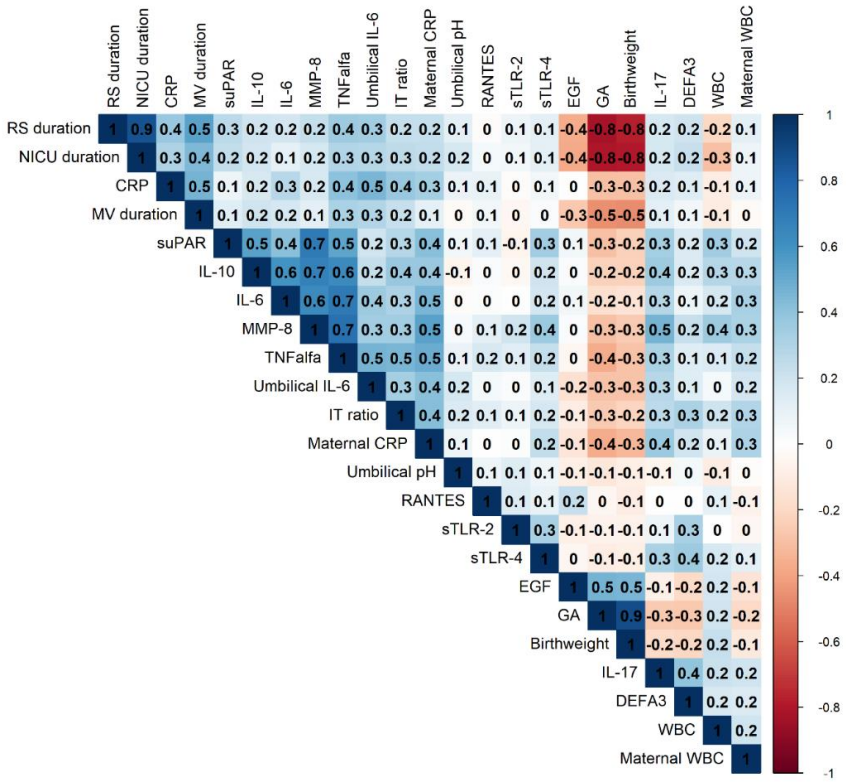


Figure 22. The correlation matrix of suPAR relationships. Blue indicates positive correlations, red indicates negative correlations and color intensity reflects the strength of these correlations. The legend provides correlation coefficients and their corresponding colors. The correlation coefficients and p values among variables are as follows: suPAR and MMP-8 (rho = 0.71, $p < 0.001$); suPAR and umbilical IL-6 (rho = 0.22, $p = 0.05$); suPAR and amniotic fluid IL-6 (rho = 0.42, $p < 0.001$); suPAR and TNF-α (rho = 0.54, $p < 0.001$); suPAR and maternal CRP (rho = 0.39, $p < 0.001$); suPAR and maternal WBC (rho = 0.21, $p = 0.04$); suPAR and GA (rho = -0.31, $p = 0.01$); suPAR and birthweight (rho = -0.24, $p = 0.01$).

4.2.4 The association of suPAR and FIRS

Figure 23 illustrates suPAR concentrations in vaginally collected amniotic fluid in the FIRS and the non-FIRS groups. The median level of suPAR was significantly higher in patients with FIRS than in the non-FIRS group, 32.36 ng/mL (IQR, 17.27–84.16) vs. 20.46 ng/mL (IQR, 11.49–36.63) (Wilcoxon, $p = 0.01$), respectively.

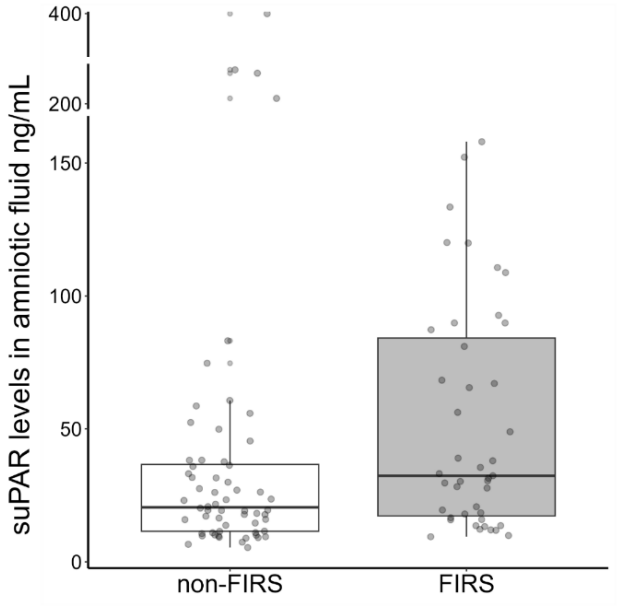


Figure 23. Comparison of suPAR levels in vaginal amniotic fluid between FIRS and non-FIRS groups. The median concentration of suPAR in the FIRS group and the non-FIRS group: median, 32.36 ng/mL, IQR, 17.27–84.16, vs. median, 20.46 ng/mL, IQR, 11.49–36.63, respectively (Wilcoxon, P = 0.01).

4.2.5 ROC curves predicting FIRS

To evaluate the predictive performance of biomarkers in non-invasively obtained amniotic fluid for FIRS in the suPAR cohort, ROC curves for suPAR and selected biomarkers were generated and depicted in Figures 24 and 25. We selected significant biomarkers, including MMP-8, TNF-α, IL-6, IL-10, IL-17, and DEFA3, based on the results of the Wilcoxon test conducted in the entire cohort (refer to Table 6). Additionally, Table 23 presents the diagnostic parameters of suPAR for FIRS, along with a comparison to other significant biomarkers of FIRS in this smaller cohort. The AUC of suPAR was 0.65 (95% CI 0.54-0.76), with a cutoff value of 27.60 ng/mL for predicting FIRS.

The DeLong test was conducted to evaluate the disparity in diagnostic characteristics (AUC) for predicting FIRS between the suPAR ROC curve and the ROC curve of selected biomarkers. The results of the DeLong test are displayed in Table 23 as p values. These findings indicate that there is no significant difference in the discriminatory power between suPAR and IL-10, IL-17, and DEFA3, all of which exhibit moderate predictive characteristics.

However, TNF-α, IL-6, and MMP-8, while marginally, were statistically significantly different in predictive performance compared to suPAR, demonstrating superior performance in identifying FIRS.

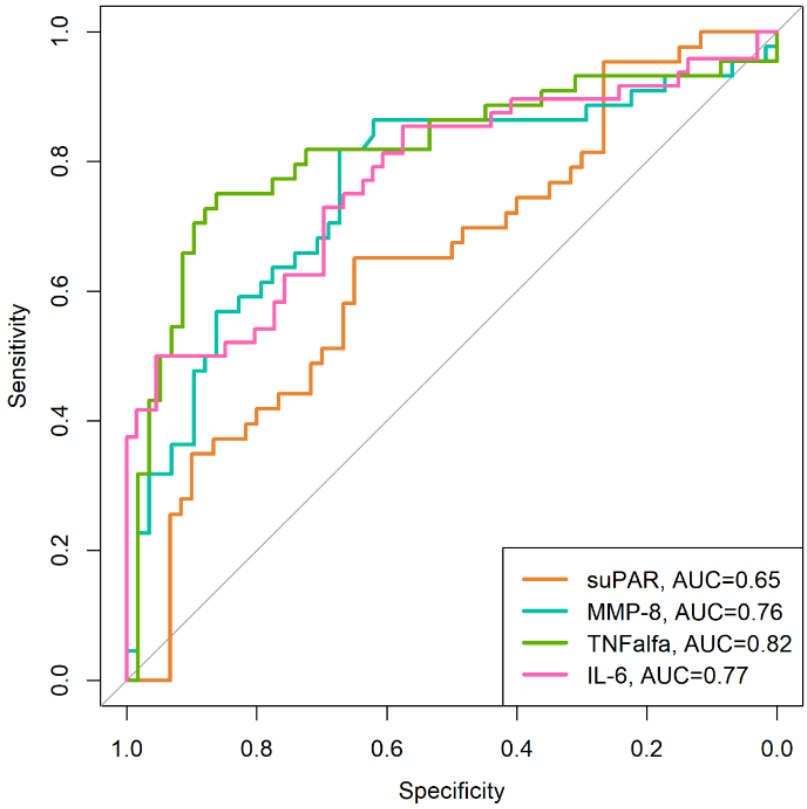


Figure 24. The suPAR ROC curve compared to MMP-8, TNFα, and IL-6 predicting FIRS.

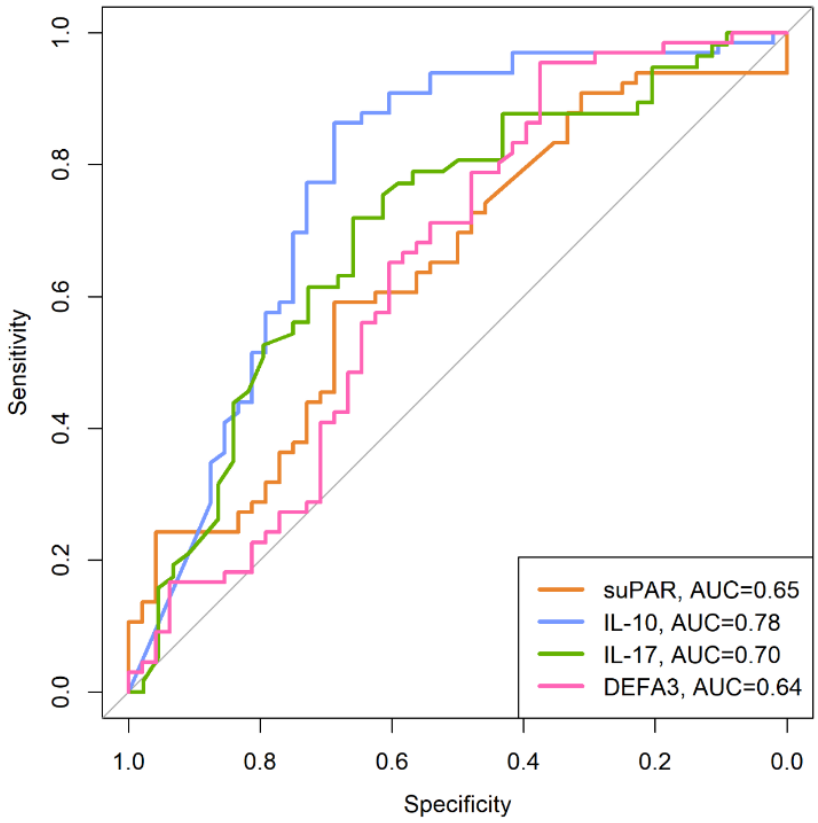


Figure 25. The suPAR ROC curve compared to IL-10, IL-17 and DEFA3 predicting FIRS.

4.2.1 Logistic regression predicting FIRS

For evaluating suPAR's potential as a predictor of FIRS, we conducted both univariate and multivariate logistic regression analyses to determine the significance of suPAR levels in vaginal amniotic fluid (Table 24). In the univariate logistic regression analysis, a one-unit increase in log-suPAR was associated with nearly a twofold increase in the odds of FIRS (OR = 1.70, 95% CI: 1.09 - 2.74, $p < 0.001$). Moreover, in this suPAR cohort, the results of univariate logistic regression for other biomarkers and clinical parameters, such as log-transformed TNF-α, MMP-8, IL-6, IL-10, IL-17, DEFA3, gestational age, and birthweight, remained consistent with those obtained from the entire cohort. However, noticeable collinearity was observed among

several variables: log-TNF-α and log-MMP-8 (rho=0.74, $p < 0.001$), log-TNF-α and log-IL-6 (rho =0.74, $p < 0.001$), log-TNF-α and log-IL-10 (rho =0.70, $p < 0.001$), and GA and birthweight (rho =0.89, $p<0.001$). Log-transformed suPAR (forced) and variables with stronger effect estimates and without collinearity, including log-TNFα, log-IL-17, log-DEFA3, and GA, were selected for further multivariate regression analysis. In the multivariate logistic regression, after adjusting for other variables, log-transformed TNF-α remained significantly associated with FIRS, with a higher odds ratio of 2.15 (95% CI: 1.57 - 3.11, $p < 0.001$). However, the associations of log-suPAR, log-IL-17, log-DEFA3, and GA with FIRS were no longer statistically significant in this model ($p > 0.05$).

Table 23. The cutoff values and diagnostic characteristics of inflammatory markers identifying FIRS in the suPAR cohort.

	Cutoff value	AUC 95% CI		Sensitivity		Specificity		PPV		NPV		Delong test P value
				%	95% CI	%	95% CI	%	95% CI	%	95% CI	
suPAR	27.60	0.65	0.54-0.76	65	51-79	65	53-77	57	48-68	72	64-82	-
MMP-8	190.91	0.76	0.66-0.86	82	68-93	67	55-79	65	57-75	34	73-93	0.054
TNF-α	86.95	0.82	0.72-0.91	75	61-86	86	78-95	81	70-91	82	75-90	0.005
IL-6	27843.18	0.77	0.68-0.86	50	35-65	95	89-100	89	77-100	72	67-79	0.049
IL-10	35.05	0.78	0.68-0.87	69	56-81	86	79-94	79	68-90	79	72-87	0.068
IL-17	1.00	0.70	0.60-0.81	66	52-80	72	60-84	65	54-76	73	65-82	0.887
DEFA3	33.90	0.64	0.53-0.75	38	23-52	95	89-100	86	70-100	58	63-74	0.727

Abbreviations: AUC, the area under the curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

Table 24. Univariate and multivariate logistic regression predicting FIRS in the suPAR cohort. Variables, that were not distributed normally, were log-transformed.

Biomarker	Univariate logistic regression			
	Estimate	P value	Odds ratio	95% CI
Log-TNF-α	0.690	<0.001	1.99	1.53 - 2.72
Log-MMP-8	0.491	<0.001	1.63	1.29 - 2.15
Log-IL-6	0.547	<0.001	1.73	1.38 - 2.23
Log-IL-10	0.560	<0.001	1.75	1.40 - 2.27
Log-IL-17	0.738	0.002	2.09	1.37 - 3.45
Log-suPAR	0.530	0.024	1.70	1.09 - 2.74
Log-DEFA3	0.775	0.002	2.17	1.19 - 4.24
GA	-0.211	0.004	0.81	0.70 - 0.92
Birthweight	-0.001	0.002	0.99	0.998 - 0.999
Multivariate logistic regression N=114, AIC=118.17				
	Estimate	P value	Odds ratio	95% CI
Log-TNF-α	0.765	<0.001	2.15	1.57 - 3.11
Log-IL-17	0.137	0.657	1.15	0.63 - 2.15
Log-DEFA3	0.678	0.095	1.97	0.91 - 4.56
GA	-0.085	0.316	0.92	0.77 - 1.09
Log-suPAR	-0.186	0.595	0.83	0.40 - 1.61

In Table 25, a stepwise logistic regression analysis was conducted to identify significant predictors of FIRS, with suPAR and GA forcibly included in the model. The analysis included a total of 114 observations, and the AIC was used to assess model fit.

The results of the stepwise logistic regression revealed the following significant predictors of FIRS: log-TNF-α and log-DEFA3. Each unit increase in log-TNF-α and log-DEFA3 was associated with a notable escalation in the odds of FIRS (OR = 2.19, 95% CI: 1.61 – 3.14, $p < 0.001$, and OR = 2.11, 95% CI: 1.02 – 4.60, $p = 0.049$, respectively). This implies that elevated levels of TNF-α and DEFA3 are independently linked with an increased likelihood of FIRS. Nonetheless, neither GA nor log-transformed suPAR emerged as significant predictors of FIRS in this stepwise regression model. The p values for both variables exceeded 0.05, indicating their negligible contribution to FIRS prediction when considering other variables.

Table 25. Stepwise (forward–backward) logistic regression for FIRS with forced suPAR and gestational age in the suPAR cohort. Variables, that were not distributed normally, were log-transformed.

Stepwise logistic regression N=114, AIC= 116.16				
Biomarker	Estimate	P value	Odds ratio	95% CI
Log-TNF-α	0.784	<0.001	2.19	1.61 – 3.14
Log-DEFA3	0.744	0.049	2.11	1.02 – 4.60
GA (forced)	-0.084	0.323	0.92	0.78 - 1.09
Log-suPAR (forced)	-0.129	0.691	0.88	0.45 – 1.62

In further analysis, we tested logistic regression models for FIRS with optimal cut-off values of suPAR and other biomarkers assessing their dose-dependent effect (Table 26). ROC curves and the Youden index were used to determine the cut-off values for amniotic fluid biomarkers (Table 23). Univariate logistic regression analysis revealed that elevated suPAR concentrations above 27 ng/mL increased the odds of having FIRS 3.47 times (95% CI; 1.55 – 8.05). The statistical evaluation of univariate logistic regression using cut-offs for other biomarkers in the suPAR cohort remained consistent with the results obtained from the full cohort.

In this multivariate logistic regression analysis, we investigated the predictive value of biomarkers and gestational age for FIRS, employing predefined cutoff values, as well. Among the biomarkers evaluated, TNF-α levels greater than 87 pg/mL showed a significant positive association with FIRS (OR = 7.13, 95% CI: 1.69 – 32.77, p = 0.008). Similarly, elevated DEFA3 levels (> 34 ng/mL) were significantly associated with an increased risk of FIRS (OR = 7.25, 95% CI: 1.57 – 42.38, p = 0.016). However, MMP-8 levels > 190 ng/mL, IL-6 levels > 27,843 pg/mL, IL-10 levels > 35 pg/mL, IL-17 levels > 1 pg/mL, and suPAR levels > 27 ng/mL did not exhibit statistically significant associations with FIRS in this multivariate analysis (p > 0.05). Additionally, gestational age was not significantly associated with FIRS (p = 0.51). These findings suggest that among the biomarkers analyzed, TNF-α and DEFA3 may serve as potential predictors of FIRS when considering predefined cutoff values, while the significance of MMP-8, IL-6, IL-10, IL-17, and suPAR in predicting FIRS may be limited in this cohort.

Table 26. Univariate and multivariate logistic regression predicting FIRS using cut-off values of biomarkers in the suPAR cohort.

Univariate logistic regression with cut-off				
Biomarker	Estimate	P value	Odds ratio	95% CI
suPAR >27	1.243	0.003	3.47	1.55 – 8.05
TNF-α >87	2.931	<0.001	18.75	7.16 – 54.82
MMP-8 > 190	2.223	<0.001	9.24	3.75 – 25.04
IL-6 >27843	3.045	<0.001	21.00	6.59 – 94.25
IL-10 >35	2.634	<0.001	13.93	5.71 – 37.18
IL-17 >1	1.600	<0.001	4.95	2.16 – 11.89
DEFA3 >34	2.53	<0.001	12.60	3.90 – 56.81
Multivariate logistic regression with cut-off N=114, AIC =102.77				
Biomarker	Estimate	P value	Odds ratio	95% CI
TNF-α > 87	1.963	0.008	7.13	1.69 – 32.77
MMP-8 > 190	0.793	0.344	2.21	0.41 – 11.57
IL-6 > 27843	1.574	0.059	4.83	1.01 – 29.22
IL-10 > 35	0.497	0.523	1.64	0.33 - 7.36
IL-17 > 1	-0.407	0.571	0.67	0.15 -2.55
DEFA3 > 34	1.981	0.016	7.25	1.57 – 42.38
GA	-0.063	0.512	0.94	0.77 - 1.13
suPAR > 27	-0.423	0.564	0.65	0.14 - 2.59

Multivariable logistic regression with stepwise (forward-backward) selection confirmed independently observed associations with FIRS while considering predefined cutoff values (Table 27). The stepwise analysis, incorporating gestational age and suPAR as a forced factor, identified TNF-α > 87, DEFA3 > 34 and IL-6 >27843 as robust and significant predictors of FIRS. However, neither gestational age nor suPAR levels >27 ng/mL demonstrated a significant association with FIRS in this stepwise regression model ($p > 0.05$).

Table 27. Stepwise (forward – backward) logistic regression for FIRS using cut-offs of biomarkers with forced gestational age and suPAR in the suPAR cohort.

Stepwise logistic regression N=114, AIC= 98.82				
Biomarker	Estimate	P value	Odds ratio	95% CI
TNF-α > 87	2.421	<0.001	11.26	3.22 - 44.25
DEFA3 > 34	2.082	0.009	8.02	1.85 - 45.02
IL-6 >27843	1.669	0.031	5.31	1.25 - 28.62
GA (forced)	-0.048	0.612	0.95	0.79 - 1.15
suPAR (forced)	-0.136	0.834	0.87	0.22 - 2.95

4.2.2 Random Forest analysis to predict FIRS

We employed Random Forest analysis to predict FIRS and rank the importance of variables using the following eight predictors: suPAR, TNF-α, MMP-8, IL-6, IL-17, IL-10, DEFA3, and GA. We tested a model with 500, 1000, 2000, and 3000 trees. The number of variables to be tested (mtry) was the square root of the total number of predictors (the square root of 8 equals 2) with values from 1 to 8. We verified the model using the OOB. Increasing the number of trees (more than 500) did not improve the OOB error. OOB errors were lowest when mtry was 4.

The test classification accuracy of this random forest model was 82% with sensitivity estimated to be 86% and specificity to be 79%. The three most important predictors of FIRS were TNF-α, IL10, and IL-6. The SHAP summary plot visualizes the impact of individual features on the model's predictions (Figure 26). Figure 26 demonstrates that out of the eight variables selected, higher values of TNF-α and IL-10 were the most important variables in predicting FIRS. Meanwhile, IL-6, IL-17, MMP-8, suPAR and DEFA3 performed moderately. GA was the least important predictor of FIRS with negative direction; there was less expectation of FIRS with higher GA. In summary, Random Forest analysis revealed that suPAR, in vaginal amniotic fluid, holds rather moderate significance in predicting FIRS compared to the other analyzed biomarkers.

With various combinations of predictive variables, we evaluated some Random Forest models to determine the best one with the highest accuracy and lowest OOB error. Nevertheless, none of the Random Forest models predicting FIRS demonstrated superior performance metrics than that reported in Figure 26.

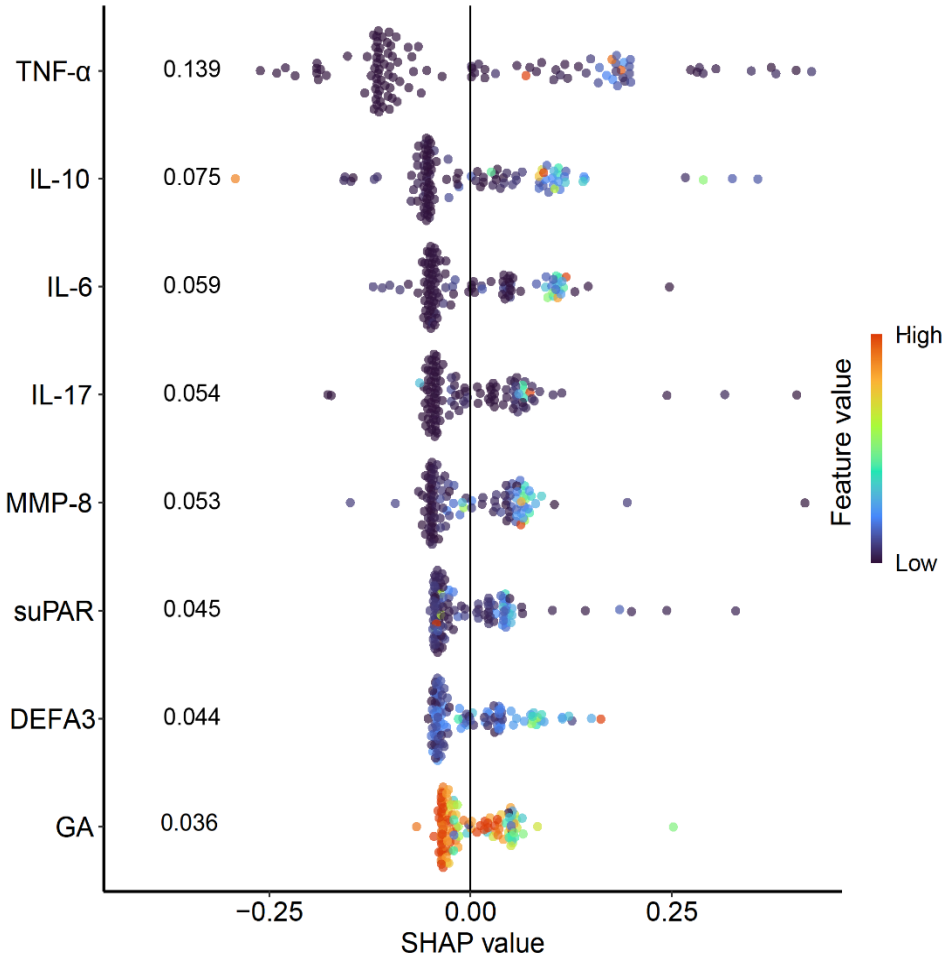


Figure 26. Variable Importance predicting FIRS in the suPAR cohort. The predictors (TNF-α, IL-10, IL-6, IL-17, MMP-8, suPAR, DEFA3, and GA) are listed in descending order of their importance in the Random Forest model.

4.2.3 SuPAR associations with adverse neonatal outcomes

Assessing suPAR associations with adverse neonatal outcomes and FIRS, we determined median levels of suPAR in the FIRS and non-FIRS groups according to the presence and absence of major neonatal outcomes. However, there was no statistically significant difference in suPAR levels based on the presence and absence of major neonatal outcomes (Table 28).

Table 28. The median concentrations of suPAR in vaginal amniotic fluid in both the FIRS and non-FIRS groups, categorized by the presence or absence of major neonatal outcomes. The results are reported as median with IQR and mean with SD, respectively.

oor	FIRS group		Non-FIRS group		P value
	Major outcomes	No outcomes	Major outcomes	No outcomes	
suPAR (ng/mL)	37.24 (20.02- 2.78)	30.36 (15.99-77.85)	28.82 (16.68-36.15)	19.81 (11.44-36.18)	0.082
Log-suPAR	3.68 ±0.86	3.52 ±0.85	3.37 ±0.96	3.15 ±0.90	0.133

4.3 Part 3: EGF and major neonatal outcomes

4.3.1 Study population characteristics

In the fourth part of the study, a cohort of 145 women and their neonates underwent analysis for EGF levels in vaginally collected amniotic fluid. The study population was categorized into three gestational age groups based on the WHO classification [182]: extremely preterm infants (22^{+0}–27^{+6} weeks GA), very preterm infants (28^{+0}–31^{+6} weeks GA), and moderate to late preterm infants (32^{+0}–34^{+6} weeks GA). Tables 29 and 30 display the demographic and clinical characteristics of the total cohort and its subgroups.

Maternal age varied slightly among the groups, while gravidity, parity, latency period, hypertensive disorders, antenatal glucocorticoids, tocolytic use, mode of delivery, and positive group B streptococcus test did not differ significantly across the subgroups. Gestational diabetes occurred more frequently in the moderate to late preterm group. Cesarean section rates were higher in extremely and very preterm neonates compared to moderate to late preterm infants. Clinical and histological chorioamnionitis, as well as funisitis, were more prevalent in patients with smaller GA.

GA groups showed statistical differences in birth weight, Apgar scores at 1 and 5 minutes, FIRS incidence, and the need for post-birth resuscitation, while umbilical cord arterial pH remained non-significant.

Table 30 presents neonatal outcomes across the three GA groups: 22^{+0}–27^{+6} weeks, 28^{+0}–31^{+6} weeks, and 32^{+0}–34^{+6} weeks. As expected, the incidence rates of respiratory disorders and other morbidities among preterm neonates, such as EOS, PDA, ROP, and early hypotension, varied among the groups. Lower GA was strongly associated with a higher incidence of adverse outcomes, with $p < 0.05$.

The frequency of all adverse neonatal outcomes decreased with increasing GA, although there was no significant difference in neonatal mortality.

Table 29. Demographic and clinical characteristics of the total study population, and three gestational age groups.

	Total cohort (n=145)	22^{+0}-27^{+6} weeks (n=24)	28^{+0}–31^{+6} weeks (n=29)	32^{+0}–34^{+6} weeks (n=92)	P value
Maternal characteristics					
Age of mother, years, mean±SD	31.25±5.62	29.8±4.1	32.9±5.74	31.1±5.82	0.048
Latency period, hours, median, IQR	71 (24-204)	113 (49-241)	63 (16-163)	70 (24-209)	0.321
Hypertensive disorders, n, %	31 (21)	4 (17)	9 (31)	18 (20)	0.358
Gestational Diabetes, n, %	34 (24)	2 (8)	4 (14)	28 (30)	0.035
Group B streptococcus test positive, n, %	17 (12)	2 (8)	4 (14)	11 (12)	0.721
Primigravida, n, %	50 (34)	8 (33)	10 (3)	32 (35)	0.991
Primipara, n, %	68 (47)	9 (38)	14 (48)	45 (49)	0.599
Antenatal glucocorticoids, n, %	125 (86)	21 (88)	28 (97)	76 (83)	0.162
Tocolytics, n, %	67 (46)	15 (63)	9 (31)	43 (47)	0.072
Mode of delivery, n, % vaginal delivery cesarean section	 112 (77) 33 (23)	 14 (58) 10 (42)	 20 (69) 9 (31)	 78 (85) 14 (15)	0.011
Induction of delivery or cesarean section n, % Spontaneous delivery n, %	 78 (54) 67 (46)	 12 (50) 12 (50)	 19 (65) 10 (35)	 47 (51) 45 (49)	0.365
Clinical chorioamnionitis, n, %	7 (5)	5 (21)	1 (3)	1 (1)	0.001
Histological chorioamnionitis, n, %	54 (37)	15 (63)	11 (38)	28 (30)	0.015
Funisitis, n, %	21 (14)	5 (21)	8 (28)	8 (9)	0.004
Neonatal characteristics					
GA at birth, weeks, median, IQR	32 (30–34)	26 (25.8–27)	30 (29–30)	33 (33–34)	<0.001
Birthweight, grams, mean ± SD	1840 ± 634	868 ± 205	1438 ± 264	2221 ± 396	<0.001
Apgar scores <7 at 1 min, n, %	22 (15)	14 (58)	5 (17)	3 (3)	<0.001
Apgar scores <7 at 5 min, n, %	7 (25)	6 (25)	1 (3)	0	<0.001
Umbilical cord arterial pH, median, IQR	7.34 (7.28–7.39)	7.38 (7.25–7.42)	7.32 (7.26–7.4)	7.34 (7.29–7.39)	0.462
The need for resuscitation after birth, n, %	22 (15)	13 (54)	4 (14)	5 (5)	<0.001
FIRS, n, %	54 (37)	13 (54)	14 (48)	27 (29)	0.032

Table 30. The analysis of neonatal outcomes according to gestational age.

Neonatal outcomes	Total cohort n=145	22^{+0}-27^{+6} weeks (n=24)	28^{+0}–31^{+6} weeks (n=29)	32^{+0}–34^{+6} weeks (n=92)	P value
Major outcomes, n, %	44 (30)	20 (87)	11 (38)	13 (14)	<0.001
Respiratory distress, n, %	121 (83)	24 (100)	29 (100)	68 (74)	<0.001
RDS grade, n, %:					
No RDS	25 (17)	0	0	25 (27)	
Mild RDS	48 (33)	2 (8)	7 (24)	39 (42)	<0.001
Moderate RDS	44 (30)	8 (33)	14 (48)	22 (24)	
Severe RDS	28 (19)	14 (58)	8 (28)	6 (67)	
Respiratory support, n, %					
None	24 (17)	0	0	24 (26)	<0.001
Mechanical ventilation	26 (18)	14 (58)	3 (10)	9 (10)	
Non-invasive respiratory therapy	95 (66)	10 (42)	26 (90)	59 (64)	
The need for surfactant, n, %	41 (28)	23 (96)	12 (41)	6 (7)	<0.001
Duration of RS, n, %:					
no RS	23 (16)	0	0	23 (25)	
≤ 24 hours	38 (26)	0	1 (3)	37 (40)	<0.001
24 – 96 hours	32 (22)	0	10 (35)	22 (24)	
≥ 96 hours	51 (35)	23 (100)[1]	18 (62)	10 (11)	
BPD	17 (12)	16 (70)	1 (3)	0	<0.001
Neonatal death, n, %	2 (1)	1 (4)	0	1 (1)	0.344
Sepsis, n, %	15 (10)	10 (42)	2 (7)	3 (3)	<0.001
Early-onset sepsis, n, %	9 (6)	6 (25)	2 (7)	1 (1)	<0.001
Early hypotension, n, %	18 (12)	12 (50)	2 (7)	4 (4)	<0.001
PDA, n %	19 (13)	12 (52)	5 (17)	2 (2)	<0.001
ROP, n, %	25 (17)	20 (87)	5 (17)	0	<0.001
Treated ROP, n, %	5 (3)	4 (17)	1 (3)	0	<0.001
IVH, n, %					
None	98 (68)	10 (42)	17 (59)	71 (77)	<0.001
1-2 grade	38 (26)	8 (33)	10 (35)	20 (22)	
3-4 grade	9 (6)	6 (25)	2 (7)	1 (1)	

[1] One neonatal death on the first day of life; thus, the total duration of RS is unknown: n=23 of 24 (100%).

4.3.2 EGF characteristics in amniotic fluid

EGF was identified in all amniotic fluid samples with a median of 81.15 pg/mL (IQR: 41.07–133.38). EGF concentrations were significantly associated with GA and decreased with GA (Figure 27). The median EGF concentration for the 22^{+0}–27^{+6} weeks GA group was 34.20 pg/mL (IQR: 19.67–50.14). In the 28^{+0}–31^{+6} weeks GA group, median EGF concentrations were 62.62 pg/mL (IQR: 34.95–

110.46) versus the median of 93.36 pg/mL (IQR: 64.89–163.53) of the 32^{+0}–34^{+6} weeks GA group. Median EGF concentrations in GA groups differed significantly ($p < 0.001$). The vaginal amniotic fluid sample of the neonate of 23 weeks GA had a minimum concentration of 3.56 pg/mL, while the sample of the infant of 34 weeks GA had a maximum concentration of 489.735 pg/mL. The median EGF concentration has almost tripled among GA groups between 22^{+0}-27^{+6} weeks and 32^{+0}–34^{+6} weeks of gestation.

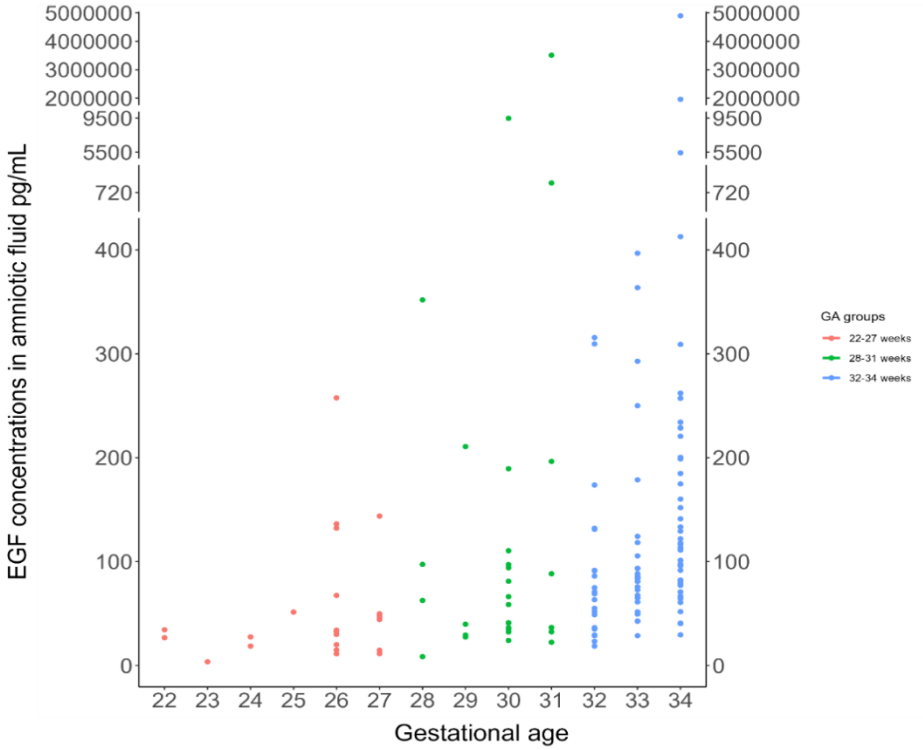


Figure 27. EGF concentrations in the amniotic fluid according to gestational age.

Spearman's correlation analysis was conducted to examine the relationship between EGF and various numerical variables, as depicted in the correlation matrix (refer to Figure 9). A favorable correlation emerged between EGF levels and both gestational age and birthweight (rho = 0.47, $p < 0.001$ and rho = 0.46, $p < 0.001$). Compared to other biomarkers, EGF had the strongest association with GA. Conversely, EGF revealed an adverse correlation with the duration of respiratory support (rho = -0.38, $p < 0.001$), mechanical ventilation (rho = -0.28, $p = 0.003$), and non-invasive respiratory support (rho = -0.38, $p < 0.001$).

Nonetheless, these correlations were of modest strength, with rho coefficients below 0.5, despite achieving statistical significance.

4.3.3 The association between EGF and FIRS

In this study, median levels of EGF did not differ between the FIRS and the non-FIRS groups: median of 80.95 pg/mL (IQR: 37.70 – 126.61) vs. median of 84.13 pg/mL (IQR: 42.67 – 147.86), p = 0.436 (Table 6). Due to the significantly higher rate of FIRS in extremely preterm and very preterm neonates, we investigated the association between median EGF levels with FIRS according to gestational age (Table 31). The analysis results confirmed that FIRS did not significantly influence EGF concentrations in non-invasively obtained amniotic fluid in different gestational age groups. This relationship was verified by evaluating Spearman's correlation between EGF and IL-6, TNF-α, and MMP-8 concentrations in amniotic fluid (Figures 9 and 10). There was no statistically significant correlation between EGF and the levels of all other inflammatory cytokines.

Table 31. FIRS effect on median EGF concentrations (pg/mL) in gestational age groups.

Gestational age groups						
	22^{+0}-27^{+6} weeks		28^{+0}–31^{+6} weeks		32^{+0}–34^{+6} weeks	
	Median EGF	n	Median EGF		Median EGF	n
FIRS	32.7	11	41.1	15	93.4	65
Non-FIRS	34.4	13	84.6	14	91.6	27
P–value	0.931		0.513		0.764	

However, we noted that there were statistically significant differences in median EGF levels comparing neonates having FIRS with major neonatal outcomes and without it (Table 14).

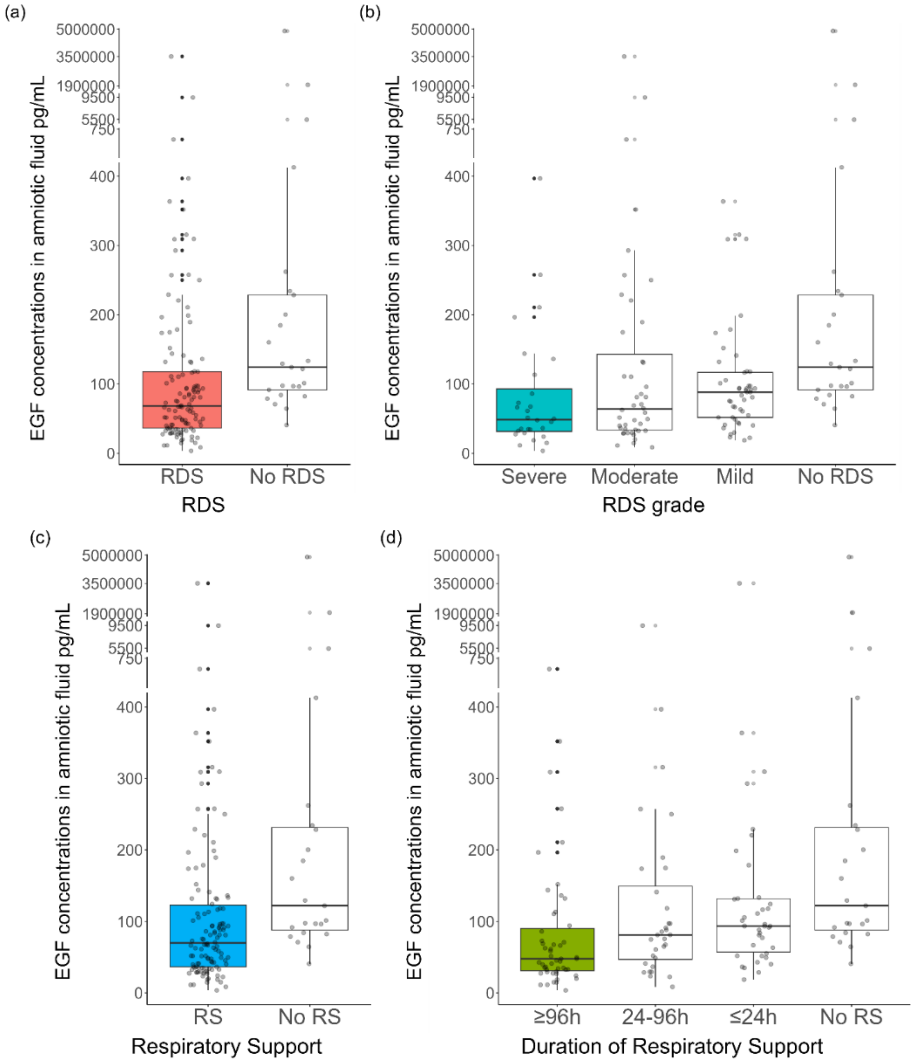
4.3.4 EGF and major neonatal outcomes

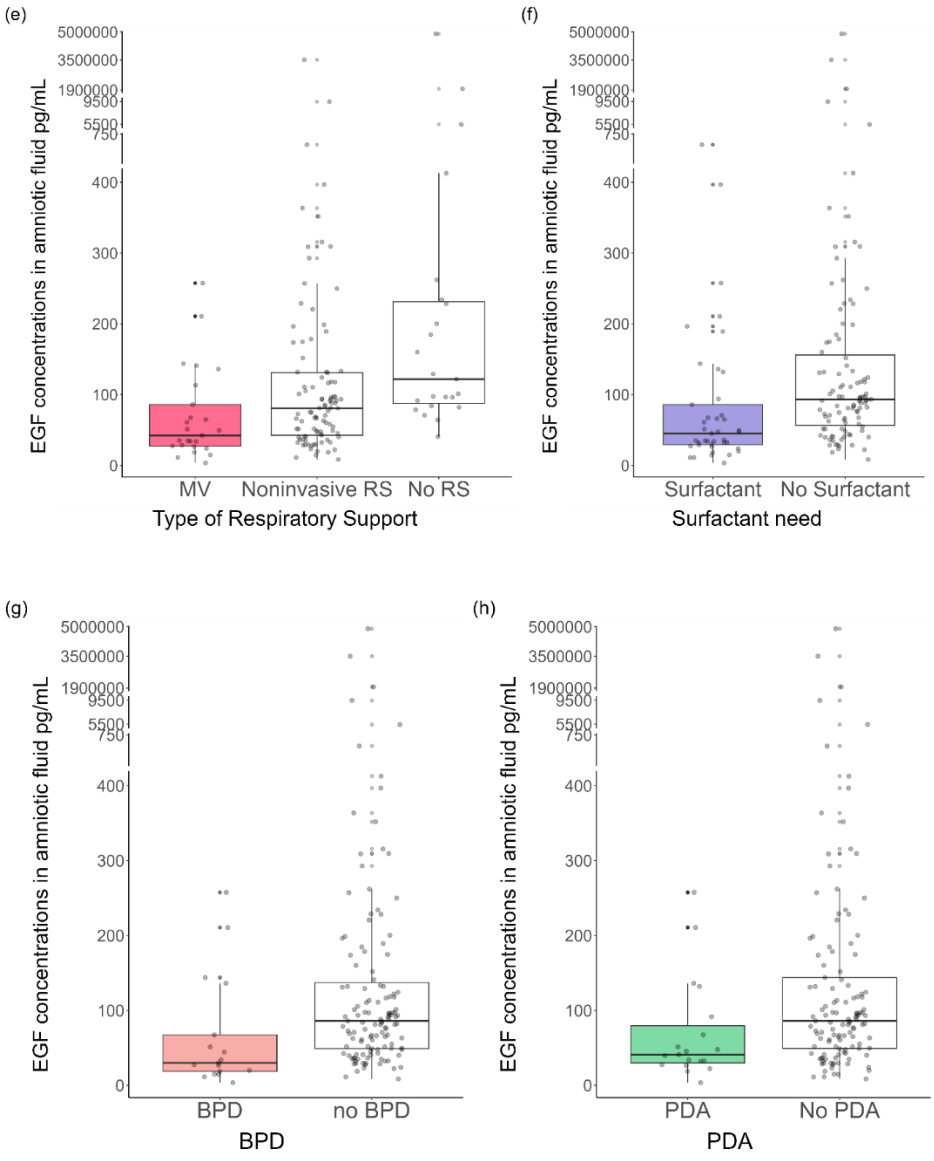
Based on our findings, we explored whether neonates experiencing various morbidities, such as respiratory distress, BPD, PDA, ROP, etc., demonstrated different EGF levels in their mothers' amniotic fluid compared to unaffected infants. Indeed, the median concentrations of amniotic fluid EGF were significantly lower in neonates with early hypotension, PDA, ROP, and respiratory

outcomes (RDS, need for respiratory support, BPD, need for surfactant) compared to infants without these conditions (Table 32 and Figure 27). Furthermore, we observed a gradual decrease in EGF levels across severity groups of RDS and respiratory support type and duration groups. However, no significant difference in EGF levels was observed in neonates with early-onset sepsis or IVH when compared to neonates without these morbidities.

