Maternal and placental inflammatory biomarkers in spontaneous preterm delivery

– predictive ability, stability and neonatal associations

by
Panagiotis Tsiartas

Department of Obstetrics and Gynecology
Institute of Clinical Sciences
The Sahlgrenska Academy
University of Gothenburg
Gothenburg, Sweden, 2017
“It’s the possibility of having a dream come true that makes life interesting”

“Όταν θέλεις πάρα πολύ κάτι, όλο το σύμπαν συνωμοτεί για να τα καταφέρεις”

Paulo Coelho
Abstract

Preterm delivery (PTD), spontaneous or iatrogenic [1], causes short- and long-term morbidity [2, 3] and underlies almost 75% of neonatal deaths [4]. The prevalence in the Nordic countries is about 6% [5] but it differs among countries. In the USA, for instance, it is around 9.6% [6]. The origin of spontaneous PTD is mostly unknown [7]. However, infection and inflammation are leading causes, mainly at early gestational ages [8]. Microbial invasion of the amniotic cavity (MIAC) occurs in 12-14% of symptomatic women with preterm labor (PTL) [9] and in 37-43% of women with preterm prelabor rupture of membranes (PPROM) [10]. MIAC elicits an inflammatory response mediated by cytokines, chemokines and other peptides, known as intra-amniotic inflammation (IAI). IAI causes early onset of symptoms, early gestational age at delivery and, consequently, worse neonatal outcome [11]. Chemokines induce chemotaxis in neutrophils and macrophages, enhancing their migration to the placenta and fetal membranes. This process, known as histological chorioamnionitis (HCA), occurs in more than half of spontaneous PTD cases. Early detection of spontaneous PTD presents a challenge because most women who deliver preterm have no obvious risk factors that can be identified early. Indeed, more than half of spontaneous PTDs occur in low-risk pregnancies.

One aim of the studies in this thesis was to study whether non-invasive strategies could predict the occurrence of spontaneous PTD within 7 days, as well as the rate of MIAC. We found that a combination of maternal serum proteins and cervical length constituted the most accurate prediction model for spontaneous PTD within 7 days of testing. However, we observed few differences between maternal serum protein levels in MIAC-positive PTL and PPROM cases.

An additional aim was to study the effect of different pre-analytical handling procedures on concentrations of interleukin-6 (IL-6), the cytokine most reported as a biomarker of IAI. We found that differences in handling procedures did not affect amniotic fluid IL-6 levels.

Furthermore, these studies investigated the relationship between neonatal outcome and placental histological findings in women with PPROM. We found that HCA and funisitis increased the risk of early-onset neonatal sepsis and retinopathy of prematurity in PPROM pregnancies.
Keywords
Preterm birth, prediction, proteins, multiplex, microbial invasion of the amniotic cavity, histological chorioamnionitis, neonatal outcome, cytokine stability, interleukin-6 (IL-6)
Sammanfattning på svenska

Förtidsbörd innebär att förlossningen sker före fullgången tid, det vill säga före graviditetsvecka 37. Förtidsbörd utgör ett betydande globalt hälsoproblem och kan vara spontan, med start av egna förlossningsvärkar, eller inducerad, det vill säga framkallad på medicinsk indikation. Dödligheten och sjukligheten är förhöjda vid förtidsbörd, men framsteg inom nyföddhetsvården har gjort att för tidigt födda barn har goda chanser att överleva och få ett bra liv. Förekomsten av förtidsbörd är cirka 6% i Sverige, att jämföra med Afrika och Nordamerika, där den ligger på 9-13%.

Orsakerna till spontan förtidsbörd är fortfarande till stora delar okända, men infektioner och inflammation är ofta involverade. Mikrober i fostervattnet förekommer hos 12-14% av kvinnor med för tidiga sammandragningar och hos 37-43% av dem med för tidig vattenavgång. Mikrobutlöst fostervatteninflammation medför ofta tidigare symtomdebut och förlossning, vilket ger högre risk för dödlighet och sjuklighet hos nyfödda. Vid infektion i fostervattnet transporteras blodcellerna neutrofiler och makrofager till moderkakan och fosterhinnorna. Denna process, som kallas korioamnionit, förekommer i mer än hälften av fallen med spontan förtidsbörd.

Tidig upptäckt av spontan förtidsbörd är en utmaning, eftersom drabbade kvinnor saknar tydliga riskfaktorer. Mer än hälften av fallen inträffar dessutom i lågriskgraviditeter, vilket innebär att inga sjukdomar hos kvinnan eller risker med graviditeten i övrigt kunnat påvisas före förlossningen.

Ett syfte med studierna i denna avhandling var att undersöka om icke-invasiva strategier (metoder utan ingrepp i livmodern) kan förutsäga spontan förtidsbörd inom sju dagar samt påvisa mikrober i fostervattnet. Vi fann att en kombination av ett påvisat äggviteämne i moderns blod och förkortad livmoderhals kan förutsäga risken för spontan förtidsbörd inom sju dagar efter blodprovstagningen. Dessutom observerade vi förändrade nivåer av vissa äggviteämnen i moderns blod när mikrober fanns i fostervattnet.

Ett annat syfte med studierna var att undersöka om olika sätt att hantera fostervatten påverkar interleukin-6 (IL-6)-koncentrationerna. IL-6 är ett äggviteämne som används för att identifiera inflammation i fostervattnet.
Vi fann att IL-6-nivåerna i fostervattnet inte påverkades av de studerade hanteringarna.
Dessutom ville vi studera vilken påverkan inflammation i moderkakan och fosterhinnorna hos kvinnor med för tidig vattenavgång har på det nyfödda barnet. Vi fann att denna inflammation ökar risken för tidig blodförgiftning samt för kärlförändringar i ögats näthinna hos för tidigt födda barn.
List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Panagiotis Tsiartas, Rose-Marie Holst, Ulla-Britt Wennerholm, Henrik Hagberg, David M. Hougaard, Kristin Skogstrand, Brad D. Pearce, Poul Thorsen, Marian Kacerovsky, Bo Jacobsson

*Prediction of spontaneous preterm delivery in women with threatened preterm labor: a prospective cohort study of multiple proteins in maternal serum*

BJOG 2012; 119:866-873

II. Teresa Cobo, Panagiotis Tsiartas, Marian Kacerovsky, Rose-Marie Holst, David M. Hougaard, Kristin Skogstrand, Ulla-Britt Wennerholm, Henrik Hagberg, Bo Jacobsson

*Maternal inflammatory response to microbial invasion of the amniotic cavity: analyses of multiple proteins in the maternal serum*


III. Panagiotis Tsiartas, Marian Kacerovsky, Maria Hallingström, Victor Liman, Teresa Cobo, Bo Jacobsson

*The effect of latency of time, centrifugation conditions, supernate filtration, and addition of protease inhibitors on amniotic fluid interleukin-6 concentrations*


IV. Panagiotis Tsiartas, Marian Kacerovsky, Ivana Musilova, Helena Hornychova, Teresa Cobo, Karin Sävman, Bo Jacobsson

*The association between histological chorioamnionitis, funisitis and neonatal outcome in women with preterm prelabor rupture of membranes*

# Contents

1. **Introduction**.................................................................................. 13  
   1.1 Preterm delivery.......................................................................... 13  
      1.1.1 Definition and background.................................................. 13  
      1.1.2 Epidemiology of PTD.......................................................... 13  
      1.1.3 Classification of PTD............................................................ 15  
      1.1.4 Subtypes of spontaneous PTD............................................. 17  
         1.1.4.1 PTL............................................................................. 17  
         1.1.4.2 PPROM................................................................. 19  
   1.1.5 Etiological factors in PTD....................................................... 19  
      1.1.5.1 Static risk factors.......................................................... 20  
      1.1.5.2 Dynamic risk factors...................................................... 21  
   1.1.6 Pathophysiology of PTD......................................................... 22  
      1.1.6.1 Inflammation and spontaneous PTD............................... 22  
         1.1.6.1.1 Infection-associated inflammation............................ 23  
         1.1.6.1.1.1 MIAC................................................................... 23  
         1.1.6.1.1.2 Microbial-associated IAI....................................... 24  
         1.1.6.1.2 Sterile inflammation............................................... 25  
      1.1.6.2 HCA and funisitis............................................................ 26  
      1.1.6.3 FIRS.............................................................................. 28  
   1.2 Outcomes in preterm-born children......................................... 29  
      1.2.1 Extremely preterm-born children........................................ 29  
      1.2.2 Moderately and late preterm-born children...................... 31  
      1.2.3 Long-term outcomes.......................................................... 32  
   1.3 Biomarkers of PTD...................................................................... 32  
2. **Aims**............................................................................................ 37  
3. **Patients and methods**................................................................. 39  
   3.1 Study I......................................................................................... 39  
      3.1.1 Ethical approval................................................................... 39  
      3.1.2 Design................................................................................. 39  
      3.1.3 Study population............................................................... 39  
      3.1.4 Clinical considerations and diagnostic approaches................... 40  
      3.1.5 Biomarker analysis methods.................................................... 42  
      3.1.6 Statistical analysis............................................................. 50  
   3.2 Study II.......................................................................................... 51
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2.1 Ethical approval</td>
<td>51</td>
</tr>
<tr>
<td>3.2.2 Design</td>
<td>52</td>
</tr>
<tr>
<td>3.2.3 Study population</td>
<td>52</td>
</tr>
<tr>
<td>3.2.4 Clinical considerations and diagnostic approaches</td>
<td>52</td>
</tr>
<tr>
<td>3.2.5 Biomarker analysis methods</td>
<td>54</td>
</tr>
<tr>
<td>3.2.6 Statistical analysis</td>
<td>54</td>
</tr>
<tr>
<td><strong>3.3 Study III</strong></td>
<td>55</td>
</tr>
<tr>
<td>3.3.1 Ethical approval</td>
<td>55</td>
</tr>
<tr>
<td>3.3.2 Design</td>
<td>55</td>
</tr>
<tr>
<td>3.3.3 Study population</td>
<td>55</td>
</tr>
<tr>
<td>3.3.4 Clinical considerations and diagnostic approaches</td>
<td>55</td>
</tr>
<tr>
<td>3.3.5 Biomarker analysis methods</td>
<td>57</td>
</tr>
<tr>
<td>3.3.6 Statistical analysis</td>
<td>58</td>
</tr>
<tr>
<td><strong>3.4 Study IV</strong></td>
<td>58</td>
</tr>
<tr>
<td>3.4.1 Ethical approval</td>
<td>58</td>
</tr>
<tr>
<td>3.4.2 Design</td>
<td>58</td>
</tr>
<tr>
<td>3.4.3 Study population</td>
<td>58</td>
</tr>
<tr>
<td>3.4.4 Clinical considerations and diagnostic approaches</td>
<td>58</td>
</tr>
<tr>
<td>3.4.5 Statistical analysis</td>
<td>61</td>
</tr>
<tr>
<td><strong>4. Results and comments</strong></td>
<td>63</td>
</tr>
<tr>
<td>4.1 Study I</td>
<td>63</td>
</tr>
<tr>
<td>4.2 Study II</td>
<td>66</td>
</tr>
<tr>
<td>4.3 Study III</td>
<td>68</td>
</tr>
<tr>
<td>4.4 Study IV</td>
<td>71</td>
</tr>
<tr>
<td><strong>5. Discussion</strong></td>
<td>75</td>
</tr>
<tr>
<td><strong>6. Conclusions and future perspectives</strong></td>
<td>83</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>85</td>
</tr>
<tr>
<td>References</td>
<td>87</td>
</tr>
</tbody>
</table>

Studies
Abbreviations

ACTH  Adrenocorticotrophic hormone
AFP   Alpha-fetoprotein
AUC   Area under the curve
BDNF  Brain-derived neurotrophic factor
BMI   Body mass index
BPD   Bronchopulmonary dysplasia
CD    Cluster of differentiation
CI    Confidence interval
CL    Cervical length
CNS   Central nervous system
CP    Cerebral palsy
CRH   Corticotropin-releasing hormone
CRP   C-reactive protein
CSF   Colony-stimulating factor
CXC   Group of chemokines with paired cysteines
DAMP  Damage-associated molecular pattern
ELISA Enzyme-linked immunosorbent assay
EOS   Early-onset sepsis
fFN   Fetal fibronectin
FIERS Fetal inflammatory response syndrome
FISH  Fluorescence in situ hybridization
G-CSF Granulocyte colony-stimulating factor
GBS   Group B Streptococcus
GM-CSF Granulocyte macrophage colony-stimulating factor
HAS   Hyaluronan synthase
HCA   Histological chorioamnionitis
HIE   Hypoxic-ischemic encephalopathy
IAI   Intra-amniotic inflammation
ICAM  Intercellular adhesion molecule
IFN   Interferon
IL    Interleukin
IVH   Intraventricular hemorrhage
LOS   Late-onset sepsis
LR    Likelihood ratio
ABBREVIATIONS

MCP  Monocyte chemotactic protein
MCP  Monocyte chemoattractant protein
MHC  Major histocompatibility complex
MIAC Microbial invasion of the amniotic cavity
MIF  Migration inhibitory factor
MIP  Macrophage inflammatory protein
MMP  Matrix metalloproteinase
nCPAP Nasal continuous positive airway pressure
NEC  Necrotizing enterocolitis
NICU Neonatal intensive care unit
NK   Natural killer
NT   Neurotrophin
NT   Neurotrophin
OR   Odds ratio
PCR  Polymerase chain reaction
POC  Point of care
PPHN Pneumonia and pulmonary hypertension of the newborn
PPROM Preterm prelabor rupture of membranes
PTD  Preterm delivery
PTL  Preterm labor
RANTES Normal T cell expressed and secreted
RDS  Respiratory distress syndrome
ROC  Receiver operating characteristics
ROP  Retinopathy of prematurity
SASP Senescence-associated secretory phenotype
SGA  Small for gestational age
S TNFR Soluble TNF receptor
T h  T helper
TGF  Transforming growth factor
TNF  Tumor necrosis factor
TTN  Transient tachypnea of the newborn
VCAM Vascular cell adhesion molecule
WBC  White blood cells
WHO  World Health Organization
1. Introduction

1.1 Preterm delivery

1.1.1 Definition and background

Preterm delivery (PTD), as defined by the World Health Organization (WHO), refers to all births occurring before 37 completed weeks (up to 36 weeks + 6 days) of gestation or at less than 259 days since the first day of the last menstrual period [12-14]. The lower limit of PTD is not clearly defined and varies internationally [15]. However, according to the WHO International Classification of Diseases (ICD-10), the perinatal period commences after 22 completed weeks of gestation. These lower and upper gestational age limits for defining PTD are arbitrary [14].

PTD is considered to be one of the most important national health indicators [16], as it is the most frequent cause of neonatal death and the second most frequent cause of death in children aged <5 years worldwide [13]. Children who survive PTD have higher rates of short- and long-term morbidity, compared to children born at term [2, 3, 17]. The serious effects of PTD, including on parents and society, makes it an important global public health issue [18].

The collaborative efforts of obstetricians and neonatologists, together with technological advances, have achieved improved survival rates over the last decades. However, PTD remains an unresolved entity with complex causes. There is an urgent need to develop effective preventive measures to reduce the worldwide incidence [15, 19].

1.1.2 Epidemiology of PTD

In 2010, the average global PTD rate (based on 184 countries) was 11%, yielding a total of 14.9 million PTD cases annually [13]. Disparities in PTD rates between countries can also be explained by differences in definition,
classification, gestational age assessment and the absence of routinely collected data [13, 14].

About 60-80% of all PTD cases occur in Africa and Asia [12, 13]. The high rates in those regions is mainly linked to the greater number of deliveries, higher infection rates, poor maternal nutrition, heavy physical work and lack of available drugs, as well as of basic obstetric and neonatal care [12, 20].

Europe has the lowest PTD rates in the world [12]. Several countries in Northern Europe, Sweden included, have rates close to 5% [13]. In the United States, a decline has occurred, to 9.63% in 2015 [6], from 2006 when the rates peaked at 12.8% [12, 15].

Most PTDs occur in the late preterm period (34 to 36+6 weeks) [6]. Although morbidity and mortality rates are relatively low among late-preterm-born babies, compared to those born at earlier gestational ages, they still exceed those of infants born at term [21, 22]. The overall decline in PTD rates observed in 2015 in the United States might be attributable in part to a reduction in late PTD. A rise in the number of iatrogenic PTDs was one of the causes of the increase in PTD rates prior to 2006. In contrast, the decline in PTD rates since 2006 has been attributed to a decrease in both spontaneous and iatrogenic PTD [23].

In 2005, the estimated annual cost of PTD in the United States was 26 billion dollars; the economic burden related to the condition was thus very high. This estimate included maternal delivery costs; medical care costs up to age 5 years for children born preterm; costs of early intervention and disability-specific lifetime medical, special education and lost productivity costs [24]. However, this cost estimate has limitations because it only includes children with cerebral palsy (CP), mental retardation, vision impairment, and hearing loss. Children born preterm are also at risk of other long-term morbidities, including asthma, learning disabilities, attention deficit disorder and emotional problems [25-28]. As adults, children born preterm may also have higher rates of insulin resistance and hypertension [29, 30].

However, the financial burden is only one aspect of the cost of PTD. The difficulties experienced by parents and extended family members after the preterm birth of a child can be severe. Maternal psychological distress, including anxiety and depression, is more often reported by women delivered preterm [31].
1.1.3 Classification of PTD

PTD can be divided into the following categories, based on gestational age at delivery [15, 32] (Table 1):

1. Extreme: delivery before 28 weeks of gestation (about 5% of PTD)
2. Severe: delivery at 28-31 weeks of gestation (about 15% of PTD)
3. Moderate: delivery at 32-33 weeks of gestation (about 20% of PTD)
4. Late: delivery at 34-36 weeks of gestation (about 60-70% of PTD)

Table 1. Classification of PTD by gestational age at delivery. Modified by Morken et al., 2005 [33]

<table>
<thead>
<tr>
<th>Subgroups (%)</th>
<th>&lt;28 weeks</th>
<th>28-31 weeks</th>
<th>32-33 weeks</th>
<th>34-36 weeks</th>
<th>&lt;37 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous PTD</td>
<td>49.5</td>
<td>35.6</td>
<td>42.6</td>
<td>60.6</td>
<td>55.2</td>
</tr>
<tr>
<td>Iatrogenic PTD</td>
<td>17.4</td>
<td>26.7</td>
<td>23.9</td>
<td>18.7</td>
<td>20.2</td>
</tr>
<tr>
<td>Intrauterine fetal death</td>
<td>2.3</td>
<td>9.0</td>
<td>4.6</td>
<td>1.4</td>
<td>2.7</td>
</tr>
<tr>
<td>Malformations</td>
<td>4.7</td>
<td>5.5</td>
<td>5.6</td>
<td>4.3</td>
<td>4.6</td>
</tr>
<tr>
<td>Multiple birth</td>
<td>16.0</td>
<td>14.7</td>
<td>16.0</td>
<td>10.1</td>
<td>11.6</td>
</tr>
<tr>
<td>Unknown onset of delivery</td>
<td>10.1</td>
<td>8.5</td>
<td>7.3</td>
<td>4.9</td>
<td>5.7</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

PTD can be also classified in the following groups, based on clinical presentation (Figure 1) [12-15, 34]:

1. Spontaneous: this category represents approximately 55% of PTD and includes preterm labor with intact membranes (PTL) and preterm prelabor rupture of the membranes (PPROM) [33].

2. Iatrogenic (medically indicated): approximately 20% of PTD is iatrogenic [33]. This category is etiologically heterogeneous and includes labor induction or cesarean section indicated by maternal or fetal complications before 37 weeks of gestation [13-15, 34]. The most common indications are
pregnancy-induced hypertension or preeclampsia, intrauterine growth restriction, non-reassuring tests of fetal wellbeing, small for gestational age (SGA) and antepartum hemorrhage [14, 32, 34-36].

3. Other subgroups: multiple pregnancies (12%), fetal malformations and intrauterine fetal deaths (7%) also contribute to PTD [33].

*Figure 1. Classification of PTD by clinical presentation. Modified by Morken et al., 2005 [33]*

Classification of PTD based only on clinical presentation has limitations because a multitude of heterogeneous conditions may be classified within the same category. For example, deliveries due to maternal hemorrhage, fetal growth abnormalities or preeclampsia are most often considered to be iatrogenic PTD. However, each of these conditions may be associated with different risk factors and pathological mechanisms [14].

Classification systems based on phenotype rather than on gestational age or clinical presentation have been proposed in order to overcome the above-mentioned limitations. Classifications based on phenotype emphasize clinical characteristics and minimize the influence of underlying etiologies. It is still likely that each phenotype may have multiple underlying etiologic pathways. The proposed systems do not classify each case as a single phenotype, since a particular case may have more than one phenotype [14, 37-39].
The first proposed phenotype classification system, published 2012, was applied in a large, international, multicenter, prospective cohort study [39]. Twelve distinct PTD phenotype clusters were defined, 11 of which were dominated by a single condition. The most frequent cluster (30%) was characterized by spontaneous contractions and/or PPROM in >70% of cases [40].

Another classification system was developed in 2015 through the identification of nine potential phenotype categories based on existing research on the pathogenesis of PTD. This classification system was tested in a prospective cohort study of women with spontaneous PTD. Maternal stress among African-American women was the most prevalent phenotype (59.8%), while decidual hemorrhage and placental dysfunction were more prevalent phenotypes among white women. Furthermore, infection/inflammation and decidual hemorrhage were more prevalent among women delivered at <28 weeks of gestation. This classification system was subsequently applied in another study to identify groups of women with similar phenotypic profiles. Women were clustered into five distinct phenotypic groups: maternal stress, PPROM, familial factors, maternal comorbidities and infection/decidual hemorrhage/placental dysfunction, conditions that may have overlapping and related pathophysiological pathways [38].

These phenotype-based classification systems provide a novel framework for the evaluation of PTD and require additional validation in other populations.

1.1.4 Subtypes of spontaneous PTD

1.1.4.1 PTL

PTL entails regular uterine contractions accompanied by cervical ripening [15], and accounts for 45-50% of all PTDs [12, 15, 32, 36, 41].

The etiology of PTL includes several epidemiologic, behavioral, environmental, and genetic factors. The major well-defined subset of PTL is associated with intra-amniotic inflammation (IAI). An overproduction of cytokines apparently occurs in PTL, enhancing local prostaglandin production that results in uterine contractions [42, 43].
When it comes to the preterm cervical remodeling associated with PTL, there is broad consensus that it is not simply an acceleration of physiological events. Most studies have been performed in animal models, demonstrating that cervical ripening can occur by more than one mechanism. One of the most important events in PTL, induced by withdrawal of progesterone, is the lack of expression of hyaluronan synthase-2 (HAS-2), one of the most important enzymes in hyaluronan synthesis. Hyaluronan is a glycosaminoglycan that is increased significantly during physiological cervical ripening. Increased expression of prostaglandin synthase-2, interleukin-6 (IL-6) and matrix metalloproteinase-8 (MMP-8) have been demonstrated in a mouse model. MMP-8 in particular might be involved in collagen matrix degradation during cervical ripening [44-46].

There is no evidence that inhibiting or arresting uterine contractility per se decreases the rate of PTD or improves neonatal outcome. While tocolytics can achieve short-term prolongation of pregnancy, which is useful for steroid administration and maternal transfer to tertiary care centers, they address a symptom rather than the underlying causes that activate the parturitional process [47].
1.1.4.2 PPROM

PPROM is defined as spontaneous rupture of the fetal membranes before 37 weeks of gestation [15] and accounts for about 25-30% of all PTD cases [12, 15, 32, 36, 41].

PPROM is considered a disease of the fetal membranes, which are weakened by proteolysis of the extracellular matrix, causing mechanical and functional disruption [48-50].

PPROM can be classified in 3 major groups [51]:
1. in the absence of cervical changes, with a longer latency to delivery
2. associated with cervical changes, more similar to PTL and with a shorter latency to delivery
3. involving bleeding disorders or coagulopathies that may be related to placental abruption

Regardless of the PPROM group, the majority of cases are associated with microbial invasion of the amniotic cavity (MIAC) and IAI, as well as clinical and histological chorioamnionitis (HCA) [52, 53].

There is controversy concerning the management of PPROM pregnancies. However, administration of antibiotics and corticosteroids to diminish the risk of respiratory disease in newborns is widely accepted. It has been shown that administration of erythromycin in PPROM pregnancies is associated with prolongation of pregnancy, reduction of neonatal surfactant treatment and decreased oxygen dependence at 28 days of age, as well as with fewer cases of neonatal bacteremia and abnormal brain scans. However, the administration of antibiotics in PPROM pregnancies has minor adverse effects on the health of children at age 7 years [54-58].

1.1.5 Etiological factors in PTD

More than one risk factor is associated with PTD, suggesting that it is the final common pathway for multiple etiologies [59]. The risk factors of PTD can be classified as static or dynamic:
1.1.5.1 Static risk factors

The static risk factors are non-modifiable during pregnancy, i.e. any modification of these factors will have minimal to no impact on pregnancy outcome:

1. PTD is familial in nature, as shown by a genetic study of heritability, according to which probands with PTD were more closely related to each other than to other members of the population [60]. Twin studies suggest that heritability for PTD ranges between 17-40% [61, 62]. It has also been shown that women who were born preterm have an increased risk of PTD [63].

2. A history of previous spontaneous PTD or repeated second-trimester pregnancy loss is among the most important risk factors, with a recurrence risk ranging from 15% to more than 50% [15, 32, 64]. This risk increases with the number of previous adverse events [64, 65].

3. Uterine anomalies are associated with higher rates of PTD, for as yet unclear reasons [66-68].

4. Black women have three times higher risk of spontaneous PTD than other ethnicities. These women are also three to four times more likely to have a very early spontaneous PTD. This racial disparity in PTD remains poorly understood [15].

5. Maternal smoking is a risk factor for spontaneous PTD and other poor pregnancy outcomes [69]. The association between spontaneous PTD and smoking is not clear but several mechanisms have been suggested. Smoking may increase the risk of infections [70] and prostaglandin production, thereby increasing the risk of PTL [69]. Moreover, smoking may increase the risk of PPROM by reducing the elastic properties of the fetal membranes, via lowered serum ascorbic acid levels, important for collagen metabolism [71].

6. Heavy alcohol consumption and drug abuse are associated with spontaneous PTD [15].

7. Maternal stress and low socioeconomic status have been found to increase the risk of spontaneous PTD [72-77].

8. Specific working conditions (long hours, hard physical work under stressful conditions) are related to spontaneous PTD [15, 64].
9. Maternal age is apparently an important risk factor, with a higher risk of spontaneous PTD among younger and older mothers [78]. Women aged under 20 have an increased risk of spontaneous PTD, a risk that seems to increase with parity [78].

10. Both low and high pre-pregnancy body mass index (BMI) have been shown to increase the risk of spontaneous PTD [79-81].

11. Maternal diet can influence the risk of spontaneous PTD. High intake of artificially sweetened beverages has been shown to be associated with increased risk of spontaneous PTD [82]. The intake of probiotic dairy products during pregnancy has been associated with a lowered risk of spontaneous PTD, due to healthier vaginal flora [83].

12. A short inter-pregnancy interval (under 6 or 12 months) has been associated with a higher risk of spontaneous PTD [84-86]. It is hypothesized that the inflammatory state in the uterus associated with the previous pregnancy requires time to resolve, but the mechanisms are still to clear [15].

