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Ultrazvukové markery infekčních komplikací u předčasného odtoku plodové vody

Ultrasonographic markers of infection-related complications in preterm prelabour rupture of membranes

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DECLARATION

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ABBREVIATIONS

CI	Confidence interval
DAMPs	Damage associated molecular patterns
ECM	Extracellular matrix
ELISA	Enzyme-linked immunoassay
IAI	Intra-amniotic inflammation
IL	Interleukin
IQR	Interquartile range
MIAC	Microbial invasion of the amniotic cavity
PAMPs	Called-pathogen associated molecular patterns
PCR	Polymerase chain reaction
PI	Pulsatile index
PPROM	Preterm prelabour rupture of membranes
PTD	Preterm delivery
PTL	Preterm labour with intact membranes
ROC	Receiver operation characteristic
TLRs	Toll-like receptors
TNF	Tumour necrosis factor

SUMMARY

Preterm prelabour rupture of membranes (PPROM) represents a serious pregnancy complication associated with approximately 30% of preterm deliveries. PPROM might be complicated by the presence of microorganisms and/or their nucleic acids in amniotic fluid termed microbial invasion of the amniotic cavity (MIAC), and the elevation of various inflammatory mediators in the amniotic fluid referred to as intra-amniotic inflammation (IAI). Based on their presence or absence, four subgroups of PPROM can be defined: i) intra-amniotic infection (presence of both MIAC and IAI), ii) sterile IAI (IAI alone), iii) colonisation of the amniotic cavity (MIAC alone), and iv) absence of both MIAC and IAI.

Although gestational age at delivery is the most important factor affecting the risk of neonatal morbidity and mortality, the presence of MIAC and/or IAI might worsen neonatal outcomes. Therefore, precise assessment of the intra-amniotic environment seems essential for ideal personalised management of PPROM pregnancies.

Modern ultrasound machines allow a very detailed examination of the foetus. The effort to identify surrogate ultrasound markers of MIAC and/or IAI represents a logical research step in this field. One of the most promising results has been found on doppler assessment on blood flow in the foetal splenic vein, a part of the foetal portal system. Therefore, the first specific aim of this study was to evaluate the pulsatile index (PI) of the splenic vein, the main portal stem, the left portal vein, and ductus venosus in the foetuses from PPROM pregnancies with respect to the presence or absence of IAI. The second specific aim of this study was to identify PI's diagnostic indices on the selected parts of the foetal portal system to predict IAI in females with PPROM.

Both specific aims were performed in the same study population, consisting of 81 females with PPROM. The presence of IAI was associated with higher PI in the splenic vein but no differences were observed in the left portal branch, ductus venosus, and portal stem between pregnancies with and without IAI. The PI value of 0.36 on the splenic vein was identified to be optimal to predict IAI in pregnancies complicated by PPROM.

Aside from the ultrasound assessment, the direct evaluation of amniotic fluid is the most precise method to investigate the intra-amniotic environment. Several promising markers of inflammation have been proposed, including a family of granzymes, particularly extracellular granzyme A. Therefore, the third specific aim of this study was to establish an association

between concentrations of extracellular granzyme A in amniotic fluid and the presence of MIAC and/or IAI. The fourth specific aim was to determine the diagnostic indices of extracellular granzyme A in amniotic fluid.

The third and fourth specific aims were performed in the same study population, consisting of 166 females with PPROM. The concentration of extracellular granzyme A in amniotic fluid was elevated in the presence of sterile IAI. A concentration of amniotic fluid extracellular granzyme A of 33.4 pg/mL was found to be an optimal cut-off value to predict the presence of sterile IAI in PPROM pregnancies.

SOUHRN

Předčasný odtok plodové vody před termínem porodu (preterm prelabor rupture of membranes, PPROM) představuje závažnou komplikaci těhotenství a je odpovědný za přibližně 30% předčasných porodů. PPROM může být komplikován přítomností mikroorganismů a/nebo jejich nukleových kyselin v plodové vodě – tento stav se nazývá mikrobiální invaze dutiny děložní (microbial invasion of the amniotic cavity, MIAC). PPROM může být také doprovázen zvýšenou hladinou různých ukazatelů zánětlivu v plodové vodě – tento stav se nazývá intra-amniální zánět (intra-amniotic inflammation, IAI). Na základě přítomnosti MIAC a IAI lze definovat čtyři podskupiny PPROM: i) intra-amniální infekce (jsou přítomny MIAC a IAI), ii) sterilní IAI (přítomen pouze IAI), iii) kolonizace amniální dutiny (přítomen pouze MIAC), iv) nepřítomnost MIAC i IAI.

Ačkoliv gestační stáří v době porodu představuje nejdůležitější faktor ovlivňující novorozeneckou morbiditu a mortalitu, přítomnost MIAC a/nebo IAI může tyto novorozenecké výsledky zhoršit. Na základě těchto informací se diagnosticko-terapeutický postup založený na precizním posouzení intra-amniálního prostředí jeví jako optimální u těhotenství komplikovaných PPROM.

Moderní ultrazvukové přístroje umožňují vyšetřit plod velice podrobně. Snaha o nalezení robustního ultrazvukového markeru predikující přítomnost MIAC a/nebo IAI tak logicky představuje další krok ve výzkumu těchto komplikací. Velice slibné výsledky přineslo dopplerovské vyšetření průtoku krve v lienální žíle plodu, která je součást portálního systému. Proto byl první cíl této práce zaměřen na porovnání hodnot pulsatilního indexu (PI) v lienální žíle, hlavním portálním kmenu, levé portální žíle a ve venozním duktu u těhotných s PPROM s přítomností a absencí IAI. Druhým cílem bylo stanovení diskriminačních hodnot PI s nejlepšími prediktivními hodnotami pro stanovení IAI.

Stanovení obou těchto cílů práce bylo provedeno na stejné kohortě pacientů. Ta se sestávala z 81 těhotných s jednočetným těhotenstvím komplikovaným PPROM. Přítomnost IAI byla spojena s vyšší hodnotou PI v lienální žíle oproti absence PI. Hodnoty PI v levém portálním kmenu, levé portální žíle a ve venozním duktu nebyly změněny mezi skupinami těhotných žen s PPROM s a bez IAI. Hodnota PI v lienální žíle 0,36 byla nalezena jako optimální k identifikaci přítomnosti IAI u těhotenství komplikovaných PPROM.

I přes slibné výsledky které přináší ultrazvukové vyšetření plodu, vyšetření plodové vody představuje nejpřesnější metodu k posouzení intra-amniálního prostředí. Bylo již navrženo mnoho potenciálních ukazatelů zánětů, včetně rodiny granzymů, především extracelulárního granzymu A. Proto byl třetí hlavní cíl této práce zaměřen na stanovení hladin extracelulárního granzymu A v plodové vodě s ohledem na přítomnosti MIAC a/nebo IAI. Čtvrtý cíl práce byl zaměřen na stanovení diskriminační hladiny extracelulární granzymu A v plodové vodě pro predikci těchto komplikací.

Třetí i čtvrtý cíl práce byly provedeny na stejné kohortě pacientů, kterou tvořilo 166 těhotných žen s jednočetným těhotenstvím komplikovaným PPROM. Zvýšená hladina extracelulárního granzymu A plodové vodě byla nalezena u skupiny těhotných žen s PPROM se sterilním IAI. Diskriminační hladina extracelulárního granzymu A v plodové vodě 33,4 pg/mL byla identifikována jako optimální pro predikci přítomnosti sterilního IAI u těhotných žen s PPROM.

1. INTRODUCTION

1.1. Preterm delivery

According to the World Health Organisation, preterm delivery (PTD) is defined as delivery before 37 weeks or 259 days of gestation are completed (1). PTD affects 5-18% of pregnancies globally, with considerable differences between various parts of the world. In Europe, the PTD rate oscillates between 5-9% (2). Despite the influx of new information (e.g., administration of vaginal progesterone to prevent preterm birth and adverse perinatal outcome) (3-5), the PTD rate has not decreased in the last decades.

PTD represents the leading cause of perinatal mortality (about 75% of perinatal mortality results from PTD) and morbidities, such as cerebral palsy, retinopathy, developmental delay, and others (3-8). There is a close relationship between these outcomes and gestational age at delivery – lower gestational age represents a higher risk for adverse perinatal outcomes and vice versa (9-11). Collectively, the reduction of the PTD rate remains the main challenge of modern perinatal medicine.

PTD represents a heterogeneous condition, a syndrome induced by many causes (12). According to this heterogeneity, PTD can be analysed from different points of view. Based on the gestational age at delivery, PTD can be divided into four subgroups: i) extreme preterm delivery, which occurs before 28 weeks of gestation, represents about 5% of PTD and is connected with extreme prematurity of newborn; ii) early preterm delivery, which occurs between 28 - 31 weeks of gestation, represents about 15 – 20% of PTD and is connected with severe prematurity of newborn; iii) moderate preterm delivery, which occurs between 32 – 33 weeks of gestation, represents about 20% of PTD and is connected with moderate prematurity of newborn; iv) late preterm delivery, which occurs between 34 – 36 weeks of gestation, represents about 60 – 70% of PTD and is connected with the lowest prematurity of newborn (13). Based on the clinical point of view, PTD can be divided into three subgroups: i.) iatrogenic preterm birth (provider-initiated), which occurs because of maternal complications or foetal disease (app. 30-35% of PTD) ii.) spontaneous preterm birth with intact membranes (PTL) (app. 40-45% of PTD); and iii.) preterm prelabour rupture of the membranes (PPROM), (app. 25 - 30% of PTD) (13-15).

1.2. PPROM

PPROM, defined as leakage of amniotic fluid before the onset of regular labour activity before the 37th week of pregnancy, occurs in 2-8% of all singleton pregnancies (16). PPROM is associated with approximately 30% of preterm deliveries and serious perinatal morbidity and mortality (4, 9, 13, 17-20). No clear consensus exists regarding a period between the leakage of amniotic fluid and onset of the labour, but many authors suggest that there should be a minimum of one hour to make a diagnosis of PPROM (21, 22).

1.2.1. Risk factors

Several epidemiological and clinical factors, like maternal reproductive tract infections (e.g., bacterial vaginosis, an infection caused by *Trichomonas vaginalis*, *Neisseria gonorrhoea*, and *Chlamydia trachomatis*), behavioural factors (e.g., cigarette smoking, substance abuse, poor nutritional status, and coitus during pregnancy), obstetric complications (e.g., multiple gestation, polyhydramnios, incompetent cervix, bleeding, prior cervical surgery, and antenatal trauma), environmental factors (e.g., stress and toxin exposure), and genetic predisposition are associated with a higher incidence of PPROM (23-30). Moreover, biochemical signals from the foetus, including endocrine signals that promote foetal membrane apoptosis, can be included in the initiation of PPROM (28-32). Yet, the understanding of the risk factors and risk-induced pathophysiology is still lacking. It is in line with the fact that PPROM represents a condition that is still unpredictable and unpreventable. This represents major difficulty in developing diagnostic markers to predict pregnancies at high risk of developing PPROM and creating adequate preventive strategies to reduce the risk of the PPROM.

1.2.2. Pathophysiology

For a better understanding of the pathophysiology, PPROM can be classified into 3 major groups: i) PPROM in the absence of cervical change; ii) PPROM associated with cervical changes, which is more similar to spontaneous preterm labour with intact membranes; and iii) PPROM involving bleeding disorders or coagulopathies that may be related to placental abruption (33).

There is a solid body of evidence that PPROM is a disease of foetal membranes (34). Foetal membranes form a barrier between the fetoplacental and maternal compartments, and their

major function is to protect the foetus during its growth and development in utero. Foetal membranes are formed from two layers – amnion and chorion. Amnion represents a single layer of amnion epithelial cells that lines the interior of the amniotic cavity, while chorion represents multilayered trophoblast cells connected to maternal decidua (35). The connection between these two layers is provided by the extracellular matrix (ECM), which is formed of various types of collagens. These collagens provide tensile strength to the membranes, and they are connected with foetal membranes by type IV collagen-rich basement membrane. Proteolysis, specifically directed to dismantle the structural integrity of the ECM, weakens the membranes and disrupts the layers resulting in PPROM (36). This process is started as a result of the interaction of many factors, e.g., oxidative stress and pathologic oxidative stress-activation of senescence (34, 37). PPROM risk factors are known as inductors of oxidative stress (38-40). Moreover, oxidative stress is an inseparable component of inflammation, and the inflammation-oxidative stress axis plays a major role in producing pathways that can lead to membrane weakening through a variety of processes (41). Similarly, abruption-associated thrombin, matrix metalloproteinase activation, and collagenolytic processes have also been reported in foetal membrane weakening and PPROM (42). Oxidative stress-induced senescence and senescence-associated changes are likely the initiators in PPROM patients without cervical change and a subset of those with cervical change.