Table 32. Median amniotic fluid EGF concentrations in neonates with major outcomes compared with neonates without outcomes.

Outcome		Median (pg/mL)	IQR	P value
RDS	RDS	68.25	36.35 - 117.90	<0.001
	No RDS	124.29	91.66 - 228.43	
Respiratory support	Need for RS	69.85	87.89 - 231.30	<0.001
	No RS	121.88	57.11 - 131.75	
Surfactant	Need for surfactant	45.36	29.25 - 86.06	<0.001
	No surfactant	93.33	56.82 - 156.01	
RS type	No RS	121.88	87.89 - 231.30	<0.001
	Non-invasive ventilation	80.83	42.81 - 131.02	
	Mechanical ventilation	42.54	27.53 - 86.06	
RDS grade	Severe	48.73	31.55 - 92.89	<0.001
	Moderate	63.82	33.69 - 142.81	
	Mild	88.24	51.78 - 116.83	
	No RDS	124.29	91.66 - 228.43	
Duration of RS	No RS	121.88	87.89 - 231.30	<0.001
	≤24 hours RS	93.50	57.11 - 131.75	
	24–96 hours RS	81.10	46.91 - 149.28	
	≥96 hours RS	47.75	31.11 - 90.05	
BPD	BPD	29.90	18.69 - 67.44	0.002
	No BPD	86.06	49.11 - 137.26	
ROP	ROP	34.02	18.69 - 49.71	<0.001
	No ROP	91.61	56.82 - 156.01	
PDA	PDA	86.06	49.37 - 143.85	0.007
	No PDA	41.05	29.92 - 79.56	
IVH	None	85.84	43.15 - 131.75	0.535
	1-2 grade	75.24	37.55 - 165.09	
	3-4 grade	58.59	34.39 - 72.77	
Early hypotension	Early hypotension	38.46	24.73 - 63.00	0.002
	No hypotension	86.06	47.79 - 144.05	
Early onset sepsis	EOS	47.75	34.39 - 67.44	0.116
	No EOS	83.89	42.37 - 137.45	


(a)
EGF concentrations in amniotic fluid pg/mL
5000000
3500000
1900000
9500
5500
750
400
300
200
100
0
RDS
No RDS
RDS
(b)
EGF concentrations in amniotic fluid pg/mL
Severe
Moderate
Mild
No RDS
RDS grade
(c)
EGF concentrations in amniotic fluid pg/mL
RS
No RS
Respiratory Support
(d)
EGF concentrations in amniotic fluid pg/mL
≥96h
24-96h
≤24h
No RS
Duration of Respiratory Support


(e)
EGF concentrations in amniotic fluid pg/mL
5000000
3500000
1900000
9500
5500
750
400
300
200
100
0
MV
Noninvasive RS
No RS
Type of Respiratory Support
(f)
EGF concentrations in amniotic fluid pg/mL
Surfactant
No Surfactant
Surfactant need
(g)
EGF concentrations in amniotic fluid pg/mL
BPD
no BPD
BPD
(h)
EGF concentrations in amniotic fluid pg/mL
PDA
No PDA
PDA

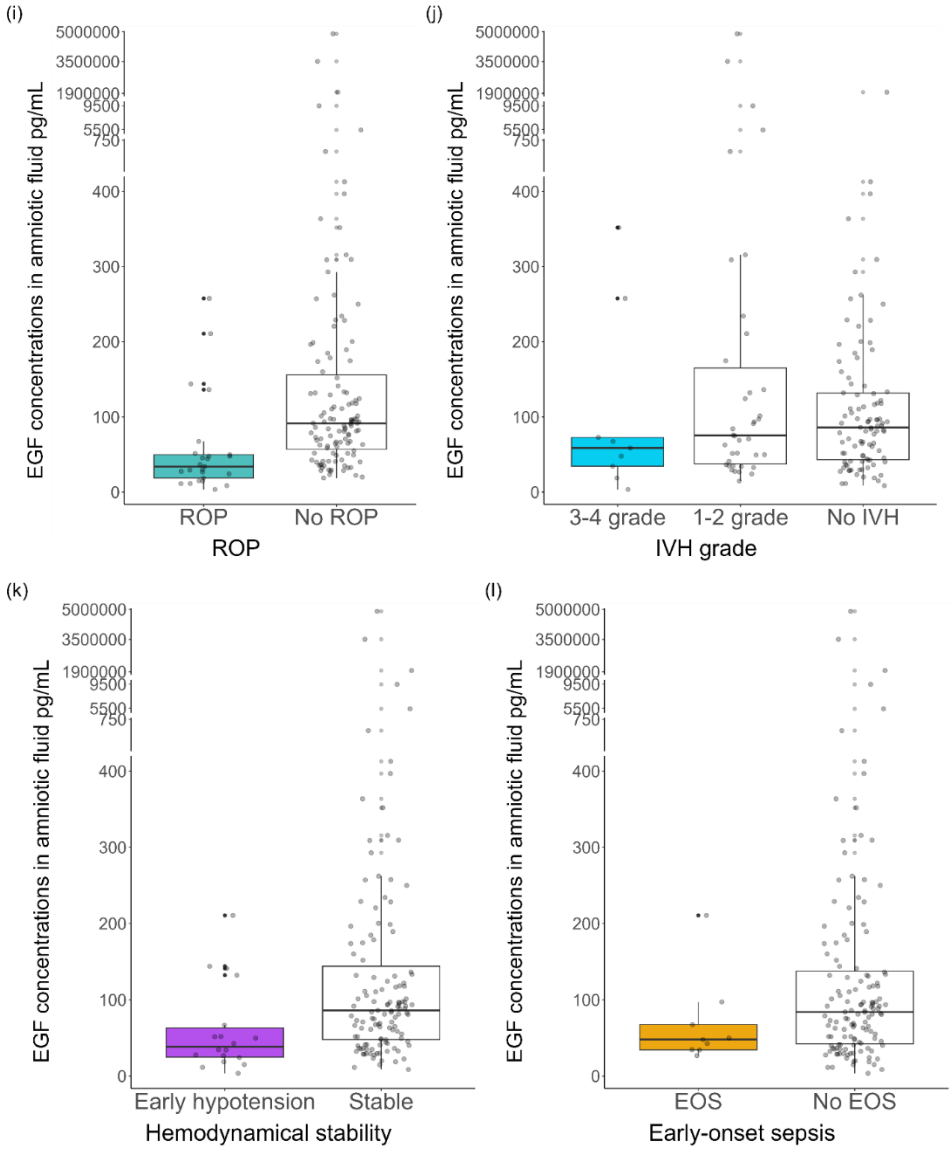


Figure 27. Median amniotic fluid EGF concentrations in infants with adverse neonatal outcomes compared with infants without outcomes.

4.3.5 Logistic regression analysis of EGF predicting adverse neonatal outcomes

We assessed the significance of EGF for predicting adverse neonatal outcomes using univariate logistic regression with different EGF cut-off values. Predictive

models were constructed for PDA, ROP, early hypotension, and severe respiratory outcomes, such as severe RDS, respiratory support (RS) for > 4 days, the need for surfactant, mechanical ventilation, and BPD. Table 33 outlines the results obtained from the univariate regression analysis. The analysis revealed that lower EGF concentrations might predict severe respiratory outcomes, ROP, PDA, and early hypotension. As the EGF cut-off value decreased, the odds ratio for neonatal outcomes increased, reaching significance for all outcomes at the EGF cut-off of 75 pg/mL. Furthermore, EGF levels below the cut-off of 35 pg/mL were associated with the highest odds of neonatal outcomes. Conversely, higher EGF cut-off values corresponded to lower odds ratios for severe neonatal morbidity, if statistically significant. In summary, in univariate logistic regression, EGF level less than 35 pg/mL in vaginal amniotic fluid significantly predicted and had the highest odds ratios for PDA, ROP, early hemodynamic instability, and respiratory outcomes, including severe RDS, RS for >4 days, the need for surfactant, mechanical ventilation, and BPD.

Table 33. The univariate regression analysis for neonatal outcomes with various cut-off values of EGF: neonatal outcomes as the outcome variable, different cut-off values of EGF as the input variable. The gray-shaded rows indicate the models that describe the odds best.

Cut-off EGF (pg/mL)	Neonatal outcomes	Estimate	P value	OR	95% CI
<200	Severe RDS	0.6004	0.361	1.82	0.57 – 8.15
	The need for Surfactant	0.8015	0.169	2.23	0.78 – 8.06
	RS for >4 days	0.8596	0.109	2.36	0.89 – 7.51
	Mechanical ventilation	1.0012	0.220	2.72	0.73 – 17.72
	BPD	0.4520	0.566	1.57	0.40 – 10.42
	PDA	0.5964	0.447	1.82	0.47 – 11.97
	ROP	0.9587	0.216	2.61	0.70 – 17.00
	Early hypotension	0.9587	0.216	3.76	0.69 – 17.00
<100	Severe RDS	0.6286	0.187	1.88	0.77 – 5.08
	The need for Surfactant	0.9354	0.029	2.55	1.14 – 6.17
	RS for >4 days	0.8972	0.022	2.45	1.14 – 6.17
	Mechanical ventilation	0.7422	0.179	2.10	0.83 – 6.09
	BPD	0.6804	0.257	1.97	0.66 – 7.32
	PDA	0.8492	0.151	2.34	0.79 - 8.57
	ROP	1.2667	0.028	3.55	1.26 - 12.74
	Early hypotension	0.7545	0.205	2.13	0.71 - 7.84

Cut-off EGF (pg/mL)	Neonatal outcomes	Estimate	P value	OR	95% CI
<90	Severe RDS	1.0815	0.023	2.95	1.21 – 7.97
	The need for Surfactant	1.2675	0.002	3.55	1.62 – 8.33
	RS for >4 days	1.3103	<0.001	3.71	1.78 – 8.08
	Mechanical ventilation	1.1872	0.026	3.28	1.29 – 9.49
	BPD	1.0999	0.066	3.00	1.001 – 11.12
	PDA	0.9496	0.085	2.58	0.93 - 8.39
	ROP	1.7087	0.003	5.52	1.96 - 19.79
	Early hypotension	1.1740	0.048	3.23	1.09 - 11.89
<75	Severe RDS	1.3146	0.004	3.72	1.56 – 9.64
	The need for Surfactant	1.6245	<0.001	5.08	2.34 – 11.71
	RS for >4 days	1.7634	<0.001	5.44	2.79 – 12.75
	Mechanical ventilation	1.1676	0.007	3.21	1.33 – 8.38
	BPD	1.5124	0.012	4.54	1.51 – 16.81
	PDA	1.3688	0.013	3.93	1.41 - 12.78
	ROP	2.1556	<0.001	8.63	3.05 - 31.04
	Early hypotension	1.5865	0.008	4.88	1.65 - 17.99
<50	Severe RDS	1.1635	0.007	3.20	1.37 – 7.59
	The need for Surfactant	1.6482	<0.001	5.20	2.41 – 11.54
	RS for >4 days	1.6942	<0.001	5.44	2.58 – 11.89
	Mechanical ventilation	1.1543	0.006	3.17	1.33 – 7.70
	BPD	1.6124	0.003	5.01	1.77 – 15.56
	PDA	1.5643	0.002	4.78	1.77 - 13.83
	ROP	2.4272	<0.001	11.33	4.31 - 33.84
	Early hypotension	1.4184	0.007	4.13	1.51 - 12.04
<35	Severe RDS	1.1170	0.017	3.06	1.19 – 7.65
	The need for Surfactant	1.9849	<0.001	7.28	3.03 – 18.47
	RS for >4 days	1.6275	<0.001	5.09	2.13 – 12.97
	Mechanical ventilation	1.4816	0.006	4.40	1.72 – 11.24
	BPD	2.2239	<0.001	9.24	3.15 – 28.76
	PDA	1.4005	0.007	4.06	1.41 - 11.41
	ROP	2.0949	<0.001	8.13	3.13 - 21.77
	Early hypotension	1.7377	0.001	5.684	1.99 - 16.45

Given the highest estimated likelihood with an EGF cut-off value of <35 pg/mL in the univariate regression, we conducted a multiple regression analysis to explore a prediction model, while adjusting for gestational age (Table 34). However, the input variable of EGF did not show statistical significance in these models. Conversely, the input variable of gestational age demonstrated a

statistically significant impact on the outcomes. Specifically, for each unit increase in weeks of gestation, the odds for outcomes decreased by 0.3–0.7 times, thereby confirming that gestational age strongly influences outcomes and determines neonatal risk.

We observed that gestational age has an impact on both EGF levels and outcomes, as indicated by the presented results. To control and investigate the impact of gestational age on the outcomes, we developed models with an EGF cut-off value of less than 35 pg/mL (compared to >35 pg/mL) across different gestational age groups (Table 34). However, when estimating the odds for infants with gestational ages of 22^{+0}–27^{+6} weeks and 32^{+0}–34^{+6} weeks, certain models could not be performed due to zero case events in specific groups: for instance, the model for BPD and ROP in the 32^{+0}–34^{+6} weeks gestational age group, and the model for RS for >4 days in the 22^{+0}–27^{+6} weeks gestational age group. In the feasible models for 22^{+0}–27^{+6} weeks gestational age, EGF did not significantly predict outcomes, except for the need for surfactant, with gestational age proving to be a more reliable predictor. In the models for mechanical ventilation and early hypotension among infants with gestational age of 32^{+0}–34^{+6} weeks the variable of EGF <35 pg/mL was close to the significance threshold, while the need for surfactant was predicted significantly by both variables: EGF <35 pg/mL and gestational age. In the model for infants with gestational age of 28^{+0}–31^{+6} weeks, EGF <35 pg/mL emerged as an independent predictor for PDA, ROP, early hypotension, and severe respiratory outcomes. Infants with GA of 28^{+0}–31^{+6} weeks and having EGF <35 pg/mL were about 3–12 times more likely to experience severe respiratory outcomes, PDA, ROP, and early hypotension compared to neonates of other gestational ages (22^{+0}–27^{+6} weeks and 32^{+0}–34^{+6} weeks). In summary, low EGF concentrations (<35 pg/mL) may serve as predictors of neonatal outcomes in univariate logistic regression. After accounting for gestational age, an EGF cut-off value <35 pg/mL proved to be a reliable predictor of PDA, ROP, early hypotension, and severe respiratory outcomes in neonates of 28^{+0}–31^{+6} weeks of GA.

Table 34. The multiple regression analysis for neonatal outcomes with EGF cut-off level <35 pg/mL, adjusted for gestational age.

Outcome variable	Input variable	Coeffi-cients	Estimate	P value	OR	95 % CI
Severe RDS	EGF <35pg/mL + GA	EGF<35	-0.3572	0.567	0.7	0.19 – 2.26
		GA	-0.4072	<0.001	0.7	0.56 – 0.78
The need for Surfactant		EGF<35	0.5996	0.345	1.82	0.51 – 6.28
		GA	-0.6879	<0.001	0.5	0.39 – 0.62
RS for >4 days		EGF<35	-0.0796	0.906	0.92	0.23 – 3.38
		GA	-0.8263	<0.001	0.44	0.32 – 0.56
Mechanical ventilation		EGF<35	0.2768	0.641	1.32	0.39 – 4.09
		GA	–0.3524	<0.001	0.7	0.59 – 0.83
BPD		EGF<35	0.3220	0.732	1.38	0.19 – 2.26
		GA	–1.286	<0.001	0.28	0.11 – 0.47
PDA		EGF<35	-0.4236	0.558	0.65	0.15 – 2.55
		GA	-0.5790	<0.001	0.56	0.43 – 0.69
ROP		EGF<35	0.5454	0.505	1.73	0.33 – 8.79
		GA	-0.9912	<0.001	0.37	0.24 – 0.51
Early hypotension		EGF<35	0.3181	0.637	1.37	0.35 – 5.00
		GA	-0.4167	<0.001	0.66	0.53 – 0.80
Severe RDS	EGF <35pg/mL+ 22^{+0}–27^{+6} weeks GA**	EGF<35	0.2361	0.669	1.27	0.38 – 3.77
		GA	2.2752	<0.001	9.73	3.38 – 30.00
The need for Surfactant		EGF<35	1.3572	0.023	3.89	1.15 – 12.47
		GA	4.5830	<0.001	97.80	18.27 – 1821.60
Mechanical ventilation		EGF<35	0.6753	0.236	1.96	0.61 – 5.84
		GA	2.2915	<0.001	9.9	3.41 – 30.19
BPD		EGF<35	1.1423	0.191	3.13	0.57 – 19.15
		GA	5.3143	<0.001	203.21	33.3 – 3997.7
PDA		EGF<35	0.3890	0.553	1.48	0.38 – 5.12
		GA	2.7311	<0.001	15.35	4.73 – 54.70
ROP		EGF<35	1.4034	0.083	4.07	0.79 – 20.49
		GA	4.7885	<0.001	120.12	29.40 – 678.8
Early hypotension		EGF<35	0.7764	0.229	2.17	0.59 – 7.54
		GA	2.6621	<0.001	14.33	4.32 - 52.15
Severe RDS	EGF <35pg/mL+ 28^{+0}–31^{+6} weeks GA	EGF<35	1.0703	0.024	2.92	1.13 – 7.35
		GA	0.5004	0.31	1.65	0.60 – 4.26
The need for Surfactant		EGF<35	1.9495	<0.001	7.03	2.90 – 17.94
		GA	0.6295	0.184	1.88	0.73 – 4.72
RS for >4 days		EGF<35	1.5894	<0.001	4.9	1.98 – 12.86

Outcome variable	Input variable	Coeffi-cients	Estimate	P value	OR	95 % CI
		GA	1.3609	0.003	3.9	1.62 – 9.76
Mechanical ventilation		EGF<35	1.6155	0.001	5.03	1.91 – 13.41
		GA	–1.0486	0.13	0.35	0.07 – 1.19
BPD		EGF<35	2.5074	<0.001	12.27	3.95 – 41.13
		GA	–2.1194	0.056	0.12	0.01 – 0.72
PDA		EGF<35	1.3739	0.010	3.95	1.36 – 11.20
		GA	0.2380	0.687	1.27	0.36 – 3.84
ROP		EGF<35	2.1425	<0.001	8.52	3.23 – 23.38
		GA	-0.3542	0.562	0.70	0.19 – 2.18
Early hypotension		EGF<35	1.8694	<0.001	6.48	2.21 – 19.48
		GA	-1.1067	0.177	0.33	0.05 – 1.37
Severe RDS		EGF<35	0.2350	0.658	1.26	0.44 – 3.54
		GA	-2.2451	<0.001	0.11	0.03 – 0.29
The need for Surfactant		EGF<35	1.1397	0.044	3.12	1.04 – 9.77
		GA	-3.0556	<0.001	0.05	0.02 – 0.12
RS for >4 days	EGF <35pg/mL + 32^{+0}–34^{+6} weeks GA**	EGF<35	0.5444	0.371	1.72	0.52 – 5.76
		GA	-3.2808	<0.001	0.04	0.01 – 0.09
Mechanical ventilation		EGF<35	1.0043	0.052	2.73	0.98 – 7.54
		GA	–1.1414	0.023	0.32	0.12 – 0.84
PDA		EGF<35	0.4506	0.438	1.57	0.49 – 4.90
		GA	-2.9451	<0.001	0.05	0.01 – 0.21
Early hypotension		EGF<35	1.0871	0.061	2.96	0.94 – 9.34
		GA	-1.7014	0.008	0.18	0.05 – 0.60

**The regression analysis was not performed due to zero events in particular groups: RS for >4 days — in 22^{+0}–27^{+6} weeks GA; ROP and BPD — in 32^{+0}–34^{+}weeks GA.

4.3.6 Random Forest analysis predicting adverse neonatal outcomes

We conducted a Random Forest analysis to forecast severe respiratory outcomes and prioritize variables based on their significance from previous analyses, including TNF-α, EGF, GA, and FIRS. We established different outcome combinations: at least one severe respiratory outcome, two or more severe respiratory outcomes, and all five severe respiratory outcomes together. We assessed models with 300, 500, 1000, 2000, and 3000 trees, adjusting the number of variables to be tested (mtry) to the square root of the total predictors (square root of 4 equals 2), with adjacent values ranging from 1 to 4. Model verification was done using the OOB. However, increasing the number of trees did not

improve the OOB error rate. The lowest OOB error occurred when mtry was 1 for the model predicting at least one respiratory outcome, 1 for the model predicting two or more respiratory outcomes, and 3 for the model predicting all five respiratory outcomes.

The model predicting at least one severe respiratory outcome achieved a test classification accuracy of 84%, with a sensitivity of 76% and a specificity of 88%. For the model predicting two or more severe respiratory outcomes, the test classification accuracy was 79%, with a sensitivity of 33% and a specificity of 97%. Meanwhile, the model predicting all five severe respiratory outcomes demonstrated a test classification accuracy of 96%, with a sensitivity of 100% and a specificity of 95%.

In Figures 28, 29, and 30, a SHAP summary plot illustrates the sequence of predictors and their impact on predicting various severe respiratory outcomes. Figures 28 and 30 reveal that out of four selected variables, GA and EGF were the most crucial in predicting at least one and all severe respiratory outcomes, respectively. GA emerged as the primary predictor, with a negative correlation implying that higher GA led to lower expectations of respiratory outcomes.

Conversely, EGF and TNF-α showed moderate predictive capability. Low EGF levels and high TNF-α values predicted severe respiratory outcomes in both models. In the model predicting two or more severe respiratory outcomes (Figure 29), the sequence of predictors remained consistent: GA, TNF-α, EGF, and FIRS, with similar impacts on outcomes. FIRS appeared as the least influential predictor in all models, yet removing it from the Random Forest analysis reduced the accuracy of the tested models. In summary, the Random Forest model demonstrated that severe respiratory outcomes could be accurately predicted using GA, EGF, and TNF-α levels in vaginal amniotic fluid.

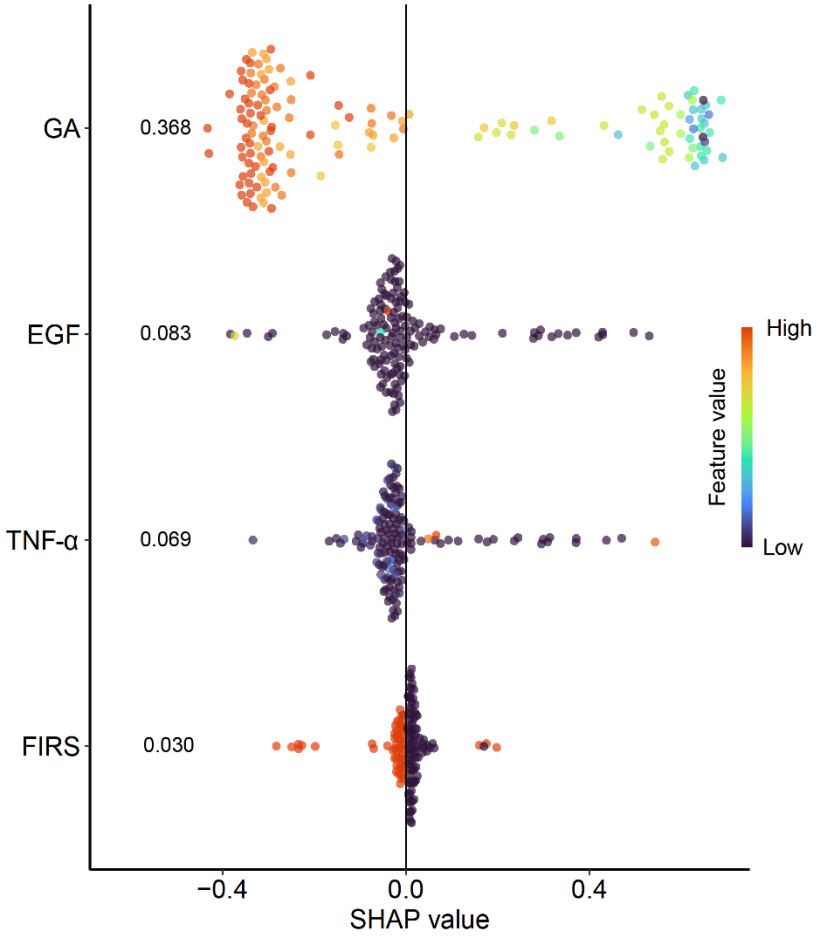


Figure 28. SHAP Summary Plot for Variable Importance predicting at least one severe respiratory outcome. The predictors (GA, EGF, TNF-α, and FIRS) are listed in descending order of their importance in the Random Forest model.

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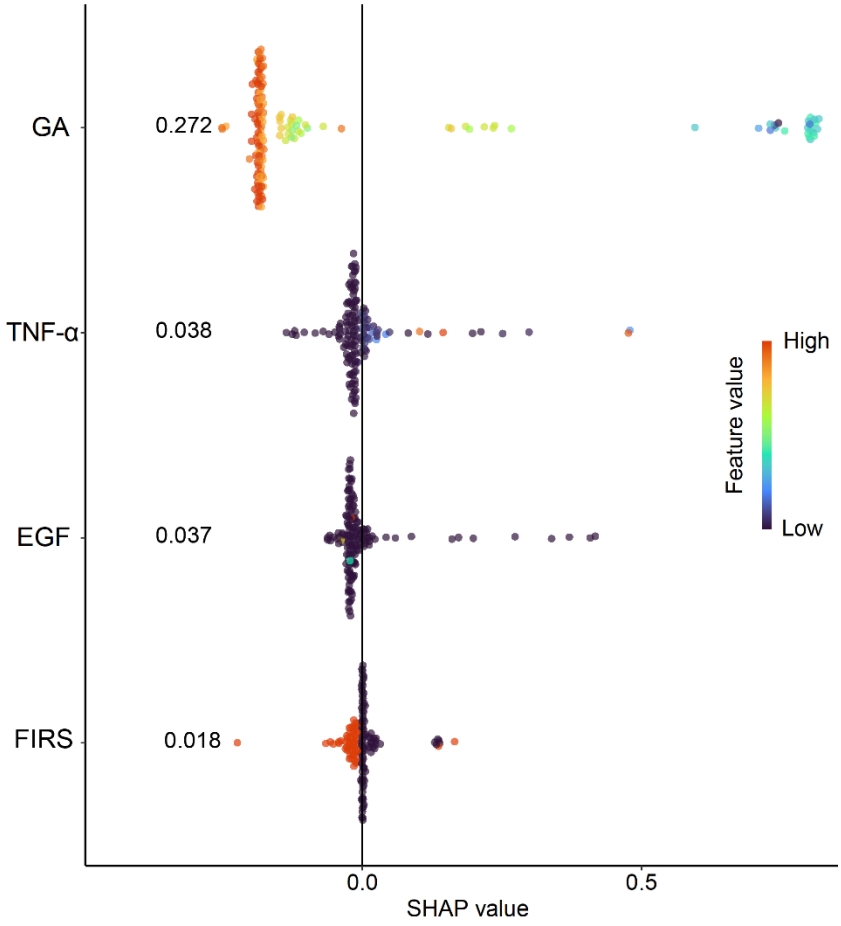

Figure 29. SHAP Summary Plot for Variable Importance predicting two or more severe respiratory outcomes. The predictors (GA, EGF, TNF-α, and FIRS) are listed in descending order of their importance in the Random Forest model.

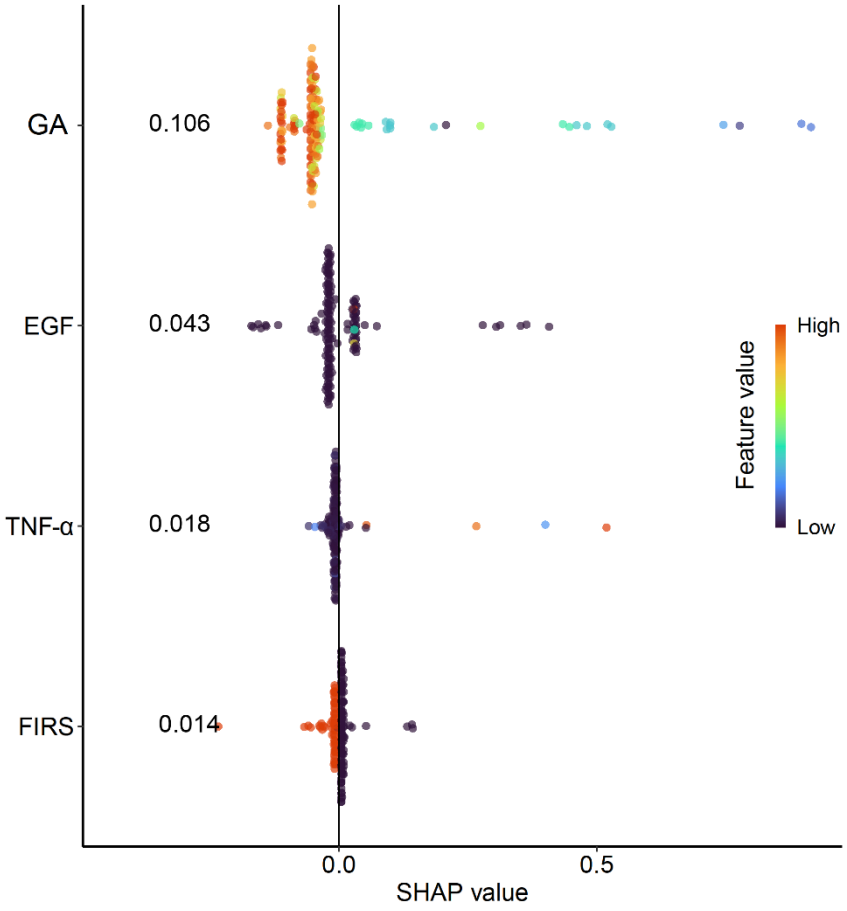

Figure 30. SHAP Summary Plot for Variable Importance predicting all five severe respiratory outcomes together. The predictors (GA, EGF, TNF-α, and FIRS) are listed in descending order of their importance in the Random Forest model.

Additionally, we employed Random Forest analysis to predict other outcomes, including PDA, ROP, and early hypotension, and to rank variable importance using the same four predictors: EGF, TNF-α, FIRS, and GA. In the Random Forest analysis for predicting PDA, we tested models with 500, 1000, 2000, and 3000 trees, and the number of variables tested (mtry) ranged from 1 to 4. Model verification was done using OOB. Increasing the number of trees beyond 500 did not enhance OOB error, with the lowest OOB errors observed when mtry was 4.

The Random Forest PDA model demonstrated a test classification accuracy of 90%, with specificity at 95% and sensitivity at 60%. GA, EGF, TNF-α, and FIRS were sequenced by importance. The SHAP summary plot (Figure 31)

demonstrated GA's paramount importance in predicting PDA, with higher values correlating with reduced PDA likelihood, while lower GA significantly increased risk. EGF and TNF-α followed GA in predicting PDA with comparable moderate performance. FIRS emerged as the least significant predictor of PDA, with both FIRS and non-FIRS cases similarly affecting PDA prediction. In summary, the Random Forest analysis revealed that PDA prediction could be accurately achieved using GA, EGF, and TNF-α levels in vaginal amniotic fluid.

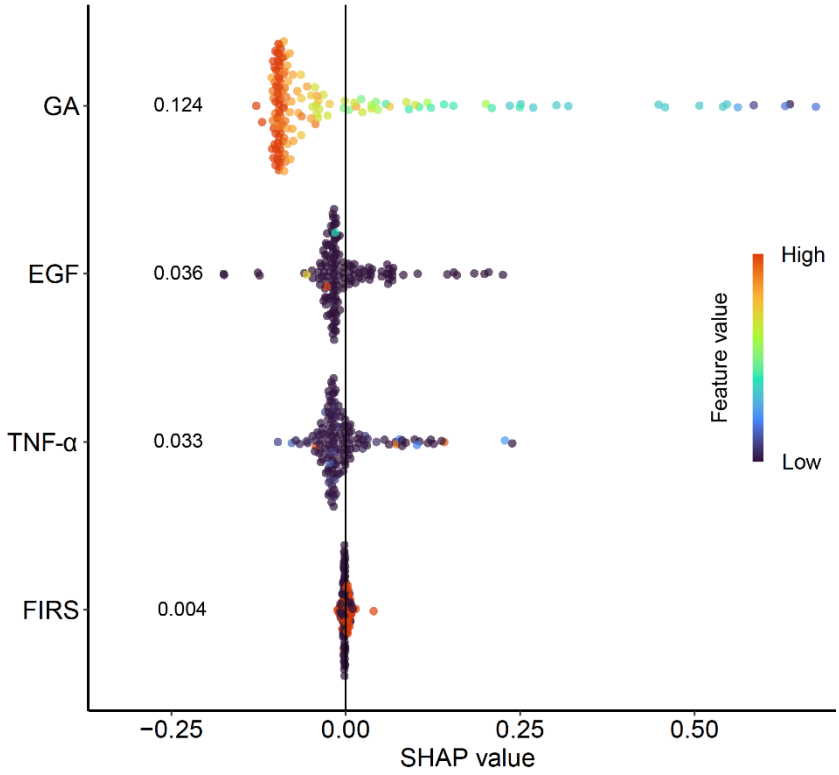

Figure 31. SHAP Summary Plot for Variable Importance predicting PDA. The predictors (GA, EGF, TNF-α, and FIRS) are listed in descending order of their importance in the Random Forest model.

For the prediction of ROP, we employed Random Forest analysis with the same predictors. The ROP model demonstrated a test classification accuracy of 95%, with specificity at 100% and sensitivity at 71%. GA, EGF, TNF-α, and FIRS were sequenced by importance in Figure 32. GA was crucial in predicting ROP, with higher values associated with decreased ROP likelihood, while EGF and TNF-α followed with moderate performance. FIRS emerged as the least significant

predictor of ROP, with its presence increasing ROP risk. In summary, the Random Forest analysis highlighted that GA, EGF, TNF-α, and FIRS in vaginal amniotic fluid could predict ROP with high accuracy.

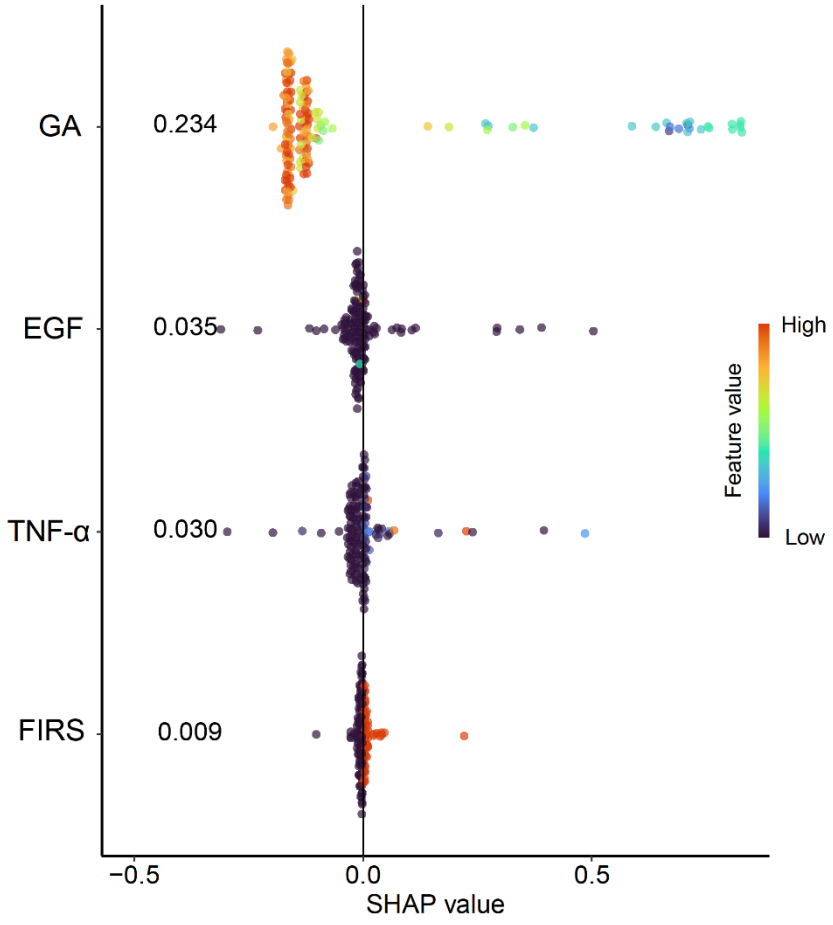

Figure 32. SHAP Summary Plot for Variable Importance predicting ROP. The predictors (GA, EGF, TNF-α, and FIRS) are listed in descending order of their importance in the Random Forest model.

Similarly, for the prediction of early hypotension, we employed Random Forest analysis with the same predictors and testing parameters as before. The model demonstrated a test classification accuracy of 91%, with specificity at 100% and sensitivity at 20%. GA, TNF-α, EGF, and FIRS were sequenced by importance in Figure 33. GA was vital in predicting hypotension, with higher values linked to decreased hypotension likelihood, while TNF-α and EGF

followed with moderate performance. FIRS emerged as the least significant predictor of hypotension, like the PDA model. In summary, the Random Forest analysis suggested that GA, TNF-α, and EGF in vaginal amniotic fluid could predict hypotension with high accuracy and specificity, albeit with lower sensitivity.

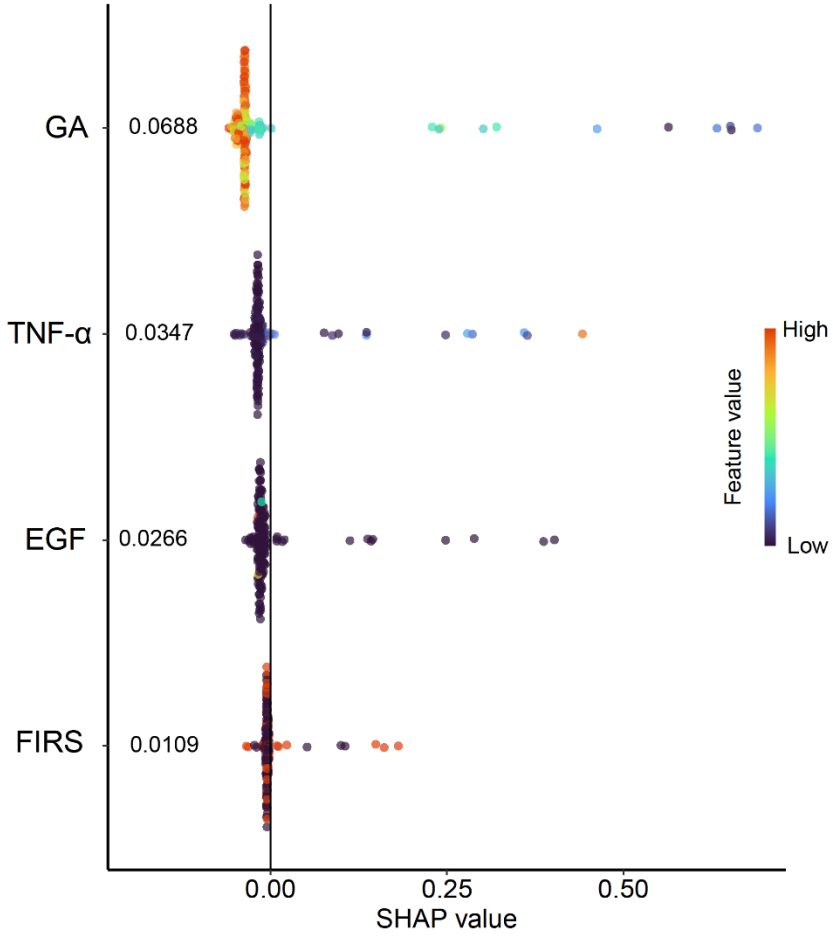

Figure 33. SHAP Summary Plot for Variable Importance predicting early hypotension. The predictors (GA, TNF-α, EGF, and FIRS) are listed in descending order of their importance in the Random Forest model.

5. DISCUSSION

In the current study, we investigated whether the concentrations of inflammatory biomarkers (TNF-α, IL-6, MMP-8, sTLR-2, sTLR-4, RANTES, suPAR, IL-10, IL-17, DEFA3, EGF) in the non-invasively collected vaginal amniotic fluid could serve as predictive markers for FIRS and major neonatal outcomes in patients after PPROM before 34 weeks of gestation.

Our study effectively employed a non-invasive sampling technique for amniotic fluid analysis. While previous research on amniotic fluid biomarkers has predominantly relied on amniocentesis [5,16–21], only a few studies have explored using vaginally obtained amniotic fluid analysis to detect FIRS [23,25,27,28]. We advocate for non-invasive amniotic fluid analysis as a viable alternative to amniocentesis for predicting FIRS and major neonatal outcomes after PPROM. In our study, concentrations of TNF-α, IL-6, MMP-8, suPAR, IL-10, IL-17, and DEFA3 in vaginally obtained amniotic fluid were strong predictors of FIRS. The incidence of major neonatal outcomes was significantly higher in the FIRS group (42%) compared to the non-FIRS group (24%). Elevated levels of TNF-α, MMP-8, IL-6, IL-10, IL-17, and DEFA3 in non-invasively collected amniotic fluid were associated with major neonatal outcomes in neonates with FIRS. Additionally, EGF was identified as a potential biomarker for functional postpartum maturity and morbidity in preterm infants. We established cut-off values for these inflammatory markers and used Random Forest analysis to rank their importance in predicting FIRS and major outcomes, thus determining the optimal predictive models.

IL-6

Nowadays, IL-6 is recognized as a marker of IAI, FIRS, and adverse neonatal outcomes. Various cut-off values have been reported with IAI, MIAC, FIRS, and outcomes, depending on the timing and type of amniotic fluid or umbilical blood sampling. The most commonly accepted cut-off value of IL-6 in amniotic fluid for IAI is 2.6 ng/mL, with mild IAI indicated by IL-6 levels of 2.6–11.2 ng/mL and severe IAI by levels of ≥ 11.3 ng/mL [8,183–185]. In our study, we found the optimal cut-off for FIRS in vaginally obtained amniotic fluid to be IL-6 >2695 pg/mL, with a sensitivity and specificity of 76%. This corresponds with a widely accepted cut-off value of >2.6 ng/mL. Kunze et al. determined a cut-off value of 1000 pg/mL for IL-6 in vaginal amniotic fluid to predict FIRS in two assays [25]. In our cohort, this 1000 pg/mL threshold demonstrated a sensitivity of 80% but a

lower specificity of 42%. Interestingly, Kacerovsky et al. reported a similar cut-off value to ours (2500 pg/mL) of vaginal fluid IL-6 for identifying MIAC and intraamniotic inflammation [23]. Mikolajczyk et al. found that an increased level of IL-6 above 26.8 pg/mL in the cervicovaginal secretions is an indirect non-invasive marker of FIRS [27]. However, this lower threshold did not significantly predict FIRS in our cohort.

