Amniotic fluid biomarkers in the diagnosis of intra-amniotic infection in preterm singleton pregnancies

-Association with microbial invasion of the amniotic cavity and histologic chorioamnionitis

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Academic dissertation

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To my family

Contents

List of original publications

The thesis is based on the following original publications, referred to by their Roman numerals I-IV in the text.

I Vaginally obtained amniotic fluid samples in the diagnosis of subclinical chorioamnionitis Myntti, T., Rahkonen, L.,Tikkanen, M., Paavonen, J., Stefanovic, V., AOGS 2016; 95:233-7

II Amniotic fluid rapid biomarkers are associated with intra-amniotic infection in preterm pregnancies regardless of the membrane status, Myntti, T., Rahkonen, L., Tikkanen, M., Pätäri-Sampo, A., Paavonen, J., Stefanovic, V., Journal of Perinatology 2016 Apr 7. doi: 10.1038/jp.2016.59.

III Comparison of Matrix Metalloproteinase-8 and a Novel Biomarker Cathelicidin in the Diagnosis of Intra-amniotic Infection, Myntti, T., Rahkonen, L., Pätäri-Sampo, A., Tikkanen, M., Sorsa, T., Juhila, J., Helve, O., Andersson, S., Paavonen, J., Stefanovic, V., Journal of Perinatology, 2016 Sept 1. doi:10.1038/jp.2016.147.

IV Amniotic fluid infection in preterm pregnancies with intact membranes, Myntti, T., Rahkonen, L., Nupponen, I., Pätäri-Sampo, A., Tikkanen, M., Sorsa, T., Juhila, J., Andersson, S., Paavonen, J., Stefanovic, V., DisMark 2017, Jan. doi:10.1155/2017/8167276

In addition, this thesis contains some unpublished data.

Abbreviations

AC	Amniocentesis
AF	Amniotic fluid
AUC	Area under the curve
BMI	Body mass index
BPD	Bronchopulmonary dysplasia
CA12-5	Cancer antigen 12-5
CI	Confidence interval
CP	Cerebral palsy
CC	Clinical chorioamnionitis
CRP	C-reactive protein
CV	Coefficient of variation
ELISA	Enzyme-linked immunosorbent assay
EONS	Early onset neonatal sepsis
FIRS	Fetal inflammatory response syndrome
HCA	Histologic chorioamnionitis
HNE	Neutrophil elastase
HOCl	Hypoclorus acid
IAI	Intra-amniotic infection
IEMA	Immunoenzymometric assay
IFCC	International Federation of Clinical Chemistry
IFMA	Immunofluorometric assay
IL	Interleukin
IVF	In vitro fertilization
IVH	Intraventricular haemorrhage
LD	Lactate dehydrogenase
MIAC	Microbial invasion of the amniotic cavity
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
NEC	Necrotizing enterocolitis
NICU	Neonatal intensive care unit

NPV	Negative predictive value
OR	Odds ratio
PCR	Polymerase chain reaction
PMN	Polymorphonuclear neutrophil
PPROM	Preterm prelabor rupture of membranes
PPV	Positive predictive value
PVL	Periventricular leukomalacia
RDS	Respiratory distress syndrome
ROC	Receiver operating characteristics
Spp.	Species
TIMP	Tissue inhibitor of metalloproteinases
WBC	White blood cells

Abstract

Chorioamnionitis, the main single cause of preterm delivery, which occurs in 10 to 13% of deliveries annually worldwide, can be subdivided into clinical and subclinical forms. The latter is more common and includes intra-amniotic infection (IAI), inflammation, and histologic chorioamnionitis (HCA). Diagnosing subclinical chorioamnionitis is necessary for optimal timing of delivery. Amniotic fluid (AF) biomarkers allow gathering of information on the inflammatory status of the uterine cavity.

The aim of the study was to evaluate AF biomarkers in the diagnosis of intra-amniotic infection.

The study was conducted at the University Hospital of Helsinki, Finland, Department of Obstetrics and Gynecology, between March 2012 and October 2015. The study population comprised 155 cases with a suspicion of IAI or preterm prelabor rupture of the membranes (PPROM) and 46 controls. Amniocentesis was performed in 105 cases between 22+0 and 36+5 weeks of gestation and in 46 controls. AF was obtained vaginally from 53 cases. In such AF samples, AF-lactate dehydrogenase (AF-LD) and AF-Glucose concentrations were determined. Determination in amniocentesis samples was of AF-LD, AF-Glucose, AF-matrix metalloproteinase (MMP)-8, AF-cathelicidin, AF-MMP-9, AF-myeloperoxidase, AF-interleukin-6, AF-neutrophil elastase (HNE), AF-elafin, AF-MMP-2, AF- tissue inhibitor of matrix metalloproteinases -1 (TIMP-1), AF-MMP-8/TIMP-1 molar ratio, and AF-C-reactive protein (CRP) levels. AF-MMP-8 measurement was by an immunoenzymometric assay, AF-LD and AF-Glucose by immunochemiluminometric assays, and others by commercial ELISA. Microbiological analyses were based on molecular microbiology and culture techniques. An experienced pathologist performed placental histopathologic examination. Data on pregnancies came from the hospital database.

