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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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Abstract of the thesis

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This thesis will be defended…….....

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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 umožnují 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 venózním 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 venózním 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.

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.

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) and has not decreased in the last decades. PTD represents the leading cause of perinatal mortality and morbidities (3-5) with 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 (6, 7).

PTD represents a heterogeneous condition, a syndrome induced by many causes (8). 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) (9, 10).

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 (11). PPROM is associated with approximately 30% of preterm deliveries and serious perinatal morbidity and mortality (3, 10). Many authors suggest that there should be a minimum of one hour period between the leakage of amniotic fluid and onset of the labour to make a diagnosis of PPROM (12, 13).

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 (14). 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 (7, 15). On the other hand, there is evidence, that the newborns from PPROM pregnancies, complicated with intra-amniotic infection and treated expectantly had the worst outcomes. (16, 17). Therefore, management of PPROM, considering the status of the intra-amniotic environment, seems to be the ideal approach for dealing with PPROM pregnancies.

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

The presence of microorganisms and/or their DNA in amniotic fluid is called microbial invasion of the amniotic cavity (MIAC) and complicates approximately 25-40% of PPROM pregnancies. It represents a pathological condition because the amniotic cavity is considered a sterile environment (18, 19).

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 (10). Of these options, an ascension of microorganisms from the lower genital tract seems to be the most important origin of microbial infection (20). The most common bacteria in PPROM pregnancies

are *Ureaplasma* spp. (21), that belongs with *Mycoplasma hominis* under an umbrella of the term - genital mycoplasmas.

Elevation of various inflammatory markers in the amniotic fluid is called intra-amniotic inflammation (IAI) (19, 22) . 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 (22, 23). 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 and amplification of cytokine production (24, 25).

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

1.2.1.1. 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% (26, 27) and is associated with the strongest intra-amniotic inflammatory response with a significantly higher level of amniotic fluid IL-6 and other inflammatory markers (28, 29) . The intensity of the inflammatory response also depends on the type of bacteria and microbial load (30, 31). 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 (14).

1.2.1.2. Sterile IAI

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, (14, 32) and is presented at a more advanced gestational age than those with intra-amniotic infection but earlier than those without IAI (14).

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, (33, 34), ii) infection of the choriodecidual space, (35), or iii) combination of those two processes (36).

1.2.1.3. 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. The colonisation frequency is about 11% in PPROM pregnancies.

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

An examination of inflammatory markers in amniotic fluid, obtained by the amniocentesis, is being considered as a "gold standard" for diagnosing intra-amniotic complications in PPROM pregnancies. However, technical difficulties connected with invasive procedure, leads research to identify some non-invasive markers of intra-amniotic complications of PPROM.

Historically, aerobic/anaerobic cultivation has been the first method to determine MIAC. 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 (20).

An elevation of white blood cell count, concentrations of lactate and 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 (37, 38) but not inferior to modern proteomic amniotic fluid markers (39). Nevertheless, there is still an effort to identify novel potential amniotic fluid markers for the detection of infection-related and inflammatory intra-amniotic complications in PPROM**.** One of these potential markers might be the granzymes family, particularly extracellular granzyme A.

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.

The most promising results brought studies, focused on the changes in the splenic vein blood flow (40, 41), which is a part of the foetal portal system. These studies 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 (40, 41). 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, and its relationship with inflammatory intra-amniotic complications, has not been performed. According to these facts, evaluation of the foetal portal system represents logical step in our effort to identify new ultrasound marker of these complications.

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. SET OF PATIENTS, METHODS AND STATISTICAL ANAYSIS

4.1. DIAGNOSIS OF PPROM

Diagnosis of PPROM was standardized for all aims of this thesis. 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. SPECIFIC AIMS 1 AND 2

4.2.1. Patients

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 10^{th} percentile, accompanied by an umbilical artery PI > 95^{th} percentile and/or a middle cerebral artery PI < 5^{th} percentile, or as an estimated foetal weight below the 3^{rd} percentile, regardless of umbilical and middle cerebral artery PI on Doppler flowmetry (42, 43). All the participants were Caucasian and provided written informed consent prior to inclusion in the study.