Elevated IL-6 concentrations can identify neonates at risk for adverse outcomes such as EOS, white matter injury, PVL, cerebral palsy, BPD, and ROP [31,66–72]. The variety of IL-6 cut-offs reported in previous studies highlights the complexity and variability of predicting neonatal outcomes. While our research, identified IL-6 levels >3999 pg/mL in vaginal amniotic fluid as a predictor of major adverse outcomes in neonates with FIRS, showing a sensitivity of 59% and specificity of 74% (OR 4.06, 95% CI, 1.60-10.73), other research has presented different thresholds in amniotic fluid obtained by amniocentesis. Yoon's study demonstrated an amniotic fluid IL-6 cut-off >17 ng/mL with a sensitivity of 69% and specificity of 79% for predicting significant neonatal morbidity (defined as neonatal sepsis, RDS, pneumonia, IVH, BPD, or NEC) and mortality [71]. Figueroa-Damián et al. reported that amniotic fluid IL-6 levels >1250 pg/mL were strongly associated with early-onset sepsis [69], whereas, another study determined amniotic fluid cut-off for EOS 17.4 ng/mL with sensitivity and specificity of 100% and 67% respectively [70]. Additionally, in predicting BPD, amniotic IL-6 levels >15 ng/mL showed high sensitivity and specificity [72]. However, a direct comparison of biomarker values between vaginal amniotic fluid and fluid obtained by amniocentesis is complex and may lead to inaccurate conclusions. It's usually more reliable to interpret biomarker values within the context of the specific sampling method and to avoid direct comparison without considering these factors. These threshold differences underscore the need for context-specific cut-offs and the importance of further research to standardize these values for clinical use.

TNF-α

TNF-α is a potent inflammatory cytokine detected in high concentrations in amniotic fluid during inflammation and/or infection in the amniotic cavity [75,76]. Several studies have noted an association between TNF-α concentrations and FIRS, consistent with our findings. Our study demonstrated that TNF-α in non-invasively collected amniotic fluid was a significant predictor of FIRS, with TNF-α levels being 10-fold higher in the FIRS group compared to the non-FIRS

group. Kunze et al. found a 10-fold difference in median TNF-α levels in vaginal amniotic fluid between FIRS cases and controls, with TNF-α enhancing the accuracy of the clinical predictive model [25]. We established a cut-off value for vaginal amniotic fluid TNF-α at 89.20 pg/mL, showing a sensitivity of 66% and specificity of 90%, demonstrating the highest diagnostic performance. Kunze et al. reported TNF-α cut-offs of 200 pg/ml and 300 pg/ml for predicting FIRS using different immunoassays in vaginal secretions [24]. In our cohort, these thresholds demonstrated high specificity – 95% and 96%, respectively – but relatively low sensitivity, at 46% and 40%. Moreover, amniotic fluid TNF-a concentrations of 6.3 pg/ml (a sensitivity of 78.4% and a specificity of 70.1%) predicted a positive amniotic fluid culture [186].

Other findings concerning TNF-α levels in the cervicovaginal fluid and intraamniotic inflammation are controversial. Mikolajczyk et al. found no association between TNF-α concentrations in the cervicovaginal fluid after PPROM and umbilical cord blood [26]. Kayem et al. studied the TNF-α bedside test (with a positivity threshold of 50 μg/L) in vaginal secretions and reported a weak relationship between TNF-α and maternal-fetal infections with a sensitivity of 47% and a specificity of 81% [27]. In both studies, vaginal samples were collected at weekly intervals until delivery, possibly affecting cytokine levels. The primary outcomes of the mentioned studies differed, and the TNF-α test threshold was rather high making result comparison difficult. Furthermore, Son et al. showed that amniotic fluid TNF-α predicted preterm birth before 32 weeks of gestation in patients with cervical insufficiency, with a cut-off value of 79.9 pg/ml (sensitivity 74%, specificity 84%) [187]. The authors pointed out that elevated inflammatory cytokines in amniotic fluid due to intraamniotic inflammation might contribute to cervical insufficiency.

TNF-α has been associated with various neonatal outcomes due to intraamniotic inflammation, corresponding to our findings. TNF-α levels in vaginal amniotic fluid were significantly higher in the FIRS group with major outcomes, such as severe RDS, a need for mechanical ventilation, death, early-onset sepsis, early hypotension, severe IVH, BPD, and severe ROP. The cut-off value of 231 pg/ml had a sensitivity of 60%, specificity of 89%, NPV of 92%, and increased the odds of having FIRS with major neonatal outcomes ninefold. This cytokine demonstrates a dose-related effect on cells, causing induction of the processes at low levels and suppression or destruction at high levels [74]. This explains the cut-off for FIRS with a major neonatal outcome of 231 pg/mL, almost three times higher than for FIRS alone.

Kunze stated the prevalence of combined neonatal morbidity in the FIRS group was 68.5% vs. 17.8% in the non-FIRS group and that vaginal amniotic fluid TNF-α was a strong predictor for FIRS and severe neonatal morbidity with cut-offs of 200 pg/ml and 300 pg/ml for two different assays [25]. In our cohort, comparable thresholds of 200 pg/mL and 300 pg/mL showed sensitivities of 60% and 55%, respectively, along with high specificities of 87% and 91% for predicting FIRS associated with major neonatal outcomes. Park et al. determined, that amniotic fluid TNF-α level above 41 pg/ml (sensitivity 82% and specificity 79%) is an independent predictor of early-onset neonatal sepsis with an odds ratio of 12.9 in patients with preterm labor and intact membranes [20]. In addition, TNF-α is associated with persistent inflammation contributing to the development of BPD in the premature lungs [78]. The levels of amniotic fluid TNF-α were found to be higher in mothers of infants with BPD with the cut-off value was ≥30 pg/mL (sensitivity of 69%, specificity 66%) [72].

TNF-α has been linked to brain injuries in preterm infants with severe chorioamnionitis and FIRS, including PVL and severe IVH [67,77]. High levels (10-fold or more increase from normal level) of TNF-α also contribute to hypotension in septic shock by various mechanisms [73] and its role is suggested in the development of PDA [79,80]. The association between TNF-α and ROP is inconsistent, with studies reporting varying TNF-α levels depending on the timing and location of sample collection. Some found increased TNF-α in plasma or umbilical cord blood linked to ROP, while others reported low levels in amniotic fluid or no significant difference in TNF-α levels between ROP and control groups [33,81–85].

MMP-8

Similarly, our study found that MMP-8 in non-invasively collected amniotic fluid was a significant predictor of FIRS, with MMP-8 levels also being 10-fold higher in the FIRS group. For example, Park et al. reported a nine-fold increase in median MMP-8 concentration in amniotic fluid associated with funisitis [19]. We established a cut-off value for vaginal amniotic fluid MMP-8 at 170.76 ng/ml, with a sensitivity of 78% and specificity of 71%, indicating its high diagnostic performance in identifying FIRS. Park et al. indicated the MMP-8 cut-off of 23 ng/ml in transabdominally obtained amniotic fluid best-indicated funisitis [19]. Other authors report an amniotic fluid MMP-8 cut-off value of 41.5 ng/mL for the diagnosis of MIAC [87]. Dorfeuille et al. analyzed MMP-8 in the vaginal fluid after PPROM and found an association between MMP-8 (with a cut-off > 500

ng/mL) and histological chorioamnionitis and adverse neonatal neurological outcomes (IVH, PVL, and hypoxic-ischemic encephalopathy), although FIRS was not the primary focus, and the patient cohort was relatively small [29]. In addition, elevated levels of MMP-8 in amniotic fluid have been associated with the development of cerebral palsy at 3 years of age [21]. In our study, we determined that MMP-8 was a good predictor of major neonatal outcomes in neonates with FIRS. The level of MMP-8 higher than 413.67 ng/mL (sensitivity 65%, specificity 72%, NPV 92%) increased the odds of having major neonatal outcomes and FIRS 4.8 times. The threshold of 500 ng/mL reported by Dorfeuille is quite close to our finding, showing a specificity of 73% and sensitivity of 60% for predicting FIRS with major neonatal outcomes in our cohort, and a specificity of 68% and sensitivity of 43% for predicting IVH. Overall, research on MMP-8 concentration in vaginal amniotic fluid is limited, and to our knowledge, cut-offs for MMP-8 in vaginal amniotic fluid predicting FIRS have not been previously reported.

DEFA3

Defensins are antimicrobial peptides involved in the innate immunity response, acting as part of the skin and mucous barrier. In cases of infection or inflammation, amniotic fluid WBCs, potentially of fetal origin, may produce these peptides. Previously, MIAC, intra-amniotic inflammation, and spontaneous preterm birth have been associated with elevated concentrations of α-defensins in amniotic fluid [62,108]. The cut-off value of defensins 1–3 > 53.2 ng/ml in amniotic fluid is related to the risk of histological chorioamnionitis and funisitis. Increased DEFA3 levels in vaginal fluids after PPROM have also been linked to adverse neonatal outcomes [109]. In the study by Lucovnik et al., higher concentrations of α-defensins in vaginal fluids were associated with histological chorioamnionitis, FIRS, and predicted neonatal mortality or neurological impairment at one year of age. The optimal cut-off value for diagnosing FIRS was >2.1 ng/mL, with a sensitivity of 100% and a specificity of 53%. For predicting infant death or neurological impairment, the cut-off was >9.4 ng/mL, with a sensitivity of 29% and a specificity of 100%. In our study, the cut-off value of DEFA3 for predicting FIRS was found to be 34.04 ng/mL (sensitivity 35%, specificity 92%), and for predicting FIRS with major neonatal outcomes, it was 35.58 ng/mL (sensitivity 36%, specificity 89%). The cut-off values reported by Lucovnik were considerably lower than ours and did not demonstrate significant diagnostic power for predicting FIRS or major outcomes in our cohort. Logistic regression analysis demonstrated that DEFA3 was independently associated with FIRS, highlighting

its potential as a predictive biomarker when controlling for gestational age. Furthermore, a Random Forest analysis produced a strong predictive model incorporating gestational age, TNF-α, and DEFA3, achieving a classification accuracy of 93%, with a sensitivity of 50% and a specificity of 100% in predicting FIRS with major outcomes.

IL-10

IL-10 is a key anti-inflammatory cytokine important for maintaining pregnancy. Levels of IL-10 are significantly higher in first and second-trimester placental tissues compared to third-trimester tissues, suggesting its down-regulation at term to prepare for labor [110]. In animal models of infection, administering IL-10 improves pregnancy outcomes, and it plays a role in controlling preterm labor associated with inflammation [188]. Reduced IL-10 expression is noted in placental tissues from pregnancies complicated by preterm labor and chorioamnionitis compared to normal controls [110].

IL-10 is linked to preterm birth, intra-amniotic infection/inflammation, and neonatal outcomes such as BPD [111,113,114]. Higher IL-10 concentrations are observed in the umbilical cord blood of neonates with FIRS [115], while increased placental IL-10 expression is considered protective against BPD [113]. Our study found that elevated IL-10 levels in vaginal amniotic fluid are associated with inflammation and adverse neonatal outcomes. An IL-10 cut-off value of >35.05 pg/mL (sensitivity 61% and specificity 87%) increased the odds of FIRS 10.2 times and the odds of FIRS with major neonatal outcomes 4.2 times (sensitivity 59% and specificity 74%). IL-10 was the second most important predictor of FIRS in Random Forest analysis, contributing significantly to the best predictive model. High IL-10 levels in amniotic fluid in cases of FIRS can be explained as a compensatory anti-inflammatory response to severe, ongoing inflammation. While a low IL-10 profile is linked with chorioamnionitis-induced preterm labor [110], elevated IL-10 levels in FIRS likely reflect the organism's effort to mitigate tissue damage and control the excessive inflammatory response. The timing of sample collection and variability in individual immune responses also contribute to these observed differences.

However, IL-10 studies results vary. Park et al. did not detect IL-10 in cervicovaginal fluid samples used to predict preterm birth [189]. Dudley et al. found no elevation in amniotic fluid IL-10 levels in infection-associated preterm birth cases [190], and Taylor also did not find IL-10 in vaginal fluid cytokine profiles predicting preterm delivery [191].

IL-17

Interleukin-17, a proinflammatory cytokine, plays a crucial role in the pathogenesis of inflammatory and autoimmune diseases. It works synergistically with other cytokines like IL-6, IL-8, and TNF-α to amplify inflammation [118]. Recent studies highlight IL-17A as a key initiator of FIRS and a contributor to chronic conditions from fetal inflammatory exposure [116]. Elevated IL-17 levels in amniotic fluid are associated with MIAC, histological chorioamnionitis, and preterm birth, potentially disrupting fetal immune development and increasing the risk of adverse neonatal outcomes, such as sepsis, BPD, PDA, NEC, IVH, ROP, PVL, and cerebral palsy [63,121,122,124,189].

In our study, an IL-17 cut-off value of 1.00 pg/mL had a sensitivity of 61% and a specificity of 75%. IL-17 levels exceeding this threshold were significantly associated with FIRS, with an odds ratio of 4.74 (95% CI: 2.21 - 10.48). A higher IL-17 cut-off value of 4.00 pg/mL (sensitivity 50%, specificity 84%) was effective in discriminating neonates with FIRS and major neonatal outcomes, with elevated IL-17 levels >4 pg/mL increasing the odds of FIRS by 5.12 times. IL-17 contributed as an important predictor of FIRS in Random Forest analysis in the best predictive model.

There is limited data regarding cut-offs of IL-17 in cervicovaginal or amniotic fluid for predicting FIRS or neonatal outcomes. Park et al. reported that cervicovaginal fluid cytokines, especially IL-6 and IL-17α, could be useful predictive markers of preterm birth in patients with preterm labor and PPROM [192], with an IL-17α cut-off value of 5.53 ng/mL showing a sensitivity of 75% and a specificity of 69%. In Jacobsson's et al. study, IL-17 (0.025 ng/mL; OR 6.1, 95% CI 1.2–31.8) significantly contributed to the prediction of MIAC in a non-invasive prediction model using cervical fluid proteins [63]. The IL-17α cut-off of 5.53 ng/mL identified by Park et al. is considerably higher than values observed in our cohort, which had a maximum level of 162.29 pg/mL. Meanwhile, Jacobsson’s threshold of 0.025 ng/mL demonstrated a high specificity of 99% but a low sensitivity of 10% in our cohort, indicating limited utility for broader clinical application in our sample.

RANTES, sTLR-2 and sTLR-4

Chemokine RANTES plays a significant role in childbirth and the host response to intrauterine infection. Elevated levels during preterm labor are linked to MIAC, and RANTES has a high predictive value for MIAC alongside IL-18, IL-1β, and IL-6. Additionally, RANTES is associated with the pathogenesis of

ROP, with lower neonatal blood levels correlating with increased ROP severity. Amniotic fluid RANTES (cut-off of 2.729 ng/ml; OR 5.4, 95% CI 1.6–17.7) significantly predicted MIAC; however, in cervicovaginal fluid, RANTES levels were not significantly different [63]. In our study, RANTES levels in vaginal amniotic fluid did not significantly differ between neonates with and without FIRS, nor did they vary according to the presence or absence of major neonatal outcomes.

Soluble Toll-like receptors (sTLR-2 and sTLR-4) are crucial for recognizing microbial components and triggering inflammatory responses [102,104]. Elevated expressions of these receptors in amniotic fluid are linked to histologic chorioamnionitis in preterm delivery, FIRS-associated dermatitis, and fetal cerebral white matter injury, underscoring their importance in MIAC and predicting adverse neonatal outcomes [59,60,103,105]. Newborns with higher amniotic fluid sTLR-4 levels have a higher incidence of BPD and a greater need for tracheal intubation [103]. In our study, the levels of sTLR-2 and sTLR-4 in vaginal amniotic fluid were not significantly different between neonates with and without FIRS and according to the presence or absence of major neonatal outcomes. Similarly, Kacerovsky et al. did not find prognostic value in sTLR-2 levels in cervicovaginal secretions for diagnosing histologic chorioamnionitis [193]. No other studies have examined sTLR-4 levels in non-invasively collected amniotic fluid or cervicovaginal secretions. Research on sTLR-2 and sTLR-4 in amniotic fluid obtained via amniocentesis has been controversial regarding their association with IAI, FIRS, and neonatal outcomes. Some studies indicate that elevated amniotic fluid sTLR-2 > 222.7 ng/ml is a promising predictor of both MIAC and histologic chorioamnionitis with high specificity in PPROM, while others, such as Dulay et al., report that sTLR-2 levels in preterm amniotic fluid are constitutive and independent of intraamniotic infection or microbial category (gram-negative vs. gram-positive) or that sTLR-4 level does not significantly indicate FIRS [102,103,105].

SuPAR

We detected suPAR in all non-invasively collected vaginal amniotic fluid samples, marking the first study to quantify suPAR levels in this fluid. The median suPAR level was 26.23 ng/mL (IQR, 15.19–51.14), much higher than previously reported blood levels of 2.02–4.4 ng/mL in pregnant women [95,96]. Uszynski et al. found 100-200 times higher concentrations of uPA and uPAR in gestational tissues and amniotic fluid obtained during cesarean sections compared to plasma

[92], indicating that higher suPAR levels in amniotic fluid might result from uPAR expression on trophoblasts and fetal membrane cells, as well as uPA/uPAR activity in fibrinolytic processes in PPROM.

Currently, suPAR is considered a nonspecific biomarker of systemic chronic inflammation, reflecting disease course, severity, and prognosis [88,194,195]. In our study, suPAR levels above 27.60 ng/mL in vaginal amniotic fluid (sensitivity 65%, specificity 65%) predicted a threefold increase in the odds of FIRS before 34 weeks of gestation. Tunc et al. found that suPAR levels in umbilical cord blood serum are predictive of BPD in preterm neonates [97]. The authors report an optimal cut-off value of 1.55 ng/mL (sensitivity 88%, specificity 85%). SuPAR was reported to be used as a prognostic factor for early and late neonatal sepsis [98,99]. However, there was no significant difference in suPAR levels based on the presence or absence of major neonatal outcomes in our study.

Furthermore, suPAR levels correlated with other inflammatory markers such as MMP-8, TNF-α, and IL-6, aligning with observations in other inflammatory conditions where blood suPAR levels were associated with TNF-α, leukocyte count, and CRP [96,194,196,197]. The strongest correlation between suPAR and MMP-8 can be explained by both systems' roles in extracellular matrix degradation and plasmin's participation in metalloproteinase activation [91].

While suPAR showed good diagnostic performance for FIRS, it was less effective than MMP-8, TNF-α, and IL-6. Random Forest analysis identified suPAR as of moderate importance for predicting FIRS. Raggam et al. found that suPAR better reflected the inflammatory state than IL-6, CRP, and procalcitonin [198]. Although suPAR has limited diagnostic value, it carries superior prognostic value or could be used to evaluate treatment response, as by measuring serum suPAR levels [88,194,195]. Most inflammatory markers are short-lived and influenced by diet, exercise, and sample handling, while suPAR is stable with minimal circadian and day-to-day variations, steady concentration after repeated freezing/thawing, and a long half-life [194,199–201].

EGF

Our study investigated the potential impact of FIRS on EGF levels in non-invasively collected amniotic fluid and analyzed the significance of EGF in predicting outcomes in preterm neonates after PPROM. To our knowledge, this is the first study to investigate EGF in vaginally collected amniotic fluid. Previous research on amniotic fluid EGF used samples obtained by amniocentesis [202–206].

We found a significant correlation between EGF concentration and gestational age, with EGF levels increasing as gestation progressed. Our findings are consistent with previous studies [202,206,207]. The rise in median EGF concentrations among GA groups was almost threefold between 22–27 weeks and 32–34 weeks of gestation. Haigh et al. reported a 10-fold rise in EGF concentrations when comparing preterm (30 weeks) and term (40 weeks) gestations [202]. These results support the notion that EGF is closely related to gestation.

Unexpectedly, there was no association between EGF and FIRS or inflammatory cytokines such as IL-6, TNF-α, MMP-8, IL-10, IL-17, etc. Shobokshi previously reported elevated EGF concentrations in cases of PROM with intraamniotic infection [13]. Our results may differ due to distinct definitions of intraamniotic infection and differences in research populations: term pregnancies with PROM versus PPROM at 22^{+0}–34^{+6} weeks of gestation. Varner's findings that amniotic fluid EGF levels did not change with chorioamnionitis confirm our results [204].

Our findings indicated that lower EGF levels in amniotic fluid were associated with higher neonatal morbidity in preterm neonates. Low EGF concentrations (<75 pg/mL) might predict severe respiratory outcomes (such as severe RDS, respiratory support for more than 4 days, the need for surfactant, mechanical ventilation, and BPD), ROP, PDA, and early hypotension. As the EGF cut-off value decreased, the odds ratio for adverse neonatal outcomes increased, with the highest odds for adverse outcomes at EGF levels below 35 pg/mL. An EGF cut-off value <35 pg/mL was a reliable predictor of PDA, ROP, early hypotension, and severe respiratory outcomes in neonates of 28^{+0}–31^{+6} weeks of GA. Currie also indicated that EGF in bronchoalveolar lavage fluid may predispose infants to chronic lung disease [207], but no data on EGF cut-off values or odds ratios were previously reported. These findings suggest that EGF may predict adverse outcomes in preterm neonates.

The current study highlights EGF as a potential biomarker for functional postpartum maturity and morbidity in preterm infants. EGF significance in neonatal respiratory morbidities, such as RDS, chronic lung disease, pulmonary hypoplasia, and congenital diaphragmatic hernia, has been reported in both animal and human research [207–211]. Currie et al. determined that the most diseased infants who later developed BPD had almost undetectable EGF levels in bronchoalveolar lavage fluid compared to controls from birth [207]. Goetzman concluded that EGF advances the histological and biochemical maturation of

lungs in rhesus monkeys, clinically improving respiratory function after preterm birth [209]. EGF and its receptor (EGFR) are linked to PDA, with EGF inducing PDA contraction [127]. EGF also protects against intestinal injury and is crucial in the pathogenesis of NEC in preterm infants [128]. Additionally, EGF enhances myelination, astrogliosis, and neurologic recovery in animal models of white matter injury [129].

New treatment options are being explored through the EGFR transactivation pathway, aiming to either close PDA in preterm neonates or keep it open in congenital heart diseases [127]. In animal studies, oral administration of EGF at physiologically relevant doses significantly decreased the incidence of NEC [128]. EGF has been identified as a factor associated with the efficacy of intranasal mesenchymal stem cell therapy in enhancing myelination following encephalopathy in premature infants [129]. Vinukonda's study revealed that recombinant EGF treatment might improve the neurological outcome of premature infants with IVH [130]. Altogether, the findings of our study and previous research suggest that EGF may be a biochemical indicator of functional postpartum maturity of preterm neonates, possibly reflecting preterm neonatal morbidity after birth.

In our study, we utilized a multivariable logistic regression and Random Forest model to predict FIRS and major neonatal outcomes ranking inflammatory biomarkers in non-invasively collected vaginal amniotic fluid. Overall, TNF-α consistently stood out as a key biomarker, independently and in the presence of other variables, highlighting its potential clinical relevance. Gestational age played a protective role, supporting the idea that shorter gestation is associated with an increased risk of FIRS with major outcomes. While other biomarkers like IL-6, MMP-8, IL-17, IL-10, and DEFA3 were individually significant, their predictive value diminished in a multivariate context, emphasizing the need to consider multiple factors for a comprehensive understanding of FIRS prediction.

In the prediction of FIRS, using Random Forest analysis, the test classification accuracy of the optimal model was 86%, (sensitivity of 81%, specificity of 89%). TNF-α, IL-10, and MMP-8 emerged as the top three predictors of FIRS, with TNF-α showing the highest significance. The model for predicting FIRS with major outcomes had an accuracy of 93% (sensitivity 50% and specificity 100%), emphasizing the significance of GA, TNF-α, and DEFA3.

When predicting specific outcomes, such as severe respiratory outcomes, PDA, ROP, and early hypotension, four variables (GA, EGF, TNF-α, and FIRS)

were selected based on good performance in the previous analysis. The model for severe respiratory outcomes achieved an impressive accuracy of 96%, with a sensitivity of 100% and a specificity of 95%, highlighting the predictive power of GA, EGF, and TNF-α levels. For PDA, the Random Forest model demonstrated a classification accuracy of 90%, with a specificity of 95% and a sensitivity of 60%. In ROP, the model achieved a classification accuracy of 95%, with a specificity of 100% and a sensitivity of 71%. Similarly, the model for early hypotension demonstrated an accuracy of 91%, with a specificity of 100% and a sensitivity of 20%. Notably, gestational age consistently emerged as a key predictor, indicating its protective effect against FIRS with major outcomes. TNF-α and EGF shared the second and the third positions.

Umbilical cord blood IL-6 levels

Umbilical cord blood IL-6 levels serve as a critical biomarker reflecting the fetal immune response and indicating intrauterine inflammatory processes. In this study, umbilical IL-6 demonstrated significant positive correlations with other pro-inflammatory amniotic fluid cytokines, such as TNF-α, IL-6, IL-17, MMP-8, and IL-10, although the correlations were weak to moderate. This implies that the levels of immunological biomarkers in vaginally collected amniotic fluid are associated with and correspond to inflammatory factors in the umbilical cord blood. Our findings are consistent with previous research that highlights the interconnection between various markers of inflammation. Recently, Ohkuma et al. reported a high correlation between IL-6 levels in amniotic fluid and umbilical cord blood [212]. In their study, amniotic fluid was obtained via amniocentesis, and the cases of preterm birth occurred within 24 hours following the procedure. This difference in methodology might explain why our study found a moderate correlation between amniotic fluid and umbilical cord blood IL-6 levels, while theirs reported a high correlation. In our study, we compared the diagnostic value of umbilical IL-6 to amniotic fluid biomarkers in predicting major neonatal outcomes. Umbilical IL-6 showed moderate predictive performance, with an AUC of 67%, and higher specificity at elevated cut-off values, outperforming some biomarkers like MMP-8, amniotic fluid IL-6, and IL-10. A Random Forest model identified GA, EGF, and TNF-α as the most significant predictors of neonatal outcomes, with lower GA, reduced EGF, and elevated TNF-α associated with worse outcomes. Both amniotic fluid IL-6 and umbilical IL-6 had moderate importance in the predictive model, reinforcing their value for risk assessment in neonatal care.

The variability in results of inflammatory biomarkers in amniotic fluid across studies can be attributed to several factors. Firstly, differences in techniques for amniotic fluid collection are notable; some countries favor amniocentesis to avoid vaginal flora contamination, resulting in lower marker levels compared to non-invasive collection methods such as tampons, sanitary pads, or speculums used in other studies. Secondly, discrepancies in collection timing, ranging from daily to sporadic sampling close to delivery or relative to the onset of maternal symptoms or clinical presentation, affect correlations with placental histology, especially when the interval from collection to delivery exceeds 48 hours. Studies that sample amniotic fluid early versus later during active infection may show varying degrees of biomarker elevation. Thirdly, variations in amniotic fluid preparation and storage methods (e.g., centrifugation, freezing) impact marker values, influencing study outcomes. Additionally, diverse gestational age ranges and subject inclusion criteria (e.g., PPROM diagnosis, exclusion of comorbidities) contribute to heterogeneity in marker levels among study cohorts. Moreover, differences in selected outcomes and their diagnostic criteria, such as for chorioamnionitis (microbiological vs. biochemical vs. histological vs. clinical) or FIRS (biochemical vs. histological), further complicate comparisons. Lastly, differences in analytical methods of immunological biomarkers, including assay sensitivities, specificities, and thresholds for biomarker detection, can lead to discrepancies in reported values across studies. Harmonizing these methodological aspects could improve the consistency and comparability of findings in future research on inflammatory biomarkers in amniotic fluid.

Our study's strengths

Our study has several strengths. Firstly, it utilized non-invasive sampling of amniotic fluid, offering a feasible and complication-free method that was both easily performed and informative. This innovative approach reduces the risk and discomfort associated with traditional amniocentesis, making it more patient-friendly and practical for broader clinical use. The analysis included a wide range of inflammatory biomarkers (TNF-α, IL-6, MMP-8, sTLR-2, sTLR-4, RANTES, suPAR, IL-10, IL-17, DEFA3, EGF), providing a comprehensive assessment of the inflammatory response associated with FIRS and major neonatal outcomes.

The study focused on FIRS as an outcome, defined by rigorous biochemical and histologic criteria, ensuring robust and reliable data. Additionally, the number of patients included was larger than most studies examining non-invasive amniotic fluid biomarkers, enhancing statistical power and generalizability of our findings.

The use of standardized hospital treatment protocols for all mothers and neonates ensured consistency in clinical care, reducing variability and potential confounding factors. The blinding of biomarker levels ensured that patient treatment was not influenced by these levels, minimizing bias and strengthening the validity of the findings.

Notably, this is the first study to evaluate EGF, suPAR, and sTLR-4, in association with FIRS and neonatal outcomes after PPROM, further contributing to its novelty and significance in the field. The study employed advanced statistical techniques, including Random Forest analysis, to identify the most significant predictors of FIRS and major neonatal outcomes, ensuring robust and reliable results.

Our study's limitations

We acknowledge several limitations in our study. Firstly, there was an imbalance in the number of newborns with FIRS compared to controls, and a statistically significant difference in gestational age between the groups, which may have affected the outcomes. The lack of a standardized non-invasive amniotic fluid sampling technique, and the fact that our non-invasive method was not validated simultaneously with the standard approach, such as amniocentesis, is another limitation. Although FIRS is associated with gestational age and lower gestational age was anticipated in the FIRS group [8], we adjusted our predictive models for this variable to minimize its effect. However, the uneven distribution of outcomes across gestational age groups, the low prevalence of each outcome, and the focus on immediate outcomes are noted limitations. Replicating the study in a homogeneous group of preterm patients with FIRS matched for gestational age would be beneficial.

The absence of a standardized non-invasive amniotic fluid sampling technique, with each previous study employing different methods [23,25,27–29,65,192], makes comparisons challenging. Although Musilova et al. previously reported a strong correlation between biomarker levels in amniotic fluid collected via amniocentesis and vaginally [23,24], our results were not validated with amniocentesis, which restricts statistical power and generalizability. Future studies with a larger sample size and longer follow-up periods are needed to enhance the reliability of our findings and evaluate the relationship between biomarkers in amniotic fluid and long-term outcomes.

Clinical implications

Over the past two decades, researchers have increasingly focused on IAI, FIRS, and their association with adverse neonatal outcomes, as well as their diagnosis. A long-standing debate persists regarding the optimal management of pregnancies complicated by PPROM – whether it is safer to prolong the pregnancy or induce delivery. The guidelines from the American College of Obstetricians and Gynecologists recommend continuing the pregnancy in the absence of intra-amniotic infection and when fetal conditions are stable, while carefully considering gestational age to minimize the risk of premature birth [10]. Accurately assessing maternal and fetal/neonatal risks in PPROM cases is crucial for guiding clinical decisions and optimizing outcomes.

Our study addresses a gap in understanding the relationship between non-invasively collected amniotic fluid biomarkers and the diagnosis of FIRS, as well as associated neonatal outcomes. After PPROM, performing immunological analysis of vaginally obtained amniotic fluid, prognosis should be evaluated in two areas: maternal condition due to IAI and fetal/neonatal outcomes related to FIRS and FIRS-associated adverse outcomes. We established cut-off thresholds for FIRS, with TNF-α being the most effective marker at 89 pg/mL, and for adverse neonatal outcomes, where GA, TNF-α >231 pg/mL, and EGF <35 pg/mL were the strongest predictors. Notably, the previously reported threshold for histological chorioamnionitis in vaginal amniotic fluid was 21.17 pg/mL [213], which is significantly lower than the thresholds we identified for FIRS, indicating a more advanced stage of inflammation in FIRS cases. In the perspective of the future, these thresholds should be refined to determine the ideal timing of induction of delivery after PPROM to optimize maternal and neonatal outcomes.

Despite significant progress, a gap remains between research and clinical practice, as the diagnosis of FIRS is still largely confined to research settings. Our findings suggest that evaluating non-invasively collected amniotic fluid could provide early risk stratification for high-risk neonates, particularly those affected by IAI and FIRS. This approach may enhance neonatal care by facilitating timely interventions that reduce morbidity and mortality. The diagnosis of FIRS is recommended as an additional assessment for preterm neonates, allowing clinicians to identify high-risk cases earlier and tailor management strategies to meet their specific needs. Simultaneously, low-risk patients may avoid unnecessary treatments, ultimately improving neonatal outcomes. Further research is essential to refine sampling techniques and optimize the timing of

specimen collection to enhance predictive accuracy, ensuring that at-risk infants receive the best possible care.

Until now, FIRS has primarily been determined either after birth or prenatally through invasive methods. Therefore, a sensitive non-invasive test for the early prediction of FIRS is needed. Evaluating vaginally obtained amniotic fluid has proven to be non-invasive, easy to perform, informative, and repeatable, if necessary, all without complications. Our findings enhance the understanding of the role of biomarkers in neonatal morbidity and may encourage further research into their use in targeted treatments. This approach could reduce the side effects of nonspecific therapies in neonates through the concept of individualized medicine. After PPROM, it is possible to collect and assess amniotic fluid non-invasively to predict neonatal outcomes before birth. This early prediction could aid in stratifying neonatal risk and influence management strategies for both mother and newborn.

We support the use of inflammatory biomarkers, specifically TNF-α, in non-invasively obtained amniotic fluid to identify FIRS after PPROM, before 34^{+6} weeks of gestation. Considering gestational age and specific biomarkers, like TNF-α and EGF, together may improve the prediction of FIRS and major neonatal outcomes, aiding in the early stratification of fetal and neonatal risk. The analysis of biomarkers before birth could significantly impact the management strategies for women with PPROM and their neonates, potentially improving outcomes through early intervention and individualized care plans.

6. CONCLUSIONS

1. The increased levels of immunological biomarkers TNF-α, MMP-8, IL-6, IL-10, IL-17, DEFA3, and suPAR in non-invasively collected amniotic fluid are reliable predictors of FIRS, with TNF-α having the highest predictive characteristics. RANTES, sTLR-2, sTLR-4, and EGF are not statistically significant for FIRS prognosis.
2. The concentrations of amniotic fluid biomarkers TNF-α, IL-6, MMP-8, and IL-17, predicting FIRS, correlate with the IL-6 concentration in umbilical cord blood.
3. Elevated concentrations of TNF-α, MMP-8, IL-6, IL-10, IL-17, and DEFA3 in non-invasively collected amniotic fluid are associated with major neonatal outcomes in preterm neonates affected by FIRS, with TNF-α being the strongest predictor of both FIRS and major neonatal outcomes. Decreased levels of EGF in amniotic fluid are associated with adverse outcomes in preterm neonates. RANTES, sTLR-2, sTLR-4, and suPAR are not statistically significant for predicting major outcomes in neonates with FIRS.
4. The levels of immunological markers predict increased odds of FIRS and adverse outcomes in preterm neonates.
    4.1 The optimal cut-off values for predicting FIRS are TNF-α > 89 pg/mL, MMP-8 > 170 ng/mL, IL-6 > 2695 pg/mL, IL-10 > 35 pg/mL, IL-17 > 1 pg/mL, DEFA3 > 34 ng/mL and suPAR > 27 ng/mL.
    4.2 The optimal cut-off values indicating increased odds of FIRS and major neonatal outcomes are TNF-α > 231 pg/mL, MMP-8 > 413 ng/mL, IL-6 > 3999 pg/mL, IL-10 > 35 pg/mL, IL-17 > 4 pg/mL, DEFA3 > 35 ng/mL.
    4.3 Decreased EGF levels < 35 pg/mL increase the odds of severe respiratory outcomes, PDA, ROP, and early hypotension by 3 to 9 times, especially in neonates born between 28^{+0} and 31^{+6} weeks of gestation.
5. Elevated TNF-α levels and decreased EGF levels, when combined with gestational age and FIRS diagnosis, enable the identification of neonates at high risk for adverse neonatal outcomes.

PRACTICAL RECOMMENDATIONS

1. Diagnosis of FIRS is recommended as an additional assessment for preterm neonates <34^{+6} weeks GA:
    1) Umbilical cord IL-6 levels above 11 pg/mL or histological funisitis indicate FIRS.
    2) Immunological analysis of amniotic fluid is recommended, whenever possible.
    3) Optimal cut-off values for non-invasively sampled amniotic fluid biomarkers are predictive of FIRS:
        - TNF-α > 89 pg/mL (sensitivity 66%, specificity 90%) indicates a 15-fold increased risk of FIRS. TNF-α is the most recommended prognostic biomarker in vaginal amniotic fluid for FIRS.
        - MMP-8 > 170 ng/mL (sensitivity 78%, specificity 71%) is associated with an 8-fold increased risk of FIRS,
        - IL-6 > 2695 pg/mL (sensitivity 76%, specificity 76%) 6-fold,
        - IL-10 > 35 pg/mL (sensitivity 61%, specificity 87%) 10-fold,
        - IL-17 > 1 pg/mL (sensitivity 61 %, specificity 75 %) 5-fold,
        - DEFA3 > 34 ng/mL (sensitivity 35 %, specificity 92 %) 7-fold,
        - suPAR > 27 ng/mL (sensitivity 65%, specificity 65%) – 3-fold.
    4) Low levels of inflammatory biomarkers help exclude the diagnosis of FIRS.
2. To identify preterm neonates <34^{+6} weeks GA at high risk for adverse neonatal outcomes:
    1) If feasible, immunological analysis of amniotic fluid is recommended.
    2) Optimal cut-off values for non-invasive amniotic fluid biomarkers allow assessment of the prognosis for FIRS and adverse neonatal outcomes:
        - TNF-α > 231 pg/mL (sensitivity 60%, specificity 89%) is associated with a 12-fold increased risk of FIRS and major neonatal outcome,

- MMP-8 > 413 ng/mL (sensitivity 65%, specificity 72%) is associated with a 5-fold increase in the risk of FIRS and major neonatal outcomes,
- IL-6 > 3999 pg/mL (sensitivity 59%, specificity 74%) 4-fold,
- IL-10 > 35 pg/mL (sensitivity 59%, specificity 74%) 4-fold,
- IL-17 > 4 pg/mL (sensitivity 50 %, specificity 84 %) 5-fold,
- DEFA3 > 35 ng/mL (sensitivity 36%, specificity 89%) 4-fold.
- EGF < 35 pg/ml increases the odds of severe respiratory outcomes, PDA, ROP and early hypotension by 3-9 fold.

3) TNF-α > 231 pg/mL and EGF < 35 pg/mL combined with gestational age and diagnosis of FIRS indicate an increased risk of adverse neonatal outcomes.

REFERENCES

1. Jung E, Romero R, Yeo L, Diaz-Primera R, Marin-Concha J, Para R, et al. The fetal inflammatory response syndrome: the origins of a concept, pathophysiology, diagnosis, and obstetrical implications. Semin Fetal Neonatal Med. 2020;25:101146.
2. Kim CJ, Romero R, Chaemsaithong P, Chaiyasit N, Yoon BH, Kim YM. Acute Chorioamnionitis and Funisitis: Definition, Pathologic Features, and Clinical Significance. Am J Obstet Gynecol. 2015;213:S29.
3. Fan SR, Liu P, Yan SM, Peng JY, Liu XP. Diagnosis and Management of Intraamniotic Infection. Matern Med. 2020;2:223–230.
4. Romero R, Gomez-Lopez N, Winters AD, Jung E, Shaman M, Bieda J, et al. Evidence that intra-amniotic infections are often the result of an ascending invasion - A molecular microbiological study. J Perinat Med. 2019;47:915–931.
5. Gomez R, Romero R, Ghezzi F, Yoon BH, Mazor M, Berry SM. The fetal inflammatory response syndrome. Am J Obstet Gynecol. 1998;179:194–202.
6. Pacora P, Chaiworapongsa T, Maymon E, Kim YM, Gomez R, Yoon BH, et al. Funisitis and chorionic vasculitis: the histological counterpart of the fetal inflammatory response syndrome. J Matern Fetal Neonatal Med. 2002;11:18–25.
7. Tang Q, Zhang L, Li H, Shao Y. The fetal inflammation response syndrome and adverse neonatal outcomes: a meta-analysis. J Matern Fetal Neonatal Med. 2021;34:3902–3914.
8. Jung E, Romero R, Yeo L, Chaemsaithong P, Gomez-Lopez N. Intra-Amniotic Infection/Inflammation and the Fetal Inflammatory Response Syndrome - ClinicalKey. . In: Polin RA, Abman SH, Rowitch DH, et al., eds. Fetal and Neonatal Physiology. Philadelphia, PA: Elsevier, 111–129.
9. Scher MS. Neurologic outcome after fetal inflammatory response syndrome: Trimester-specific considerations. Semin Fetal Neonatal Med. 2020;25:101137.
10. American College of Obstetritians and Gynecologist. Prelabor Rupture of Membranes: ACOG Practice Bulletin, Number 217. Obstet Gynecol. 2020;135:e80–e97.
11. Bastek JA, Weber AL, McShea MA, Ryan ME, Elovitz MA. Prenatal inflammation is associated with adverse neonatal outcomes. Am J Obstet

Gynecol. 2014;210:450.e1-450.e10.
12. McCartney SA, Kapur R, Liggitt HD, Baldessari A, Coleman M, Orvis A, et al. Amniotic fluid interleukin 6 and interleukin 8 are superior predictors of fetal lung injury compared with maternal or fetal plasma cytokines or placental histopathology in a nonhuman primate model. Am J Obstet Gynecol. 2021;225:89.e1-89.e16.
13. Shobokshi A, Shaarawy M. Maternal serum and amniotic fluid cytokines in patients with preterm premature rupture of membranes with and without intrauterine infection. Int J Gynecol Obstet. 2002;79:209–215.
14. Mercer BM, Crouse DT, Goldenberg RL, Miodovnik M, Mapp DC, Meis PJ, et al. The antibiotic treatment of PPROM study: Systemic maternal and fetal markers and perinatal outcomes. Am J Obstet Gynecol. 2012;206:145.e1-145.e9.
15. Nakahara M, Goto S, Kato E, Nojiri S, Itakura A, Takeda S. Maternal risk score for the prediction of fetal inflammatory response syndrome after preterm premature rupture of membranes. J Obstet Gynaecol Res. 2020;46:2019–2026.
16. Maymon E, Romero R, Pacora P, Gomez R, Athayde N, Edwin S, et al. Human neutrophil collagenase (matrix metalloproteinase 8) in parturition, premature rupture of the membranes, and intrauterine infection. Am J Obstet Gynecol. 2000;183:94–99.
17. Kim KW, Romero R, Park HS, Park CW, Shim SS, Jun JK, 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;197:292.e1-292.e5.
18. Maymon E, Ghezzi F, Edwin SS, Mazor M, Bo Hyun Yoon, Gomez R, et al. The tumor necrosis factor α and its soluble receptor profile in term and preterm parturition. Am J Obstet Gynecol. 1999;181:1142–1148.
19. Park JS, Romero R, Yoon BH, Moon J Bin, Oh SY, Han SY, et al. The relationship between amniotic fluid matrix metalloproteinase-8 and funisitis. Am J Obstet Gynecol. 2001;185:1156–1161.
20. Park KH, Yoon BH, Shim SS, Jun JK, Syn HC. Amniotic fluid tumor necrosis factor-alpha is a marker for the prediction of early-onset neonatal sepsis in preterm labor. Gynecol Obstet Invest. 2004;58:84–90.
21. Moon JB, Kim JC, Yoon BH, Romero R, Kim G, Oh SY, et al. Amniotic fluid matrix metalloproteinase-8 and the development of cerebral palsy. J Perinat Med. 2002;30:301–306.