1.2.3. Diagnosis of PPROM

The diagnosis of PPROM is usually suspected from patient history and subsequently confirmed during a clinical examination or using biochemical tests (21). Patient history plays an important role in the diagnosis algorithm, and with its accuracy, about 90% should not be overlooked (43). The Temesvary test represents the most common verification of amniotic fluid leakage. This test is based on changing the acidic vaginal pH to alkaline in the event of PPROM. This change can be shown by the colour reaction of bromthymol from yellow to green in an alkaline environment. Despite its wide availability, a high false-positive rate (related to contamination with vaginal discharge, urine, blood or semen) makes this test inappropriate to determine the diagnosis of PPROM (44). The gold standard for the diagnosis of PPROM is represented by visualisation of amniotic fluid leakage from the cervix or pooling amniotic fluid in the posterior vaginal fornix. This can be visualised during the speculum examination and remains the most objective method for diagnosing PPROM (9, 18, 44, 45). In case of diagnostic doubts, some modern tests can be used. These tests are based on confirmation of insulin-like growth factor-

binding protein 1 (Actim^{TM} PROM test) and placental alpha macroglobulin 1 (Amnisure ROM test) in cervicovaginal discharge, and both are very sensitive and specific (46, 47).

1.2.4. Clinical management

The clinical management of PPROM pregnancies represents balancing the benefits of prolonging gestation to reduce adverse events related to prematurity against the risk of potential complications (abruption, secondary infection, umbilical cord prolapse, etc.) (48). Based on the evidence that gestational age is the most important factor in neonatal morbidity and mortality, expectant management is recommended in PPROM pregnancies before 34 weeks of gestation (11, 49). More problematic is the management beyond the 34 weeks. Yet, the recent data shows that expectant management might be safe for mother and child and can improve neonatal outcomes even in PPROM beyond 34 weeks. (50, 51). These data encourage clinicians to be expectant after 34 weeks as well. Conversely, to make the PPROM management safer for both mother and foetus, it would be very useful to have knowledge about the intra-amniotic environment available since there is evidence that the newborns from PPROM pregnancies, complicated with intra-amniotic infection (the presence of both microorganisms and inflammation in amniotic fluid) and treated expectantly had the worst outcomes. (52-54). Therefore, management of PPROM, considering the status of the intra-amniotic environment, seems to be the ideal approach for dealing with PPROM pregnancies.

1.3. Infection-related and inflammatory intra-amniotic complications in PPROM

1.3.1. Microbial invasion of the amniotic cavity

MIAC is defined as the presence of microorganisms and/or their DNA in amniotic fluid. It represents a pathological condition because the amniotic cavity is considered a sterile environment (55, 56). Microorganisms may gain access to the amniotic cavity irrespective of the status of foetal membranes in PPROM; MIAC can be either cause or consequence of PPROM (57-59). MIAC complicates approximately 25-40% of PPROM, and it depends on the gestational age at sampling, ethnicity, and detection techniques (56, 60). MIAC can induce an inflammatory response leading to the production of cytokines (61-83), chemokines (84-90), other inflammatory mediators (60, 91-99), and thrombin (100-105). Moreover, the production of matrix-degrading enzymes in the presence of MIAC has been established (106-113). These matrix-degradation enzymes can degrade the ECM, and this process is described as a part of the mechanism of membrane rupture (114-120). Several routes exist how microorganisms might reach the amniotic cavity: i) to ascend from the vagina and cervix, ii) to spread hematogenously through the placenta, iii) to disseminate retrogradely from the peritoneal cavity through the fallopian tubes, and iv) iatrogenic inoculation during invasive intrauterine procedures (13). Of these options, an ascension of microorganisms from the lower genital tract seems to be the most important origin of microbial infection (121). These microorganisms can move through the endocervical canal, invade the decidua and chorioamnion, cross intact membranes, and enter the amniotic cavity (122, 123). The most common bacteria in PPROM pregnancies are *Ureaplasma* sp*p.* (124-126), that belongs with *Mycoplasma hominis* under an umbrella of the term - genital mycoplasmas. These small, low virulent bacteria do not have their own cell wall; however, they have the capability to trigger a strong inflammatory response in the intra-amniotic environment (127-129). Hematogenous spread theory is supported by the fact that oral bacteria such as *Fusobacterium nucleatum, Porphyromonas gingivalis,* and group A *Streptococcus* have been found in the amniotic fluid (130, 131).

1.3.2. Intra-amniotic inflammation

IAI represents a condition characterised by the elevation of various inflammatory markers in the amniotic fluid. These markers include cytokines, chemokines, antimicrobial proteins and lipids (56, 60). The development of IAI is usually a consequence of the activation of the intra-amniotic innate immune response through the system of pattern recognition receptors (60, 132-134). This activation involves recognition of specific components of microorganisms (called pathogen-associated molecular patterns; PAMPs) or endogenous molecules called alarmin (damage-associated molecular patterns; DAMPs) via Toll-like receptors (TLRs), activation of nuclear factor kappa B (a transcription factor that regulates many pro-inflammatory and labour-associated genes) and amplification of cytokine production including the tumour necrosis factor (TNF-α) and interleukins (IL-1β, IL-6 and IL-8) (135-139). Moreover, microorganisms can also induce the production of matrix-degrading enzymes, which are responsible for membrane rupture (106-111). Matrix metalloproteinases, elastases, cathepsin, etc., can degrade the ECM, weakening the membranes (112, 114-119). Cytokines, which induce apoptosis, such as members of the TNF-α superfamily, may also participate in the mechanisms responsible for the membrane rupture, as they can induce programmed cell death, FAS and FAS ligand (140).

1.3.3. Intra-amniotic complications based on the presence or absence of MIAC and IAI

Based on the presence or absence of MIAC and IAI, pregnancies complicated by PPROM can be divided into one of four subgroups: i) intra-amniotic infection (the presence of both MIAC and IAI), ii) sterile IAI (the presence of IAI per se), iii) colonisation of the amniotic cavity (the presence of MIAC per se), and the absence of both MIAC and IAI

1.3.3.1. Intra-amniotic infection

The presence of MIAC and IAI characterises Intra-amniotic infection. The frequency of intra-amniotic infection in patients with PPROM in the absence of labour is 20-40% (124, 141-148). In contrast, the prevalence of intra-amniotic infection is about 75% at the time of the onset of labour in PPROM pregnancies (141).

The intra-amniotic infection is associated with the strongest intra-amniotic inflammatory response. This is supported by the observation of a significantly higher level of amniotic fluid

IL-6 and other inflammatory markers in cases of intra-amniotic infection (149, 150). The intensity of the inflammatory response also depends on the type of bacteria and microbial load (127, 151, 152). Moreover, patients with PPROM in very low weeks of gestation (before 25 weeks) have a stronger intensity of the inflammatory response in the presence of intra-amniotic infection (48). Besides, Romero et al. observed a significantly higher rate of acute placental inflammatory lesions (histologic chorioamnionitis and funisitis) in patients with intra-amniotic infection (48). On the other hand, some authors did not find significant differences in the short-term neonatal morbidity regarding the presence of intra-amniotic infection, sterile IAI and MIAC alone (17).

1.3.3.2. Sterile intra-amniotic inflammation

Sterile IAI is characterised by the presence of IAI in the absence of detectable microorganisms. The frequency of sterile IAI is about 5–29% in PPROM pregnancies, depending on the population (17, 48). Sterile IAI in PPROM pregnancies is presented at a more advanced gestational age than those with intra-amniotic infection but earlier than those without IAI (48).

The mechanisms responsible for the induction of sterile IAI in PPROM pregnancies remain undetermined. More explored is the mechanism in the case of sterile IAI and preterm labour with intact membranes, where two possible ways are proposed: i) damage of foetal membranes leading to the release of alarmins into the amniotic fluid, resulting in a subsequent inflammatory response through pattern recognition receptors (17, 48, 106, 153-155), ii) infection of the choriodecidual space, leading to the release of inflammatory mediators from the foetal membranes into the amniotic fluid (156), or iii) combination of those two processes (157). The theory of damage to foetal membranes is supported by the amniotic fluid concentration of the proteotypic alarmin and high mobility group box-1, observed in patients with sterile IAI and PTL (149). The theory of choriodecidual space infection is supported by several studies showing that the presence of microbes in chorioamnion was associated with an increased level of IL-6 in the amniotic fluid without confirmation of MIAC (156, 158).

1.3.3.3. Colonisation of the amniotic cavity

The colonisation of the amniotic cavity represents a condition where a small amount of microorganisms with low virulent potential is present in amniotic fluid. This small amount of microorganism cannot activate the intra-amniotic innate immune response, and inflammatory markers are not elevated (83). The colonisation frequency is about 11% in PPROM pregnancies (48). Two possible ways are proposed as mechanisms of this condition's origin: i) contamination of the amniotic fluid with skin bacteria during amniotic fluid sampling and/or during pre-analytical processing of amniotic fluid samples, or ii) colonisation of the amniotic cavity with microorganisms from low genital tracts representing an early stage of MIAC, with a weak intra-amniotic inflammatory response that is not intense enough to pass a threshold for IAI (159-161).

1.4. Methods to detect infection-related and inflammatory intra-amniotic complications in PPROM

Through extensive research, an examination of inflammatory markers in amniotic fluid, obtained by the invasive procedure – amniocentesis, is being considered as a "gold standard" for diagnosing intra-amniotic complications in PPROM pregnancies. Although amniocentesis is an invasive approach, recent studies clearly show that this procedure is considered safe and reliable. However, to perform amniocentesis under all clinical scenarios, irrespective of the amount of amniotic fluid, requires a high level of expertise. This fact prevents this procedure from being a routine part of managing pregnancies with PPROM. Therefore, it is of utmost importance to reveal a non-invasive approach for assessing intra-amniotic complications of PPROM.

1.4.1. Microbial invasion of the amniotic cavity

From a historic perspective, aerobic/anaerobic cultivation has been the first method to determine MIAC. However, using just cultivation suffers from low sensitivity due to the fact that some bacteria found in amniotic fluid are difficult to cultivate or non-cultivable. Therefore, incorporating polymerase chain reaction (PCR)-based techniques that detect nucleic acids from bacteria in the process of MIAC detection dramatically improves sensitivity and can improve our detection of MIAC (121, 133, 162). PCR techniques can be specific (specific microorganism targeted, for example, presence of genital mycoplasmas) and non-specific (focusing on detection of the 16S rRNA gene, usable in detecting other microorganisms) (151, 162). On the other hand, the assessment of 16S rRNA cannot reveal yeast in amniotic fluid. This is why a combination of both cultivation and non-cultivation methods is needed.

1.4.2. Intra-amniotic inflammation

An elevation of white blood cell count in amniotic fluid and/or increased concentrations of lactate in amniotic fluid and/or decreased level of glucose in amniotic fluid were traditionally

considered as markers of IAI. However, IL-6 in amniotic fluid has been shown to be superior to these markers (163, 164) but not inferior to modern proteomic amniotic fluid markers (cangranulins, neutrophil defensins) (165). In addition, the assessment of IL-6 in amniotic fluid is inexpensive and broadly accessible, even in the bed-site manner.

Since IAI is considered a binary variable (present vs absent), a threshold for the concentration of IL-6 in amniotic fluid must exist. Various methods focusing on different epitopes of IL-6 molecules have been used to assess IL-6 in amniotic fluid. It is necessary to have an appropriate threshold for each method used. The classical threshold for concentrations of IL-6 in amniotic fluid distinguishing between the presence and absence of IAI was 2600 pg/mL. This threshold value was developed for IL-6 assessed by the enzyme-linked immunosorbent assays (ELISA) from the R&D company. Unfortunately, ELISA is not optimal to assess IL-6 in amniotic fluid in clinical practice since it would be time and money consuming. Therefore, other thresholds have been suggested for point-of-care tests: 745 pg/mL when IL-6 is assessed by the test kit from Milenia company (lateral flow immunoassay method), and 3000 pg/mL when IL-6 is analysed by the test kit from Roche company (automated eletrochemiluminescene immunoassay method). Both methods can deliver the result within 20 minutes, making these methods clinically relevant and reliable.

Along with the point-of-care test for IL-6, a method for assessing matrix metalloproteinase-8 in amniotic fluid as a marker of IAI in a bedside manner has been suggested (166). In addition, several other proteins and lipids have been suggested as possible markers of IAI with various diagnostic indices (167-169).

1.5. Ultrasound assessment and the detection of infection-related and inflammatory intra-amniotic complications in PPROM

Today, ultrasonography represents an inseparable part of prenatal care for the mother and foetus. Moreover, the development of modern ultrasound machines allows for very detailed observation of the foetus. Therefore, searching for the strong ultrasound marker of inflammatory intra-amniotic complications in PPROM pregnancies represents a logical step in researching these complications.