### 1.1.5.2 Dynamic risk factors

The dynamic risk factors are clinical risks or pathological entities associated with adverse obstetric outcomes that the static risk factors, independently or in combination, could predispose to or cause. Complex interactions between various risk factors during pregnancy can generate epigenetic changes and consequent altered gene expression that contribute to dynamic clinical risks [59]. The phenotypes caused by these interactions include the following:

1. Infection is the only pathological process with an established causal link with spontaneous PTD [15, 43]. Infection plays a more prominent role in the pathogenesis of spontaneous PTD at early gestational ages. The lower the gestational age at PTD, the higher the frequency of infection-associated inflammation and HCA [8, 15, 87].

2. A subset of spontaneous PTD is characterized by maternal anti-fetal cellular and antibody-mediated rejections [88, 89]. This fetal systemic inflammatory response in cases with evidence of maternal anti-fetal rejection has recently been described [89].

3. Multifetal pregnancies and pregnancies with polyhydramnios are expected to be shorter due to an abnormal increase in uterine volume or limited capacity
of the uterus to expand [15]. Stretching of myometrial cells is associated with increased expression of gap junctions that are required for propagation and synchronization of uterine contractions [90, 91].

4. Placental abruption and consequent bleeding may lead to uterine contractions [92] and are associated with PTD in more than half of cases [93]. Altered placental vascular flow and chorio-decidual hemorrhage may represent a possible pathophysiological pathway initiating uterine contractions and PTD [94].

5. Short cervical length, diagnosed by vaginal ultrasonography during pregnancy, is associated with a high risk of spontaneous PTD [95].

Static and dynamic risk factors initiate pathways with unique biomarker profiles, contributing to labor-inducing changes and resulting in PTD.

1.1.6 Pathophysiology of PTD

PTD is considered to be a complex disease, with multiple pathways leading to a common pathway including inflammation and oxidative stress. The maternal-fetal signals and their causal origins are still to be identified [96].

1.1.6.1 Inflammation and spontaneous PTD

Regardless of the underlying risk factor, spontaneous PTD is associated with inflammatory changes (leukocyte activation; increased levels of inflammatory cytokines and chemokines; degradation by matrix metalloproteinases of the myometrial, cervical and fetal membrane extracellular matrix) [42, 97, 98]. These inflammatory events result in loss of membrane structural integrity, myometrial activation and cervical ripening. PTL and PPROM are host responses in which an overwhelming immune reaction can trigger the laboring process [99].

1.1.6.1.1 Infection-associated inflammation

Under physiological conditions, the amniotic cavity is regarded as sterile. The isolation of microorganisms in the amniotic fluid, defined as MIAC, is
1. INTRODUCTION

considered a pathological finding. It is estimated that 25-40% of spontaneous PTD is associated with infection [98].

1.1.6.1.1 MIAC

Approximately 25-40% of PPROM cases are complicated by MIAC, depending on gestational age at sampling, ethnicity and microbiological detection technique [10, 52]. Some studies suggest that this rate may be even higher [10, 49, 100]. MIAC occurs in 12-14% of women with PTL [9] and, more specifically, in 20-60% of women with PTL at <28 weeks of gestation and in 10-25% with PTL at 28-32 weeks of gestation [101, 102].

Culture-proven MIAC has been associated with early onset of symptoms, early gestational age at delivery and shorter latency to delivery. Consequently, higher neonatal morbidity has been reported [103]. Exceptionally, MIAC can occur in women without symptoms [104].

Microorganisms may gain access to the amniotic cavity by any of the following pathways: ascending from the vagina or cervix (most commonly), hematogenous dissemination through the placenta, retrograde from the peritoneal cavity via the fallopian tubes or accidentally during invasive procedures (amniocentesis, cordocentesis, chorionic villi sampling) [8].

Microorganisms that gain access to the amniotic cavity from the lower genital tract are first localized in the supravisceral decidua. Subsequent propagation to the chorioamniotic space can lead to MIAC [105, 106]. There is evidence that bacteria can cross intact membranes, so membrane rupture is not necessary for access to the amniotic cavity [107]. It has been shown that the chorion-decidua is not extensively involved in MIAC. Bacteria are primarily found in the amnion in cases of IAI, indicating that MIAC is a prerequisite for invasion of the membranes. Specifically, bacteria are detected more frequently in the amniotic fluid than in the membranes of patients with MIAC [108].

The most common bacteria found in the amniotic fluid in PPROM cases are genital mycoplasmas (Ureaplasma urealyticum, Ureaplasma parvum and Mycoplasma hominis) [10, 109]. Polymicrobial invasion of the amniotic cavity occurs in approximately 30% of cases [110-114]. Currently, non-cultivation techniques should be considered the gold standard in MIAC diagnosis because many amniotic fluid microorganisms are difficult to cultivate [115, 116]. The
presence of genital mycoplasmas in the amniotic cavity can elicit either an intense IAI response, comparable to that generated by other aerobic and anaerobic bacteria, or no inflammatory response at all [117, 118]. It has been shown that the inflammatory response to different bacteria varies. Exposure in vitro of human fetal membranes to *Escherichia coli* and *Gardnerella vaginalis* (also commonly found in intra-amniotic isolates) produces a stronger cytokine response, compared to genital mycoplasmas [119].

**1.1.6.1.1.2 Microbial-associated IAI**

The presence of bacteria in the amniotic fluid may activate an innate host immune response through pattern recognition receptors, which detect the specific motifs on the microorganisms' surface and initiate the inflammatory response resulting in microbial-associated IAI. Microbial-associated IAI is defined as MIAC with IAI [9, 10, 52, 120]. The intensity of IAI depends on the microbial load and the types of bacteria in the amniotic fluid [53, 121]. The presence of a small amount of bacteria with low virulence, such as *Ureaplasma spp.*, is unlikely to elicit IAI. This condition is considered instead to be a colonization of the amniotic fluid [11].

Microbial-associated IAI is associated with spontaneous PTD [8, 42, 43]. Women with PTL often have IAI (evidenced by elevated amniotic fluid levels of inflammatory cytokines and chemokines), even if amniotic fluid cultures are negative [102, 122-125]. IAI is associated with short latency to delivery and high rates of perinatal morbidity and mortality, regardless of whether amniotic fluid cultures are positive [9, 102, 122, 126-129]. One possible explanation for the perinatal morbidity associated with culture-negative IAI is that the cultures are falsely negative in these cases. Polymerase chain reaction (PCR) amplification has revealed prokaryotic 16S subunit ribosomal RNA in amniotic fluid in several culture-negative PTL cases, indicating infection and not only microbial degradation products. PTL cases with 16S rRNA PCR-verified MIAC have similar outcomes as cases with culture-verified MIAC [113, 130, 131].

Amniotic fluid culture results may take days, while IAI can be rapidly diagnosed by analysis of amniotic fluid IL-6. Of a variety of tests, IL-6 performs best in detecting IAI, as well as in the identification of patients at imminent risk of PTD and adverse neonatal outcomes [127, 129, 132]. Based on recent studies, IAI is diagnosed when the amniotic fluid IL-6 concentration, determined by enzyme-linked immunosorbent assay (ELISA), is $\geq 2600 \text{ pg/ml}$ [102, 110, 111].
However, the results take time (an ELISA requires at least eight hours) and are often not available in time for clinical decisions. Furthermore, laboratories often run these assays only a few times per week, limiting the availability of results for acute clinical decisions. In order to address this issue, a lateral flow-based immunoassay point of care (POC) test for IL-6 was developed, with results available within 20 minutes; IAI is diagnosed when the amniotic fluid IL-6 concentration is \( \geq 745 \) pg/ml [133-135]. The results of this test correlate significantly with IL-6 levels assessed by ELISA [136].

MIAC and IAI are distinct entities, each of which can be either present or absent: both present, IAI present but MIAC absent, MIAC present but IAI absent or both absent [11]. However, the inflammatory process is a continuum, not simply present or absent. Clinical outcomes can be correlated with different grades in the inflammatory response (amniotic inflammatory response syndrome), with categories defined by the number of biomarkers in the amniotic fluid [11, 137].

1.1.6.1.2 Sterile inflammation

IAI can be associated either with MIAC or with conditions in which cellular stress induce the release of mediators that activate the innate immune system [98, 138, 139]. The term “sterile IAI” refers to an inflammatory process in which microorganisms cannot be detected [140-144]. The precise causes have not been identified but damage-associated molecular patterns (DAMP) could be responsible stimuli for this inflammatory process. These cell-derived molecules initiate immunity in response to trauma, ischemia and tissue damage, through the activation of Toll-like receptors [143-146].

Sterile IAI is diagnosed when the amniotic fluid IL-6 concentration is \( \geq 2600 \) pg/mL and there is no evidence of MIAC (negative amniotic fluid culture and no PCR detection of microbial footprints) [120].

It has been shown that sterile IAI is more common (26%) than microbial-associated IAI (11%) in women with PTL. Furthermore, women with sterile IAI have similar rates of placental inflammation and adverse perinatal outcomes as women with microbial-associated IAI who give birth at the corresponding gestational ages. In pregnancies complicated by PPROM, the incidences of sterile IAI and microbial-associated IAI are equal (approximately 30%) [111, 120].
1. INTRODUCTION

1.1.6.1.3 Oxidative stress

Risk factors for PTD can cause oxidative stress (important component of the inflammatory process), generation of reactive oxygen species and cellular damage. These damaged cells respond with cytokine production as part of their remodeling mechanism. Cells protect themselves against oxidative stress by regulation of redox (balance between pro- and antioxidant) status, by enzymes and antioxidants. A redox imbalance could compromise a biological system’s ability to detoxify these highly reactive molecules or to repair the damages caused by them [59, 99].

There is evidence of varying degrees of oxidative stress and inflammation in fetal tissues and amniotic fluid in spontaneous PTD cases. The pathophysiological pathways of spontaneous PTD are mediated by oxidative stress-induced damage to the fetal membranes, not by oxidative stress alone [99, 147, 148].

It has been reported that oxidative stress-associated cellular damage can cause fetal membrane senescence. Senescence is an irreversible form of cell cycle arrest in which cells persist in the tissues and produce sterile inflammation called senescence-associated secretory phenotype (SASP). During this inflammatory process, molecules with uterotonic activity are produced, triggering the labor process as well as matrix-degrading enzymes causing membrane degradation and rupture. Aging of fetal membranes is premature in spontaneous PTD, especially in PPROM, where redox imbalance and oxidative stress-associated cellular damage are more dominant, causing DNA injury that can trigger cell cycle arrest and SASP [99, 149-151]. In early PTL, oxidative stress causing cellular damage is more balanced, and DNA damage is likely to be repaired [152-154].

1.1.6.2 HCA and funisitis

Acute chorioamnionitis is a frequent diagnosis in placental pathology reports and represents IAI [155-157], which be either microbial-associated or sterile (40-60% of cases) [110, 111, 120].

A small subset of spontaneous PTD patients without IAI has acute inflammatory lesions of the placenta. This may be because chorioamniotic inflammation is a host defense mechanism against danger signals of non-microbial origin or because non-viable microorganisms may release chemotactic factors, leading to placental inflammation in these cases [155].
Acute inflammatory lesions of the placenta are characterized by the infiltration of neutrophils into the placental disc, the chorioamniotic membranes and the umbilical cord. When the inflammatory process affects the chorion and amnion, it is termed acute chorioamnionitis; if it affects the villous tree, it is termed acute villitis. If the inflammatory process involves the umbilical cord, it is referred to as acute funisitis, the histological counterpart of the fetal inflammatory response syndrome (FIRS) [156, 158].

In clinical practice, the term chorioamnionitis refers to a clinical syndrome characterized by fever, maternal or fetal tachycardia, uterine tenderness and foul-smelling amniotic fluid. This clinical syndrome is frequently associated with HCA on microscopic examination of the placenta [159]. On the other hand, the majority of HCA cases are asymptomatic.

The frequency of HCA in women delivered at 21-24 weeks of gestation is approximately 94%, emphasizing the role of acute inflammation in early PTD. The prevalences of HCA are 40% in women delivered at 25-28 weeks of gestation, 35% at 29-32 weeks of gestation and 11% at 33-36 weeks of gestation [160]. HCA is commonly (50-80%) found in pregnancies complicated by PPROM. The rate of HCA in PTL is lower (approximately 30%), possibly because HCA is associated with MIAC, which is more frequent in PPROM pregnancies [109, 161].

HCA and funisitis are associated with adverse maternal and neonatal outcomes. The latter include PTD, perinatal death, neonatal sepsis, retinopathy of prematurity (ROP), chronic lung disease and fetal brain injury (intraventricular hemorrhage (IVH), cerebral white matter damage), as well as long-term sequelae leading to CP [162-167].

Neutrophils are not normally present in the chorioamniotic membranes; they migrate from the maternal decidua into the fetal membranes in cases of HCA. Chemotactic molecules induce the migration of neutrophils toward the amniotic cavity, from the intervillous space into the chorionic plate of the placenta. Thus, inflammation of the chorionic plate is a maternal inflammatory response. Studies performed with fluorescence in situ hybridization (FISH) and immunohistochemistry have shown that approximately 90% of neutrophils found in the membranes are of maternal origin. In contrast, inflammation of the umbilical cord and chorionic vessels in the chorionic plate of the placenta is of fetal origin [168-170].
Several grading (intensity of the acute inflammatory process at a particular site) and staging (progression of the process, based on the anatomical regions infiltrated by neutrophils) systems have been proposed to describe the severity of HCA. In this thesis, the system proposed by Salafia et al. [171] is used to describe the grade and stage of the inflammatory process. According to this study, more than one focus with at least 5 neutrophils or at least one focus with 5-20 neutrophils in the chorion-decidua and the chorionic plate were found in 95-98% and 84%, respectively, of uncomplicated term deliveries. Therefore, only intense neutrophil infiltration can be regarded as pathological and definitively diagnostic for HCA. The reproducibility of the grading and staging of maternal and fetal inflammation has been investigated in a study concluding that there is more agreement among pathologists in identifying the presence or absence of inflammation than in grading and staging [156].

Inflammation of the umbilical vessels begins in the vein (phlebitis) and is followed by inflammation of the arteries (arteritis). Infiltration of neutrophils into Wharton’s jelly is a common finding in acute funisitis. Differences have been found in the genes expressed by the umbilical artery and vein walls, suggesting that the wall of the vein is more prone to an inflammatory response than that of the arteries. Arteritis is evidence of a more advanced fetal inflammatory process. The umbilical cord plasma concentrations of IL-6 and the frequency of adverse neonatal outcome are higher in cases of arteritis. Acute funisitis begins as a multifocal process along the umbilical cord, with merging of the foci as the inflammatory process progresses. The severity of funisitis significantly correlates with fetal plasma and amniotic fluid IL-6 concentrations [172, 173].

1.1.6.3 FIRS

MIAC can gradually progress to fetal invasion. Access ports for bacteria into the fetus include the respiratory and gastrointestinal tract, skin, conjunctiva and ear. Once bacteria gain access to the fetus, they are recognized by Toll-like receptors, and a localized (and subsequently systemic) inflammatory response can be elicited. For example, fetuses exposed to bacteria can have severe dermatitis or pneumonitis and microorganisms reaching the fetal circulation can subsequently lead to a systemic inflammatory response [174]. FIRS is characterized by elevated fetal plasma concentrations of IL-6 and is associated with severe neonatal complications [89, 158, 175-182].
FIRS can also be caused by non-microbial-related insults, for example in cases of sterile inflammation. The precise nature of the causing stimuli in these cases has not been elucidated [110].

1.2 Outcomes in preterm-born children

PTD is the commonest cause of neonatal death worldwide, with about 3.1 million children per year dying due to complications. Most cases of PTD (about 84%) occur after 32 completed weeks of gestation. Infants born after this gestational age generally survive in high-income countries, while those in low-income countries are less fortunate. Ninety percent of infants born before 28 weeks of gestational age survive in high-income countries, compared to 10% in low-income countries [183, 184].

1.2.1 Extremely preterm-born children

Increased survival in extremely preterm neonates is associated with an increased rate of severe acute and chronic morbidities, e.g. bronchopulmonary dysplasia (BPD), necrotizing enterocolitis (NEC), ROP, hearing impairment, hypoxic-ischemic encephalopathy (HIE), IVH and early- (EOS) and late-onset (LOS) sepsis [185, 186].

BPD is one of the most frequent chronic lung diseases in infants [187]. One commonly accepted definition includes oxygen dependence at 28 postnatal days [188]. According to the Swedish National Registry for BPD, 80-90% of extremely preterm neonates contract this condition. The prevalence decreases to approximately 30% at 27 gestational weeks. Both prenatal and postnatal growth restriction and inflammation have been suggested as keystones in BPD pathogenesis [189]. In a large study of preterm infants with acute respiratory disease, an increased risk of BPD associated with long-duration PPROM was found, supporting the association between HCA and BPD [190]. Even with adequate therapy (e.g. prenatal steroids and antibiotics, early surfactant treatment, “gentle” ventilatory strategies, meticulous monitoring of oxygen administration), BPD remains a major cause of both short- and long-term morbidity [186].
NEC is seen almost exclusively in preterm-born neonates and is one of the most common gastrointestinal emergencies in this group. Mortality rates range from 15% to 30%. The estimated incidence ranges between 4 and 7%, with approximately one-third of the most severe cases needing surgical management. The pathophysiology of this condition is still unclear. However, immature intestinal motility and digestion might predispose preterm babies to NEC [191]. Infants surviving NEC are at higher risk of the long-term complications associated with PTD (impaired gut function and adverse motor, sensory and cognitive outcomes), compared with unaffected children born at a similar gestational age [192].

ROP is a widespread complication of PTD. It is the second leading cause of childhood blindness in the world [193]. ROP occurs in up to 3% of children born before 28 weeks of gestation and leads to severe visual impairment in up to 8% of those born before 26 weeks. Furthermore, children born before 28 weeks of gestation are at six-fold risk of myopia and hypermetropia, and 25% of this group require glasses by the age of 6 [194]. According to the Swedish National Register for Retinopathy of Prematurity (SWEDROP), 16% had mild ROP and 9% had severe ROP in two cohorts of infants born before 32 weeks of gestation [195, 196].

Preterm-born children are also at increased risk of HIE, with an estimated incidence of 1.5 per 1000 live births. HIE may be related either to congenital brain damage, predisposing to birth asphyxia, or to primary birth asphyxia. Over 25% of these children have long-term neurodevelopmental problems [197]. Therapeutic cooling has been shown to improve outcomes, with a 9% reduction in mortality and a 13% reduction in neurodevelopmental disability [198].

In the United States, about 12 000 preterm-born children develop IVH every year. The pathogenesis of IVH is complex and heterogeneous. An inherent fragility of the germinal matrix vasculature predisposes to hemorrhage and cerebral blood flow fluctuation induces vascular rupture [199]. The incidence of the condition has remained almost stationary during the last two decades [200, 201]. IVH is a major problem in preterm-born children, as a large number of them develop neurologic sequelae [202]; approximately 50–75% contract CP, mental retardation and/or hydrocephalus [202, 203].

Sepsis is one of the leading causes of morbidity and mortality among preterm-born children [204]. EOS is most consistently defined as occurring in the first three days of life. It is caused by bacterial pathogens transmitted vertically from
1. INTRODUCTION

1.1 Mother to infant before or during delivery [205]. *Group B streptococcus* (GBS) and *Escherichia coli* account for about 70% of EOS cases [206, 207]. The incidence of culture-proven EOS in the United States is estimated to be 0.77 to 1 per 1,000 live births [208, 209], with a case fatality rate of 16% [209]. A Swedish cohort study reported an incidence of GBS-generated EOS of 0.4 per 1,000 live births, and the total morbidity burden was approximately three times higher [210]. LOS is defined as sepsis occurring after 72 hours, and up to the age of 90 or 120 days, in preterm neonates. The condition may be caused by vertically or horizontally acquired pathogens [205, 211]. Gram-positive organisms cause 70% of initial LOS episodes, with coagulase-negative *Staphylococci* accounting for 48% of the infections [212]. The incidence of LOS has increased with improved survival in premature infants, especially those with very low birth weight, indicating the role of hospitalization and life-sustaining medical interventions in its pathogenesis [206, 213]. One study showed that 36.3% of neonates born before 28 weeks of gestation had at least one episode of LOS, compared with 29.6% of moderately preterm, 17.5% of late preterm and 16.5% of term infants [214]. An increased risk of morbidity and mortality has been found among infants with LOS, compared to unaffected infants [205, 212].

1.2.2 Moderately and late preterm-born children

Late preterm babies, often considered to be normal newborns by parents and care providers, have 3.5 times more morbidity during the perinatal hospitalization period than term-born babies, and neonatal mortality is 4.6 times higher. A continuous relationship exists between gestational age and neonatal morbidity and mortality at between 32 and 36 weeks of gestation [215]. Infants born at 32-36 weeks of gestation contract respiratory distress syndrome (RDS), transient tachypnea of the newborn (TTN), pneumonia and pulmonary hypertension of the newborn (PPHN) at higher rates than term infants [216, 217]. Moderate and late preterm infants are more likely to develop severe infections such as sepsis, meningitis and pneumonia than term infants [21, 208]. A population-based Swedish study including infants born at between 30-34 weeks of gestation found that acute lung disorders were diagnosed in 28%, hypoglycemia in 16%, bacterial infection in 15% and hyperbilirubinemia in 59% [218].
1.2.3 Long-term outcomes

Long-term sequelae in children born extremely preterm, demonstrated in the EpiCure studies, included neurological and psychiatric impairment, adverse cardiovascular outcome and impaired lung function [219-224].

The commonest long-term neurodevelopmental disability associated with PTD is CP, affecting an estimated 12% of preterm-born children according to a recent systematic review. The same review showed that an additional 19% had motor and co-ordination problems not formally classified as CP [225]. A population-based study performed in Sweden reported that 9.5% of children born extremely preterm had CP at the age of 6.5 years [226]. The risk of CP increases with decreasing gestational age at delivery, from approximately 1% at 34 weeks to 20% at ≤ 26 weeks of gestation [227-231]. The pathophysiological mechanisms underlying neurodevelopmental disability in PTD survivors are still poorly understood and complex. Multiple risk factors may contribute to brain injury and abnormal brain development in the extremely preterm-born child. Inflammatory cytokines associated with PTD pathways may in combination with genetic factors render the preterm infant’s brain vulnerable to injury [232]. Some studies have shown that clinical and histological chorioamnionitis are risk factors for CP [233, 234], while others have also reported a significantly higher incidence of CP in infants exposed to funisitis [235].

Preterm-born infants have a higher prevalence of neonatal seizures, with an estimated prevalence at about 31% [236], and outcomes are worse in this group, compared to term-born babies [237]. Seizures may alter brain development by affecting cell division, differentiation, migration, and synaptogenesis [238].

Approximately one-third of all preterm-born children have some cognitive impairment and about 7% are severely impaired [236]. These children are 1.3-2.8- times more likely to require special education [231].

1.3 Biomarkers of PTD

Prediction and prevention remain a challenge in modern obstetrics, mainly because spontaneous PTD is considered to be a syndrome with multiple origins. The multifactorial nature of this condition requires strategies simultaneously identifying multiple markers and assessing clinical and biophysical risk factors. Clinical risk factors alone lack the sensitivity required to effectively identify the majority of patients at risk of spontaneous PTD [239].
A highly effective risk-predicting system would identify high-risk patients, in order to avoid overtreatment of low-risk patients. A tool making it possible to detect a PTD process in progress or to assess a woman’s risk early in pregnancy is required. The early detection of spontaneous PTD is difficult because most women who deliver preterm have no obvious risk factors and more than half of spontaneous PTDs occur in low-risk pregnancies. Moreover, the initial signs and symptoms are most often mild, making early detection difficult [240].

Biomarkers have been defined as parameters that can be measured in a biological sample, and that provide information on an exposure, or on the actual or potential effects of that exposure in an individual or group [241].

The ideal clinical biomarker to predict spontaneous PTD would:

1. Identify women presenting with all subtypes of the condition

2. Be measurable in a biological sample that is easily obtained, with minimal maternal and fetal risk

The biomarker test should ideally be inexpensive, reproducible and easy to perform early in pregnancy in order to allow for potential interventions. It should also have a high positive likelihood ratio (+ LR), increasing the probability that women with a positive test result are actually at risk of spontaneous PTD, and a low negative likelihood ratio (- LR), in order to confidently rule out the disorder with a negative test result. Lastly, the required technological platform for the analysis of the samples should be widely available [242, 243].

Recent systematic reviews have suggested that single biomarkers are neither sensitive indicators of spontaneous PTD nor predictors of spontaneous PTD risk. The most frequently reported biomarkers for spontaneous PTD in the literature, among 116 candidates investigated between 1965-2008, are IL-6, corticotropin-releasing hormone (CRH), IL-8, C-reactive protein (CRP), beta-human chorionic gonadotropin (beta-HCG), alpha fetoprotein (AFP), IL-1β, tumor necrosis factor-α (TNF-α), cortisol, ferritin, adrenocorticotropic hormone (ACTH), estriol, MMP-9, IL-2 and relaxin. Heterogeneities in study design, sampling procedures, study populations, assays and other analyses, as well as improper definitions, were limiting factors in the reported studies [244].

The hypothesis of potential interactions between biomarkers as a possible predictor of spontaneous PTD has been also investigated. The conclusion was
that biomarker interactions have better predictive potential than single biomarkers, even in the absence of a main effect of single biomarkers [245]. For instance, the association between cervical length and cervico-vaginal fibronectin in asymptomatic women is limited and more capable of detecting women at lower risk than those at higher risk of spontaneous PTD [246, 247].

Proteomics is a newly developed field of research that studies the global set of proteins and their expression, function, and structure in a tissue, cell, or organism at a given moment [248]. In recent years, proteomics has been extensively applied to search for biologically relevant biomarkers and to generate protein profiles characteristic of IAI and spontaneous PTD [249-251]. Numerous publications have described maternal and fetal biomarker changes in biological samples, based on proteomic technologies [137, 251-254]. A systematic review of the literature has been conducted to identify proteomic biomarkers for spontaneous PTD. Of a total of 64 identified proteins, none was reproducible or capable of correctly predict spontaneous PTD. The conclusion of this review was that a proteome-based biomarker panel is unlikely to predict spontaneous PTD risk. It has been also reported that population-specific panels are more likely to be better predictors of spontaneous PTD [255].

Technological advances have further modernized biomarker discovery and multiplex technology, entailing simultaneous analysis of a broad panel of biomarkers based on known pathways of spontaneous PTD, has been used in research. Multiplex technology permits the use of a small sample volume, it is cost effective and it presents minimal technological challenges, compared to proteomics. A systematic review of the literature on spontaneous PTD biomarkers identified with multiplex approaches has been performed. According to this review, 40% of the studies used Luminex technology to identify biomarkers in the same sample and the number of analytes reported in the studies ranged from 2 to 44. A total of 42 biomarkers were identified as being associated to spontaneous PTD. Of the 31 spontaneous PTD-related biomarkers assessed in maternal serum, the ones most frequently identified were Regulated on activation, normal T cell expressed and secreted (RANTES) and IL-10. This systematic review concluded that multiplex assays provide a potential technological platform for identifying biomarkers for spontaneous PTD, although no single or combination of biomarkers that yielded a better prediction of the spontaneous PTD risk was identified [256].
A standardized approach is essential in biomarker research and guidelines have been created, aimed at designing combinable biomarker studies in future. According to these guidelines [244, 256]:

1. Biomarker studies should properly define the outcome phenotype (iatrogenic PTD or spontaneous PTD with PTL or PPROM onset), based on gestational age at delivery.