22. Romero R, Chaemsaithong P, Docheva N, Korzeniewski SJ, Tarca AL, Bhatti G, et al. Clinical chorioamnionitis at term V: Umbilical cord plasma cytokine profile in the context of a systemic maternal inflammatory response. J Perinat Med. 2016;44:53–76.
23. Musilova I, Bestvina T, Hudeckova M, Michalec I, Cobo T, Jacobsson B, et al. Vaginal fluid interleukin-6 concentrations as a point-of-care test is of value in women with preterm prelabor rupture of membranes. Am J Obstet Gynecol. 2016;215:619.e1-619.e12.
24. Oh KJ, Lee JH, Romero R, Park HS, Hong JS, Yoon BH. A new rapid bedside test to diagnose and monitor intraamniotic inflammation in preterm PROM using transcervically collected fluid. Am J Obstet Gynecol. 2020;223:423.e1-423.e15.
25. Kunze M, Klar M, Morfeld CA, Thorns B, Schild RL, Markfeld-Erol F, et al. Cytokines in noninvasively obtained amniotic fluid as predictors of fetal inflammatory response syndrome. Am J Obstet Gynecol. 2016;215:96.e1-96.e8.
26. Romero R, Maymon E, Pacora P, Gomez R, Mazor M, Yoon BH, et al. Further observations on the fetal inflammatory response syndrome: a potential homeostatic role for the soluble receptors of tumor necrosis factor alpha. Am J Obstet Gynecol. 2000;183:1070–1077.
27. Mikołajczyk M, Wirstlein P, Adamczyk M, Skrzypczak J, Wender-Ozegowska E. Value of cervicovaginal fluid cytokines in prediction of fetal inflammatory response syndrome in pregnancies complicated with preterm premature rupture of membranes (pPROM). J Perinat Med. 2020;48:249–255.
28. Kayem G, Batteux F, Girard N, Schmitz T, Willaime M, Maillard F, et al. Predictive value of vaginal IL-6 and TNFα bedside tests repeated until delivery for the prediction of maternal-fetal infection in cases of premature rupture of membranes. Eur J Obstet Gynecol Reprod Biol. 2017;211:8–14.
29. Dorfeuille N, Morin V, Tétu A, Demers S, Laforest G, Gouin K, et al. Vaginal Fluid Inflammatory Biomarkers and the Risk of Adverse Neonatal Outcomes in Women with PPROM. Am J Perinatol. 2016;33:1003–1007.
30. Muraskas J, Astrug L, Amin S. FIRS: Neonatal considerations. Semin Fetal Neonatal Med. 2020;25:101142.
31. Chiesa C, Pacifico L, Natale F, Hofer N, Osborn JF, Resch B. Fetal and early neonatal interleukin-6 response. Cytokine. 2015;76:1–12.
32. Musilova I, Andrys C, Drahosova M, Soucek O, Stepan M, Bestvina T, et

al. Intraamniotic inflammation and umbilical cord blood interleukin-6 concentrations in pregnancies complicated by preterm prelabor rupture of membranes. J Matern Fetal Neonatal Med. 2017;30:900–910.
33. Bartkevičienė D, Pilypienė I, Serapinas D, Vaigauskaitė B, Vankevičiūtė RA, Bartkevičiūtė A, et al. Umbilical Blood Levels of IL-6 and TNF-α as Predictors of the Central Nervous System Damage and Retinopathy in Preterm Infants. Am J Perinatol. 2021;38:1036–1041.
34. Redline RW, Faye-Petersen O, Heller D, Qureshi F, Savell V, Vogler C. Amniotic Infection Syndrome: Nosology and Reproducibility of Placental Reaction Patterns. Pediatr Dev Pathol. 2003;6:435–448.
35. Kim CJ, Yoon BH, Kim M, Park JO, Cho SY, Chi JG. Histo-topographic distribution of acute inflammation of the human umbilical cord. Pathol Int. 2001;51:861–865.
36. Romero R, Gomez R, Ghezzi F, Bo Hyun Yoon, Mazor M, Edwin SS, et al. A fetal systemic inflammatory response is followed by the spontaneous onset of preterm parturition. Am J Obstet Gynecol. 1998;179:186–193.
37. Keelan JA. Intrauterine inflammatory activation, functional progesterone withdrawal, and the timing of term and preterm birth. J Reprod Immunol. 2018;125:89–99.
38. DiGiulio DB, Romero R, Amogan HP, Kusanovic JP, Bik EM, Gotsch F, et al. Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: A molecular and culture-based investigation. PLoS One. 2008;3:1–10.
39. Romero R, Miranda J, Kusanovic JP, Chaiworapongsa T, Chaemsaithong P, Martinez A, et al. Clinical chorioamnionitis at term I: Microbiology of the amniotic cavity using cultivation and molecular techniques. J Perinat Med. 2015;43:19–36.
40. Wang X, Buhimschi CS, Temoin S, Bhandari V, Han YW, Buhimschi IA. Comparative microbial analysis of paired amniotic fluid and cord blood from pregnancies complicated by preterm birth and early-onset neonatal sepsis. PLoS One. 2013;8:e56131–e56131.
41. Salafia CM, Misra DP. Histopathology of the fetal inflammatory response to intra-amniotic pathogens. Semin Fetal Neonatal Med.;25. Epub ahead of print 1 August 2020. DOI: 10.1016/J.SINY.2020.101128.
42. Starzyńska A, Wychowański P, Nowak M, Sobocki BK, Jereczek-Fossa BA, Słupecka-Ziemilska M. Association between Maternal Periodontitis and Development of Systematic Diseases in Offspring. Int J Mol Sci.;23.

Epub ahead of print 2022. DOI: 10.3390/ijms23052473.
43. Lee J, Romero R, Chaiworapongsa T, Dong Z, Tarca AL, Xu Y, 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. Am J Reprod Immunol. 2013;70:265–284.
44. Gibbs RS. Diagnosis of intra-amniotic infection. Semin Perinatol. 1977;1:71–7.
45. Higgins RD, Saade G, Polin RA, Grobman WA, Buhimschi IA, Watterberg K, et al. Evaluation and Management of Women and Newborns with a Maternal Diagnosis of Chorioamnionitis: Summary of a Workshop. Obstet Gynecol. 2016;127:426–436.
46. Romero R, Espinoza J, Kusanovic JP, Gotsch F, Hassan S, Erez O, et al. The preterm parturition syndrome. BJOG An Int J Obstet Gynaecol. 2006;113:17–42.
47. Maki Y, Furukawa S, Nakayama T, Oohashi M, Shiiba N, Furuta K, et al. Clinical chorioamnionitis criteria are not sufficient for predicting intra-amniotic infection. J Matern Neonatal Med. 2022;35:52–57.
48. Vintzileos AM, Smulian JC. Abnormal fetal heart rate patterns caused by pathophysiologic processes other than fetal acidemia. Am J Obstet Gynecol. 2023;228:S1144–S1157.
49. Vintzileos AM, Campbell WA, Nochimson DJ, Connolly ME, Fuenfer MM, Hoehn GJ. The fetal biophysical profile in patients with premature rupture of the membranes—An early predictor of fetal infection. Am J Obstet Gynecol. 1985;152:510–516.
50. Lear CA, Westgate JA, Ugwumadu A, Nijhuis JG, Stone PR, Georgieva A, et al. Understanding Fetal Heart Rate Patterns That May Predict Antenatal and Intrapartum Neural Injury. Semin Pediatr Neurol. 2018;28:3–16.
51. Witt RG, Blair L, Frascoli M, Rosen MJ, Nguyen QH, Bercovici S, et al. Detection of microbial cell-free DNA in maternal and umbilical cord plasma in patients with chorioamnionitis using next generation sequencing. PLoS One. 2020;15:1–15.
52. Newberry DM. Comparison of Placental and Neonatal Admission Complete Blood Cell Count and Blood Cultures. Adv Neonatal Care. 2018;18:215–222.
53. Moore SP, Newberry DM, Jnah AJ. Use of Placental/Umbilical Blood

Sampling for Neonatal Admission Blood Cultures: Benefits, Challenges, and Strategies for Implementation. Neonatal Netw. 2017;36:152–159.
54. Abdel-Razeq SS, Buhimschi IA, Bahtiyar MO, Rosenberg VA, Dulay AT, Han CS, et al. Interpretation of Amniotic Fluid White Blood Cell Count in "Bloody Tap" Amniocenteses in Women With Symptoms of Preterm Labor. Obstet Gynecol. 2010;116:344.
55. Jung E, Romero R, Suksai M, Gotsch F, Chaemsaithong P, Erez O, et al. Clinical chorioamnionitis at term: definition, pathogenesis, microbiology, diagnosis, and treatment. Elsevier Inc. Epub ahead of print 2024. DOI: 10.1016/j.ajog.2023.02.002.
56. Kacerovsky M, Holeckova M, Stepan M, Gregor M, Vescicik P, Lesko D, et al. Amniotic fluid glucose level in PPROM pregnancies: a glance at the old friend. J Matern Neonatal Med. 2022;35:2247–2259.
57. Prairie E, Côté F, Tsakpinoglou M, Mina M, Quiniou C, Leimert K, et al. The determinant role of IL-6 in the establishment of inflammation leading to spontaneous preterm birth. Cytokine Growth Factor Rev. 2021;59:118–130.
58. Kemp MW. Preterm birth, intrauterine infection, and fetal inflammation. Front Immunol. 2014;5:574.
59. Kim YM, Romero R, Chaiworapongsa T, Kim GJ, Kim MR, Kuivaniemi H, 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. 2004;191:1346–1355.
60. Kim YM, Romero R, Chaiworapongsa T, Espinoza J, Mor G, Kim CJ. Dermatitis as a component of the fetal inflammatory response syndrome is associated with activation of Toll-like receptors in epidermal keratinocytes. Histopathology. 2006;49:506–514.
61. Espinoza J, Chaiworapongsa T, Romero R, Edwin S, Rathnasabapathy C, Gomez R, 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. 2003;13:2–21.
62. Heine RP, Wiesenfeld H, Mortimer L, Greig PC. Amniotic fluid defensins: Potential markers of subclinical intrauterine infection. Clin Infect Dis. 1998;27:513–518.
63. Holst RM, Hagberg H, Wennerholm UB, Skogstrand K, Thorsen P,

Jacobsson B. Prediction of microbial invasion of the amniotic cavity in women with preterm labour: Analysis of multiple proteins in amniotic and cervical fluids. BJOG An Int J Obstet Gynaecol. 2011;118:240–249.
64. Chaiyasit N, Romero R, Chaemsaithong P, Docheva N, Bhatti G, Kusanovic JP, et al. Clinical chorioamnionitis at term VIII: A rapid MMP-8 test for the identification of intra-amniotic inflammation. J Perinat Med. 2017;45:539–550.
65. Lee SM, Romero R, Park JS, Chaemsaithong P, Jun JK, Yoon BH. A Transcervical Amniotic Fluid Collector: A New Medical Device for The Assessment of Amniotic Fluid in Patients with Ruptured Membranes. J Perinat Med. 2015;43:381.
66. Bo Hyun Yoon, Jong Kwan Jun, Romero R, Kyo Hoon Park, Gomez R, Choi H, et al. Amniotic fluid inflammatory cytokines (interleukin-6, interleukin-1β, and tumor necrosis factor-α), neonatal brain white matter lesions, and cerebral palsy. Am J Obstet Gynecol. 1997;177:19–26.
67. Bo Hyun Yoon, Romero R, Chong Jai Kim, Ja Nam Koo, Choe G, Hee Chul Syn, et al. High expression of tumor necrosis factor-α and interleukin-6 in periventricular leukomalacia. Am J Obstet Gynecol. 1997;177:406–411.
68. Park YJ, Woo SJ, Kim YM, Hong S, Lee YE, Park KH. Immune and inflammatory proteins in cord blood as predictive biomarkers of retinopathy of prematurity in preterm infants. Investig Ophthalmol Vis Sci. 2019;60:3813–3820.
69. Figueroa-Damián R, Arredondo-García JL, Mancilla-Ramírez J. Amniotic fluid interleukin-6 and the risk of early-onset sepsis among preterm infants. Arch Med Res. 1999;30:198–202.
70. Oshima Y, Tanaka S, Tsumura K, Tsuda S, So K, Shinagawa T, et al. Amniotic fluid Gram stain and interleukin-6 can predict early-onset neonatal sepsis. J Obstet Gynaecol Res. 2021;47:726–733.
71. Yoon BH, Romero R, Kim CJ, Jun JK, Gomez R, Choi JH, et al. Amniotic fluid interleukin-6: A sensitive test for antenatal diagnosis of acute inflammatory lesions of preterm placenta and prediction of perinatal morbidity. Am J Obstet Gynecol. 1995;172:960–970.
72. Bo Hyun Yoon, Romero R, Jong Kwan Jun, Kyo Hoon Park, June Dong Park, Ghezzi F, et al. Amniotic fluid cytokines (interleukin-6, tumor necrosis factor-α, interleukin-1β, and interleukin-8) and the risk for the development of bronchopulmonary dysplasia. Am J Obstet Gynecol.

1997;177:825–830.
73. Ramseyer VD, Garvin JL. Tumor necrosis factor-α: Regulation of renal function and blood pressure. Am J Physiol - Ren Physiol. 2013;304:1231–1242.
74. Laha D, Grant R, Mishra P, Nilubol N. The Role of Tumor Necrosis Factor in Manipulating the Immunological Response of Tumor Microenvironment. Front Immunol. 2021;12:1415.
75. Bo Hyun Yoon, Romero R, Joong Shin Park, Jae Won Chang, Young Ah Kim, Ju Cheol Kim, et al. Microbial invasion of the amniotic cavity with Ureaplasma urealyticum is associated with a robust host response in fetal, amniotic, and maternal compartments. Am J Obstet Gynecol. 1998;179:1254–1260.
76. Novy MJ, Duffy L, Axthelm MK, Sadowsky DW, Witkin SS, Gravett MG, et al. Ureaplasma parvum or Mycoplasma hominis as sole pathogens cause chorioamnionitis, preterm delivery, and fetal pneumonia in rhesus macaques. Reprod Sci. 2009;16:56–70.
77. Lu H yan, Zhang Q, Wang Q xia, Lu J ying. Contribution of Histologic Chorioamnionitis and Fetal Inflammatory Response Syndrome to Increased Risk of Brain Injury in Infants With Preterm Premature Rupture of Membranes. Pediatr Neurol. 2016;61:94-98.e1.
78. Hayes D, Feola DJ, Murphy BS, Shook LA, Ballard HO. Pathogenesis of bronchopulmonary dysplasia. Respiration. 2010;79:425–436.
79. Park HW, Choi YS, Kim KS, Kim SN. Chorioamnionitis and patent ductus arteriosus: A systematic review and meta-analysis. PLoS One. 2015;10:1–14.
80. Wei YJ, Hsu R, Lin YC, Wong TW, Kan CD, Wang JN. The Association of Patent Ductus Arteriosus with Inflammation: A Narrative Review of the Role of Inflammatory Biomarkers and Treatment Strategy in Premature Infants. Int J Mol Sci.;23. Epub ahead of print 2022. DOI: 10.3390/ijms232213877.
81. Wu PY, Fu YK, Lien RI, Chiang MC, Lee CC, Chen HC, et al. Systemic Cytokines in Retinopathy of Prematurity. J Pers Med. 2023;13:1–40.
82. Hellgren G, Löfqvist C, Hansen-Pupp I, Gram M, Smith LE, Ley D, et al. Increased postnatal concentrations of pro-inflammatory cytokines are associated with reduced IGF-I levels and retinopathy of prematurity. Growth Horm IGF Res. 2018;39:19–24.
83. Jang JH, Kim JG, Lee YH, Bae JG, Park JH. The association between

amniotic fluid-derived inflammatory mediators and the risk of retinopathy of prematurity. Med (United States). 2022;101:E29368.
84. Ryu A, Park KH, Oh KJ, Lee SY, Jeong EH, Park JW. Predictive value of combined cervicovaginal cytokines and gestational age at sampling for intra-amniotic infection in preterm premature rupture of membranes. Acta Obstet Gynecol Scand. 2013;92:517–524.
85. Ling XC, Huang PH, Chen HC, Hsueh YJ, Lee CW, Lien R, et al. Association of serum levels of inflammatory cytokines with retinopathy of prematurity in preterm infants. Front Pediatr.;11. Epub ahead of print 2023. DOI: 10.3389/fped.2023.1195904.
86. Cabral-Pacheco GA, Garza-Veloz I, Castruita-De La Rosa C, Ramirez-Acuña JM, Perez-Romero BA, Guerrero-Rodriguez JF, et al. The Roles of Matrix Metalloproteinases and Their Inhibitors in Human Diseases. Int J Mol Sci. 2020;21:1–53.
87. Myntti T, Rahkonen L, Pätäri-Sampo A, Tikkanen M, Sorsa T, Juhila J, et al. Comparison of amniotic fluid matrix metalloproteinase-8 and cathelicidin in the diagnosis of intra-amniotic infection. J Perinatol. 2016;36:1049–1054.
88. Desmedt S, Desmedt V, Delanghe JR, Speeckaert R, Speeckaert MM. The intriguing role of soluble urokinase receptor in inflammatory diseases. Crit Rev Clin Lab Sci. 2017;54:117–133.
89. Dinesh P, Rasool MK. uPA/uPAR signaling in rheumatoid arthritis: Shedding light on its mechanism of action. Pharmacol Res. 2018;134:31–39.
90. Loosen SH, Breuer A, Tacke F, Kather JN, Gorgulho J, Alizai PH, et al. Circulating levels of soluble urokinase plasminogen activator receptor predict outcome after resection of biliary tract cancer. JHEP reports Innov Hepatol. 2020;2:100080.
91. Thunø M, Macho B, Eugen-Olsen J. suPAR: The molecular crystal ball. Dis Markers. 2009;27:157–172.
92. Uszyński M, Perlik M, Uszyński W, Zekanowska E. Urokinase plasminogen activator (uPA) and its receptor (uPAR) in gestational tissues: Measurements and clinical implications. Eur J Obstet Gynecol Reprod Biol. 2004;114:54–58.
93. Higham JP, Stahl-Hennig C, Heistermann M. Urinary suPAR: a non-invasive biomarker of infection and tissue inflammation for use in studies of large free-ranging mammals. R Soc Open Sci.;7. Epub ahead of print 1

February 2020. DOI: 10.1098/RSOS.191825.
94. Tzanakaki G, Paparoupa M, Kyprianou M, Barbouni A, Eugen-Olsen J, Kourea-Kremastinou J. Elevated soluble urokinase receptor values in CSF, age and bacterial meningitis infection are independent and additive risk factors of fatal outcome. Eur J Clin Microbiol Infect Dis. 2012;31:1157–1162.
95. Odden N, Henriksen T, Mørkrid L. Serum soluble urokinase plasminogen activator receptor (suPAR) in early pregnancy prior to clinical onset of preeclampsia. Acta Obstet Gynecol Scand. 2012;91:1226–1232.
96. Toldi G, Bíró E, Szalay B, Stenczer B, Molvarec A, Rigó J, et al. Soluble urokinase plasminogen activator receptor (suPAR) levels in healthy pregnancy and preeclampsia. Clin Chem Lab Med. 2011;49:1873–1876.
97. Tunc T, Cekmez F, Yildirim S, Bulut O, Ince Z, Saldir M, et al. Predictive value of soluble urokinase plasminogen activator receptor, soluble ST2, and IL-33 in bronchopulmonary dysplasia. Pediatr Res. 2014;75:788–792.
98. Nurhayati R, Yudani T, Raras M, Kawurjan L. Role of soluble urokinase plasminogen activator receptor (suPAR) as prognosis markers of neonatal sepsis. IOSR J Pharm Biol Sci (IOSR-JPBS.;6:6–14.
99. Okulu E, Arsan S, Akin IM, Ates C, Alan S, Kilic A, et al. Serum Levels of Soluble Urokinase Plasminogen Activator Receptor in Infants with Late-onset Sepsis. J Clin Lab Anal. 2015;29:347–352.
100. Kawasaki T, Kawai T. Toll-like receptor signaling pathways. Front Immunol. 2014;5:1–8.
101. Hagberg H, Peebles D, Mallard C. Models of white matter injury: Comparison of infectious, hypoxic-Ischemic, and excitotoxic insults. Ment Retard Dev Disabil Res Rev. 2002;8:30–38.
102. Dulay AT, Buhimschi CS, Zhao G, Oliver EA, Mbele A, Jing S, et al. Soluble TLR2 Is Present in Human Amniotic Fluid and Modulates the Intraamniotic Inflammatory Response to Infection. J Immunol. 2009;182:7244–7253.
103. Kacerovsky M, Andrys C, Hornychova H, Pliskova L, Lancz K, Musilova I, et al. Amniotic fluid soluble Toll-like receptor 4 in pregnancies complicated by preterm prelabor rupture of the membranes. J Matern Neonatal Med. 2012;25:1148–1155.
104. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol. 2001;2:675–680.
105. Andrys C, Kacerovsky M, Drahosova M, Musilova I, Pliskova L,

Hornychova H, et al. Amniotic fluid soluble Toll-like receptor 2 in pregnancies complicated by preterm prelabor rupture of membranes. J Matern Neonatal Med. 2013;26:520–527.
106. Schneider JJ, Unholzer A, Schaller M, Schäfer-Korting M, Korting HC. Human defensins. J Mol Med. 2005;83:587–595.
107. Fu J, Zong X, Jin M, Min J, Wang F, Wang Y. Mechanisms and regulation of defensins in host defense. Signal Transduct Target Ther.;8. Epub ahead of print 2023. DOI: 10.1038/s41392-023-01553-x.
108. Espinoza J, Chaiworapongsa T, Romero R, Edwin S, Rathnasabapathy C, Gomez R, 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 Neonatal Med. 2003;13:2–21.
109. Lucovnik M, Kornhauser-Cerar L, Premru-Srsen T, Gmeiner-Stopar T, Derganc M. Neutrophil defensins but not interleukin-6 in vaginal fluid after preterm premature rupture of membranes predict fetal/neonatal inflammation and infant neurological impairment. Acta Obstet Gynecol Scand. 2011;90:908–916.
110. Hanna N, Bonifacio L, Weinberger B, Reddy P, Murphy S, Romero R, et al. Evidence for interleukin-10-mediated inhibition of cyclo-oxygenase-2 expression and prostaglandin production in preterm human placenta. Am J Reprod Immunol. 2006;55:19–27.
111. Cheng S-B, Sharma S. Interleukin-10: A Pleiotropic Regulator in Pregnancy Shi-Bin. Am J Reprod Immunol. 2015;73:487–500.
112. Ouyang W, Rutz S, Crellin NK, Valdez PA, Hymowitz SG. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. Annu Rev Immunol. 2011;29:71–109.
113. McGowan EC, Kostadinov S, McLean K, Gotsch F, Venturini D, Romero R, et al. Placental IL-10 dysregulation and association with bronchopulmonary dysplasia risk. Pediatr Res. 2009;66:455–460.
114. Gotsch F, Romero R, Kusanovic JP, Erez O, Espinoza J, Kim CJ, et al. The anti-inflammatory limb of the immune response in preterm labor, intra-amniotic infection/inflammation, and spontaneous parturition at term: A role for interleukin-10. J Matern Neonatal Med. 2008;21:529–547.
115. Madsen-Bouterse SA, Romero R, Tarca AL, Kusanovic JP, Espinoza J, Kim CJ, et al. The transcriptome of the fetal inflammatory response

syndrome. Am J Reprod Immunol. 2010;63:73–92.
116. Lawrence SM, Ruoss JL, Wynn JL. IL-17 in Neonatal Health and Disease. Am J Reprod Immunol. 2018;79:e12800.
117. Ruiz de Morales JMG, Puig L, Daudén E, Cañete JD, Pablos JL, Martín AO, et al. Critical role of interleukin (IL)-17 in inflammatory and immune disorders: An updated review of the evidence focusing in controversies. Autoimmun Rev. 2020;19:102429.
118. Jain VG, Willis KA, Jobe A, Ambalavanan N. Chorioamnionitis and neonatal outcomes. Pediatr Res. 2022;91:289–296.
119. Ito M, Nakashima A, Hidaka T, Okabe M, Bac ND, Ina S, et al. A role for IL-17 in induction of an inflammation at the fetomaternal interface in preterm labour. J Reprod Immunol. 2010;84:75–85.
120. Rito DC, Viehl LT, Buchanan PM, Haridas S, Koenig JM. Augmented Th17-type immune responses in preterm neonates exposed to histologic chorioamnionitis. Pediatr Res. 2017;81:639–645.
121. Wynn JL, Wilson CS, Hawiger J, Scumpia PO, Marshall AF, Liu JH, et al. Targeting IL-17A attenuates neonatal sepsis mortality induced by IL-18. Proc Natl Acad Sci U S A. 2016;113:E2627–E2635.
122. Sood BG, Madan A, Saha S, Schendel D, Thorsen P, Skogstrand K, et al. Perinatal systemic inflammatory response syndrome and retinopathy of prematurity. Pediatr Res. 2010;67:394–400.
123. Lawrence SM, Wynn JL. Chorioamnionitis, IL-17A, and Fetal Origins of Neurologic Disease. Am J Reprod Immunol. 2018;79:e12803.
124. Carlo WA, McDonald SA, Tyson JE, Stoll BJ, Ehrenkranz RA, Shankaran S, et al. Cytokines and Neurodevelopmental Outcomes in Extremely Low Birth Weight Infants. J Pediatr. 2011;159:919.
125. Appay V, Rowland-Jones SL. RANTES: A versatile and controversial chemokine. Trends Immunol. 2001;22:83–87.
126. Athayde N, Romero R, Maymon E, Gomez R, Pacora P, Araneda H, et al. A role for the novel cytokine RANTES in pregnancy and parturition. Am J Obstet Gynecol. 1999;181:989–994.
127. Hong Z, Cabrera JA, Mahapatra S, Kutty S, Weir EK, Archer SL. Activation of the EGFR/p38/JNK pathway by mitochondrial-derived hydrogen peroxide contributes to oxygen-induced contraction of ductus arteriosus. J Mol Med. 2014;92:995–1007.
128. Dvorak B, Khailova L, Clark JA, Hosseini DM, Arganbright KM, Reynolds CA, et al. Comparison of epidermal growth factor and heparin-

binding epidermal growth factor-like growth factor for prevention of experimental necrotizing enterocolitis. J Pediatr Gastroenterol Nutr. 2008;47:11–18.
129. Vaes JEG, van Kammen CM, Trayford C, van der Toorn A, Ruhwedel T, Benders MJNL, et al. Intranasal mesenchymal stem cell therapy to boost myelination after encephalopathy of prematurity. Glia. 2021;69:655–680.
130. Vinukonda G, Hu F, Mehdizadeh R, Dohare P, Kidwai A, Juneja A, et al. Epidermal growth factor preserves myelin and promotes astrogliosis after intraventricular hemorrhage. Glia. 2016;64:1987–2004.
131. Musilova I, Hornychova H, Kostal M, Jacobsson B, Kacerovsky M. Ultrasound measurement of the transverse diameter of the fetal thymus in pregnancies complicated by the preterm prelabor rupture of membranes. J Clin Ultrasound. 2013;41:283–289.
132. Musilova I, Spacek R, Stranik J, Jacobsson B, Kacerovsky M. Fetal Portal System Flowmetry and Intra-Amniotic Inflammation in Preterm Prelabor Rupture of Membranes. Fetal Diagn Ther. 2019;46:323–332.
133. Musilova I, Kacerovsky M, Andrys C, Kostal M, Slaba K, Jacobsson B. The fetal splenic vein flow pattern and fetal inflammatory response in the preterm prelabor rupture of membranes. J Matern Fetal Neonatal Med. 2014;27:770–774.
134. Mastrolia SA, Erez O, Loverro G, Di Naro E, Weintraub AY, Tirosh D, et al. Ultrasonographic approach to diagnosis of fetal inflammatory response syndrome: A tool for at-risk fetuses? Am J Obstet Gynecol. 2016;215:9–20.
135. Hall M, Hutter J, Suff N, Avena Zampieri C, Tribe RM, Shennan A, et al. Antenatal diagnosis of chorioamnionitis: A review of the potential role of fetal and placental imaging. Prenat Diagn. 2022;42:1049–1058.
136. Aksakal SE, Kandemir O, Altinbas S, Esin S, Muftuoglu KH. Fetal tyhmus size as a predictor of histological chorioamnionitis in preterm premature rupture of membranes. J Matern Neonatal Med. 2014;27:1118–1122.
137. Hutter J, Jackson L, Ho A, Avena Zampieri C, Hajnal J V., Al-Adnani M, et al. The use of functional placental magnetic resonance imaging for assessment of the placenta after prolonged preterm rupture of the membranes in vivo: A pilot study. Acta Obstet Gynecol Scand. 2021;100:2244–2252.
138. Khong TY, Mooney EE, Ariel I, Balmus NCM, Boyd TK, Brundler MA, et al. Sampling and Definitions of Placental Lesions: Amsterdam Placental

Workshop Group Consensus Statement. Arch Pathol Lab Med. 2016;140:698–713.
139. Kim CJ, Yoon BH, Romero R, Moon J Bin, Kim M, Park SS, et al. Umbilical arteritis and phlebitis mark different stages of the fetal inflammatory response. Am J Obstet Gynecol. 2001;185:496–500.
140. Gotsch F, Romero R, Kusanovic JP, Mazaki-Tovi S, Pineles BL, Erez O, et al. The fetal inflammatory response syndrome. Clin Obstet Gynecol. 2007;50:652–683.
141. Romero R, Savasan ZA, Chaiworapongsa T, Berry SM, Kusanovic JP, Hassan SS, et al. Hematologic profile of the fetus with systemic inflammatory response syndrome. J Perinat Med. 2012;40:19–32.
142. Chaiworapongsa T, Romero R, Berry SM, Hassan SS, Yoon BH, Edwin S, et al. The role of granulocyte colony-stimulating factor in the neutrophilia observed in the fetal inflammatory response syndromegsa. J Perinat Med. 2011;39:653–666.
143. Toti P, De Felice C, Stumpo M, Schürfeld K, Leo L Di, Vatti R, et al. Acute thymic involution in fetuses and neonates with chorioamnionitis. Hum Pathol. 2000;31:1121–1128.
144. Sciaky-Tamir Y, Hershkovitz R, Mazor M, Shelef I, Erez O. The use of imaging technology in the assessment of the fetal inflammatory response syndrome-imaging of the fetal thymus. Prenat Diagn. 2015;35:413–419.
145. Toti P, Felice C De, Occhini R, Schuerfeld K, Stumpo M, Epistolato MC, et al. Spleen depletion in neonatal sepsis and chorioamnionitis. Am J Clin Pathol. 2004;122:765–771.
146. Bo Hyun Yoon, Romero R, Jong Kwan Jun, Maymon E, Gomez R, Mazor M, et al. An increase in fetal plasma cortisol but not dehydroepiandrosterone sulfate is followed by the onset of preterm labor in patients with preterm premature rupture of the membranes. Am J Obstet Gynecol. 1998;179:1107–1114.
147. Nathanielsz PW, Berghorn KA, Derks JB, Giussani DA, Docherty C, Unno N, et al. Life before birth: Effects of cortisol on future cardiovascular and metabolic function. Acta Paediatr Int J Paediatr. 2003;92:766–772.
148. De Felice C, Bagnoli F, Toti P, Musarò MA, Peruzzi L, Paffetti P, et al. Transient hypothyroxinemia of prematurity and histological chorioamnionitis. J Perinat Med. 2005;33:514–518.
149. Thébaud B, Goss KN, Laughon M, Whitsett JA, Abman SH, Steinhorn RH, et al. Bronchopulmonary dysplasia. Nat Rev Dis Prim. 2019;5:78.

150. Watterberg KL, Demers LM, Scott SM, Murphy S. Chorioamnionitis and Early Lung Inflammation in Infants in Whom Bronchopulmonary Dysplasia Develops. Pediatrics. 1996;97:210–215.
151. Villamor-Martinez E, Álvarez-Fuente M, Ghazi AMT, Degraeuwe P, Zimmermann LJI, Kramer BW, et al. Association of Chorioamnionitis with Bronchopulmonary Dysplasia among Preterm Infants: A Systematic Review, Meta-analysis, and Metaregression. JAMA Netw Open. 2019;2:1–21.
152. Romero R, Espinoza J, Gonçalves LF, Gomez R, et al. Fetal cardiac dysfunction in preterm premature rupture of membranes. J Matern Fetal Neonatal Med. 2004;16:146–157.
153. Di Naro E, Cromi A, Ghezzi F, Giocolano A, Caringella A, Loverro G. Myocardial dysfunction in fetuses exposed to intraamniotic infection: New insights from tissue Doppler and strain imaging. Am J Obstet Gynecol. 2010;203:459.e1-459.e7.
154. Yanowitz TD, Jordan JA, Gilmour CH, Towbin R, Bowen A, Roberts JM, et al. Hemodynamic disturbances in premature infants born after chorioamnionitis: association with cord blood cytokine concentrations. Pediatr Res. 2002;51:310–316.
155. Been J V., Lievense S, Zimmermann LJI, Kramer BW, Wolfs TGAM. Chorioamnionitis as a Risk Factor for Necrotizing Enterocolitis: A Systematic Review and Meta-Analysis. J Pediatr. 2013;162:236-242.e2.
156. Andrews WW, Goldenberg RL, Faye-Petersen O, Cliver S, Goepfert AR, Hauth JC. The Alabama Preterm Birth study: polymorphonuclear and mononuclear cell placental infiltrations, other markers of inflammation, and outcomes in 23- to 32-week preterm newborn infants. Am J Obstet Gynecol. 2006;195:803–808.
157. Miranda RN, Omurtag K, Castellani WJ, De Las Casas LE, Quintanilla NM, Kaabipour E. Myelopoiesis in the Liver of Stillborns With Evidence of Intrauterine Infection. Arch Pathol Lab Med. 2006;130:1786–1791.
158. Pfisterer C, Faber R, Horn LC. Chorioamnionitis-induced changes of fetal extramedullar hematopoiesis in the second trimester of gestation. Is diagnosis from fetal autopsy possible? Virchows Arch. 2005;446:150–156.
159. Bieghs V, Vlassaks E, Custers A, Van Gorp PJ, Gijbels MJ, Bast A, et al. Chorioamnionitis induced hepatic inflammation and disturbed lipid metabolism in fetal sheep. Pediatr Res. 2010;68:466–472.

160. Yoon BH, Kim YA, Romero R, Kim JC, Park KH, Kim MH, et al. Association of oligohydramnios in women with preterm premature rupture of membranes with an inflammatory response in fetal, amniotic, and maternal compartments. Am J Obstet Gynecol. 1999;181:784–788.
161. Lee SE, Romero R, Lee SM, Yoon BH. Amniotic fluid volume in intra-amniotic inflammation with and without culture-proven amniotic fluid infection in preterm premature rupture of membranes. J Perinat Med. 2010;38:39–44.
162. Azpurua H, Dulay AT, Buhimschi IA, Bahtiyar MO, Funai E, Abdel-Razeq SS, et al. Fetal renal artery impedance as assessed by Doppler ultrasound in pregnancies complicated by intraamniotic inflammation and preterm birth. Am J Obstet Gynecol. 2009;200:203.e1-203.e11.
163. Galinsky R, Moss TJM, Gubhaju L, Hooper SB, Jane Black M, Polglase GR. Effect of intra-amniotic lipopolysaccharide on nephron number in preterm fetal sheep. Am J Physiol - Ren Physiol. 2011;301:280–285.
164. Nguyen DN, Thymann T, Goericke-Pesch SK, Ren S, Wei W, Skovgaard K, et al. Prenatal Intra-Amniotic Endotoxin Induces Fetal Gut and Lung Immune Responses and Postnatal Systemic Inflammation in Preterm Pigs. Am J Pathol. 2018;188:2629–2643.
165. Villamor-Martinez E, Cavallaro G, Raffaeli G, Rahim OMMM, Gulden S, Ghazi AMT, et al. Chorioamnionitis as a risk factor for retinopathy of prematurity: An updated systematic review and meta-analysis. PLoS One. 2018;13:1–20.
166. Leung JC, Cifra CL, Agthe AG, Sun CCJ, Viscardi RM. Antenatal factors modulate hearing screen failure risk in preterm infants. Arch Dis Child Fetal Neonatal Ed. 2016;101:F56–F61.
167. Shim YJ, Choi BY, Park KH, Lee H, Jung YM, Kim YM. Inflammatory and Immune Proteins in Umbilical Cord Blood: Association with Hearing Screening Test Failure in Preterm Neonates. Mediators Inflamm.;2018. Epub ahead of print 2018. DOI: 10.1155/2018/4209359.
168. Cristobal R, Oghalai JS. Hearing loss in children with very low birth weight: current review of epidemiology and pathophysiology. Arch Dis Child - Fetal Neonatal Ed. 2008;93:F462–F468.
169. Lithuanian obstetric guidelines. Preterm premature rupture of membranes. Website of Ministry of Health of the Republic of Lithuania, https://sam.lrv.lt/uploads/sam/documents/files/Akušerinė metodika_Priešlaikinis neišnešioto vaisiaus dangalų

plyšimas_SAM_2019-07-20.pdf (2019).
170. VUHSK Protocol. Preterm premature rupture of membranes. 2017.
171. Sweet DG, Carnielli V, Greisen G, Hallman M, Ozek E, te Pas A, et al. European Consensus Guidelines on the Management of Respiratory Distress Syndrome – 2019 Update. Neonatology. 2019;115:432–450.
172. Jensen EA, Mong DA, Biko DM, Maschhoff KL, Kirpalani H. Common etiologies of respiratory distress in infants. . In: Goldsmith JP, Karotkin EH, Keszler M, et al., eds. Assisted ventilation of the neonate : an evidence-based approach to newborn respiratory care. Philadelphia: Elsevier, 2016, 72.
173. Dempsey E, Ting J, Van Heijst A, Joynt C, Cheung P-Y. Treating Hypotension in Preterm Neonates With Vasoactive Medications. Front Pediatr. 2018;6:86.
174. Mullaly R, El-Khuffash AF. Haemodynamic assessment and management of hypotension in the preterm. Arch Dis Child Fetal Neonatal Ed. 2024;109:120–127.
175. Puopolo KM, Benitz WE, Zaoutis TE. Management of neonates born at ≤34 6/7 weeks' gestation with suspected or proven early-onset bacterial sepsis. Pediatrics.;142. Epub ahead of print 1 December 2018. DOI: 10.1542/peds.2018-2896.
176. VUHSK management protocol. Diagnosis and treatment of early bacterial infection in newborns. 2021.
177. Papile LA, Burstein J, Burstein R, Koffler H. Incidence and evolution of subependymal and intraventricular hemorrhage: A study of infants with birth weights less than 1,500 gm. J Pediatr. 1978;92:529–534.
178. Volpe JJ. Neurology of the Newborn. Neurol Newborn. 2008;1–1094.
179. VUHSK Protocol. Diagnosis and management of patent ductus arteriosus in preterm neonates. 2019.
180. Nick Evans, Malcolm G, Osborn D, Kluckow M. Patent ductus arteriosus in preterm infants. Neoreviews. 2004;5:e86–e97.
181. Chiang MF, Quinn GE, Fielder AR, Ostmo SR, Paul Chan R V., Berrocal A, et al. International Classification of Retinopathy of Prematurity, Third Edition. Ophthalmology. 2021;128:e51–e68.
182. March of Dimes, PMNCH, Save the Children, World Health Organisation. Born Too Soon: the global action report on preterm birth. Geneva, https://www.who.int/pmnch/media/news/2012/201204_borntoosoon-report.pdf (2012, accessed 20 May 2020).

183. C. Andrew Combs, Gravett M, Garite TJ, Hickok DE, Lapidus J, Porreco R, et al. Amniotic fluid infection, inflammation, and colonization in preterm labor with intact membranes. Am J Obstet Gynecol. 2014;210:125.e1-125.e15.
184. Romero R, Miranda J, Chaiworapongsa T, Korzeniewski SJ, Chaemsaithong P, Gotsch F, et al. Prevalence and Clinical Significance of Sterile Intra-amniotic Inflammation in Patients with Preterm Labor and Intact Membranes. Am J Reprod Immunol. 2014;72:458–474.
185. Romero R, Yoon BH, Mazor M, Gomez R, Diamond MP, Kenney JS, et al. The diagnostic and prognostic value of amniotic fluid white blood cell count, glucose, interleukin-6, and Gram stain in patients with preterm labor and intact membranes. Am J Obstet Gynecol. 1993;169:805–816.
186. Thomakos N, Daskalakis G, Papapanagiotou A, Papantoniou N, Mesogitis S, Antsaklis A. Amniotic fluid interleukin-6 and tumor necrosis factor-α at mid-trimester genetic amniocentesis: Relationship to intra-amniotic microbial invasion and preterm delivery. Eur J Obstet Gynecol Reprod Biol. 2010;148:147–151.
187. Son GH, You YA, Kwon EJ, Lee KY, Kim YJ. Comparative Analysis of Midtrimester Amniotic Fluid Cytokine Levels to Predict Spontaneous Very Pre-term Birth in Patients with Cervical Insufficiency. Am J Reprod Immunol. 2016;75:155–161.
188. Sadowsky DW, Novy MJ, Witkin SS, Gravett MG. Dexamethasone or interleukin-10 blocks interleukin-1β-induced uterine contractions in pregnant rhesus monkeys. Am J Obstet Gynecol. 2003;188:252–263.
189. Park S, You YA, Yun H, Choi SJ, Hwang HS, Choi SK, et al. Cervicovaginal fluid cytokines as predictive markers of preterm birth in symptomatic women. Obstet Gynecol Sci. 2020;63:455–463.
190. Dudley DJ, Hunter C, Mitchell MD, Varner MW. Amniotic fluid interleukin-10 (IL-10) concentrations during pregnancy and with labor. J Reprod Immunol. 1997;33:147–156.
191. Taylor BD, Holzman CB, Fichorova RN, Tian Y, Jones NM, Fu W, et al. Inflammation biomarkers in vaginal fluid and preterm delivery. Hum Reprod. 2013;28:942–952.
192. Park S, You YA, Yun H, Choi SJ, Hwang HS, Choi SK, et al. Cervicovaginal fluid cytokines as predictive markers of preterm birth in symptomatic women. Obstet Gynecol Sci. 2020;63:455–463.
193. Kacerovsky M, Musilova I, Jacobsson B, Drahosova M, Hornychova H,

Rezac A, et al. Cervical and vaginal fluid soluble Toll-like receptor 2 in pregnancies complicated by preterm prelabor rupture of membranes. J Matern Neonatal Med. 2015;28:1116–1122.
194. Rasmussen LJH, Petersen JEV, Eugen-Olsen J. Soluble Urokinase Plasminogen Activator Receptor (suPAR) as a Biomarker of Systemic Chronic Inflammation. Front Immunol. 2021;12:5051.
195. Eugen-Olsen J, Giamarellos-Bourboulis EJ. suPAR: The unspecific marker for disease presence, severity and prognosis. Int J Antimicrob Agents. 2015;46:S33–S34.
196. Andersen O, Eugen-Olsen J, Kofoed K, Iversen J, Haugaard SB. Soluble urokinase plasminogen activator receptor is a marker of dysmetabolism in HIV-infected patients receiving highly active antiretroviral therapy. J Med Virol. 2008;80:209–216.
197. Slot O, Brünner N, Locht H, Oxholm P, Stephens RW. Soluble urokinase plasminogen activator receptor in plasma of patients with inflammatory rheumatic disorders: increased concentrations in rheumatoid arthritis. Ann Rheum Dis. 1999;58:488–492.
198. Raggam RB, Wagner J, Prüller F, Grisold A, Leitner E, Zollner-Schwetz I, et al. Soluble urokinase plasminogen activator receptor predicts mortality in patients with systemic inflammatory response syndrome. J Intern Med. 2014;276:651–658.
199. Zhou X, Fragala MS, McElhaney JE, Kuchel GA. Conceptual and methodological issues relevant to cytokine and inflammatory marker measurements in clinical research. Curr Opin Clin Nutr Metab Care. 2010;13:541.
200. Chew-Harris J, Appleby S, Richards AM, Troughton RW, Pemberton CJ. Analytical, biochemical and clearance considerations of soluble urokinase plasminogen activator receptor (suPAR) in healthy individuals. Clin Biochem. 2019;69:36–44.
201. Siahanidou T, Margeli A, Tsirogianni C, Charoni S, Giannaki M, Vavourakis E, et al. Clinical Value of Plasma Soluble Urokinase-Type Plasminogen Activator Receptor Levels in Term Neonates with Infection or Sepsis: A Prospective Study. Mediators Inflamm. 2014;375702.
202. Haigh R, D'souza SW, Micklewright L, Gregory H, Butler SJ, Hollingsworth M, et al. Human amniotic fluid urogastrone (epidermal growth factor) and fetal lung phospholipids. BJOG An Int J Obstet Gynaecol. 1989;96:171–178.