A couple of studies have been conducted to define ultrasound markers of intra-amniotic inflammatory complications in PPROM. Historically, the changes in biophysical profile or amniotic fluid were investigated (170-175). Moreover, a detailed examination of the foetal heart (heart morphology and Doppler velocimetry heart parameters) was conducted to obtain promising results (176, 177). Some publications evaluated the role of Doppler velocimetry of the fetoplacental circulation in the determination of microbial and inflammatory intra-amniotic complications of PPROM (178-181). Apart from the foetal heart, the foetal spleen, as the organ of the immune and hematopoietic systems, seems to be affected by intra-amniotic inflammatory complications and plays a role in responding to stimulus.

Our research group has reported promising results from the ultrasound assessment of the splenic vein. The studies focused on the changes in the splenic vein blood flow (182, 183), a part of the foetal portal system, showing that the presence of pulsatile flow pattern in the splenic vein was associated with higher rates of acute inflammatory lesions in the placentas, higher concentrations of IL-6 in umbilical cord blood and risk of the subsequent development of early-onset neonatal sepsis independent of gestational age (182, 183). However, blood flow in the splenic vein was only assessed in a dichotomous manner, differentiating between the presence and absence of a pulsatile flow. Moreover, evaluation of other parts of the foetal portal system, which could shed more light on this phenomenon, has not been performed. Finally, inflammatory changes in the placenta seem to be a relevant outcome only when there is a short temporal association between ultrasound evaluation and delivery. From this point of view, the presence of IAI, defined as the elevation of the amniotic fluid level of IL-6, appears to be a more appropriate outcome reflecting the actual situation in the amniotic cavity.

1.6. Novel potential amniotic fluid markers for the detection of infection-related and inflammatory intra-amniotic complications in PPROM

The development of IAI in PPROM can be characterised as a direct consequence of the activation of innate immune response representing the first-line defence mechanism against MIAC (97, 184, 185). IAI can be characterised by elevated pro- and anti-inflammatory proteins and lipids markers in the amniotic fluid and a higher number of immune cells in amniotic fluid (186). Several proteins, lipids, and cells involved in well-orchestrated intra-amniotic inflammatory response have been thoroughly investigated to reveal their diagnostic indices for IAI (124, 163, 164, 166-169, 187, 188). Nevertheless, other mediators being deeply engaged in the development process of inflammation have yet to be assessed whether they might be promising biomarkers of IAI. One of these potential markers might be the granzymes family, particularly extracellular granzyme A.

2. HYPOTHESIS OF THE THESIS

The main hypotheses of this thesis were:

1) the presence of IAI in pregnancies complicated by PPROM affects blood flow in the foetal portal system.

2) intra-amniotic infection and sterile IAI in pregnancies with PPROM are associated with different concentrations of inflammatory mediators in amniotic fluid.

3) the assessment of blood flow on the foetal portal system and concentration of inflammatory mediator in amniotic fluid has diagnostic value for predicting IAI or its phenotypes.

3. OBJECTIVE OF THE THESIS

The main aims of this thesis were: i) to evaluate pulsatile index (PI) in selected parts of the foetal portal system regarding the presence or absence of IAI in females with PPROM, ii) to assess the concentration of thoroughly selected inflammatory mediator in amniotic fluid from PPROM pregnancies, and iii) to test their diagnostic indices to predict IAI (ultrasound markers) or the phenotypes of IAI (amniotic fluid marker). The specific aims were identified and selected to fulfil the main aims of the thesis and are enumerated below.

Specific aims:

1) To evaluate pulsatile index (PI) of the splenic vein, the main portal stem, the left portal vein, and the ductus venosus in the foetuses from PPROM pregnancies with respect to the presence or absence of IAI.

2) To identify diagnostic indices of PI on the selected parts of the foetal portal system to predict IAI in females with PPROM.

3) To measure the concentrations of granzyme A in amniotic fluid obtained by amniocentesis from PPROM pregnancies with respect to the presence of intra-amniotic infection and sterile IAI.

4) To assess diagnostic indices of the concentration of granzyme A to predict intra-amniotic infection and sterile IAI in females with PPROM.

4. MATERIALS AND METHODS

4.1. PATIENTS

4.1.1. Specific aims 1 and 2

A prospective study was conducted between October 2015 and August 2018. Females with singleton pregnancies complicated by PPROM at gestational ages ranging from 24+0 to 36+6 weeks admitted to the Department of Obstetrics and Gynaecology, University Hospital in Ostrava and the Department of Obstetrics and Gynaecology, University Hospital in Hradec Kralove were recruited. Only females aged 18 years and above were eligible for the study. Gestational age was determined for all pregnancies based on first-trimester biometry. Females with hypertension, preeclampsia, gestational diabetes, gross vaginal bleeding, foetal growth restriction, or foetal structural or chromosomal abnormalities were excluded from the study. Foetal growth restriction was defined as an estimated foetal weight below the 10th percentile, accompanied by an umbilical artery PI > 95th percentile and/or a middle cerebral artery PI < 5th percentile, or as an estimated foetal weight below the 3rd percentile, regardless of umbilical and middle cerebral artery PI on Doppler flowmetry (189, 190). All the participants were Caucasian and provided written informed consent prior to inclusion in the study. This study protocol was approved by the Ethics Committee of University Hospital in Ostrava (August 01; No. 348/2015) and University Hospital in Hradec Kralove (June 01, 2015; No. 201506 I96L).

4.1.2. Specific aims 3 and 4

A retrospective cohort study was performed in the Department of Obstetrics and Gynaecology of the University Hospital, Hradec Králové, between May 2014 and June 2017. The inclusion criteria were: i) singleton pregnancies complicated by PPROM and ii) age ≥ 18 years. The exclusion criteria were: i) foetal growth restriction; ii) congenital or chromosomal foetal abnormalities; iii) gestational or pregestational diabetes; iv) gestational hypertension; v) preeclampsia; vi) signs of foetal hypoxia; vii) significant vaginal bleeding. All the participants were Caucasian and provided written informed consent prior to inclusion in the study. The collection of amniotic fluid samples for research purposes was approved by the Ethics Committee of the University Hospital in Hradec Kralove (June 2015; No 201506 I96L).

4.2. METHODS

4.2.1. Specific aims 1 and 2

4.2.1.1. Diagnosis of PPROM

PPROM was defined as leakage of amniotic fluid before the onset of labour and was diagnosed visually by using a sterile speculum examination to confirm the pooling of amniotic fluid in the vagina. In case of clinical doubt, PPROM was confirmed by the presence of insulin-like growth factor–binding protein (ACTIM PROM test; Medix Biochemica, Kauniainen, Finland) in the vaginal fluid.

4.2.1.2 Ultrasound examination of the foetal portal system

Ultrasound examination was performed by one operator (RS) in the University Hospital, Ostrava using a GE VOLUSON E8 ultrasound machine (GE Healthcare, Milwaukee, WI) with 4–8 MHz curved transducer and one operator (IKM) in the University Hospital, Hradec Kralove using an EPIQ 7 ultrasound machine (Philips, Seattle, WA, USA) with 9-2 MHz and 5-2 MHz curved transducers. Ultrasound examination was performed at the time of admission or within 24 hours of admission before administering corticosteroids, antibiotics, and tocolytics.

Spectral Doppler parameters were acquired in the absence of uterine contractions and during foetal quiescence. The angle of insonation was kept below 30°, the Doppler shift was corrected at non-zero angles, and the sample volume was adapted to the vessel size. PI was obtained automatically from at least a 1.5-second steady-state velocity profile. A high-pass filter was set at 50 Hz. Mechanical and thermal indices did not exceed 1.0. The splenic vein was identified in an axial view of the foetal abdomen behind the stomach as a vessel leaving the splenic hilum and continuing into the portal vein and was evaluated close to the splenic hilum. The portal stem was assessed in an axial abdominal view at the point at which it divides into the left and right branches (191), and the left portal branch was assessed in an axial abdominal view at the extension of the umbilical vein after the branching site of the ductus venosus (192). The ductus venosus was evaluated in an oblique or mid-sagittal abdominal view at the isthmus (193).

4.2.1.3. Assessment of the amniotic fluid

4.2.1.3.1. Amniotic fluid sampling

Ultrasound-guided, free-hand transabdominal amniocentesis was performed at the time of admission before administering corticosteroids, antibiotics, and tocolytics. Approximately 2-3 mL of amniotic fluid was aspirated.

4.2.1.3.2. Evaluation of concentrations of interleukin-6 in amniotic fluid

Concentrations of IL-6 in the fresh, unprocessed amniotic fluid samples were assessed using a Milenia QuickLine IL-6 lateral flow immunoassay using a Milenia POCScan reader (Milenia Biotec, GmbH, Giessen, Germany) (194). The measurement range was 50–10,000 pg/mL. The intra- and inter-assay variations were 12.1% and 15.5%, respectively (194).

4.2.2. Specific aims 3 and 4

4.2.2.1. Diagnosis of PPROM

PPROM was defined as leakage of amniotic fluid prior to the onset of labour and was diagnosed visually using a sterile speculum examination to confirm the pooling of amniotic fluid in the vagina. In case of clinical doubt, PPROM was confirmed by the presence of insulin-like growth factor–binding protein (ACTIM PROM test; Medix Biochemica, Kauniainen, Finland) in the vaginal fluid.

4.2.2.2. Assessment of the amniotic fluid

4.2.2.2.1. Amniotic fluid sampling

Ultrasound-guided, free-hand transabdominal amniocentesis was performed at the time of admission before administering corticosteroids, antibiotics, and tocolytics. Approximately 2–3 mL of amniotic fluid was aspirated and divided into four tubes. The first tube was used to evaluate IL-6 directly at the labour ward, while the second and third tubes were immediately sent to a microbiology laboratory to assess MIAC. The last tube was centrifuged for 15 minutes at 2000g to remove cells and debris, divided into aliquots stored at -80C until analysis.

4.2.2.2.2. Evaluation of concentrations of interleukin-6 in amniotic fluid

Concentrations of IL-6 in the fresh, unprocessed amniotic fluid samples were assessed using a Milenia QuickLine IL-6 lateral flow immunoassay using a Milenia POCScan reader (Milenia

Biotec, GmbH, Giessen, Germany) (194). The measurement range was 50–10,000 pg/mL. The intra- and inter-assay variations were 12.1% and 15.5%, respectively (194).

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

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

4.2.2.2.4. Detection of other bacteria in amniotic fluid

Bacterial DNA was identified by PCR targeting *16S rRNA* using the following primers: 5′-CCAGACTCCTACGGGAGGCAG-3′ (V3 region) and 5′-ACATTTCACAACACGAGC-GACGA-3′ (V6 region) (131). Each reaction contained 3 µL target DNA, 500 nM forward and reverse primers, and Q5 High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA) in a total volume of 25 µL. Amplification was performed using a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA). The products were visualised on an agarose gel. Positive reactions yielded 950-bp products that were subsequently analysed by sequencing. The 16S PCR products were purified and sequenced by PCR using the above primers and BigDye Terminator kit v3.1 (Thermo Fisher Scientific, Waltham, MA). The bacteria were then typed using the sequences obtained from BLAST® and SepsiTest™ BLAST.

4.2.2.2.5. Aerobic and anaerobic cultures of amniotic fluid

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

4.2.2.2.6. Evaluation of extracellular granzyme A in amniotic fluid

Concentrations of extracellular granzyme A in amniotic fluid were assessed by ELISA using the ELISA Kit for Granzyme A (GZMA) (Cloud-Clone Corp., Houston, TX) according to the manufacturer's instructions. The detection range of the kit is from 8 to 500 pg/mL. The absorbance values were read at 450 nm on a Multiskan RC ELISA reader (Thermo Fisher Scientific, Waltham, MA, USA).

4.3. STATISTICAL ANALYSES

4.3.1. Specific aim 1

The normality of the data was tested using the D'Agostino-Pearson omnibus normality test and the Shapiro-Wilk test. The maternal and neonatal characteristics were compared with the non-parametric Mann-Whitney U test for continuous variables and are presented as median values [interquartile range (IQR)] and with Fischer's exact test for categorical variables and are presented as numbers (%). The comparison of vessel PI values was performed with the non-parametric Mann-Whitney U test, and Spearman's partial correlation was used to adjust the results for gestational age at examination. The reproducibility of the measurements between operators was assessed by interobserver variations calculated with the interclass correlation. Differences were considered significant at $p < 0.05$. All p values were obtained using two-sided tests, and all statistical analyses were performed using GraphPad Prism 7.0 software for Mac OS X (GraphPad Software, San Diego, CA, USA) or the SPSS 19.0 statistical package for Mac OS X (SPSS Inc., Chicago, IL, USA).