The most optimal cut-off value based on the ROC-curve for AF-LD in vaginally obtained AF against HCA was 1029 IU/L with a sensitivity of 65% and specificity of 69%. In such samples, glucose concentrations did not differ between women with or without HCA. In amniocentesis samples, AF-LD and AF-Glucose correlated with HCA and MIAC, and the most optimal cut-off values for both end-points were a respective 429 IU/L and 0.7 mmol/L. When AF-LD and AF-Gluc concentrations were adjusted by gestational age at amniocentesis, the association disappeared. The concomitant use of AF-LD and AF-Glucose provided no additional value. AF-MMP-8, AF-cathelicidin, AF-MMP-9, AF-MPO, AF-IL-6, AF-Elafin, AF-HNE, and AF-TIMP-1 were associated with MIAC, but AF-MMP-2 and AF-CRP were not. The results were similar also when adjusted by gestational age at amniocentesis. Neutrophil-produced biomarkers were associated with IAI. MIAC occurred equally often in pregnancies with PPROM and with intact membranes. Infection and inflammation were more common at lower gestational ages.

In conclusion, the accuracies of AF-LD and AF-Glucose were quite poor, meaning that better biomarkers for IAI diagnostics are essential. None of the other biomarkers studied out-performed others, and larger studies are needed to confirm and further extend our results. However, IAI seemed to be associated with neutrophil activation. The usefulness of each biomarker for clinical purposes depends more on local circumstances, laboratory method availability, and the clinicians´ familiarity with each biomarker than on exact differences in accuracy.

Introduction

Intra-amniotic infection or inflammation, one form of chorioamnionitis, is the main etiologic factor in preterm delivery (Goldenberg *et al.* 2008). This association was first described in 1974 (Buhimschi *et al.* 2013). Chorioamnionitis may appear in both clinical and subclinical forms. Clinical chorioamnionitis with maternal fever as one of the essential signs occurs in 5 to 10% (Edwards 2005) of all preterm deliveries, but still represents only the tip of the iceberg. Subclinical chorioamnionitis, occurring more frequently *(*Wu *et al.* 2009, Galinsky *et al.* 2013), can be subdivided into histologic chorioamnionitis (HCA), intra-amniotic infection (IAI), and intra-amniotic inflammation. IAI is usually defined as intra-amniotic inflammation in the presence of microbial invasion of the amniotic cavity (MIAC). However, in the current literature, overlapping and incoherence occurs in the definitions and criteria of chorioamnionitis and IAI.

Chorioamnionitis plays a key role in neonatal morbidity and mortality both in pregnancies complicated by preterm prelabor rupture of the membranes (PPROM) and in those with intact fetal membranes *(Yoon et al.* 2001, Kacerovsky *et al.* 2014, Liu *et al.* 2014, Roescher *et al.* 2014, Kim *et al.* 2015b). IAI causes adverse neonatal outcomes similar to those of sterile inflammation (Combs *et al.* 2014). One of the most significant risk factors for chorioamnionitis is PPROM and the following prolonged latency, *i.e.* time interval between the PPROM and the labor (Fishman, Gelber 2012). Although routine prophylactic antibiotics after PPROM have reduced the incidence of chorioamnionitis and neonatal infections (Tita, Andrews 2010, Kenyon *et al.* 2013), attempts at prevention of adverse outcomes in pregnancies with clinical chorioamnionitis may be ineffective (Yoon *et al.* 2001, Kim *et al.* 2015b).

Among the main challenges in modern obstetric practice are early diagnosis of subclinical chorioamnionitis and appropriate timing of delivery (weighing the benefits of pregnancy prolongation against risk of fetal infection).

Traditionally, a general inflammation marker in the chorioamnionitis diagnosis has been maternal plasma C-reactive protein (CRP), although it has proven a poor marker for HCA, MIAC (Stepan *et al.* 2016), and clinical chorioamnionitis (Trochez-Martinez *et al.* 2007). Due to a lack of exact cut-off levels and a wide range of confidence intervals, this marker exhibits very limited clinical usefulness (Buhimschi *et al.* 2013, Dulay *et al.* 2015). No other biomarkers from maternal serum samples are in clinical use.

A plethora of studies cover AF biomarkers obtained by amniocentesis (AC) in the diagnosis of IAI, but only few have undergone clinical validation. Currently in wide use in intra-amniotic infection (IAI) diagnostics are AF lactate dehydrogenase (LD) (Garry *et al.* 1996), glucose (Gluc) (Romero *et al.* 1990, Greig *et al.* 1994), interleukin-6 , (Romero *et al.* 1993a, 1993c), and matrix metalloproteinase-8 (Maymon *et al.* 2000b, *Park et al.* 2013a), but their low accuracy, particularly of AF-LD and AF-Gluc, has limited their clinical use.

Studies concerning AF biomarkers obtained non-invasively from vaginal samples are few. They have revealed an association of AF-LD and AF-Gluc with MIAC, but sample size has been limited (Magloire *et al.* 2006b, Buhimschi *et al.* 2006). Pregnancies with preterm prelabor rupture of membranes (PPROM) are frequently associated with oligohydramnion, making AC technically difficult or impossible. In these cases, AF sampling vaginally from leaking AF would be of great value.

Taking into consideration the fact that in pregnancies with preterm labor and intact fetal membranes IAI and subclinical chorioamnionitis are frequent (Romero *et al.* 2014c), we wanted to explore the value of

traditional and novel biomarkers in the diagnosis of these entities both in pregnancies with PPROM and with intact fetal membranes. The inconsistent and limited results from the biomarkers obtained vaginally in PPROM pregnancies led us to investigate them further.

Review of the literature

Preterm birth

Preterm delivery is defined by the World Health Organization (WHO) as delivery at less than 37 weeks of gestation (Goldenberg *et al.* 2008, Harrison, Goldenberg 2016), without any limit for neonatal weight. The lower limit for delivery varies between countries, but in Finland it is set at 22+0 weeks of gestation or weight of the neonate over 500 g (THL 2016).