2. The objective of the study must be stated clearly in order to understand the rationale for selecting the included biomarkers. Biomarkers can be mainly classified as (1) those that help understand the biology and mechanistic aspects of labor and delivery and (2) risk predictors and indicators.

3. Testing a hypothesis regarding a mechanistic factor or risk indicator requires a proper epidemiological study design. Designing the study with appropriate epidemiological methods is essential to properly recruit subjects and collect and analyze samples.

4. Studies should clearly report essential study details (type of facility for conducting studies, participant recruitment, consent methods, gestational age at recruitment, enrollment period), baseline characteristics of study population in terms of \textit{a priori} risk status for PTD, race and ethnicity and clinical status at sample collection.

5. Studies should provide a detailed report of sample type and collection timing, sample processing procedures and storage details. The success of a biomarker study is directly dependent on specimen collection quality and on processing before assay, as well as on appropriate storage conditions, in order to avoid variations in biomarker level.

6. Biomarker discovery depends on the quality of the selected assay for sample analysis. Prior to its selection, investigators must judge the appropriateness of an assay. Assay procedure and methodology, biomarker recovery methods from the biological specimen, sensitivity, specificity and inter- and intra-assay variations should be reported in detail.

7. Statistical analysis should address the hypothesis and objectives established prior to the study performance and design. Statistical assessment of biomarker concentration distribution (normal or non-normal); selection of the appropriate data presentation form, based on distribution (mean and standard deviation or median and interquartile range), identification and adjustment for potential confounders; and adjustment for multiple testing are recommended.
8. If the discovery assay evaluates a large number of potential biomarkers and results indicate that only a small number are associated with PTD risk, it might make sense to only test the smaller number in validation efforts.

9. Prior to the validation studies using the predictive mathematical algorithm combining the assay results of each biomarker from the exploratory studies, an independent sample set can be tested for algorithm optimization.

10. A prospective validation, using a new, independent set of specimens, is also required to confirm the performance of the biomarker panel.
The primary aims of the studies reported in this thesis were to investigate whether it could be possible to predict the occurrence of spontaneous PTD within 7 days (Study I) as well as to investigate MIAC rates (Study II) by non-invasive sample strategies (maternal serum).

A second aim was to study the effect of different handling procedures on the concentrations of IL-6, often used to identify IAI (Study III).

The third aim was to study the associations of placental histological findings with neonatal outcome in women with PPROM (Study IV).
3. Patients and Methods

3.1 Study I

3.1.1 Ethical approval
The local Ethics Committee at the University of Gothenburg approved the study (Nos. 349-95, 476-05).

3.1.2 Design
Prospective cohort study

3.1.3 Study population
This study included 142 healthy women without major medical problems and with singleton pregnancies, presenting at Sahlgrenska University Hospital, Gothenburg, Sweden, with imminent PT L at 22+0 - 33+6 gestational weeks, from 1996 to 2005. The majority of enrolled women was of non-Hispanic, white ethnicity and had a stable family situation (Table 2).

Table 2. Ethnicity and marital-cohabitation status

<table>
<thead>
<tr>
<th></th>
<th>Delivery ≤7 days</th>
<th>Delivery &gt;7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Hispanic black</td>
<td>1%</td>
<td>2%</td>
</tr>
<tr>
<td>Non-Hispanic white</td>
<td>37%</td>
<td>57%</td>
</tr>
<tr>
<td>Hispanic</td>
<td>1%</td>
<td>2%</td>
</tr>
<tr>
<td>Cohabiting with baby’s father</td>
<td>37%</td>
<td>56%</td>
</tr>
<tr>
<td>Living alone</td>
<td>2%</td>
<td>4%</td>
</tr>
</tbody>
</table>
3.1.4 Clinical considerations and diagnostic approaches

In this study, imminent PTL was defined as:

1. Regular uterine contractions (at least two contractions per 10 minutes for ≥ 30 minutes, confirmed by external tocometry), in combination with at least one of three cervical changes, verified by digital examination:
   a. Length ≤ 2 cm and dilatation ≥ 1 cm
   b. Length ≤ 2 cm and softening
   c. Dilation ≥ 1 cm and softening and/or

2. Cervical length < 30 mm

Cervical ripening was assessed by digital examination and cervical length was measured by transvaginal sonography using a standardized method [257], with the woman in the dorsal lithotomy position and with an empty bladder. When the cervical canal was visualized, the probe was withdrawn to avoid pressure, distortion or elongation of the cervix. A sagittal view showing the entire cervix (endocervix and vaginal cervix), including the echogenic endocervical mucosa along the length of the cervical canal, was obtained. Calipers were used to measure the distance between the notches made by the junction of the anterior and posterior cervical walls at the internal and external os. Three measurements were performed and the shortest distance in mm was noted.

The interval of 7 days between sampling and delivery was chosen since it makes sense from a clinical point of view. Seven days gives the clinician the possibility to admit the woman, administer corticosteroids and tocolytics and let her give birth, if it is unavoidable. On the other hand, the 7-day interval is an arbitrarily determined period without biological basis or confirmation; it simply represents the actual average length of the hospital stay and monitoring of patients with imminent PTL.

Because of limited resources, we did not recruit women during the night, on weekends, during vacations, over Christmas or during busy periods on the delivery ward. This obviously limited the number of women enrolled. Inclusion in the study was constant throughout the year, with the exception of a lower inclusion rate during the summer for these reasons (Table 3).
Table 3. Seasonal variation in inclusions/deliveries

<table>
<thead>
<tr>
<th></th>
<th>Delivery ≤7 days</th>
<th>Delivery &gt;7 days</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st quarter</td>
<td>16%</td>
<td>18%</td>
<td>34%</td>
</tr>
<tr>
<td>2nd quarter</td>
<td>4%</td>
<td>11%</td>
<td>15%</td>
</tr>
<tr>
<td>3rd quarter</td>
<td>10%</td>
<td>16%</td>
<td>26%</td>
</tr>
<tr>
<td>4th quarter</td>
<td>11%</td>
<td>15%</td>
<td>26%</td>
</tr>
</tbody>
</table>

Tocolytics (intravenous terbutaline and/or indomethacin, the latter at < 28 weeks of gestation) was administered, according to local protocol.

Corticosteroids were administered at 24+0 - 33+6 gestational weeks, according to local protocol, in order to stimulate fetal lung maturity. Corticosteroids were administered in the majority of the cases (80%), and 78% were given two doses, as shown in Table 4.

Table 4. Corticosteroid administration

<table>
<thead>
<tr>
<th></th>
<th>Delivery ≤7 days</th>
<th>Delivery &gt;7 days</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosteroids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>34%</td>
<td>46%</td>
<td>80%</td>
</tr>
<tr>
<td>No</td>
<td>6%</td>
<td>14%</td>
<td>20%</td>
</tr>
<tr>
<td>Double dose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>31%</td>
<td>46%</td>
<td>78%</td>
</tr>
<tr>
<td>No</td>
<td>9%</td>
<td>14%</td>
<td>23%</td>
</tr>
</tbody>
</table>

The majority (65%) was sampled after corticosteroid administration, due to limited resources (Table 5). The serum samples were placed in a refrigerator (+4 °C) within 10 minutes, and processed within 6 hours before freezing (-80 °C) for later analysis. The aliquots were not thawed until analysis and none were re-frozen and re-thawed for later analysis.

Table 5. Timing of maternal serum sampling and corticosteroid administration

<table>
<thead>
<tr>
<th></th>
<th>Delivery ≤7 days</th>
<th>Delivery &gt;7 days</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sampling before</td>
<td>16%</td>
<td>19%</td>
<td>35%</td>
</tr>
<tr>
<td>Sampling after</td>
<td>25%</td>
<td>41%</td>
<td>65%</td>
</tr>
</tbody>
</table>
3.1.5 Biomarker analysis methods

Assays of maternal serum were performed at Statens Serum Institut, Department of Clinical Biochemistry in Denmark, using a multiplex sandwich immunoassay based on Luminex flowmetric xMAP technology [258].

The maternal serum samples were diluted 1:10 in extraction buffer (phosphate-buffered saline containing a complete protease inhibitor cocktail with EDTA - Roche, Basel, Switzerland; one tablet dissolved per 25 mL of assay buffer (phosphate-buffered saline containing 5 mL/L Tween-20 and 10 g/L bovine serum albumin)) and analyzed in duplicate. A sample was added to each filter plate well with a suspension of capture antibody-conjugated beads. After an incubation of 1.5 hours, the beads were washed twice and subsequently reacted for 1.5 hours with a mixture of relevant detection antibodies. Next, a quantity of streptavidin-phycoerythrin was added to the wells, after which incubation continued for an additional 30 minutes. Finally, the beads were washed twice, re-suspended in buffer and analyzed [258].

Luminex xMAP (multiple analyte profiling) technology enables multiplexing of biological assays, reducing time, labor and costs, compared with traditional methods such as ELISA, Western blotting, PCR and traditional arrays. Systems using xMAP Technology are based on microbeads, i.e. microspheres that have been color-coded to generate about 100 distinct sets (Figure 3). Each bead can be coated with thousands of antibodies (capture antibodies), allowing the capture and sensitive detection of the specific analyte of interest from a sample. Another antibody (reporter antibody), marked with a fluorophore, is then added and binds to the capturing antibody in a complex with unique spectral qualities. Inside the Luminex 100 analyzer, which can read 50-100 beads per analyte, a red laser identifies the bead type while a green laser excites the fluorophore on the reporter antibody and quantifies the fluorescent signal corresponding to the concentration of the analyte in the test sample (Data from https://www.luminexcorp.com/).
Concentrations of the following markers (Table 6), chosen from a candidate protein approach to PTD pathways, were measured in maternal serum (symbols assigned by the HUGO Gene Nomenclature Committee are in parenthesis): IL1b, IL-2 (IL2), IL-4 (IL4), IL-5 (IL5), IL-6 (IL6/IFNb2), IL-8 (IL8), IL-10 (IL10), IL-12 (IL-12A – natural killer cell stimulatory factor 1), IL-17 (IL17A), IL-18 (IL-18 – interferon-gamma-inducing factor), Soluble IL-6 receptor a (sIL6R), Interferon-γ (IFNG), Tumor necrosis factor-α (TNF, TNF superfamily, member 2), TNF-β (LTA – lymphotoxin alpha – TNF superfamily, member 1), Monocyte chemotactic protein-1 [MCP-1, CCL2 – chemokine (C–C motif) ligand 2], Transforming growth factor-β (TGFβ1), Macrophage inflammatory protein-1α [CCL3 – chemokine (C–C motif) ligand 3], Macrophage inflammatory protein-1β [CCL4 – chemokine (C–C motif) ligand 4], MMP-9 (MMP9), Triggering receptor expressed on myeloid cells-1 (TREM1), Brain-derived neurotrophic factor (BDNF), Granulocyte-macrophage-colony-stimulating factor (CSF2 – colony stimulating factor 2), Neurotrophin-3 (NTF3), Neurotrophin-4 (NTF4), Soluble TNF receptor I (sTNFR1A), Migration inhibitory factor (MIF), RANTES [CCL5 – chemokine (C–C motif) ligand 5].
### Table 6. Source, target and function of cytokines, chemokines and other proteins analyzed in the study (Data from http://www.cells-talk.com/)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Secreted/expressed by</th>
<th>Target cells/tissue - function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pro-inflammatory</strong></td>
<td>Monocytes/macrophages</td>
<td>Co-stimulates activation of T helper cells and promotes inflammation, maturation and clonal expansion</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelial cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Th1 cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IL-2</strong></td>
<td>T cells</td>
<td>Activates antigen–primed Th1 cells</td>
</tr>
<tr>
<td><strong>Immunoregulatory</strong></td>
<td></td>
<td>Induces proliferation, supports long-term growth and enhances activity of naive T cells</td>
</tr>
<tr>
<td><strong>IL-4</strong></td>
<td>T cells</td>
<td>Facilitates production of antibodies from antigen-primed B cells and stimulates Th2 cells</td>
</tr>
<tr>
<td>Immunoregulatory</td>
<td>NK cells</td>
<td>Down-regulates Th1 response</td>
</tr>
<tr>
<td>Mast cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IL-5</strong></td>
<td>T cells</td>
<td>Activates eosinophils and stimulates eosinophil production</td>
</tr>
<tr>
<td><strong>Proliferative</strong></td>
<td>Mast cells</td>
<td>Promotes adherence to VCAM-1</td>
</tr>
<tr>
<td>Eosinophils</td>
<td></td>
<td>Matures Th2 cells</td>
</tr>
<tr>
<td>IL-6</td>
<td>Mixed pro- and anti-inflammatory</td>
<td>Many different cells, mainly macrophages and monocytes</td>
</tr>
<tr>
<td>------</td>
<td>----------------------------------</td>
<td>------------------------------------------------------</td>
</tr>
<tr>
<td>IL-8</td>
<td>Pro-inflammatory, chemoattractant</td>
<td>Monocytes/macrophages, Fibroblasts, Endothelial cells, Epithelial cells, Others</td>
</tr>
<tr>
<td>IL-10</td>
<td>Anti-inflammatory</td>
<td>T and B cells, Macrophages, Dendritic cells</td>
</tr>
<tr>
<td>IL-12</td>
<td>Immunoregulatory</td>
<td>Macrophages, Dendritic cells, B cells</td>
</tr>
<tr>
<td>IL-17</td>
<td>Pro-inflammatory and immunoregulatory</td>
<td>Th1 cells</td>
</tr>
</tbody>
</table>
### IL-18

**Pro-inflammatory**
- Macrophages
- Monocytes
- Keratinocytes

**Immunoregulatory**

**Important mediator of chronic inflammation**

- Enhances the inflammatory process by stimulating production of IFN-γ, TNF-α and IL-1β by NK cells and macrophages
- Interacts with IL-12
- Stimulates cytotoxic activity of T cells and NK cells

### Soluble IL-6 Ra

**Mixed pro- and anti-inflammatory**
- Unknown

**Mediates both local and systemic IL-6-mediated events**

### IFN-γ

**Anti-viral and immunoregulatory**
- T helper cells
- Cytotoxic T cells
- NK cells
- Macrophages
- Dendritic cells

**Anti-viral and anti-parasitic activity**

- Increases MHC I and II expression
- Stimulates antigen presentation, cytokine production and has effector functions on monocytes
- Stimulates killing by NK cells and neutrophils
- Inhibits IL-4 production and action

### TNF-α

**Pro-inflammatory**
- Macrophages
- Dendritic cells
- Neutrophils
- Activated lymphocytes
- NK cells
- Endothelial cells
- Mast cells

**Increases adhesion molecules on endothelial cells (ICAM-1, VCAM-1) and chemoattractants**

- Activates neutrophils and phagocytosis
- Induces edema, vascular leakage and coagulation
<table>
<thead>
<tr>
<th>Pathway</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mediator of apoptosis, tissue injury, toxic shock and sepsis</td>
<td>Induces production of IL-1β, IL-6 and IL-10</td>
</tr>
<tr>
<td><strong>TNF-β</strong></td>
<td><strong>Pro-inflammatory</strong></td>
</tr>
<tr>
<td>T cells</td>
<td>Induces synthesis of GM-CSF, G-CSF, IL-1, collagenases, prostaglandins</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>Promotes proliferation of fibroblasts</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Others</td>
</tr>
<tr>
<td><strong>MCP-1</strong></td>
<td><strong>Chemotactic, pro-inflammatory</strong></td>
</tr>
<tr>
<td>Monocytes</td>
<td>Chemotactic for monocytes and T cells</td>
</tr>
<tr>
<td>Macrophages</td>
<td>Activates macrophages</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Induces basophil histamine release</td>
</tr>
<tr>
<td>B cells</td>
<td>Keratinocytes</td>
</tr>
<tr>
<td>TGF-β</td>
<td><strong>Immunoregulatory, growth-modulatory</strong></td>
</tr>
<tr>
<td>Macrophages</td>
<td>Inhibition of monocyte/macrophage MHC class expression and pro-inflammatory cytokine synthesis</td>
</tr>
<tr>
<td>Mast cells</td>
<td>Suppression of proliferation and cell growth but can also promote cell production, e.g. neurogenesis</td>
</tr>
<tr>
<td>Platelets</td>
<td>Stimulates formation of matrix proteins and inhibits MMPs</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Smooth muscle cells</td>
</tr>
<tr>
<td><strong>MIP-1α</strong></td>
<td><strong>Chemotactic, pro-inflammatory</strong></td>
</tr>
<tr>
<td>T and B cells</td>
<td>Anti-viral defense</td>
</tr>
<tr>
<td>Mast cells</td>
<td>Chemotactic for monocytes, T cells, neutrophils and eosinophils</td>
</tr>
<tr>
<td>Monocytes</td>
<td>Activates production of IL-1, IL-6 and TNF by</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Neutrophils</td>
</tr>
</tbody>
</table>
### MIP-1β

**Chemotactic, pro-inflammatory**

- T and B cells
- Monocytes
- Mast cells
- Fibroblasts
- Neutrophils
- Endothelial cells

- Chemotactic for monocytes and T cells
- Activates production of IL-1, IL-6 and TNF by monocytes and expression of β1-integrins on endothelial cells
- Enhances the cytolytic responses of cytotoxic T and NK cells

### TREM-1

**Pro-inflammatory**

- Neutrophils

- Stimulates neutrophil- and monocyte-mediated inflammatory responses through triggering and release of IL-8, TNF-α and IL-1α

### BDNF

**Trophic**

- CNS neurons

- Expressed in hippocampus, cortex and synapses of the basal forebrain
- Supports survival of primary sensory neurons
- Additive effect with NT-3

### GM-CSF

**Pro-inflammatory**

- T cells (Th2)
- Fibroblasts
- Endothelial cells
- Monocytes
- Macrophages
- Mast cells
- Neutrophils

- Maturates dendritic cells, neutrophils and macrophages
- Activates mature neutrophils and mononuclear phagocytic cells
- Prolongs survival and contributes to activity of eosinophils
<table>
<thead>
<tr>
<th><strong>NT-3</strong></th>
<th><strong>NT-4</strong></th>
<th><strong>Soluble TNF RI</strong></th>
<th><strong>MIF</strong></th>
<th><strong>RANTES</strong></th>
<th><strong>MMP-9</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trophic</strong></td>
<td><strong>Trophic</strong></td>
<td><strong>Trophic</strong></td>
<td><strong>Immunoregulatory</strong></td>
<td><strong>Chemotactic, pro-inflammatory</strong></td>
<td><strong>Extracellular matrix enzyme</strong></td>
</tr>
<tr>
<td>CNS neurons</td>
<td>CNS neurons</td>
<td>Multiple cell lines</td>
<td>T cells</td>
<td>T cells</td>
<td>Monocytes</td>
</tr>
<tr>
<td>Supports survival of neuronal cells Additive effect with BDNF</td>
<td>Similar activity as NT-3</td>
<td>Binds to TNF trimers in the circulation, preventing membrane-bound TNF receptor-TNF ligand interaction</td>
<td>Inhibits macrophage migration and stimulates macrophage activation</td>
<td>Chemotactic for monocytes, eosinophils and basophils Mediates histamine release from basophils Activates T cells Chronic inflammation</td>
<td>Is tightly bound to tissues and utilizes heparan sulfate proteoglycans as extracellular docking molecules Degrades fibrillin, the principal structural component of the extracellular matrix</td>
</tr>
</tbody>
</table>
Calibration curves were prepared in a 1:1 mixture of guinea pig serum (Jackson ImmunoResearch, www.jacksonimmuno.com) and pig serum (Dako, www.dako.com). The means of the intra-assay and inter-assay coefficients of variation were 11% and 17%, respectively.

Skogstrand et al. have previously described the working ranges in serum [258]. The detection level in maternal serum was set at half the lowest concentrations in the working range, as described by Skogstrand et al. [259]: 17 IL-1 (40 pg/ml), IL-2 (4 pg/ml), IL-4 (4 pg/ml), IL-5 (4 pg/ml), IL-6 (40 pg/ml), IL-8 (40 pg/ml), IL-10 (10 pg/ml), IL-12 (4 pg/ml), IL-17 (4 pg/ml), IL-18 (40 pg/ml), sIL-6Rα (2500 pg/ml), IFN-γ (4 pg/ml), TNF-α (4 pg/ml), TNF-β (4 pg/ml), MCP-1 (156 pg/ml), TGF-β (4 pg/ml), MIP-1α (40 pg/ml), MIP-1β (40 pg/ml), MMP-9 (5000 pg/ml), TREM-1 (100 pg/ml), BDNF (10 pg/ml), GM-CSF (4 pg/ml), NT-3 (40 pg/ml), NT-4 (4 pg/ml), s TNF RI (156 pg/ml), MIF (100 pg/ml) and RANTES (40 pg/ml). The proteins with undetectable serum levels in more than 50% of the samples were subsequently excluded from further analyses.

3.1.6 Statistical analysis

Women who did and did not deliver within 7 days were compared using a Mann–Whitney U-test for continuous variables and Fisher’s exact test for dichotomous variables.

Receiver operating characteristic (ROC) curves and the area under the curve (AUC) were computed for each protein, cervical length and the background variables for the prediction of delivery within 7 days. The best cut-off values for each protein and cervical length were determined based on three parameters:

1. Calculation of the highest sum of sensitivity plus specificity for each coordinate point in the ROC curve, after SPSS 17.0 analysis

2. Use of the statistical software MedCalc 11.0.1.0, that automatically indicates the best cut-off on the ROC curve, using the highest average of sensitivity plus specificity

3. A visual/individual method based on selection of the closest point on the ROC curve to the top left-hand corner of the curve with the visually best AUC
In this study, an attempt was made to determine the best cut-off value for each variable using these methods, as well as to check whether the sensitivity was higher than the specificity before determining the best cut-off value. This approach was chosen in order to avoid missing women that would deliver preterm. In more detail: for every coordinate point on the ROC curve, using the SPSS software, there is one sensitivity value and one 1-specificity value; it was thus not difficult to select the cut-off value based on the methods mentioned above. However, in cases in which there was one sensitivity value and more than one 1-specificity value for determination of the best cut-off value on the ROC curve, the lowest 1-specificity value of the 2-3 values indicated by the SPSS was chosen. This approach was adopted in order to attribute more importance to sensitivity and to select as the best cut-off point that with the highest sensitivity and the lowest specificity. Continuous variables were dichotomized from the ROC curve to obtain the optimal prediction of delivery within 7 days. Crosstabs were used to obtain odds ratios (ORs).

The significant variables in univariate analyses (p <0.05) were entered into a backward stepwise logistic regression. The limit 0.05 in the univariate analyses was selected in order to avoid the problem of mass significance and a large number of predictors exceeding 10. Choosing a different limit than 0.05, for example 0.25, would have resulted in the inclusion of 20 variables as possible predictors in the stepwise selection, inappropriate according to common statistical standards. All analyses were performed for each serum marker, cervical length and the combinations.

The Hosmer-Lemeshow test was performed for the goodness of fit of the models. A p-value of <0.05 or a 95% confidence interval (95% CI) that did not include 1.0 was considered to be statistically significant.

All calculations were made using the software SPSS 17.0 (SPSS Inc., Chicago, IL, USA) and MedCalc 11.0.1.0 (MedCalc Software, Mariakerke, Belgium).

3.2 Study II

3.2.1 Ethical approval

The local Ethics Committee at the University of Gothenburg approved the study (Dnr 349-95, Ö 506-99 and Dnr 476-05).
3.2.2 Design

Prospective cohort study

3.2.3 Study population

Women with PPROM (n=73) or PTL (n=116) at gestational age 22+0 to 33+6 weeks, admitted to the Department of Obstetrics and Gynecology at Sahlgrenska University Hospital in Gothenburg, Sweden, between 1996 and 2005, were enrolled. It must be noted that 104 PTL cases in this study were also included in Study I (n= 104/116; 90%). In both the PPROM and PTL groups, the majority of enrolled women was of non-Hispanic, white ethnicity and had a stable family situation (Table 7).

Table 7. Ethnicity and marital-cohabitation status

<table>
<thead>
<tr>
<th></th>
<th>PPROM</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIAC+</td>
<td>MIAC-</td>
<td></td>
</tr>
<tr>
<td>Non-Hispanic black</td>
<td>0%</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>Non-Hispanic white</td>
<td>25%</td>
<td>72%</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>0%</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>Cohabiting with baby’s father</td>
<td>22%</td>
<td>69%</td>
<td></td>
</tr>
<tr>
<td>Living alone</td>
<td>3%</td>
<td>6%</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>PTL</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIAC+</td>
<td>MIAC-</td>
<td></td>
</tr>
<tr>
<td>Non-Hispanic Black</td>
<td>0%</td>
<td>3%</td>
<td></td>
</tr>
<tr>
<td>Non-Hispanic White</td>
<td>18%</td>
<td>75%</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>0%</td>
<td>4%</td>
<td></td>
</tr>
<tr>
<td>Cohabiting with baby’s father</td>
<td>18%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Living alone</td>
<td>0%</td>
<td>6%</td>
<td></td>
</tr>
</tbody>
</table>

3.2.4 Clinical considerations and diagnostic approaches

Inclusion in the study was homogeneous throughout the year, except for a lower inclusion rate during the summer months, for the reasons mentioned for Study I (Table 8).
Table 8. Seasonal variation in inclusions/deliveries

<table>
<thead>
<tr>
<th></th>
<th>MIAC +</th>
<th>MIAC -</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PPROM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1\textsuperscript{st} quarter</td>
<td>12%</td>
<td>25%</td>
<td>37%</td>
</tr>
<tr>
<td>2\textsuperscript{nd} quarter</td>
<td>8%</td>
<td>14%</td>
<td>22%</td>
</tr>
<tr>
<td>3\textsuperscript{rd} quarter</td>
<td>3%</td>
<td>14%</td>
<td>16%</td>
</tr>
<tr>
<td>4\textsuperscript{th} quarter</td>
<td>1%</td>
<td>23%</td>
<td>25%</td>
</tr>
<tr>
<td><strong>PTL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1\textsuperscript{st} quarter</td>
<td>6%</td>
<td>29%</td>
<td>35%</td>
</tr>
<tr>
<td>2\textsuperscript{nd} quarter</td>
<td>4%</td>
<td>10%</td>
<td>14%</td>
</tr>
<tr>
<td>3\textsuperscript{rd} quarter</td>
<td>3%</td>
<td>24%</td>
<td>27%</td>
</tr>
<tr>
<td>4\textsuperscript{th} quarter</td>
<td>6%</td>
<td>17%</td>
<td>23%</td>
</tr>
</tbody>
</table>

Corticosteroids were administered at between 24+0 and 33+6 gestational weeks in the majority of cases (96% in PPROM vs 85% in PTL), most of whom were given two doses (90% in PPROM vs 83% in PTL) (Table 9).

