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Intraamniální infekční a zánětlivé komplikace spojené s předčasným odtokem plodové vody

Infection-related intra-amniotic complications in women with preterm prelabor rupture of membranes

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Abstract of the thesis

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The thesis will be defended ..... …

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1 Souhrn

Předčasný odtok plodové vody před 37. týdnem těhotenství (PPROM) je zodpovědný zhruba za třetinu všech předčasných porodů. Infekční a zánětlivé komplikace u PPROM jsou představovány přítomností mikrobiální invaze do amniální dutiny (MIAC), intraamniálním zánětem (IAI) a histologickou chorioamnionitidou (HCA). Jejich prevalence u PPROM se pohybuje mezi 20-50%. Jejich přítomnost je také spojena se zhoršenou perinatální mortalitou a morbiditou.

Prvním specifickým cílem dizertační práce bylo hodnocení prediktivního potenciálu C- reaktivního proteinu (CRP) v mateřské krvi při přijetí k diagnostice MIAC a/nebo HCA. Do studie bylo zařazeno 386 žen s PPROM. Hladina CRP byla nejvyšší u žen s přítomností obou MIAC a HCA.

Druhým specifickým cílem bylo hodnocení novorozenecké morbidity u žen s PPROM do 34. týdne gravidity. Do studie bylo zařazeno celkem 122 žen s PPROM v gestačním stáří 24^{+0}-34^{+0} týdnů. U novorozenců byla sledována morbidita a mortalita, které byly hodnoceny vzhledem k přítomnosti MIAC a/nebo HCA. Přítomnost MIAC a HCA byla spojena s vyšším rizikem rozvoje časné novorozenecké sepse.

Třetím a čtvrtým specifickým cílem bylo zhodnocení vztahu stavu periodontu a přítomností intraamniálních infekčních a zánětlivých komplikací spojených s PPROM, a také zhodnocení vztahu lokální zánětlivé odpovědi v gingiválním tekutině a intraamniální či mateřské systémové zánětlivé odpovědi. Do studie bylo zařazeno celkem 78 žen s PPROM v gestačním stáří 24^{+0}-36^{+6} týdnů. Ve třetí cíli nebyl prokázán vztah mezi stavem periodontu a intraamniálními infekčními a zánětlivými komplikacemi. V posledním specifickém cíli práce nebyl nalezen vztah mezi lokální zánětlivou odpovědí v gingiválním tekutině a intraamniální či mateřskou zánětlivou odpovědí..

Hlavním závěry dizertační práce jsou zjištění, že podskupina PPROM komplikovaná současnou přítomností MIAC a HCA je spojena s nejvyšší mateřskou systémovou zánětlivou odpovědí, měřenou hladinou CRP v mateřské krvi, a nejvyšším rizikem rozvoje časné novorozenecké sepse. Nebyl prokázán vztah mezi klinickým stavem periodontu a přítomností intraamniálních infekčních či zánětlivých komplikací u pacientek s PPROM.

2 Summary

Preterm prelabor rupture of membranes before 37. weeks of gestation (PPROM) is responsible for approximately one third of all preterm deliveries. Infection-related complications in PPROM are represented by the presence of microbial invasion of the amniotic cavity (MIAC), intra-amniotic inflammation (IAI) and histological chorioamnionitis (HCA). The presence of these complications is found in approximately 20-50% of all cases of PPROM and is associated with worse neonatal outcomes.

The first specific aim was to evaluate whether maternal serum CRP has a diagnostic value for diagnosis of MIAC and HCA. The study population consisted 386 women. CRP level was the highest in PPROM pregnancies complicated with MIAC and HCA.

The second specific aim was to evaluate short-term neonatal outcome in women with PPROM before 34 weeks of gestation. The study population consisted 122 women with PPROM at gestation age 24^{+0}-34^{+0} weeks. In neonates was evaluated the influence of MIAC and HCA on neonatal outcome. The presence of both MIAC and HCA increased the risk of early onset sepsis.

The third and fourth specific aim was evaluation of an association between periodontal status and infection-related intra-amniotic complications in pregnancies complicated by PPROM and/or evaluation of association between the local inflammatory response in gingival crevicular fluid and maternal and intra-amniotic inflammatory responses, respectively. The study consisted 78 women with PPROM. In both studies was not proven the association between periodontinum and intraamniotic environment and/or maternal inflammatory response.

The main findings of the thesis are that (i) the presence of both MIAC and HCA is associated with the highest maternal inflammatory response, measured by CRP, and increased the risk of early onset sepsis in pregnancies complicated by PPROM, (ii) the periodontal status is not related to the infection-related and inflammatory intra-amniotic complications in women with PPROM.

3 Introduction

Preterm delivery is defined by the World Health Organization (WHO) as delivery at less than 37 weeks of gestation(1). Preterm delivery results from various disorders related to the mother and/or fetus, some of which are explained, and some that are of unknown etiology(2). Worldwide, the rate of preterm birth is approximately 11%, and almost 15 million preterm births occur annually(3). In the US, preterm births represent 11-13% of all deliveries; in Europe and other developed countries, that number is approximately 5-9% (4).

Preterm birth is responsible for 75% of perinatal mortality and more than half of the long-term morbidity (5). Improvement in neonatal outcome has occurred since antenatal corticosteroids and magnesium neuroprophylaxes have been implemented into clinical management (6). The crucial point with regard to perinatal adverse outcomes is represented by the gestational age at delivery, when late preterm-born neonates have significantly lower mortality and morbidity rates compared with those born at earlier gestational ages (7, 8).

Preterm delivery represents a rather heterogeneous condition that can be subdivided according to various conditions and aspects.
First, based on the phenotypes, it can be divided into the following categories: i) spontaneous preterm delivery with intact membranes (PTL), ii) preterm prelabor rupture of membranes (PPROM), and iii) iatrogenic preterm delivery for maternal or fetal indications. Approximately 30% to 35% of preterm births are iatrogenic, 40% to 45 % are PTL, and 25% to 30% are PPROM.