203. Pesonen K, Viinikka L, Koskimies A, Banks AR, Nicolson M, Perheentupa J. Size heterogeneity of epidermal growth factor in human body fluids. Life Sci. 1987;40:2489–2494.
204. Varner M, Dildy G, Hunter C, Dudley D, Clark S, Mitchell M. Amniotic fluid epidermal growth factor levels in normal and abnormal pregnancies. J Soc Gynecol Investig. 1996;3:17–19.
205. Scott S, Buenaflor G, Orth D. Immunoreactive human epidermal growth factor concentrations in amniotic fluid, umbilical artery and vein serum, and placenta in full-term and preterm infants. Biol Neonate. 1989;56:246–251.
206. Hofmann G, Abramowicz J. Epidermal growth factor (EGF) concentrations in amniotic fluid and maternal urine during pregnancy. Acta Obstet Gynecol Scand. 1990;69:217–221.
207. Currie AE, Vyas JR, MacDonald J, Field D, Kotecha S. Epidermal growth factor in the lungs of infants developing chronic lung disease. Eur Respir J. 2001;18:796–800.
208. Yoshimura S, Masuzaki H, Miura K, Gotoh H, Moriyama S, Fujishita A, et al. Effect of epidermal growth factor on lung growth in experimental fetal pulmonary hypoplasia. Early Hum Dev. 2000;57:61–69.
209. Goetzman B, Read L, Plopper C, Tarantal A, George-Nascimento C, Merritt T, et al. Prenatal exposure to epidermal growth factor attenuates respiratory distress syndrome in rhesus infants. Pediatr Res. 1994;35:30–36.
210. Candilera V, Bouchè C, Schleef J, Pederiva F. Lung growth factors in the amniotic fluid of normal pregnancies and with congenital diaphragmatic hernia. J Matern Neonatal Med. 2016;29:2104–2108.
211. Zhu D, Tan J, Maleken AS, Muljadi R, Chan ST, Lau SN, et al. Human amnion cells reverse acute and chronic pulmonary damage in experimental neonatal lung injury. Stem Cell Res Ther. 2017;8:1–24.
212. Ohkuma K, Ono T, Oshima Y, So K, Tsumura K, Yamasaki F, et al. Diagnostic accuracy of amniotic fluid interleukin-6 for fetal inflammatory response syndrome. J Obstet Gynaecol Res. 2023;49:1498–1505.
213. Balciuniene G, Gulbiniene V, Dumalakiene I, Viliene V, Bartkeviciene D, Pilypiene I, et al. Prognostic Markers for Chorioamnionitis: IL-6, TNF-α, and MMP-8 in Vaginally Obtained Amniotic Fluid. J Clin Med. 2021;10:1–9.

SUPPLEMENTS

Supplement 1.

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LEIDIMAS
ATLIKTI BIOMEDICININĮ TYRIMĄ

2017-07-04 Nr.158200-17-931-434

Tyrimo pavadinimas:
Biocheminių ir imunologinių žymenų vaisiaus vandenyse reikšmė, prognozuojant intrauterinę infekciją

Protokolo Nr.:	VVT
Versija:	02
Data:	2017-06-29
Informuoto asmens sutikimo forma:	neščiajai
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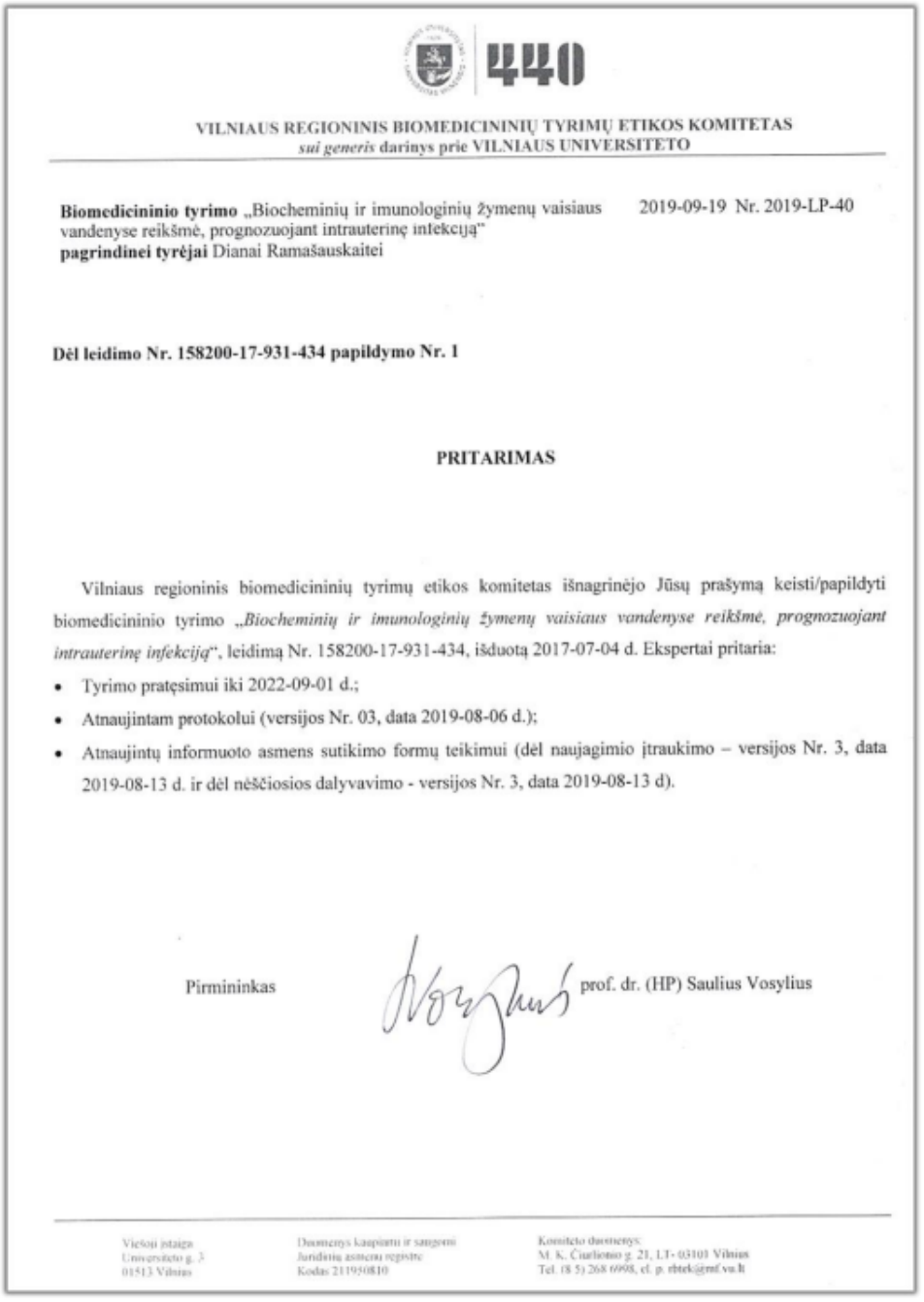
versija	2
data	2017-06-29
Pagrindinis tyrėjas:	**Diana Ramašauskaitė**
Įstaigos pavadinimas:	VUL Santaros klinikos
Adresas:	Santariškių g. 2, Vilnius
Leidimas galioja iki:	**2021-09**

Leidimas išduotas Vilniaus regioninio biomedicininių tyrimų etikos komiteto posėdžio (protokolas Nr. 158200-2017/07), vykusio 2017 m. liepos 4 d. sprendimu.

Pirmininkas  Saulius Vosylius

Supplement 2.



440

VILNIAUS REGIONINIS BIOMEDICININIŲ TYRIMŲ ETIKOS KOMITETAS
sui generis darinys prie VILNIAUS UNIVERSITETO

Biomedicininio tyrimo „Biocheminių ir imunologinių žymenų vaisiaus vandenyse reikšmė, prognozuojant intrauterinę infekciją"
pagrindinei tyrėjai Dianai Ramašauskaitei

2019-09-19 Nr. 2019-LP-40

Dėl leidimo Nr. 158200-17-931-434 papildymo Nr. 1

PRITARIMAS

Vilniaus regioninis biomedicininių tyrimų etikos komitetas išnagrinėjo Jūsų prašymą keisti/papildyti biomedicininio tyrimo *„Biocheminių ir imunologinių žymenų vaisiaus vandenyse reikšmė, prognozuojant intrauterinę infekciją"*, leidimą Nr. 158200-17-931-434, išduotą 2017-07-04 d. Ekspertai pritaria:

- Tyrimo pratęsimui iki 2022-09-01 d.;
- Atnaujintam protokolui (versijos Nr. 03, data 2019-08-06 d.);
- Atnaujintų informuoto asmens sutikimo formų teikimui (dėl naujagimio įtraukimo – versijos Nr. 3, data 2019-08-13 d. ir dėl nėščiosios dalyvavimo - versijos Nr. 3, data 2019-08-13 d).

Pirmininkas prof. dr. (HP) Saulius Vosylius

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Supplement 3.

VALSTYBINĖ DUOMENŲ APSAUGOS INSPEKCIJA

VšĮ Vilniaus universiteto ligoninės Santaros klinikoms
(per E. pristatymo informacinę sistemą
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SPRENDIMAS
DĖL LEIDIMO VŠĮ VILNIAUS UNIVERSITETO LIGONINĖS SANTAROS KLINIKOMS ATLIKTI ASMENS DUOMENŲ TVARKYMO VEIKSMUS

2018 m. kovo 9 d. Nr. 2R-476(2.6-1.)
Vilnius

Valstybinė duomenų apsaugos inspekcija, išnagrinėjusi VšĮ Vilniaus universiteto ligoninės Santaros klinikų 2018-03-06 raštu Nr. SR-1172 pateiktą Pranešimą dėl išankstinės patikros Nr. SR-1172 (toliau – Pranešimas) (Inspekcijoje gauta 2018-03-07, reg. Nr. 1R-1774),

n u s t a t ė,

kad Pranešime nurodyti asmens duomenų tvarkymo veiksmai atitinka Lietuvos Respublikos asmens duomenų teisinės apsaugos įstatyme nustatytus asmens duomenų tvarkymo ir duomenų subjektų teisių įgyvendinimo reikalavimus bei numatytos tinkamos organizacinės ir techninės duomenų saugumo priemonės.

Valstybinė duomenų apsaugos inspekcija, vadovaudamasi Lietuvos Respublikos asmens duomenų teisinės apsaugos įstatymo 33 straipsniu, Išankstinės patikros atlikimo taisyklių, patvirtintų Valstybinės duomenų apsaugos inspekcijos direktoriaus 2016 m. birželio 22 d. įsakymu Nr.1T-23(1.12.E), 12 ir 19.2 punktais,

n u s p r e n d ž i a

VšĮ Vilniaus universiteto ligoninės Santaros klinikoms išduoti leidimą atlikti Pranešime nurodytų asmens duomenų apie *sveikatą* tvarkymo mokslinio medicininio tyrimo „Biocheminių ir imunologinių žymenų vaisiaus vandenyse reikšmė, prognozuojant intrauterinę infekciją“, prot. Nr. VVT, tikslu veiksmus.

Šis sprendimas Administracinių bylų teisenos įstatymo nustatyta tvarka per vieną mėnesį nuo jo įteikimo dienos gali būti skundžiamas Vilniaus apygardos administraciniam teismui.

Direktorius Raimondas Andrijauskas

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SANTRAUKA

SANTRUMPOS

AUC, plotas po ROC kreive.
BGS, B grupės β hemolizinio Streptokoko nustatymas.
BPD, bronchopulmoninė displazija.
CPO, cezario pjūvio operacija.
CRB, C reaktyvusis baltymas.
DEFA3, α defensinas 3.
DPV, dirbtinė plaučių ventiliacija.
EGF, epidermio augimo faktoriaus.
GA, gestacijos amžius.
GS, galimybių santykis.
IAI, intraamnioninė infekcija ir (arba) uždegimas.
IL-10, interleukinas-10.
IL-17, interleukinas-17.
IL-6, interleukinas-6.
IQR, interkvartilinis intervalas.
ISK, intraskilvelinė kraujosruva.
KSS, kvėpavimo sutrikimo sindromas.
KT, kvėpuojamoji terapija.
MMP-8, matrikso metaloproteinazė-8.
NNR, neišnešiotų naujagimių retinopatija.
NPV, neigiama prognozinė vertė.
OOB, *Out-of-Bag error* paklaida.
PI, pasikliautinis intervalas.
PNVDP, priešlaikinis neišnešioto vaisiaus dangalų plyšimas.
SD, standartinis nuokrypis.
sTLR-2, tirpus Toll-like receptorius 2.
sTLR-4, tirpus Toll-like receptorius 4.
suPAR, tirpus urokinazės tipo plazminogeno aktyvatoriaus receptorius.
TNF-α, auglio nekrozės faktorius-α.
TPV, teigiama prognozinė vertė.
VUAS, vaisiaus uždegiminio atsako sindromas.
VULSK, Vilniaus universiteto ligoninės Santaros klinikos.

6.1 Įvadas

6.1.1 Mokslinio darbo naujumas ir aktualumas

Vaisiaus uždegiminio atsako sindromas (VUAS) yra sisteminė vaisiaus reakcija į intraamnioninę infekciją ir (arba) uždegimą (IAI), apibūdinama įvairių citokinų kiekio padidėjimu [1,2]. Intraamnioninė infekcija ir (arba) uždegimas yra viena iš pagrindinių savaiminio priešlaikinio gimdymo priežasčių, sukelianti 40-70 proc. visų priešlaikinių gimdymų [3]. VUAS apibrėžiamas kaip citokinų kiekio padidėjimas vaisiaus kraujyje [5] arba histologiškai nustatomas kaip ūminis funizitas [6]. VUAS dažniausiai yra lydimas priešlaikinio neišnešioto vaisiaus dangalų plyšimo (PNVDP) ir priešlaikinio gimdymo, todėl didėja nepalankių naujagimio baigčių rizika [4]. VUAS lemia padidėjusį naujagimių sergamumą, mirtingumą ir artimąsias bei atokiąsias baigtis: kvėpavimo sutrikimo sindromą (KSS), sepsį, bronchopulmoninę displaziją (BPD), intraskilvelines kraujosruvas (ISK), periventrikulinę leukomaliaciją, neišnešiotų naujagimių retinopatiją (NNR), neurosensorinį klausos sutrikimą ir neurologinės raidos sutrikimus, įskaitant cerebrinį paralyžių [1,5,7–9]. Dėl galimų motinos ir vaisiaus rizikų ir komplikacijų, IAI ir VUAS diagnozė dažniausiai lemia nėštumo užbaigimą neatidėliotinu gimdymu [10,11].

IAI ir VUAS diagnostika, ypač po PNVDP, yra sudėtinga. Šiuo metu VUAS diagnozė grindžiama tik biocheminiais ir histologiniais kriterijais. Histologiškai virkštelė tyriama tik po gimimo [8] Tuo tarpu motinos kraujo biocheminiai žymenys pasižymi ribotu jautrumu ir specifiškumu nustatant VUAS ir suteikia daugiau informacijos apie motinos uždegimo atsaką, o ne vaisiaus [12–15]. Žymenų, esančių kordocentezės būdu paimtame vaisiaus kraujyje arba amniocentezės metu paimtuose vaisiaus vandenyse, analizė yra labai jautri ir specifiška [5,16–21]. Šie metodai yra invaziniai, priešingai nei saugus ir patikimas virkštelės kraujo žymenų nustatymas iš karto po gimimo, tačiau netinkamas antenatalinei diagnostikai diagnosis [12,14,22]. Šie apribojimai apsunkina VUAS diagnostiką klinikinėje praktikoje. Pastaruoju metu moksliniais tyrimais siekiama patvirtinti greitą, lengvai atliekamą, specifinį ir jautrų ankstyvo VUAS diagnozavimo metodą, todėl dėmesys krypsta į neinvazinės diagnostikos galimybes, siekiant išspręsti prenatalinės VUAS diagnostikos spragą.

Įrodyta, kad vaisiaus vandenų uždegimo žymenų analizė yra optimalus metodas IAI ir VUAS diagnozuoti [1,17,23–25]. Nors įvairūs vaisiaus vandenų žymenys, kaip pavyzdžiui, interleukinas-6 (IL-6), matrikso metaloproteinazė-8 (MMP-8), auglio nekrozės faktorius-α (TNF-α), tirpus Toll-like receptorius 2

(sTLR-2), α defensinai (DEFA3), interleukinas-10 (IL-10,), interleukinas-17 (IL-17), buvo plačiai tyrinėti kaip prognoziniai IAI, VUAS ir nepalankių naujagimio baigčių rodmenys, tačiau dauguma mokslinių tyrimų analizavo transabdominalinės amniocentezės būdu paimtų vaisiaus vandenų mėginius [16–21] arba kordocentezės būdu paimtus vaisiaus kraujo mėginius [5,26]. Tik keliuose tyrimuose buvo tiriami neinvaziniu būdu gautų vaisiaus vandenų žymenys diagnozuojant VUAS, ir dar mažiau prognozuojant VUAS ir naujagimių baigtis [23,25,27-29]. Be to, mūsų žiniomis, nėra atlikta tyrimų, nagrinėjančių vaisiaus vandenų tirpaus urokinazės tipo plazminogeno aktyvatoriaus receptoriaus (suPAR), tirpaus Toll-like receptoriaus 4 (sTLR-4) ir epidermio augimo faktoriaus (EGF) ir VUAS ryšį, ypač neinvaziniu būdu paimtuose vaisiaus vandenyse.

Žymenys šiam tyrimui buvo atrinkti atsižvelgiant į jų vaidmenį IAI ir VUAS patogenezėje, taip pat į jų ir nepalankių naujagimių baigčių ryšį. Daugelis šių žymenų, pavyzdžiui, IL-6, TNF-α, MMP-8, IL-10, IL-17 ir DEFA3, yra pripažinti svarbiais uždegimo mediatoriais IAI atveju, todėl jie naudingi ir VUAS diagnostikai. Be šių gerai ištirtų IAI žymenų, buvo įtraukti suPAR ir sTLR-4. SuPAR yra žinomas kaip sisteminio uždegimo prognozinis žymuo kitų ligų atveju, o sisteminis uždegiminis atsakas yra vienas iš pagrindinių VUAS komponentų. Kita vertus, EGF buvo pasirinktas dėl jo svarbos vaisiaus ir naujagimio augimui, vystymuisi ir audinių atsistatymui. EGF įtraukimas suteikia vertingų žinių apie jo galimą poveikį naujagimių augimui ir vystymuisi VUAS kontekste.

Šio tyrimo metu siekėme nustatyti VUAS ir nepalankių naujagimio baigčių riziką, vertindami įvairius imunologinius žymenis, tokius kaip IL-6, TNF-α, MMP-8, sTLR-2, sTLR-4, RANTES, IL-10, IL-17, DEFA3, suPAR ir EGF, neinvaziniu būdu paimtuose vaisiaus vandenyse įvykus PNVDP iki 34^{+6} nėštumo savaičių. Tikimės, kad šis neinvazinis vaisiaus vandenų ištyrimo metodas, kuris yra paprastai atliekamas, nereikalaujantis specialių įgūdžių ir neturintis komplikacijų, taps klinikinės praktikos dalimi. Geresnė neinvazinė antenatalinė VUAS diagnostika galėtų padėti vertinant naujagimių riziką ir mažinant perinatalines pasekmes.

6.1.2 Tikslas ir uždaviniai

Darbo tikslas yra įvertinti neinvaziniu būdu paimtų vaisiaus vandenų imunologinių žymenų reikšmę, prognozuojant vaisiaus uždegiminio atsako sindromą ir neišnešiotų naujagimių baigtis.

Darbo uždaviniai:

1. Įvertinti vaisiaus vandenų imunologinių žymenų koncentracijų ir VUAS tarpusavio ryšį.
2. Nustatyti vaisiaus vandenų imunologinių žymenų, prognozuojančių VUAS, ir virkštelės kraujo uždegimo žymens IL-6 tarpusavio ryšį.
3. Įvertinti vaisiaus vandenų imunologinių žymenų koncentracijų ir neišnešioto naujagimio baigčių: KSS, surfaktanto poreikio, kvėpuojamosios terapijos trukmės ir tipo, BPD, sepsio, ankstyvos hipotenzijos, AAL, ISK, NNR ir mirties, tarpusavio ryšį.
4. Nustatyti nepalankių neišnešioto naujagimio baigčių riziką, vertinant vaisiaus vandenų imunologinių žymenų koncentracijų ribines vertes.
5. Papildyti neišnešioto naujagimio priežiūros rekomendacijas, remiantis vaisiaus uždegiminio atsako ir naujagimio baigčių prognoze, vertinant neinvaziniu būdu paimtų vaisiaus vandenų imunologinių žymenų koncentracijų pokyčius.

Tyrimo hipotezė: neinvaziniu būdu paimtų vaisiaus vandenų imunologiniai žymenys prognozuoja vaisiaus uždegiminio atsako sindromą ir neišnešiotų naujagimių baigtis.

6.1.3 Ginamieji teiginiai

1. Imunologinių žymenų TNF-α, IL-6, MMP-8, sTLR-2, sTLR-4, RANTES, IL-10, IL-17, DEFA3, suPAR ir EGF koncentracijos neinvaziniu būdu paimtuose vaisiaus vandenyse yra susijusios su VUAS.
2. Vaisiaus vandenų imunologinių žymenų, prognozuojančių VUAS, koncentracijos koreliuoja su virkštelės kraujo uždegimo žymens IL-6 koncentracija.
3. Vaisiaus vandenų imunologinių žymenų koncentracijų pokyčiai yra susiję su nepalankiomis neišnešiotų naujagimių baigtimis: KSS, surfaktanto poreikiu, kvėpuojamosios terapijos trukme ir tipu, BPD, sepsiu, ankstyva hipotenzija, AAL, ISK, NNR ir mirtimi.
4. Imunologinių žymenų TNF-α, IL-6, MMP-8, sTLR-2, sTLR-4, RANTES, IL-10, IL-17, DEFA3, suPAR ir EGF koncentracijų ribinės vertės prognozuoja didesnę VUAS ir nepalankių neišnešioto naujagimio baigčių tikimybę.
5. Neinvaziniu būdu paimtų vaisiaus vandenų imunologinių žymenų analizė gali pagerinti VUAS antenatalinę diagnostiką ir padėti numatyti naujagimių nepalankių baigčių riziką.

6.2 Tyrimo metodai

6.2.1 Tyrimo struktūra

Visa disertacijos analizė grindžiama tyrimo „Neinvazinis imunologinių žymenų nustatymas vaisiaus vandenyse priešlaikinio gimdymo metu“, finansuoto Lietuvos mokslo tarybos, duomenimis (Nr. P-MIP-19-66, 2017-2022 m.).

Šis perspektyvusis kohortinis tyrimas, inicijuotas Vilniaus universiteto Medicinos fakulteto (pagrindinė tyrėja prof. Diana Ramašauskaitė), buvo atliktas Vilniaus universiteto ligoninės Santaros klinikose (VUL SK) 2017-2022 m. Vaisiaus vandenų imunologiniai tyrimai buvo atliekami Valstybiniame mokslinių tyrimų institute, Inovatyvios medicinos centre. Tyrimui atlikti gauti Vilniaus regioninio biomedicininių tyrimų etikos komiteto leidimai (2017-07-04 Nr. 158200-17-931-434 ir papildymas 2019-09-19 Nr.2019-LP-40) ir Valstybinės duomenų apsaugos inspekcijos leidimas (2018-03-09, Nr. 2R-1765 (2.6-1.)).

Tyrime perspektyviai dalyvavo nėščios moterys, kurios buvo hospitalizuotos į VULSK dėl PNVDP 22^{+0}–34^{+6} vienvaisio nėštumo savaitę. Po gimimo į tyrimą taip pat buvo įtraukti neišnešioti naujagimiai. Visos tyrimo dalyvės pasirašė informuoto asmens sutikimo formą. Informuoto asmens sutikimo formą dėl naujagimio dalyvavimo tyrime pasirašė abu tėvai.

Įtraukimo kriterijai:

- Moters amžius ≥18 m.
- Vienvaisis nėštumas.
- Nėštumo trukmė 22^{+0}-34^{+6} sav.
- Diagnozuotas PNVDP.

Neįtraukimo kriterijai:

- Daugiavaisis nėštumas.
- Placentos pirmeiga.
- Kraujavimas iš makšties.
- Diagnozuotos vaisiaus vystymosi ydos.
- Nestabili vaisiaus būklė.

6.2.2 Surinkti motinų duomenys

Po įtraukimo buvo renkami motinų duomenys, įskaitant amžių, ankstesnių nėštumų ir gimdymų skaičių, gretutines ligas, paskutinio menstruacinio ciklo datą, PNVDP laiką, su nėštumu susijusias ligas, B grupės streptokoko (BGS) tyrimo ir

kraujo laboratorinių tyrimų (C reaktyvusis baltymas (CRB), bendras leukocitų skaičius) rezultatus. Taip pat buvo surinkta informacija apie taikytą gydymą (antibiotikai, kortikosteroidai, tokolizė). Po gimdymo buvo registruojami papildomi motinos duomenys, tokie kaip gimdymo būdas, gimdymo sužadinimas, bevandenis laikotarpis ir gestacijos amžius gimdymo metu.

Nėštumo trukmė buvo nustatoma pagal pirmąją paskutinių mėnesinių dieną, jei mėnesinių ciklas buvo reguliarus. Jei menstruacijų ciklas buvo nereguliarus arba atlikus ultragarsinį tyrimą $11^{+0} – 13^{+6}$ savaitę skirtumas didesnis nei 5 dienos, GA buvo nustatomas pagal ultragarso tyrimą. PNVDP buvo diagnozuojamas, jei apžiūros per makštį metu buvo stebimas vaisiaus vandenų tekėjimas. Neaiškiais atvejais PNVDP buvo patvirtintas naudojant AmniSure® (QIAGEN, Džermantaunas, JAV) testą placentos α mikroglobulino-1 baltymui nustatyti. Bevandenis laikotarpis buvo apibrėžtas kaip laikotarpis nuo PNVDP iki gimdymo.

Pagal Lietuvos akušerių ginekologų draugijos patvirtintas akušerijos metodikas (2019 m.) bei VUL SK patvirtiną gydymo protokolą (2017 m.), moterims, kurioms buvo nustatyta PNVDP iki 34 nėštumo savaitės, buvo taikoma laukimo taktika. Pacientėms skirta antibiotikų profilaktika, vaisiaus plaučių brandinimas kortikosteroidais ir, jei reikėjo, tokolitikai. Antibiotikų terapijai buvo skiriama intraveninis ampicilinas ir eritromicinas 2 paras, vėliau geriamasis amoksicilinas ir eritromicinas 5 dienas. Plaučių brandinimui buvo švirkščiamas deksametazonas į raumenis 12 mg 2 kartus kas 12 valandų. Po vaisiaus plaučių brandinimo gimdymas prasidėdavo spontaniškai arba buvo sužadintas. Gimdymo sužadinimo indikacijos buvo intrauterinė infekcija, kraujavimas arba prasta vaisiaus būklė. Dalyvavimas tyrime nepakeitė įprastos klinikinės praktikos.

Klinikinis chorioamnionitas buvo diagnozuojamas remiantis Gibso kriterijais: motinos karščiavimas ≥ 38 °C ir bent 2 iš šių požymių: moters tachikardija (> 100 k./min.), vaisiaus tachikardija (> 160 k./min.), nemalonaus kvapo pūlingos išskyros arba vaisiaus vandenys, gimdos jautrumas (skausmingumas), leukocitozė (> $15x10^9$/l) ir CRB > 40 mg/l [44].

6.2.3 Vaisiaus vandenų mėginiai ir imunologinė analizė

Apžiūros per makštį metu laisvai tekantys vaisiaus vandenys buvo renkami steriliais makšties skėtikliais į sterilius centrifugavimo mėgintuvėlius kas antrą dieną. Į imunologinę analizę buvo įtraukti mėginiai, paimti per 48 valandas iki gimdymo. Pasirinkome trumpesnį nei 48 valandų mėginių ėmimo laikotarpį, kad išlaikytume reikšmingą laiko ryšį tarp vaisiaus vandenų tyrimų rezultatų ir histologinių placentos bei virkštelės radinių. Mėginiai su gausia kraujo ir gleivių

priemaiša arba kurių kiekis buvo nepakankamas, buvo pašalinti iš tyrimo. Siekiant gauti skaidrius mėginius, mėginiai buvo centrifuguojami 3000 aps/min. greičiu 5 min. 4 °C temperatūroje ir užšaldomi –80 °C temperatūroje.

Vienas Valstybinio mokslinių tyrimų instituto tyrėjas atliko imunologinius tyrimus su saugomais vaisiaus vandenų mėginiais. Šie mėginiai buvo atšildyti ir paruošti pagal komercinių rinkinių gamintojo instrukcijas, visi mėginiai buvo tiriami po du kartus. Optinis tankis buvo matuojamas spektrofotometru (*BioTek Instruments,* Vinuskis, JAV), o žymenų koncentracijos apskaičiuotos naudojant standartines kreives ir specializuotą programinę įrangą *Gen5 Microplate Data Collection & Analysis Software* (*BioTek Instruments*, Vinuskis, JAV).

Imunologiniai žymenys buvo matuojami naudojant fermentinius imunosorbentinius tyrimus (ELISA). IL-6 koncentracija buvo matuojama naudojant rinkinį "*Human IL-6 ELISA kit*" (*Bender MedSystems GmbH*, Viena, Austrija), kurio jautrumas <1 pg/ml. TNF-α buvo matuojamas "*Human TNFα ELISA Kit*" (*Life Technologies*, Valtamas, JAV), kai mažiausia aptinkama dozė yra <2 pg/ml. MMP-8 koncentracija buvo tiriama 0,013 ng/ml jautrumo rinkiniu "*Human Total MMP-8, Quantikine ELISA*" (*R&D Systems*, Mineapolis, JAV), o sTLR-2 ir sTLR-4 - 0,112 ir 0,118 ng/ml jautrumo rinkiniais "*Enzyme-linked Immunosorbent Assay Kit for Toll Like Receptor 2 (TLR2)*" ir "*Enzyme-linked Immunosorbent Assay Kit For Toll Like Receptor 4 (TLR4)"* (*Cloud-clone Corp.,* Keitis, JAV), atitinkamai. SuPAR koncentracijos buvo nustatomos "*Human uPAR Immunoassay, Quantikine ELISA*" (*R&D Systems*, Mineapolis, JAV), DEFA3 - "*Human Defensin Alpha 3, Neutrophil Specific (DEFA3) ELISA Kit*" (*Abbexa Ltd*, Kembridžas, JK), IL-10 - *"Human IL-10 ELISA^{PRO} kit"* (*Mabtech*, Stokholmas, Švedija), EGF - "*Human EGF ELISA kit*" (*Bender MedSystems*, Viena, Austrija), IL-17 - „*LEGEND MAX Human IL-17A ELISA Kit*" (*BioLegend*, San Diegas, JAV) ir RANTES - "*Human CCL5/RANTES DuoSet ELISA*" (*R&D Systems*, Mineapolis, JAV). Jei optinis tankis viršijo didžiausias standartines vertes, mėginiai buvo skiedžiami, o tikslumas tikrinamas pakartotinai. Žymenų koncentracijos nebuvo atskleistos nei tyrėjams, nei klinikiniam personalui, išlaikant nešališką analizę.

6.2.4 Surinkti naujagimių duomenys

Po gimimo naujagimiai buvo įtraukiami į tyrimą ir vertinama jų būklė nuo gimimo ir iki išrašymo iš ligoninės. Buvo renkami naujagimių duomenys: gimimo svoris, Apgar balai, gaivinimo poreikis po gimimo ir laboratorinių tyrimų rezultatai, tokie kaip virkštelės arterinio kraujo pH, virkštelės kraujo IL-6,

leukocitų skaičius, nesubrendusių ir bendras neutrofilų santykis 6–24 val. po gimimo, CRB, kraujo pasėlis ir krūtinės ląstos rentgenografija. Virkštelės IL-6 koncentracija buvo nustatyta naudojant automatizuotą chemiliuminescencinį fermentinį imuninį testą (DPC, Los Andželas, JAV).

Registruotos šios nepalankios naujagimio baigtys: KSS, surfaktanto poreikis, kvėpuojamosios terapijos tipas ir trukmė, BPD, ankstyvasis sepsis, ankstyva hipotenzija, gydymo trukmė Naujagimių intensyviosios terapijos skyriuje , AAL ir jo gydymas, ISK, NNR ir mirtis.

Pagal Europos bendro sutarimo gaires [171], KSS diagnozuotas pagal kvėpavimo sutrikimo simptomus ir krūtinės ląstos rentgenogramosduomenis, skirstant į sunkumo laipsnius nuo sveikų iki sunkaus KSS [172]. Surfaktanto terapija buvo taikoma pagal Europos bendro sutarimo gaires [171]: profilaktiškai – naujagimiams, gimusiems iki 26 nėštumo sav., o virš 26 sav. naujagimiams – pagal klinikinį vertinimą. BPD buvo diagnozuota, jei išliko deguonies ar kvėpavimo palaikymo poreikis 28 dienų amžiuje, 36 savaičių postmenstruaciniame amžiuje ar iki išrašymo.

Ankstyva hipotenzija buvo nustatoma, kai kraujospūdis nukrisdavo žemiau 3 procentilio pagal GA pereinamuoju laikotarpiu ir reikėjo vazoaktyvių vaistų [173,174]. Ankstyvasis sepsis nustatomas remiantis klinikiniais ir (arba) laboratoriniais sepsio požymiais atsiradusiais per pirmąsias 72 valandas po gimimo, esant teigiamam ar neigiamam kraujo pasėliui, pagal VUL SK ankstyvojo sepsio protokolą [175,176].

ISK diagnozuota remiantis galvos smegenų ultragarsiniu tyrimu, o sunkumo laipsnis buvo nustatomas pagal Papile ir Volpe sistemą: I laipsnio (subependiminis kraujavimas), II laipsnio (ISK be skilvelių išsiplėtimo), III laipsnio (ISK su skilvelių išsiplėtimu) ir IV laipsnio (ISK su išplitimu į parenchimą). III ir IV laipsnio ISK laikoma sunkia ISK [177,178].

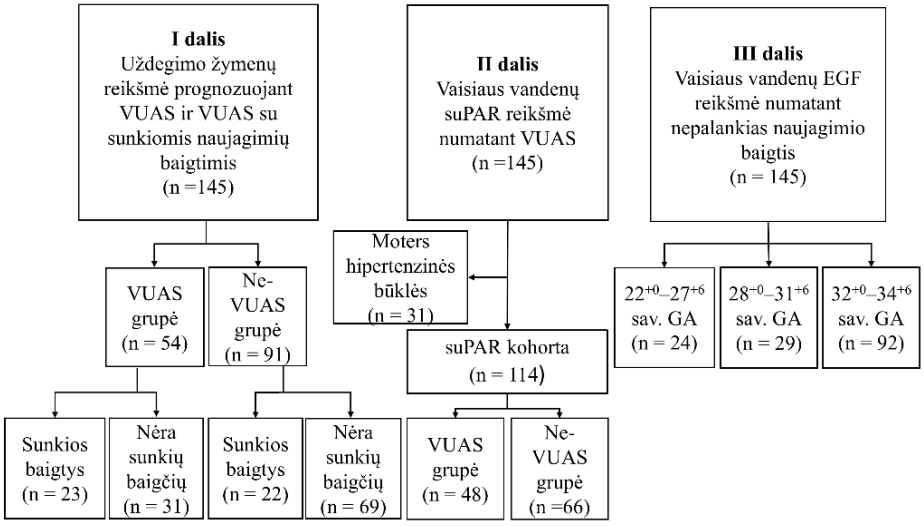
AAL buvo apibrėžiamas kaip hemodinamiškai reikšmingas, jei jis išlikdavo ilgiau nei 72 valandas po gimimo, esant ultragarsiniams diagnostiniams kriterijams pagal VUL SK protokolą: AAL skersmuo > 1,5 mm, kairiojo prieširdžio ir aortos santykis > 1,4 parasternalinėje ilgosios ašies projekcijoje, plaučių arterijos ir aortos santykis > 1,2, kairiojo skilvelio ir kairiojo prieširdžio išsiplėtimas, o nusileidžiančioje aortoje buvo atvirkštinis holodiastolinis srautas [179,180]. Pagal VUL SK protokolą NNR buvo diagnozuota gydytojo oftalmologo pagal tinklainės kraujagyslių pakitimus, remiantis tarptautine NNR klasifikacija, o sunki NNR buvo apibrėžiama, kai reikėjo operacinio gydymo [181].

Dėl mažo atskirų naujagimių baigčių dažnio buvo naudojamas sudėtinis mirštamumo ir sunkaus sergamumo rodiklis – sunkios naujagimių baigtys. Jis buvo nustatomas, esant bent vienai iš šių būklių: sunkus KSS, dirbtinė plaučių ventiliacija, mirtis, ankstyvas sepsis, ankstyva hipotenzija, sunki ISK, BPD ir sunki NNR.

Visos placentos buvo vertintos histologiškai, o uždegiminiai placentos, membranų ir virkštelės pažeidimai įvertinti pagal Amsterdamo placentos darbo grupės ir Pediatrijos patologijos draugijos Perinatalinės sekcijos diagnostikos kriterijus [34,138]. Histologinis chorioamnionitas buvo nustatomas radus neutrofilinę infiltraciją choriono ir decidualinėje membranose bei amnione. Funizitas buvo diagnozuotas pagal neutrofilų infiltraciją virkštelės kraujagyslėse [34,138], o VUAS buvo nustatomas pagal virkštelės kraujo IL-6 koncentraciją > 11 pg/ml ir/arba histologinio funizito buvimą [5,6,32].

6.2.5 Tyrimo metodika

Tyrimas buvo suskirstytas į 3 dalis (1 pav.).

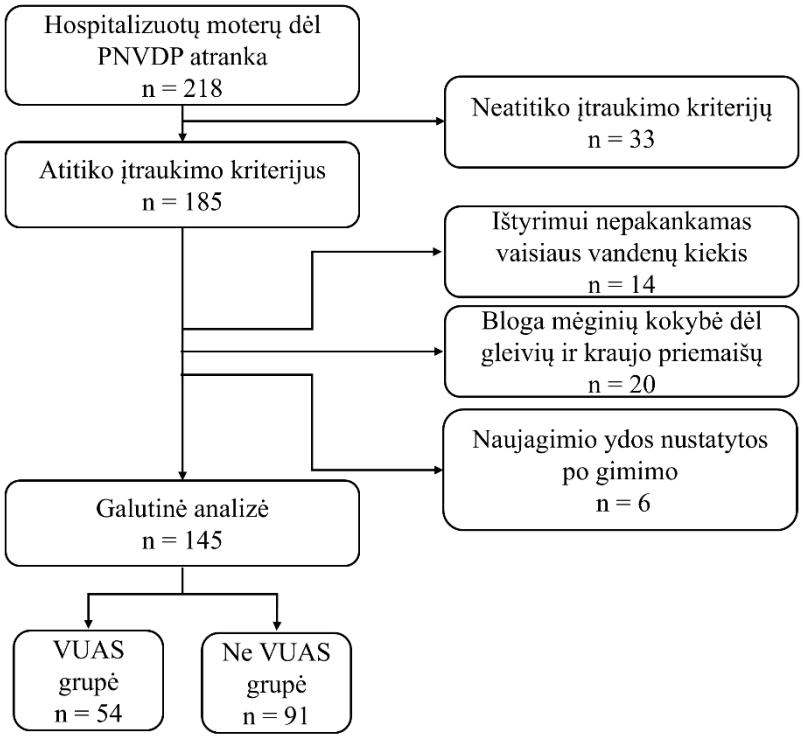


1 paveikslas. Disertacijos dalių schema.

6.2.6 I dalis. Imunologinių žymenų ir VUAS bei VUAS su sunkiomis naujagimio baigtimis ryšys

Pirmoje tyrimo dalyje siekėme įvertinti uždegimo žymenų reikšmę numatant VUAS ir VUAS su sunkiomis naujagimių baigtimis. 2 pav. pateikta pacientų

srauto diagrama, kurioje pavaizduoti 145 galutinės analizės dalyviai. Pagal VUAS diagnozę tiriamoji populiacija buvo suskirstyta į VUAS grupę (n=54) ir ne-VUAS grupę (n=91).

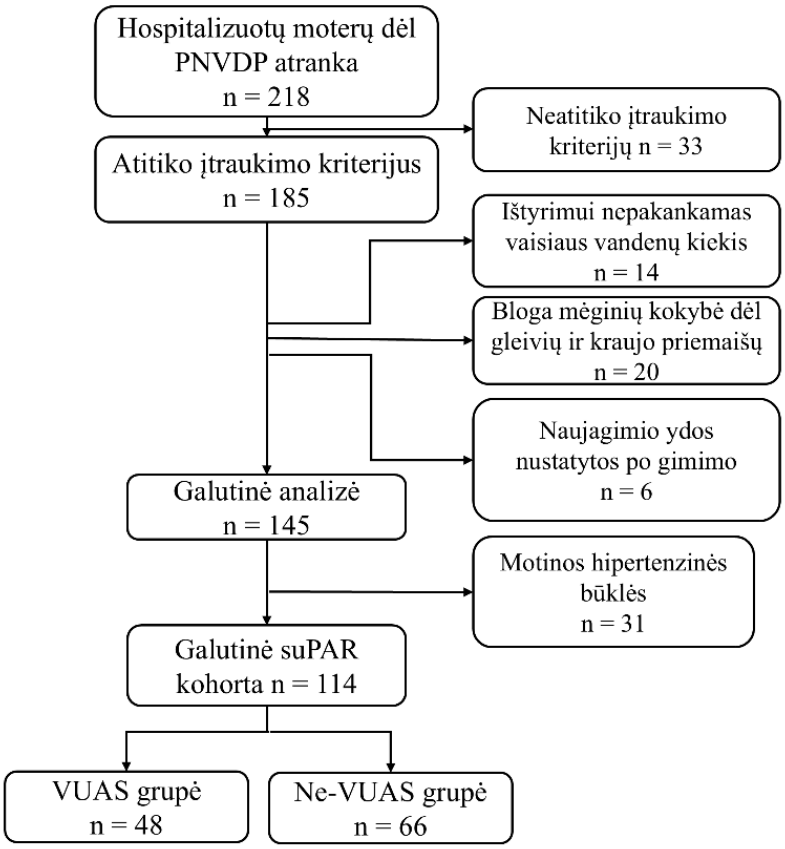


2 paveikslas. I dalies tiriamųjų atrankos schema.

Analizuojant VUAS su sunkiomis naujagimio baigtimis ir žymenų ryšį, VUAS ir ne-VUAS grupės toliau buvo vertinamos pagal sunkių naujagimių baigčių buvimą ar nebuvimą.