Table 9. Corticosteroid administration

<table>
<thead>
<tr>
<th></th>
<th>MIAC +</th>
<th>MIAC -</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PPROM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corticosteroids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>24%</td>
<td>72%</td>
<td>96%</td>
</tr>
<tr>
<td>No</td>
<td>0%</td>
<td>0%</td>
<td>4%</td>
</tr>
<tr>
<td>Double dose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>23%</td>
<td>68%</td>
<td>90%</td>
</tr>
<tr>
<td>No</td>
<td>1%</td>
<td>9%</td>
<td>10%</td>
</tr>
<tr>
<td><strong>PTL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corticosteroids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>17%</td>
<td>68%</td>
<td>85%</td>
</tr>
<tr>
<td>No</td>
<td>2%</td>
<td>13%</td>
<td>15%</td>
</tr>
<tr>
<td>Double dose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>16%</td>
<td>68%</td>
<td>83%</td>
</tr>
<tr>
<td>No</td>
<td>3%</td>
<td>14%</td>
<td>17%</td>
</tr>
</tbody>
</table>

Amniocentesis, with aspiration of 30–50 mL of amniotic fluid, was performed within 24 hours of admission. The majority of the PPROM cases (64%) were sampled before, while the majority of PTL cases (53%) were sampled after, corticosteroid administration (Table 10).
Table 10. Timing of amniocentesis and corticosteroid administration

<table>
<thead>
<tr>
<th>PPROM</th>
<th>MIAC +</th>
<th>MIAC -</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amniocentesis before</td>
<td>18%</td>
<td>47%</td>
<td>64%</td>
</tr>
<tr>
<td>Amniocentesis after</td>
<td>7%</td>
<td>29%</td>
<td>36%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PTL</th>
<th>MIAC +</th>
<th>MIAC -</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amniocentesis before</td>
<td>10%</td>
<td>37%</td>
<td>47%</td>
</tr>
<tr>
<td>Amniocentesis after</td>
<td>10%</td>
<td>44%</td>
<td>53%</td>
</tr>
</tbody>
</table>

After sampling, the amniotic fluid was immediately placed in a refrigerator (4 °C) and processed within five hours. A sample of uncentrifuged amniotic fluid was analyzed with PCR for *Ureaplasma urealyticum* and *Mycoplasma hominis* and cultured for aerobic and anaerobic bacteria. Blood samples were obtained simultaneously with the amniocentesis.

3.2.5 Biomarker analysis methods

Similar methodological considerations as in Study I

3.2.6 Statistical analysis

The Mann-Whitney U-test was applied for the comparison of demographic and clinical characteristics and Fisher’s exact test for the categorical variables. The differences were considered statistically significant at a p<0.05 with two-sided alternative hypotheses. Statistical analyses were performed using SPSS 19.0 (IBM Corporation, Armonk, NY, USA).
3.3 Study III

3.3.1 Ethical approval

The local Ethics Committee at the University of Gothenburg approved the study (Dnr 991-11).

3.3.2 Design

Cross-sectional cohort study

3.3.3 Study population

From June to August 2012, amniotic fluid was collected at between gestational weeks 38+0 and 41+6 from 21 women without chronic disease, who were undergoing elective cesarean section at Sahlgrenska University Hospital, Gothenburg, Sweden.

Inclusion criteria for the study were: maternal age ≥18 years, singleton pregnancy, intact fetal membranes and absence of uterine contractions at admission. Exclusion criteria were: known fetal malformations or chromosome abnormalities and cesarean section indicated by medical complications such as hypertension, pre-eclampsia or intrauterine growth restriction.

3.3.4 Clinical considerations and diagnostic approaches

Amniotic fluid samples were obtained using a sterile syringe to which a sterile aspiration tube was attached. The aspiration tube was inserted deep into the amniotic cavity through a small fenestration of the fetal membranes, prior to complete uterotomy, and 45 mL of amniotic fluid was aspirated (Figure 4).
Each amniotic fluid sample was immediately transferred to the laboratory in a capped sterile syringe and separated into nine 4.5-mL- aliquots. Figure 5 shows the different handling of the aliquots.

The samples centrifuged at 2000g for 10 minutes at 4°C within 2 hours of sampling, without addition of protease inhibitor and without supernate filtration,
were defined as the standard group. This corresponds to our research group’s previous pre-analytical handling of amniotic fluid [260, 261].

In the other groups (test groups), one variable at a time was changed in order to evaluate:

1. The effect of latency from sampling to centrifugation (Group 1). Group 1 was divided into the subgroups 1a and 1b, stored at 4°C for 5 and 24 hours, respectively, before centrifugation.

2. The effect of different centrifugal forces (Group 2). Group 2 was divided into subgroups 2a and 2b, centrifuged at 300g and 12000g, respectively.

3. The effect of centrifugation duration (Group 3). The samples were centrifuged for 20 minutes.

4. The effect of centrifugation temperature (Group 4). The samples were centrifuged at the room temperature of 20°C.

5. The effect of supernatant filtration through a syringe filter (Syringe filter, TPP, Trasadingen, Switzerland, 0.22 μm) (Group 5).

6. The effect of adding 200 μL of protease inhibitor to amniotic fluid directly after sampling (Complete Mini EDTA-free Protease inhibitor Cocktail Tablets, Roche Diagnostics GmbH, Mannheim, Germany) (Group 6).

3.3.5 Biomarker analysis methods

After processing, aliquots from each group were immediately stored at -80°C until analysis with ELISA (R&D Systems, Minneapolis, MN), within 8 months of sampling. Samples were assayed in duplicate for IL-6 and the mean value was used for analysis.

Each ELISA performed was specific for IL-6 (Human IL-6 Quantikine ELISA, R&D Systems, Minneapolis, MN), with a detection rate of 15.6-1500 pg/mL. The inter- and intra-assay coefficients of variation were <10% and <5%, respectively.
3.3.6 Statistical analysis

Results were calculated by pairing IL-6 concentrations in the standard group with those in each test group (Groups 1-6). Wilcoxon signed ranks test was used to calculate p-values. A p-value <0.05 was considered significant. All analyses were performed using SPSS 20.0.0 (SPSS Inc., Chicago, IL, USA).

3.4 Study IV

3.4.1 Ethical approval

The Ethics Committee at University Hospital Hradec Kralove in the Czech Republic approved the study protocol (19 March 2008; No. 200804 SO1P).

3.4.2 Design

Prospective cohort study

3.4.3 Study population

In this study, 231 women were enrolled. Participants were diagnosed with PPROM at gestational age 24+0 - 36+6 weeks and had been admitted to the Department of Obstetrics and Gynecology at the University Hospital Hradec Kralove in the Czech Republic between July 2008 and October 2010.

3.4.4 Clinical considerations and diagnostic approaches

PPROM was diagnosed by a sterile speculum examination, confirming amniotic fluid pooling in the vagina, and a positive test for insulin-like growth factor-binding protein (ACTIM PROM test; MedixBiochemica, Kauniainen, Finland) in the vaginal fluid.

Management of PPROM in the Czech Republic is active, except in cases at <28 weeks of gestation, which are handled expectantly. Timing of induction of labor or elective cesarean section depends on gestational age:
1. Within 24 h at >34+0 weeks

2. Within 48 h at 32+0 - 33+6 weeks

3. Within 72 h at 28+0 - 31+6 weeks

The placenta was fixed in 10% neutral buffered formalin directly after delivery and tissue blocks from the placenta, umbilical cord and placental membranes were processed according to routine and embedded in paraffin. A single pathologist, blinded to the clinical status, performed the histological examination. In this study, a second pathologist did not review the histological diagnosis of the placenta. However, according to Simmonds et al. [262] and Kramer et al. [263], there is high intra- and inter-observer agreement between one and two pathologists examining the same placenta for membrane inflammation, funisitis, and umbilical cord vasculitis. The degree of polymorphonuclear leukocyte infiltration was evaluated separately in the free membranes (amnion and chorion-decidua), the chorionic plate and the umbilical cord, according to the criteria proposed by Salafia et al. [171]. HCA was diagnosed based on histological grade (grades 3–4 in the chorion-decidua and/or 3–4 in the chorionic plate and/or 1–4 in the amnion and/or 1–4 in the umbilical cord). Funisitis was diagnosed based on histological grades 1–4 in the umbilical cord (Table 11).

Table 11. Grading system for HCA and funisitis. Modified by Salafia et al., 1989 [171]

<table>
<thead>
<tr>
<th>Amnion and chorion-decidua</th>
<th>Grade 1</th>
<th>One focus of at least 5 neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 2</td>
<td>More than one focus of grade-1 inflammation, or at least one focus of 5-20 neutrophils</td>
<td></td>
</tr>
<tr>
<td>Grade 3</td>
<td>Multiple and/or confluent foci of grade-2 inflammation</td>
<td></td>
</tr>
<tr>
<td>Grade 4</td>
<td>Diffuse and dense acute inflammation</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Umbilical cord</th>
<th>Grade 1</th>
<th>Neutrophils within the inner third of the umbilical vein wall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 2</td>
<td>Neutrophils within the inner third of at least two umbilical vessel walls</td>
<td></td>
</tr>
<tr>
<td>Grade 3</td>
<td>Neutrophils in the perivascular Wharton’s jelly</td>
<td></td>
</tr>
<tr>
<td>Grade 4</td>
<td>Panvasculitis and funisitis extending deep into the Wharton’s jelly</td>
<td></td>
</tr>
</tbody>
</table>
Chorionic plate

Grade 1  One focus of at least 5 neutrophils in subchorionic fibrin
Grade 2  Multiple foci of grade-1 inflammation in subchorionic fibrin
Grade 3  Few neutrophils in connective tissue or chorionic plate
Grade 4  Numerous neutrophils in chorionic plate and chorionic vasculitis

For the study of the neonatal outcomes, an entity called “composite neonatal morbidity” was defined and included:

1. Tracheal intubation and/or

2. RDS, defined by two or more of the following criteria:
   a. Evidence of respiratory compromise and persistent oxygen requirement for >24 hours
   b. Administration of exogenous surfactant
   c. Radiographic evidence of hyaline membrane disease, and/or

3. IVH, diagnosed by cranial ultrasound and graded 1–4, according to the criteria defined by Papile et al. [264], and/or

4. NEC, defined as a radiologic finding of either intramural gas or free intra-abdominal gas, and/or

5. ROP, identified by retinoscopy, and/or

6. EOS or LOS, defined as any systemic bacterial infection evidenced by clinical symptoms in association with either a positive blood culture or elevated CRP and/or affected WBC count during the first 72 hours of life or at 4–120 days of life, respectively, and/or

7. BPD in a preterm infant, defined by oxygen dependency at 28 days of life, and/or

8. Pneumonia, diagnosed by an abnormal chest X-ray finding, and/or

9. Perinatal death, defined as death before hospital discharge
3.4.5 Statistical analysis

Continuous variables (demographic and clinical characteristics) were compared using the Mann-Whitney U-test and categorical variables were compared using the Pearson Chi-square test. Spearman’s partial correlation was applied to adjust data for gestational age at delivery. Differences were considered to be statistically significant at $p < 0.05$, with two-sided alternative hypotheses. Statistical analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL).
4. Results and comments

4.1 Study I

Fifty-seven women (40%) of the 142 included in the study delivered within 7 days of sampling.

Only proteins with detectable maternal serum levels in more than 50% of the samples were included in the analyses. Thus, among the 27 initially quantified serum proteins, 22 were included in the further analyses and IL-1β, IL-2, IL-5, IL-6 and IL-8 were excluded. Women who delivered within 7 days had significantly higher levels of IL-10, soluble IL-6Ra, TNF-β MIP-1β, MMP-9, BDNF, soluble TNF receptor I, MIF and RANTES than those who delivered later. After adjustment for corticosteroid administration and sampling time with logistic regression, statistically significant effects were only found for TGF-β and BDNF levels (Table 12).

Table 12. Effect of corticosteroid administration and sampling time on maternal serum cytokine/chemokine levels

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Crude p-value</th>
<th>Adjusted p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>0.827</td>
<td>0.896</td>
</tr>
<tr>
<td>IL-10</td>
<td>&lt;0.001</td>
<td>0.378</td>
</tr>
<tr>
<td>IL-12</td>
<td>0.357</td>
<td>0.942</td>
</tr>
<tr>
<td>IL-17</td>
<td>0.331</td>
<td>0.187</td>
</tr>
<tr>
<td>IL-18</td>
<td>0.950</td>
<td>0.923</td>
</tr>
<tr>
<td>Soluble</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6Ra</td>
<td>0.050</td>
<td>0.101</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.336</td>
<td>0.539</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.920</td>
<td>0.531</td>
</tr>
<tr>
<td>TNF-β</td>
<td>0.043</td>
<td>0.262</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.699</td>
<td>0.594</td>
</tr>
<tr>
<td>TGF-β</td>
<td>0.217</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Crude p-value</th>
<th>Adjusted p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIP-1α</td>
<td>0.379</td>
<td>0.338</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>0.024</td>
<td>0.872</td>
</tr>
<tr>
<td>MMP-9</td>
<td>&lt;0.001</td>
<td>0.107</td>
</tr>
<tr>
<td>TREM-1</td>
<td>0.773</td>
<td>0.539</td>
</tr>
<tr>
<td>BDNF</td>
<td>0.008</td>
<td>0.025</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>0.042</td>
<td>0.627</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Crude p-value</th>
<th>Adjusted p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soluble</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF RI</td>
<td>0.004</td>
<td>0.692</td>
</tr>
<tr>
<td>MIF</td>
<td>&lt;0.001</td>
<td>0.818</td>
</tr>
<tr>
<td>RANTES</td>
<td>0.018</td>
<td>0.271</td>
</tr>
</tbody>
</table>
Cervical length was significantly shorter among women who delivered within 7 days (5.5 mm vs 21 mm; p <0.001), compared to women who delivered later.

The ROC curve analysis used to calculate the best cut-off value and the AUC for every single protein and cervical length showed that the highest AUC values were for IL-10 (AUC = 0.69; p <0.001), MMP-9 (AUC = 0.74; p <0.001), MIF (AUC = 0.71; p <0.001) and cervical length (AUC = 0.77; p <0.001).

The highest crude ORs for delivery within 7 days were for IL-10 (OR 4.6; C.I. 2.2-9.7), MMP-9 (OR 6.0; C.I. 2.9-12.7), MIF (4.7; C.I. 2.2-9.9), RANTES (OR 3.9; C.I. 1.8-8.6) and cervical length (OR 21.2; C.I. 6.0-74.7).

Based on a stepwise multivariable logistic regression of dichotomous variables, univariate analyses were performed for the most significant serum proteins alone, then cervical length alone and, lastly, the combination of serum proteins and cervical length, in order to construct the best prediction model.

We found that the model combining proteins and cervical length had the best predictive capacity. In the multivariable model combining maternal serum proteins and cervical length, high levels of IL-10 (≥ 48 pg/ml; OR 5.0; 95% CI 1.7–14.3), RANTES (≥ 49293 pg/ml; OR 9.9; 95% CI 2.5–39.2) and short cervical length (≤18 mm; OR 43.5; 95% CI 8.9–211.3) were statistically significant contributors to the prediction of spontaneous PTD within 7 days of sampling.

Using this combined model, the ROC curve analysis showed an AUC of 0.88, sensitivity 74%, specificity 87%, positive predictive value 76%, negative predictive value 86%, + LR 5.83 and - LR 0.30.

The Hosmer-Lemeshow test of goodness of fit of the combined model yielded a p-value of 0.91. The regression equation for the combined model is
\[
\text{Logit } P = -4.71 + 1.62xIL-10 + 2.29xRANTES + 3.77xCL
\]
(CL is cervical length).

In the regression equation, all the variables are binary values. In order to ascertain whether adding continuous variables to the regression model would be a more powerful approach, an analysis with the variables as continuous variables in the combined model was performed (Table 13).
Table 13. Predictive capacity of the combined model, comparison of binary and continuous variables

<table>
<thead>
<tr>
<th></th>
<th>Binary approach</th>
<th>Continuous approach</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correctly predicted</td>
<td>82.6%</td>
<td>77.6%</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>73.8%</td>
<td>73.8%</td>
</tr>
<tr>
<td>Specificity</td>
<td>87.3%</td>
<td>82.2%</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>75.6%</td>
<td>68.9%</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>86.2%</td>
<td>85.5%</td>
</tr>
<tr>
<td>+LR</td>
<td>5.83</td>
<td>4.16</td>
</tr>
<tr>
<td>-LR</td>
<td>0.30</td>
<td>0.32</td>
</tr>
<tr>
<td>AUC</td>
<td>0.88 (0.84-0.95)</td>
<td>0.86 (0.79-0.92)</td>
</tr>
</tbody>
</table>

The binary approach has a predictive capacity resembling that of the continuous approach, as shown above. Moreover, the binary approach would be easier for clinicians to use bedside. Since the continuous variables in this study can have very extreme values, OR estimation can be very uncertain. For a more precise estimation of the continuous variables, more observations including many extreme values would be required.

In order to validate the combined model in this study, two resampling methods were used, i.e. bootstrapping (Table 14) and cross-validation. In practice, resampling is based on repeatedly drawing samples from a training set of observations and refitting a model on each sample in order to obtain additional information about the fitted model.

Table 14. Validation of the combined prediction model with bootstrapping, simulation of 1000 studies

<table>
<thead>
<tr>
<th></th>
<th>Combined model OR (95% CI)</th>
<th>Bootstrapping Mean OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervical length</td>
<td>43.5 (8.9-211.3)</td>
<td>54.9</td>
</tr>
<tr>
<td>High RANTES, plasma</td>
<td>9.9 (2.5-39.2)</td>
<td>11.1</td>
</tr>
<tr>
<td>High IL-10, plasma</td>
<td>5.0 (1.7-14.3)</td>
<td>5.6</td>
</tr>
<tr>
<td><strong>AUC (95% CI)</strong></td>
<td>0.88 (0.84-0.95)</td>
<td>0.89 (0.84-0.94)</td>
</tr>
</tbody>
</table>
According to these results, the mean AUC resulting from bootstrapping is approximately the same as the AUC from the original analysis. The mean ORs for cervical length, RANTES and IL-10 are higher than those found in the original analysis.

The cross-validation method (leave-one-out method) yielded an AUC of 0.83 (0.76-0.91). This indicates that performing the same study on another cohort of women with imminent spontaneous PTD might have led to a somewhat lower AUC for the same combined predictive model.

However, the results with the resampling methods suggest that the combined model is robust in predicting spontaneous PTD within 7 days of sampling.

4.2 Study II

As in Study I, only proteins with detectable maternal serum levels in more than 50% of the samples were included in the analyses. Thus, among the 27 initially quantified serum proteins, 25 were included in the further analyses and IL-2 and IL-5 were excluded.

The median gestational age at sampling and delivery were 32+0 (range 22+6–33+6) and 34+5 weeks (range 32+1–43+0), respectively. The overall rate of MIAC was 29% (21 of 73) in women with PPROM and 19% (22 of 116) in women with PTL.

To evaluate the effect of the interval (days) from blood sample storage to multiple immunoassay analysis on the outcome presence or absence of MIAC, a linear regression (one-way ANOVA) was performed, revealing no differences, either for the PPROM group (F=0.304; p=0.583) or the PTL group (F=1.122; p=0.292).

Women with PPROM and PTL were evaluated separately. In both groups, the data were classified according to the whole study population group (all PPROM and all PTL), women with gestational age at admission <32+0 weeks and women with gestational age at admission ≥32+0 weeks.

High levels of maternal serum IL-18 (451 vs 335; p=0.031) and low levels of MCP-1 (139 vs 213, P=0.039) were observed in the PPROM with MIAC group. In the subgroup of women with PPROM at < 32+0 weeks, maternal serum IL-18
levels were significantly higher (654 vs 361; p=0.003) and levels of IL-1β were significantly lower (9 vs 20; p=0.008) in women with MIAC than in those without. No significant association was found between MIAC and any of the cytokine levels at over 32+0 weeks of gestation.

In the group of women with PTL, there were no significant differences in the inflammatory response in those with MIAC. Only IL-6 levels were significantly higher (21 vs 14, P=0.019) in the subgroup of women with PTL at <32+0 weeks of gestation. No significant association was observed with any cytokine at over 32+0 weeks of gestation.

The stratification into two gestational age subgroups (less and more than 32 weeks) in this study was based on the finding from a study by Kacerovsky et al. [52] that the intra-amniotic inflammatory response to bacteria in pregnancies complicated by PPROM seems to be different at gestational ages above and below 32 weeks.

After adjustment for corticosteroid administration and sampling time using logistic regression, the only significant effect was on IL-6 levels in the PPROM group (Table 15).

Table 15. Effect of corticosteroid administration and sampling time on maternal serum cytokine/chemokine levels

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>PTL Crude p-value</th>
<th>PTL Adjusted p-value</th>
<th>PPROM Crude p-value</th>
<th>PPROM Adjusted p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>0.441</td>
<td>0.176</td>
<td>0.135</td>
<td>0.226</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.974</td>
<td>0.577</td>
<td>0.244</td>
<td>0.227</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.124</td>
<td>0.289</td>
<td>0.226</td>
<td>0.037</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.178</td>
<td>0.680</td>
<td>0.386</td>
<td>0.278</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.249</td>
<td>0.295</td>
<td>0.316</td>
<td>0.801</td>
</tr>
<tr>
<td>IL-12</td>
<td>0.635</td>
<td>0.345</td>
<td>0.168</td>
<td>0.209</td>
</tr>
<tr>
<td>IL-17</td>
<td>0.050</td>
<td>0.946</td>
<td>0.873</td>
<td>0.461</td>
</tr>
<tr>
<td>IL-18</td>
<td>0.082</td>
<td>0.129</td>
<td>0.031</td>
<td>0.071</td>
</tr>
<tr>
<td>Soluble IL-6Ra</td>
<td>0.116</td>
<td>0.090</td>
<td>0.888</td>
<td>0.954</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.598</td>
<td>0.184</td>
<td>0.709</td>
<td>0.533</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.572</td>
<td>0.214</td>
<td>0.484</td>
<td>0.270</td>
</tr>
<tr>
<td>TNF-β</td>
<td>0.266</td>
<td>0.216</td>
<td>0.939</td>
<td>0.976</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.779</td>
<td>0.460</td>
<td>0.039</td>
<td>0.051</td>
</tr>
</tbody>
</table>
4. RESULTS AND COMMENTS  

No statistically significant differences were found between the PPROM and PTL groups regarding the type of microorganism isolated from the amniotic fluid in MIAC-positive cases (p=0.250) (Table 16).

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>PPROM</th>
<th>PTL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungi</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Gram-negative bacilli</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Gram-positive bacilli</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Gram-positive cocci</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Ureaplasma urealyticum</td>
<td>9</td>
<td>3</td>
</tr>
</tbody>
</table>

4.3 Study III  

In this study, IL-6 concentrations were assayed in 21 amniotic fluid samples, divided into nine groups that were handled with different protocols. The demographics of the study population are displayed in Table 17.

<table>
<thead>
<tr>
<th>Maternal age, years (median, Q1–Q3)</th>
<th>34 (30–36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nulliparous</td>
<td>9 (43%)</td>
</tr>
</tbody>
</table>
Gestational age at cesarean section (median, Q1–Q3)  39+0 (38+5-39+2)
Smoker  2 (10%)
Maternal BMI (median, Q1–Q3)  25 (22–27)

The median and quartile (Q1 and Q3) values of the amniotic fluid IL-6 for each group are listed in Table 18 and presented graphically in Figure 6.

Table 18. Amniotic fluid IL-6. Values are shown as median, 25th (Q1) and 75th (Q3) quartiles

<table>
<thead>
<tr>
<th>Median (pg/mL)</th>
<th>Q1 (pg/mL)</th>
<th>Q3 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard group</td>
<td>615</td>
<td>342</td>
</tr>
<tr>
<td>Group 1a</td>
<td>585</td>
<td>354</td>
</tr>
<tr>
<td>Group 1b</td>
<td>610</td>
<td>351</td>
</tr>
<tr>
<td>Group 2a</td>
<td>610</td>
<td>355</td>
</tr>
<tr>
<td>Group 2b</td>
<td>615</td>
<td>358</td>
</tr>
<tr>
<td>Group 3</td>
<td>580</td>
<td>350</td>
</tr>
<tr>
<td>Group 4</td>
<td>605</td>
<td>346</td>
</tr>
<tr>
<td>Group 5</td>
<td>605</td>
<td>343</td>
</tr>
<tr>
<td>Group 6</td>
<td>606</td>
<td>349</td>
</tr>
</tbody>
</table>

Figure 6. Amniotic fluid IL-6 concentrations (pg/mL), presented by group
The IL-6 concentrations in the standard group ranged between 211 and 2005 pg/mL, with a median value of 615 pg/mL. No significant differences were found, in the median or dispersion values of the IL-6 concentrations, between the standard group and the six test groups.

The differences between IL-6 concentrations in each test group and IL-6 concentrations in the standard group were computed (Figure 7).

Figure 7. Schematic presentations of the differences in IL-6 concentrations between the test groups and the standard group. p-values are calculated with Wilcoxon signed-rank test. The markers (o) in the diagram represent outliers and (☆) extreme values [265]

There were no significant differences in amniotic fluid IL-6 concentrations between the test groups and the standard group.
4.4 Study IV

A total of 231 women with diagnosed PPROM were included in the analyses. One hundred forty-two cases (61%) were HCA-positive and 89 cases (39%) were HCA-negative. Among the HCA-positive cases, 19% were also positive for funisitis.

In this study, gestational age at delivery, latency from PPROM to delivery and birth weight cannot be considered as outcomes, because of the active management of PPROM pregnancies in the Czech Republic.

Women with HCA had neonates with significantly lower birth weight (1,840 g vs 2190 g; p<0.001) and higher rates of composite neonatal morbidity (75 cases vs 30 cases; p=0.005), RDS (52 cases vs 18 cases; p=0.008), BPD (18 cases vs 2 cases; p=0.006) and EOS (15 cases vs 1 case; p=0.001), than those without HCA.

After adjustment for gestational age at delivery, EOS differed significantly between these two groups [15 (11%) vs 1 (1%); p=0.011]. After adjustment for corticosteroid and antibiotics administration, EOS (p=0.027) and birth weight (p=0.032) differed significantly between the two groups. This significant difference remained only for EOS (p=0.029) after adjustment for corticosteroids and antibiotics administration and gestational age at delivery (Table 19).