PPROM occurs in between 2-8% from all pregnancies (9). PPROM is responsible for approximately one third of preterm births, and it substantiality contributes to significant perinatal morbidity and mortality (8, 10). PPROM has a multifactorial etiology and might be diagnosed as one or more etiologic processes in different patients (8, 11, 12). Infectious etiology used to be considered to play a crucial role in the pathophysiology of PPROM; however, recent studies have shown that the majority of cases show no presence of bacteria in the amniotic fluid, nor intra-amniotic inflammation (13-16).

Based on the pathophysiologic processes leading to PPROM, it might be classified into three major groups: i) PPROM with the absence of cervical change and longer latency to delivery, ii) PPROM involving bleeding disorders or coagulopathies, and iii) PPROM associated with cervical changes and shorter latency to delivery (17).

Pregnancies affected by PPROM might be complicated by microbial invasion of the amniotic cavity (MIAC) and intra-amniotic inflammation (IAI). The presence of these two complications is found in approximately 30% to 60% of cases of PPROM, respectively (18, 19). Both these complications might result in the development of histological chorioamnionitis (HCA). The presence of MIAC is thought to activate intra-amniotic immune response through the system of pattern recognition receptors, which results in the development of microbial-associated IAI (19-22). Moreover, the intensity of IAI depends on the type of bacteria and their microbial load (23-25). Therefore, the presence of a small amount of bacteria with a low virulent potential, such *Ureaplasma* species, in the amniotic fluid is unlikely to elicit IAI (25, 26).

Therefore, this condition is not associated with worse pregnancy and neonatal outcomes. This scenario is characterized as a colonization of amniotic fluid (23, 27).

In contrast, some endogenous mediators called alarmins (e.g., high mobility group box-1 protein) are released from necrotic cells into the amniotic fluid and can trigger IAI through the same system of pattern recognition receptors as in the infectious scenario (15, 28). This scenario leads to the development of sterile IAI (the presence of IAI without any proven microorganisms in the amniotic fluid).

The presence of both MIAC and HCA is associated with the most intensive IAI in PPROM compared to the PPROM pregnancies complicated by HCA alone (sterile HCA), MIAC alone (colonization), and PPROM pregnancies without both MIAC and HCA (29, 30). Similar results were observed in terms of the intensity of the fetal inflammatory response, measured by umbilical cord blood IL-6 concentrations (31). Moreover, pregnancies complicated with MIAC and HCA are prone to subsequent development of early-onset sepsis in newborns (32).

On the other hand, the presence of HCA alone or MIAC alone has similar intra-amniotic and fetal inflammatory responses as in women without either MIAC or HCA. Likewise, the newborns from these pregnancies do not have a higher risk of the development of early-onset sepsis (29, 30).

The presence of MIAC and HCA might be considered to be outcomes only when PPROM is managed actively owing to a short interval between the amniotic fluid and the placenta samplings. Therefore, in PPROM managed expectantly, when a longer latency is expected, the presence of MIAC and HCA cannot be taken as an outcome. In women with PPROM managed expectantly, the knowledge about MIAC and IAI more appropriately describe an intra-amniotic environment.

The presence of both MIAC and IAI (microbial-associated IAI) has been shown to be related to the highest intra-amniotic, cervical, and maternal inflammatory responses (33-35). Interestingly, no difference has been shown between the intensity of fetal inflammatory responses triggered by microbial-associated and sterile IAI (36). It means that the fetal inflammatory response is driven by IAI regardless of the presence or absence of bacteria in amniotic fluid.

Currently, the evaluation of amniotic fluid obtained by transabdominal amniocentesis is still considered as the gold standard to show infection-related and inflammatory intra-amniotic complications. Therefore, there is an urgent need for surrogate markers from noninvasively obtained body fluids such as cervical and vaginal fluids or maternal blood. Nevertheless, their diagnostic value still has not reached the level of the amniotic fluid markers because of their limited sensitivity and specificity.

In general, two different management approaches of PPROM are considered, the expectant and the active approaches. Since the gestational age of delivery has been clearly shown to be a main determinant of neonatal morbidity and mortality, the expectant management is recommended that the expectant management might be a method of choice in women with PPROM beyond 34 weeks as well (37-41). However, the presence of IAI and microbial-associated IAI is associated with short-term and long-term consequences (36, 42). Archabald et al have shown that newborns from pregnancies with PPROM complicated by microbial-associated IAI and treated

expectantly had the worst outcome (43). Therefore, the individualized management of PPROM based on the knowledge about the presence of MIAC and IAI might be the best option.

PPROM might be characterized as a pregnancy complication associated with a relatively high rate of the neonatal morbidity and mortality. The morbidity and mortality are mainly related to gestational age of delivery (10, 12, 44-46). However, the neonatal outcome from PPROM has been shown to be worse than that from PTL.

Because a majority of PPROM might be complicated by the presence of MIAC, IAI, and HCA, it is possible to anticipate that newborns from these pregnancies are jeopardized by the development of fetal inflammatory response syndrome (FIRS) (31). This condition is characterized by the elevation of umbilical cord blood IL-6 greater than 11 pg/mL. The presence of funisitis and/or chorionic plate vasculitis represents the histopathological hallmark (47). It is well known that fetuses affected by FIRS are at higher risk of multi-organ disorders and serious neonatal morbidity (48, 49). However, there is a lack of information about whether and how the presence of MIAC and HCA might affect the short-term neonatal outcome.