4.2.2. Methods

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. 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) (44).

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 (45), 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 (46). The ductus venosus was evaluated in an oblique or mid-sagittal abdominal view at the isthmus (47).

4.2.3. Statistical analysis

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).

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. SPECIFIC AIMS 3 AND 4

4.3.1. Patients

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.

4.3.2. Methods

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.

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) (44).

Detection of microorganisms proceeded as follows: i) PCR analysis of *Ureaplasma spp*., *Mycoplasma hominis*, and *Chlamydia trachomatis*; ii) sequencing of the 16S rRNA gene; iii) aerobic and anaerobic cultivation.

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.

4.3.3. Statistical analysis

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).

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

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.

IAI was defined as amniotic fluid IL-6 concentrations ≥ 745 pg/mL when IL-6 was measured using lateral flow-based immunoassay point-of-care test (48, 49). Intra-amniotic infection was defined as the concurrent presence of MIAC and IAI. Sterile IAI was defined as the presence of IAI without MIAC. The colonisation of the amniotic cavity was defined as the presence of MIAC without IAI. Negative amniotic fluid was defined as the absence of MIAC and IAI.

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 presence of IAI was observed in 27% of the patients (22/81). 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.

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; $p = 0.003$), as well as after the adjustment for gestational age at examination ($p = 0.003$).

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; $p = 0.17$).

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; $p = 0.88$).

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; $p = 0.86$).

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).

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.

5.3.2. 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; $p = 0.02$). 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.

5.3.3. 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; $p = 0.006$), as well as after the adjustment for gestational age at sampling ($p = 0.002$).

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;

6. DISCUSSION

6.1. SPECIFIC AIMS 1 AND 2

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.

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 (45, 50). The left portal branch exhibits a continuum from continuous flow through monophasic to biphasic pulsation, with pulsatile patterns as predominant types (46). 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 (45, 51).

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 (40). 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 (52). 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 (51). 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 considered (53). 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 (54, 55). Changes in the splenic circulation induced by sepsis were described in an adult animal model (56, 57). Endotoxemia modifies the pre- and post-capillary vascular tone in the spleen, increases hydrostatic pressure in the splenic capillaries, and leads to fluid extravasation (58, 59). 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 (60, 61). 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 (61).

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 a goal to reduce the number of transabdominal amniocenteses required for the detection of IAI.

6.2. SPECIFIC AIMS 3 AND 4

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.

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 (62-64). 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) (65, 66).

Extracellular granzyme A has been identified in plasma/serum circulation (67-78), as well as in the local body fluid (75, 79-81). 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 (63), 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) (63) 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 (82) 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 (83); and v) granzymes B positive cells were found in the normal placentas from the first trimester of the pregnancy (84). 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.

The frequency of sterile IAI condition represents between 5% and 29% of PPROM pregnancies (14, 32). 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 (32, 85, 86); ii) infection in amniochorial niche triggering the release of the inflammatory mediators from the foetal membranes into the amniotic fluid (35). 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 (14, 32) and lower numbers of immunocompetent cells (87) 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 further supports the hypothesis 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.

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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9. PUBLICATIONS AND LECTURES

9.1 ORIGINAL SCIENTIFIC PAPERS PUBLISHED IN THE JOURNALS WITH IMPACT FACTOR

1. **Spacek R,** Musilova I, Andrys C, Soucek O, Burckova H, Pavlicek J, et al. Extracellular granzyme A in amniotic fluid is elevated in the presence of sterile intra-amniotic inflammation in preterm prelabor rupture of membranes. *J Matern Fetal Neonatal Med. [IF$_{2020}$ 2.398]. 2020:1-10.* PMID: 32912008.
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3. Matulova J, Kacerovsky M, Hornychova H, Stranik J, Mls J, **Spacek R**, et al. Acute Histological Chorioamnionitis and Birth Weight in Pregnancies With Preterm Prelabor Rupture of Membranes: A Retrospective Cohort Study. *Front Pharmacol.* [*IF$_{2021}$ 5,988] 2022;13:861785.* PMID: 35308217.
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10. Musilova I, Andrys C, Drahosova M, Soucek O, Kutova R, Pliskova L, **Spacek R** et al. Amniotic fluid calreticulin in pregnancies complicated by the preterm prelabor rupture of membranes. *J Matern Fetal Neonatal Med.[IF$_{2016}$ 1.411]. 2016;29(24):3921-9.* PMID: 26953684.