Worldwide, the rate of preterm birth is about 11% (Harrison, Goldenberg 2016), and 15 million preterm births occur annually (Galinsky *et al.* 2013). The rate has stayed almost stable during recent decades (Norman, Shennan 2013), or has even risen (Galinsky *et al.* 2013). In Finland, the rate of preterm birth is 5.3% (THL 2016), similar to other European countries' (Cappelletti *et al.* 2016), and it has remained quite stable over 15 years (THL 2016).

Preterm birth can be classified as spontaneous or induced preterm birth. The former can be further subdivided into preterm birth with intact membranes, and preterm birth starting with preterm prelabor rupture of the membranes (PPROM). Preterm delivery starting with PPROM comprises 30 to 40% of all preterm deliveries, while the number of term deliveries with PPROM is only 2 to 3.5% (Henderson *et al.* 2012, Erdemir *et al.* 2013). Most PPROM cases occur between 34 and 37 weeks of pregnancy (Kacerovsky *et al.* 2014).

Infection or inflammation is the main single cause of preterm birth, and is responsible for approximately 25% of all preterm births (Goldenberg *et al.* 2008, Cappelletti *et al.* 2016). Infections can be subdivided into intrauterine and extrauterine infections, the latter including malaria, pyelonephritis, pneumonia, and periodontitis (Parthiban, Mahendra 2015). Moreover, chronic inflammatory conditions in the mother, like obesity, autoimmune diseases such as systemic lupus erythematosus, multiple sclerosis, and type 1 diabetes (Cappelletti *et al.* 2016), are associated with increased risk for preterm birth. Furthermore, an altered maternal microbiome in the oral cavity or placenta can also cause an increased risk for preterm birth (Jain, Gyamfi-Bannerman 2016, Vinturache *et al.* 2016). Microbe-associated infection is not a necessary cause of increased risk for preterm birth, because sterile intra-amniotic inflammation also elevates the risk for preterm labor at <34 weeks of gestation (Romero *et al.* 2014b). The most important risk factor for preterm birth is, however, a maternal history of preterm birth, especially in the early weeks of gestation (Vinturache *et al.* 2016).

Preterm birth is the leading cause of neonatal mortality (Slattery, Morrison 2002), and it causes approximately 35% of neonatal deaths during the first four weeks of life (Norman, Shennan 2013, Harrison, Goldenberg 2016), and altogether 75% of total perinatal mortality (Slattery, Morrison 2002, Goldenberg *et al.* 2008). Improvement in neonatal outcome has occurred since antenatal corticosteroids and magnesium neuroprophylaxis have been incorporated into clinical management. Antenatal corticosteroid administration between 24 and 34 weeks of gestation in the setting of threatening preterm labor has reduced the risk for respiratory distress syndrome (RDS) for over 20 years in high-income countries. In low-income countries, accessibility of antenatal corticosteroids is less frequent (Harrison, Goldenberg 2016). Administration of magnesium sulfate to the mother with threatening preterm delivery <32 weeks of

gestation has shown beneficial effects on the newborn´s neurodevelopmental outcome, mainly by reducing the incidence of cerebral palsy (CP) (Kamyar *et al.* 2016). The number needed to treat to prevent one case of CP is 63 (95% CI 43-155) (Doyle *et al.* 2009). However, in one recent study, magnesium neuroprophylaxis had no favorable effect on neonatal outcome in neonates born at <32 weeks of gestation in the setting of chorioamnionitis (Kamyar *et al.* 2016).

Economic consequences of preterm birth

One approximation of annual costs of prematurity in the USA, in 2007, was 26.2 billion dollars (Behrman, Butler 2007). Costs of prematurity are not only due to hospitalization in the neonatal period, but also due to a lifelong need of special help for those with handicaps, along with increased need for healthcare and social services (Saigal, Doyle 2008, Platt 2014). In PPROM pregnancies, between 34 and 37 weeks of gestation, induction of labor costs more than does expectant management (Vijgen *et al.* 2014). Delayed labor from 34 weeks to 35 weeks can achieve a 42% decrease in neonatal costs. Moreover, delay of one more week yields a 38% extra decrease in neonatal costs (Loftin *et al.* 2010). The incremental costs of prematurity over those of full-term infants from birth to age 18 years have been approximated to be for preterm (<37 weeks of gestation) infants 1.5 times as high, for very preterm (28-32 weeks) infants 2.5 times as high, and for extremely preterm (<28 weeks) infants 3.2 times as high (Mangham *et al.* 2009).

Chorioamnionitis

Chorioamnionitis refers to the inflammatory changes of the chorion-plate, amniotic membranes, or both (Higgins *et al.* 2016). Inflammation may be subsequent to microbial invasion of the amniotic cavity (MIAC), or it may occur without proven microbiologic etiology (Romero *et al.* 2014c). Microbes leading to chorioamnionitis, according to current knowledge, consist of bacteria, viruses, and yeasts (Ramos Bde *et al.* 2015). Those microbes may reach the amniotic cavity by the following routes: 1) ascent from the vagina, 2) hematogenous spread, 3) iatrogenic spread during an invasive procedure, such as amniocentesis (AC) or chorion villus sampling, and 4) retrograde invasion through the fallopian tubes (Figure 1) (Kim *et al.* 2015a).