Periodontal disease represents one of the most frequent chronic inflammatory conditions in the adult population. The reported prevalence of chronic periodontal disease is approximately 10% to 90% in the adult population (50). Periodontal disease is often caused by periopathogenic bacteria such as *Fusobacterium* sp., *Prevotella* species, *Porphyromonas* species, and other anaerobic gram-negative bacilli. It has been suggested that the presence of periodontal disease in pregnancy might cause translocation of periopathogenic bacteria to uteroplacental circulation, damage the placenta, and potentially lead to preterm delivery (51). Periodontal disease is often caused by periopathogenic bacteria such as *Fusobacterium* species, *Prevotella* species, *Porphyromonas* species, and other anaerobic Gram-negative bacilli. It has been suggested that the presence of periodontal disease in pregnancy might cause translocation of periopathogenic bacteria to uteroplacental circulation, damage the placenta, and potentially lead to preterm delivery (52, 53). In addition, periopathogenic bacteria have been demonstrated as a cause of MIAC in women with PPROM (54).
Periodontitis, a chronic local inflammation, might be associated with elevation of pro-inflammatory cytokines in the cervical fluid, such as IL-6 (55-58). These inflammatory mediators may reach the systemic circulation, which could lead to a low-grade systemic inflammation (59, 60). However, there is a lack of knowledge regarding whether this originally local inflammatory response localized in gingival cervical fluid may affect the intra-amniotic compartment via the spreading of bacteria or their products or through the systemic inflammatory response in women with PPROM and lead to the development of sterile IAI.

4 Objective of the thesis

1. To evaluate whether maternal serum CRP has a diagnostic value for infection-related intra-amniotic complications in PPROM pregnancies. Next aim was to evaluate whether there was a correlation between maternal inflammatory response, measured by maternal serum CRP, and microbial load of *Ureaplasma* species.

2. To identify whether the presence of infection-related intra-amniotic complications is associated with worse short term neonatal outcomes in pregnancies complicated by PPROM in women below 34 weeks of gestation.

3. To investigate an association between the periodontal status and infection-related intra-amniotic complications in pregnancies complicated by PPROM.

4. To identify the association between the local inflammatory response in gingival crevicular fluid measured by the levels of multiple proteins and maternal and intra-amniotic inflammatory responses measured by maternal CRP and amniotic fluid IL-6 concentrations, respectively, in women with PPROM.

5 Materials and methods

5.1 Patients

5.1.1 Specific aim I.

A prospective cohort study was performed. The study population consisted 386 women with singleton pregnancies at gestational ages between 24+0 and 36+6 weeks who were admitted for PPROM to the Department of Obstetrics and Gynecology in Hradec Kralove between January 2008 to December 2013.

5.1.2 Specific aim II.

Prospective observational cohort study was performed. The study population consisted of 122 women with PPROM at gestation age 24+0-34+0 weeks who were admitted to the Department of Obstetrics and Gynecology, University Hospital Hradec Kralove, Czech Republic between June 2008 and February 2012. All women included in specific aim II. were part of the specific aim I.

5.1.3 Specific aim III. and IV.

A prospective study of pregnant women between 24+0-36+6 weeks gestation with PPROM, who were admitted to the Department of Obstetrics and Gynecology at the University Hospital Hradec Kralove in the Czech Republic between December 2014 and April 2016, was conducted. A total of 78 women with PPROM met inclusion criteria to the study.

5.2 Inclusion criteria

Inclusion criteria were: singleton pregnancies complicated by PPROM between 24+0 to 36+6 weeks of gestation and age of 18 years and more.

5.3 Exclusion criteria

Women were excluded from the study if they had signs of clinical chorioamnionitis, fetuses with an estimated weight $<10^{th}$ percentile, the presence of either congenital or chromosomal fetal abnormalities, gestational or pre-gestational diabetes, gestational hypertension, preeclampsia, signs of fetal hypoxia, or significant vaginal bleeding.

5.4 Sample collection

5.4.1 Amniotic fluid

Ultrasonography-guided transabdominal amniocentesis was carried out, and approximately 5 mL of amniotic fluid was aspirated and divided into three tubes. The first and second tubes with uncentrifuged amniotic fluid were transported immediately to the laboratory for DNA extraction, polymerase chain reaction for

Ureaplasma species, *Mycoplasma hominis*, and *Chlamydia trachomatis* and 16S rRNA gene. Uncentrifuged amniotic fluid from the third tube was used to the assessment of IL-6 concentrations and after that was centrifuged for 15 minutes at 300 *g* immediately after collection in order to remove debris and cells, divided into aliquots and stored at -70°C until analysis.

5.4.2 Maternal blood

Maternal blood was obtained through venipuncture of the cubital vein at admission. Maternal serum CRP was measured using an immunoturbidimetric analysis (Modular PP analyzer, Roche, Basel, Switzerland), with a method sensitivity of 0.3 mg/L. (61)

5.4.3 Gingival crevicular fluid

Gingival crevicular fluid was collected using standard sterile paper strips during a dental examination (MM Absorbent Paper Points, Medin a.s., Czech Republic). The deepest gingival pocket in each individual woman was selected for sampling. The selected tooth was separated and cleaned by cotton rolls and dried by air. The strip was inserted into the pocket using sterile tweezers. The strip was left in situ for 30 seconds. The strips were subsequently placed into the tubes containing 0.5 mL of sterile phosphate-buffered saline. The tube with the strip was shaken at 1400 moves/minute for 20 minutes. Strip was then removed using sterile tweezers. Tube was subsequently centrifuged 300 × *g* for 15 minutes at room temperature. The supernatant was aliquoted and stored at −70°C until analyses.