4.3.2. Specific aim 2

The receiver operation characteristic (ROC) curve was constructed to assess the predictive value of the splenic vein PI for the presence of IAI. The cut-off value was determined based on the Youden index. Statistical analyses were performed using GraphPad Prism 7.0 software for Mac OS X (GraphPad Software, San Diego, CA, USA).

4.3.3. Specific aim 3

The normality of the data was tested using the D'Agostino-Pearson omnibus normality test and the Shapiro-Wilk test. The demographic characteristics were compared by a non-parametric Kruskal-Wallis test for the continuous variables and chi-square test for the categorical variables and presented as a median (IQR) and numbers (%), respectively. Since levels of granzyme A in amniotic fluid were not normally distributed, the non-parametric Mann-Whitney U-test and Kruskal-Wallis tests were used for analyses, as appropriate. The Spearman partial correlation was used to adjust the results for gestational age at the sampling. The Spearman correlation was used to assess the association between amniotic fluid granzyme A levels and IL-6 levels and gestational age at the sampling. Differences were considered statistically significant at $p < 0.05$. All p-values were obtained from two-sided tests, and all statistical analyses were performed using GraphPad Prism 8.4.3 for Mac (GraphPad Software, San Diego, CA, USA).

4.3.4. Specific aim 4

The ROC curve was constructed to assess the predictive value of extracellular granzyme A in amniotic fluid for the presence of IAI. The cut-off value was determined based on the Youden index. Statistical analyses were performed using GraphPad Prism 7.0 software for Mac OS X (GraphPad Software, San Diego, CA, USA).

4.4. CLINICAL DEFINITIONS

4.4.1. Microbial invasion of the amniotic cavity

MIAC was determined based on positive PCR analysis of *Ureaplasma* species, *M. hominis*, *C trachomatis*, a combination of these species, positivity for 16S rRNA assay, cultivation of microbes under aerobic/anaerobic from the amniotic fluid, or a combination of these parameters.

3.4.2. Intra-amniotic inflammation

IAI was defined as amniotic fluid IL-6 concentrations $\geq$ 745 pg/mL when IL-6 was measured using lateral flow-based immunoassay point-of-care test (195, 196).

4.4.3. Intra-amniotic infection

Intra-amniotic infection was defined as the concurrent presence of MIAC and IAI

4.4.4. Sterile intra-amniotic inflammation

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

4.4.5. Colonisation of the amniotic cavity

The colonisation of the amniotic cavity was defined as the presence of MIAC without IAI.

4.4.6. Negative amniotic fluid

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

4.5. CLINICAL MANAGEMENT OF PPROM

Gestational age was determined by first-trimester foetal biometry. Participants with PPROM at less than 35 weeks of gestation were treated with antibiotics and corticosteroids to accelerate lung maturation; those with PPROM beyond 35 weeks of gestation were only treated with antibiotics. Participants with IAI (a concentration of bedside IL-6 $\geq$ 745 pg/mL) received clarithromycin intravenously, 500 mg every 12 h, for 7 days, unless delivery occurred, while those without IAI (a concentration of bedside IL-6 < 745 pg/mL) were treated with benzylpenicillin 5.0 million IU intravenous start dose and 2.5 million IU every 6 h intravenously for 7 days unless delivery occurred. In case of a penicillin allergy, patients were treated with clindamycin, 900 mg intravenously every 8 h for 7 days unless delivery occurred. Once the results of MIAC were available, the antibiotic therapy was eventually modified accordingly. Participants with PPROM were treated conservatively, except those with proven intra-amniotic infection beyond 28 weeks of gestation. They were induced for birth, or an elective caesarean section was performed within 72 h of admission.

5. RESULTS

5.1. SPECIFIC AIM 1

5.1.1. Demographic and clinical characteristics of the study population

Eighty-one singleton pregnant females were included in the analysis; 48% (39/81) and 52% (42/81) of the participants were admitted and assessed at the Department of Obstetrics and Gynaecology, University Hospital of Ostrava and the Department of Obstetrics and Gynaecology, University Hospital of Hradec Kralove, respectively. All the participants underwent the assessment of PI on the splenic vein, the main portal stem, the left portal branch, and the ductus venosus.

Doppler parameters were obtained from all foetuses, except for the left portal branch in one foetus from the pregnancy without IAI at the Hradec Kralove's since the vessel could not be visualised appropriately.

The interobserver intraclass correlation coefficients for PI measurements, based on 10 pairs of measurements for each vein, were 0.90 [95% confidence interval (CI): 0.67-0.98] for the splenic vein, 0.88 (95% CI: 0.62-0.97) for the portal stem, 0.80 (95% CI: 0.15-0.95) for the left portal branch, and 0.89 (95% CI: 0.62-0.97) for the ductus venosus.

The presence of IAI was observed in 27% of the patients (22/81). The demographic and clinical characteristics of participants according to the presence and absence of IAI are shown in Table 1.

Table 1. Specific aims 1 and 2 - demographical and clinical characteristics of females with preterm prelabour rupture of the membranes with and without intra-amniotic inflammation.

Characteristic	With intra-amniotic inflammation (n=22)	Without intra-amniotic inflammation (n=59)	*p*-value
Maternal age [years, median (IQR)]	32 (26-36)	30 (26-35)	0.52
Primiparous [number (%)]	7 (32%)	33 (56%)	0.08
Pre-pregnancy body mass index [kg/m^2, median (IQR)]	24.7 (22.5-27.2)	23.8 (22.2-25.9)	0.47
Gestational age at examination [weeks + days, median (IQR)]	30+4 (25+4-32+2)	33+2 (31+1-34+2)	**0.003**
Gestational age at delivery [weeks + days, median (IQR)]	30+6 (25+6-32+6)	33+6 (32+0-35+0)	**0.001**
Latency from PPROM to ultrasound examination [hours, median (IQR)]	18 (6-98)	9 (4-36)	0.14
Latency from ultrasound examination to delivery [days, median (IQR)]	160 (69-185)	211 (140-225)	0.01
Amniotic fluid IL-6 concentrations [pg/mL, median (IQR)]	10000 (1339-10000)	221 (110-347)	**<0.0001**
Smoking [number (%)]	8 (36%)	8 (14%)	**0.03**
Administration of corticosteroids [number (%)]	20 (92%)	47 (80%)	0.33
Administration of antibiotics [number (%)]	20 (92%)	54 (92%)	1.00
Administration of tocolytics [number (%)]	9 (41%)	19 (32%)	0.60
Spontaneous delivery [number (%)]	10 (46%)	42 (71%)	**0.04**
Caesarean section [number (%)]	12 (55%)	14 (24%)	**0.01**
Forceps/vacuum extraction delivery [number (%)]	0 (0%)	3 (5%)	0.56
Birth weight [grams, median (IQR)]	1525 (968-1970)	2150 (1590-2510)	**0.0002**
Apgar score; 5 minutes [number, median (IQR)]	10 (9-10)	10 (9-10)	0.97
Apgar score; 10 minutes [number, median (IQR)]	10 (10-10)	10 (10-10)	0.83

Abbreviations:

IL: interleukin

PPROM: preterm prelabour rupture of membranes

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

5.1.2. The pulsatility degree of the splenic vein and IAI

Foetuses from PPROM pregnancies with IAI had higher PI values in the splenic vein than those from pregnancies without IAI in crude analysis (with IAI: median 0.35, IQR: 0.22-0.67 vs without IAI: median 0.24, IQR: 0.17-0.30; $p = 0.003$; Figure 1), as well as after the adjustment for gestational age at examination ($p = 0.003$).

Figure 1.

The pulsatility degree of the splenic vein in women with PPROM
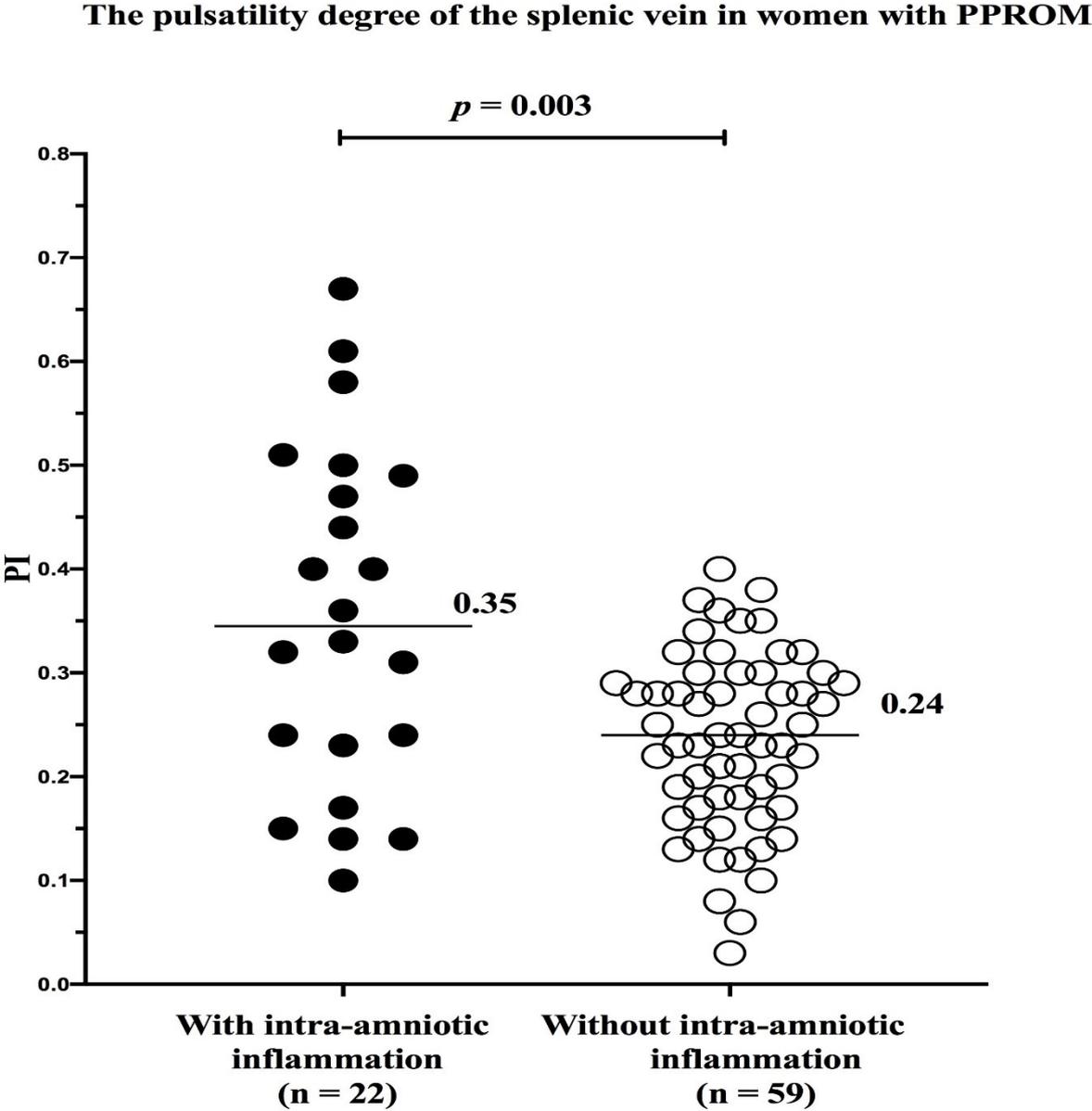
5.1.3. The pulsatility degree of the main portal stem and IAI

No difference was identified in PI of the main portal stem between foetuses from PPROM pregnancies with and without IAI (with IAI: median 0.41, IQR 0.33-0.42 vs without IAI: median 0.35, IQR 0.25-0.42; $p = 0.17$; Figure 2).

Figure 2.