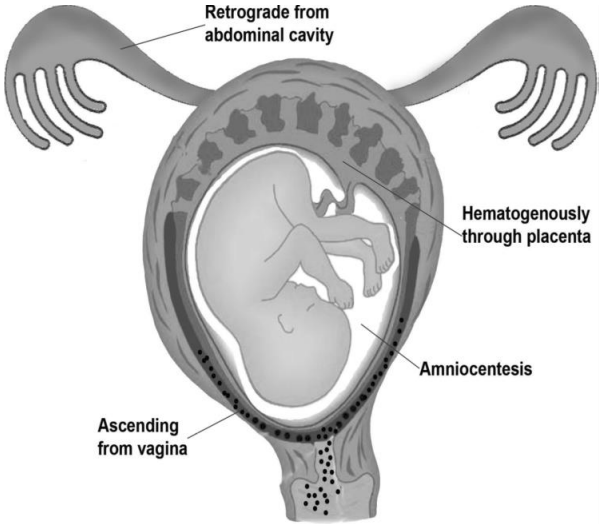


Figure 1. Routes of microbes to reach the amniotic cavity; from. L. Rahkonen thesis 2010 with the kind permission of the author.

Intra-uterine infection may exist in amniotic fluid (AF) (amnionitis), in the fetal membranes (chorioamnionitis), between maternal and fetal tissues (choriodeciduitis), in the placenta, in the umbilical cord (funisitis), or in the fetus (Figure 2) (Goldenberg *et al.* 2000).

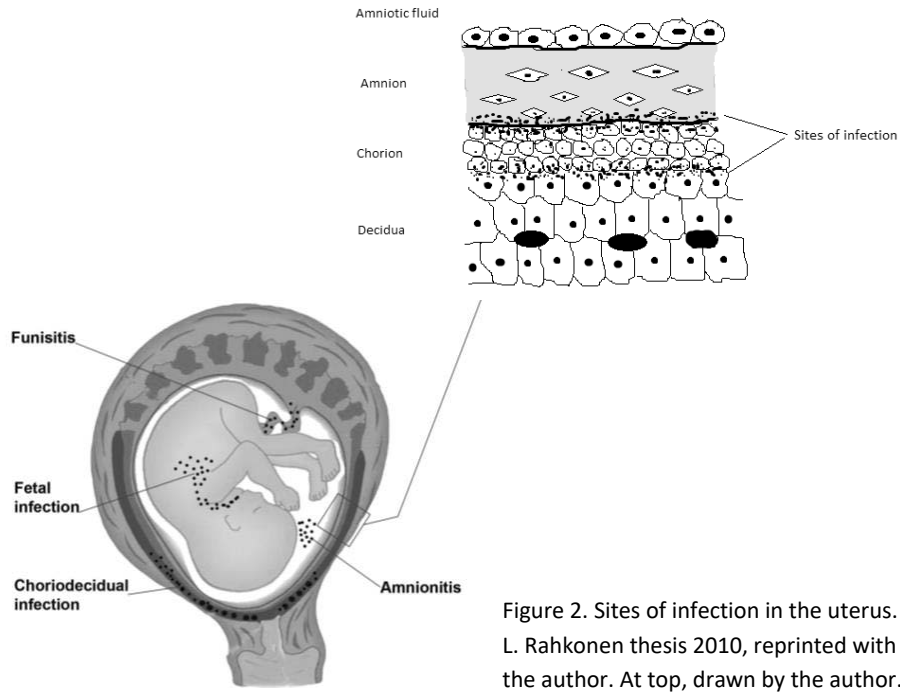


Figure 2. Sites of infection in the uterus. At bottom, from L. Rahkonen thesis 2010, reprinted with permission of the author. At top, drawn by the author.

The mechanism behind infection and preterm labor consists of microbes releasing endotoxins and exotoxins which are recognized by a maternal host response, *i.e.* Toll-like receptors existing on the surface of leukocytes, and epithelial and trophoblast cells. This leads to production of pro-inflammatory cytokines, such as interleukin-6 (IL-6), and chemokines in the decidua and fetal membranes. Cytokines then activate an inflammatory cascade, in which prostaglandins are produced, and acute-phase reactants like C-reactive protein (CRP) are released into the circulation, and neutrophils are activated. Neutrophils migrating to the infectious site release proteases such as matrix metalloproteinases (MMPs) and their regulators, like tissue inhibitors of metalloproteinase -1 (TIMP-1) (Alfakry *et al.* 2016). Prostaglandins stimulate uterine contractions, and metalloproteinases stimulate collagen degradation, leading to cervical softening and preterm labor or rupture of the amniotic membranes (Galinsky *et al.* 2013, Fox, Eichelberger 2015) (Figure 3).

The mechanism behind sterile inflammation is, instead, considered to involve stimuli leading to release of endogenous molecules, called alarmins, which evoke a host response through Toll-like receptors, leading to an inflammatory process and causing sterile HCA (Kacerovsky *et al.* 2014, Romero *et al.* 2014c).