Gingival crevicular fluid levels of the following proteins were analysed at the Statens Serum Institute (Department of Clinical Biochemistry and Immunology, Copenhagen, Denmark) using a multiple sandwich immunoassay based on flowmetric Meso-Scale technology. The gingival fluid levels of IL-1β, IL-6, IL-8, IL-10, IL-12, IL-17, IL-18, adiponectin, brain-derived neurotropic factor (BDNF), CRP, heat shock protein 70 (HSP 70), high mobility group box protein-1 (HMGB-1), granulocyte macrophage colony stimulating factor (GM-CSF), insulin-like growth factor-binding protein (IGFBP)-1, macrophage inhibitory protein (MIP) -1α, MIP-1β, MMP-8, MMP-9, monocyte chemotactic protein-1 (MCP-1), S100A8, T-cell-specific protein (RANTES), tumor necrosis factor (TNF)-α, TNF-β, tumor growth factor-β1 (TGF-β1), soluble TNF receptor-1 (sTNF-R1), and trombospondin-1 were assessed

5.4.4 Placenta and fetal membranes

After delivery, each placenta was collected and fixed in formalin, and tissue samples with placental membranes were inserted in paraffin. Tissue sections of placentas were stained with hematoxylin-eosin for histological examination. The degree of neutrophil infiltration was assessed separately in the free fetal membranes (amnion and chorion-decidua), in the placenta (amnion and chorionic plate), and in the umbilical cord according to the criteria given by Salafia et al. (62).

5.5 Dental examination

Periodontal examination was performed within 72 hours of admission. A full-mouth recording was used to determine the periodontal and oral hygiene status. Probing pocket depth (PPD), defined as the distance from the marginal gingiva to the bottom of the pockets, and clinical attachment loss (CAL), defined as the distance from the cementoenamel junction to the bottom of the pockets, were measured at four sites (medial, buccal, oral, and vestibular) on each fully erupted tooth. Third molars and retained roots were not included. Probing depth was measured by a calibrated periodontal probe PCPUNC156 (Hu-Friedy, Chicago, IL) with fine calibration with single millimeter grading. No other probes were used. A Decayed, Missing, and Filled (DMF) index was recorded for each subject. Gingival status was evaluated by gingival index (GI) as adapted from Silness and Löe and the plaque index (PLI) was evaluated according to Löe et al (63, 64).

5.6 Neonatal outcomes

Short-term neonatal outcome was evaluated on the presence of the following outcomes: need for tracheal intubation, respiratory distress syndrome (defined by the presence of two or more of the following criteria: evidence of respiratory compromise, a persistent oxygen requirement for >24 h, administration of exogenous surfactant and evidence of hyaline membrane disease on X-ray), intraventricular hemorrhage grade I-IV (diagnosed by cranial ultrasound examinations based on criteria defined by Papile et al.) (65), necrotizing enterocolitis at least grade IIA according to modified Bell´s criteria (66), retinopathy of prematurity (identified by retinoscopy), early onset sepsis (EOS - defined as evidence of positive blood culture during the first 72 h of life and either the presence of clinical symptoms or strong clinical suspicions of sepsis [presence of symptoms and elevated CRP and/or affected white blood cell count], late-onset sepsis (defined as evidence of positive blood culture between 4-120 days of life and either the presence of clinical symptoms or strong clinical suspicions of sepsis [presence of symptoms and elevated CRP and/or affected white blood cell count]), bronchopulmonary dysplasia (defined by the infant's oxygen requirement and/or ventilator support at 28 days of life and in the 36th postmenstrual week), pneumonia (diagnosed by abnormal findings on chest X-rays), or neonatal death, defined as death before hospital discharge.

5.7 Definitions

5.7.1 MIAC

MIAC was determined based on a positive result of the PCR analysis for *Ureaplasma* species, *Mycoplasma hominis* and *Chlamydia trachomatis* and/or positivity for the 16S rRNA gene with subsequent microorganism identification by sequencing.

5.7.2 IAI

Diagnosis of IAI was confirmed with an amniotic fluid IL-6 concentration of 745 pg/mL or higher, measured by a point of care test. Sterile IAI was defined as IAI without MIAC (61, 67).

5.7.3 HCA

Diagnosis of HCA was based on the presence of neutrophil infiltration in the chorion-decidua (Grades 3-4), the chorionic plate (Grades 3-4), the umbilical cord (Grades 1-4), and/or the amnion (Grades 1-4). The classification of histological chorioamnionitis is presented in Table 1. A single perinatal pathologist, who was blinded to the clinical status of the women, reviewed all of the histopathological samples

5.7.4 Periodontal disease

Severe periodontal disease was defined as having two or more sites with ≥ 6 mm CAL (not on the same tooth) or one or more site(s) with ≥ 5 mm PPD. Second, 'other' periodontal disease comprised two lesser levels of disease: moderate periodontal disease, defined as two or more sites with ≥ 4 mm clinical CAL (not on the same tooth) or two or more sites with PPD ≥ 5 mm, also not on the same tooth; and mild periodontal disease, defined as ≥ 2 sites with ≥ 3 mm CAL and ≥ 2 sites with ≥ 4 mm PPD (not on the same tooth), or 1 site with ≥ 5 mm PPD. Subjects not meeting either criterion were considered as having a healthy periodontium. This definition was adopted and simplified for four site probing from the CDC/AAP definition published by Eke et al. (68). and recommended by the consensual statement published by Holtfreter et al. (69).

5.8 Statistical analyses

The demographic and clinical characteristics were compared using non-parametric Kruskal-Wallis test and data was presented as the median [interquartile range (IQR)] and using the Chi-square test and presented as number (%). The normality of the data was tested using the D'Agostino and Pearson omnibus normality test. Maternal serum CRP concentrations were compared with using non-parametric Kruskal-Wallis test and data was presented as the median [interquartile range (IQR)]. A Spearman partial correlation was used to adjust the data for gestational age at admission, at delivery, administration of corticosteroids, administration of antibiotics and smoking. Spearman rank correlation test was used for analysis of correlation between CRP concentrations and the microbial load of *Ureaplasma* species and for analysis of association between levels of proteins in gingival crevicular fluid and maternal serum CRP and amniotic fluid IL-6 concentrations Differences were considered statistically significant at $p<0.05$. All p values were from two-sided tests, and all the statistical analyses were performed using SPSS 19.0 for Mac OS X (SPSS Inc., Chicago, IL).