The pulsatility degree of the main portal stem in women with PPROM
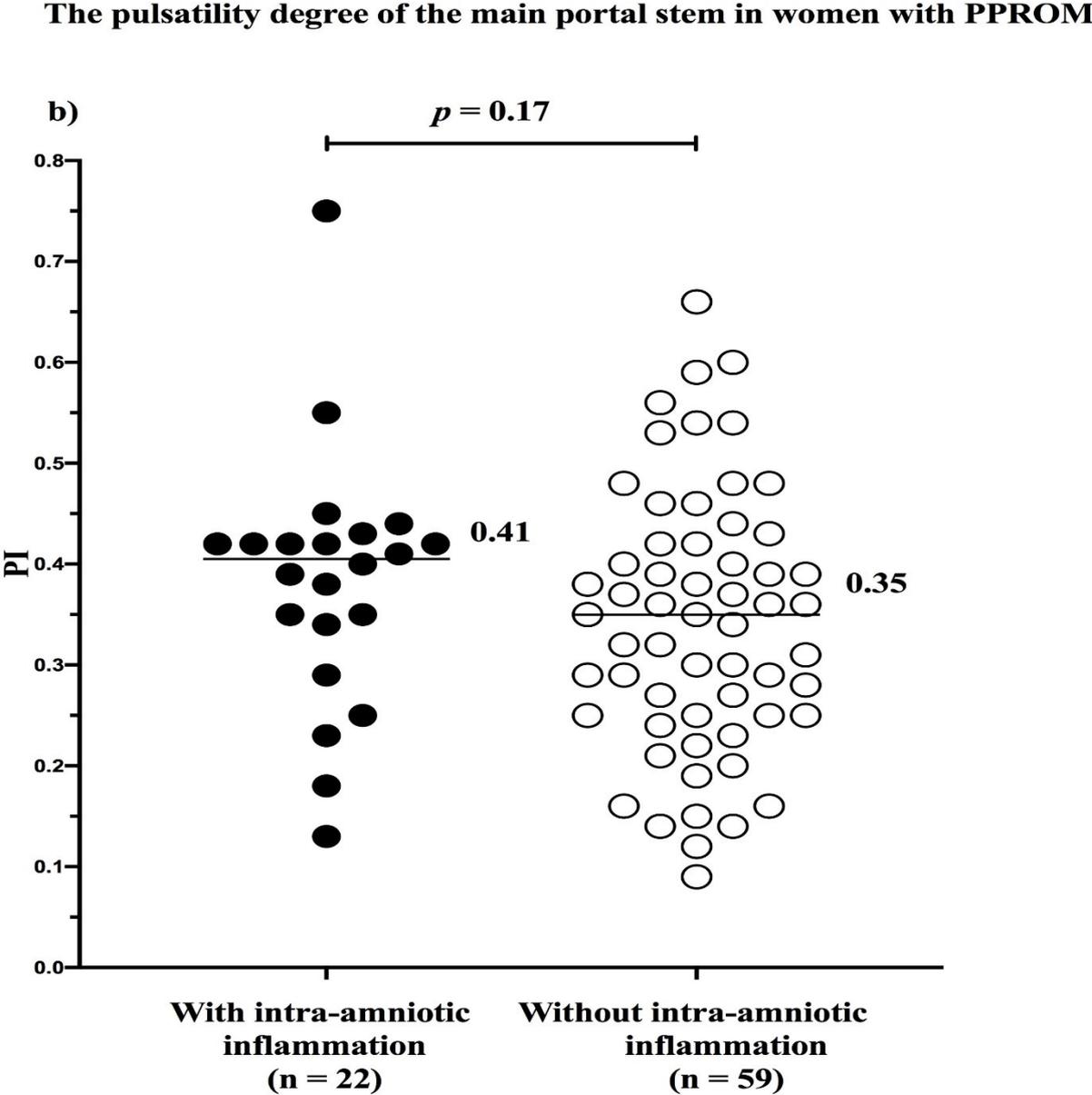
5.1.4. The pulsatility degree of the left portal vein and IAI

No difference in PI of the left portal vein was observed between foetuses from PPROM pregnancies with and without IAI (with IAI: median 0.34, IQR 0.24-0.43 vs without IAI: median 0.32, IQR 0.25-0.44; $p = 0.88$; Figure 3).

Figure 3.

The pulsatility degree of the left portal vein in women with PPROM
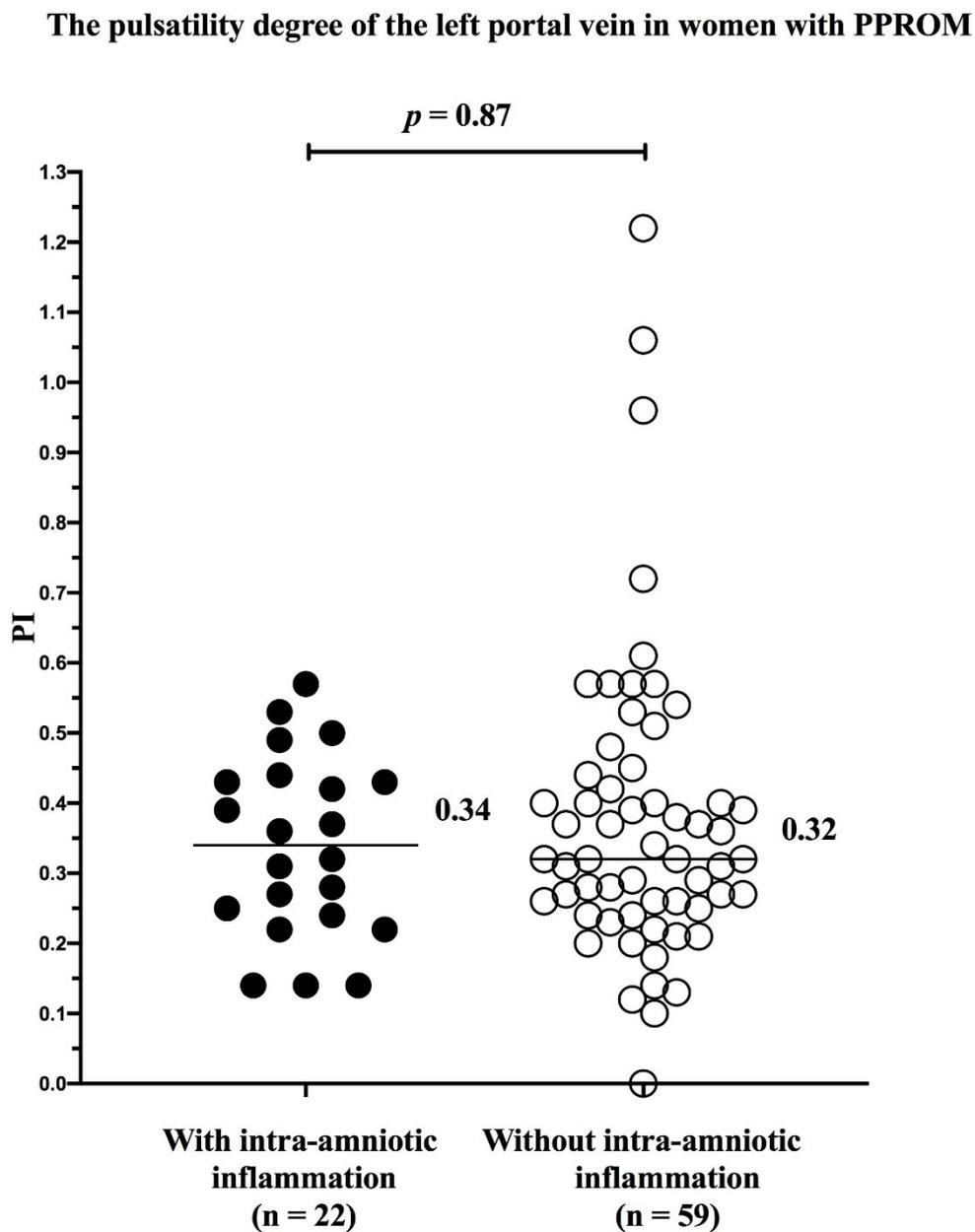
5.1.5. The pulsatility degree of the ductus venosus and IAI

No difference in PI of the main portal stem was found between foetuses from PPROM pregnancies with and without IAI (with IAI: median 0.48, IQR 0.32-0.65 vs without IAI: median 0.46, IQR 0.37-0.59; $p = 0.86$; Figure 4).

Figure 4.

The pulsatility degree of the ductus venosus in women with PPROM

5.2. SPECIFIC AIM 2

A cut-off value of 0.36 was found to be an optimal PI of the splenic vein to predict IAI in females with PPROM with a sensitivity of 50% (11/22; 95% CI 31-69), specificity of 93% (4/49; 95% CI 84-97), positive predictive value of 73% (11/15; 95% CI 48-89), negative predictive value of 83% (55/66; 95% CI 73-90), positive likelihood ratio of 7.4 (95% CI 2.6-20.8), negative likelihood ratio of 0.5 (95% CI 0.4-0.8), odds ratio of 13.8 (95% CI 3.5-43.6) and area under ROC curve of 0.72 (95% CI 0.56-0.87; Figure 5).

Figure 5.

AUC = 0.72; p = 0.003

5.3. SPECIFIC AIM 3

5.3.1. Demographic and clinical characteristics of the study population

One hundred and sixty-six females with PPROM between gestational ages 24+0 and 33+6 weeks were included in this study. MIAC and IAI were reported in 30% (50/166) and 20% (33/166) of the patients, respectively. Intra-amniotic infection, sterile IAI, colonisation of the amniotic cavity, and negative amniotic fluid were identified in 15% (25/166), 5% (8/166), 15% (25/166), and 65% (108/166) of the participants, respectively.

The demographic and clinical data of the patients with PPROM based on these four subgroups are shown in Table 2. The microbial species identified in amniotic fluid obtained from the patients with intra-amniotic infection and colonisation of the amniotic cavity are presented in Table 3.

Table 2. Specific aims 3 and 4 - demographical and clinical characteristics of the women with preterm prelabor rupture of the membranes with intra-amniotic infection, sterile intra-amniotic inflammation, colonization of the amniotic cavity, and negative amniotic fluid.

Characteristic	Intra-amniotic infection (n=25)	Sterile intra-amniotic inflammation (n=8)	Colonization of the amniotic cavity (n=25)	Negative amniotic fluid (n=108)	p-value
Maternal age [years, median (range)]	30.5 (17-42)	28.0 (21-35)	32.0 (18-42)	31.0 (21-40)	0.34
Prepregnancy body mass index [kg/m², median (range)]	24.6 (16.5-38.0)	24.1 (19.3-37.8)	22.3 (16.0-33.5)	22.7 (15.8-39.0)	0.67
Gestational age at admission [weeks, median (range)]	31+6 (24+2-36+5)	33+6 (25+1-36+6)	33+3 (25+3-36+6)	34+0 (25+3-36+6)	**0.03**
Gestational age at delivery [weeks, median (range)]	32+1 (24+5-36+5)	34+1 (25+1-36+6)	34+2 (26+5-36+6)	34+4 (25+2-36+6)	**0.005**
Latency from PPROM to amniocentesis [hours, median (range)]	9 (1-97)	8 (3-432)	6 (1-35)	6 (1-68)	0.15
Latency from PPROM to delivery [hours, median (range)]	36,5 (3-106)	45 (17-768)	28 (7-390)	32,5 (4-452)	0.72
CRP levels at admission [mg/L, median (range)]	15.5 (1.5-106.3)	8.4 (2.3-59.1)	4 (0.2-13.2)	5.6 (0.1-47)	**<0.0001**
WBC count at admission [x10⁹ L, median (range)]	13.8 (9.2-23.5)	15.3 (8.9-19.9)	11.9 (6.5-21.6)	11.5 (5.7-24.2)	**0.001**
Amniotic fluid IL-6 at admission [pg/mL, median (range)]	8478 (831-10000)	996 (801-1446)	199 (50-673)	194 (50-678)	**<0.0001**
Administration of corticosteroids [number (%)]	21 (84%)	5 (63%)	16 (64%)	77 (70%)	0.40
Administration of antibiotics [number (%)]	25 (100%)	8 (100%)	24 (96%)	101 (95%)	0.66
Spontaneous vaginal delivery [number (%)]	19 (76%)	7 (88%)	20 (80%)	80 (73%)	0.86
Cesarean section [number (%)]	6 (24%)	1 (12%)	5 (20%)	30 (27%)	0.67
Forceps delivery [number (%)]	0 (0%)	0 (0%)	0 (0%)	1 (1%)	0.91
Birth weight [grams, median (range)]	1790 (550-2840)	2215 (990-3320)	2285 (780-3250)	2240 (700-3550)	**0.005**
Acute histological chorioamnionitis [number (%)]	23 (92%)	7 (88%)	17 (68%)	57 (52%)	**0.002**
Funisitis [number (%)]	15 (60%)	2 (25%)	10 (40%)	23 (21%)	**<0.0001**
Apgar score <7; 5 minutes [number (%)]	2 (8%)	1 (13%)	0 (0%)	2 (2%)	0.12
Apgar score <7; 10 minutes [number (%)]	1 (4%)	1 (13%)	0 (0%)	1 (1%)	0.08

Continuous variables were compared using a nonparametric Kruskal-Wallis test.
Categorical variables were compared using the chi-square test.
Continuous variables are presented as median (IQR) and categorical as number (%).
Statistically significant results are marked in bold.

Abbreviations:
CRP: C-reactive protein
IL: interleukin
PPROM: preterm prelabor rupture of membranes
WBC: white blood cells

Table 3. Specific aims 3 and 4 - bacterial species identified in the amniotic fluid from women with preterm prelabor rupture of membranes with intra-amniotic infection and colonization of the amniotic cavity.