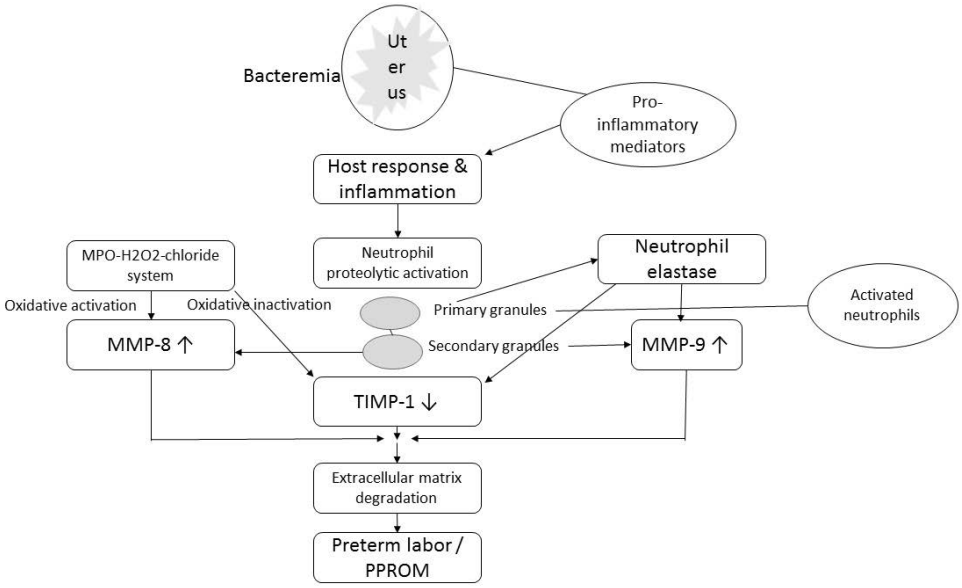


Figure 3. Inflammation cascade. Modified from Alfakry 2016, with the kind permission of SAGE Publishing.

MPO, myeloperoxidase; H2O2-chloride, hypochlorus-acid; MMP-8, matrix metalloproteinase-8, TIMP-1, tissue inhibitor of metalloproteinases-1; MMP-9, matrix metalloproteinase-9; PPROM, preterm prelabor rupture of the membranes.

Several mechanical and functional barriers protect the amniotic cavity from an external microbial load (King *et al.* 2007b). This is crucial in maintaining pregnancy continuation. The placenta and amniotic membranes form a mechanical barrier (Tita, Andrews 2010) including syncytiotrophoblasts surrounding the villi (Redline 2004). Amnion epithelium and trophoblasts also act as a functional barrier by producing antimicrobial

peptides (King *et al.* 2007a, 2007b, Stock *et al.* 2007). The mucus plug in the closed cervical canal is also both an anatomical and functional barrier to microbial invasion from the vagina (Kim *et al.* 2015a). Functional barriers also include vaginal lactobacilli flora affecting the virulence of micro-organisms (Tita, Andrews 2010), and cervical epithelial cells´ microbicidal products (cathelicidin, calgranulins, and defensins like elafin) participating in blocking ascending infections (Hein *et al.* 2002, Stock *et al.* 2009, Buhimschi *et al.* 2013, Frew *et al.* 2014), and endometrial glandular cells producing antibacterial proteins (Redline 2004).

In the chorioamniotic unit, neutrophils and decidua originate from maternal side, but the villus tree and chorioamniotic membranes from the fetal side (Kim *et al.* 2015a). In a normal, non-infectious situation neutrophils are absent from the chorioamniotic membranes (Kim *et al.* 2015a), but the number of maternal neutrophils increases shortly before upcoming labor in the decidua and myometrium (Keski-Nisula *et al.* 2000, 2003, Gomez-Lopez *et al.* 2014). Inflammation in such tissues is physiological phenomena occurring during normal labor.

Definition and classification

Although the term “chorioamnionitis” in clinical practice often refers to several signs and symptoms, for example to uterine tenderness, infectious discharge from the uterine cervix, or increased infection parameters along with maternal fever, chorioamnionitis can be subdivided into two distinctive subgroups: clinical and subclinical. The latter consists of HCA and intra-amniotic inflammation with or without microbiologic etiology (Figure 4).

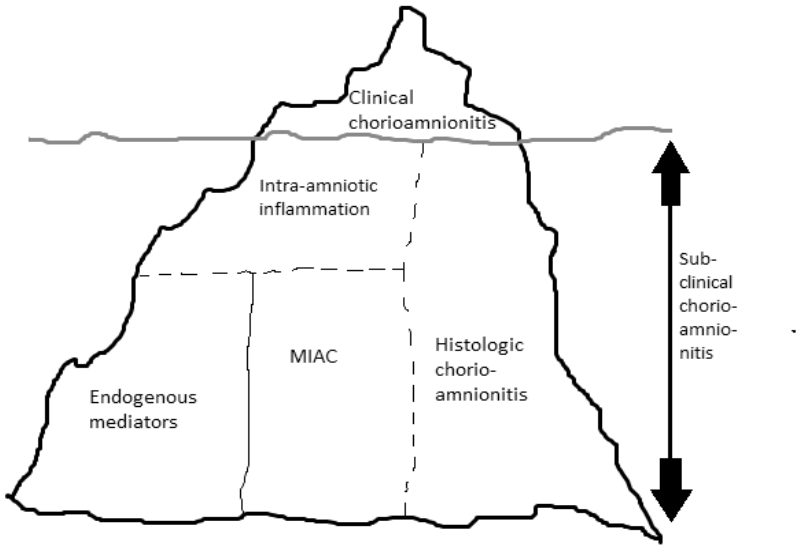


Figure 4. Schematic diagram of chorioamnionitis types. Subclinical chorioamnionitis can be subdivided into histologic chorioamnionitis (HCA), intra-amniotic infection (IAI), and intra-amniotic inflammation. IAI means intra-amniotic inflammation in the presence of microbial invasion of the amniotic cavity (MIAC). Drawn by the author.

Clinical chorioamnionitis

Clinical chorioamnionitis has been traditionally defined by Gibbs criteria since the 1970s (Gibbs 1977, Sung *et al.* 2016). It includes maternal fever ≥ 38 ºC with at least one or two of the following criteria: uterine tenderness, maternal or fetal tachycardia, foul-smelling or infectious discharge from the uterine cervix, or total maternal white blood cell (WBC) count > 20 x 10^9/L (Fishman, Gelber 2012, Romero *et al.* 2015).