Table 19. Neonatal outcomes in the groups with and without HCA. Crude p-value (P1); adjusted for gestational age at delivery (P1); adjusted for corticosteroid and antibiotics administration (P3) and adjusted for corticosteroid and antibiotics administration and gestational age at delivery (P4)

<table>
<thead>
<tr>
<th></th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight (g)</td>
<td>&lt;0.001</td>
<td>0.153</td>
<td>0.032</td>
<td>0.158</td>
</tr>
<tr>
<td>5-min Apgar score &lt;7</td>
<td>0.109</td>
<td>0.890</td>
<td>0.159</td>
<td>0.608</td>
</tr>
<tr>
<td>10-min Apgar score &lt;7</td>
<td>0.155</td>
<td>0.788</td>
<td>0.179</td>
<td>0.620</td>
</tr>
<tr>
<td>Affected neonatal WBC count</td>
<td>0.615</td>
<td>0.229</td>
<td>0.117</td>
<td>0.120</td>
</tr>
<tr>
<td>Positive hemoculture</td>
<td>0.259</td>
<td>0.526</td>
<td>0.386</td>
<td>0.461</td>
</tr>
<tr>
<td>Neonatal composite morbidity</td>
<td>0.005</td>
<td>0.527</td>
<td>0.077</td>
<td>0.423</td>
</tr>
<tr>
<td>NICU stay ≥1 d</td>
<td>0.116</td>
<td>0.659</td>
<td>0.593</td>
<td>0.706</td>
</tr>
<tr>
<td>Tracheal intubation</td>
<td>0.014</td>
<td>0.530</td>
<td>0.077</td>
<td>0.303</td>
</tr>
<tr>
<td>Duration tracheal intubation ≥1 d</td>
<td>0.011</td>
<td>0.472</td>
<td>0.348</td>
<td>0.554</td>
</tr>
</tbody>
</table>
Neonates with funisitis had significantly lower birth weight (1,630 g vs 2,080 g; p <0.001) and Apgar scores at 5 (10 cases vs 12 cases; p=0.001) and 10 min (6 cases vs 5 cases; p=0.002). The neonates with funisitis had higher rates of composite neonatal morbidity (33 cases vs 72 cases; p <0.001), perinatal death (11% vs 3%; p=0.001), RDS (24 cases vs 46 cases; p <0.001), ROP (10 cases vs 7 cases; p <0.001), BPD (11 cases vs 9 cases; p <0.001), EOS (18% vs 4%; p=0.001) and LOS (11% vs 4%; p=0.040).

After adjustment for gestational age at delivery, ROP [10 (23%) vs 7 (4%); p=0.014] and EOS [8 (18%) vs 8 (4%); p=0.002] differed significantly between these two groups. After adjustment for corticosteroid and antibiotics administration, birth weight (p<0.001), 5-min Apgar score <7 (p=0.004), neonatal composite morbidity (p<0.001), NICU stay ≥1 day (p=0.017), tracheal intubation (p=0.014), duration tracheal intubation ≥1 day (p=0.020), duration nCPAP ≥1 day (p=0.013), perinatal death (p=0.046), ROP (p=0.002), BPD (p=0.004) and EOS (p=0.001) differed significantly between the two groups. This significant difference remained only for ROP (p=0.018) and EOS (p=0.001) after adjustment for corticosteroid and antibiotics administration and gestational age at delivery (Table 20).
Table 20. Neonatal outcomes in the group of women with and without funisitis. Crude p-value (P1); adjusted for gestational age at delivery (P2); adjusted for corticosteroid and antibiotics administration (P3) and corticosteroid and antibiotics administration and gestational age at delivery (P4)

<table>
<thead>
<tr>
<th>Outcome</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight (g)</td>
<td>&lt;0.001</td>
<td>0.255</td>
<td>&lt;0.001</td>
<td>0.339</td>
</tr>
<tr>
<td>5-min Apgar score &lt;7</td>
<td>0.001</td>
<td>0.331</td>
<td>0.004</td>
<td>0.516</td>
</tr>
<tr>
<td>10-min Apgar score &lt;7</td>
<td>0.002</td>
<td>0.426</td>
<td>0.007</td>
<td>0.646</td>
</tr>
<tr>
<td>Affected neonatal WBC count</td>
<td>0.725</td>
<td>0.204</td>
<td>0.168</td>
<td>0.170</td>
</tr>
<tr>
<td>Positive hemoculture</td>
<td>0.042</td>
<td>0.193</td>
<td>0.124</td>
<td>0.317</td>
</tr>
<tr>
<td>Neonatal composite morbidity</td>
<td>&lt;0.001</td>
<td>0.220</td>
<td>&lt;0.001</td>
<td>0.110</td>
</tr>
<tr>
<td>NICU stay ≥1 d</td>
<td>0.002</td>
<td>0.827</td>
<td>0.017</td>
<td>0.578</td>
</tr>
<tr>
<td>Tracheal intubation</td>
<td>0.001</td>
<td>0.436</td>
<td>0.014</td>
<td>0.684</td>
</tr>
<tr>
<td>Duration tracheal intubation ≥1 d</td>
<td>0.003</td>
<td>0.886</td>
<td>0.020</td>
<td>0.152</td>
</tr>
<tr>
<td>Duration nCPAP ≥1 d</td>
<td>0.002</td>
<td>0.722</td>
<td>0.013</td>
<td>0.510</td>
</tr>
<tr>
<td>RDS</td>
<td>&lt;0.001</td>
<td>0.378</td>
<td>0.009</td>
<td>0.527</td>
</tr>
<tr>
<td>Perinatal death</td>
<td>0.111</td>
<td>0.897</td>
<td>0.046</td>
<td>0.850</td>
</tr>
<tr>
<td>IVH</td>
<td>0.394</td>
<td>0.756</td>
<td>0.813</td>
<td>0.848</td>
</tr>
<tr>
<td>IVH grade 1-2</td>
<td>0.792</td>
<td>0.529</td>
<td>0.787</td>
<td>0.646</td>
</tr>
<tr>
<td>IVH grade 2-4</td>
<td>0.112</td>
<td>0.694</td>
<td>0.285</td>
<td>0.831</td>
</tr>
<tr>
<td>NEC</td>
<td>0.228</td>
<td>0.738</td>
<td>0.470</td>
<td>0.801</td>
</tr>
<tr>
<td>Intestinal perforation</td>
<td>0.228</td>
<td>0.993</td>
<td>0.470</td>
<td>0.941</td>
</tr>
<tr>
<td>Patent ductus arteriosus</td>
<td>0.101</td>
<td>0.965</td>
<td>0.318</td>
<td>0.961</td>
</tr>
<tr>
<td>ROP</td>
<td>&lt;0.001</td>
<td>0.014</td>
<td>0.002</td>
<td>0.018</td>
</tr>
<tr>
<td>BPD</td>
<td>&lt;0.001</td>
<td>0.066</td>
<td>0.004</td>
<td>0.271</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>0.192</td>
<td>0.686</td>
<td>0.471</td>
<td>0.639</td>
</tr>
<tr>
<td>Pulmonary hypertension</td>
<td>0.515</td>
<td>0.843</td>
<td>0.988</td>
<td>0.758</td>
</tr>
<tr>
<td>EOS</td>
<td>0.001</td>
<td>0.002</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>LOS</td>
<td>0.040</td>
<td>0.949</td>
<td>0.199</td>
<td>0.837</td>
</tr>
</tbody>
</table>

HCA: Histological chorioamnionitis; WBC: White blood cells; NICU: Neonatal intensive care unit; nCPAP: Nasal continuous positive airway pressure; RDS: Respiratory distress syndrome; IVH: Intraventricular hemorrhage; NEC: Necrotizing enterocolitis; ROP: Retinopathy of prematurity; BPD: bronchopulmonary dysplasia; EOS: early-onset sepsis; LOS: late-onset sepsis
PTD, accounting worldwide for more than 15 million preterm neonates annually, is a major perinatal health problem, not only in terms of associated mortality, but also with regard to morbidity and its economic implications [12].

Despite significant advances in obstetric and perinatal care, there has been little progress in reducing the global PTD rate [15]. Because of the complex nature of spontaneous PTD and the involvement of several pathways in its pathogenesis, prediction and prevention remain difficult and pose a continuing and significant challenge in maternal and fetal medicine.

Major effort has been devoted to identifying inflammatory biomarkers to predict spontaneous PTD in both asymptomatic and symptomatic women and to improve our understanding of the mechanisms and pathways leading to this condition. While several biomarkers have been tested as predictors of spontaneous PTD, few have proven useful for clinical purposes [15, 244]. So far, among the biomarkers evaluated, fetal fibronectin (fFN) in the cervico-vaginal secretion, together with short cervical length on transvaginal ultrasound has been found to be the most powerful and consistent predictor of spontaneous PTD (sensitivity around 80%, specificity around 61%, positive predictive value around 17% and negative predictive value around 97%) [41, 95, 266-269]. The clinical usefulness of both tests lies primarily in their negative predictive value, aiding in avoiding unnecessary interventions and guiding clinicians in decision-making regarding in utero transfer, administration of antenatal corticosteroids and/or tocolysis.

To progress in our understanding and prediction of spontaneous PTD, we must acknowledge that it is not one disease with a single solution or cure, but rather the product of overlapping pathophysiological pathways [39]. Indeed, it is has been suggested that a single biomarker cannot accurately predict spontaneous PTD [244, 270, 271]. Since the pathophysiology of spontaneous PTD is multifactorial with different pathways and biomarkers involved, multiplex immunoassay analysis have been introduced in the last decade, with the aim of simultaneously using several biomarkers from different and distinct biological pathways to better predict the risk [272]. These multiplex analyses of biomarker
panels thus constitute the future direction of biomarker research [244]. Studies have shown that a combination of biomarkers in minimally invasive samples (cervical fluid, serum) and cervical length measurement could help to better predict MIAC [273] and spontaneous PTD in women with PPROM [274] and PTL [260, 275]. The development of predictive models based on the analysis of multiple biomarkers seems feasible and allows early identification, and potentially early intervention, before the onset of labor, in women at risk.

A perfect predictor of spontaneous PTD must have good specificity, sensitivity and high accuracy in order to increase the probability of diagnosis in women with a positive test result, as well as to rule out the condition when test results are negative [243, 272, 276]. Feasibility, accessibility, patient convenience and minimally invasive sampling are also important. Although a non-invasive approach is preferable for predicting spontaneous PTD, it is currently unclear which non-invasively sampled body fluid best represents the intra-amniotic environment and whether biomarkers in non-invasive samples reflect the intra-amniotic compartment adequately [272]. The accuracy of serum or plasma biomarkers for predicting spontaneous PTD in asymptomatic women has been shown to be very low [270].

In the following, the main findings of the studies in this thesis and the main findings concerning the analyzed biomarkers are briefly discussed. More detailed discussion is found in the Discussion section of each publication.

**Study I**

In the last decade, there have been efforts to predict spontaneous PTD in women with PTL using cervical length, alone [257, 277-284] or in combination with cervical biomarkers [260, 266, 285-291]. A cervical length of <15 mm (found in less than 10% of symptomatic women) detects approximately 60% of those who will deliver within 7 days and represents a 5.7-fold increased risk of delivering within 7 days [292]. Few studies have investigated the relationship between biomarkers in maternal serum and spontaneous PTD in women with PTL, suggesting that effective prediction of spontaneous PTD may be possible with a non-invasive approach [293-302]. In Study I, it was found that a multivariable prediction model, combining maternal serum biomarkers (IL-10 and RANTES) with cervical length, had predictive power equal to that of invasive models for delivery within 7 days from the beginning of labor [275].
IL-10 and RANTES were statistically significant contributors to the prediction of spontaneous PTD within 7 days of sampling in Study I. IL-10 is likely to be an important cytokine because it suppresses the production of pro-inflammatory cytokines by other cells and numerous studies have documented its production at the maternal-fetal interface [303-305]. Pregnancy is not dependent on IL-10 production, however; IL-10 (-/-) mice are still fertile [306]. Up-regulation of this anti-inflammatory cytokine leads to suppression of NK cells and T lymphocytes against fetal alloantigens [307, 308]. In Study I, maternal serum IL-10 levels were significantly higher in women who delivered preterm (within 7 days of sampling) than in women who delivered later. We can therefore hypothesize that IL-10 can lessen the pathological effect of inflammation prevalent in spontaneous PTD. The other cytokine in our prediction model is RANTES. Maternal serum RANTES levels were significantly higher in women who delivered preterm (within 7 days of sampling), compared with women who delivered later. This pro-inflammatory cytokine that chemoattracts monocytes, with additional activity toward T-cells, has been found in the decidua of pregnancies complicated with spontaneous PTD. Its expression in this tissue is correlated with both macrophage and neutrophil abundance, suggesting that these immune cell subtypes are a major production source [309]. However, there are few studies describing the association between high levels of RANTES in maternal serum and spontaneous PTD [309-311].

Study II

There are few studies, with contradictory results, on the role of maternal serum biomarkers as predictors of IAI and fetal membrane inflammation [249, 312-316]. In Study II, the maternal serum inflammatory response was evaluated according to the presence of MIAC in women with PTL and PPROM. We found that the inflammatory status of the amniotic cavity is not accurately reflected in maternal serum. Studies have shown that sampling from maternal serum and other non-invasive procedures is effective in predicting PTD [293-302], but it seems that maternal serum sampling is not so effective in predicting MIAC. A weak maternal inflammatory response in women with MIAC was found and differences in the biomarker levels were only evident at early gestational ages (<32 weeks). This finding is in accordance with previous studies showing that the intra-amniotic inflammatory response to bacteria is different below and above 32 weeks of gestation. This process seems to be heterogeneous as a result of a broad spectrum of factors that can affect cytokine production in amniotic
fluid, including the duration of MIAC, the type of bacteria, the microbial burden and bacterial virulence [52, 317].

In Study II, a higher but weak expression of serum IL-6 in women with PTL and MIAC was observed. Despite the fact that IL-6 is a classical marker of inflammation, no differences in the concentration of this serum protein were found between PPROM cases with or without MIAC. Higher IL-6 concentrations have been observed in the amniotic fluid of asymptomatic women delivering preterm, compared to those with term delivery [244], but a temporal relationship between IL-6 and interval until delivery has yet not been established [318]. The test accuracy of IL-6 in amniotic fluid seems to be especially good for both asymptomatic and symptomatic women, but is generally poor in other biological fluids (e.g. blood, cervico-vaginal fluid) [270, 276]. However, from a clinical point of view, the main limitation of this biomarker is its lack of specificity in predicting disease, because it is overexpressed in maternal serum in both infectious and non-infectious conditions [319, 320].

There have been no previous studies on the role of maternal serum IL-18 in women with MIAC. In Study II, higher levels of maternal serum IL-18 were found in women with PPROM who had MIAC, which suggests a pro-inflammatory effect of IL-18 in amniotic fluid and maternal serum in the presence of infection. It has previously been shown that IL-18 in amniotic fluid is associated with MIAC and spontaneous PTD in women with PTL [321]. IL-18 is a cytokine with pleiotropic qualities that regulate both the innate and acquired immune responses and it can stimulate both Th 1 and 2 responses, depending on the local cytokine environment [322, 323]. IL-18 is synthesized as a pro-form and is activated through cleavage by caspase-1 [324]. It is important in the host defense against severe infections via induction of other cytokines and effector cells and molecules. It enhances the inflammatory process by stimulating the production of IFN-γ, TNF-α and IL-1β [323]. IL-18 can also activate apoptosis by enhancing Fas ligand and Fas expression [325] and is found in both amniotic fluid and maternal and fetal plasma [326-328].

There is a lack of previous studies evaluating the importance of IL-1β and MCP-1 as risk factors for MIAC in maternal serum. In Study II, low levels of IL-1β and MCP-1 were observed in women with PPROM who had MIAC, indicating only a minor maternal serum inflammatory response mediated by those two cytokines. IL-1 plays a central role in the pro-inflammatory response. It is a prototypical pro-inflammatory cytokine that regulates a wide spectrum of immunological processes and it exists in two forms, IL-1α and IL-1β, both of
which bind to two distinct membrane receptors [329]. IL-1β induces a systemic and local response to pathogenic invasion or tissue injury by inducing transcription or enhancing mRNA stability of a wide variety of pro-inflammatory genes. It has been shown to be a major factor in the initiation of infection-related PTL and spontaneous PTD [330]. IL-1β levels are elevated in the amniotic fluid of pregnancies complicated by microbial-associated IAI and it may be capable of causing PTL by up-regulating prostaglandin production, leading to myometrial contractions [331-333]. IL-1β also enhances MMP-1 and MMP-3 expression in decidual cells, possibly leading to PPROM [334]. MCP-1 stimulates chemotaxis of monocytes and several associated cellular events, including Ca²⁺ flux and integrin expression. It is also a weak inducer of cytokine expression in monocytes and, at high concentrations, elicits a respiratory burst leading to generation of reactive oxygen species [335].

Studies I and II have several strengths. We evaluated several biomarkers in maternal serum that can be easily tested during pregnancy. A non-invasive approach for predicting spontaneous PTD is relevant for clinical practice. Moreover, we adjusted for a possible confounding effect of gestational age at sampling and corticosteroid and antibiotics administration, in order to determine the respective effect of those covariates on serum levels of the inflammatory cytokines. Despite these strengths, an important limitation of these studies arises from the pre-analytical variability in sampling and storage procedures that has been shown to affect assayed biomarker concentrations. The best way to limit the effect of pre-analytical factors is to start sample processing immediately and to standardize it as much as possible. This approach was not, however, feasible in our studies for logistic reasons.

Study III

It is known that cytokines are fragile, that plasma and serum samples should be frozen promptly for long-term storage and that repeated freeze-thaw cycles should be avoided [336]. Some evidence indicates that a partial degradation of proteins occurs over time, even when stored at -80°C [337]. Amniotic fluid specimens are commonly frozen and stored for research purposes. During the last two decades, several studies of poor obstetric outcomes have been based on data from analysis of cytokine levels in frozen amniotic fluid [314, 338, 339]. Some studies have examined the stability of certain cytokines stored in amniotic fluid samples for prolonged periods of time [337, 340], revealing some degradation despite optimal freezing conditions. However, the impact of sample
age and storage time on levels of inflammatory biomarkers is often inadequately addressed in studies.

On the other hand, little is known about how pre-analytical handling procedures affect cytokine levels. Skogstrand et al. [341] showed that trustworthy measurements of various inflammatory proteins in serum, plasma and whole blood relies on preparation and interval before long-term storage. According to this study, 4\(^\circ\)C was the best temperature at which to preserve blood before separation into plasma and serum. It should, however, be noted that, even at this low temperature, measurable concentrations of several of the inflammatory markers increased significantly during the storage period. However, there are no data evaluating cytokine stability in amniotic fluid after different pre-analytical handling procedures. Study III showed that the investigated pre-analytical handling procedures – latency between sampling and analysis, centrifugal conditions (centrifugal force, time, and temperature), supernatant filtration and addition of protease inhibitor – did not seem to affect IL-6 concentrations in amniotic fluid.

The strength of Study III is the analysis of amniotic fluid samples after a short storage time in the freezer. Nonetheless, a limitation of the study is that only amniotic fluid from uncomplicated term pregnancies was used, instead of from second- and/or early third-trimester pregnancies, or from women with symptoms of PTL or with PPROM. Moreover, this study does not allow prediction of the degree of change in IL-6 levels in the samples after a prolonged storage time. The influence of the studied handling procedures on other amniotic fluid cytokines and the effect of storage time on the IL-6 concentrations after different pre-analytical handling procedures were not evaluated in this study and thus require investigation in future.

**Study IV**

The few studies evaluating the influence of MIAC on short-term neonatal outcome in women with PPROM have found a negative impact on neonatal morbidity [342-347]. However, a recent study showed that gestational age at delivery seems to be more important for short-term neonatal outcome than MIAC or IAI in PPROM pregnancies [348]. Furthermore, HCA and funisitis have been linked with EOS in many studies [349-353]. Both MIAC and HCA have also been associated with increased risk of EOS in pregnancies complicated by PPROM before 34 weeks of gestation [346]. It has been also shown that the
higher the stage (or grade) of either HCA or funisitis and the earlier the gestational age at delivery, the higher the incidence of neonatal morbidity [354-356].

Study IV confirmed that some adverse neonatal outcomes (EOS and ROP) are associated with HCA and funisitis in PPROM pregnancies. The results of this study are in accordance with the results of a nationwide population-based study by Morken et al. [2], based on data from the Swedish Medical Birth Register and the Swedish Hospital Discharge Register. This study showed that preterm infants were at increased risk of sepsis at gestational age 32-33 weeks (Hazard Ratio 1.58; 95% CI: 1.12, 3.10) [13].

One of the strengths of Study IV is the relatively large number of women included, with a clearly defined phenotype of exclusively PPROM-induced PTD and a well-defined classification of HCA and funisitis. The active management of women with PPROM in the Czech Republic gave us the ability to compare short-term neonatal outcome, without the influence of long latencies on the evaluation of HCA and funisitis. Another strength of this study is the fact that the same pathologist examined all placentas, thus eliminating inter-observer variability. Limitations of the study are the facts that the infectious or non-infectious nature of the HCA was confirmed with non-cultivation techniques in amniotic fluid and that not all EOS cases were culture-positive.
6. Conclusions and future perspectives

1. A combined prediction model, including non-invasive serum biomarkers and cervical length measurement by transvaginal sonography, is potentially useful for clinicians in determining which women with PTL symptoms have higher risk to spontaneously deliver preterm. This model must, however, be tested in a new cohort of high-risk women in order to confirm its predictive ability. If the moderately high positive predictive value and negative predictive value are thus confirmed, it could help identify women at considerable risk of delivering preterm, who could then be targeted for treatment (tocolytics to delay delivery, enabling transfer to a specialist unit, and corticosteroids to reduce neonatal morbidity and mortality). Likewise, women at low risk could be sent home without over-treatment.

2. The identification of inflammatory status in maternal serum in PPROM and PTL cases is not as accurate as analyzing amniotic fluid in predicting the occurrence of MIAC. A weak maternal inflammatory response in maternal serum is evident only at early gestational ages. This indicates the need for further research to establish the potential contribution of inflammatory cytokines that can be determined with non-invasive methods, in combination with those identified in amniotic fluid, to detect microbial-associated IAI in women with PPROM and PTL.

3. Amniotic fluid IL-6 levels appear to be stable regardless of sample handling prior to analysis, when analyses are performed after a short storage period.

4. HCA and funisitis in PPROM pregnancies are associated with increased rates of EOS and funisitis is associated with increased rates of EOS and ROP. From a clinical perspective, it is important that future studies clarify the role of placental pathology, MIAC, IAI, type of microorganism and gestational age for short- and long-term neonatal outcomes in spontaneous PTD with either PPROM or PTL onset.
Acknowledgements

I wish to express my sincere gratitude to:

Bo Jacobsson, my supervisor and colleague, for your never-ending enthusiasm, ideas, encouragement and support. Thank you for introducing me to the fascinating world of medical and clinical research and for staying with me throughout the whole journey! I also thank you for providing me with resources during my PhD studies and the possibility to meet so many interesting researchers during these years.

Karin Sävman, my co-supervisor and colleague, for kind support and encouragement. Designing studies with you has been a fascinating and engaging experience!

Marian Kacerovsky, my co-supervisor, for support, good advice and valuable input. Thank you for being so generous, both in research and privately, for creating such a great atmosphere and for advising me on small and big issues! Your prompt responses have been so important to me during my research journey.

Teresa Cobo, my co-supervisor, for endless support, advice and inspiring discussions, for generously sharing your extensive knowledge of preterm delivery, for practical help with research issues and, of course, for top-quality guidance in the different phases of my PhD studies.

Maria Hallingström, head of the Perinatal Laboratory, for kind and prompt support, help with practical issues and valuable input in organizing and analyzing samples and databases.

Jaroslava Johansson, colleague, friend and tutor during my residency, for listening and giving valuable support and advice. I would especially like to thank you for establishing the first contact with my research supervisor.
Rose-Marie Holst, co-author and colleague, for the enthusiastic support that you gave me during the years we worked together, both concerning research and clinical issues.

Jonas Bacelis, PhD student, for inspiring discussions and important statistical ideas and input during my studies.

Verena Sengpiel and Linda Englund-Ögge, wonderful research and clinical colleagues, for support, good advice and practical help with research issues.

All other co-authors, no one to be forgotten!

Collaborators and colleagues at the Perinatal Laboratory, for supportive talks and help with getting things done.

Ali Khatibi, friend and colleague, for good advice on small and big issues.

Andreas Fredriksson, friend and colleague, for help with the Swedish abstract of the thesis.

All friends and colleagues at the Department of Obstetrics and Gynecology, Sahlgrenska University Hospital, Gothenburg.

Vasilis, my best friend, for walking by my side all these years, encouraging and inspiring me in my life choices, and for kind and endless support whenever I needed it. Your life philosophy is going to accompany me for the rest of my life.

My parents, grandmother and brother, for always encouraging, inspiring and supporting me during my life. Thank you for making me who I am!

Dimitris, my friend and teacher, for support, advice and inspiration for my life as an adult.
References


REFERENCES

48. Mercer, B.M., et al., The Preterm Prediction Study: prediction of preterm premature rupture of membranes through clinical findings and


112. Romero, R., et al., *Infection and labor. V. Prevalence, microbiology, and clinical significance of intraamniotic infection in women with


167. Tsiartas, P., et al., The association between histological chorioamnionitis, funisitis and neonatal outcome in women with


279. Melamed, N., et al., *Is measurement of cervical length an accurate predictive tool in women with a history of preterm delivery who present
 REFERENCES


325. Hashimoto, W., et al., *Differential antitumor effects of administration of recombinant IL-18 or recombinant IL-12 are mediated primarily by


339. Bamberg, C., et al., Correlation of midtrimester amniotic fluid cytokine concentrations with adverse pregnancy outcome in terms of


Prediction of spontaneous preterm delivery in women with threatened preterm labour: a prospective cohort study of multiple proteins in maternal serum

P Tsiartas, a,b RM Holst, b UB Wennerholm, b H Hagberg, b,c DM Hougaard, d K Skogstrand, d BD Pearce, e P Thorsen, f M Kacerovsky, b,a B Jacobsson b,h

a Department of Obstetrics and Gynaecology, Papageorgiou University Hospital, Thessaloniki, Greece b Department of Obstetrics and Gynaecology, Institute for Clinical Sciences, Sahlgrenska Academy, Sahlgrenska University Hospital/Ostra, Göteborg, Sweden c Institute of Reproductive and Developmental Biology, Hammersmith Campus, Imperial College, London, UK d Department of Clinical Biochemistry and Immunology, Statens Serum Institute, Copenhagen, Denmark e Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA, USA f Department of Obstetrics and Gynaecology, Lillebaelt Hospital, Kolding, Denmark g Department of Obstetrics and Gynaecology, Charles University in Prague, Faculty of Medicine Hradec Královen, University Hospital Hradec Královen, Královy, Czech Republic h Institute of Public Health, Oslo, Norway

Correspondence: Dr P Tsiartas, Department of Obstetrics and Gynaecology, Papageorgiou University Hospital, Ring Road, Nea Efkarpia, Thessaloniki 56429, Greece. Email tsiaartspanos@gmail.com

Accepted 29 February 2012. Published Online 24 April 2012.

Objective To analyse whether specific proteins in maternal serum and cervical length, alone or in combination, can predict the likelihood that women with intact membranes with threatened preterm labour will deliver spontaneously within 7 days of sampling.

Design Cohort study.

Setting Sahlgrenska University Hospital, Gothenburg, Sweden.

Population Women at between 22 and 33 weeks of gestation with threatened preterm labour (n = 142) admitted to the Sahlgrenska University Hospital, Gothenburg, Sweden, in 1995–2005.

Methods Maternal serum was tested for 27 proteins using multiplex xMAP technology. Individual levels of each protein were compared, and calculations were performed to investigate potential associations between different proteins, cervical length and spontaneous preterm delivery. Receiver operating characteristic curves were used to find the best cut-off values for continuous variables in relation to spontaneous preterm delivery within 7 days of sampling. Prediction models were created based on a stepwise logistic regression using binary variables.

Main outcome measure Spontaneous preterm delivery within 7 days.