Intra-amniotic infection (n=25)	Colonization of the amniotic cavity (n=25)
Ureaplasma spp. (n=14)	*Ureaplasma* spp. (n=15)
Ureaplasma spp. + *Chlamydia trachomatis* (n=2)	*Ureaplasma* spp. + *Mycoplasma hominis* (n=5)
Ureaplasma spp. + *Lactobacillus* spp. (n=1)	*Ureaplasma* spp. + *Chlamydia trachomatis* (n=2)
Ureaplasma spp. + *Sneathia sanguinegens* (n=1)	*Ureaplasma* spp. + *Leptotrichia amnionii* (n=1)
Ureaplasma spp. + *Veilonella* spp. (n=1)	*Enterococcus faecium* (n=1)
Mycoplasma hominis +*Peptococcus* spp. + *Propionibacterium* spp. + *Bacteroides* spp. (n=1)	*Streptococcus pneumonia* (n=1)
Chlamydia trachomatis (n=1)	
Fusobacterium nucleatum (n=1)	
Streptococcus agalactiae (n=2)	
Streptococcus spp. (n=1)	

5.3.2. Concentrations of extracellular granzyme A in amniotic fluid

The concentrations of extracellular granzyme A were measurable in all samples from PPROM pregnancies. No correlation between granzyme A concentration and gestational age at the sampling was identified (rho = -0.14; p = 0.07).

5.3.3. Extracellular granzyme A in amniotic fluid based on the presence and absence of MIAC

Participants with MIAC had lower concentrations of extracellular granzyme A in amniotic fluid than those without this complication (with MIAC: median 15.9 pg/mL, IQR 6.9-26.1 vs without MIAC: median 19.9, IQR 12.7-29.5; p = 0.03; Figure 6) in crude analysis, as well as after the adjustment for the gestational age at sampling (p = 0.02).

Figure 6.

Amniotic fluid extracellular granzyme A
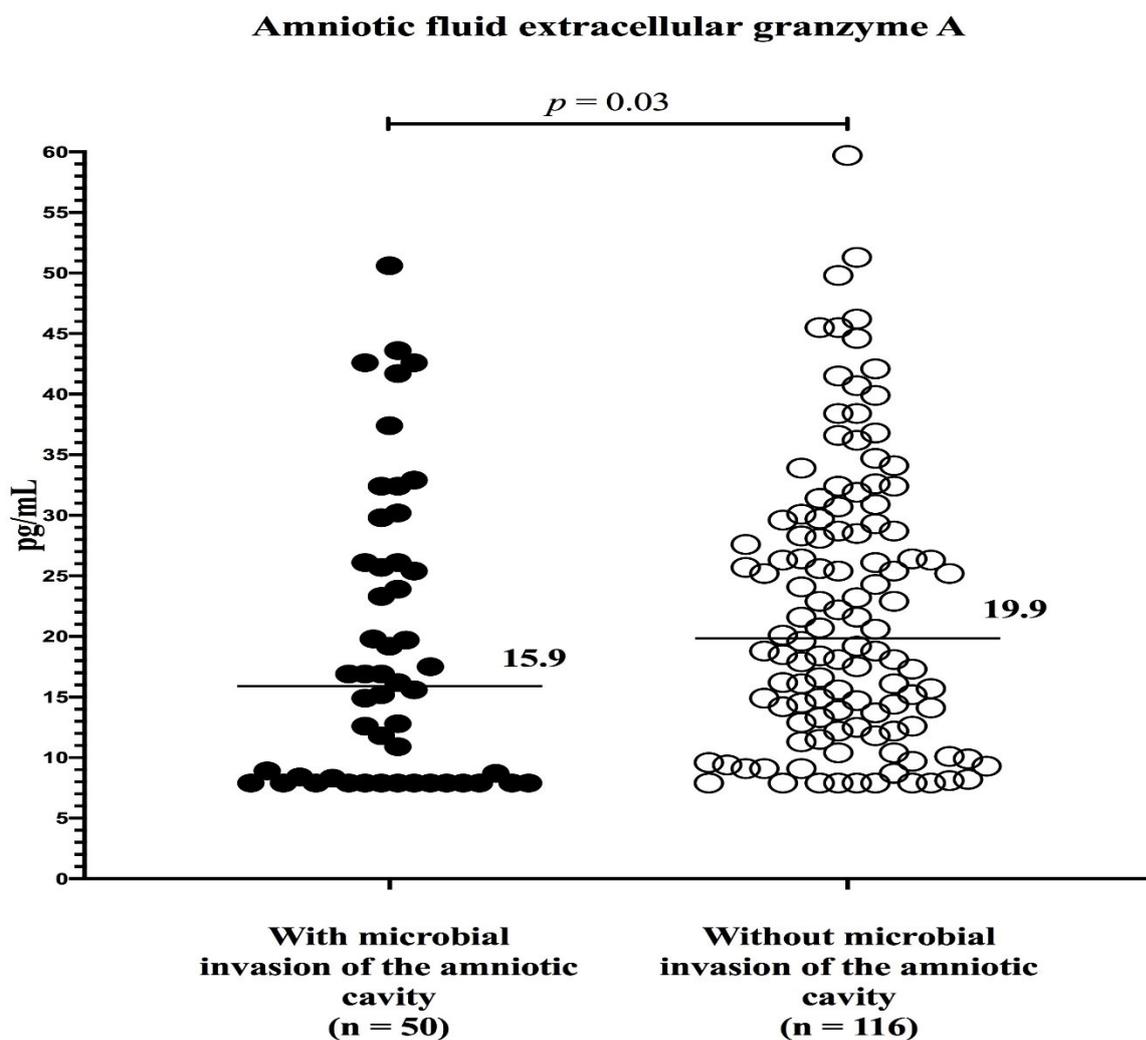
5.3.4. Extracellular granzyme A in amniotic fluid based on the presence and absence of IAI

No difference was observed in the concentrations of extracellular granzyme A in the amniotic fluid between participants with and without IAI (with IAI: median 16.9 pg/mL, IQR 11.4-26.9 vs without IAI: median 18.5 pg/mL, IQR 10.9-28.7; $p = 0.93$; Figure 7).

Figure 7.

Amniotic fluid extracellular granzyme A
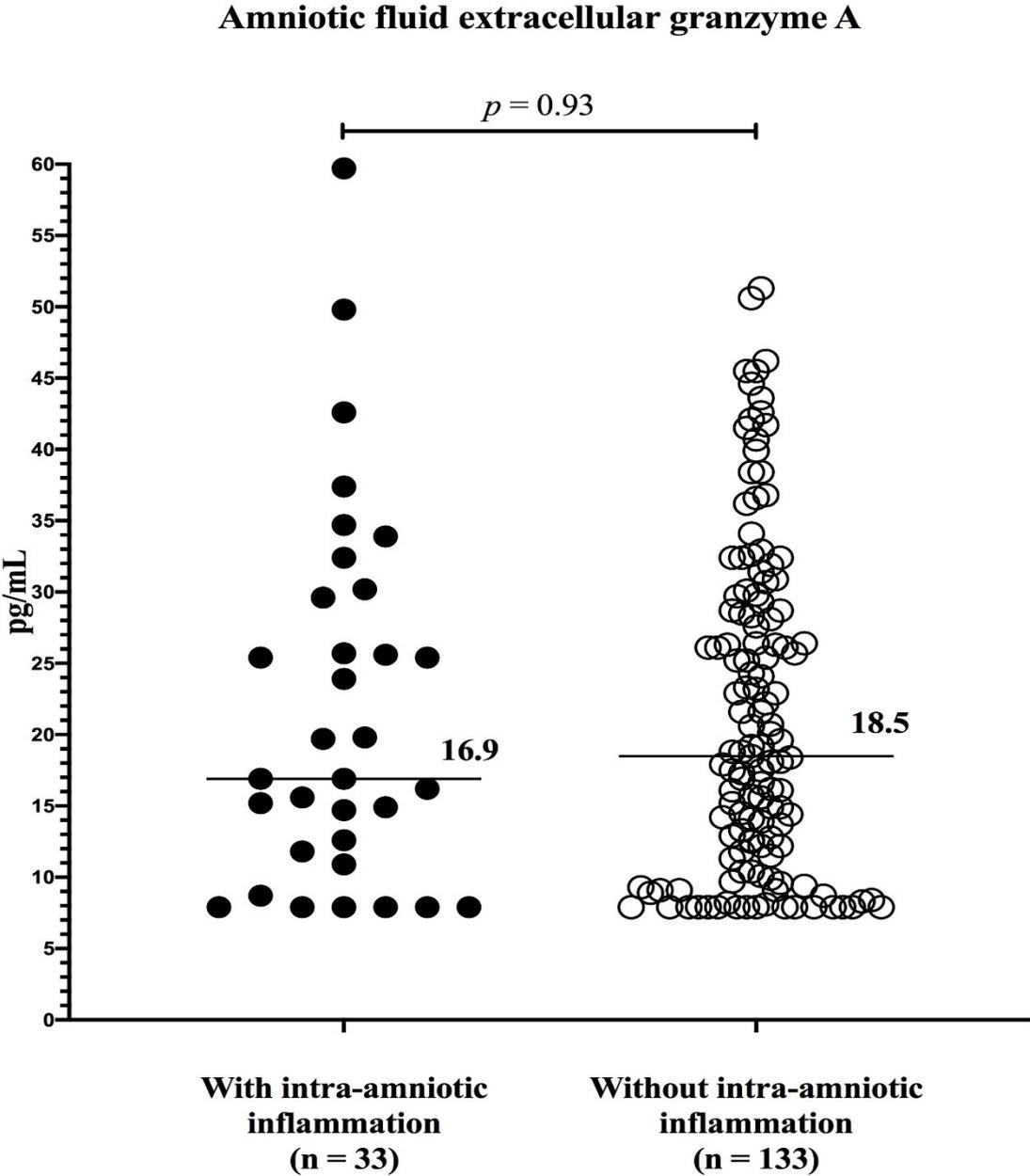
5.3.5. Extracellular granzyme A in amniotic fluid based on the presence of intra-amniotic infection, sterile intra-amniotic inflammation, colonisation of the amniotic cavity and negative amniotic fluid

A difference in the concentrations of extracellular granzyme A in the amniotic fluid was found among the participants with intra-amniotic infection, sterile IAI, colonisation and with negative amniotic fluid (intra-amniotic infection: median 15.6 pg/mL, IQR 7.5-24.7; sterile IAI: median 31.8 pg/mL, IQR 25.5-46.0; colonisation of the amniotic cavity: median 16.9 pg/mL, IQR 6.5-31.1; negative amniotic fluid: median 18.8 pg/mL, IQR 12.3-28.7; p = 0.02; Figure 8). The patients with sterile IAI had higher concentrations of extracellular granzyme A in amniotic fluid than those with intra-amniotic infection, colonisation, and negative amniotic fluid in crude and adjusted analyses (Table 4).

Figure 8.

Amniotic fluid extracellular granzyme A

p = 0.02
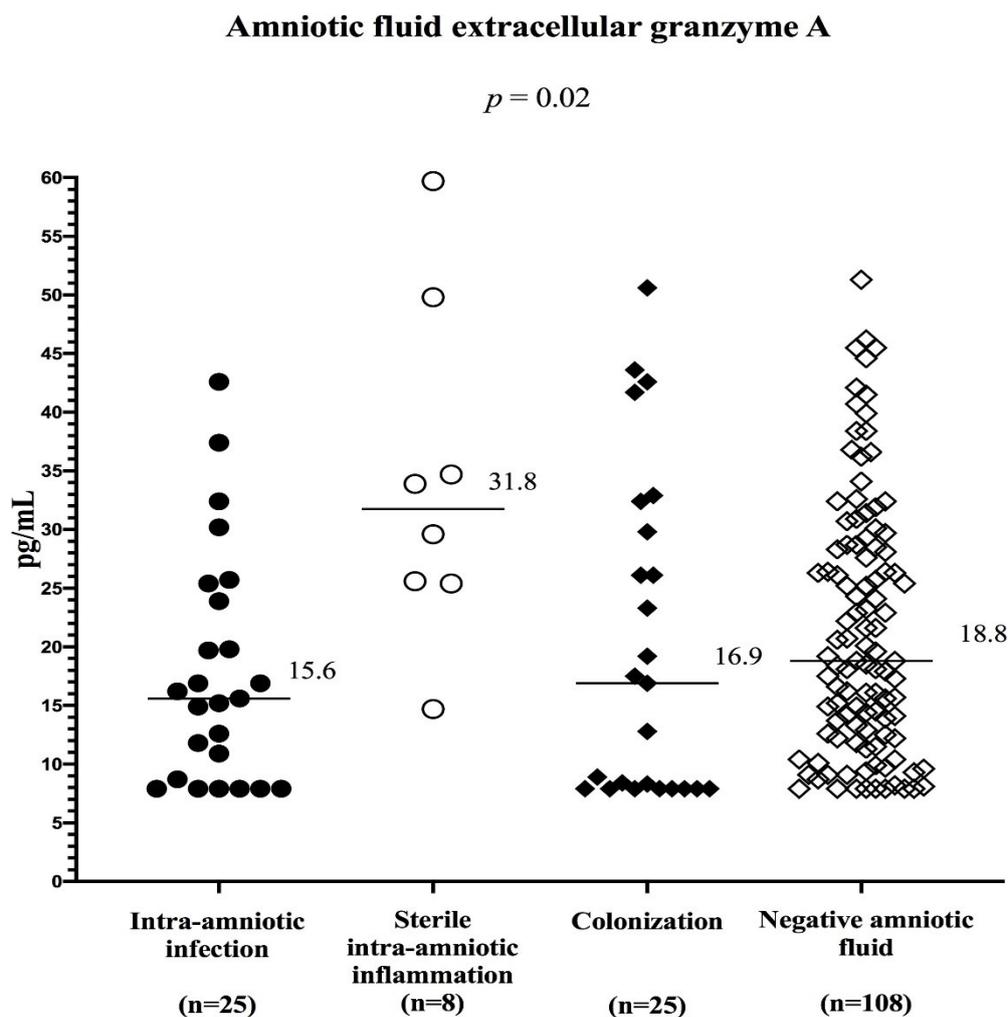
Table 4. Specific aims 3 and 4 - extracellular granzyme A in amniotic fluid among the subgroups of the women with intra-amniotic infection, sterile intra-amniotic inflammation, colonization of the amniotic cavity and negative amniotic fluid.