Intra-amniotic infection and inflammation

Intra-amniotic infection (IAI) is defined as elevated concentration of inflammatory markers, such as IL-6, and matrix metalloproteinase -8 (MMP-8) in AF in the presence of MIAC. The microbial colonization, *per se*, is not considered consistent with infection; an inflammatory component is necessary (Combs *et al.* 2014). Intra-amniotic inflammation in the absence of MIAC results from activation of endogenous mediators which evoke a host response and lead to progression of an inflammatory process.

Histologic chorioamnionitis

The placenta consists of three parts: the chorionic plate, the chorioamniotic membranes, and the umbilical cord. The term HCA refers to inflammatory changes, i.e. neutrophil infiltration, mainly polymorphonuclear leukocytes, in any of these parts.

HCA can be subdivided according to inflammatory changes in the villus tree (villitis), vessels (vasculitis), or umbilical cord (funisitis). Funisitis is defined as a neutrophil infiltration into the vessel walls of the umbilical cord or into Wharton´s jelly (Park *et al.* 2016). It is considered a more severe stage of HCA, since it reflects the spread of inflammation to the umbilical cord, and is the fetal counterpart to the maternal infection (Tita, Andrews 2010).

HCA covers two subtypes of chorioamnionitis: with MIAC (infectious) and without MIAC (sterile) (Romero *et al.* 2014c). Inflammatory changes in the latter are thought to be caused by the host immune response (Redline 2004) or by meconium (Menon *et al.* 2010). Manifestation of the infection depends on the virulence of microbes in the chorioamniotic space: subsequent clinical chorioamnionitis follows infection by highly virulent microbes, whereas subclinical HCA usually occurs in the presence of low virulence microbes (Galinsky *et al.* 2013).

Risk factors for chorioamnionitis

PPROM and the latency time between membrane rupture and delivery are among the most important risk factors for chorioamnionitis (Tita, Andrews 2010, Fishman, Gelber 2012) (Table 1).

Table 1. Risk factors for chorioamnionitis.

Nulliparity	Digital examinations during delivery
Prolonged PPROM	Meconium-stained amniotic fluid
Bacterial vaginosis	Prolonged duration of delivery
Sexually transmitted genital infections	Intrauterine monitoring (CTG, IUP)
Alcohol- or drug abuse	Epidural use
Tobacco smoking	GBS colonization in maternal vagina or perineum
Immunosuppression	
African-American ethnicity	
Altered placental or vaginal microbiome	

Based on data from Tita 2010, Fishman 2012, Johnson 2014, Prince 2014
CTG, cardiotocography; GBS, *Group B Streptococcus*; IUP, intrauterine pressure; PPROM, preterm prelabor rupture of the membranes

Microbiome

Women with preterm labor have a diminished amount of protective *Lactobacillus* spp. in their vaginal flora and a predominance of *Gardnerella* or *Ureaplasma* species (DiGiulio *et al.* 2015). Moreover, pre-existing viral load may induce inflammation or preterm birth (Ramos Bde *et al.* 2015).

Formerly, the genital tract beyond the cervix was presumed to be sterile, but the placenta harbors its own microbiome, which is more related to an oral microbiome than to a vaginal microbiome (Aagaard *et al.* 2014, Mysorekar, Cao 2014, Prince *et al.* 2014, Fox, Eichelberger 2015, Prince *et al.* 2016). The placental microbiome differs between women with preterm labor or a history of antenatal infection and those achieving full-term pregnancies (Prince *et al.* 2016) (Table 2). The most common bacterium of the placenta is *E.coli* (Aagaard *et al.* 2014). Placental microbes are usually not pathogenic. However, the altered immune system during pregnancy may lead to hematogenous colonization, changes in the community of placental microbes, and thereafter to dysbiosis, which can evoke an inflammatory response leading to preterm labor or PPROM (Prince *et al.* 2014).

Table 2. Maternal microbiome by pregnancy outcome.

Preterm labor, intact membranes	**Preterm labor, PPROM**	**Term labor without chorioamnionitis**	**Term labor with chorioamnionitis**
Oral:		Oral, Non-pregnant:	
Fusobacterium *Bergeyella* spp. *Streptococcus* spp.	NA	*Firmicutes* *Actinobacteria* *Bacteroidetes* *Proteobacteria* *Fusobacterium*	NA
Amniotic fluid:	Amniotic fluid:	Amniotic fluid:	Amniotic fluid:
Ureaplasma spp.	*Ureaplasma* spp.	*Ureaplasma spp.*	*Ureaplasma spp.*
Mycoplasma spp.	*Mycoplasma* spp.	*Mycoplasma*	*Mycoplasma*
Fusobacterium	*Fusobacterium*	*Coagulase negative Staphylococcus*	*Fusobacterium*
Sneathia	*Sneathia*	*Streptococcus agalactiae*	*Sneathia*
Bacteroides	*Bacteroides*	*Lactobacillus* spp.	*Bacteroides*
Prevotella	*Prevotella*		*Acinetobacter* spp.
Leptotrichia	*Leptotrichia*		*Lactobacillus* spp.
Peptostreptococcus	*Enterococcus*		*Enterococcus*
Escherichia coli	*Hemophilus*		*Gardnerella*
Gardnerella	*Streptococcus*		*Streptococcus*
Bacillus	*Staphylococcus*		*Escherichia coli*
Placenta:	Placenta:	Placenta:	Placenta:
Ureaplasma spp.↑	*Ureaplasma spp.*↑	*Enterobacter*	*Enterobacter*
Acinetobacter↓	*Acinetobacter*↓	*Acinetobacter*	*Acinetobacter*
E.coli	*E.coli*	*E.coli*	*E.coli*
Enterobacter	*Enterobacter*	*Lactobacillus* spp.	*Lactobacillus* spp.
Fusobacterium ↑	*Fusobacterium* ↑		
Lactobacillus crispatus ↓	*Lactobacillus crispatus* ↓		
Vagina:		Vagina:	
Lactobacillus crispatus		*Lactobacillus* spp.	