6 Results

6.1 Specific aim I.

Women with both MIAC and HCA exhibited the highest concentration of CRP [median: 9.0 mg/L (IQR: 3.9-20.1)] vs. women with HCA alone [median: 6.4 mg/L (IQR: 3.6–12.1)] or MIAC alone [median: 4.3 mg/L (IQR: 3.6-15.6)] or with neither MIAC nor HCA [median: 4.5 mg/L (IQR: 2.0-8.6)]; in crude analysis ($p < 0.0001$), as well as after adjustment for gestational age of sampling ($p < 0.0001$).
A positive correlation between the microbial burden of *Ureaplasma* species in the amniotic fluid and maternal CRP concentrations was found (Spearman rho = 0.33, $p = 0.002$).

6.2 Specific aim II.

The short-term neonatal outcome according to the presence or absence of MIAC and/or HCA is presented in Table 4. In the unadjusted analysis, a higher need to administrate surfactant and a higher incidence of EOS were observed in women with both MIAC and HCA. However, after adjusting the analysis for gestational age at delivery, only EOS remained significant (p=0.001). No other significant differences were observed for short-term neonatal outcomes among the subgroups.

6.3 Specific aim III.

A total of 45% (35/78) women had periodontal disease. Mild, moderate, and severe periodontal disease was present in 19% (15/78), 19% (15/78), and 6% (5/78) of women, respectively. In women with mild periodontal disease, 67% (10/15) had MIAC and 67% (10/15) had IAI. In women with moderate periodontal disease, 26% (4/15) had MIAC and 33% (5/15) had IAI.
Based on the presence of MIAC and or IAI, women were split in four subgroups: 1) with both MIAC and IAI (microbial-associated IAI; 15% [12/78]), 2) with IAI alone (sterile IAI; 10% [8/78]), with MIAC alone (colonization; 13% [10/78]), and without both MIAC and IAI (62% [48/78]). No associations between periodontal status and any particular subgroup of women were found.

6.4 Specific aim IV.

No correlations between the levels of proteins in the gingival crevicular fluid and maternal serum CRP and amniotic fluid IL-6 concentrations were observed, except for a weak positive correlation between the GM-CSF and CRP (Spearman rho = 0.26, $p = 0.02$). No correlations between levels of proteins in gingival crevicular fluid and amniotic fluid IL-6 concentrations were observed, except the correlation with BDNF (rho= 0.73; p=0.05).

7 Discussion

7.1 Specific aim I.

The main findings of the specific aim I are as follows: i) the presence MIAC and HCA was associated with the highest maternal inflammatory response measured by maternal serum CRP; ii) microbial load of *Ureaplasma* species in the amniotic fluid was related to the intensity of maternal inflammatory response.

The worst scenario in PPROM is the presence of MIAC and HCA (19, 30, 70). Previously was demonstrated, the presence of MIAC and HCA represented the condition with the highest inflammatory response in the amniotic cavity and fetal compartment (29, 31). However, the only method to diagnose MIAC is an invasive procedure, and histopathological information of HCA is not available antenatally. Therefore, the present study was focused on the prediction of MIAC and HCA using a non-invasive approach, which involved the determination of a classical marker of infection - maternal serum CRP.

Previous studies reported the influence of CRP in pregnancies complicated with HCA (71-73), but few studies focused on the relationship with MIAC (18). Howman et al. observed a significantly higher maternal inflammatory response when evaluating CRP in women with HCA (71). The study population also included women with clinical chorioamnionitis. Therefore, the differences in CRP could partially reflect the inclusion of women with severe and late stage infections, when the maternal inflammatory response is primarily activated (74). Contrary to Howman et al., Laar et al. found that the inclusion of women with clinical chorioamnionitis did not exhibit differences when the role of CRP was evaluated to detect HCA (72). Similarly, Martinez et al. conducted a review based on the evaluation of eight studies and did not observe data to support an influence of HCA on CRP (73). A lack of association was also observed when evaluating maternal serum CRP between PPROM women with or without MIAC (18).

The data obtained in the present study revealed differences in CRP levels between different infectious subgroups of women with PPROM. CRP concentrations are gestational age-dependent (75), and differences in gestational age at sampling were observed between groups. Therefore, CRP concentrations were adjusted for this confounding factor. CRP remained significantly higher in the subgroup of women with MIAC and HCA after adjusting by gestational age at sampling. However, the predictive value of CRP to identify the worst infectious scenario was weak, even at early gestational ages. These findings revealed that assessment of CRP levels was a poor predictor of MIAC and HCA, which highlights the importance of considering the amniocentesis as the only accurate method to identify this infectious condition. Therefore, these findings are interesting and clinically relevant.

The previous studies clearly demonstrated that the intraamniotic and maternal inflammatory responses were depended on the microbial load of *Ureaplasma* species (23, 25, 26). The present study confirmed an association between maternal CRP and the amount of *Ureaplasma* species in the amniotic fluid.

7.2 Specific aim II.

The main finding of the specific aim II was the fact that PPROM pregnancies before 34 weeks of gestation complicated by the presence of both MIAC and HCA are at increased risk of subsequent development of EOS.

There are few studies evaluating the influence of MIAC on short-term neonatal outcome in women with PPROM, and they report a negative impact on neonatal morbidity (76-78). In this regard, Shim et al. reported a lower 1-min Apgar score and a higher risk of respiratory distress syndrome, intraventricular hemorrhage and bronchopulmonary dysplasia in newborns from women with MIAC (77). A higher risk of EOS in newborns exposed to intraamniotic inflammation and MIAC was also observed by Buhimschi et al. (76). Finally, Cobo et al. found that the presence of specific proteomic biomarkers (neutrophil defensins and calgranulins A, C) were found to be independent predictors of MIAC and neonatal composite morbidity in women with PPROM (78).