6.2.7 II dalis. SuPAR kohorta

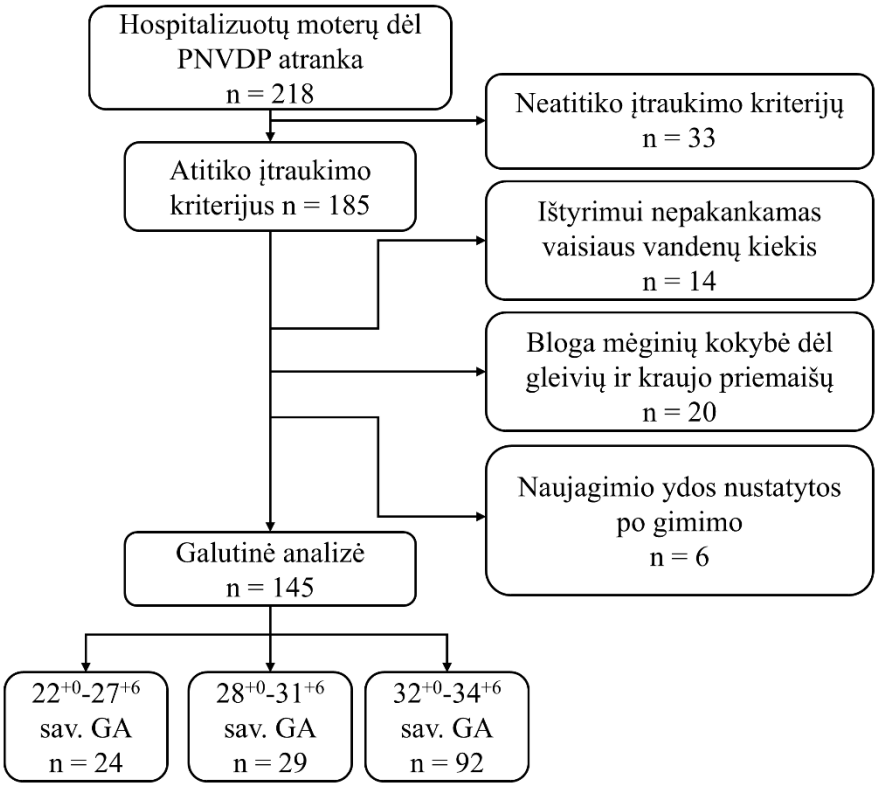
Antroje tyrimo dalyje siekėme įvertinti suPAR reikšmę prognozuojant VUAS ir VUAS su sunkiomis naujagimių baigtimis. Dėl suPAR sąsajų su hipertenzinėmis ligomis retrospektyviai atmetėme visus motinos hipertenzinių būklių atvejus. 3 pav. parodyta tiriamųjų atrankos schema. Galutinėje analizėje dalyvavo 114 tiriamųjų, kurie buvo suskirstyti į VUAS grupę (n=48) ir ne-VUAS grupę (n=66).



3 paveikslas. II dalies tiriamųjų atrankos schema.

6.2.8 III dalis. Vaisiaus vandenų EGF ir nepalankių naujagimio baigčių ryšys

Trečioje tyrimo dalyje siekėme įvertinti EGF reikšmę prognozuojant nepalankias naujagimių baigtis. Tyrimo populiacija, kurią sudarė 145 moterys ir jų naujagimiai, pagal PSO klasifikaciją [182] buvo suskirstyta į tris GA grupes: ypač neišnešioti naujagimiai (22^{+0}-27^{+6} savaičių GA), labai neišnešioti naujagimiai (28^{+0}-31^{+6} savaičių GA) ir vidutiniškai ir vėlyvi neišnešioti naujagimiai (32^{+0}-34^{+6} savaičių GA). 4 paveiksle pateikta III dalies tiriamųjų atrankos schema.



4 paveikslas. III dalies tiriamųjų atrankos schema.

6.2.9 Doktorantės indėlis

Doktorantė buvo viena iš tyrimo tyrėjų, atsakinga už tyrimo proceso organizavimą, protokolo ir dokumentų, reikalingų tyrimo leidimams gauti, parengimą, dalyvių įtraukimą į tyrimą, motinos ir naujagimio duomenų rinkimą, statistinės analizės atlikimą ir publikacijų rašymą.

6.2.10 Tyrimo imties apskaičiavimas

Tyrimo imties skaičiavimui naudota „G*Power“ (versija 3.1.9.4) programa. Kriterijaus reikšmingumo lygmuo pasirinktas $\alpha = 0,05$, o kriterijaus galia – $0,8 < \beta < 0,95$. Pasirinktas imčių dydis atitiko reikalavimus.

6.2.11 Statistinė duomenų analizė

Statistinė analizė buvo atlikta naudojant R programinę įrangą (versija 4.0.5). Kintamųjų skirstinių normalumas patikrintas pagal pasiskirstymą, lyginant vidurkius ir medianas, vizualiai vertinant histogramas bei įvertinus asimetriją. Duomenys buvo pateikti kaip vidurkiai su standartiniu nuokrypiu (SD) arba medianos su interkvartiliniu intervalu (IQR), priklausomai nuo kintamųjų tipo. Nenormaliai pasiskirstę duomenys buvo logaritmuojami tolesnei analizei. Kategoriniai kintamieji pateikti absoliučiais skaičiais ir procentais. Lyginant tęstinius kintamuosius tarp dviejų grupių, normaliai pasiskirsčiusiems kintamiesiems naudotas Studento t-testas, o nenormaliai pasiskirsčiusiems – *Mann-Whitney-Wilcoxon* testas, o ANOVA arba *Kruskal-Wallis* testai – lyginant daugiau nei 2 grupes.

Spearmano koreliacinė analizė buvo atlikta siekiant ištirti ryšius tarp žymenų ir motinos bei naujagimio parametrų. ROC kreivės buvo braižomos siekiant įvertinti žymenų diagnostinį tikslumą VUAS prognozuoti, o *Youden* indeksas buvo naudojamas nustatyti optimalią ribinę vertę.

Vienanarės ir daugianarės logistinės regresijos modeliai buvo taikomi siekiant įvertinti žymenis kaip prognozinius rodmenis, apskaičiuojant galimybių santykius (GS) su pasikliautiniais intervalais (PI). Logistinės regresijos kintamieji su p reikšme < 0,05 buvo įtraukti į daugianario modelio kūrimą, ir naudojant žingsninę logistinę regresiją, kuri, savo ruoštu atlikta remiantis AIC kriterijumi. Taip pat buvo atlikta atsitiktinių medžių klasifikatoriaus (*Random Forest*) analizė, skirta prognozuoti VUAS ir naujagimio baigtis, įvertinant prognozinių rodmenų svarbą. *Shapley Additive Explanations* (SHAP) verčių vaizdavimas buvo taikytas siekiant vizualizuoti prognozinių žymenų poveikį atsitiktinių medžių modelio rezultatams. Atsitiktinių medžių analizė dažnai turi didesnį prognozavimo tikslumą nei logistinė regresija esant nelinijiniams ryšiams, o logistinė regresija išlieka naudinga individualiai kintamojo interpretacijai. Statistiniai rezultatai buvo laikomi reikšmingais, jei p reikšmė < 0,05.

6.3 Rezultatai

6.3.1 I dalis. Imunologinių žymenų ir VUAS bei VUAS su sunkiomis naujagimio baigtimis ryšys

6.3.1.1 Tiriamosios populiacijos charakteristika

Tyrimo populiacija buvo suskirstyta į dvi grupes pagal VUAS diagnozę: VUAS grupė (n=54) ir ne-VUAS grupė (n=91). 1 lentelėje pateikti tiriamųjų grupių demografiniai ir klinikiniai ypatumai. Motinos veiksniai statistiškai nesiskyrė tarp grupių, išskyrus dažnesnius hipertenzinius sutrikimus ne-VUAS grupėje. VUAS grupėje buvo dažnesnis savaiminis gimdymas, taip pat dažnesnis klinikinio ir histologinio chorioamnionito paplitimas. VUAS grupės naujagimiai buvo mažesnio svorio, mažesnio GA ir dažniau gavo <7 balus pagal Apgar, todėl jiems dažniau prireikė gaivinimo veiksmų po gimimo. Jiems taip pat dažniau nustatytas KSS, ypač sunkus, ir dažniau prireikė kvėpavimo palaikymo, įskaitant DPV. VUAS grupėje dažniau nustatytas ankstyvasis sepsis ir NNR. Virkštelės pH, naujagimių mirties, ankstyvosios hipotenzijos, AAL, BPD ir ISK rodikliai tarp grupių reikšmingai nesiskyrė.

1 lentelė. Tiriamųjų demografiniai ir klinikiniai ypatumai

Charakteristika	VUAS grupė (n=54)	Ne-VUAS grupė (n=91)	P reikšmė
Motinos charakteristika			
Motinos amžius, m., vidurkis ± SD	31.22 ±5.5	31.26 ±5.7	0.966
Bevandenis laikotarpis, val., mediana, IQR	64 (16–200)	75 (34–216)	0.408
Hipertenzinės būklės, n, %	6 (11)	25 (28)	0.020
Gestacinis diabetas, n, %	12 (22)	22 (24)	0.761
Teigiamas BGS testas, n, %	7(13)	10 (11)	0.444
Pirmas nėštumas, n, %	20 (37)	30 (33)	0.618
Pirmas gimdymas, n, %	23 (43)	45 (50)	0.424
Kortikosteroidai antenataliai, n, %	47 (87)	78 (86)	0.823
Tokolitikai, n, %	23 (43)	41 (48)	0.501
Gimdymo būdas, n, % Makštinis gimdymas CPO	 46 (85) 8 (15)	 68 (75) 23 (25)	0.079

Charakteristika	VUAS grupė (n=54)	Ne-VUAS grupė (n=91)	P reikšmė
Indukuotas gimdymas arba CPO, n, % Savaiminis gimdymas, n, %	22 (41) 32 (59)	56 (61) 35 (39)	0.015
Klinikinis chorioamnionitas, n, %	6 (11)	1 (1)	0.011
Histologinis chorioamnionitas, n, %	38 (70)	16 (18)	<0.001
Funizitas, n, %	21 (39)	0	<0.001
Naujagimių charakteristika			
GA, sav., mediana, IQR	31.5 (28–33)	33.0 (31–34)	0.003
Gimimo svoris, g., vidurkis ± SD	1675 ± 640	1938 ± 613	0.020
Apgar balai <7 1 min., n, %	14 (26)	8 (9)	0.005
Apgar balai <7 5 min., n, %	6 (11)	1 (1)	0.007
Virkštelės arterinio kraujo pH, vidurkis ± SD	7.34 ± 0.09	7.32 ± 0.09	0.331
Gaivinimo poreikis po gimimo, n, %	14 (26)	8 (9)	0.008
Sunkios baigtys, n, %	22 (42)	22 (24)	0.029
Kvėpavimo sutrikimas, n, %	51 (94)	71 (78)	0.009
Sunkus KSS, n, %	12 (22)	16 (18)	0.049
Kvėpuojamoji terapija, n, % Netaikyta Dirbtinė ventiliacija Neinvazinė KT	 3 (6) 12 (22) 39 (72)	 20 (22) 13 (14) 58 (64)	0.025
Surfaktanto poreikis, n, %	16 (30)	25 (28)	0.812
Naujagimio mirtis, n, %	2 (4)	0	0.193
Sepsis, n, %	8 (15)	7 (8)	0.173
Ankstyvasis sepsis, n, %	7 (13)	2 (2)	0.009
Ankstyva hipotenzija, n, %	10 (19)	8 (9)	0.086
Bronchopulmoninė displazija, n, %	9 (17)	8 (9)	0.142
Atviras arterinis latakas, n %	9 (17)	10 (11)	0.305
Neišnešiotų naujagimių retinopatija, n, %	16 (30)	9 (10)	0.003
Gydyta NNR, n, %	3 (6)	2 (2)	0.358
Intraskilvelinės kraujosruvos, n, % Nėra 1-2 laipsnio 3-4 laipsnio	 34 (63) 15 (28) 5 (9)	 64 (70) 23 (25) 4 (4)	0.437

6.3.1.2 Imunologinių žymenų ir VUAS ryšys

6.3.1.2.1 Žymenų koncentracijų palyginimas VUAS ir ne-VUAS grupėse

VUAS grupėje vaisiaus vandenų žymenų MMP-8, TNF-α, IL-6, IL-10, IL-17 ir DEFA3 koncentracijos buvo statistiškai patikimai didesnės nei ne-VUAS grupėje, o sTLR-2, sTLR-4, EGF ir RANTES koncentracijos statistiškai reikšmingai nesiskyrė tarp grupių (2 lentelė).

2 lentelė. Vaisiaus vandenų žymenų koncentracijų palyginimas VUAS ir ne-VUAS grupėse. Vaisiaus vandenų žymenų kintamieji, kurie nebuvo normaliai pasiskirstę, buvo transformuoti logaritmu arba kvadratu.

Žymuo	VUAS grupė		Ne-VUAS grupė		P reikšmė
	Mediana	IQR	Mediana	IQR	
MMP-8 (ng/ml)	673.30	175.02-1886.61	65.57	15.69-34.54	<0.001
TNF-α (pg/ml)	136.43	17.65-18.59	14.35	6.71-9.58	<0.001
IL-6 (pg/ml)	4079.09	2761.82-97294.34	1425.45	437.53-3301.46	<0.001
IL-10 (pg/ml)	59.43	5.21-170.35	6.26	0-21.24	<0.001
IL-17 (pg/ml)	1.80	0.56-5.88	0.50	0.21-1.09	<0.001
sTLR-2 (ng/ml)	0.22	0.06-0.64	0.197	0.06-0.39	0.472
sTLR-4 (ng/ml)	0.29	0.15-0.56	0.30	0.08-0.56	0.453
Virkštelės IL-6 (pg/ml)	52.70	18.15-184.25	4.16	2.97-5.87	<0.001
EGF (pg/ml)	80.95	37.70-126.61	84.13	42.67-147.86	0.436
RANTES (pg/ml)	335.45	160.75-935.30	476.32	108.28-1223.25	0.501
DEFA3 (ng/ml)	27.63	14.87-36.65	19.94	15.23-29.47	0.025
	Vidurkis	SD	Vidurkis	SD	P reikšmė
Log-TNF-α	4.95	2.20	2.95	1.37	<0.001
Log-MMP-8	5.93	2.14	4.20	1.86	<0.001
Log-IL-6	9.15	2.38	7.21	1.72	<0.001
Log-IL-10	3.53	2.17	1.92	1.71	<0.001
Log-IL-17	1.38	1.26	0.67	0.79	<0.001
Log-EGF	4.35	1.10	4.73	2.13	0.159
Log-TLR2	0.32	0.34	0.25	0.25	0.352
Sqrt-TLR4	1.19	0.16	1.18	0.18	0.563
Log-RANTES	5.71	1.40	5.82	1.87	0.693
Log-DEFA3	3.19	0.70	2.94	0.58	0.032

6.3.1.2.2 ROC kreivių analizė ir ribinės vertės VUAS prognozei

Pritaikę ROC analizę, įvertinome kiekvieno žymens diagnostines charakteristikas ir nustatėme optimalias ribines vertes VUAS prognozuoti (3 lentelė). TNF-α ir MMP-8 pademonstravo geras prognozines VUAS charakteristikas, jų AUC vertės atitinkamai buvo 0,77 ir 0,75. Prognozinės IL-6, IL-10 ir IL-17 charakteristikos buvo vidutinės. DEFA3, sTLR-2, sTLR-4, RANTES ir EGF pasižymėjo silpnomis prognozinėmis VUAS savybėmis, todėl jie nebuvo įtraukti į tolesnę analizę.

3 lentelė. Vaisiaus vandenų žymenų ribinės vertės ir diagnostinės charakteristikos, numatant VUAS.

Žymuo	Ribinė vertė	AUC 95%PI		Jautrumas		Specifiškumas		TPV		NPV	
				%	95%PI	%	95%PI	%	95%PI	%	95%PI
MMP-8	170.76	0.75	0.65-0.84	78	64-88	71	61-81	63	55-72	84	76-91
TNF-α	89.20	0.77	0.67–0.86	66	52-78	90	84-96	81	69-92	81	74-87
IL-6	2695.68	0.74	0.66-0.83	76	65-87	76	65-87	58	50-67	82	75-90
IL-10	35.05	0.72	0.63-0.81	61	48-74	87	80-93	73	63-85	79	74-85
IL-17	1.003	0.69	0.60-0.79	61	47-76	75	64-84	61	51-73	75	68-83
sTLR-2	0.64	0.54	0.43- 0.65	27	13-40	84	75-92	50	32-68	66	62-71
sTLR-4	1.55	0.46	0.36-0.56	98	94-100	6	1-12	40	38-41	83	50-100
EGF	91.63	0.54	0.44-0.64	63	50-76	47	36-57	41	34-49	68	59-77
RANTES	493.45	0.54	0.44-0.63	65	53-80	48	38-59	42	34-49	72	62-80
DEFA3	34.04	0.61	0.51-0.71	35	22-48	92	87-98	73	57-89	71	66-75

Santrumpos: AUC - plotas po ROC kreive, PI – pasikliautinis intervalas, TPV - teigiama prognozinė vertė; NPV- neigiama prognozinė vertė.

6.3.1.2.3 Logistinė regresija numatant VUAS

Buvo atlikta vienanarė ir daugianarė logistinė regresinė analizė siekiant įvertinti žymenų reikšmę prognozuojant VUAS (4 lentelė). Atliekant vienanarę analizę, logaritminiai TNF-α, MMP-8, IL-6, IL-10, IL-17, DEFA3, gestacinis amžius, hipertenzinės būklės ir savaiminis gimdymas buvo reikšmingai susiję su VUAS. Atliekant daugialypę analizę nustatyta, kad logaritmuotas TNF-α yra statistiškai reikšmingas VUAS prognozinis veiksnys (GS 1,83, $p < 0,001$), o DEFA3 – arti reikšmingumo ribos. Žingsninė logistinė regresija (5 lentelė) patvirtino, kad didesnės TNF-α ir DEFA3 koncentracijos buvo nepriklausomai susijusios su didesne VUAS tikimybe. Gestacinis amžius neturėjo reikšmingos įtakos šioms sąsajoms.

4 lentelė. Vaisiaus vandenų žymenų logistinė regresija numatant VUAS. Nenormaliai pasiskirstę kintamieji buvo logaritmuoti analizei.

	Vienanarė logistinė regresija			
Kintamieji	Koeficientas	P reikšmė	GS	95% PI
Log-TNF-α	0.608	<0.001	1.84	1.46-2.38
Log-MMP-8	0.446	<0.001	1.56	1.28-1.95
Log-IL-6	0.463	<0.001	1.59	1.33-1.95
Log-IL-10	0.424	<0.001	1.53	1.27-1.87
Log-IL-17	0.702	<0.001	2.02	1.37-3.14
Log-DEFA3	0.664	0.026	1.94	1.10-3.60
GA	-0.170	0.004	0.84	0.75-0.94
Gimimo svoris	-0.001	0.0171	0.99	0.998-0.999
Hipertenzinės būklės (ref. taip)	-1.109	0.024	0.33	0.12-0.82
Savaiminis gimdymas (ref. taip)	0.845	0.016	2.33	1.18-4.68
Daugianarė logistinė regresija N=145, AIC=158.95				
Log-TNF-α	0.635	<0.001	1.83	1.39-2.50
Log-IL-17	0.072	0.793	1.01	0.61-1.69
Log-DEFA3	0.662	0.062	1.94	0.98- 4.02
GA	-0.064	0.440	0.95	0.83-1.11
Hipertenzinės būklės (ref. taip)	0.429	0.546	0.76	0.23- 2.31
Savaiminis gimdymas (ref. taip)	0.416	0.435	1.27	0.51- 3.11

5 lentelė. Žingsninė logistinė regresija (kaupiamoji-šalinamoji) logaritmuotų žymenų prognozuojat VUAS ir kontroliuojant pagal GA.

Žingsninė logistinė regresija N=145, AIC=153.68				
Žymuo	Koeficientas	P reikšmė	GS	95% PI
Log-TNF-α	0.655	<0.001	1.93	1.51-2.53
Log-DEFA3	0.653	0.051	1.92	1.01-3.81
GA (įtrauktas)	-0.037	0.614	0.96	0.84-1.11

Tyrime įvertinti VUAS prognoziniai modeliai, taikant optimalias žymenų ribines vertes, nustatytas pagal ROC kreives ir Youdeno indeksą (6 lentelė). Taikant vienanarę logistinę regresiją, nustatėme reikšmingų sąsajų su šiais žymenimis: TNF-α kiekis >89 pg/ml, MMP-8 >170 ng/ml, IL-6 >2695 pg/ml, IL-10 >35 pg/ml, IL-17 >1 pg/ml ir DEFA3 >34 ng/ml. Atlikus daugialypę analizę, TNF-α ir DEFA3 buvo vieninteliai reikšmingi nepriklausomi VUAS prognozuojantys veiksniai. Žingsninė regresija patvirtino šias sąsajas (7 lentelė).

6 lentelė. Žymenų ribinių verčių logistinė regresija prognozuojant VUAS.

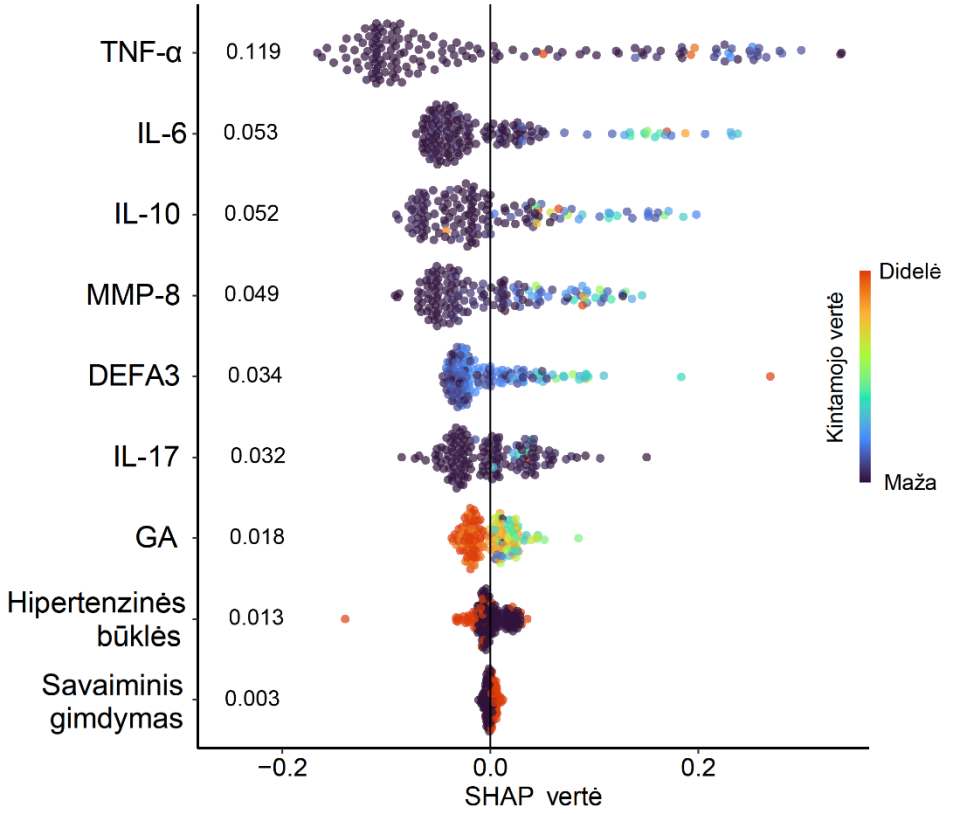
Vienanarė logistinė regresija				
Žymuo	Koeficientas	P reikšmė	GS	95% PI
TNF-α >89	2.70	<0.001	14.92	5.91- 41.79
MMP-8 >170	2.03	<0.001	7.62	3.37- 18.32
IL-6 >2695	1.89	<0.001	6.41	3.06-14.16
IL-10 >35	2.32	<0.001	10.21	4.63-23.94
IL-17 >1	1.56	<0.001	4.74	2.21-10.48
DEFA3 >34	1.87	<0.001	6.51	2.61-17.97
Daugianarė logistinė regresija; N=145, AIC =143.49				
TNF-α >89	2.44	0.001	11.50	2.73-57.32
MMP-8 >170	1.07	0.138	2.90	0.70-12.22
IL-6 >2695	-0.55	0.424	0.47	0.06-2.61
IL-10 >35	0.52	0.410	2.12	0.55-7.67
IL-17 >1	0.11	0.837	1.33	0.41-4.06
DEFA3 >34	1.43	0.025	3.42	0.84-16.25
GA	-0.002	0.970	0.96	0.80-1.14
Hipertenzinės būklės (ref. taip)	-0.10	0.885	2.09	0.39-12.14
Savaiminis gimdymas (ref. taip)	-0.50	0.385	0.80	0.20-2.80

7 lentelė. Žymenų ribinių verčių žingsninė (kaupiamoji-šalinamoji) logistinė regresija prognozuojat VUAS ir kontroliuojant pagal GA.

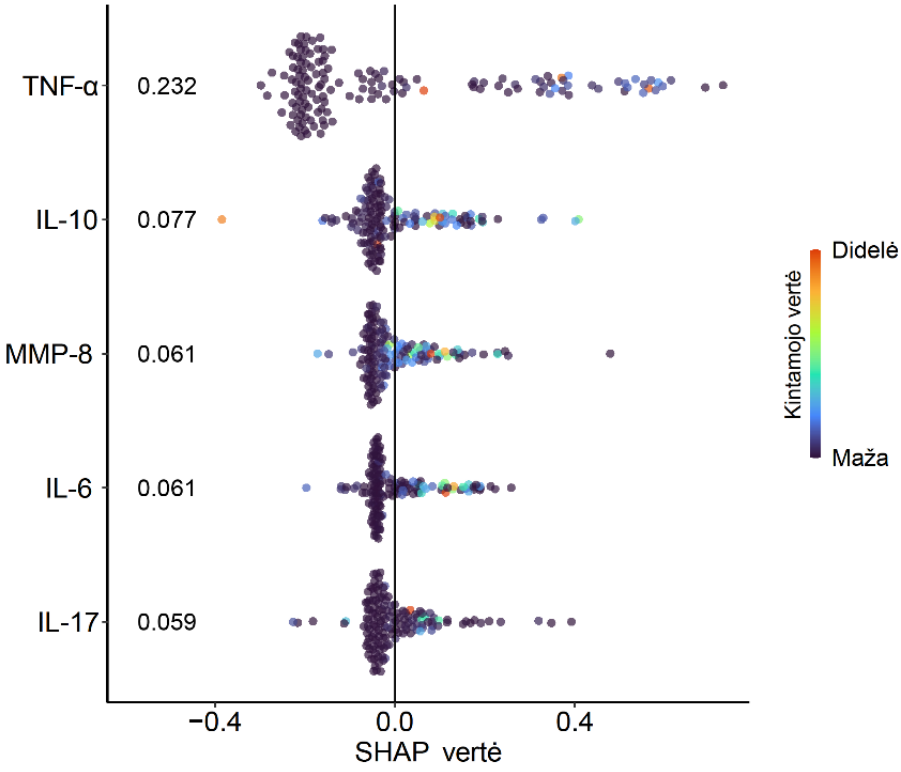
Žingsninė logistinė regresija N=145, AIC=135.44				
Žymuo	Koeficientas	P reikšmė	GS	95% PI
TNF-α > 89	2.297	<0.001	9.94	3.18-34.69
DEFA3 >34	1.482	0.015	4.40	1.38-15.16
MMP-8 >170	0.904	0.101	2.47	0.81-7.23
GA (įtrauktas)	-0.005	0.4392	0.99	0.85-1.16

6.3.1.2.4 Atsitiktinių medžių klasifikatoriaus analizė prognozuojant VUAS

Siekiant įvertinti žymenų reikšmę tarpusavyje numatant VUAS taikyta atsitiktinių medžių analizė. Buvo įvertinti devyni veiksniai: TNF-α, MMP-8, IL-6, IL-10, IL-17, DEFA3, GA, hipertenzinės būklės ir savaiminis gimdymas. Išbandėme modelį su 300, 500, 1000, 2000 ir 3000 medžių, o testuojamų kintamųjų skaičius (mtry) buvo nustatytas kaip kvadratinė šaknis iš bendro prognozinių veiksnių skaičiaus (kvadratinė šaknis iš 9 lygi 3) su artimiausiomis reikšmėmis nuo 1 iki 6. Modelį tikrinome naudodami OOB paklaidą (*Out-of-Bag error*, OOB). OOB paklaida buvo mažiausia, kai mtry buvo 3, o didėjant medžių skaičiui modelis nepagerėjo. Modelis pasiekė 80 proc. klasifikavimo tikslumą, jo jautrumas buvo 63 proc., o specifiškumas – 92 proc., nustatant, kad TNF-α, IL-10 ir IL-6 yra svarbiausi prognozavimo veiksniai (5 paveikslas). Toliau optimizavus modelį su penkiais kintamaisiais (TNF-α, MMP-8, IL-6, IL-17 ir IL-10), tikslumas pagerėjo iki 86 proc., jautrumas padidėjo iki 81 proc., o specifiškumas išliko 89 proc. Apibendrinant, vaisiaus vandenų TNF-α yra reikšmingiausias žymuo prognozuojant VUAS.



5 paveikslas. Prognozinių veiksnių reikšmė numatant VUAS SHAP suvestinės diagramoje. X ašyje SHAP vertės rodo veiksnių įtaką modelio rezultatams: teigiamos vertės didina VUAS tikimybę, o neigiamos - mažina. Y ašyje prognoziniai veiksniai išdėstyti pagal svarbą, o spalvos rodo veiksnių vertes: raudonai oranžinė spalva reiškia didesnes vertes, o tamsiai mėlyna - mažesnes.

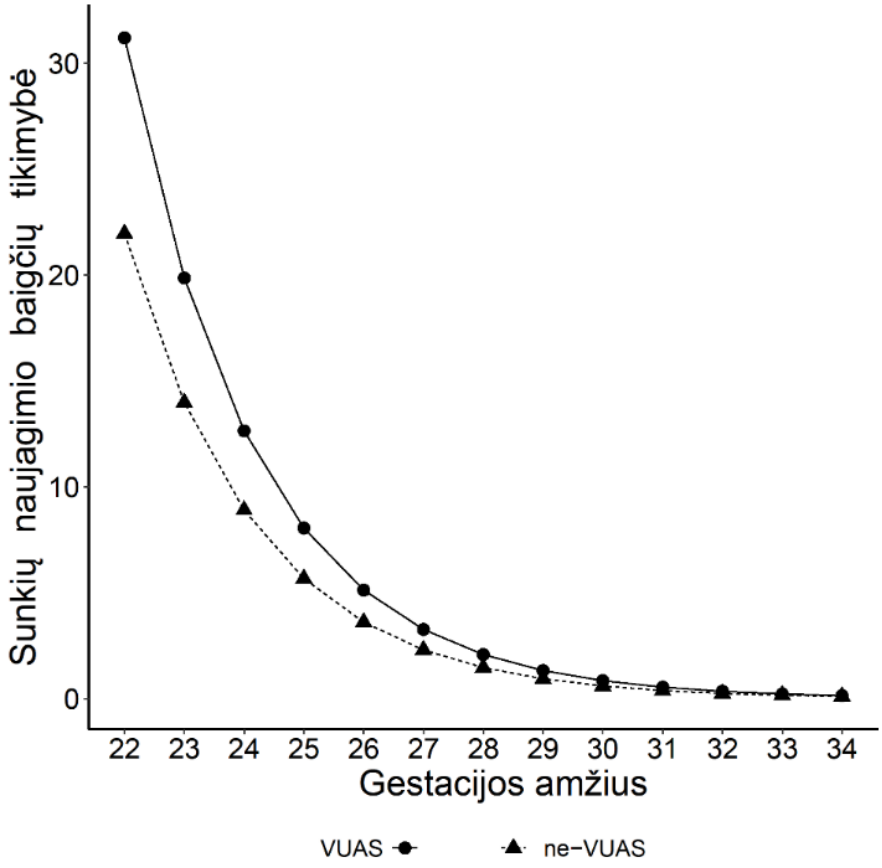


6 paveikslas. Optimalaus VUAS prognozinio modelio SHAP suvestinė. Penki prognoziniai veiksniai (TNF-α, IL-10, MMP-8, IL-6, IL-17) išvardyti mažėjančia tvarka pagal jų svarbą atsitiktinių medžių modelyje.

6.3.1.3 Imunologinių žymenų reikšmė numatant VUAS ir sunkias naujagimio baigtis

6.3.1.3.1 Sunkių naujagimių baigčių paplitimas

Nustatėme, kad VUAS grupės naujagimiams buvo daugiau nepalankių baigčių nei ne-VUAS grupės naujagimiams (42 proc. vs. 24 proc., p = 0,02, 1 lentelė). 7 paveiksle pavaizduota prognozuojama sunkių naujagimių baigčių tikimybė priklausomai nuo gestacijos amžiaus ir VUAS buvimo ar nebuvimo. Atsižvelgiant į gestacijos amžių, sunkių naujagimių baigčių tikimybė buvo didesnė VUAS grupėje nei ne-VUAS grupėje.



7 paveikslas. Sunkių naujagimių baigčių tikimybių prognozė

6.3.1.3.2 Žymenų koncentracijų palyginimas VUAS ir ne-VUAS grupėse pagal sunkių baigčių buvimą

Palyginome vaisiaus vandenų žymenų koncentracijas VUAS ir ne-VUAS grupėse pagal sunkių naujagimio baigčių buvimą ir nebuvimą (8 lentelė). VUAS grupės naujagimių, kuriems nustatytos sunkios baigtys, vaisiaus vandenų TNF-α, MMP-8, IL-6, IL-10, IL-17, EGF, DEFA3 koncentracija statistiškai patikimai skyrėsi nuo ne-VUAS grupės naujagimių ar VUAS grupės, bet be sunkių baigčių.

8 lentelė. Vaisiaus vandenų žymenų koncentracijų palyginimas VUAS ir ne-VUAS grupėse pagal sunkių naujagimio baigčių buvimą ir nebuvimą. Duomenys pateikti medianomis su 95 proc. PI ir vidurkiais su SD, atitinkamai. Vaisiaus vandenų žymenų kintamieji, kurie nebuvo normaliai pasiskirstę, buvo transformuoti logaritmu arba kvadratu.

	VUAS grupė		Ne-VUAS grupė		
Žymuo	Sunkios baigtys	Nėra baigčių	Sunkios baigtys	Nėra baigčių	P reikšmė
TNF-α (pg/ml)	462.16 (15.66 - 1191.02)	104.80 (17.77 - 302.91)	17.74 (6.19 - 74.30)	12.83 (6.83 - 30.46)	<0.001
MMP-8 (ng/ml)	801.82 (134.28 - 2259.07)	515.81 (177.20 - 1282.03)	16.90 (9.05 - 147.18)	76.40 (22.66 - 302.78)	<0.001
IL-6 (pg/ml)	4079.09 (1949.43 - 99180.39)	3920.91 (2808.86 - 70164.47)	871.23 (111.06 - 3751.62)	1562.55 (630.91 - 3294.89)	<0.001
IL-10 (pg/ml)	53.18 (2.30 - 198.02)	59.62 (6.28 - 146.76)	6.51 (0 - 23.88)	3.61 (0 - 20.02)	<0.001
IL-17 (pg/ml)	3.20 (0.60 - 9.38)	1.69 (0.54 - 5.31)	0.49 (0.16 - 0.91)	0.50 (0.24 - 1.30)	0.004
sTLR-2 (ng/ml)	0.27 (0.10 - 0.68)	0.19 (0.05 - 0.50)	0.24 (0.08 - 0.36)	0.17 (0.04 - 0.39)	0.664
sTLR-4 (ng/ml)	0.51 (0.23 - 0.77)	0.25 (0.12 - 0.48)	0.41 (0.09 - 0.58)	0.27 (0.09 - 0.56)	0.383
uIL-6 (pg/ml)	120.50 (25.00 - 1000.00)	33.00 (16.10 - 99.80)	4.98 (3.68 - 5.56)	3.97 (2.86 - 6.57)	<0.001
EGF (pg/ml)	48.73 (30.51 - 109.29)	88.24 (65.81 - 131.51)	55.18 (30.67 - 71.44)	96.02 (51.59 - 189.35)	0.004
RANTES (pg/ml)	395.30 (231.96-935.30)	190.32 (78.08 - 1010.85)	477.00 (75.52 - 1779.07)	413.26 (114.580 - 1138.93)	0.752
DEFA3 (ng/ml)	28.41 (19.14-37.72)	27.72 (11.76-35.85)	20.23 (16.43-31.49)	19.89 (14.72-29.05)	0.049
log-TNF-α	5.39±2.54	4.56±1.91	3.44±1.97	2.81±1.13	<0.001
log-IL-6	9.08±2.61	9.11±2.22	6.82±2.39	7.34±1.44	<0.001
log-IL-10	3.36±2.36	3.56±2.03	1.88±1.89	1.94±1.66	<0.001
log-IL-17	1.57±1.40	1.25±1.18	0.66±0.85	0.67±0.78	0.002
log-MMP-8	5.78±2.64	5.99±1.79	3.63±2.27	4.36±1.72	<0.001
log-RANTES	5.99±1.13	5.50±1.59	5.71±2.22	5.86±1.76	0.759
log-TLR2	0.35±0.34	0.31±0.35	0.23±0.27	0.26±0.21	0.535
sqrt-TLR4	1.22±0.15	1.18±0.17	1.19±0.17	1.18±0.19	0.786
log-EGF	4.12±0.94	4.54±1.19	3.82±0.93	5.02±2.32	0.024
log-DEFA3	3.37±0.58	3.07±0.56	3.05±0.79	2.91±0.55	0.029

6.3.1.3.3 ROC kreivių analizė ir ribinės vertės numatant VUAS ir sunkias baigtis

Įvertinome vaisiaus vandenų imunologinių žymenų diagnostines charakteristikas ir ribines vertes VUAS ir sunkių baigčių prognozei, naudodami ROC analizę. TNF-α turėjo didžiausią diskriminacinę galią (AUC 0,73) nustatant VUAS su sunkiomis baigtimis, pasižymėjo dideliu specifiškumu, bet vidutiniu jautrumu. MMP-8, IL-6, IL-10, IL-17, EGF ir DEFA3 prognozinė galia buvo vidutinė (AUC 0,62-0,67), pasižymėjo dideliu specifiškumu, bet maža teigiama prognozine verte, o tai rodo, kad gali būti klaidingai teigiamų rezultatų.

9 lentelė. Vaisiaus vandenų žymenų ribinės vertės ir diagnostinės charakteristikos, numatant VUAS ir sunkias baigtis.

Žymuo	Ribinė vertė	AUC 95% PI		Jautrumas		Specifiškumas		TPV		NPV	
				%	95% PI	%	95% PI	%	95% PI	%	95% PI
MMP-8	413.67	0.67	0.51 - 0.82	65	45 – 85	72	63 - 80	30	21 - 40	92	87 - 96
TNF-α	231.13	0.73	0.59 - 0.87	60	35 - 80	89	82 - 94	50	35 - 68	92	88 - 96
IL-6	3999.42	0.67	0.53 - 0.81	59	41 - 77	74	66 - 81	29	20 - 39	91	87 - 95
IL-10	35.05	0.64	0.49 - 0.78	59	36 - 77	74	66 - 82	30	20 - 40	91	87 - 95
IL-17	4.00	0.66	0.51 - 0.81	50	30 - 70	84	76 - 90	37	23 - 52	90	86 – 94
EGF	51.49	0.62	0.48 - 0.76	59	41 - 77	73	65 - 80	28	19 - 38	91	87 - 95
DEFA3	35.58	0.66	0.53 - 0.79	36	18 - 55	89	83 - 93	36	19 - 55	89	85 - 92

Santrumpos: AUC - plotas po ROC kreive, PI – pasikliautinis intervalas, TPV - teigiama prognozinė vertė; NPV - neigiama prognozinė vertė.

6.3.1.3.4 Logistinė regresija, numatant VUAS ir sunkias naujagimio baigtis

Siekiant įvertinti vaisiaus vandenų uždegimo žymenų prognozinę VUAS ir sunkių naujagimio baigčių reikšmę, buvo atlikta vienanarė ir daugianarė logistinė regresinė analizė. Vienanarės analizės metu tokie žymenys kaip TNF-α, MMP-8, IL-6, IL-10, IL-17 ir DEFA3 kartu su klinikiniais veiksniais, kaip GA ir gimimo svoris, buvo reikšmingai susiję su padidėjusia VUAS bei sunkių baigčių tikimybe. Dėl kolineariškumo į daugianarį modelį atrinkti stipresni prognoziniai veiksniai. TNF-α išliko reikšmingas prognozinis veiksnys, o GA turėjo apsauginį poveikį, mažindamas VUAS ir sunkių baigčių tikimybę. Log-DEFA3 didino VUAS ir sunkių baigčių tikimybę, tačiau nepasiekė statistinio patikimumo. Žingsninė regresinė analizė patvirtino, kad TNF-α, DEFA3 ir GA buvo pagrindiniai prognoziniai VUAS ir sunkių naujagimio baigčių veiksniai, o didesnis GA mažino VUAS bei sunkių naujagimio baigčių riziką.

10 lentelė. Vaisiaus vandenų žymenų logistinė regresija numatant VUAS ir sunkias naujagimio baigtis. Nenormaliai pasiskirstę kintamieji buvo logaritmuoti analizei.

Vienanarė logistinė regresija				
Įvesties kintamieji	Koeficientas	P reikšmė	GS	95% PI
Log-TNF-α	0.474	<0.001	1.61	1.27-2.09
Log-MMP-8	0.263	0.038	1.30	1.03-1.69
Log-IL-6	0.292	0.007	1.34	1.09-1.67
Log-IL-10	0.252	0.033	1.29	1.03-1.63
Log-IL-17	0.535	0.008	1.71	1.15- 2.57
Log-EGF	-0.333	0.163	0.72	0.43-1.05
Log-DEFA3	1.170	0.009	3.22	1.41-8.25
GA	-0.319	< 0.001	0.72	0.62- 0.84
Gimimo svoris	-0.002	< 0.001	0.99	0.97-0.99
Hipertenzinės būklės (ref. taip)	-0.635	0.334	0.53	0.12-1.70
Savaiminis gimdymas (ref. taip)	-0.018	0.969	0.98	0.39-2.45
Daugianarė logistinė regresija N=145, AIC= 155.26				
Log-TNF-α	0.392	0.013	1.48	1.09-2.05
Log-IL-17	-0.100	0.720	0.90	0.51-1.54
Log-DEFA3	0.887	0.069	2.43	0.99- 6.85
GA	-0.260	0.003	0.77	0.65- 0.91

11 lentelė. Žingsninė logaritmuotų žymenų logistinė regresija (kaupiamoji-šalinamoji) prognozuojat VUAS ir sunkias naujagimio baigtis bei kontroliuojant pagal GA.

Žingsninė logistinė regresija N=145, AIC=100.98				
Žymuo	Koeficientas	P reikšmė	GS	95% PI
GA	-0.268	0.002	0.77	0.64-0.90
Log-TNF-α	0.323	0.024	1.38	1.05-1.85
Log-DEFA3	0.857	0.057	2.36	1.03-6.12

Tolimesnėje logistinės regresijos analizėje, buvo naudojamos optimalios ribinės vertės prognozuojant VUAS ir sunkias naujagimių baigtis, remiantis nuo dozės priklausančiu žymenų poveikiu. Vienanarė logistinė regresija parodė, kad tokie žymenys kaip TNF-α, MMP-8, IL-6, IL-10, IL-17, DEFA3 ir EGF buvo reikšmingai susiję su VUAS ir sunkiomis naujagimių baigtimis. Tačiau taikant daugianarę regresiją, tik TNF-α išliko reikšmingu prognoziniu rodmeniu, o tai rodo jo patikimumą. Žingsninė regresija dar kartą patvirtino, kad TNF-α, kurio riba yra > 231 pg/ml, yra stiprus VUAS ir sunkių naujagimių baigčių prognozinis veiksnys, o gestacinis amžius turėjo apsauginį poveikį, nes jam didėjant rizika mažėjo.

12 lentelė. Žymenų ribinių verčių logistinė regresija prognozuojant VUAS ir sunkias naujagimio baigtis.