9.2 OTHER PAPERS PUBLISHED IN THE JOURNALS WITH IMPACT FACTOR

9.3 ORIGINAL SCIENTIFIC PAPERS PUBLISHED IN THE JOURNALS WITHOUT IMPACT FACTORS

1. **Spacek R**, Kacerovsky M, Andrys C, Soucek O, Kukła R, Bolehovska R, Musilova I. Amniotic fluid soluble CD93 is elevated in the presence of intra-amniotic inflammation in preterm prelabor rupture of the fetal membranes. *Česká Gynekologie, 2022, accepted for publication 14.7.2022*

9.4 OTHER PAPERS PUBLISHED IN THE JOURNALS WITHOUT IMPACT FACTORS

1. **Špaček R**., Musilová I., Magdová K., Šimetka O., Kacerovský M.; Ultrazvuková diagnostika syndromu fetální zánětlivé odpovědi u žen s předčasným odtokem plodové vody; *Česká Gynekologie, 2017, 82, č. 1, s. 145–151*; PMID: 28585848.
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6. Šimetka O, **Špaček R**, Vašek P, Lattová V, Michalec I, Procházka M.; Srovnání efektivity misoprostolu a dinoprostu při indukci druhotrimestrálního abortu; *Česká Gynekologie, 2011, 76, č. 6 , s. 72-76;* PMID: 22312845.

9.5. LECTURES CZECH

1. Vaginální porod po dvou předchozích císařských řezech, *XXXVII. celostátní konference Sekce perinatologie a fetomaternální medicíny ČGPS ČLS JEP - Štemberovy dny, Praha 2022.*
2. Komplexní management preeklampsie, *XL. Regionální pracovní dny klinické biochemie, Karlova Studánka 2021.*
3. Zajištění rodičky a novorozence z pohledu porodníka, *XXXVI. Neonatologické dny ČNS ČLS JEP, Ostrava 2021.*
4. PPROM v previabilním období a v období pozdní prematurity, *Regionální perinatologické setkání MSK za rok 2020, Ostrava 2021.*
5. Preeclampsie a HELLP syndrom z pohledu praktického lékaře, *7. kongres medicíny pro praxi v Ostravě – Kongres praktických lékařů, Ostrava 2020.*
6. Stanovení rizika preeklampsie v rámci prvotrimestrálního screeningu pomocí markeru PIGF, *Setkání soukromých gynekologů Brno 2020.*
7. Management inkompatibility v krevních systémech matky a plodu, Hanákovy dny, Ostravice 2019.
8. Odložený podvaz pupečníku u císařského řezu, *Hanákovy dny, Ostravice 2019.*

9. Porodník, porodní asistentka a dula*, Edukační den porodní asistence, Ostrava 2019.*
10. Komplikace vícečetné gravidity, *Pětrošův den prenatální diagnostiky, Ostrava 2018.*
11. Ultrazvukové vyšetření žilního systému plodu u těhotných s PPROM, *XXXIV. celostátní konference Sekce perinatologie a fetomaternální medicíny ČGPS ČLS JEP, Karlovy Vary 2017.*
12. Diabetes v těhotenství a jeho důsledky na zdraví žen, *Kongres praktických lékařů, Ostrava 2017.*
13. Vedení a výsledky porodů SGA plodů ve FN Ostrava v letech 2014 až 2015, *XXXIII. celostátní konference Sekce perinatologie a fetomaternální medicíny ČGPS ČLS JEP, Ústí nad Labem 2016.*
14. Ultrazvuk a předčasný porod, *Čechova ultrazvuková konference, Olomouc 2016.*
15. PPROM současný management, *Pětrošův den prenatální diagnostiky, Ostrava 2016.*
16. Ultrazvuk v šestinedělí, *36. celostátní konference Sekce ultrazvukové diagnostiky ČGPS ČLS JEP, Brno 2015.*