Results In order to determine the best prediction model, we analysed models of serum proteins alone, cervical length alone, and the combination of serum proteins and cervical length. We found one multivariable combined model through the data analysis that more accurately predicted spontaneous preterm delivery within 7 days. This model was based on serum interleukin-10 (IL-10) levels, serum RANTES levels and cervical length (sensitivity 74%, specificity 87%, positive predictive value 76%, negative predictive value 86%, likelihood ratio 5.8 and area under the curve 0.88).

Conclusions A combination of maternal serum proteins and cervical length constituted the best prediction model, and would help determine whether women with threatened preterm labour are likely to deliver within 7 days of measurement.

Keywords Maternal serum proteins, prediction, preterm delivery, xMAP technology.

Introduction

Preterm delivery (PTD) (birth before 37 weeks of gestation) remains a cause of substantial perinatal mortality and long-term morbidity. Fetal and neonatal morbidity and mortality rates are strongly associated with gestational age at birth. Specifically, infants born before 32 weeks of gestation are at risk of sequelae. In many countries, like the USA, the rates of PTD (12–13%) continue to rise. However, in Sweden, the rate has been stable at around 5.5% for the past 30 years. PTD aetiological factors operate through multiple pathophysiological pathways that overlap and include molecular factors, creating pathophysiological heterogeneity.

More than 70% of women presenting with symptoms of preterm labour (PTL) do not progress to active labour and delivery. It is essential to identify pregnant women with threatened PTL who will deliver preterm, and differentiate them from the women who will continue their pregnancies to full term. Unnecessary hospital stays and potential harmful treatments can thus be avoided, and attention can instead be focused on women who are truly at high risk of spontaneous PTD. Attempts to predict PTD based on maternal and biochemical data, and interventions to reduce PTD rates, have been largely unsuccessful. The necessity of finding reliable prediction models is urgent, and a multiple-markers test indicative of the multifactorial aetiology of PTD is likely to be more successful.

Studies indicate that an increased production of cytokines in maternal and fetal serum, and amniotic and cervical fluid, is involved in both labour at term and PTL. The study of such proteins is of great importance because the pathophysiology of PTD most likely involves the disruption of molecular networks, as opposed to an isolated abnormality in an individual gene or protein. Thus, it is important to measure multiple biomarkers simultaneously and place these into a biological context. A multiplexed sandwich immunoassay has been developed based on flowmetric xMAP technology. This technology makes it possible to analyse an array of proteins simultaneously using small sample volumes. The xMAP technology has been used to analyse multiple inflammatory markers and neurotrophins in maternal serum in women with threatened PTL.

In the current study, we aimed to test the predictive ability of 27 maternal serum proteins alone and in combination with the cervical length for delivery within 7 days of sampling in women with threatened PTL at various gestational ages, using xMAP technology.

Methods

In this prospective cohort study, we enrolled 142 healthy women without major medical problems (i.e. diabetes mellitus, hypertension or pre-eclampsia) and with singleton pregnancies presenting with threatened PTL between 22 weeks plus 0–7 days and 33 weeks plus 6–7 days of gestation at Sahlgrenska University Hospital, Gothenburg, Sweden, from 1996 to 2005. Threatened PTL was defined as regular uterine contractions (at least two uterine contractions every 10 minutes for ≥30 minutes, as confirmed by external tocometry), in combination with one of three cervical changes, documented by digital examination (1, length ≤2 cm and dilatation ≥1 cm; 2, length ≤2 cm and cervical softening; 3, dilatation ≥1 cm and cervical softening), and/or cervical length <30 mm measured by transvaginal sonography. All women had intact membranes at enrolment.

Cervical ripening was assessed by digital examination and cervical length was measured by transvaginal sonography using a standardised method. The cervical length was measured with the woman adopting the dorsal lithotomy position with an empty bladder. When the cervical canal was visualised, the probe was withdrawn to avoid pressure, distortion and elongation of the cervix. A sagittal view showing the entire cervix (endocervix and vaginal cervix), with the echogenic endocervical mucosa along the length of the cervical canal, was obtained. Calipers were used to measure the distance between the notches made by the junction of the anterior and posterior cervical walls at the internal and external os. Three measurements were performed (in millimetres), and the shortest distance was noted.

Women with preterm prelabour rupture of membranes, known uterine malformations, fetal malformations, significant vaginal bleeding, imminent delivery, cervical cerclage or fetal distress were excluded from the study. Because of limited resources, we did not recruit women during the night, at weekends, at Christmas or in the summer months, as well as during busy hours on the delivery ward, which limited the number of women enrolled in the study.

Gestational age was determined by routine ultrasound in the second trimester (at 16–19 weeks of gestation) in all women, with the exception of three in which the gestational ages were determined by the date of their last menstrual periods. Tocolytic therapy (intravenous terbutaline and/or indomethacin, the latter used at <28 weeks of gestation) was administered according to local protocol. A corticosteroid was administered (betamethasone 12 mg × 1 dose × 2 days) to stimulate fetal lung maturity.

Serum samples were drawn from the mother at the time of hospital admission, after informed consent to participate in the study was obtained. The serum samples were placed in a refrigerator (+4°C) within 10 minutes, and were processed within 6 hours before freezing (~80°C) for later analyses. Concentrations of the following markers were measured (symbols assigned by the HUGO Gene Nomenclature Committee are in parenthesis): interleukin-1β (IL1b), IL-2 (IL2), IL-4 (IL4), IL-5 (IL5), IL-6 (IL6/INFγ), IL-8 (IL8), IL-10 (IL10), IL-12 (interleukin 12A – natural killer cell
stimulatory factor 1), IL-17 (IL17A), IL-18 (IL18 – interferon-gamma-inducing factor), soluble IL-6 receptor (sIL6R), interferon-γ (IFNG), tumour necrosis factor-α (TNF, TNF superfamily, member 2), tumour necrosis factor-β (LTA – lymphotoxin alpha – TNF superfamily, member 1), monocytic chemotactic protein-1 [CCL2 – chemokine (C–C motif) ligand 2], transforming growth factor-β (TGFβ1), macrophage inflammatory protein-1α [CCL3 – chemokine (C–C motif) ligand 3], macrophage inflammatory protein-1β [CCL4 – chemokine (C–C motif) ligand 4], matrix metalloproteinase-9 (MMP9), triggering receptor expressed on myeloid cells-1 (TREM1), brain-derived neurotrophic factor (BDNF), granulocyte-macrophage-colony-stimulating factor (CSF2 – colony stimulating factor 2), neurotrophin-3 (NTF3), neurotrophin-4 (NTF4), soluble TNF receptor I (sTNFRI1), migration inhibitory factor (MIF), regulated on activation normal T-expressed and secreted RANTES [CCL5 – chemokine (C–C motif) ligand 5]. Assays of maternal serum were analysed at Statens Serum Institute, Department of Clinical Biochemistry, Denmark, using a multiplex sandwich immunoassay based on flowmetric xMAP technology, as previously described.17,18

The maternal serum was measured in duplicates in a diluted assay buffer. A sample was added to each filter plate well with a suspension of capture-antibody-conjugated beads. After an incubation of 1.5 hours the beads were washed twice and subsequently reacted for 1.5 hours with a mixture of relevant detection antibodies. Next, a quantity of streptavidin-phycocerythrin was added to the wells. Incubation then was continued for an additional 30 minutes. Finally, the beads were washed twice, re-suspended in buffer and analysed.

The detection level in the maternal serum was set as half the lowest concentrations in the working range:17 IL-1β (40 pg/ml), IL-2 (4 pg/ml), IL-4 (4 pg/ml), IL-5 (4 pg/ml), IL-6 (40 pg/ml), IL-8 (40 pg/ml), IL-10 (10 pg/ml), IL-12 (4 pg/ml), IL-17 (4 pg/ml), IL-18 (40 pg/ml), sIL-6Rα (2500 pg/ml), IFN-γ (4 pg/ml), TNF-α (4 pg/ml), TNF-β (4 pg/ml), MCP-1 (156 pg/ml), TGF-β (4 pg/ml), MIP-1α (40 pg/ml), MIP-1β (40 pg/ml), MMP-9 (5000 pg/ml), TREM-1 (100 pg/ml), BDNF (10 pg/ml), GM-CSF (4 pg/ml), NT-3 (40 pg/ml), NT-4 (4 pg/ml), soluble TNF RI (156 pg/ml), MIF (100 pg/ml) and RANTES (40 pg/ml). The staff member who performed the analyses (K.S.) did not have any clinical information on the measure of outcome.

The study was approved by the local ethics committee at the University of Gothenburg (nos 349-95, 476-05).

Statistical analysis

Women who did and did not deliver within 7 days were compared using a Mann–Whitney U-test for continuous variables and Fisher’s exact test for dichotomous variables. Receiver operating characteristic (ROC) curves and the area under the curve (AUC) were computed for each protein, cervical length and the background variables for the prediction of delivery within 7 days. The best cut-off values for every protein and cervical length were determined with SPSS 17.0 and Medcalc 11.0.1.0 for ROC curve analysis, based mainly on the calculation of the highest summary of sensitivity plus specificity for each coordinate point in the ROC curve, but at the same time a higher weight was attributed to the sensitivity for the determination of the best cut-off point for every variable. Continuous variables were dichotomised from the ROC curve to obtain the optimal prediction of delivery within 7 days. Crosstabs were used to obtain odds ratios (ORs). The significant variables in univariate analyses (P < 0.05) were entered into a backward stepwise logistic regression. We used 0.05 as our limit in the univariate analyses in order to avoid the problem of mass significance and the use of a large number of predictors exceeding 10. Choosing a different limit than 0.05, for example 0.25, would result in the inclusion of 20 variables as possible predictors in the stepwise selection, which would not be appropriate to common statistical standards. All analyses were performed for each serum marker, the cervical length and the combinations. We also used a Hosmer and Lemeshow test for the goodness of fit of the models. A P value of less than 0.05 or a 95% confidence interval (95% CI) that did not include 1.0 was considered to be statistically significant. All analyses were computed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) and Medcalc 11.0.1.0 (MedCalc Software, Mariakerke, Belgium).

Results

In our cohort of 142 women with singleton pregnancies, 40% (57/142) delivered within 7 days. Demographic characteristics are presented in Table 1. Women who delivered within 7 days of sampling did not differ from those who delivered later with regard to the number of previous PTDs, body mass index (BMI), smoking status or gestational age at study inclusion. The women who delivered within 7 days were more often nulliparous (70%) than women who delivered later (56%).

The median concentrations of all proteins in maternal serum and the cervical length in relation to delivery within 7 and after 7 days, together with the P values, are presented in Table 2. Only proteins with detectable maternal serum levels in more than 50% of the samples were included in the analyses. Thus, IL-1β, IL-2, IL-5, IL-6 and IL-8 were excluded from further analyses.

Women who delivered within 7 days had significantly higher levels of IL-10, soluble IL-6Rα, TNF-β, MIP-1β, MMP-9, BDNF, soluble TNF receptor I, MIF and RANTES than did the women who delivered later (Table 2). The highest values of AUC were detected for IL-10 (AUC = 0.69, P < 0.001), MMP-9 (AUC = 0.74, P < 0.001), MIF
Table 1. Clinical background variables in women delivering within or after 7 days of assessment

<table>
<thead>
<tr>
<th>Variable</th>
<th>Women delivering ≤7 days n = 57</th>
<th>Women delivering &gt;7 days n = 85</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years, median, Q1–Q3)</td>
<td>30.5 (28–33.5)</td>
<td>29 (24–33)</td>
<td>0.190</td>
</tr>
<tr>
<td>Nulliparous (n = 88) (61.9%)</td>
<td>40 (70%)</td>
<td>48 (56%)</td>
<td>0.079</td>
</tr>
<tr>
<td>Gestational age at sampling (weeks + days, median, range)</td>
<td>30 + 0 (22 + 3 to 33 + 5)</td>
<td>30 + 5 (22 + 2 to 33 + 5)</td>
<td>0.077</td>
</tr>
<tr>
<td>Gestational age at delivery (weeks + days, median, range)</td>
<td>30 + 5 (23 + 2 to 34 + 0)</td>
<td>36 + 6 (24 + 2 to 43 + 0)</td>
<td>0.000</td>
</tr>
<tr>
<td>Latency in days (median, range, Q1–Q3)</td>
<td>2 (0–7, 1–3.5)</td>
<td>42 (8–125, 23–61.5)</td>
<td>0.000</td>
</tr>
<tr>
<td>Previous spontaneous preterm delivery (n = 27) (19%)</td>
<td>8 (14%)</td>
<td>19 (22%)</td>
<td>0.278</td>
</tr>
<tr>
<td>Smoking at the beginning of pregnancy (n = 22) (15.4%)</td>
<td>8 (14%)</td>
<td>14 (16.4%)</td>
<td>0.815</td>
</tr>
<tr>
<td>BMI at the beginning of pregnancy (median, Q1–Q3)</td>
<td>22 (20–24)</td>
<td>21 (20–26)</td>
<td>0.787</td>
</tr>
</tbody>
</table>

P value calculated by a Mann–Whitney U-test or a Fisher's exact test. Q1–Q3 represents the values of the 25th and 75th percentiles. Significant results are marked in bold.

Table 2. Levels of cytokines and neuropeptides (median, Q1–Q3, 25th and 75th percentiles, pg/ml) in maternal serum and the cervical length (median, Q1–Q3, 25th and 75th percentiles, mm) measured at sampling, in relation to delivery within 7 days.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Delivery within 7 days median, (Q1–Q3) (n = 57)</th>
<th>Delivery after 7 days median, (Q1–Q3) (n = 85)</th>
<th>% above detection limit</th>
<th>P1</th>
<th>AUC</th>
<th>Cut-off value</th>
<th>% above cut-off</th>
<th>P2</th>
<th>Delivery within 7 days crude OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>5 (4–7)</td>
<td>5 (4–7)</td>
<td>59</td>
<td>0.827</td>
<td>0.48</td>
<td>1 pg/ml</td>
<td>47</td>
<td>0.9 (0.4–1.7)</td>
<td>0.864</td>
</tr>
<tr>
<td>IL-10</td>
<td>93 (38.5–176.5)</td>
<td>30 (4–71.5)</td>
<td>74</td>
<td>&lt;0.001</td>
<td>0.69</td>
<td>48 pg/ml</td>
<td>50</td>
<td>4.6 (2.2–9.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-12</td>
<td>11 (4–25)</td>
<td>13 (4.5–32.5)</td>
<td>74</td>
<td>0.357</td>
<td>0.45</td>
<td>19 pg/ml</td>
<td>35</td>
<td>0.6 (0.3–1.3)</td>
<td>0.288</td>
</tr>
<tr>
<td>TNFα</td>
<td>18 (4–51)</td>
<td>14 (4–39)</td>
<td>61</td>
<td>0.331</td>
<td>0.54</td>
<td>34 pg/ml</td>
<td>32</td>
<td>1.8 (0.6–3.7)</td>
<td>0.104</td>
</tr>
<tr>
<td>IL-1B</td>
<td>389 (242–528)</td>
<td>353 (264–484.5)</td>
<td>100</td>
<td>0.950</td>
<td>0.50</td>
<td>268 pg/ml</td>
<td>69</td>
<td>0.5 (0.2–1.1)</td>
<td>0.139</td>
</tr>
<tr>
<td>Soluble</td>
<td>26 545</td>
<td>24 941</td>
<td>100</td>
<td>0.050</td>
<td>0.59</td>
<td>22 576 pg/ml</td>
<td>64</td>
<td>2.7 (1.2–5.8)</td>
<td>0.008</td>
</tr>
<tr>
<td>IL-6Rα</td>
<td>(23 000.5–38912.5)</td>
<td>(18 879.5–31 172.5)</td>
<td>100</td>
<td>0.001</td>
<td>0.74</td>
<td>749 pg/ml</td>
<td>69</td>
<td>3.7 (1.6–8.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>12 (4–34.5)</td>
<td>10 (4–36.5)</td>
<td>63</td>
<td>0.336</td>
<td>0.54</td>
<td>10 pg/ml</td>
<td>50</td>
<td>1.6 (0.8–3.1)</td>
<td>0.175</td>
</tr>
<tr>
<td>TNF-α</td>
<td>18 (4–27)</td>
<td>14 (3–33.5)</td>
<td>72</td>
<td>0.920</td>
<td>0.50</td>
<td>17 pg/ml</td>
<td>43</td>
<td>1.6 (0.8–3.2)</td>
<td>0.171</td>
</tr>
<tr>
<td>TNF-β</td>
<td>64 (19–99)</td>
<td>45 (19.5–68)</td>
<td>100</td>
<td>0.043</td>
<td>0.60</td>
<td>68 pg/ml</td>
<td>33</td>
<td>2.9 (1.4–6)</td>
<td>0.004</td>
</tr>
<tr>
<td>MCP-1</td>
<td>176 (10–414.5)</td>
<td>178 (58–433)</td>
<td>57</td>
<td>0.699</td>
<td>0.48</td>
<td>25 pg/ml</td>
<td>76</td>
<td>0.5 (0.2–1)</td>
<td>0.108</td>
</tr>
<tr>
<td>TGFβ</td>
<td>106 (43–188)</td>
<td>89 (40–161.5)</td>
<td>100</td>
<td>0.217</td>
<td>0.56</td>
<td>111 pg/ml</td>
<td>39</td>
<td>1.7 (0.8–3.4)</td>
<td>0.120</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>157 (98–274)</td>
<td>141 (92.5–229.5)</td>
<td>95</td>
<td>0.379</td>
<td>0.54</td>
<td>197 pg/ml</td>
<td>33</td>
<td>1.7 (0.8–3.4)</td>
<td>0.149</td>
</tr>
<tr>
<td>MMP-9</td>
<td>542 061</td>
<td>301 680</td>
<td>100</td>
<td>&lt;0.001</td>
<td>0.74</td>
<td>469 645 pg/ml</td>
<td>41</td>
<td>6.0 (2.9–12.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TREM-1</td>
<td>1525 (517.0–2563.5)</td>
<td>1204 (610–2894)</td>
<td>100</td>
<td>0.773</td>
<td>0.48</td>
<td>1472 pg/ml</td>
<td>46</td>
<td>1.5 (0.7–2.9)</td>
<td>0.236</td>
</tr>
<tr>
<td>BDNF</td>
<td>5483 (3969.5–8195.0)</td>
<td>4581 (3317.0–6156.5)</td>
<td>100</td>
<td>0.000</td>
<td>0.63</td>
<td>6950 pg/ml</td>
<td>25</td>
<td>3.7 (1.6–8.2)</td>
<td>0.001</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>13 (10–36)</td>
<td>24 (10–53)</td>
<td>100</td>
<td>0.042</td>
<td>0.40</td>
<td>21 pg/ml</td>
<td>55</td>
<td>0.4 (0.2–0.8)</td>
<td>0.016</td>
</tr>
<tr>
<td>NT-4</td>
<td>10 (4–19)</td>
<td>12 (4–25)</td>
<td>65</td>
<td>0.382</td>
<td>0.45</td>
<td>19 pg/ml</td>
<td>29</td>
<td>0.4 (0.2–1)</td>
<td>0.091</td>
</tr>
<tr>
<td>NT-3</td>
<td>82 (37.5–178.5)</td>
<td>52 (10–144)</td>
<td>66</td>
<td>0.076</td>
<td>0.58</td>
<td>60 pg/ml</td>
<td>54</td>
<td>2.3 (1.1–4.7)</td>
<td>0.017</td>
</tr>
<tr>
<td>Soluble</td>
<td>1129 (790.5–1129.0)</td>
<td>902 (622.5–1253.5)</td>
<td>99</td>
<td>0.004</td>
<td>0.64</td>
<td>749 pg/ml</td>
<td>69</td>
<td>3.7 (1.6–8.5)</td>
<td>0.002</td>
</tr>
<tr>
<td>TNFRI</td>
<td>56 320</td>
<td>21934 (4806–43 160)</td>
<td>95</td>
<td>&lt;0.001</td>
<td>0.71</td>
<td>45 695 pg/ml</td>
<td>35</td>
<td>4.7 (2.2–9.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RANTES</td>
<td>42 013</td>
<td>38 504</td>
<td>100</td>
<td>0.018</td>
<td>0.61</td>
<td>49 293 pg/ml</td>
<td>27</td>
<td>3.9 (1.8–8.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cervical length</td>
<td>5.5 (0–15)</td>
<td>21 (15–26)</td>
<td>–</td>
<td>&lt;0.001</td>
<td>0.77</td>
<td>18 mm</td>
<td>57</td>
<td>21.2 (6.0–74.7)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Predicting spontaneous preterm delivery

© 2012 The Authors BJOG An International Journal of Obstetrics and Gynaecology © 2012 RCOG 869
Based on a stepwise multivariable logistic regression of dichotomous variables, we first analysed the most significant serum proteins from the univariate analyses alone, then cervical length alone and, lastly, the combination of serum proteins along with cervical length to construct the best prediction model. We found that the combined model with proteins and cervical length had the best prediction capacity (Tables 3 and 4). In the combined multivariable model of maternal serum proteins and cervical length, high levels of IL-10 (≥48 pg/ml; OR 5.0, 95% CI 1.7–14.3), RANTES (≥49293 pg/ml; OR 9.9, 95% CI 2.5–39.2) and short cervical length (≤18 mm; OR 43.5, 95% CI 8.9–211.3) were statistically significant contributions to the prediction of spontaneous PTD within 7 days of sampling (Table 3). Using this combined model, the ROC curve (Figure 1) had an AUC of 0.88, sensitivity 74%, specificity 87%, positive predictive value 76%, negative predictive value 86%, positive likelihood ratio 5.83 and negative likelihood ratio 0.30 (Table 3).

**Discussion**

The necessity of finding good prediction models for imminent PTD is urgent, and a multiple-markers test indicative of the multifactorial aetiology of PTD is likely to be the most successful. Our best multivariable prediction model was based on maternal serum IL-10, RANTES and cervical length, and demonstrated a good capacity for the prediction of delivery within 7 days from the beginning of labour.

Few studies have investigated the relationship between inflammatory cytokines in the maternal serum and spontaneous PTD in women before and during PTL. Vogel et al. developed a combined prediction model using both serum and vaginal inflammatory markers in the early second trimester (at 12–25 weeks of gestation) in women with a prior PTD and without symptoms at enrolment: this model predicted 69% of all recurrent spontaneous PTD at a 5% false-positive rate. In a previous study, we analysed the same 27 proteins from amniotic and cervical fluid in 89 women, and found one combined multivariable prediction model based on amniotic macrophage inflammatory protein-1beta, cervical interferon-gamma and monocyte chemotactic protein-1. We showed that a combination of proteins from amniotic fluid and cervical fluid could help determine which women will deliver within 7 days. In the present study we used maternal serum, which is easier to collect and less invasive for the patient compared with amniotic fluid and cervical fluid.

Transvaginal cervical length measurement is commonly used to predict PTD in women with threatened PTL. A cervical length measurement of 25 mm or less is generally considered...
The fetal fibronectin test is also used in predicting spontaneous PTD within 7–10 days of testing among women with symptoms of threatened PTD. Several studies have reported that the fetal fibronectin test and cervical length measurement provided similar results in predicting the risk of PTD. However, it remains unclear whether the combination of the fetal fibronectin test and cervical length measurement improves the prediction of PTD, and how these tests should be combined.

In our multivariable prediction model, levels of maternal serum IL-10 were significantly higher in women who delivered preterm (within 7 days of sampling) compared with women who delivered later. Using explant cultures of gestational membranes, Mitchell et al. found that IL-10 exerts anti-inflammatory effects on choriodecidua, but that in the adjacent amnion it has a remarkable pro-inflammatory action. These findings suggest that the fetal membranes can exhibit opposing responses to IL-10, depending on whether the inflammation occurs at the maternal or fetal surface. Aaltonen et al. also found that in vitro stimulation of choriodecidual tissue with high levels of *Ureaplasma urealyticum* induced an inflammatory response, followed by the secretion of IL-10.

Another cytokine in our prediction model was RANTES. We found that levels of maternal serum RANTES were statistically higher in women who delivered preterm (within 7 days of sampling), compared with women who delivered later. However, there are few studies describing the association between RANTES in maternal serum and the PTD. Chow et al. showed that RANTES levels in maternal serum were elevated in the second trimester in asymptomatic women. Using a murine model, Yang et al. also showed that the production of several pro-inflammatory cytokines, including RANTES, occurred very early in the cascade of events that lead to PTL.

The interval of 7 days was chosen to represent an average number of days of hospital stay and monitoring of such women with symptoms of preterm labour. During this period of time the clinician has the possibility to admit the woman, provide corticosteroids and tocolytics, and let her deliver.

**Conclusion**

In our study population of women with threatened PTL, we defined a combined prediction model that uses small serum volumes and cervical length measurements. This model has the potential to be useful to clinicians to determine which symptomatic women will deliver preterm. This model would, however, need to be tested in a new cohort of high-risk women in order to confirm its predictive ability. If such studies confirm the moderately high positive predictive value and negative predictive value, this could help to identify women with a considerable risk of delivering preterm, who can be targeted for treatment, and likewise women who are at low risk that could be sent home without treatment. A non-invasive, low-cost test with a high positive predictive value could assist in the timing of corticosteroid administration, for example. On the market today, there are tests with high negative predictive values that make it possible to refrain from hospitalisation, bed rest and repeated monitoring; however, none are available with a high positive predictive value. Furthermore, improved tests with high positive predictive value can help to target novel treatment for high-risk groups in clinical trials, as we have so far not been able to improve the overall neonatal outcome in this group of women with symptoms of threatened PTL.

**Disclosure of interests**

All authors declare that there are no conflicts of interest to disclose.

**Contribution to authorship**

The contributions of individual authors to this paper are as follows: planning research (PT and BJ); performing
research (PT, BJ, R-MH, HH, UBW); analysing data (PT, BJ, KS, DH, PTH, MK); interpreting data (PT, BJ, BP, KS, DH, PTH, MK); and writing the first draft of the article (PT and BJ).

Details of ethics approval
The study was approved by the local ethics committee at the University of Gothenburg (nos 349-95 and 476-05). All patients gave informed consent before enrolment.

Funding
This work was supported by grants from the Swedish Medical Society (SLS 2008–21198), Swedish government grants to researchers in the public health service (ALFGBG-2863 and ALFGBG-11522) and Swedish Medical Research Council (VR 2006–3396), NIMH grant 5R21-MH68513-3 and by and ALFGBG-11522) and Swedish Medical Research Council to researchers in the public health service (ALFGBG-2863 and 476-05). All

Details of ethics approval
The study was approved by the local ethics committee at the University of Gothenburg (nos 349-95 and 476-05). All patients gave informed consent before enrolment.

Acknowledgement
We thank Mattias Molin for his assistance with the statistical analyses.