Similar results have been reporting regarding the occurrence of HCA (79-81). In this regard, Rusell et al. observed higher rates of perinatal death and EOS in the first 48 hours when HCA was present (81). Moreover, Tsiartas et al. observed an association between HCA and the occurrence of EOS (80). Erdemir et al. observed higher rates of EOS, surfactant requirements, bronchopulmonary dysplasia and mortality when pregnancies exhibited HCA. None of these studies, except that of Erdemir et al., considered gestational age at delivery when evaluating short-term neonatal outcome. Erdemir et al. suggested that the effect of HCA on neonatal morbidity and mortality was more prominent than the effect of low gestational age alone (79).

The fact that in the current study was used active management allowed to explore clearly defined subgroups of women with PPROM according to the presence of MIAC and/or HCA. Thus, in the current study, a significantly higher risk of EOS when both MIAC and HCA were present in women with PPROM prior to 34 weeks was observed.

Similar results were reported in women with PPROM after 34 weeks of gestation; however, gestational age was not considered as a potential confounder factor (70). In the current study, not only in the crude analysis but also after adjusting the analysis for gestational age at delivery, the risk of EOS remained significantly higher in women with both MIAC and HCA. This highlights the importance of considering the presence of both MIAC and HCA as the worst-case scenario for the fetus. This infectious exposition increased the risk of EOS regardless of the gestational age at which newborns are born.

Finally, no significant differences in the short-term neonatal outcome were observed when compared women with HCA alone, MIAC alone or with women without either MIAC or HCA. Similarly, although in women with PTL, Combs et al. reported that women with MIAC alone with low inflammatory response in the amniotic cavity presented a similar short-term neonatal outcome compared to women without infection (27). Altogether, it seems that it is the infectious nature of the combined MIAC and HCA condition that is responsible for the increased risk of EONS and not

the activation of the intra-amniotic inflammatory response given that there was no difference on the composite neonatal outcome.

Altogether, it seems crucial an antenatal prediction of those women with both MIAC and HCA. Whether MIAC and HCA are associated with the highest intra-amniotic inflammatory response (mediated by not only a multiplex approach (78) but also by bedside IL-6 (30) and whether the presence of MIAC and HCA is associated with a significant risk of EOS, for further clinical perspective, defining the best cut-off of IL-6 for prediction of the occurrence of EOS in women with MIAC and HCA should be given.

7.3 Specific aim III.

The principal findings of this specific aim are as follows: i) periodontal disease was found in 45% women with PPROM; ii) women with MIAC did not have a worse periodontal status than women without MIAC; iii) women with IAI did not have a worse periodontal status than women without IAI; iv) the presence MIAC and/or IAI was not associated with a different periodontal status in women with PPROM.

In this cohort of women with PPROM, the prevalence of periodontal disease reached 45%. Given that the prevalence of periodontal disease in women in PPROM has been rarely studied, observed results are unique. An epidemiological study from Italy comparing the periodontal status of healthy pregnant women and women with selected pregnancy pathologies included women with PPROM (74 out of 230 women with pregnancy pathologies). Overall, periodontal disease was diagnosed in 82% of women with pregnancy pathologies (82). Nevertheless, this study did not include specific details of periodontal health in the women with PPROM, so it remains unclear how many women with PPROM had periodontal disease (82). Another study from Croatia found periodontal disease in 62% of women with spontaneous preterm delivery. Even though women with PPROM were included, the rates of periodontal disease in women with PPROM were not provided (83). The only study specifying periodontal health of women with PPROM is a study from Chile (84). However, only eight women with PPROM were included and therefore it is impossible to draw a conclusion about the prevalence of periodontal disease in PPROM from such a small cohort.

The mechanisms by which periodontal disease can affect pregnancy and trigger preterm delivery are still poorly understood. It has been hypothesized that cytokines produced by periodontal inflammation are released into systemic circulation and may affect the placenta and fetus, and may be a cause of sterile IAI. Alternatively, oral organisms can be disseminated into amniotic fluid, placental circulation, and the fetus itself, causing MIAC. The presence of MIAC may trigger an intra-amniotic inflammatory response and lead to preterm delivery. This hypothesis is supported by data from an animal model. Mice infected with *Porphyromonas gingivalis* (periopathogenic bacteria) had increased IL-6 levels and exhibited preterm delivery. Moreover, *Porphyromonas gingivalis* was found in the placental tissue in situ (85). A clear translation of these data to humans is a challenging issue. In this study, no associations between periodontal disease and MIAC were found. Given MIAC is a heterogeneous group containing different bacterial species having different microbial

loads in amniotic fluid, it would be important to know whether some periopathogenic bacteria were found in women with PPROM. In this study, periopathogenic bacteria in amniotic fluid was found in 4% (3/78) of women (2x *Streptococcus intermedius* and 1x *Fusobacterium nucleatum*). In two out of these three women, the presence of periopathogenic bacteria in amniotic fluid led to the development of IAI. Interestingly, none of the women with periopathogenic bacteria in amniotic fluid had severe periodontal disease (one had none, one mild, one was moderate). The rate of amniotic fluid periopathogenic bacteria identified in this study appears to be lower than the rates presented in previous studies by Gauthier et al. (11% [3/27] of women with spontaneous preterm delivery) and by León et al. (31% [8/28] of women with spontaneous preterm delivery) (84, 86). However, the fact that the above-mentioned studies evaluated the presence of periopathogenic bacteria in cohorts of women containing both phenotypes of preterm delivery, as well as with gestational ages less than 34 weeks must be taken in the consideration. The latter is important information since the rate of MIAC is much higher in spontaneous preterm delivery prior to 34 weeks than beyond 34 weeks. In this cohort, women with gestational age of less than 34 weeks represented just 60% (47/78) of women. Moreover, the above-mentioned studies evaluated the presence of periopathogenic bacteria in amniotic fluid with genus-specific PCR. This specific approach is able to reveal lower amniotic microbial loads of bacteria than standard PCR evaluation of 16S rRNA followed by Sanger sequencing.