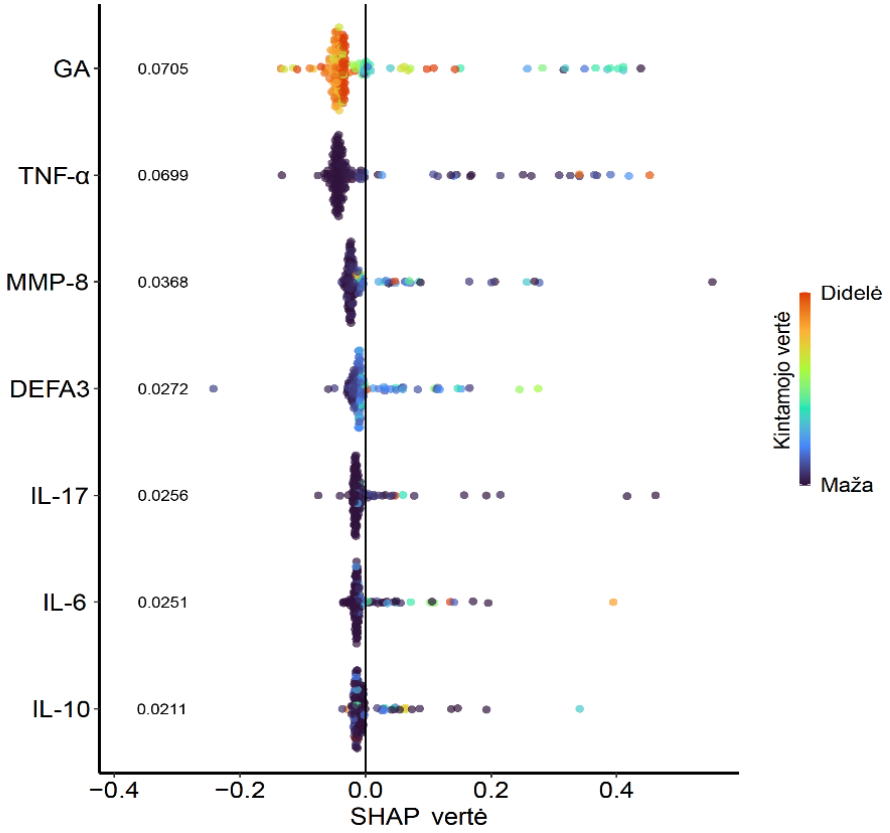
Vienanarė ribinių verčių logistinė regresija				
žymuo	Koeficientas	P reikšmė	GS	95% PI
TNF-α >231	2.485	< 0.001	12.00	4.18-36.78
MMP-8 >413	1.575	0.002	4.82	1.76-13.27
IL-6 > 3999	1.402	0.004	4.06	1.60-10.73
IL-10 >35	1.434	0.003	4.19	1.65-11.10
IL-17 >4	1.633	0.002	5.12	1.84-14.43
DEFA3 >35	1.331	0.010	3.79	1.33-10.39
EGF <51	1.175	0.013	3.24	1.28-8.37
Daugianarė ribinių verčių logistinė regresija N=145, AIC =103.2				
TNF-α >231	2.221	0.013	9.21	1.82-65.04
MMP-8 >413	0.4094	0.630	1.50	0.27-7.89
IL-6 > 3999	-0.337	0.677	0.71	0.12-3.13
IL-10 >35	0.263	0.724	1.30	0.29-5.61
IL-17 >4	0.411	0.603	1.51	0.31-7.15
DEFA3 >35	0.450	0.510	1.57	0.39- 5.83
EGF<51	0.832	0.245	2.30	0.57- 9.77
GA	-0.179	0.089	0.83	0.68-1.02

13 lentelė. Žymenų ribinių verčių **ž**ingsninė (kaupiamoji-šalinamoji) logistinė regresija prognozuojat VUAS ir sunkias naujagimio baigtis bei kontroliuojant pagal GA

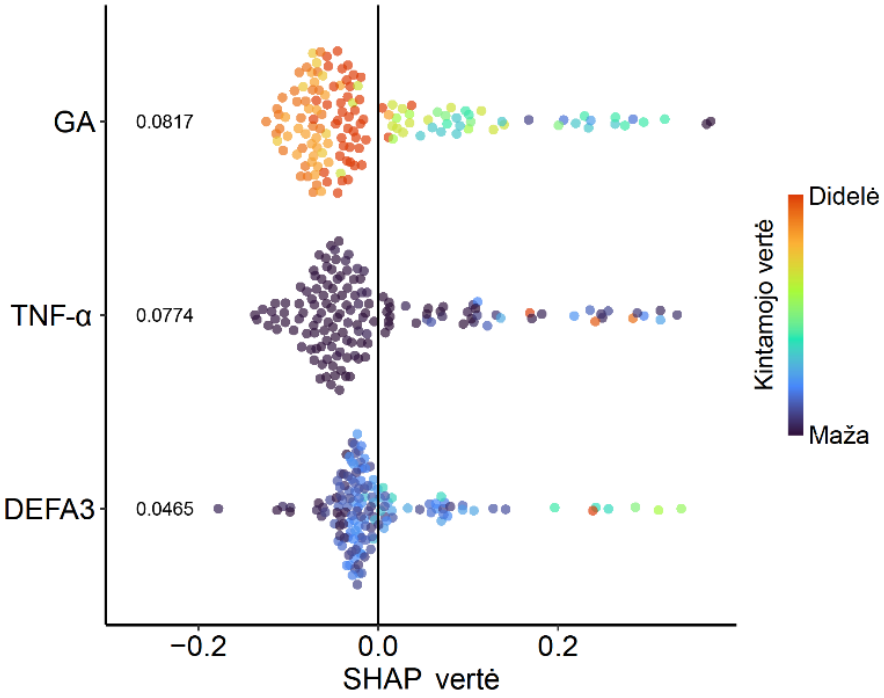
Žingsninė logistinė regresija N=145, AIC=94.415				
Žymuo	Koeficientas	P reikšmė	GS	95% PI
TNF-α >231	2.335	<0.001	10.33	3.38-33.30
GA	-0.269	0.001	0.76	0.64-0.89

6.3.1.3.5 Atsitiktinių medžių klasifikatoriaus analizė prognozuojant VUAS ir sunkias naujagimio baigtis

Atsitiktinių medžių klasifikatoriaus analizė buvo atlikta siekiant įvertinti septynis prognozinius veiksnius (TNF-α, MMP-8, IL-6, IL-17, IL-10, DEFA3 ir GA) numatant VUAS ir sunkias naujagimio baigtis. Modelis išbandytas su 300, 500, 1000, 2000 ir 3000 medžių. Nustatyta mažiausia OOB paklaida, kai mtry lygus 2. Modelio efektyvumas pasiekė 88 proc. tikslumą, 33 proc. jautrumą ir 97 proc. specifiškumą. Didžiausios įtakos turėjo GA, TNF-α ir MMP-8 (8 paveikslas). Toliau testuotas patobulintas modelis, sudarytas iš 3 prognozinių veiksnių GA, TNF-α ir DEFA3 (9 paveikslas). Modelis bandytas su 300, 500, 1000, 2000 ir 3000 medžių. Mažiausia OBB paklaida nustatyta, kai mtry lygus 1. Pasiektas 93 proc. modelio tikslumas, 50 proc. jautrumas ir 100 proc. specifiškumas. Apibendrinant, GA ir TNF-α kiekis vaisiaus vandenyse yra svarbiausi veiksniai, padedantys prognozuoti VUAS ir sunkias naujagimio baigtis.



8 paveikslas. Prognozinių veiksnių reikšmė numatant VUAS ir sunkias naujagimio baigtis SHAP diagramoje. Y ašyje prognoziniai veiksniai (GA, TNF-α, MMP-8, DEFA3, IL-17, IL-6 ir IL-10) išdėstyti pagal svarbą mažėjimo tvarka.



9 paveikslas. Optimalaus VUAS ir sunkių naujagimio baigčių prognozinio modelio SHAP diagrama. Trys prognoziniai veiksniai (GA, TNF-α ir DEFA3) išvardyti mažėjančia tvarka pagal jų svarbą modelyje.

6.3.1.4 Vaisiaus vandenų uždegimo žymenų ir virkštelės kraujo IL-6 kiekio ryšys

Virkštelės kraujyje IL-6 kiekis nustatytas 0-40000 pg/ml intervale, mediana - 8,21 pg/ml (IQR: 3,79-38,83). Koreliacinė analizė atlikta siekiant įvertinti virkštelės IL-6 ir vaisiaus vandenų žymenų ryšį. Virkštelės IL-6 vidutiniškai arba silpnai teigiamai koreliuoja su kitais uždegiminiais vaisiaus vandenų žymenimis, tokiais kaip TNF-α (rho = 0,4, p < 0,001), IL-6 (rho = 0,3, p < 0,001), IL-17 (rho = 0,3, p = 0,01), MMP-8 (rho = 0,3, p <0,001) ir IL-10 (rho = 0,2, p = 0,03), o tai reiškia, kad imunologinių žymenų kiekis tiek virkštelės kraujyje, tiek pro makštį paimtuose vaisiaus vandenyse atitinka.

Siekdami palyginti virkštelės IL-6 diagnostines charakteristikas su vaisiaus vandenų žymenų charakteristikomis, pirmiausiai įvertinome virkštelės IL-6 ROC kreivę sunkioms naujagimių baigtims prognozuoti. VUAS į analizę neįtrauktas,

kadangi, remiantis virkštelės IL-6 kiekiu, nustatyta VUAS diagnozė. AUC buvo 67 % (95% PI 57-80), o esant ribinei vertei ≥19,3 pg/ml specifiškumas buvo 77 proc., jautrumas – 53 proc., TPV – 47 proc., o NPV – 81 proc. Kai ribinė vertė 100,5 pg/ml, specifiškumas padidėjo iki 92 proc., jautrumas – 34 proc., PPV – 61 proc., o NPV – 78 proc. Tuomet palyginome virkštelės IL-6 diagnostinį veiksmingumą su vaisiaus vandenų žymenų charakteristikomis, pritaikę *Delong* testą. Remiantis AUC skirtumais, virkštelės IL-6 pranoko šiuos vaisiaus vandenų žymenis numatant sunkias naujagimių baigtis ($P < 0.05$): MMP-8 ($P = 0,012$), vaisiaus vandenų IL-6 ($P = 0,037$) ir IL-10 ($P = 0,035$). Tačiau nepastebėta reikšmingų AUC skirtumų lyginant su TNF-α ($P = 0,245$), DEFA3 ($P = 0,376$), IL-17 ($P = 0,106$) ir EGF ($P = 0,886$).

Sunkioms naujagimių baigtims prognozuoti buvo atlikta atsitiktinių medžių klasifikatoriaus analizė, kurioje vaisiaus vandenų žymenys buvo lyginami su virkštelės IL-6 ir GA. Į modelį buvo įtraukti devyni prognostiniai veiksniai: TNF-α, MMP-8, IL-6, IL-17, IL-10, DEFA3, virkštelės IL-6, EGF ir GA. VUAS į modelį neįtrauktas, nes jo diagnozė yra paremta virkštelės IL-6 kiekiu, kuris jau įtrauktas į modelį. Modelis išbandytas su 500–3000 medžių skaičiumi. Nustatyta mažiausia OOB paklaida, kai mtry lygus 2. Tolesni medžių skaičiaus ir `mtry` koregavimai nepagerino OOB paklaidos. Galutinis modelis pasiekė 84 proc. klasifikavimo tikslumą, jo jautrumas buvo 54 proc., specifiškumas – 97 proc., PPV – 86 proc., o NPV - 83 proc. Prognostiniai veiksniai buvo suskirstyti pagal svarbą remiantis SHAP vertėmis, o GA, EGF ir TNF-α buvo nustatyti kaip didžiausią įtaką naujagimių baigčių prognozei turintys veiksniai. Mažesnis GA, sumažėjusi EGF ir padidėjusi TNF-α koncentracija buvo stipriausi prognoziniai veiksniai. Vaisiaus vandenų IL-6, virkštelės IL-6 ir MMP-8 reikšmė prognoziniame modelyje buvo panaši ir vidutinė.

6.3.2 II dalis. SuPAR kohorta

6.3.2.1 SuPAR populiacijos charakteristika

Antros dalies tyrimo kohortą sudarė 114 tiriamųjų, kurie buvo suskirstyti į VUAS (n=48) ir ne-VUAS grupes (n=66). Tiriamųjų charakteristika pateikiama 14 lentelėje. VUAS grupėje dažniau pasireiškė klinikinis ir histologinis chorioamnionitas, mažesnis gimimo svoris, gestacinis amžius ir Apgar balai. Taip pat VUAS grupės naujagimiams statistiškai patikimai dažniau nustatytos nepalankios baigtys tokios kaip ankstyvasis sepsis, kvėpavimo sistemos

problemos, ankstyva hipotenzija, BPD, NNR. Virkštelės pH, naujagimių mirties, AAL, surfaktanto poreikio ir ISK rodikliai tarp grupių nesiskyrė.

14 lentelė. Tiriamųjų demografiniai ir klinikiniai ypatumai

Charakteristika	VUAS grupė (n=48)	Ne-VUAS grupė (n=66)	P reikšmė
Motinos charakteristika			
Motinos amžius, m., vidurkis ± SD	31.2 ± 5.5	31.1 ± 5.7	0.932
Bevandenis laikotarpis, val., mediana, IQR	93 (17-205)	106 (44-236)	0.199
Gestacinis diabetas, n, %	10 (21)	13 (20)	0.907
Teigiamas BGS testas, n, %	7 (24)	10 (22)	0.814
Pirmas nėštumas, n, %	17 (46)	31 (40)	0.566
Pirmas gimdymas, n, %	20 (39)	28 (44)	0.566
Kortikosteroidai antenataliai, n, %	41 (85)	55 (83)	0.764
Tokolitikai, n, %	23 (48)	38 (58)	0.312
Gimdymo būdas, n, % Makštinis gimdymas CPO	 43 (90) 5 (10)	 55 (83) 11 (17)	0.423
Indukuotas gimdymas arba CPO, n, % Savaiminis gimdymas, n, %	17 (35) 31 (65)	33 (50) 33 (50)	0.279
Klinikinis chorioamnionitas, n, %	6 (13)	1 (2)	0.024
Histologinis chorioamnionitas, n, %	38 (79)	15 (23)	<0.001
Funizitas, n, %	21 (44)	0	<0.001
Naujagimių charakteristika			
GA, sav., mediana, IQR	31 (27-33)	33 (32-34)	0.001
Gimimo svoris, g., vidurkis ± SD	1688± 669	2070± 555	0.012
Apgar balai <7 1 min., n, %	14 (29)	6 (9)	0.014
Apgar balai <7 5 min., n, %	6 (13)	1 (2)	0.037
Virkštelės arterinio kraujo pH, vidurkis ± SD	7.35 (7.29-7.42)	7.36 (7.32-7.40)	0.774
Gaivinimo poreikis po gimimo, n, %	14 (29)	6 (9)	0.005
Sunkios baigtys, n, %	19 (40)	11 (17)	0.014
Kvėpavimo sutrikimas, n, %	46 (96)	48 (73)	0.001
Sunkus KSS, n, %	10 (21)	8 (12)	0.005
Kvėpuojamoji terapija, n, % Netaikyta	 2 (4)	 18 (27)	0.002

Charakteristika	VUAS grupė (n=48)	Ne-VUAS grupė (n=66)	P reikšmė
Dirbtinė ventiliacija Neinvazinė KT	11 (23) 35 (73)	6 (9) 42 (64)	
Surfaktanto poreikis, n, %	15 (31)	14 (21)	0.224
Naujagimio mirtis, n, %	1 (2)	0	0.422
Sepsis, n, %	8 (17)	2 (3)	0.017
Ankstyvasis sepsis, n, %	7 (17)	1 (2)	0.007
Ankstyva hipotenzija, n, %	10 (21)	5 (8)	0.039
Bronchopulmoninė displazija, n, %	9 (19)	4 (6)	0.044
Atviras arterinis latakas, n %	8 (17)	8 (12)	0.462
Neišnešiotų naujagimių retinopatija, n, %	15 (32)	6 (9)	0.003
Gydyta NNR, n, %	3 (6)	1(2)	0.168
Intraskilvelinės kraujosruvos, n, % Nėra 1-2 laipsnio 3-4 laipsnio	 29 (60) 14 (29) 5 (10)	 46 (70) 18 (27) 2 (3)	0.243

6.3.2.2 Vaisiaus vandenų suPAR ir VUAS ryšys

SuPAR kiekis nustatytas visuose vaisiaus vandenų mėginiuose 5,36–399,98 ng/ml intervale, mediana – 26,23 ng/ml (IQR: 15,19-51,14). VUAS grupėje suPAR koncentracija buvo statistiškai reikšmingai didesnė nei ne-VUAS grupėje: 32,36 ng/ml (IQR, 17,27–84,16) ir 20,46 ng/ml, (IQR, 11,49–36,63), p = 0,01, atitinkamai.

6.3.2.3 SuPAR ROC kreivių analizė ir ribinės vertės VUAS prognozei

Siekdami įvertinti suPAR diagnostines charakteristikas ir ribinę vertę VUAS prognozuoti, atlikome ROC analizę (15 lentelė). Buvo analizuojami šie statistiškai reikšmingi VUAS žymenys suPAR kohortoje: suPAR, MMP-8, TNF-α, IL-6, IL-10, IL-17 ir DEFA3. Vaisiaus vandenų suPAR AUC buvo 0,65, o ribinė vertė VUAS prognozei - 27,60 ng/ml. *DeLong* testas neparodė statistiškai reikšmingo skirtumo tarp suPAR ir IL-10, IL-17 bei DEFA3 diagnostinių charakteristikų, visi šie rodikliai turėjo vidutinę prognozinę galią. Tačiau TNF-α, IL-6 ir MMP-8 parodė geresnius rezultatus nei suPAR, nustatant VUAS.

15 lentelė. Vaisiaus vandenų žymenų ribinės vertės ir diagnostinės charakteristikos, numatant VUAS, suPAR kohortoje.

	Ribinės vertės	AUC 95% PI		Jautrumas		Specifiškumas		TPV		NPV		Delong testo P reikšmė
				%	95% PI	%	95% PI	%	95% PI	%	95% PI	
suPAR	27.60	0.65	0.54-0.76	65	51-79	65	53-77	57	48-68	72	64-82	-
MMP-8	190.91	0.76	0.66-0.86	82	68-93	67	55-79	65	57-75	34	73-93	0.054
TNF-α	86.95	0.82	0.72-0.91	75	61-86	86	78-95	81	70-91	82	75-90	0.005
IL-6	27843.18	0.77	0.68-0.86	50	35-65	95	89-100	89	77-100	72	67-79	0.049
IL-10	35.05	0.78	0.68-0.87	69	56-81	86	79-94	79	68-90	79	72-87	0.068
IL-17	1.00	0.70	0.60-0.81	66	52-80	72	60-84	65	54-76	73	65-82	0.887
DEFA3	33.90	0.64	0.53-0.75	38	23-52	95	89-100	86	70-100	58	63-74	0.727

Santrumpos: AUC - plotas po ROC kreive, PI – pasikliautinis intervalas, TPV – teigiama prognozinė vertė; NPV – neigiama prognozinė vertė.

6.3.2.4 Logistinė regresija numatant VUAS

Vienanarė logistinė regresinė analizė parodė, kad didesnė suPAR koncentracija vaisiaus vandenyse reikšmingai didina VUAS tikimybę, o log-suPAR padidėjimas vienetu didina VUAS tikimybę beveik du kartus (GS = 1.70, 95% PI: 1.09 - 2.74, p < 0,001) (16 lentelė). Tačiau atlikus daugialypę analizę, tik log-TNF-α reikšmingai prognozavo VUAS, o suPAR, IL-17, DEFA3 ir GA nebuvo statistiškai reikšmingi (16 ir 17 lentelės).

16 lentelė. SuPAR kohortos vaisiaus vandenų žymenų logistinė regresija numatant VUAS. Nenormaliai pasiskirstę kintamieji buvo logaritmuoti analizei.

	Vienanarė logistinė regresija			
Kintamieji	Koeficientas	P reikšmė	GS	95% PI
Log-TNF-α	0.690	<0.001	1.99	1.53-2.72
Log-MMP-8	0.491	<0.001	1.63	1.29-2.15
Log-IL-6	0.547	<0.001	1.73	1.38-2.23
Log-IL-10	0.560	<0.001	1.75	1.40-2.27
Log-IL-17	0.738	0.002	2.09	1.37-3.45
Log-suPAR	0.530	0.024	1.70	1.09-2.74
Log-DEFA3	0.775	0.002	2.17	1.19-4.24
GA	-0.211	0.004	0.81	0.70-0.92
Gimimo svoris	-0.001	0.002	0.99	0.998-0.999
Daugianarė logistinė regresija N=114, AIC=118.17				
Log-TNF-α	0.765	<0.001	2.15	1.57-3.11
Log-IL-17	0.137	0.657	1.15	0.63-2.15
Log-DEFA3	0.678	0.095	1.97	0.91-4.56
GA	-0.085	0.316	0.92	0.77-1.09
Log-suPAR	-0.186	0.595	0.83	0.40-1.61

17 lentelė. Žingsninė logistinė regresija (kaupiamoji-šalinamoji) prognozuojat VUAS, įvedus log-suPAR ir kontroliuojant pagal GA.

Žingsninė logistinė regresija N=114, AIC= 116.16				
Kintamieji	Koeficientas	P reikšmė	GS	95% PI
Log-TNF-α	0.784	<0.001	2.19	1.61 – 3.14
Log-DEFA3	0.744	0.049	2.11	1.02 – 4.60
GA (įtrauktas)	-0.084	0.323	0.92	0.78 – 1.09
Log-suPAR (įtrauktas)	-0.129	0.691	0.88	0.45 – 1.62

Atliekant tolesnę analizę buvo išbandyti logistinės regresijos modeliai, taikant suPAR ir kitų žymenų ribines vertes VUAS prognozuoti. Vienanarė analizė atskleidė, kad suPAR kiekis, viršijantis 27 ng/ml, reikšmingai 3,47 karto didino VUAS tikimybę (18 lentelė). Atlikus daugianarę analizę nustatyta, kad TNF-α (>87 pg/ml) ir DEFA3 (>34 ng/ml) yra reikšmingi VUAS prognoziniai veiksniai, o suPAR ir kiti žymenys, įskaitant MMP-8, IL-6, IL-10 ir IL-17, reikšmingo ryšio neparodė (18 lentelė). Žingsninė regresija patvirtino TNF-α, DEFA3 ir IL-6 svarbą, tačiau GA ir suPAR koncentracija nebuvo reikšmingi prognoziniai veiksniai (19 lentelė).

18 lentelė. Žymenų ribinių verčių logistinė regresija prognozuojant VUAS suPAR kohortoje.

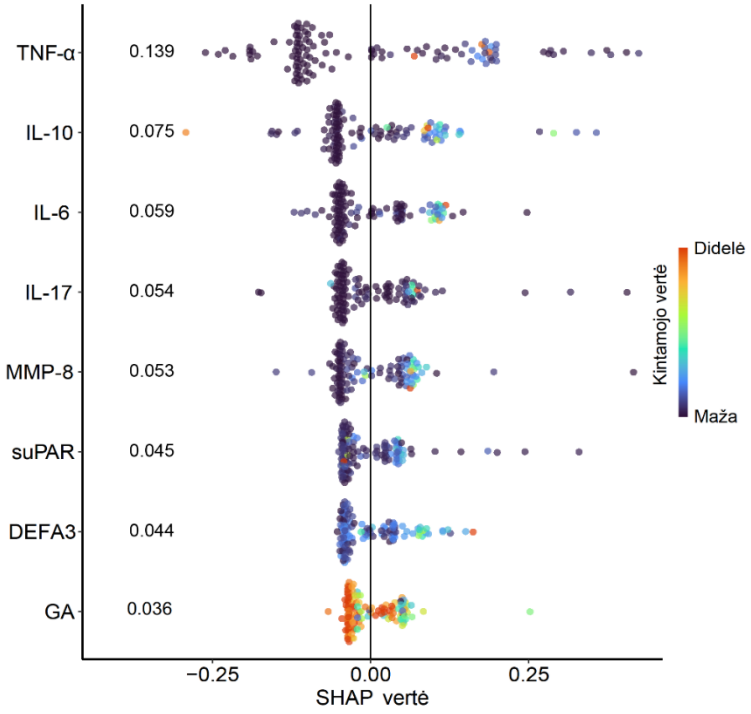
Vienanarė ribinių verčių logistinė regresija				
Kintamieji	Koeficientas	P reikšmė	GS	95% PI
suPAR >27	1.243	0.003	3.47	1.55 – 8.05
TNF-α >87	2.931	<0.001	18.75	7.16 – 54.82
MMP-8 >190	2.223	<0.001	9.24	3.75 – 25.04
IL-6 >27843	3.045	<0.001	21.00	6.59 – 94.25
IL-10 >35	2.634	<0.001	13.93	5.71 – 37.18
IL-17 >1	1.600	<0.001	4.95	2.16 – 11.89
DEFA3 >34	2.53	<0.001	12.60	3.90 – 56.81
Daugianarė ribinių verčių logistinė regresija N=114, AIC =102.77				
TNF-α > 87	1.963	0.008	7.13	1.69 – 32.77
MMP-8 >190	0.793	0.344	2.21	0.41 – 11.57
IL-6 >27843	1.574	0.059	4.83	1.01 – 29.22
IL-10 >35	0.497	0.523	1.64	0.33-7.36
IL-17 >1	-0.407	0.571	0.67	0.15 -2.55
DEFA3 >34	1.981	0.016	7.25	1.57 – 42.38
GA	-0.063	0.512	0.94	0.77-1.13
suPAR >27	-0.423	0.564	0.65	0.14-2.59

19 lentelė. Žymenų ribinių verčių žingsninė (kaupiamoji-šalinamoji) logistinė regresija prognozuojant VUAS suPAR kohortoje. SuPAR ir GA yra įtraukti kintamieji.

Žingsninė logistinė regresija N=114, AIC= 98.82				
Kintamieji	Koeficientas	P reikšmė	GS	95% PI
TNF-α >87	2.421	<0.001	11.26	3.22-44.25
DEFA3 >34	2.082	0.009	8.02	1.85-45.02
IL-6 >27843	1.669	0.031	5.31	1.25-28.62
GA (įtrauktas)	-0.048	0.612	0.95	0.79-1.15
suPAR (įtrauktas)	-0.136	0.834	0.87	0.22-2.95

6.3.2.5 Atsitiktinių medžių klasifikatoriaus analizė prognozuojant VUAS suPAR kohortoje

VUAS prognozei buvo naudojama atsitiktinių medžių analizė, įvertinant aštuonis prognozinius veiksnius: suPAR, TNF-α, MMP-8, IL-6, IL-17, IL-10, DEFA3 ir GA. Išbandėme modelį su 300, 500, 1000, 2000 ir 3000 medžių, o mtry buvo nustatytas kaip kvadratinė šaknis iš 8 lygu 2 su reikšmėmis nuo 1 iki 8. Modelis tikrintas remiantis OOB paklaidą, kuri buvo mažiausia, kai mtry lygus 4. Šio modelio tikslumas – 82 proc., jautrumas – 86 proc. ir specifiškumas – 79 proc. TNF-α, IL-10 ir IL-6 buvo svarbiausi VUAS prognoziniai rodmenys, suPAR buvo vidutinio reikšmingumo, o GA – mažiausiai reikšmingas (10 paveikslas). Naudodami įvairius prognozinių veiksnių derinius, įvertinome kitus atsitiktinių medžių klasifikatoriaus modelius, siekdami rasti modelį, kurio tikslumas yra didžiausias, o OOB paklaida – mažiausia. Nei vienas iš testuotų modelių, prognozuojančių VUAS, neprilygo aukščiau minėtam modeliui.



10 paveikslas. SuPAR kohortos prognozinių veiksnių reikšmė numatant VUAS SHAP diagramoje. Y ašyje prognoziniai veiksniai (TNF-α, IL-10, IL-6, IL-17, MMP-8, suPAR, DEFA3 ir GA) išdėstyti pagal svarbą mažėjimo tvarka.

6.3.2.6 SuPAR ir VUAS bei sunkių naujagimio baigčių ryšys

Vertindami suPAR ir VUAS bei sunkių baigčių ryšį, nenustatėme statistiškai reikšmingo suPAR koncentracijų skirtumo VUAS ir ne-VUAS grupėse pagal sunkių baigčių buvimą ar nebuvimą. (20 lentelė).

20 lentelė. SuPAR koncentracijų (medianos ir IQR|) palyginimas VUAS ir ne-VUAS grupėse atsižvelgiant į sunkias naujagimių baigtis.

Žymuo	VUAS grupė		Ne-VUAS grupė		P reikšmė
	Sunkios baigtys	Nėra sunkių baigčių	Sunkios baigtys	Nėra sunkių baigčių	
suPAR (ng/ml)	37.24 (20.02- 2.78)	30.36 (15.99-77.85)	28.82 (16.68-36.15)	19.81 (11.44-36.18)	0.082

6.3.3 III dalis. Vaisiaus vandenų EGF ir nepalankių naujagimio baigčių ryšys

6.3.3.1 EGF populiacijos charakteristika

Trečioje tyrimo dalyje buvo analizuota visa 145 tiriamųjų kohorta, suskirstyta į 3 GA grupes pagal PSO klasifikaciją [182]: ypač neišnešioti (22^{+0}-27^{+6} sav.), labai neišnešioti (28^{+0}-31^{+6} sav.) ir vidutiniškai ir vėlyvi neišnešioti naujagimiai (32^{+0}-34^{+6} sav.). Tarp tiriamųjų grupių, stebėti motinos amžiaus skirtumai. Taip pat nėščiųjų diabetas nustatytas dažniau 32^{+0}–34^{+6} GA sav. grupėje, o CPO dažnis buvo didesnis ypač ir labai neišnešiotų naujagimių grupėje. Klinikinis ir histologinis chorioamnionitas nustatytas dažniau yapč neišnešiotų naujagimių grupėje. Kitų statistiškai reikšmingų motinos charakteristikos skirtumų tarp tiriamųjų grupių nenustatėme (21 lentelė). Kaip ir tikėtasi, stebėti statistiškai reikšmingi klinikinės naujagimių charakteristikos skirtumai lyginat šias GA grupes. Mažesnis GA buvo susijęs su mažesniu gimimo svoriu, žemesniais Apgar balais, dažnesniu VUAS ir didesniu sergamumu. Naujagimių mirtingumo rodikliai tarp grupių reikšmingai nesiskyrė (22 lentelė).

21 lentelė. Tiriamosios populiacijos charakteristika pagal gestacijos amžiaus grupes.

	Visa kohorta (n=145)	22^{+0}-27^{+6} sav. (n=24)	28^{+0}-31^{+6} sav. (n=29)	32^{+0}–34^{+6} sav. (n=92)	P reikšmė
Motinos charakteristika					
Motinos amžius, m., vidurkis ± SD	31.25±5.62	29.8±4.1	32.9±5.74	31.1±5.82	0.048
Bevandenis laikotarpis, val., mediana, IQR	71 (24-204)	113 (49-241)	63 (16-163)	70 (24-209)	0.321
Hipertenzinės būklės, n, %	31 (21)	4 (17)	9 (31)	18 (20)	0.358
Gestacinis diabetas, n, %	34 (24)	2 (8)	4 (14)	28 (30)	0.035
Teigiamas BGS testas, n, %	17 (12)	2 (8)	4 (14)	11 (12)	0.721
Pirmas nėštumas, n, %	50 (34)	8 (33)	10 (3)	32 (35)	0.991
Pirmas gimdymas, n, %	68 (47)	9 (38)	14 (48)	45 (49)	0.599
Kortikosteroidai antenataliai, n, %	125 (86)	21 (88)	28(97)	76 (83)	0.162
Tokolitikai, n, %	67 (46)	15 (63)	9 (31)	43 (47)	0.072
Gimdymo būdas, n, % Makštinis gimdymas CPO	 112 (77) 33 (23)	 14 (58) 10 (42)	 20 (69) 9 (31)	 78 (85) 14 (15)	0.011
Indukuotas gimdymas arba CPO, n, % Savaiminis gimdymas, n, %	78 (54) 67 (46)	12 (50) 12 (50)	19 (65) 10 (35)	47 (51) 45 (49)	0.365
Klinikinis chorioamnionitas, n, %	7 (5)	5 (21)	1 (3)	1 (1)	0.001
Histologinis chorioamnionitas, n, %	54 (37)	15 (63)	11 (38)	28 (30)	0.015
Funizitas, n, %	21 (14)	5 (21)	8 (28)	8 (9)	0.004
Naujagimių charakteristika					
GA, sav., mediana, IQR	32 (30–34)	26 (25.8–27)	30 (29–30)	33 (33–34)	<0.001
Gimimo svoris, g., vidurkis ± SD	1840±634	868±205	1438 ±264	2221 ±396	<0.001
Apgar balai <7 1 min., n, %	22 (15)	14 (58)	5 (17)	3 (3)	<0.001
Apgar balai <7 5 min., n, %	7 (25)	6 (86)	1 (3)	0	<0.001
Virkštelės arterinio kr. pH, vidurkis ± SD	7.34 (7.28–7.39)	7.38 (7.25–7.42)	7.32 (7.26–7.4)	7.34 (7.29–7.39)	0.462
Gaivinimo poreikis po gimimo, n, %	22 (15)	13 (54)	4 (14)	5 (5)	<0.001
VUAS, n, %	54 (37)	13 (54)	14 (48)	27 (29)	0.032

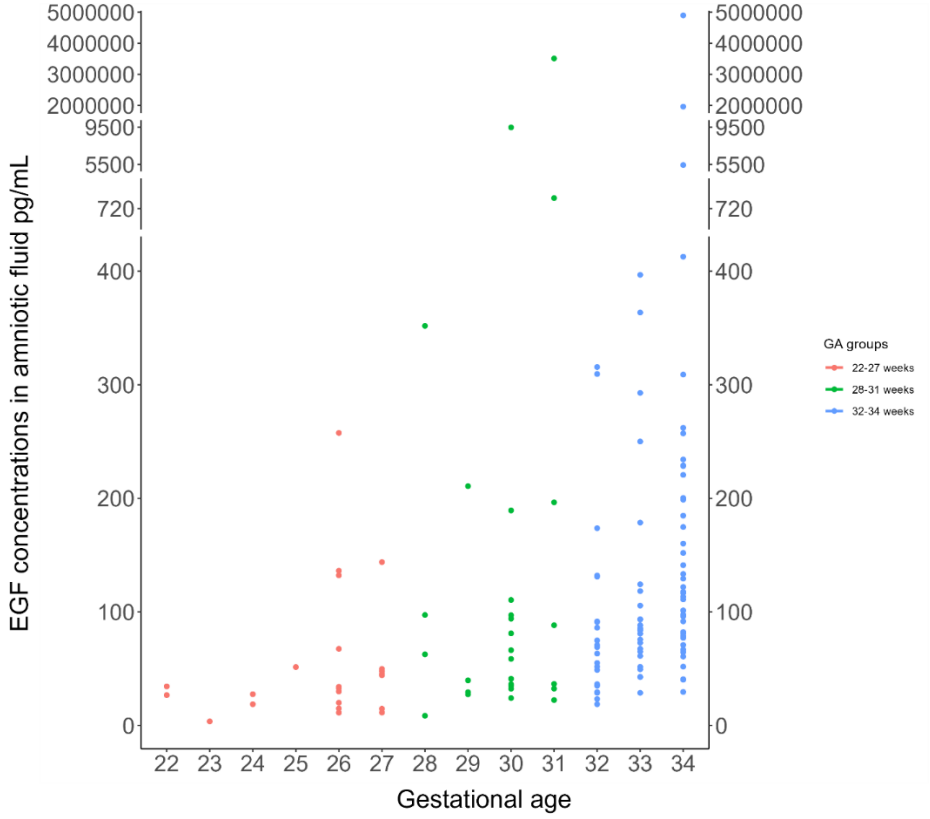
22 lentelė. Nepalankių naujagimių baigčių pasiskirstymas pagal GA grupes.

Naujagimių baigtys	Visa kohorta (n=145)	22^{+0}-27^{+6} sav. (n=24)	28^{+0}-31^{+6} sav. (n=29)	32^{+0}–34^{+6} sav. (n=92)	P reikšmė
Sunkios baigtys, n, %	44 (30)	20 (87)	11 (38)	13 (14)	<0.001
Kvėpavimo sutrikimas, n, %	121 (83)	24 (100)	29 (100)	68 (74)	<0.001
KSS stadijos, n, %:					
Nėra KSS	25 (17)	0	0	25 (27)	
Lengvas KSS	48 (33)	2 (8)	7 (24)	39 (42)	<0.001
Vidutinis KSS	44 (30)	8 (33)	14 (48)	22 (24)	
Sunkus KSS	28 (19)	14 (58)	8 (28)	6 (67)	
Kvėpuojamoji terapija, n, %					
Netaikyta	24 (17)	0	0	24 (26)	<0.001
DPV	26 (18)	14 (58)	3 (10)	9 (10)	
Neinvazinė KT	95 (66)	10 (42)	26 (90)	59 (64)	
Surfaktanto poreikis, n, %	41 (28)	23 (96)	12 (41)	6 (7)	<0.001
KT trukmė, n, %:					
Nėra KT	23 (16)	0	0	23 (25)	
≤24 val.	38 (26)	0	1 (3)	37 (40)	<0.001
24–96 val.	32 (22)	0	10 (35)	22 (24)	
≥96 val.	51 (35)	23 (100)	18 (62)	10 (11)	
BPD	17 (12)	16 (70)	1 (3)	0	<0.001
Naujagimio mirtis, n, %	2 (1)	1 (4)	0	1 (1)	0.344
Sepsis, n, %	15 (10)	10 (42)	2 (7)	3 (3)	<0.001
Ankstyvasis sepsis, n, %	9 (6)	6 (25)	2 (7)	1 (1)	<0.001
Ankstyva hipotenzija, n, %	18 (12)	12 (50)	2 (7)	4 (4)	<0.001
AAL, n %	19 (13)	12 (52)	5 (17)	2 (2)	<0.001
NNR n, %	25 (17)	20 (87)	5 (17)	0	<0.001
Gydyta NNR, n, %	5 (3)	4 (17)	1 (3)	0	<0.001
ISK, n, %					
Nėra	98 (68)	10 (42)	17 (59)	71 (77)	<0.001
1-2 laipsnio	38 (26)	8 (33)	10 (35)	20 (22)	
3-4 laipsnio	9 (6)	6 (25)	2 (7)	1 (1)	

6.3.3.2 Vaisiaus vandenų EGF charakteristika

EGF buvo aptiktas visuose vaisiaus vandenų mėginiuose, o jo koncentracija buvo statistiškai reikšmingai susijusi su gestacijos amžiumi ir mažėjo didėjant GA (11 paveikslas). EGF koncentracijos mediana reikšmingai skyrėsi GA grupėse: 34,20 pg/ml – 22^{+0}-27^{+6} sav., 62,62 pg/ml – 28^{+0}-31^{+6} sav. ir 93,36 pg/ml – 32^{+0}-34^{+6} sav. ($p < 0,001$), atitinkamai. EGF kiekis teigiamai

koreliavo ir su GA, ir su gimimo svoriu (atitinkamai rho = 0,47 ir 0,46, p < 0.001).



11 paveikslas. Vaisiaus vandenų EGF koncentracijos pagal GA.

6.3.3.3 EGF ir VUAS ryšys

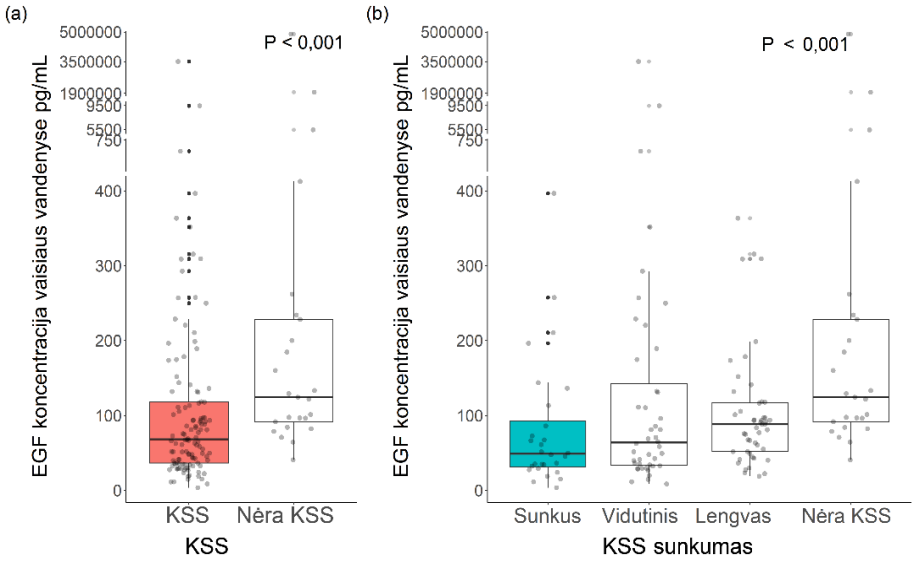
EGF koncentracijų medianos statistiškai patikimai nesiskyrė VUAS ir ne-VUAS grupėse. Dėl EGF ir GA ryšio, palyginome EGF koncentracijas VUAS ir ne-VUAS grupėse, atsižvelgę į gestacijos amžių (23 lentelė). Nustatėme, kad VUAS neturėjo statistiškai reikšmingos įtakos EGF koncentracijų pokyčiams vaisiaus vandenyse skirtingose GA grupėse. Be to, neradome reikšmingo EGF ir uždegimo citokinų, tokių kaip IL-6, TNF-α ir MMP-8, ryšio. Tačiau pastebėjome, kad EGF koncentracijos statistiškai reikšmingai skyrėsi lyginant VUAS ir ne-VUAS grupės naujagimius pagal sunkių baigčių buvimą.

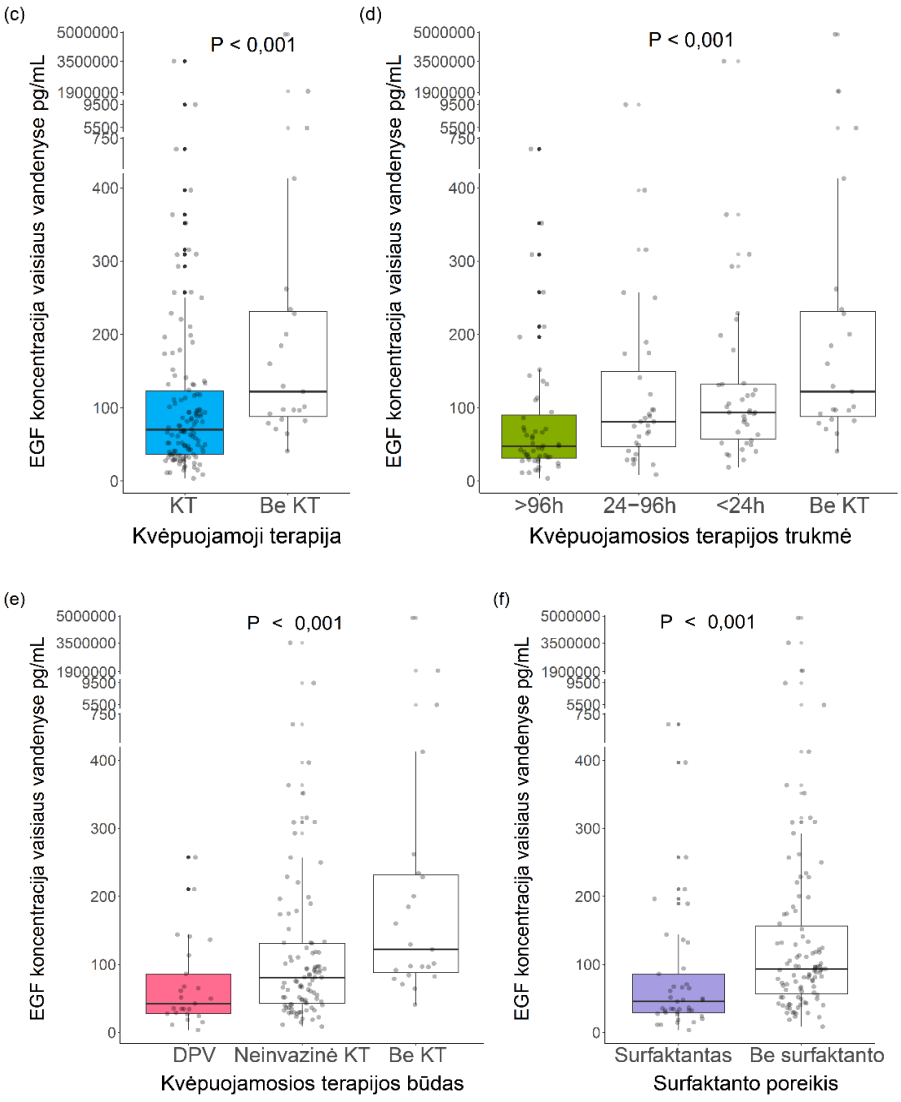
23 lentelė. VUAS įtaka EGF koncentracijų (pg/ml) medianoms gestacijos amžiaus grupėse.