References
Maternal inflammatory response to microbial invasion of the amniotic cavity: analyses of multiple proteins in the maternal serum

TERESA COBO1, PANAGIOTIS TSIARTAS2,3, MARIAN KACEROVSKY4, ROSE-MARIE HOLST3, DAVID M. HOUGAARD5, KRISTIN SKOGSTRAND5, ULLA-BRITT WENNERHOLM3, HENRIK HAGBERG3 & BO JACOBSSON3,6

1Maternal Fetal Medicine Department, Hospital Clinic, Institute for Biomedical Investigations August Pi i Sunyer (IDIBAPS), University of Barcelona, Barcelona, Spain, 2Department of Obstetrics and Gynecology, Papageorgiou Hospital, Thessaloniki, Greece, 3Department of Obstetrics and Gynecology, Sahlgrenska University Hospital, Gothenburg, Sweden, 4Department of Obstetrics and Gynecology, University Hospital, Hradec Kralove, Czech Republic, 5Department of Clinical Biochemistry and Immunology, Statens Serum Institut, Copenhagen, Denmark, and 6Institute of Public Health, Oslo, Norway

Key words
Maternal serum inflammation, gestational age, microbial invasion of the amniotic cavity, preterm prelabor rupture of membranes, preterm labor

Correspondence
Dr Teresa Cobo, Prematurity Unit, Department of Maternal-Fetal Medicine, Hospital Clinic, Sabino de Arana 1, Barcelona, Barcelona 08028, Spain. E-mail: tcobo@clinic.ub.es

Conflict of interest
The authors have stated explicitly that there are no conflicts of interest in connection with this article.


Received: 8 February 2012 Accepted: 1 October 2012
DOI: 10.1111/aogs.12028

Abstract
Objective. To evaluate the maternal inflammatory response to microbial invasion of the amniotic cavity (MIAC) in women with preterm labor and preterm prelabor rupture of membranes using selected proteins in the maternal serum. Design. A prospective cohort study. Setting. Labor ward from Salgrensga University Hospital. The evaluation of the maternal inflammatory response in the presence of MIAC in preterm labor and preterm prelabor rupture of membranes. Population. One hundred and sixteen women with preterm labor and 73 women with preterm prelabor rupture of membranes between the gestational ages of 22+0 and 33+6 weeks. Methods. Twenty-seven maternal serum proteins were assayed by a multiple immunoassay. Main outcome measures. The maternal serum inflammatory response was evaluated according to the presence of MIAC. Data were stratified by gestational age. Results. There were few differences in the maternal serum protein levels when MIAC was present in both preterm labor and preterm prelabor rupture of membranes. In preterm prelabor rupture of membranes, higher levels of interleukin-18 (median 654 vs. 361 pg/mL, \( p = 0.003 \)) and lower levels of interleukin-1 \( \beta \) (9.5 vs. 19.9 pg/mL, \( p = 0.008 \)) and monocyte chemotactic protein-1 (139.1 vs. 212.6 pg/mL, \( p = 0.039 \)) were observed in women with MIAC. Interleukin-6 (20.8 vs. 13.9 pg/mL, \( p = 0.019 \)) was the only biomarker that increased significantly in preterm labor complicated with MIAC. All of the differences between preterm labor and preterm prelabor rupture of membranes were observed at less than 32+0 weeks of gestation. Conclusions. A weak maternal inflammatory response in the serum was observed in women with MIAC.

Abbreviations: BDNF, brain-derived neurotrophic factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN\( \gamma \), interferon-\( \gamma \); IL, interleukin; MCP-1, monocyte chemotactic protein-1; MIAC, microbial invasion of the amniotic cavity; MIF, migration inhibiting factor; MIP, macrophage inflammatory protein; MMP-9, matrix metalloproteinase-9; NT3, neurtropin-3; NT4, neurtropin-4; PPROM, preterm prelabor rupture of membranes; PTL, preterm labor; RANTES, regulated on activation normal T-expressed and secreted proteins; sIL6r, soluble IL-6 receptor; sTNFR1, soluble tumor necrosis factor receptor 1; TGF-\( \beta \), transforming growth factor-\( \beta \); TNF, tumor necrosis factor; TREM-1, triggering receptor expressed on myeloid cells-1.
Introduction

Intra-amniotic inflammation has been proposed to be the strongest predictor of microbial invasion of the amniotic cavity (MIAC), and it has an important role in the occurrence of neonatal morbidity and the pathogenesis of spontaneous preterm delivery, including preterm prelabor rupture of membranes (PPROM) and preterm labor (PTL; 1).

Several inflammatory biomarkers have been reported as predictors of MIAC in amniotic fluid (2,3); however, there are few studies on the role of these inflammatory cytokines as markers of infection in non-invasive samples [e.g. the cervix (4–7) or the maternal serum (8,9)]. Maternal serum interleukin (IL)-6 was previously reported to be a predictor of MIAC and infectious and non-infectious neonatal morbidity (8,10,11). Thus, Gravett et al. (9) suggested that selected proteomic biomarkers, such as calgranulin B and insulin-like growth factor binding protein-1 (IGFBP-1), may be detectable in the maternal serum of women affected by MIAC.

The prediction of MIAC using a non-invasive approach is relevant to clinical practice because of the feasibility and easy accessibility. We hypothesized that there is a different expression pattern of maternal cytokines as a result of MIAC in women with PPROM and PTL as determined by a multiple immunoassay, the Flowmetric Luminex xMAP Technology (2,12–15). This novel technological approach provides precise and accurate detection of the inflammatory response, with the capability to measure a broad panel of proteins simultaneously in invasive (amniocentesis) and non-invasive samples (maternal serum samples).

The aim of this study was to evaluate the differences in the expression of a broad spectrum of proteins in the maternal serum in women with PPROM and PTL with or without MIAC. The findings from this study may help to elucidate the maternal inflammatory response to the presence of micro-organisms in amniotic fluid and determine whether the identification of a higher inflammatory response in non-invasive samples accurately reflects the status of the amniotic cavity in pregnancies complicated with PPROM and PTL.

Material and methods

A prospective cohort study was conducted on pregnant women with a diagnosis of PPROM and PTL who were admitted to the Department of Obstetrics and Gynecology at Sahlgrenska University Hospital in Gothenburg, Sweden between 1996 and 2005. Gestational age was established according to a departmental protocol. Non-antenatal antibiotic treatment was used only if the uterine contractions were identified according to a departmental protocol. Non-antenatal antibiotic treatment was used except in women with clinical symptoms of chorioamnionitis.

Ultrasound-guided transabdominal amniocentesis was performed in antiseptic conditions within 24 h of admission, and 30–50 mL of amniotic fluid was aspirated. After sampling, the amniotic fluid was immediately placed in a refrigerator (4°C) and processed within five hours. The samples were refrigerated at 855°C until analysis. A sample of uncentrifuged amniotic fluid was immediately transported to the microbiological laboratory for PCR analysis of Ureaplasma urealyticum and Mycoplasma hominis and for aerobic and anaerobic culture. The results were available for clinical management.

Microbial invasion of the amniotic cavity was defined as a positive PCR for genital mycoplasmas and/or a positive amniotic fluid culture; however, coagulase-negative Staphylococcus was considered to be a skin contamination, except for women with high levels of amniotic fluid IL-6. Blood samples were obtained simultaneously to amniocentesis sampling and
were centrifuged and were frozen immediately at −70°C until analysis.

Informed consent was obtained from all of the women. The regional ethical committee approved the collection and use of the samples and information for research purposes (Dnr 349-95/Ö 506-99/Dnr 476-05).

Maternal serum analyses

The maternal serum IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, IL-18, soluble IL-6 receptor (sIL6r), interferon-γ (IFNγ), tumor necrosis factor (TNF)-α, TNF-β, monocyte chemotactic protein-1 (MCP-1), transforming growth factor-β (TGF-β), macrophage inflammatory protein-1α (MIP-1α), MIP-1β, matrix metalloproteinase-9 (MMP-9), triggering receptor expressed on myeloid cells-1 (TREM-1), brain-derived neurotrophic factor (BDNF), granulocyte macrophage colony-stimulating factor (GM-CSF), neutropin-3 (NT3), neutropin-4 (NT4), soluble TNF receptor 1 (sTNFR1), migration inhibiting factor (MIF) and regulated on activation normal T-expressed and secreted (RANTES) proteins were analysed at the Statens Serum Institute (Department of Clinical Biochemistry and Immunology, Copenhagen, Denmark) using a multiple sandwich immunoassay based on Flowmetric Luminex xMAP Technology according to the previously published workflow (2,12–15). The samples of maternal serum were diluted 1:10 in extraction buffer (phosphate-buffered saline containing Complete protease inhibitor cocktail with EDTA (Roche, Basel, Switzerland; one tablet dissolved per 25 mL of assay buffer, phosphate-buffered saline containing 5 mL/L Tween 20 and 10 g/L bovine serum albumin) and measured in duplicate. Calibration curves were prepared in a 1:1 mixture of guinea pig serum (Jackson ImmunoResearch, www.jacksonimmuno.com) and pig serum (Dako, www.dako.com). The means of the intra-assay and interassay coefficients of variation were 11 and 17%, respectively. The working ranges in serum are described by Skogstrand et al. (15). Values below the lowest concentrations in the working ranges were set as half the lowest concentrations as described by Skogstrand et al. (14). The analyses were performed by an investigator who was blinded to the clinical status of the women. Two proteins (IL-2 and IL-5) were present at undetectable serum levels in more than 50% of the samples and were subsequently excluded from further analyses.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 19.0 for Windows XP OS (IBM Corporation, Armonk, NY, USA). The demographic and clinical characteristics were compared using the nonparametric Mann–Whitney U-test and are presented as the medians (range). The categorical variables were compared using Fisher’s exact test and are presented as the number (%). The differences were considered statistically significant at a confidence level of \( p < 0.05 \) with two-sided alternative hypotheses.

Results

Between 1996 and 2005, a total of 189 women with a diagnosis of PPROM (\( n = 73 \)) and PTL (\( n = 116 \)) between 22\(^{+6}\) and 33\(^{+6}\) weeks of gestation who were admitted to the Department of Obstetrics and Gynecology of Salgrenska University Hospital in Gothenburg, Sweden and who met the inclusion criteria were included in the study. The gestational age (median) at sampling and delivery were 32\(^{+6}\) (range 22\(^{+6}\)–33\(^{+6}\)) and 34\(^{+5}\) weeks (range 32\(^{+1}\)–43\(^{+6}\)), respectively. The overall rate of MIAC was 29% (21 of 73) in women complicated with PPROM and 19% (22 of 116) in women with PTL.

The maternal characteristics according the presence of MIAC in the group of women with PPROM and PTL are summarised in Tables 1 and 2, respectively. The latency from sampling to delivery was significantly lower in women with MIAC. No differences were observed in relation to the presence or absence of MIAC regarding the latency period from sampling to delivery to multiple immunoassay.

Women with PPROM and PTL were evaluated separately in two different groups. In both groups, the data were classified according to the whole study population group (all PPROM and PTL patients), women with a gestational age at admission of <32\(^{+6}\) weeks and women with a gestational age at admission of ≥32\(^{+6}\) weeks. A comparison of the levels of maternal serum cytokines and neuropeptides in all of the subgroups is shown in Tables 3 and 4.

High levels of maternal serum IL-18 and low levels of MCP-1 were observed in the group of women with PPROM who had MIAC. In the subgroup of women with PPROM below 32\(^{+6}\) weeks, the maternal serum IL-18 levels were significantly higher, and the levels of IL-1β were significantly lower in women with MIAC in comparison to those without. No significant association was observed between MIAC and any of the cytokines over 32\(^{+6}\) weeks of gestation (Table 3).

In the group of women with PTL, there was no significant difference in the inflammatory response in those complicated with MIAC. Only IL-6 was significantly higher in the subgroup of women with PTL at <32\(^{+6}\) weeks of gestation. No significant association was observed with any cytokine over 32\(^{+6}\) weeks of gestation (Table 4).

Discussion

The maternal serum inflammatory response in women with subclinical MIAC was weak. The differences in the expression of maternal serum biomarkers were observed only at early gestational ages. There was no maternal inflammatory response due to the presence of infection over 32\(^{+6}\) weeks of gestation in women with PPROM or PTL.
In contrast to the strong evidence that the presence of subclinical MIAC activates an inflammatory response in amniotic fluid (16–19), the maternal response in serum was not pronounced. According to our data, only IL-18, MCP-1, IL-1β and IL-6 presented different expression levels in the presence of MIAC in women with PPROM.

Interleukin-18 has been recognized as an important regulator of innate and acquired immune responses, and it is expressed in autoimmune diseases, a variety of cancers and numerous infectious diseases (20–22). There have been several studies on the role of IL-18 in pregnancy and the expression of this cytokine in amniotic fluid. Interleukin-18 has been demonstrated to have a pro-inflammatory effect in the presence of MIAC and a protective effect against preterm delivery when it is overexpressed in amniotic fluid (2,4). Studies on the effect of IL-18 on preterm delivery have indicated that low levels of IL-18 in the maternal serum were associated with a longer interval to delivery (23). There have been no previous studies on the role of maternal serum IL-18 in women with MIAC. According to our data, higher levels of maternal serum IL-18 were present in women with PPROM who had MIAC than in women without MIAC, which suggests a pro-inflammatory effect of IL-18 in amniotic fluid and in maternal serum in the presence of infection.

Regarding the expression of other cytokines and chemokines in the maternal serum, low levels of MCP-1 and IL-1β were observed in women with PPROM who had MIAC. Contrasting results have been reported on the levels of MCP-1 and IL-1β in the amniotic fluid compartment (24–26) of women with PPROM and PTL complicated with MIAC. In non-invasive samples, Torbé et al. (27) suggested a relation between high levels of IL-1β in the maternal serum and the presence of histological chorioamnionitis and the occurrence of early onset neonatal sepsis in women with PTL. Likewise, maternal serum MCP-1 has been considered to be a chemokine that is elevated in pro-inflammatory

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Presence of microbial invasion of the amniotic cavity (n = 21)</th>
<th>Absence of microbial invasion of the amniotic cavity (n = 52)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>28 (20–34)</td>
<td>28 (19–42)</td>
<td>0.691</td>
</tr>
<tr>
<td>Nulliparity</td>
<td>11 (52)</td>
<td>34 (65)</td>
<td>0.597</td>
</tr>
<tr>
<td>Smoking</td>
<td>7 (33)</td>
<td>12 (23)</td>
<td>0.226</td>
</tr>
<tr>
<td>Prior preterm delivery</td>
<td>2 (9)</td>
<td>4 (8)</td>
<td>0.631</td>
</tr>
<tr>
<td>Corticosteroids prior to amniocentesis</td>
<td>11 (52)</td>
<td>40 (77)</td>
<td>0.812</td>
</tr>
<tr>
<td>Gestational age at sampling (weeks)</td>
<td>32 ± 2 (32 ± 33 ± 3)</td>
<td>32 ± 2 (23 ± 33 ± 3)</td>
<td>0.590</td>
</tr>
<tr>
<td>latency from sampling to delivery (days)</td>
<td>3 (1–9)</td>
<td>11 (0–117)</td>
<td>0.001</td>
</tr>
<tr>
<td>Gestational age at delivery (weeks)</td>
<td>33 ± 2 (32 ± 33 ± 3)</td>
<td>33 ± 2 (31 ± 41 ± 3)</td>
<td>0.069</td>
</tr>
</tbody>
</table>

Note: The continuous variables were compared using a nonparametric Mann–Whitney U-test [presented as the range]. The categorical variables were compared using Fisher’s exact test and are presented as the number (%).
T. Cobo et al.

Overall study population (22–31 weeks) = 18) amniotic cavity (n = pn = 30)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Micr. of the amniotic cavity (n = 18)</th>
<th>Absence of micr. of the amniotic cavity (n = 55)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>16 (4–294.9)</td>
<td>20 (4–294.9)</td>
<td>0.008</td>
</tr>
<tr>
<td>IL-4</td>
<td>5 (3.8–8.6)</td>
<td>6 (4–24.1)</td>
<td>0.307</td>
</tr>
<tr>
<td>IL-6</td>
<td>19 (3.9–10.62)</td>
<td>19 (4–10.62)</td>
<td>0.161</td>
</tr>
<tr>
<td>IL-8</td>
<td>4 (3.6–8.1)</td>
<td>4 (19–13.5)</td>
<td>0.386</td>
</tr>
<tr>
<td>IL-10</td>
<td>31 (4–229.6)</td>
<td>24 (7–36.2)</td>
<td>0.143</td>
</tr>
<tr>
<td>IL-12</td>
<td>11 (2.5–20.2)</td>
<td>20 (5.9–20.2)</td>
<td>0.259</td>
</tr>
<tr>
<td>IL-17</td>
<td>3 (6.6–11.62)</td>
<td>2 (8.9–78.1)</td>
<td>0.254</td>
</tr>
<tr>
<td>IL-18</td>
<td>451 (1884–1054.5)</td>
<td>335 (763–13380)</td>
<td>0.031</td>
</tr>
<tr>
<td>IL-19</td>
<td>25 870 (1883.4–467 175.8)</td>
<td>25 245 (12 527.5–47 507.7)</td>
<td>0.888</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>4 (4–53)</td>
<td>6 (4–484.1)</td>
<td>0.709</td>
</tr>
<tr>
<td>TNF-α</td>
<td>16 (4–296.8)</td>
<td>18 (3.6–219.4)</td>
<td>0.484</td>
</tr>
<tr>
<td>TNF-β</td>
<td>45 (10–148.9)</td>
<td>44 (9.9–163.1)</td>
<td>0.939</td>
</tr>
<tr>
<td>MCP-1</td>
<td>139 (10–477.4)</td>
<td>213 (10–4037.6)</td>
<td>0.039</td>
</tr>
<tr>
<td>TGF-β</td>
<td>76 (6–40 1170.9)</td>
<td>111 (40–522.9)</td>
<td>0.538</td>
</tr>
<tr>
<td>MMP-1</td>
<td>129 (237–236.1)</td>
<td>138.5 (10–715.2)</td>
<td>0.334</td>
</tr>
<tr>
<td>MMP-3</td>
<td>225 (1083–4805)</td>
<td>231 (841–1000.5)</td>
<td>0.788</td>
</tr>
<tr>
<td>MMP-9</td>
<td>425 961 (65 754.2–1 182.777.2)</td>
<td>341 596 (500–988 507.4)</td>
<td>0.636</td>
</tr>
<tr>
<td>TREM-1</td>
<td>1349 (50–36 461.5)</td>
<td>1373 (500–137 519.4)</td>
<td>0.611</td>
</tr>
<tr>
<td>BDNF</td>
<td>5910 (1144–11 181.1)</td>
<td>4953 (1886–9.9–11370.8)</td>
<td>0.183</td>
</tr>
<tr>
<td>GACSF</td>
<td>14 (10–66.6)</td>
<td>18 (10–198.9)</td>
<td>0.418</td>
</tr>
<tr>
<td>NT-1</td>
<td>8 (3.5–46.5)</td>
<td>14 (4–71.2)</td>
<td>0.176</td>
</tr>
<tr>
<td>NT-3</td>
<td>32 (4–541.8)</td>
<td>46 (35–1274.7)</td>
<td>0.238</td>
</tr>
<tr>
<td>NTNF1</td>
<td>952 (395.2–1740.8)</td>
<td>934 (2084–3376.8)</td>
<td>0.573</td>
</tr>
<tr>
<td>MT-1</td>
<td>24 333 (3933–246 175.1)</td>
<td>37 316 (50–1 134 149.3)</td>
<td>0.384</td>
</tr>
<tr>
<td>SMARTES</td>
<td>38 501 (12 064–129 149.9)</td>
<td>39 462 (1964–6–16 160.0)</td>
<td>0.711</td>
</tr>
</tbody>
</table>

*Note: The continuous variables were compared using a nonparametric Mann-Whitney U test (presented as the median [range]). Abbreviations: BDNF, brain-derived neurotrophic factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL-1, interleukin-1; MCP-1, monocyte chemotactic protein-1; IFN-γ, interferon-γ; IL-1, interleukin-1; MCP-1, monocyte chemotactic protein-1; MIF, migration inhibiting factor; MIP, macrophage inflammatory protein; MMP-9, matrix metalloproteinase-9; NT-1, neutrophin-1; NT-4, neutrophin-4; RANTES, regulated on activation normal T-expressed and secreted protein; sIL-2R, soluble interleukin-2 receptor; STNF-1, soluble tumor necrosis factor receptor 1; TGF-β, transforming growth factor-β; TNF, tumor necrosis factor; and TREM-1, triggering receptor expressed on myeloid cells-1.*
Maternal inflammatory proteins predict infection

---

Table 4. Comparison of the levels of serum proteins in women with and without microbial invasion of the amniotic cavity according to gestational age at preterm labor.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Overall study population (22–34+6 weeks)</th>
<th>Gestational age (22–34+6 weeks)</th>
<th>p-Value</th>
<th>Gestational age (22–34+6 weeks)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Microbial invasion of the amniotic cavity</td>
<td>Absence of microbial invasion of the amniotic cavity</td>
<td></td>
<td>Microbial invasion of the amniotic cavity</td>
<td>Absence of microbial invasion of the amniotic cavity</td>
</tr>
<tr>
<td>IL-1β</td>
<td>11 (0.8–36.5)</td>
<td>14 (3.1–1996.7)</td>
<td>0.441</td>
<td>11 (3.8–25.7)</td>
<td>14 (3.1–1996.7)</td>
</tr>
<tr>
<td>IL-6</td>
<td>6.0 (6.1–11.2)</td>
<td>5.3 (3.6–17.5)</td>
<td>0.974</td>
<td>6.0 (6.1–11.2)</td>
<td>5.3 (3.6–17.5)</td>
</tr>
<tr>
<td>IL-8</td>
<td>18 (4–400)</td>
<td>16 (4–347.6)</td>
<td>0.124</td>
<td>21 (12–4000)</td>
<td>14 (4–1011.5)</td>
</tr>
<tr>
<td>IL-10</td>
<td>51.0 (3.9–9)</td>
<td>43 (3.5–49.9)</td>
<td>0.178</td>
<td>5.4 (3.8–9)</td>
<td>4.5 (3.5–49.9)</td>
</tr>
<tr>
<td>IL-12</td>
<td>71 (44–400)</td>
<td>40 (12–427.9)</td>
<td>0.649</td>
<td>72 (22–2851.5)</td>
<td>112 (12–2752)</td>
</tr>
<tr>
<td>L-17</td>
<td>8.0 (4–400)</td>
<td>9 (4–1752)</td>
<td>0.146</td>
<td>10 (4–400)</td>
<td>11 (4–1752)</td>
</tr>
<tr>
<td>L-18</td>
<td>412 (28–106)</td>
<td>314 (70–2268.3)</td>
<td>0.082</td>
<td>403 (218–1056)</td>
<td>316 (70–2268.3)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>14 (3.1–9.6)</td>
<td>24 (4.0–91.9–972 006.6)</td>
<td>0.116</td>
<td>24 (14.0–91.9–972 006.6)</td>
<td>24 (9.6–131.444.7)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>14 (3–48.9)</td>
<td>13 (4–460.0)</td>
<td>0.598</td>
<td>14 (3–48.9)</td>
<td>13 (4–460.0)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>14 (10–340.0)</td>
<td>138 (10–2958.9)</td>
<td>0.779</td>
<td>222 (10–540.0)</td>
<td>124 (9–2938.9)</td>
</tr>
<tr>
<td>TGF-β</td>
<td>81 (40–362.9)</td>
<td>97 (39–6919.6)</td>
<td>0.740</td>
<td>79 (40–2073)</td>
<td>108 (40–8119.6)</td>
</tr>
<tr>
<td>MIP-1</td>
<td>140 (32.9–39171)</td>
<td>140 (10–2146.0)</td>
<td>0.297</td>
<td>163 (32.9–39171)</td>
<td>138 (10–1948.1)</td>
</tr>
<tr>
<td>MMP-9</td>
<td>247 (13.4–3413.7)</td>
<td>220 (7–3471.9)</td>
<td>0.290</td>
<td>242 (13.4–3413.7)</td>
<td>220 (7–3471.9)</td>
</tr>
<tr>
<td>MMP-13</td>
<td>417 (40–927.9–318 318.2–438 962.5)</td>
<td>373 (18–2840.2–139 800.6)</td>
<td>0.373</td>
<td>423 (473 (140 927.9–318 318.2–438 962.5)</td>
<td>473 (248.0–21 463 800.6)</td>
</tr>
<tr>
<td>TREM-1</td>
<td>951 (500-2565.6)</td>
<td>1998 (257–57 671.8)</td>
<td>0.174</td>
<td>1071 (900–2565.6)</td>
<td>988 (257–57 671.8)</td>
</tr>
<tr>
<td>sIL6rα</td>
<td>3215 (955–2466.9)</td>
<td>4886 (125–59 345.6)</td>
<td>0.157</td>
<td>5204 (966–12 696.6)</td>
<td>4881 (125–59 345.6)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>15.1 (10–58.8)</td>
<td>16 (10–460.0)</td>
<td>0.470</td>
<td>14 (10–58.3)</td>
<td>12 (10–460)</td>
</tr>
<tr>
<td>NT4</td>
<td>7.9 (3–28.1)</td>
<td>114 (685.0)</td>
<td>0.214</td>
<td>6.3 (38–1)</td>
<td>12 (4–68)</td>
</tr>
<tr>
<td>NT3</td>
<td>68.9 (6.5–394.3)</td>
<td>49 (15–634.5)</td>
<td>0.509</td>
<td>72 (4–394)</td>
<td>44 (4–15 634.5)</td>
</tr>
<tr>
<td>TNFRl1</td>
<td>998 (550–1878.5)</td>
<td>901 (78–13 388.6)</td>
<td>0.330</td>
<td>1079 (550–1878.5)</td>
<td>891 (78–13 388.4)</td>
</tr>
<tr>
<td>MIF</td>
<td>41200 (5–3899 2047)</td>
<td>26 442 (90–128 491.1)</td>
<td>0.122</td>
<td>41450 (30–899 2047)</td>
<td>32 271 (50–128 491.1)</td>
</tr>
<tr>
<td>RANTES</td>
<td>35 935 (21 881.4–160 000)</td>
<td>38 521 (15 596.8–160 000)</td>
<td>0.412</td>
<td>34 004 (21 981.4–160 000)</td>
<td>38 843 (20 520.3–160 000)</td>
</tr>
</tbody>
</table>

Note: The continuous variables were compared using a non-parametric Mann–Whitney U-test (presented as the median [range]). Abbreviations are defined in the footnote to Table 3.
environments, such as pre-eclampsia (28). In contrast to this finding (27,28), our data supported a lower maternal response that was mediated by IL-1β and MCP-1 in women with MIAC. This finding is novel because there is a lack of previous studies that evaluated the importance of these two biomarkers as risk factors for MIAC in the maternal serum. Whether the different outcomes may partly explain the differences in our study remains unknown. Further studies are needed to confirm these preliminary results.

A higher but weak expression of maternal serum IL-6 was observed in women with PTL who had MIAC, which is consistent with previous findings. However, from a clinical point of view, the main limitation of this biomarker is its lack of specificity in predicting disease, because it is overexpressed in the maternal serum during infectious and non-infectious conditions (8,10,11).

Regarding the onset of the inflammatory response, significant differences in the maternal serum biomarkers were observed in PPROM and PTL only at less than 32+0 weeks of gestation. This finding is clinically relevant and emphasizes that the inflammatory response to the presence of microorganisms in amniotic fluid is gestational age dependent and is more pronounced at early gestational ages. We have previously reported, in a data set from the Czech Republic, the influence of gestational age as the main determinant of the inflammatory response in the amniotic fluid in women with PPROM complicated with MIAC (29). According to those data (29), there was no association between the levels of amniotic fluid cytokines and the occurrence of MIAC in women with PPROM over 32+0 weeks of gestation. Taken together, our data suggest that the maternal and intra-amniotic inflammatory responses are not responsible for the pathogenesis of MIAC at late gestational ages. Finally, the maternal inflammatory response due to MIAC was weaker than that reported in amniotic fluid (2). From a clinical point of view, therefore, the inflammatory status of the amniotic cavity is more relevant as a predictor of MIAC than the inflammatory response in the maternal serum.