↑ increased microbe level compared to that of term pregnancy without chorioamnionitis

↓decreased microbe level compared to that of term pregnancy without chorioamnionitis

NA, not available

Data modified from Prince 2014, DiGiulio 2012, Keski-Nisula 1997, Kim 2015, Aagaard 2016, Prince 2016

Incidence

Clinical chorioamnionitis occurs in 1 to 4% of all pregnancies in developed countries (Johnson *et al.* 2014). Data of developing countries are lacking. The rate is higher in preterm pregnancies (5-15%) than in term pregnancies (1-2%) (Edwards 2005). Chorioamnionitis cannot develop until the amnion and chorion are fused (on average 11 weeks of gestation), and it occurs rarely before fusion of the placental membranes with the uterine cavity (on average at 19 weeks of gestation) (Redline 2012). In pregnancies complicated with PPROM clinical chorioamnionitis occurs in up to 26% (van de Laar *et al.* 2009). Of women with clinical chorioamnionitis, 62% have HCA, and 60% have funisitis (Tita, Andrews 2010). Subclinical chorioamnionitis is more common than clinical chorioamnionitis, which occurs in only 18% of pregnancies with intra-amniotic inflammation (Buhimschi *et al.* 2013).

IAI (57% vs 42%) and intra-amniotic inflammation (29% vs 21%) occur more frequently in PPROM pregnancies than in preterm pregnancies with intact membranes (Park *et al.* 2013d) (Table 3).

Table 3. The rate of chorioamnionitis

Study	n	Setting	Term/ Preterm	Outcome	Prevalence %
Romero et al 2014	135	Intact membranes	Preterm	Sterile inflammation IAI	26 11
Musilova et al 2015	166	PPROM	Preterm	Sterile inflammation IAI	4 21
Romero et al 2014	100	PPROM and intact membranes	Term and preterm	MIAC Inflammation	34 40
Romero et al 2015	59	PPROM	Preterm	Sterile inflammation IAI HCA among those without inflammation HCA among those with IAI	29 29 38 93.3
Romero et al 2014	231	Intact membranes cx<25 mm without symptoms	Preterm <30 GW	Inflammation IAI HCA without inflammation HCA among those with inflammation HCA among those with IAI	10.4 2.2 46.9 41.7 100
Kim et al 2015		Review	Term and preterm	MIAC with CC at term MIAC with preterm labor and intact membrans MIAC with PPROM without labor	61 8.7-34 17-57.7
Horvath et al 2014	4237	Intact membranes; retrospective analysis	Term Preterm	HCA	5.1 30
Lahra et al 2004	3928	Cohort of 24-34 GW	20-24 GW 34 GW	HCA	66 16
Erdemir et al 2013	43	PPROM and intact membranes	<35 GW	HCA CC	23.2 8.3
Kim et al 2016	146	PPROM	20-33 GW	HCA CC MIAC	50.7 8.9 34.9

Kim SM et al 2014	412	Intact membranes	24-35 GW	HCA Inflammation HCA among those with inflammation HCA without inflammation HCA among those with MIAC	44.4 42.0 72.7 20.9 31.1
Roberts et al 2012	195	Intact membranes; low-risk women	Term	HCA	34
Ovalle et al 1998	71	PPROM	24-34 GW	HCA HCA with MIAC	58 51
Odibo et al 1999	88	Intact membranes	22-36GW	HCA MIAC	60.2 17
Seong et al 2008	884	Intact membranes	Term	MIAC not in labor MIAC in active labor	1 13
Cobo et al 2012	66	PPROM	22-33+6 GW	MIAC	20
Romero et al 2014	142	Intact membranes	20-35 GW	MIAC By PCR/ESI-MS MIAC by culture HCA in culture+, PCR+ HCA in PCR+ HCA in culture-, PCR-	12 7 100 70 35

GW, gestational weeks; HCA, histologic chorioamnionitis; MIAC, microbial invasion of the amniotic cavity; IAI, intra-amniotic infection; CC, clinical chorioamnionitis; PPROM, preterm prelabor rupture of the membranes; PCR, polymerase chain reaction; ESI-MS, electrospray ionization time-of-flight mass spectrometry

The prevalence of **MIAC** in preterm labor or in PPROM varies greatly among studies. The discovery of bacteria in AF was first reported in 1927 (DiGiulio 2012). In PPROM cases, MIAC occurs in 20 to 50%, while in preterm labor with intact membranes, MIAC is detectable in 6 to 20% (Yoon *et al.* 1998, Ovalle *et al.* 2006, DiGiulio *et al.* 2010, Lisonkova *et al.* 2014, Romero *et al.* 2014c). The occurrence of preterm contractions in the latter situation increases the prevalence of MIAC to as high as 50% (Genc, Ford 2010). MIAC also occurs in 7 to 22% of women having a short cervix (Gomez *et al.* 2005, Hassan *et al.* 2006, Kusanovic *et al.* 2007). Furthermore, bacteria and viruses may be detectable in AF also in uneventful pregnancies (Ramos Bde *et al.* 2015). Microbes are reported in AF in only about 1% of women at term pregnancy before parturition without any signs of infection (Seong *et al.* 2008).