The increase in systemic proinflammatory cytokines produced by periodontal inflammation is possibly the likely event (58) that leads to the development of IAI. It has been shown that IL-6 levels tend to be higher in patients with periodontal disease in serum, as well as in gingival crevicular fluid (87). In this study, no differences in periodontal status between women with and without IAI were observed. Moreover, no differences were revealed when women were split into four subgroups based on the presence of MIAC and/or IAI in order to more precisely specify the background of IAI (microbial-associated vs. sterile). On the other hand, in this study results were in line with the recent study conducted by Soucy-Giguére et al., where no differences in amniotic fluid IL-6 and matrix metalloproteinase 8 concentrations were identified in women with and without periodontal disease (88). In addition, they observed no association between spontaneous preterm delivery and periodontal disease.

The other issue with comparing data from other cohorts previously published is a lack of standardization in periodontal disease reporting. There has been a secondary analysis published by Manau et al. comparing 14 individual periodontal disease definitions and more than 50 continuous measurements on 1296 pregnant women(89). The conclusion of this study was quite simple. Out of those 14 definitions applied on the same sample, 14 different prevalences of periodontal disease were reported, ranging from 14.5 to 70.8%. This fact clearly complicates the comparison of the data. The first large consensual statement for periodontal disease reporting was published in 2015 by Holtfreter et al. (69). This statement suggested the standardized use of periodontal disease reporting according to CDC/AAP definitions published previously by Eke et al. (68). Even though these recommendations were published after the initiation of this study, these definitions with negligible changes were adopted.

7.4 Specific aim IV.

The main findings of the specific aim IV are as follows: i) no correlations between local inflammatory response in gingival crevicular fluid, characterized by levels of multiple proteins, and maternal and intra-amniotic inflammatory responses, measured by maternal serum and amniotic fluid IL-6 concentrations, respectively, were found; ii) no correlations between local inflammatory response in gingival crevicular fluid and intra-amniotic inflammatory response in women with sterile IAI was revealed; and iii) the presence of sterile IAI was not related to different levels of mediators in gingival crevicular fluid.

Gingival crevicular fluid represents complex fluid derived from a variety of sources, mainly from a maternal serum transudate and an inflammatory exudate (90-93). Therefore, gingival crevicular fluid in women with PPROM contains constituents from the host, from the bacteria colonizing subgingival and supragingival plaque, as well as from the cells and tissues of periodontium (90-93). A plethora of various protein markers, including cytokines, chemokines, neuropeptides, and enzymes, has been found in this fluid (90, 94). Dozens of them have been suggested to be potential markers of periodontal disease so far (94, 95). In addition, analysis of gingival crevicular fluid might be used to assess the process whether and how periodontal disease influences other specific systemic disease and vice versa (90, 96, 97).

In this study, the associations between local inflammatory response in gingival crevicular fluid, measured by levels of multiple proteins and maternal and intra-amniotic inflammatory response in women with PPROM, were evaluated. To assess the intensity of the local inflammatory response in gingival crevicular fluid, the Meso-Scale technology was employed.

Severity of periodontal disease has been proposed to be a main reason for the elevation of inflammatory mediator levels in gingival crevicular fluid. (56, 98, 99) These locally produced inflammatory mediators in gingival crevicular fluid are able enter systemic circulation and induce an acute-phase response in the liver leading to an elevation of maternal serum CRP concentrations. (100, 101) Given this information as basic, the correlation between proteins levels in gingival crevicular fluid and maternal serum CRP concentrations was evaluated. In this study, no association was observed. Likewise, no association between proteins levels in gingival crevicular fluid and amniotic fluid IL-6 concentrations was observed. These results confirm that intra-amniotic complications in PPROM, but not periodontal disease are responsible for systemic and intra-amniotic inflammatory response in women with PPROM. This is supported by the fact, that the subgroup of women with microbial-associated IAI, where the systemic (maternal serum CRP concentrations) and intra-amniotic (amniotic fluid IL-6 concentrations) inflammatory responses were the highest, did not have different levels of inflammatory mediators in gingival crevicular fluid than women in the others subgroups.

The pathophysiology of sterile IAI has not been fully explained yet (34, 102-104). Therefore, it is hypothesized that a low-grade systemic inflammation, which may occur due to high inflammatory mediator levels in gingival crevicular fluid, might led to the presence of sterile IAI. Nevertheless, this theory has not been proven in this study, since there was no association between gingival crevicular fluid levels and

amniotic fluid IL-6 concentrations in women with sterile IAI. In addition the subgroup of women with sterile IAI did not have different levels than remaining women groups.

The information from this study might be a clinically relevant, because it clearly shown local inflammation in gingival crevicular fluid doesn't lead to the development of sterile IAI in women PPROM. Therefore the information from gingival crevicular fluid would not be helpful in the process of selection of women who are at high or at low risk of sterile IAI.

8 Conclusion

The presence of both MIAC and HCA is associated with the highest maternal inflammatory response in women with PPROM. The microbial load of *Ureaplasma* species affects the intensity of maternal inflammatory response.

The presence of both MIAC and HCA increases the risk of early onset sepsis in pregnancies complicated by preterm prelabor rupture of membranes before 34 weeks of gestation.

The presence of MIAC and IAI is not related to the periodontal status of women with PPROM.

The local inflammatory response in the gingival crevicular fluid is not related to the maternal and intra-amniotic inflammatory responses in women with PPROM.