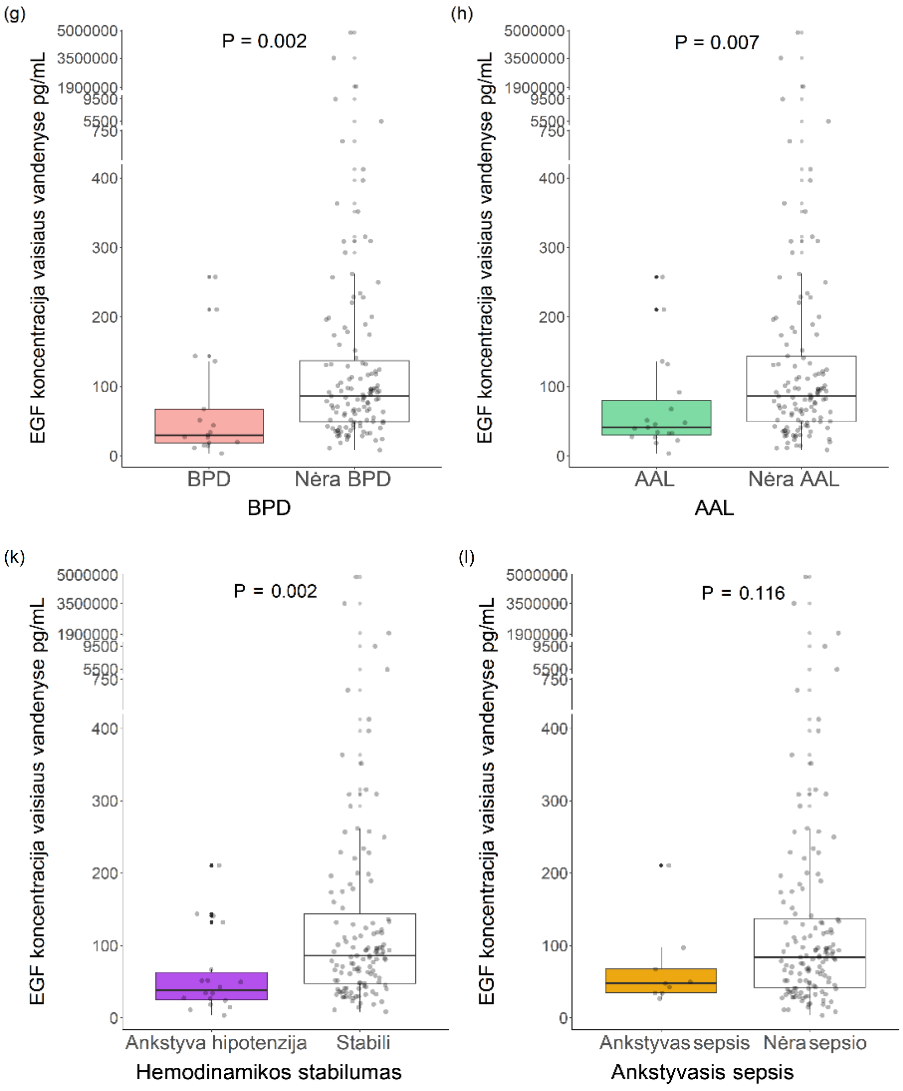
Gestacijos amžiaus grupės						
	22^{+0}–27^{+6} sav.		28^{+0}–31^{+6} sav.		32^{+0}–34^{+6} sav.	
	Mediana EGF	n	Mediana EGF		Mediana EGF	n
VUAS	32.7	11	41.1	15	93.4	65
Ne-VUAS	34.4	13	84.6	14	91.6	27
P reikšmė	0.931		0.513		0.764	

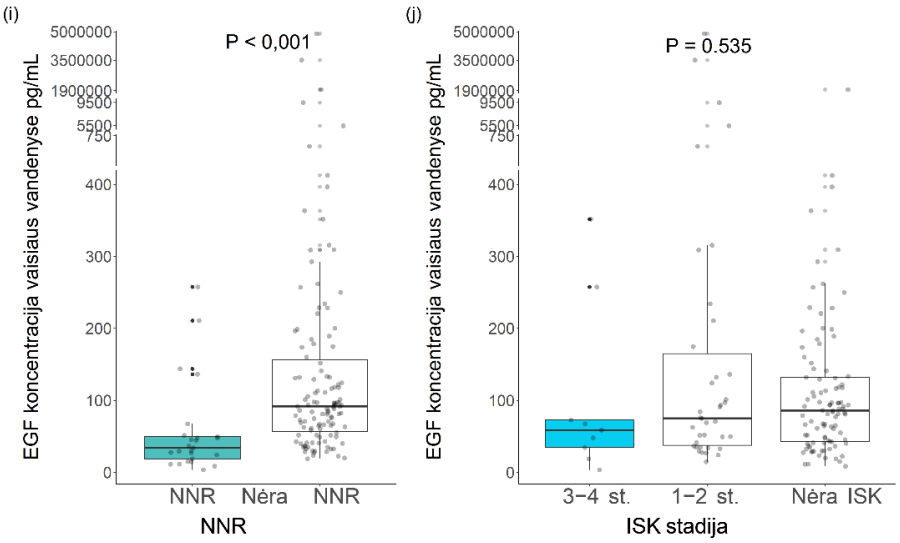
6.3.3.4 EGF ir nepalankios naujagimių baigtys

Nagrinėjome, ar vaisiaus vandenų EGF kiekis skiriasi lyginant naujagimius, kuriems nustatytos nepalankios baigtys, ir naujagimius be nepalankių baigčių. Naujagimių, kuriems nustatyta ankstyva hipotenzija, AAL, NNR, KSS ir kuriems reikėjo kvėpuojamosios terapijos ar surfaktanto, vaisiaus vandenų EGF koncentracijos buvo reikšmingai mažesnės nei naujagimių, kuriems šių būklių nebuvo (12 paveikslas a, b, c, d, e, f, g, h, i, k). Be to, EGF koncentracija mažėjo didėjant KSS sunkumui ir ilgėjant kvėpuojamosios terapijos trukmei. Nenustatėme reikšmingų EGF koncentracijos skirtumų tarp naujagimių, kuriems diagnozuotas ankstyvasis sepsis ar ISK, ir naujagimių, be šių būklių (12 paveikslas j ir l).




(c)
P < 0,001
EGF koncentracija vaisiaus vandenyse pg/mL
5000000
3500000
1900000
9500
5500
750
400
300
200
100
0
KT
Be KT
Kvėpuojamoji terapija
(d)
P < 0,001
EGF koncentracija vaisiaus vandenyse pg/mL
>96h
24−96h
<24h
Be KT
Kvėpuojamosios terapijos trukmė
(e)
P < 0,001
EGF koncentracija vaisiaus vandenyse pg/mL
DPV
Neinvazinė KT
Be KT
Kvėpuojamosios terapijos būdas
(f)
P < 0,001
EGF koncentracija vaisiaus vandenyse pg/mL
Surfaktantas
Be surfaktanto
Surfaktanto poreikis


(g)
P = 0.002
EGF koncentracija vaisiaus vandenyse pg/mL
5000000
3500000
1900000
9500
5500
750
400
300
200
100
0
BPD
Nėra BPD
BPD
(h)
P = 0.007
AAL
Nėra AAL
AAL
(k)
P = 0.002
Ankstyva hipotenzija
Stabili
Hemodinamikos stabilumas
(l)
P = 0.116
Ankstyvas sepsis
Nėra sepsio
Ankstyvasis sepsis



12 paveikslas. Vaisiaus vandenų EGF koncentracijų palyginimas tarp naujagimių, kuriems nustatytos ir nenustatytos nepalankios baigtys (a-j).

6.3.3.5 Logistinė EGF regresija numatant nepalankias naujagimių baigtis

Tyrime naudota vienanarė logistinė regresija, siekiant įvertinti EGF koncentraciją kaip svarbiausių naujagimių baigčių, tokių kaip AAL, NNR, ankstyva hipotenzija ir sunkios kvėpavimo būklės, prognozinį veiksnį (24 lentelė). Nustatyta, kad mažesnė EGF koncentracija leidžia prognozuoti nepalankias naujagimio baigtis, o mažėjant EGF koncentracijai šių baigčių tikimybė didėja. EGF riba 75 pg/ml, turėjo statistiškai patikimą prognozinę galią visoms baigtims, o EGF koncentracija <35 pg/ml buvo susijusi su didžiausia nepalankių naujagimių baigčių, sunkių kvėpavimo sutrikimų, AAL, NNR ir ankstyvos hipotenzijos rizika.

24 lentelė. Vienanarė EGF ribinių verčių logistinė regresija prognozuojant nepalankias naujagimio baigtis. Pilka spalva pažymi modelius, kurių galimybių santykis (GS) didžiausias.

EGF ribinė vertė (pg/ml)	Naujagimio baigtis	Koefi-cientas	P reikšmė	GS	95% PI
<100	Sunkus KSS	0.6286	0.187	1.88	0.77 – 5.08
	Surfaktanto poreikis	0.9354	0.029	2.55	1.14 – 6.17
	KT >4 d.	0.8972	0.022	2.45	1.14 – 6.17
	DPV	0.7422	0.179	2.10	0.83 – 6.09
	BPD	0.6804	0.257	1.97	0.66 – 7.32
	AAL	0.8492	0.151	2.34	0.79 - 8.57
	NNR	1.2667	0.028	3.55	1.26 - 12.74
	Ankstyva hipotenzija	0.7545	0.205	2.13	0.71 - 7.84
<75	Sunkus KSS	1.3146	0.004	3.72	1.56 – 9.64
	Surfaktanto poreikis	1.6245	<0.001	5.08	2.34 – 11.71
	KT >4 d.	1.7634	<0.001	5.44	2.79 – 12.75
	DPV	1.1676	0.007	3.21	1.33 – 8.38
	BPD	1.5124	0.012	4.54	1.51 – 16.81
	AAL	1.3688	0.013	3.93	1.41 - 12.78
	NNR	2.1556	<0.001	8.63	3.05 - 31.04
	Ankstyva hipotenzija	1.5865	0.008	4.88	1.65 - 17.99
<35	Sunkus KSS	1.1170	0.017	3.06	1.19 – 7.65
	Surfaktanto poreikis	1.9849	<0.001	7.28	3.03 – 18.47
	KT >4 d.	1.6275	<0.001	5.09	2.13 – 12.97
	DPV	1.4816	0.006	4.40	1.72 – 11.24
	BPD	2.2239	<0.001	9.24	3.15 – 28.76
	AAL	1.4005	0.007	4.06	1.41 - 11.41
	NNR	2.0949	<0.001	8.13	3.13 - 21.77
	Ankstyva hipotenzija	1.7377	0.001	5.684	1.99 - 16.45

Tolimesnė daugianarė regresinė analizė parodė (25 lentelė), kad, nors EGF kiekis <35 pg/ml, buvo stiprus naujagimių nepalankių baigčių prognozės veiksnys vienanarėje analizėje, gestacinis amžius pasirodė esąs svarbesnis baigčių prognozės veiksnys. Kiekviena papildoma nėštumo savaitė mažino 0,3-0,7 karto nepalankių naujagimio baigčių riziką. Atlikus analizę skirtingo GA grupėse, EGF <35 pg/ml išliko patikimas AAL, NNR, ankstyvosios hipotenzijos ir sunkių kvėpavimo baigčių prognozinis rodmuo naujagimiams, gimusiems 28^{+0}-31^{+6} nėštumo savaitę. Šios grupės naujagimių, EGF stoka didino nepalankių baigčių tikimybę 3-12 kartų. Tačiau kitose GA grupėse gestacijos amžius buvo patikimesnis prognozinis veiksnys nei EGF.

25 lentelė. Daugianarė EGF <35 pg/ml logistinė regresija numatant nepalankias naujagimio baigtis, atsižvelgus į gestacijos amžių.

Priklausomas kintamasis	Įvesties kintamieji	Kinta-masis	Koefi-cientas	P reikš-mė	GS	95 % PI
Sunkus KSS	EGF <35pg/ml + GA	EGF<35	-0.3572	0.567	0.7	0.19 – 2.26
		GA	-0.4072	<0.001	0.7	0.56 – 0.78
Surfaktanto poreikis		EGF<35	0.5996	0.345	1.82	0.51 – 6.28
		GA	-0.6879	<0.001	0.5	0.39 – 0.62
KT >4 d.		EGF<35	-0.0796	0.906	0.92	0.23 – 3.38
		GA	-0.8263	<0.001	0.44	0.32 – 0.56
DPV		EGF<35	0.2768	0.641	1.32	0.39 – 4.09
		GA	–0.3524	<0.001	0.7	0.59 – 0.83
BPD		EGF<35	0.3220	0.732	1.38	0.19 – 2.26
		GA	–1.286	<0.001	0.28	0.11 – 0.47
AAL		EGF<35	-0.4236	0.558	0.65	0.15 – 2.55
		GA	-0.5790	<0.001	0.56	0.43 – 0.69
NNR		EGF<35	0.5454	0.505	1.73	0.33 – 8.79
		GA	-0.9912	<0.001	0.37	0.24 – 0.51
Ankstyva hipotenzija		EGF<35	0.3181	0.637	1.37	0.35 – 5.00
		GA	-0.4167	<0.001	0.66	0.53 – 0.80
Sunkus KSS	EGF <35pg/ml+ 22^{+0}–27^{+6} sav. GA**	EGF<35	0.2361	0.669	1.27	0.38 – 3.77
		GA	2.2752	<0.001	9.73	3.38 – 30.00
Surfaktanto poreikis		EGF<35	1.3572	0.023	3.89	1.15 – 12.47
		GA	4.5830	<0.001	97.80	18.27 – 1821.60
DPV		EGF<35	0.6753	0.236	1.96	0.61 – 5.84
		GA	2.2915	<0.001	9.9	3.41 – 30.19
BPD		EGF<35	1.1423	0.191	3.13	0.57 – 19.15
		GA	5.3143	<0.001	203.21	33.3 – 3997.7
AAL		EGF<35	0.3890	0.553	1.48	0.38 – 5.12
		GA	2.7311	<0.001	15.35	4.73 – 54.70
NNR		EGF<35	1.4034	0.083	4.07	0.79 – 20.49
		GA	4.7885	<0.001	120.12	29.40 – 678.8
Ankstyva hipotenzija		EGF<35	0.7764	0.229	2.17	0.59 – 7.54
		GA	2.6621	<0.001	14.33	4.32 - 52.15
Sunkus KSS	EGF <35pg/ml+28^{+0}–31^{+6} sav. GA	EGF<35	1.0703	0.024	2.92	1.13 – 7.35
		GA	0.5004	0.31	1.65	0.60 – 4.26
Surfaktanto poreikis		EGF<35	1.9495	<0.001	7.03	2.90 – 17.94
		GA	0.6295	0.184	1.88	0.73 – 4.72
KT >4 d.		EGF<35	1.5894	<0.001	4.9	1.98 – 12.86

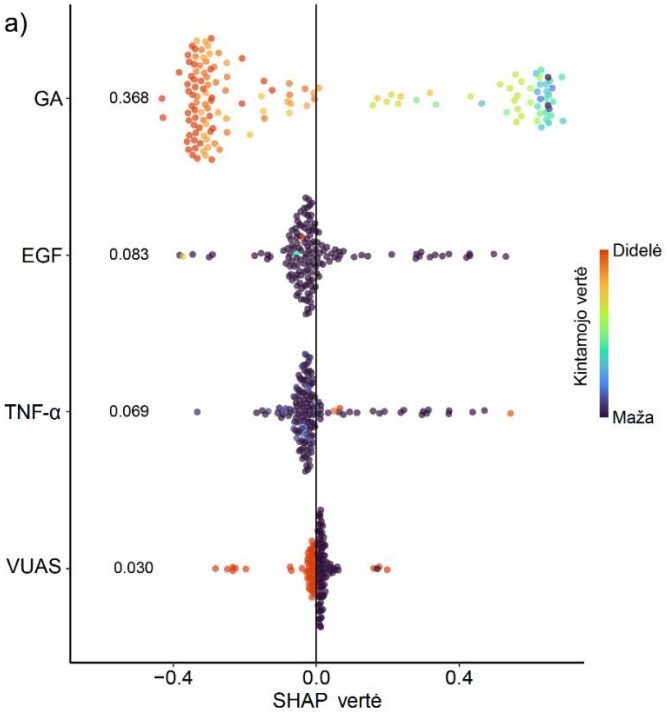
Priklausomas kintamasis	Įvesties kintamieji	Kinta-masis	Koefi-cientas	P reikš-mė	GS	95 % PI
		GA	1.3609	0.003	3.9	1.62 – 9.76
DPV		EGF<35	1.6155	0.001	5.03	1.91 – 13.41
		GA	–1.0486	0.13	0.35	0.07 – 1.19
BPD		EGF<35	2.5074	<0.001	12.27	3.95 – 41.13
		GA	–2.1194	0.056	0.12	0.01 – 0.72
AAL		EGF<35	1.3739	0.010	3.95	1.36 – 11.20
		GA	0.2380	0.687	1.27	0.36 – 3.84
NNR		EGF<35	2.1425	<0.001	8.52	3.23 – 23.38
		GA	-0.3542	0.562	0.70	0.19 – 2.18
Ankstyva hipotenzija		EGF<35	1.8694	<0.001	6.48	2.21 – 19.48
		GA	-1.1067	0.177	0.33	0.05 – 1.37
Sunkus KSS		EGF<35	0.2350	0.658	1.26	0.44 – 3.54
		GA	-2.2451	<0.001	0.11	0.03 – 0.29
Surfaktanto poreikis		EGF<35	1.1397	0.044	3.12	1.04 – 9.77
		GA	-3.0556	<0.001	0.05	0.02 – 0.12
KT >4 d.	EGF <35pg/ml + 32^{+0}–34^{+6} sav. GA **	EGF<35	0.5444	0.371	1.72	0.52 – 5.76
		GA	-3.2808	<0.001	0.04	0.01 – 0.09
DPV		EGF<35	1.0043	0.052	2.73	0.98 – 7.54
		GA	–1.1414	0.023	0.32	0.12 – 0.84
AAL		EGF<35	0.4506	0.438	1.57	0.49 – 4.90
		GA	-2.9451	<0.001	0.05	0.01 – 0.21
Ankstyva hipotenzija		EGF<35	1.0871	0.061	2.96	0.94 – 9.337
		GA	-1.7014	0.008	0.18	0.05 – 0.60

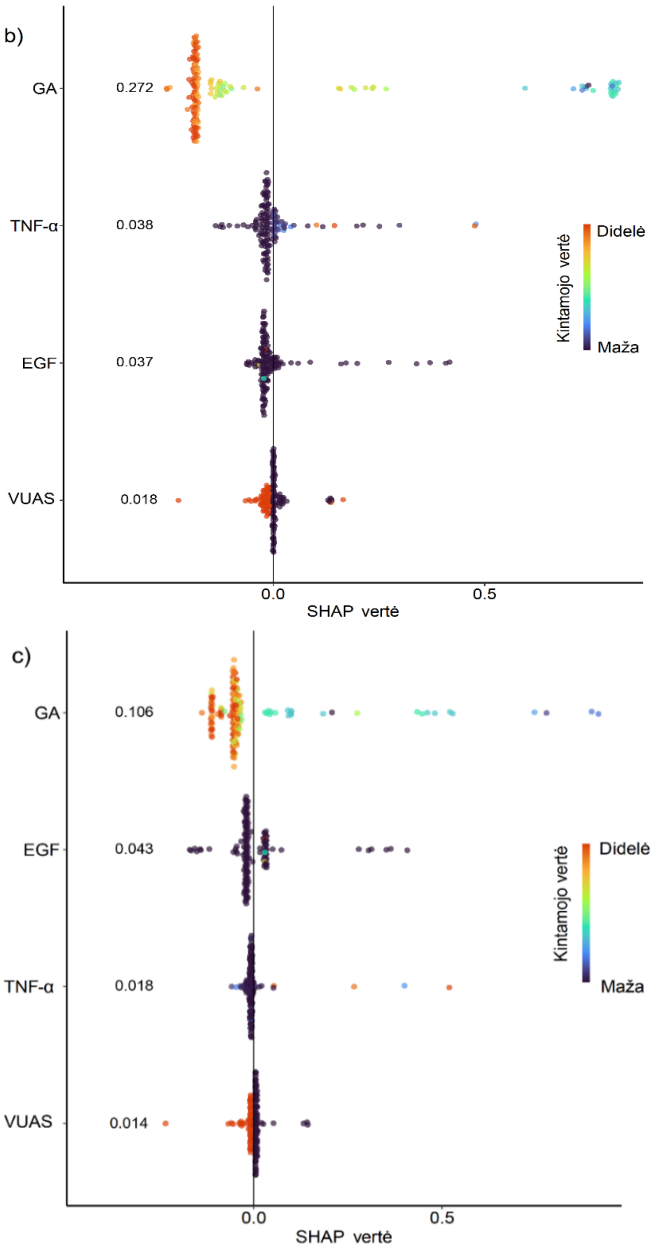
** Regresinė analizė neatlikta dėl nulinių verčių tam tikrose grupėse: KT >4 d. — 22^{+0}–27^{+6} sav. GA; NNR ir BPD – 32^{+0} –34^{+6} savaičių GA.

6.3.3.6 Atsitiktinių medžių klasifikatoriaus analizė prognozuojant nepalankias naujagimio baigtis

Siekdami prognozuoti nepalankių naujagimių baigčių, tokių kaip sunkus kvėpavimo sutrikimas, AAL, NNR ir ankstyva hipotenzija, riziką ir modeliuose įvertinti pagrindinius prognozinius rodmenis (GA, VUAS, vaisiaus vandenų EGF ir TNF-α), atlikome atsitiktinių medžių klasifikatoriaus analizę. Įvertinome tris modelius prognozuojančius sunkias kvėpavimo baigtis (13 paveikslas): sunkų KSS, surfaktanto poreikį, kvėpuojamoji terapija >4 d., DPV, BPD. Modelio, prognozuojančio bent vieną sunkią kvėpavimo takų būklę (13 paveikslas a), klasifikavimo tikslumas buvo 84 proc., jautrumas - 76 proc. ir specifiškumas 88 proc. Prognozuojant dvi ir daugiau sunkių

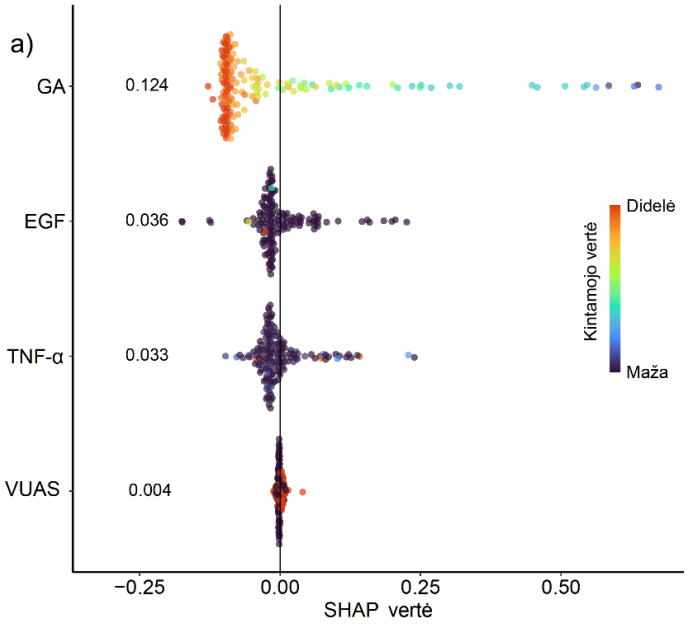
respiracinių baigčių (13 paveikslas b), tikslumas buvo 79 proc., tačiau jautrumas sumažėjo iki 33 proc., o specifiškumas - 97 proc.. Didžiausio tikslumo pasiekė modelis, prognozuojantis visas penkias sunkias kvėpavimo sistemos baigtis (13 paveikslas c) - 96 proc., jo jautrumas buvo puikus - 100 proc., o specifiškumas - 95 proc. GA buvo stipriausias prognozinis veiksnys, o didesnis GA buvo susijęs su mažesne neigiamų pasekmių rizika. EGF ir TNF-α taip pat turėjo vidutinę prognozavimo galią, o VUAS turėjo mažiausią įtaką, tačiau prisidėjo prie modelio tikslumo.

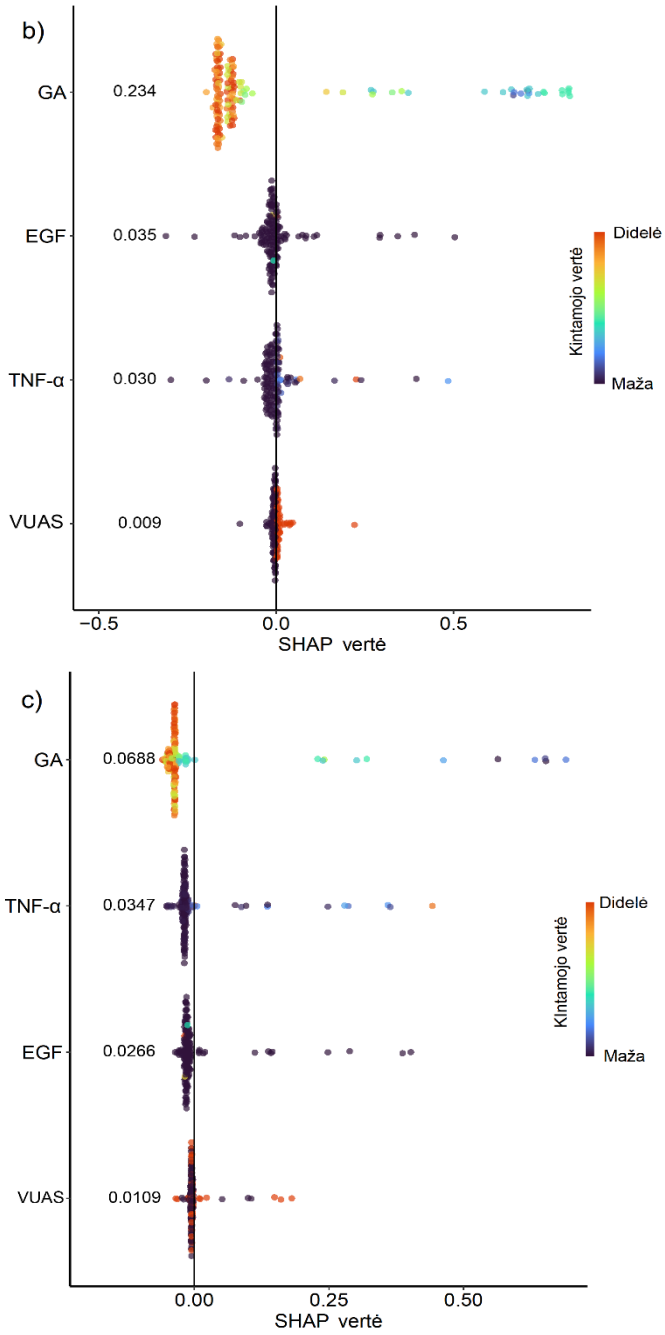




13 paveikslas. Prognozinių veiksnių reikšmė numatant kvėpavimo sutrikimų riziką SHAP diagramoje: a) bent vieno sunkaus kvėpavimo sutrikimo prognozė; b) 2 ir daugiau sunkių kvėpavimo baigčių prognozė; c) visų 5 sunkių kvėpavimo baigčių prognozė. Y ašyje prognoziniai veiksniai (GA, EGF, TNF-α ir VUAS) išdėstyti pagal svarbą mažėjimo tvarka.

Atsitiktinių medžių klasifikatoriaus AAL, NNR ir ankstyvosios hipotenzijos modeliai pasižymėjo stipriomis prognozavimo galimybėmis, dideliu tikslumu ir specifiškumu, tačiau įvairiu jautrumu. AAL prognozinio modelio klasifikavimo tikslumas siekė 90 proc., specifiškumas – 95 proc., o jautrumas – 60 proc. NNR prognozinis modelis pasiekė 95 proc. klasifikavimo tikslumą, 100 proc. specifiškumą ir 71 proc. jautrumą. Ankstyvosios hipotenzijos modelio tikslumas buvo 91 proc., specifiškumas – 100 proc., o jautrumas - tik 20 proc. Prognozinių veiksnių GA, TNF-α, EGF ir VUAS eiliškumas pagal svarbą pavaizduotas 14 paveiksle (a, b ir c). GA buvo svarbiausias prognozinis rodmuo, o didesnis GA mažino šių nepalankių baigčių tikimybę. EGF ir TNF-α turėjo vidutinę prognostinę vertę. VUAS buvo mažiausiai įtakingas prognozinis veiksnys, tačiau jo pašalinimas iš modelių mažino modelių tikslumą.





14 paveikslas. Prognozinių veiksnių reikšmė, numatant AAL, NNR ir ankstyvos hipotenzijos riziką SHAP diagramoje: a) AAL modelis; b) NNR modelis c) ankstyvos hipotenzijos modelis. Y ašyje prognoziniai veiksniai (GA, EGF, TNF-α ir VUAS) išdėstyti pagal svarbą mažėjimo tvarka.

6.4 Išvados

1. Padidėjusios imunologinių žymenų TNF-α, MMP-8, IL-6, IL-10, IL-17, DEFA3 ir suPAR koncentracijos neinvaziniu būdu paimtuose vaisiaus vandenyse yra patikimi VUAS prognoziniai rodmenys, o TNF-α turi didžiausią prognozinę vertę. RANTES, sTLR-2, sTLR-4 ir EGF nėra statistiškai reikšmingi VUAS prognozei.
2. Vaisiaus vandenų imunologinių žymenų TNF-α, IL-6, MMP-8 ir IL-17, prognozuojančių VUAS, koncentracijos koreliuoja su virkštelės kraujo IL-6 koncentracija.
3. Padidėjusi TNF-α, MMP-8, IL-6, IL-10, IL-17 ir DEFA3 koncentracija neinvaziniu būdu paimtuose vaisiaus vandenyse yra susijusi su sunkiomis neišnešiotų naujagimių, sergančių VUAS, baigtimis, o TNF-α yra stipriausias VUAS ir sunkių naujagimių baigčių prognozinis rodmuo. Sumažėjęs EGF kiekis vaisiaus vandenyse yra susijęs su nepalankiomis neišnešiotų naujagimių baigtimis. RANTES, sTLR-2, sTLR-4, ir suPAR neturi statistiškai reikšmingo ryšio su VUAS paveiktų naujagimių sunkiomis baigtimis.
4. Imunologinių žymenų koncentracijų pokyčiai prognozuoja padidėjusią VUAS ir nepalankių neišnešiotų naujagimių baigčių tikimybę:
    a. Optimalios ribinės vertės VUAS prognozuoti yra šios: TNF-α > 89 pg/ml, MMP-8 > 170 ng/ml, IL-6 > 2695 pg/ml, IL-10 > 35 pg/ml, IL-17 > 1 pg/ml, DEFA3 > 34 ng/ml ir suPAR > 27 ng/ml.
    b. Optimalios ribos vertės, rodančios padidėjusią VUAS ir sunkių naujagimio baigčių tikimybę, yra šios: TNF-α > 231 pg/ml, MMP-8 > 413 ng/ml, IL-6 > 3999 pg/ml, IL-10 > 35 pg/ml, IL-17 > 4 pg/ml, DEFA3 > 35 ng/ml.
    c. Sumažėjęs EGF kiekis < 35 pg/ml 3-9 kartų didina sunkių kvėpavimo baigčių, AAL, NNR ir ankstyvosios hipotenzijos tikimybę, ypač 28^{+0}-31^{+6} gestacijos savaičių naujagimiams.
5. Padidėjęs TNF-α kiekis ir sumažėjęs EGF kiekis neinvazyviai paimtuose vaisiaus vandenyse kartu su gestacijos amžiumi ir VUAS diagnoze leidžia antenataliai numatyti neišnešiotų naujagimių nepalankių baigčių riziką.

1. VUAS diagnozės nustatymas yra rekomenduojamas kaip papildomas neišnešiotų naujagimių <34^{+6} sav. GA būklės įvertinimas.
    1) Po gimimo nustatytas virkštelės kraujo IL-6 kiekis > 11 pg/ml ir (arba) histologinis funizitas, rodo VUAS.
    2) Esant galimybei, naudinga atlikti imunologinę vaisiaus vandenų analizę.
    3) Neinvaziniu būdu paimtų vaisiaus vandenų žymenų optimalios ribinės vertės leidžia prognozuoti VUAS:
        - Rekomenduojamas atlikti VUAS prognozinis žymuo yra TNF-α. TNF-α > 89 pg/ml (jautrumas 66 proc., specifiškumas 90 proc.) rodo 15 kartų padidėjusią VUAS riziką.
        - MMP-8 > 170 ng/ml (jautrumas 78 proc., specifiškumas 71 proc.) 8 kartus didina VUAS galimybę,
        - IL-6 > 2695 pg/ml (jautrumas 76 proc., specifiškumas 76 proc.) – 6 kartus,
        - IL-10 > 35 pg/ml (jautrumas 61 proc., specifiškumas 87 proc.) – 10 kartų,
        - IL-17 > 1 pg/ml (jautrumas 61 proc., specifiškumas 75 proc.) – 5 kartus,
        - DEFA3 > 34 ng/ml (jautrumas 35 proc., specifiškumas 92 proc.) - 7 kartus,
        - SuPAR > 27 ng/ml (jautrumas 65 proc., specifiškumas 65 proc.) – 3 kartus.
    4) Mažos uždegimo žymenų koncentracijos leidžia atmesti VUAS diagnozę.
2. Neišnešiotų naujagimių <34^{+6} sav. GA nepalankių baigčių rizikos įvertinimas:
    1) Jei įmanoma, naudinga atlikti imunologinę vaisiaus vandenų analizę.
    2) Neinvaziniu būdu paimtų vaisiaus vandenų žymenų optimalios ribinės vertės leidžia įvertinti VUAS ir sunkių naujagimio baigčių prognozę:
        - TNF-α > 231 pg/ml (jautrumas 60 proc., specifiškumas 89 proc.) 12 kartų didina VUAS ir sunkių naujagimio baigčių riziką,

- MMP-8 > 413 ng/ml (jautrumas 65 proc., specifiškumas 72 proc.) 5 kartus didina VUAS ir sunkių naujagimio baigčių galimybę,
- IL-6 > 3999 pg/ml (jautrumas 59 proc., specifiškumas 74 proc.) – 4 kartus,
- IL-10 > 35 pg/ml (jautrumas 59 proc., specifiškumas 74 proc.) – 4 kartus,
- IL-17 > 4 pg/ml (jautrumas 50 proc., specifiškumas 84 proc.) – 5 kartus,
- DEFA3 > 35 ng/ml (jautrumas 36 proc., specifiškumas 89 proc.) – 4 kartus,
- EGF < 35 pg/ml 3-9 kartų didina sunkių kvėpavimo baigčių, AAL, NNR ir ankstyvosios hipotenzijos tikimybę.

3) TNF-α > 231 pg/ml ir EGF < 35 pg/ml kartu su gestacijos amžiumi ir VUAS diagnoze rodo padidėjusią nepalankių naujagimio baigčių riziką.

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PUBLICATION LIST

Publications related to the topic of the thesis

1. Gulbinienė Violeta, Balčiūnienė Greta, Dumalakienė Irena, Vilienė Rita, Pilypienė Ingrida, Ramašauskaitė Diana. (2022). The significance of TNF-α and MMP-8 concentrations in non-invasively obtained amniotic fluid predicting fetal inflammatory response syndrome. International Journal of Gynecology and Obstetrics, 160(2), 476–482. https://doi.org/10.1002/ijgo.14478
2. Gulbinienė Violeta, Dumalakienė Irena, Balčiūnienė Greta, Pilypienė Ingrida, Narkevičiūtė Ieva, Novickij Vitalij, Vyšniauskis Gintautas, Ramašauskaitė Diana. (2024). Soluble urokinase plasminogen activator receptor in vaginally collected amniotic fluid predicting fetal inflammatory response syndrome: a prospective cohort study. BMC Pregnancy and Childbirth, 24(1:54), 1–12. https://doi.org/10.1186/s12884-023-06221-0
3. Gulbinienė Violeta, Balčiūnienė Greta, Petronienė Justina, Vilienė Rita, Dumalakienė Irena, Pilypienė Ingrida, Ramašauskaitė Diana. (2022). The significance of epidermal growth factor in noninvasively obtained amniotic fluid predicting respiratory outcomes of preterm neonates. International Journal of Molecular Sciences, 23(6:2978), 1–15. https://doi.org/10.3390/ijms23062978

Other publications

1. Balčiūnienė G, Kvederaitė-Budrė G, Gulbinienė V, Dumalakienė I, Vilienė R, Pilypienė I, Drąsutienė GS, Ramašauskaitė D. (2021). Neutrophil-lymphocyte ratio for the prediction of histological chorioamnionitis in cases of preterm premature rupture of membranes: a case-control study. BMC Pregnancy and Childbirth, 21(1), 656–656. https://doi.org/10.1186/s12884-021-04101-z
2. Balčiūnienė G, Gulbinienė V, Kvederaitė-Budrė G, Dumalakienė I, Vilienė R, Pilypienė I, Drąsutienė GS, Ramašauskaitė D. (2021). A value of soluble Toll-like receptor 2 and 4 in vaginally obtained amniotic fluid for the prediction of histological chorioamnionitis. Acta Obstetricia et Gynecologica Scandinavica, 100(12), 2209–2215. https://doi.org/10.1111/aogs.14228

3. Balčiūnienė G, Gulbinienė V, Dumalakienė I, Vilienė R, Bartkevičienė D, Pilypienė I, Drąsutienė GS, Ramašauskaitė D. (2021). Prognostic Markers for Chorioamnionitis: IL-6, TNF-α, and MMP-8 in Vaginally Obtained Amniotic Fluid. Journal of Clinical Medicine, 10(5), 1136. https://doi.org/10.3390/jcm10051136
4. Balčiūnienė G, Kvederaitė-Budrė G, Gulbinienė V, Pilypienė I, Drąsutienė GS, Ramašauskaitė D. (2021). Antenatalinė chorioamnionito diagnostika. Lithuanian Obstetrics and Gynaecology. 3:190-194
5. Gulbinienė V, Pilypienė I, Navarackaitė I, Dauengauer-Kirlienė S, Balčiūnienė G, Ramašauskaitė D. (2020). Bevandenio laikotarpio trukmės įtaka neišnešiotų naujagimių, gimusių 24–32 gestacijos savaitę, baigtims. Lithuanian Obstetrics and Gynaecology. 23:26-32
6. Dauengauer-Kirlienė S, Prunskienė H, Lisauskienė L, Lisauskas V, Pilypienė I, Gulbinienė V. (2020). Įgimtos citomegalo viruso infekcijos diagnostika atliekant galvos smegenų echoskopiją: klinikinis atvejis ir literatūros apžvalga. Laboratorinė medicina, 22(1), 15–18.
7. Vita Langienė, Arūnas Liubšys, Violeta Drejerienė, Violeta Gulbinienė. Naujagimio įgimta odos aplazija. Klinikinis atvejis. 2021, Lithuanian Obstetrics & Gynecology; 24(1):79-82. DOI: 10.37499/LAG.611.

Presentations on the topic of the thesis

1. 5th JENS Congress, September 18-23rd 2023, Rome, Italy. V. Gulbinienė, G. Balčiūnienė, I. Dumalakienė, R. Vilienė, I. Pilypienė, D. Ramašauskaitė. The association between TNF-α in non-invasively obtained amniotic fluid and outcomes in preterm infants with fetal inflammatory response syndrome.
2. 28th EBCOG Congress, May 18-20th 2023, Krakow, Poland. V. Gulbinienė, I. Dumalakienė, G. Balčiūnienė, I. Pilypienė, D. Ramašauskaitė. Soluble urokinase-type plasminogen activator receptor as biomarker of intra-amniotic inflammation in vaginally collected amniotic fluid.
3. 12th International Congress of UENPS; Krakow (Poland); September 2nd-4th, 2022. V. Gulbinienė, G.Balčiūnienė, R.Vilienė, I.Dumalakienė, I.Pilypienė, D.Ramašauskaitė. Predictive markers for

bronchopulmonary dysplasia in noninvasively obtained amniotic fluid in preterm neonates. The winner of the best abstract. https://doi.org/10.7363/120104

4. 4th Summer discussions on Neonatology congress, July 15-18th 2022, Avignon, France. V. Gulbinienė, G.Balčiūnienė, R.Vilienė, I.Dumalakienė, I.Pilypienė, D.Ramašauskaitė. Predictive markers for fetal inflammatory response syndrome in noninvasively obtained amniotic fluid.
5. 23rd FIGO congress, October 21st-28th 2021, Sidney, Australia. V. Gulbinienė, G. Balčiūnienė, J. Petronienė, R. Vilienė, I. Dumalakienė, I. Pilypienė, D. Ramašauskaitė. The Significance of TNF-α, MMP-8 Concentrations in Non-Invasively Obtained Amniotic Fluid Predicting Fetal Inflammatory Response Syndrome. Selected as one of the five best presentations and invited for publication in Q1.
6. 23rd FIGO congress, October 21st-28th 2021, Sidney, Australia. G. Balčiūnienė, V. Gulbinienė, I. Dumalakienė, R. Vilienė, I. Pilypienė, G. Drąsutienė, D. Ramašauskaitė. Predictive value of suPAR on Chorioamnionitis.
7. 27th EBCOG congress, September 2nd-4th 2021, Athens, Greece. V. Gulbinienė, G. Balčiūnienė, J. Petronienė, R. Vilienė, I. Dumalakienė, I. Pilypienė, D. Ramašauskaitė. The significance of epidermal growth factor concentrations in non-invasively obtained amniotic fluid predicting respiratory outcomes of preterm neonates.
8. 27th EBCOG congress, September 2nd-4th 2021, Athens, Greece. G. Balčiūnienė, V.Gulbinienė, I.Dumalakienė, R.Vilienė, I.Pilypienė, G.Drąsutienė, D. Ramašauskaitė. Neutrophil-lymphocyte ratio for the prediction of chorioamnionitis.
9. Conference of Lithuanian Neonatology Association “Innovations in neonatology and other relevant issues”, May 31st, 2024, Vilnius, Lithuania. V. Gulbinienė. Innovations and opportunities in the diagnosis of fetal inflammatory response syndrome.
10. Scientific-practical conference “Obstetrics and Gynaecology Clinic 220: past, present, future”, November 24th, 2023, Vilnius, Lithuania. V. Gulbinienė. Non-invasive analysis of amniotic fluid biomarkers and its implications for fetal/newborn health prognosis.

CONCISE INFORMATION ABOUT THE DOCTORAL STUDENT

PhD candidate Violeta Gulbinienė is a neonatologist and pediatrician at Vilnius University Hospital Santaros Klinikos, where she works in the Neonatology Department, Neonatology Center and Obstetrics and Gynecology Center. Since 2020, she has also been a lecturer at Vilnius University Faculty of Medicine, mentoring medical students and serving as a junior assistant in neonatology and pediatrics.

Her qualifications are bolstered by extensive postgraduate training and certifications in neonatal care both in Lithuania and abroad, including Italy, Poland, France, Australia, Greece, Austria, and Germany. She earned her medical degree from Vilnius University Faculty of Medicine in 2008, completed an internship there in 2009, and pursued a residency in pediatrics and neonatology from 2009 to 2017. Beginning in 2020, she commenced her doctoral studies in medical sciences at Vilnius University.

Her research activities include roles as an investigator in several biomedical studies focusing on neonatal intensive care, infection, and advanced neonatal therapies. She has been certified in Good Clinical Practice for researchers since 2012. From 2010 to 2015, she worked as a researcher in European multicenter clinical trials (Phase III Neomero 1 and Phase I-II Neomero 2) evaluating the pharmacokinetics, safety, and efficacy of Meropenem for treating neonatal sepsis and meningitis. Additionally, from 2017 to 2020, she was a researcher in the multicenter randomized controlled trial "CORSAD28", which compared new versus old systems with a T-Piece device for the stabilization of infants born before 28 weeks of gestation. Between 2013 and 2016, she coordinated biomedical research projects at VULSK Children’s Hospital, underscoring her commitment to advancing neonatal care through both clinical practice and research.

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