	Intra-amniotic infection	Sterile intra-amniotic inflammation	Colonization	Negative amniotic fluid
Intra-amniotic infection	X	$p = 0.004$; adj. $p = 0.02$	$p = 0.73$; adj. $p = 0.44$	$p = 0.09$; adj. $p = 0.03$
Sterile intra-amniotic inflammation	$p = 0.004$; adj. $p = 0.02$	X	$p = 0.02$; adj. $p = 0.02$	$p = 0.01$; adj. $p = 0.004$
Colonization	$p = 0.73$; adj. $p = 0.44$	$p = 0.02$; adj. $p = 0.02$	X	$p = 0.23$; adj. $p = 0.36$
Negative amniotic fluid	$p = 0.09$; adj. $p = 0.03$	$p = 0.01$; adj. $p = 0.004$	$p = 0.23$; adj. $p = 0.36$	X

p-value: a comparison between two subgroups (a nonparametric Mann-Whitney U test)

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

5.3.6. Extracellular granzyme A in amniotic fluid and sterile IAI

The participants with sterile IAI had higher concentrations of extracellular granzyme A in amniotic fluid than those without sterile IAI in crude analysis (with sterile IAI: median 31.8 pg/mL, IQR 25.5-46.0 vs without sterile IAI: median 18.0 pg/mL, IQR 10.4-28.4; $p = 0.006$; Figure 9), as well as after the adjustment for gestational age at sampling ($p = 0.002$).

5.3.7. Extracellular granzyme A and interleukin-6 in amniotic fluid

No correlation was found between the concentrations of extracellular granzyme A and IL-6 in amniotic fluid (rho = -0.04; $p = 0.58$).

Figure 9.
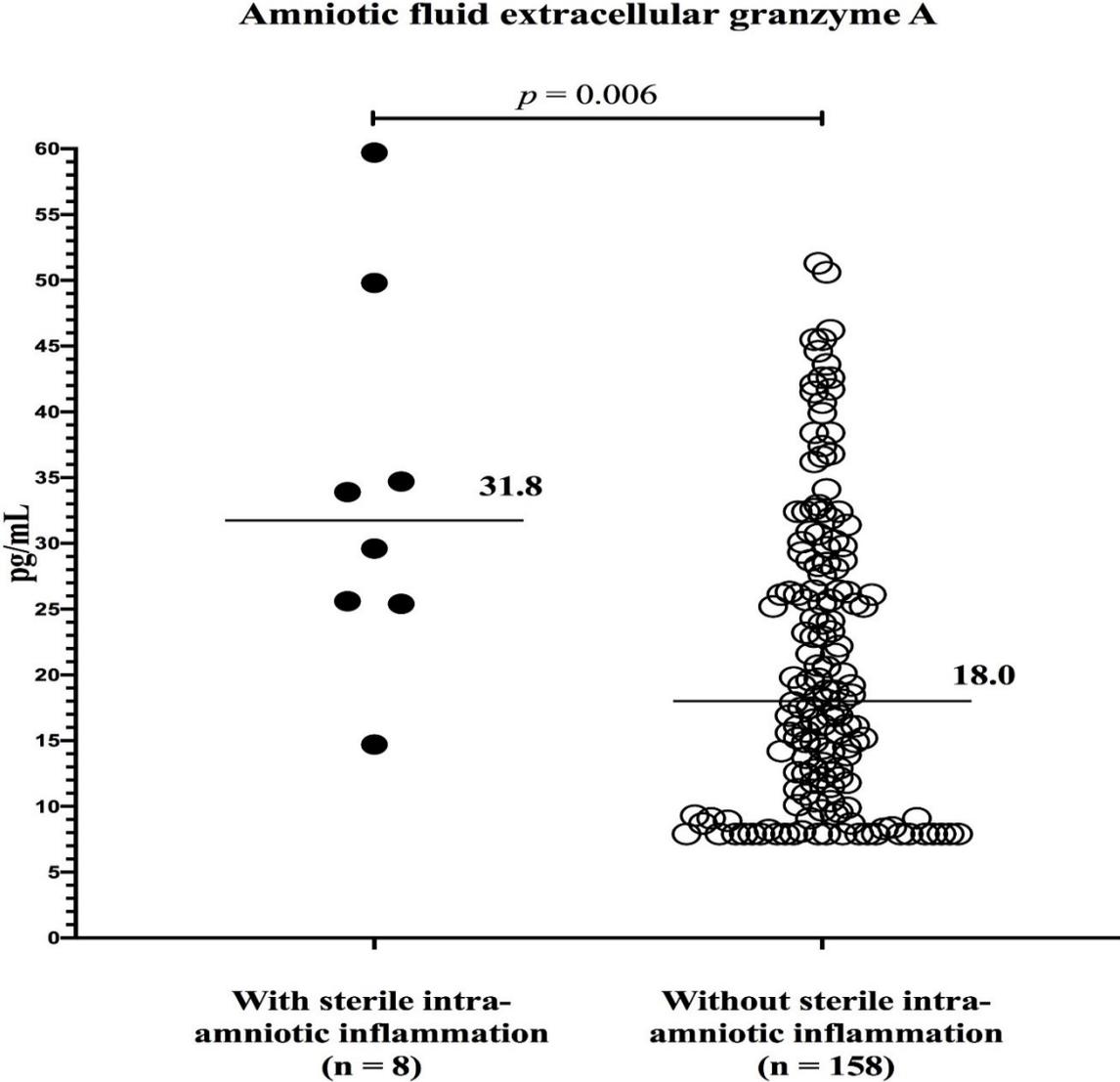
5.4. SPECIFIC AIM 4

A cut-off value of 33.4 pg/mL was found to be ideal to predicting sterile IAI in females with PPROM with sensitivity of 50% (4/8; 95% CI 22-79), specificity of 87% (137/158; 95% CI 81-91), positive predictive value of 16% (4/25; 95% CI 6-35), negative predictive value of 97% (137/141; 95% CI 93-99), positive likelihood ratio of 3.8 (95% CI 1.7-8.4), negative likelihood ratio of 0.6 (95% CI 0.3-1.2), odds ratio of 6.5 (95% CI 1.8-23.4) and area under ROC curve of 0.78 (95% CI 0.64-0.92; Figure 10).

Figure 10.
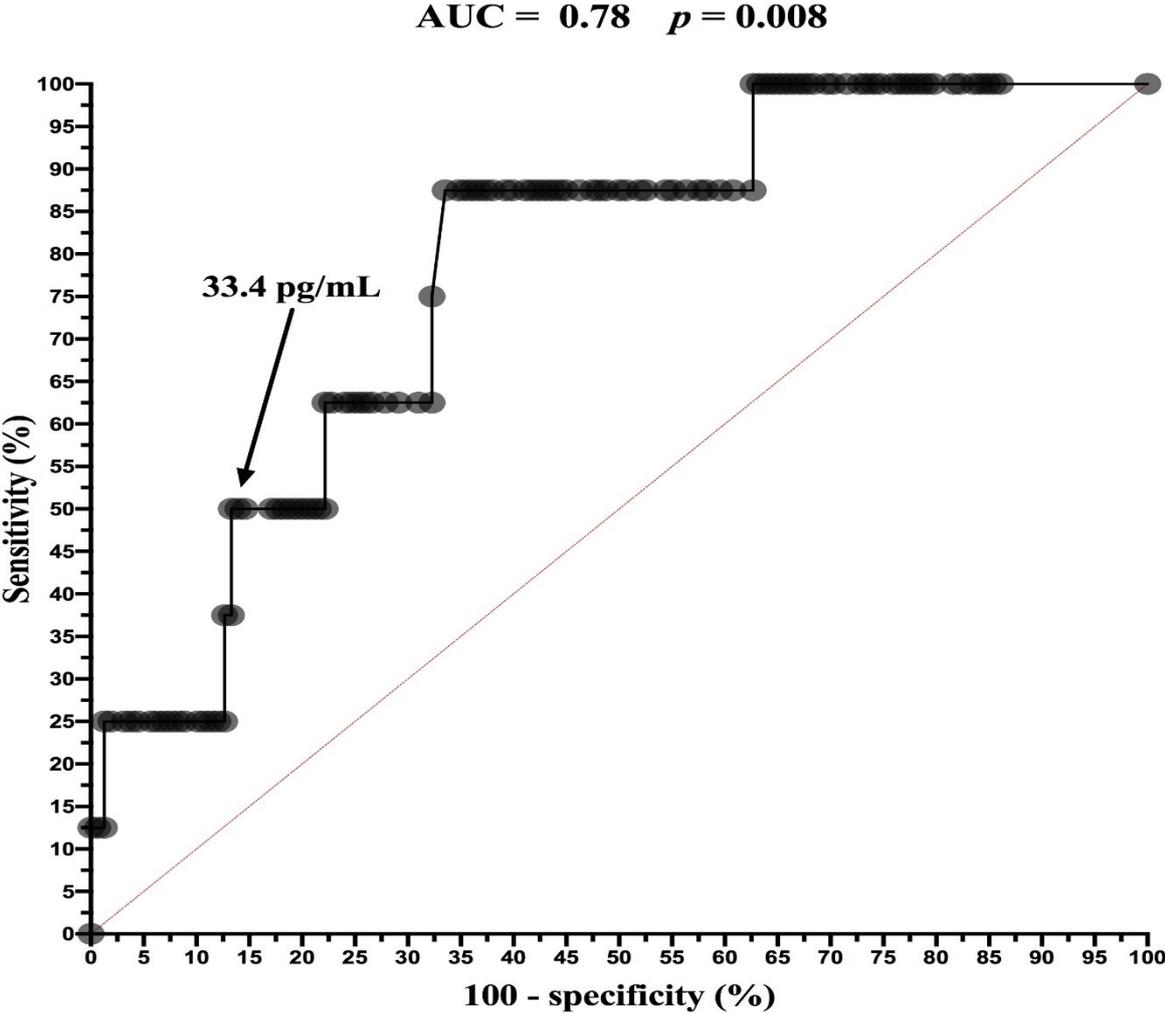
6. DISCUSSION

6.1. SPECIFIC AIMS 1 AND 2

6.1.1. Main observations

The main findings of these aims are as follows:

1) foetuses from PPROM pregnancies complicated by IAI exhibited a higher pulsatility degree in the splenic vein than those from pregnancies without IAI;
2) no differences in the pulsatility degree between foetuses from PPROM pregnancies with and without IAI were observed in the main portal stem, left portal vein and ductus venosus;
3) PI of the splenic vein cut-off value of 0.36 was optimal to non-invasively predict the presence of IAI in pregnancies complicated by PPROM.

6.1.2. Meaning of the observations

A range of different flow patterns is displayed in the foetal portal system in uncomplicated pregnancies. Continuous flow is dominant in the splenic vein at its origin, while monophasic pulsation is present in the portal stem in almost all foetuses (191, 197). The left portal branch exhibits a continuum from continuous flow through monophasic to biphasic pulsation, with pulsatile patterns as predominant types (192). It has been accepted that two different sources of pulsation are involved in the origin of the pulsatile flow patterns in this part of the foetal circulation. The adjacent hepatic artery is considered a probable source of pulsation observed in the portal stem, while pulsation in the left portal branch reflects the transmission of the pulse wave from the ductus venosus (191, 198).