One of the strengths of the study was the ability to assess the most relevant inflammatory biomarkers, which enabled a complete assessment and improved knowledge of the maternal inflammatory response compared with what was previously reported. Thus, we have evaluated the maternal expression of selected cytokines in women with PTL and PPROM separately. In addition, our study has limitations. The study was conducted at a single institution, which prevented the use of a larger sample size. Short- and long-term neonatal outcomes were not evaluated. A comparison of the serum and amniotic fluid levels of cytokines was not performed.

From a future perspective, the assessment of inflammatory biomarkers for MIAC in the maternal serum may not be warranted in future studies to determine its clinical relevance, because it does not accurately reflect the status of the amniotic cavity.

In summary, the identification of the inflammatory status in the maternal serum in women with PPROM and PTL is not as accurate as the identification of the inflammatory status in amniotic fluid to predict the occurrence of microbial invasion of the amniotic cavity. The maternal inflammatory response is evident only at early gestational ages. As a result of the weak contribution of maternal serum biomarkers as predictors of MIAC, these results support the need for further research to establish the potential contribution of these non-invasive inflammatory cytokines in combination with those identified in amniotic fluid to detect intrauterine infection in patients with PPROM and PTL.

**Funding**

This study was supported by the Swedish Medical Society (SLS 2008-21198), Swedish government grants to researchers in the public health service (ALFFGBG-2863, ALFFGBG-11522 and ALFFGBG-136431) and Instituto de Salud Carlos III in Spain for the Rio Hortega grant (CM09/00213). Additional support came from grants from the Spanish government (FIS PI041258).

**References**


The effect of latency of time, centrifugation conditions, supernate filtration, and addition of protease inhibitors on amniotic fluid interleukin-6 concentrations

OBJECTIVE: Amniotic fluid specimens are commonly frozen and stored for research purposes. A previous study examined the stability of certain cytokines in stored amniotic fluid samples for prolonged periods of time. They showed some degradation of the cytokines despite optimal freezing conditions. On the other hand, little is known about how preanalytical handling procedures affect cytokine levels. Since amniotic fluid interleukin (IL)-6 is the most reported biomarker to identify intraamniotic inflammation, the purpose of this study was to evaluate the effect of different handling procedures on immunoassay measurable concentrations of IL-6 in amniotic fluid.

STUDY DESIGN: From June through August 2012, amniotic fluid was collected from 21 women between gestational weeks 38+0 and 41+6, undergoing elective cesarean delivery at Sahlgrenska University Hospital/Östra, Gothenburg, Sweden. The study was approved by the local ethical committee at the University of Gothenburg (Dnr 991-11). Samples were obtained before the complete hysterotomy was performed, and 45 mL of amniotic fluid was aspirated. Each amniotic fluid sample was separated into 9 groups of 4.5 mL each. The standard group was defined as the group with centrifugation of the sample at 2000 g, for 10 minutes, in 4°C within 2 hours from sampling, without addition of protease inhibitor and without supernate filtration. This approach was the preanalytical procedure of the amniotic fluid that our research group has previously used. In the other groups (test groups), one variable was changed to evaluate the influence of latency of time from sampling to centrifugation (5 hours of storage in group 1 and 24 hours in group 2), the centrifugal force (300g in group 3 and 12,000g in group 4),

FIGURE
Schematic presentation of the differences in IL-6 concentrations between the test groups and the standard group

$P$ values are calculated with Wilcoxon signed ranks test. The markers (○) in the diagram represent outliers and (★) extreme values. A value of $P < .05$ was considered significant.

IL-6, interleukin-6.
centrifugation time (20 minutes in group 5), centrifugation temperature (20°C in group 6), supernate filtration (group 7), and serine-, cysteine-, and metalloproteases inhibitor (group 8). After processing, aliquots from each group were immediately stored at −80°C until analysis with enzyme-linked immunosorbent assay within 8 months from sampling. Each enzyme-linked immunosorbent assay performed was specific for IL-6 with a detection rate of 15.6-1500 pg/mL. The inter assay and intra assay coefficient of variation was <10% and <5%, respectively. Results were calculated by pairing the concentrations of IL-6 in the standard group with those in each test group using Wilcoxon signed ranks test. A value of P < .05 was considered significant.

RESULTS: A total of 21 amniotic fluid samples were handled with different approaches and IL-6 concentrations were assayed. No significant differences were observed on median and dispersion values of IL-6 concentrations between the standard group and the test groups (groups 1-8) as presented graphically in the Figure.

CONCLUSION: Differences of the preanalytical handling procedures do not seem to interfere on amniotic fluid IL-6 concentration. Since most studies in the literature use frozen, banked amniotic fluid samples for the evaluation of a wide variety of obstetrical outcomes,4,5 to our knowledge, no published studies have evaluated amniotic fluid cytokine stability after different preanalytical handling procedures of the samples. In this study, different latency between sampling and analysis, centrifugal conditions (centrifugal force, time, and temperature), supernate filtration, and addition of protease inhibitor had no influence on the amniotic fluid IL-6 levels. However, we consider as a limitation of the study the lack of a fresh IL-6 assessment as control of frozen determinations. Whether a possible explanation of the lack of differences among different handling procedures is due to a short freezing time of amniotic fluid samples also remains unknown in this study.

Panagiotis Tsiartas, MD
Department of Obstetrics and Gynecology
Sahlgrenska University Hospital
Gothenburg, Sweden
tsiartaspanos@gmail.com

Marian Kacerovsky, MD, PhD
Department of Obstetrics and Gynecology
University Hospital Hradec Kralove
Hradec Kralove
Czech Republic

Maria Hallingström, BSc
Department of Obstetrics and Gynecology
Sahlgrenska University Hospital
Gothenburg, Sweden

Victor Liman, BSc
Department of Obstetrics and Gynecology
Sahlgrenska University Hospital
Gothenburg, Sweden

Teresa Cobo, MD, PhD
BCNatal—Barcelona Center for Maternal-Fetal and Neonatal Medicine (Hospital Clinic and Hospital Sant Joan de Deu)
Fetal i+D Fetal Medicine Research Center
Institut d’Investigacions Biomediques Agusti Pi i Sunyer
University of Barcelona
Center for Biomedical Research on Rare Diseases
Barcelona, Spain

Bo Jacobsson, MD, PhD
Department of Obstetrics and Gynecology
Sahlgrenska University Hospital
Gothenburg, Sweden
Department of Genes and Environment
Division of Epidemiology
Norwegian Institute of Public Health
Oslo, Norway

The authors report no conflict of interest.

REFERENCES


The association between histological chorioamnionitis, funisitis and neonatal outcome in women with preterm prelabor rupture of membranes

Panagiotis Tsiartas1, Marian Kacerovsky2,3, Ivana Musilova3,4, Helena Hornychova5, Teresa Cobo6, Karin Sävman7,8, and Bo Jacobsson1,9

1Department of Obstetrics and Gynecology, Sahlgrenska University Hospital, Gothenburg, Sweden, 2Biomedical Research Center, University Hospital Hradec Kralove, Czech Republic, 3Department of Obstetrics and Gynecology, Charles University in Prague, Faculty of Medicine Hradec Kralove, Czech Republic, 4Department of Obstetrics and Gynecology, University Hospital, Pardubice, Czech Republic, 5Fingerland’s Department of Pathology, University Hospital Hradec Kralove, Hradec Kralove, Czech Republic, 6Maternal-Fetal Medicine Department, Hospital Clinic, Institut d’Investigacions Biomediques August Pi i Sunyer (IDIBAPS), Universitat de Barcelona, Barcelona, Spain, 7Perinatal Center, Department of Physiology and Neuroscience and 8Department of Pediatrics, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, and 9Institute of Public Health, Oslo, Norway

Abstract

Objective: To determine the impact of histological chorioamnionitis (HCA) and funisitis on neonatal outcome in preterm prelabor rupture of membranes (PPROM) pregnancies.

Methods: Women with PPROM between 24 +0 to 36 +6 weeks of gestation, admitted to the Department of Obstetrics and Gynecology at the University Hospital Hradec Kralove in the Czech Republic, between July 2008 and October 2010, were enrolled in the study (n = 231). The incidence of early-onset sepsis (EOS) differed significantly in neonates born to women with and without HCA, after adjustment for gestational age (11% versus 1%, p = 0.011). The incidence of EOS in neonates was also significantly different, after adjustment for gestational age, in cases with and without funisitis (18% versus 4%, p = 0.002). The same was also found for retinopathy of prematurity (ROP) cases with and without funisitis (23% versus 4%, p = 0.014), after adjustment for gestational age.

Conclusions: HCA and funisitis increase the risk of adverse perinatal outcome in PPROM pregnancies.

Keywords

Intra-uterine inflammation, neonatal morbidity, placenta, preterm birth

Introduction

Infants born preterm continue to have high rates of morbidity and mortality, despite improvements in neonatal care [1]. Preterm prelabor rupture of membranes (PPROM) occurs approximately in one-third of all preterm deliveries and is strongly associated with intra-uterine infection/inflammation [2,3]. Histological chorioamnionitis (HCA) has been found more frequently in placentas from preterm deliveries than in those from term deliveries [4]. Neonatal mortality and morbidity in the first 48 h of life are higher among infants whose antenatal course was complicated by HCA and funisitis [5]. Intra-uterine infection/inflammation is characterized by activation of pro-inflammatory cytokines in umbilical cord blood and by funisitis (polymorphonuclear leukocyte infiltration along the umbilical cord in response to infection). Fetal response to intra-uterine infection/inflammation is associated with substantial neonatal mortality and severe morbidity [6–9].

The aim of this study was to examine the association of neonatal outcome with HCA and funisitis in pregnancies complicated by PPROM.

Material and methods

Women with singleton pregnancies, diagnosed with PPROM at gestational age 24 +0–36 +6 weeks and admitted to the Department of Obstetrics and Gynecology at the University Hospital Hradec Kralove in the Czech Republic between July 2008 and October 2010, were enrolled in the study. Gestational age was established according to the first-trimester ultrasound scan. Women with multiple pregnancies, structural and chromosomal anomalies, major pregnancy-related medical problems (hypertension, diabetes mellitus, preeclampsia), as well as women with clinical signs of chorioamnionitis or vaginal bleeding at admission, were excluded.
PPROM, defined as spontaneous rupture of membranes before 37 weeks of gestation that precedes the onset of uterine contractions, was diagnosed by a sterile speculum examination confirming amniotic fluid pooling in the vagina and a positive test for insulin-like growth factor-binding protein (ACTIM PROM test; MedixBiochemica, Kauniainen, Finland) in the vaginal fluid.

Tocolysis for 48 h was considered in the absence of clinical chorioamnionitis, placental abruption and fetal compromise at gestational ages $<34+0$ weeks. A complete course of steroids, two 14-mg i.m. betamethasone doses with a 24-h interval, was administered when PPROM occurred at $24+0–33+6$ weeks. Prophylactic parenteral azithromycin was started at admission and continued for 7 d. Maternal and fetal status were closely monitored until delivery. Maternal serum C-reactive protein (CRP) and white blood cell (WBC) count were assayed upon admission to the hospital and daily until the delivery.

Management of PPROM in the Czech Republic is active except in cases at $<28$ weeks of gestation, which are handled expectantly. Timing of induction of labor or elective cesarean section depends on gestational age, i.e. within 24 h at gestational age $>34+0$ weeks, within 48 h at gestational age $32+0–33+6$ weeks and within 72 h at gestational age $28+0–31+6$ weeks.

The placenta was fixed in 10% neutral buffered formalin directly after delivery and tissue blocks from the placenta (at least two samples), umbilical cord (usually one sample) and placental membranes (at least two samples) were processed according to routine and embedded in paraffin. Three-micrometer thick sections were cut from the paraffin blocks and stained with hematoxylin and eosin for standard histological examination, performed by a single pathologist (HH), blinded to the clinical status. The degree of polymorphonuclear leukocyte infiltration was evaluated separately in the free membranes (amnion and chorion-decidual), the chorionic plate and the umbilical cord, according to criteria proposed by Salafia et al. [10]. The diagnosis of HCA was determined based on histological grades 3–4 in the chorionic-decidual, and/or 3–4 in the chorionic plate, and/or 1–4 in the amnion and/or 1–4 in the umbilical cord. The diagnosis of funisitis was determined based on histological grades 1–4 in the umbilical cord [10].

Medical records for all neonates, regarding gestational age at delivery, morbidity and mortality, were reviewed (MK, IM). An entity called “composite neonatal morbidity” was defined and included tracheal intubation; respiratory distress syndrome (RDS), defined by the presence of two or more of the following criteria: evidence of respiratory compromise and persistent oxygen requirement for $>24$ h, administration of exogenous surfactant or radiographic evidence of hyaline membrane disease; intraventricular hemorrhage (IVH), diagnosed by cranial ultrasound examination and graded 1–4. According to criteria defined by Papile et al. [11]; necrotizing enterocolitis (NEC), defined as a radiologic finding of either intramural gas or free intra-abdominal gas; retinopathy of prematurity (ROP), identified by retinoscopy; early (EOS) and late (LOS) onset sepsis, defined as any systemic bacterial infection evidenced by clinical symptoms in association with either a positive blood culture or an elevated CRP and/or affected WBC count during the first 72 h of life or at 4–120 d of life, respectively; bronchopulmonary dysplasia (BPD) in a pre-term-born infant, defined by oxygen dependency at 28 d of life; pneumonia, diagnosed by an abnormal chest X-ray finding; or perinatal death, defined as death before hospital discharge.

Data were analyzed in relation to neonatal outcome and in two sub-groups of the population: women with and without HCA and women with and without funisitis.

Written informed consent was obtained from all subjects. The Institutional Review Board approved the collection and use of these samples and information for research purposes (Ethics Committee of University Hospital Hradec Kralove, Sokolska 581, 500 05, Hradec Kralove, 19 March 2008; No. 200804 S01P). A sub-cohort of women included in this study is presented in another publication studying umbilical cord blood IL-6 and funisitis in relation to EOS [12]. This publication deals with a larger cohort of women and investigates HCA and funisitis in relation to more extensive neonatal outcome.

Statistical analysis
Statistical analyses were performed using SPSS 20.0 for Mac OS X (SPSS Inc., Chicago, IL). Demographic and clinical characteristics were compared using the Mann–Whitney $U$-test for continuous variables [values are presented as median and interquartile range (IQR)]. Categorical variables were compared using the Pearson Chi-square test and were presented as count with the proportion. Spearman’s partial correlation was used to adjust data for gestational age at delivery. Differences were considered to be statistically significant at a confidence level of $p<0.05$ with two-sided alternative hypotheses.

Results
A total of 231 women with a diagnosis of PPROM were the objects of this analysis. The characteristics of women with ($n=142$, 61.4%) and without HCA ($n=89$, 38.5%), and the characteristics of women with ($n=45$, 19.4%) and without funisitis ($n=186$, 80.5%), are presented in Table 1. Women with HCA had a longer latency from PPROM to delivery, higher CRP at admission, higher body mass index and higher rates of antenatal corticosteroid administration, compared to those without HCA. Women with funisitis had a longer latency from PPROM to delivery, higher WBC count at admission and higher rates of antenatal corticosteroid administration, compared to those without funisitis. In our study, gestational age at delivery, latency from PPROM to delivery and birth weight cannot be considered as outcomes because of the above mentioned active management of PPROM pregnancies in the Czech Republic.

A comparison of the neonatal outcome in women with and without HCA is presented in Table 2. Women with HCA had neonates with significantly lower birth weight and higher rates of composite neonatal morbidity, RDS, BPD and EOS. After adjustment for gestational age at delivery, EOS differed significantly between these two groups [15 (11%) versus 1 (1%)].
Table 1. Maternal characteristics among the different groups. $p$ and $p^*$ values were calculated using the Mann–Whitney $U$-test for continuous variables and the Pearson Chi square test for categorical variables.

<table>
<thead>
<tr>
<th></th>
<th>The presence of HCA (n = 142)</th>
<th>The absence of HCA (n = 89)</th>
<th>$p$</th>
<th>The presence of funisitis (n = 45)</th>
<th>The absence of funisitis (n = 186)</th>
<th>$p^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>32 (10)</td>
<td>30 (6)</td>
<td>0.066</td>
<td>32 (7)</td>
<td>31 (9)</td>
<td>0.422</td>
</tr>
<tr>
<td>(median; IQR)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational age at admission (weeks + days) (median; IQR)</td>
<td>32 + 0 (33)</td>
<td>33 + 6 (22)</td>
<td>&lt;0.001</td>
<td>30 + 5 (39)</td>
<td>33 + 3 (25)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gestational age at delivery (weeks + days) (median; IQR)</td>
<td>32 + 3 (31)</td>
<td>34 + 0 (22)</td>
<td>&lt;0.001</td>
<td>31 + 0 (38)</td>
<td>33 + 5 (22)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Latency from PPROM to delivery (hours) (median; IQR)</td>
<td>56 (57)</td>
<td>21.5 (52)</td>
<td>&lt;0.001</td>
<td>65 (55)</td>
<td>33 (60)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Nulliparity (n = 110, 48%)</td>
<td>63 (44%)</td>
<td>47 (53%)</td>
<td>0.354</td>
<td>15 (34%)</td>
<td>95 (51%)</td>
<td>0.113</td>
</tr>
<tr>
<td>CRP at admission (mg/L) (median; IQR)</td>
<td>7 (10)</td>
<td>4 (5)</td>
<td>&lt;0.001</td>
<td>6 (9)</td>
<td>5 (8)</td>
<td>0.156</td>
</tr>
<tr>
<td>WBC count at admission ($\times 10^9$/L) (median; IQR)</td>
<td>12 (5)</td>
<td>12 (4)</td>
<td>0.491</td>
<td>13 (4)</td>
<td>12 (5)</td>
<td>0.047</td>
</tr>
<tr>
<td>Positive hemoculture (3%)</td>
<td>23 (6)</td>
<td>21 (5)</td>
<td>0.023</td>
<td>21 (5)</td>
<td>22 (6)</td>
<td>0.240</td>
</tr>
<tr>
<td>Neutrophils at admission ($\times 10^9$/L) (median, IQR)</td>
<td>13 (7)</td>
<td>11 (5)</td>
<td>0.615</td>
<td>0.229</td>
<td>0.526</td>
<td>0.205</td>
</tr>
<tr>
<td>Antibiotics (n = 222, 96%)</td>
<td>135 (95%)</td>
<td>87 (98%)</td>
<td>0.005</td>
<td>0.237</td>
<td>0.972</td>
<td>0.005</td>
</tr>
<tr>
<td>Neonatal outcomes for women with and without funisitis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Neonatal outcomes between the group of women with HCA and without HCA. $p$ values were calculated using the Mann–Whitney $U$-test for continuous variables and the Pearson Chi square test for categorical variables. Spearman’s partial correlation was performed to adjust by gestational age at delivery ($p^*$).

<table>
<thead>
<tr>
<th></th>
<th>Presence HCA (n = 142)</th>
<th>Absence HCA (n = 89)</th>
<th>$p$</th>
<th>$p^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birthweight (g) (median, IQR)</td>
<td>1840 (918)</td>
<td>2190 (688)</td>
<td>&lt;0.001</td>
<td>0.153</td>
</tr>
<tr>
<td>5 min Apgar score &lt;7 (9%)</td>
<td>17 (12%)</td>
<td>5 (6%)</td>
<td>0.109</td>
<td>0.890</td>
</tr>
<tr>
<td>10 min Apgar score &lt;7 (5%)</td>
<td>9 (6%)</td>
<td>2 (2%)</td>
<td>0.155</td>
<td>0.788</td>
</tr>
<tr>
<td>Newborn WBC count ($\times 10^9$/L) (median, IQR)</td>
<td>13 (7)</td>
<td>11 (5)</td>
<td>0.615</td>
<td>0.229</td>
</tr>
<tr>
<td>Positive hemoculture (3%)</td>
<td>5 (4%)</td>
<td>1 (1%)</td>
<td>0.259</td>
<td>0.526</td>
</tr>
<tr>
<td>Neonatal composite morbidity (45%)</td>
<td>75 (53%)</td>
<td>30 (34%)</td>
<td>0.005</td>
<td>0.527</td>
</tr>
<tr>
<td>Permanence NICU ≥1 d (40%)</td>
<td>61 (43%)</td>
<td>29 (33%)</td>
<td>0.116</td>
<td>0.659</td>
</tr>
<tr>
<td>Tracheal intubation (15%)</td>
<td>28 (20%)</td>
<td>7 (8%)</td>
<td>0.014</td>
<td>0.530</td>
</tr>
<tr>
<td>Duration tracheal intubation ≥1 d (17%)</td>
<td>31 (22%)</td>
<td>8 (9%)</td>
<td>0.011</td>
<td>0.472</td>
</tr>
<tr>
<td>Duration nCPAP ≥1 d (34%)</td>
<td>55 (39%)</td>
<td>24 (27%)</td>
<td>0.067</td>
<td>0.887</td>
</tr>
<tr>
<td>RDS (30%)</td>
<td>52 (37%)</td>
<td>18 (20%)</td>
<td>0.008</td>
<td>0.574</td>
</tr>
<tr>
<td>Perinatal death (4%)</td>
<td>9 (6%)</td>
<td>1 (1%)</td>
<td>0.058</td>
<td>0.810</td>
</tr>
<tr>
<td>IVH (20%)</td>
<td>31 (22%)</td>
<td>16 (18%)</td>
<td>0.479</td>
<td>0.959</td>
</tr>
<tr>
<td>IVH grade 1–2 (19%)</td>
<td>29 (20%)</td>
<td>15 (17%)</td>
<td>0.501</td>
<td>0.904</td>
</tr>
<tr>
<td>IVH grade 2–4 (2%)</td>
<td>3 (2%)</td>
<td>1 (1%)</td>
<td>0.575</td>
<td>0.764</td>
</tr>
<tr>
<td>NEC (2%)</td>
<td>4 (3%)</td>
<td>1 (1%)</td>
<td>0.389</td>
<td>0.797</td>
</tr>
<tr>
<td>Intestinal perforation (2%)</td>
<td>3 (2%)</td>
<td>2 (2%)</td>
<td>0.945</td>
<td>0.342</td>
</tr>
<tr>
<td>Patent ductus arteriosus (6%)</td>
<td>11 (8%)</td>
<td>3 (3%)</td>
<td>0.175</td>
<td>0.780</td>
</tr>
<tr>
<td>ROP (7%)</td>
<td>13 (9%)</td>
<td>4 (4%)</td>
<td>0.187</td>
<td>0.798</td>
</tr>
<tr>
<td>Bronchopulmonary dysplasia (9%)</td>
<td>18 (13%)</td>
<td>2 (2%)</td>
<td>0.006</td>
<td>0.332</td>
</tr>
<tr>
<td>Pneumonia (9%)</td>
<td>16 (4%)</td>
<td>1 (1%)</td>
<td>0.181</td>
<td>0.490</td>
</tr>
<tr>
<td>Pulmonary Hypertension (3%)</td>
<td>6 (4%)</td>
<td>1 (1%)</td>
<td>0.181</td>
<td>0.426</td>
</tr>
<tr>
<td>Early onset sepsis (7%)</td>
<td>15 (11%)</td>
<td>1 (1%)</td>
<td>0.006</td>
<td>0.011</td>
</tr>
<tr>
<td>Late onset sepsis (5%)</td>
<td>8 (6%)</td>
<td>4 (4%)</td>
<td>0.704</td>
<td>0.234</td>
</tr>
</tbody>
</table>

IQR is the interquartile range. Significant values are marked in bold.
HCA: Histological chorioamnionitis; WBC: White blood cells; NICU: Neonatal intensive care unit; nCPAP: Nasal continuous positive airway pressure; RDS: Respiratory distress syndrome; IVH: Intraventricular hemorrhage; NEC: Necrotizing enterocolitis; ROP: Retinopathy of prematurity.

Neonatal outcomes for women with and without funisitis are presented in Table 3. Neonates with funisitis had significantly lower birth weight and Apgar scores at 5 and 10 min. The neonates with funisitis had higher rates of composite neonatal morbidity, perinatal death, RDS, ROP, BPD, EOS and LOS. After adjustment for gestational age at delivery, ROP [10 (23%) versus 7 (4%)] and EOS [8 (18%) versus 8 (4%)] differed significantly between these two groups.

**Discussion**
HCA is often diagnosed in preterm-delivered placenta after PPROM and has been considered to be a consequence of an...
intra-amniotic inflammatory process which induces the preterm parturition syndrome [9,13]. Moreover, HCA is independently associated with lower gestational age and increases major neonatal morbidity [14]. In this study, we confirmed that placental inflammatory status is associated with adverse neonatal outcome in PPROM pregnancies. We found that the rate of EOS was significantly higher in the group with HCA and that the rates of ROP and EOS were significantly higher in the group with funisitis.

The incidence of infectious diseases among preterm neonates is inversely correlated with gestational age [15,16]. Several studies have shown that there is markedly increased incidence of EOS after prenatal exposure to HCA, mainly in preterm neonates [17,18]. This study demonstrates a significantly higher incidence of EOS among the neonates prenatally exposed to HCA and funisitis. This difference in EOS rates among neonates remained statistically significant after adjustment for gestational age at delivery, showing that HCA and/or funisitis are independent risk factors for the development of EOS in PPROM pregnancies.

ROP is a disorder characterized by vasoproliferation in the retina of preterm infants; it can cause long-term visual disability [19]. Gestational age and perinatal placental inflammation are associated with the development of ROP [20–23]. Sato et al. showed that many genes associated with inflammation and neovascularization were up-regulated in an oxygen-induced retinopathy model in mice [24]. In this study, we found an increased incidence of ROP in neonates with funisitis, irrespective of gestational age at delivery. Prenatal inflammatory stimuli might exaggerate local retinal inflammation with consequent neovascularization in the developing retina and subsequent ROP [25].

One of the strengths of this study is the relatively large number of women included with a clearly defined phenotype of exclusively PPROM-induced preterm delivery. Moreover, we have used a well-defined classification of HCA and funisitis. Another strength of this study is the fact that the same pathologist examined all placentas. However, the study is limited by the fact that we did not confirm the infectious or non-infectious nature of the HCA with microbial cultures and that not all EOS cases were culture-positive. The latter is a common finding in very preterm infants in whom prophylactic antibiotic treatment may actually precede clinical symptoms or blood volumes for cultures may be limited. Clinical symptoms in association with laboratory tests supporting systemic infection are thus commonly used to define clinical sepsis, as has been the case in this study.

In conclusion, our study suggests that HCA and funisitis in PPROM pregnancies is associated with increased rates of EOS and that funisitis is associated with increased rates of EOS and ROP. Controversy still exists in the literature regarding the mechanisms by which HCA and funisitis impact neonatal outcome and future studies focusing on the impact of placental pathology on neonatal outcome are warranted.

Declaration of interest

This work was supported by Charles University in Prague, Faculty of Medicine, Hradec Kralove, Czech Republic (project “PRVOUK” P37/10) and the Faculty Hospital in Hradec Kralove (long-term organization development plan).
The authors report no conflict of interest. The authors are exclusively responsible for the content and writing of the paper.

References