9 Literature

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10 Publications and lectures

10.1 Original papers published in the journals with impact factor

1. **STEPAN, M.** – COBO, T. – MALY, J. – NAVRATILOVA, M. – MUSILOVA, I. –HORNYCHOVA, H. – JACOBSSON, B. – KACEROVSKY, M. Neonatal outcomes in subgroups of women with preterm prelabor rupture of membranes before 34 weeks., J Matern Fetal Neonatal Med. 7/2016; IF 1,36.
2. **STEPAN, M.** – COBO, T. – MUSILOVA, I. – HORNYCHOVA, H. – JACOBSSON, B. – KACEROVSKY M. Maternal Serum C-Reactive Protein in Women with Preterm Prelabor Rupture of Membranes., PLoS One. 3/2016; IF 3,23
3. MUSILOVA, I. – KACEROVSKY, M. – **STEPAN, M.** – BESTVINA, T. – PLISKOVA, L. – ZEDNIKOVA, B. – JACOBSSON, B. Maternal serum C-reactive protein concentration and intra-amniotic inflammation in women with preterm prelabor rupture of membranes. PLoS One. 8/2017; IF 3,23.
4. KACEROVSKY, M. – MUSILOVA, I. – BESTVINA, T. - **STEPAN, M.** – COBO, T. - JACOBSSON, B. Preterm Prelabor Rupture of Membranes between 34 and 37 Weeks: A Point-of-Care Test of Vaginal Fluid Interleukin-6 Concentrations for a Noninvasive Detection of Intra-Amniotic Inflammation. Fetal Diagnosis and Therapy. 8/2017; IF 2,7.
5. RADOCHOVA, V. – KACEROVSKA MUSILOVA, I. – **STEPAN, M.** – VESCICIK, P. – SLEZAK, R. - JACOBSSON, B. - KACEROVSKY, M. Periodontal disease and intra-amniotic complications in women with preterm prelabor rupture of membranes. J Matern Fetal Neonatal Med. 8/2017; IF 1,36.
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7. MUSILOVA, I. – BESTVINA, T. – STRANIK, J. – **STEPAN, M.** – JACOBSSON, B. – KACEROVSKY, M. Transabdominal amniocentesis is a feasible and safe procedure in preterm prelabor rupture of membranes. Fetal Diagnosis and Therapy. in press 2017; IF 2,7.
8. SLEHA, R. – BOSTIKOVA, V. – HAMPL, R. – SALAVEC, M. – HALADA, P. – **STEPAN, M.** – NOVOTNA, S. – KUKLA, R. – SLEHOVA, E. – KACEROVSKY, M. – BOSTIK, P. Prevalence of Mycoplasma hominis and Ureaplasma urealyticum in women undergoing an initial infertility evaluation. Epidemiol Mikrobiol Imunol. 2016; IF 0,3.
9. MUSILOVA, I. – ANDRYS, C. – DRAHOSOVA, M. – SOUCEK, O. – PLISKOVA, L. – **STEPAN, M.** – BESTVINA, T. – MALY, J. – JACOBSSON, B. – KACEROVSKY, M. Amniotic fluid clusterin in pregnancies complicated by the preterm prelabor rupture of membranes. J Matern Fetal Neonatal Med. 11/2016; IF 1,36.
10. MUSILOVA, I. – ANDRYS, C. – DRAHOSOVA, M. – SOUCEK, O. – PLISKOVA, L. – **STEPAN, M.** – BESTVINA, T. – MALY, J. – JACOBSSON, B.– KACEROVSKY, M. Amniotic fluid cathepsin-G in pregnancies complicated by the preterm prelabor rupture of membranes., J Matern Fetal Neonatal Med. 10/2016; IF 1,36.

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13. MUSILOVA, I. – TOTHOVA, L. – MENON, R. – VLKOVA, B. – CELEC, P. – HORNYCHOVA, H. – KUTOVA, R. – PLISKOVA, L. – ANDRYS, C. – **STEPAN, M.** – KACEROVSKY, M. Umbilical cord blood markers of oxidative stress in pregnancies complicated by preterm prelabor rupture of membranes., . J Matern Fetal Neonatal Med. 6/2016; IF 1,36.
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10.2 Original papers published in the journals without impact factor

1. HAMPL R., **ŠTĚPÁN M**. Variability in timing of human embryos cleavage monitored by time-lapse system in relation to patient age, Czech Gynecology 2013; 78, č. 6, s. 531-536.

10.3 Lectures – international

1. Bedside assesment of amniotic fluid IL-6 in women with PPROM. RECOOP consortium TriNet Meeting, Split, Croatia, May 15-17, 2014.

2. Diagnostic value of maternal serum C-reactive protein to identify the infectious subgroup of women with preterm prelabor rupture of the membranes. Sahlgrenska University hospital, May 12, 2015.

10.4 Lectures – Czech

1. PPROM: možnosti využití stanovení genitálních mykoplasmat v pupečníkové krvi. 14. celostátní konference fetální medicíny. Praha, 10. leden, 2014.

2. PPROM: možnosti využití stanovení genitálních mykoplasmat. 5. Konference nemocničních gynekologů a porodníků. Ostrava, 30. listopadu, 2014

3. PPROM: prediktivní potenciál C-reaktivního proteinu v mateřské krvi. 16. celostátní konference fetální medicíny. Praha, 16. leden, 2016.

4. Gestační diabetes mellitus- rizika pro plod. III. ročník mezioborového postgraduálního sympozia se zaměřením na: „Diabetes mellitus a životní etapy“. Hradec Králové, 14. října, 2016.

5. PPROM: komplement a intra-amniální zánět. 17. celostátní konference fetální medicíny. Praha, 20. leden, 2017.

6. Onemocnění periodontu a PPROM. 8. Konference nemocničních gynekologů a porodníků. České Budějovice, 1. prosince, 2017.

7. PPROM – Ovlivňuje stav dutiny ústní intraamniální prostředí? 18. celostátní konference fetální medicíny. Praha, 19. leden, 2018.