A higher pulsatility degree in the splenic vein was observed when IAI was present in this study. The increased pulsatility degree in the splenic vein agrees with the previously reported appearance of pulsatile flow in this foetal vessel in pregnancies complicated by acute histological chorioamnionitis and funisitis. However, the flow was only previously assessed qualitatively, distinguishing between two dichotomous variables, i.e., continuous and pulsatile flow patterns (182). The flow of all examined veins was quantified by calculating PI to accurately determine the pulsatility degree within the range from continuous flow through mild to marked pulsations.

In terms of pulsatility degree in the portal stem, the result did not follow our expectation, based on our previous study (199). The absence of difference in PI in the portal stem in foetuses from pregnancies with IAI may be explained by the fact that the splenic vein represents only one of its three affluent branches. Therefore, these gentle changes in PI in the splenic vein can be overlapped in the portal stem flow after the junction of all three vessels.

From the pathophysiology point of view, the absence of differences in the pulsatility degree in the left portal branch and the ductus venosus is important. The ductus venosus enables the transmission of hemodynamic alterations of the precordial venous system into the left portal branch. This phenomenon has been previously demonstrated by an increasing pulsatility degree and even by the appearance of flow reversal in the left portal branch when severe foetal compromise associated with growth restriction or non-immune hydrops was present (198, 200). The retrograde transmission of increased pulsatility degree from the left portal branch into other parts of the foetal portal system has not been reported. However, the possible interference of this hemodynamic alteration with the flow in other parts of the foetal portal system up to the level of the splenic vein might be expected. Based on the evidence for foetal cardiac dysfunction in the foetal inflammatory response, the potential influence of hemodynamic changes in precordial veins had been taken into account (201, 202). However, the absence of differences between groups in the pulsatility degree in the left portal branch and the ductus venosus excluded this mechanism and supported a local cause for an increase in the pulsatility degree affecting only the foetal splenic vein and portal stem in IAI.

Although the spleen and liver are among the organs involved in the foetal inflammatory response, there is a lack of knowledge about hemodynamic changes in these organs during IAI; we can only hypothesise about the mechanism of our findings (203-207). Changes in the splenic circulation induced by sepsis were described in an adult animal model (208, 209). Endotoxemia modifies the pre- and post-capillary vascular tone in the spleen, increases hydrostatic pressure in the splenic capillaries, and leads to fluid extravasation (210, 211). The extravasated fluid accumulates in the connective tissue surrounding the splenic vascular arcade. This might increase the stiffness of this tissue and might consequently decrease the splenic vein compliance, in turn contributing to an increase in the pulsatility degree (205, 212, 213). Nevertheless, in addition to changes in mechanical vascular properties, the low vascular cross-sectional area is the most crucial factor contributing to an increase in the pulsatility degree. Thus, the small diameter of the splenic vein might have significantly contributed to our findings (213).

In this study, the diagnostic indices of the PI on the splenic vein to predict IAI was assessed. The cut-off value of 0.36 was identified as optimal with excellent specificity and a very good negative predictive value. Consequently, this cut-off value after a proper validation on the independent cohort of females with PPROM might be a non-invasive ultrasound tool to stratify PPROM pregnancies in the subsets with and without risk of IAI with an ultimate goal to reduce the number of transabdominal amniocenteses required for the detection of IAI.

6.1.3. Strengths and limitations

The main strength is that only females with a well-defined subtype of preterm birth were included. Secondly, ultrasound examination was performed at the time of amniocentesis, and therefore the results were related to the actual amniotic fluid status. However, limitations should also be mentioned. First, regardless of the fact that the recruitment and ultrasound assessment of the females with PPROM were concurrently performed at the two large Obstetrics and Gynaecology clinics in the Czech Republic, the sample size of participants with IAI is still limited. This poor sample size might increase the potential for a type II error. Secondly, the results of ultrasound examinations were assessed with respect to IAI, which is defined based on the amniotic fluid IL-6 concentrations, but not with respect to foetal inflammatory response, which is characterised based on the concentrations of IL-6 concentration in umbilical cord plasma. We are aware that the latter would be a more precise determinant of the foetal inflammatory status. Nevertheless, this would require the performance of cordocentesis at the time of admission, which we considered ethically unacceptable. On the other hand, the association of IAI with higher intensity of foetal inflammatory response was shown in two previous studies and supported the use of amniotic IL-6 levels as an acceptable approach (52, 214).

6.2. SPECIFIC AIMS 3 AND 4

6.2.1 Main observations

The main findings of these aims are as follows:

1) extracellular granzyme A is a constituent of amniotic fluid from PPROM pregnancies;

2) amniotic fluid concentrations of extracellular granzyme A are diminished when MIAC is present;

3) amniotic fluid concentrations of extracellular granzyme A are elevated when sterile IAI is present;

4) amniotic fluid extracellular granzyme A cut-off value of 33.4 is optimal to predict sterile IAI in females with PPROM;

5) amniotic fluid concentrations of extracellular granzyme A and IL-6 do not correlate.

6.2.2. Meaning of the observations

IAI and/or intra-amniotic infection have been shown to be associated with a higher number of B and T lymphocytes and natural killer cells in amniotic fluid (186, 215, 216). Cytotoxic subsets of T lymphocytes and natural killer cells, the immunocompetent cells playing an important role in defence against virally infected and tumour cells, express a family of homologous serine proteases called granzymes, consisting of five members (A, B, H, K, and M) (217-219).

Granzyme A is the most abundant serine protease constitutively present in the granules of cytotoxic T lymphocytes (220-222). Granzyme A has a unique quaternary structure among serine proteases consisting of a disulphide-linked homodimer of 60 kDa linked via Cys93 (223). Dimerisation creates a high degree of specificity for granzyme A due to an extended site for its substrates (224). With its tryptase-like activity, granzyme A has been shown to activate caspase-independent cell death pathways with the cleavage of the mitochondrial protein NDUFS3 resulting in reactive oxygen species generation. Granzyme A is stored in granules inside the immunocompetent cells, but they may be released: i) through immunological synapse into a targeted cell, leading to cell death, or ii) extracellularly.

Extracellular granzyme A has been identified in plasma/serum circulation (225-242), as well as in the local body fluid (229, 230, 237-240, 243). In this study, extracellular granzyme A was assessed in the samples of amniotic fluid obtained from singleton pregnancies complicated by PPROM between gestational ages 24 and 36 weeks. Extracellular granzyme A was measurable

in all samples. The exact source of extracellular granzyme A in amniotic fluid is not clear. It is likely that immunocompetent cells in amniotic fluid, mainly T lymphocytes and natural killer cells, are an important source of extracellular granzyme. However, it can be hypothesised that various sources contribute (e.g. the placenta, the foetal membranes) to the presence of extracellular granzyme A in amniotic fluid due to the following reasons: i) regardless of the fact that a number of T cells and natural killers cells is the highest between gestational 15-30 and decreases toward the term (215), no correlation between extracellular granzyme A in amniotic fluid and gestational age at sampling was found; ii) intra-amniotic infection is associated with the elevation of the numbers of all amniotic fluid immunocompetent cells (except innate lymphoid cells) (215) but females with intra-amniotic infection did not have different levels of extracellular granzyme A in amniotic fluid than those without either MIAC or IAI; iii) MIAC in PPROM is associated with higher numbers of total T cells, CD4+ T cells, CD8+ T cells, neutrophils, and monocytes/macrophages (244) but females with MIAC did not have higher concentrations of extracellular granzyme A levels; iv) granzyme B and K positive cells were found in the human placentas with and without villitis unknown aetiology (245); and v) granzymes B positive cells were found in the normal placentas from the first trimester of the pregnancy (246). Collectively, the information above provides indirect evidence that the placenta and/or the foetal membranes should contribute to the production of extracellular granzyme A in amniotic fluid.

Approximately one-third of PPROM pregnancies were associated with MIAC at the time of diagnosis of PPROM (17). MIAC in PPROM represents a very heterogeneous condition due to: i) various microorganisms present in amniotic fluid (17, 247); ii) the possibility of the concomitant presence of multiple microorganisms in amniotic fluid (17, 247, 248); and iii) a broad range of microbial loads in amniotic fluid (17, 152, 249). Since extracellular granzyme A has been shown to be elevated in the systemic circulation of patients with tuberculosis (227), melioidosis (caused by gram-negative bacteria *Burkholderia pseudomallei*)(225), and typhoid fever (caused by *Salmonella enterica*) (226), as well as in bronchoalveolar lavage from patients with pneumonia (caused by *Streptococcus pneumoniae*) (229), a similar trend of the concentrations of extracellular granzyme A in amniotic fluid was expected in participants with MIAC. Surprisingly, participants with MIAC did not have higher concentrations of extracellular granzyme A in amniotic fluid than those without this complication but even lower concentrations. The explanation of this unexpected observation is unclear. However, when a subset of the participants with sterile IAI, having the highest extracellular granzyme A

concentrations in amniotic fluid, were examined from a cohort of the participants without MIAC, no difference in the concentrations of extracellular granzyme A levels was observed between those with and without MIAC (data not shown). This finding supports our speculation that other sources than amniotic fluid immunocompetent cells contribute to the amniotic fluid's extracellular granzyme A.

Employment of both cultivation and molecular biology methods to assess MIAC gave us a unique opportunity to identify a subset of the PPROM with sterile IAI. The frequency of this condition represents between 5% and 29% of PPROM pregnancies (17, 48). The underlying pathology leading to the development of sterile IAI has yet to be understood; however, two main mechanisms (or their combination) are considered: i) damage of the foetal membranes that leads to the release of endogenous molecules (alarmins) into amniotic fluid with a subsequent inflammatory response through the system of the pattern recognition receptors (17, 106, 155); ii) infection in amniochorial niche triggering the release of the inflammatory mediators from the foetal membranes into the amniotic fluid (156). Sterile IAI in pregnancies is usually associated with a milder intensity of intra-amniotic inflammatory response than intra-amniotic infection in terms of lower levels of inflammatory mediators (17, 48) and lower numbers of immunocompetent cells (250) in amniotic fluid.

In this thesis, a subset of participants with sterile IAI had higher concentrations of extracellular granzyme A in amniotic fluid than the remaining participants. Even higher than participants with intra-amniotic infection. This observation, along with the absence of a correlation between concentrations of IL-6 and granzyme A in amniotic fluid, further supports the hypothesis mentioned above that the placenta or foetal membranes contribute intensively to the extracellular concentrations of granzyme A in amniotic fluid.

This thesis brings a piece of new information that a cut-off value of 33.4 pg/mL can be used as an optimal tool for predicting sterile IAI in females with PPROM pregnancies. Especially its negative predictive value of 97% can be used routinely to distinguish between sterile IAI and intra-amniotic infection. On the other hand, the finding that sterile IAI is related to the highest concentrations of extracellular granzyme A in amniotic fluid should be taken with caution, owing to the small sample size of this subset of females.

6.2.3 Strengths and limitations

The main strength of this study is the assessment of MIAC with a very extensive and thorough approach combining aerobic/anaerobic cultivation, a specific PCR for *Ureaplasma* spp., *Mycoplasma hominis* and *Chlamydia trachomatis*, and a non-specific PCR for the 16S rRNA gene. Secondly, a relatively large cohort of females with singleton pregnancies with well-defined phenotypes of spontaneous preterm delivery (PPROM), in whom an amniocentesis was performed at the time of admission, was used in this study. This part of the thesis also suffers from some important limitations. For instance, the results on an elevation of the concentrations of amniotic fluid extracellular granzyme A in a subset of females with sterile IAI were not replicated in an independent cohort of the females. Also, the presence of granzyme A positive cells in the placenta and the foetal membranes were not evaluated.

7. CONCLUSION

7.1. SPECIFIC AIM 1

IAI was associated with increased pulsatility degree in the splenic vein in PPROM. The absence of differences in the pulsatility degree in the left portal branch, ductus venosus and portal stem between pregnancies with and without IAI excludes the transmission of hemodynamic changes from the precordial venous system and supports a local cause of the findings mentioned above.

7.2. SPECIFIC AIM 2

PI of the splenic vein of 0.36 was identified as optimal for predicting IAI in pregnancies complicated by PPROM.

7.3. SPECIFIC AIM 3

Extracellular granzyme A is a constituent of amniotic fluid in singleton pregnancies with PPROM between gestational ages 24 and 36 weeks. Concentrations of amniotic fluid extracellular granzyme A are elevated in the presence of sterile IAI. Concentrations of amniotic fluid extracellular granzyme A are comparable among the subsets of participants with intra-amniotic infection, colonisation of the amniotic cavity and negative amniotic fluid.

7.4. SPECIFIC AIM 4

The concentration of amniotic fluid extracellular granzyme A of 33.4 pg/mL was found to be optimal for predicting sterile IAI in females with PPROM pregnancies.

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