The rate of polymicrobial MIAC cases shows a wide variability between 9 and 67% (Tita, Andrews 2010, DiGiulio 2012, Romero *et al.* 2014a, Kim *et al.* 2015a). The most common microbe found in AF is *Ureaplasma urealyticum /parvum*, a typical microbe of the lower genital tract (Combs *et al.* 2014, Murtha, Edwards 2014). The spectrum of microbes differs between women with preterm labor with intact membranes and PPROM (DiGiulio 2012).

The role of viruses in the field of IAI is not yet fully understood, though their presence in the AF is more frequent than previously thought. (Baschat *et al.* 2003). Viruses isolated from AF include *parvovirus B19, human herpesvirus-6, cytomegalovirus, Epstein-Barr* virus*, enterovirus, adenovirus, syncytial* virus*, and zika* virus (Ramos Bde *et al.* 2015, Calvet *et al.* 2016), though no proven association exists between AF viruses and adverse outcomes or pregnancy loss (Miller *et al.* 2009, Bopegamage *et al.* 2013, Ramos Bde *et al.* 2015). However, *adenovirus* has been detected in 40% of preterm placentas, and HCA also occurs more frequently in placentas containing *adenovirus* (Tsekoura *et al.* 2010).

Candida albicans is the most common of the *Candida species* detected from AF (DiGiulio 2012). The rate of *Candida* in AF is quite low, but robust neonatal infections have been reported, often related to early gestational age (DiGiulio 2012).

Archaea also have the pathogenic potential to promote MIAC (DiGiulio 2012), but with no IAI cases reported. Protozoa, like *Toxoplasma gondii* and *Trypanosoma cruzi,* have also been detected in the AF (DiGiulio 2012), but studies concerning *Plasmodium malariae* in AF are lacking, although placental malaria is, however, quite common (DiGiulio 2012). No IAI resulting from these zoonoses has been reported.

The lower the gestational age, the higher the frequency of **HCA** (Lahra, Jeffery 2004). Variation in HCA frequency is great from 94% at a lower gestational age to 5% at full term (Horvath *et al.* 2014, Kim *et al.* 2015a). However, the absolute number of HCA cases in term pregnancies is high (Roberts *et al.* 2012). Racial variability has also been reported in the frequency of HCA (Nadeau *et al.* 2016). The rate of HCA varies markedly, from 24 to 80% with PPROM (Menon *et al.* 2010, Lee *et al.* 2013a, Xie *et al.* 2015), and up to 88% in women with intact membranes and preterm labor (Greig *et al.* 1994, Menon *et al.* 2010). In term pregnancies, HCA is mainly non-infectious, instead is just an inflammatory phenomenon *(Roberts et al.* 2012). Labor itself is an inflammatory process (Keski-Nisula *et al.* 2000), which is evident in term pregnancies as a higher rate of HCA in women in labor than prior to labor (Keski-Nisula *et al.* 2000, *Kim et al.* 2015a). Sterile HCA is more common than MIAC-related HCA in preterm pregnancies with intact membranes, and it occurs more frequently at a lower gestational age, as does microbe-related HCA (Romero *et al.* 2014c). In PPROM pregnancies, the rate of sterile HCA has been reported at 44% (75/167 cases) (Vajrychova 2016).

Funisitis occurring in 60% of pregnancies with HCA (Tita, Andrews 2010) is present in about 1% of term pregnancies with intact membranes without clinical signs of chorioamnionitis and in 7% with prelabor rupture of the membranes before parturition (Lee *et al.* 2006). During labor, funisitis occurs in 3% if membranes are intact and in 4% if membranes are ruptured (Lee *et al.* 2006). In preterm pregnancies, funisitis occurs in 31% in PPROM pregnancies with HCA, but in pregnancies with intact membranes it does not occur without IAI, even in the presence of HCA (Park *et al.* 2016).

Diagnosis

Clinical chorioamnionitis

The use of maternal fever as an obligatory criterion for the diagnosis of clinical chorioamnionitis originally described by Gibbs in 1977 is puzzling. Recent evidence show that the removal of fever as an essential criterion for chorioamnionitis enhances the sensitivity of prediction of neonatal sepsis in all preterm pregnancies between 24 and 34 weeks of gestation (Sung *et al.* 2016). The symptoms and signs of chorioamnionitis can also result from physiological reactions to delivery, making, however, the clinical criteria of chorioamnionitis neither sensitive nor specific. In the diagnosis of IAI, it is unacceptable to use such method with a maximal accuracy of only 57% (Romero *et al.* 2016b).

Maternal fever as an isolated finding occurs in about 15% of term deliveries (Evers *et al.* 2012). Other common conditions causing fever during labor include epidural anesthesia, dehydration, extrauterine infections, hyperthyreosis, and prostaglandins used for labor induction (Greenwell *et al.* 2012, Curtin *et al.*

2015, Higgins *et al.* 2016, Sultan *et al.* 2016). After clinical protocol to administrate antibiotic prophylaxis to all women positive for Group B Streptococcus and to women with PPROM, the role of fever as a sign of chorioamnionitis in the presence of other symptoms of chorioamnionitis has become disputed (Sung *et al.* 2016). However, intrapartum fever, *per se*, can cause changes in fetal immune response (Mazaki-Tovi, Vaisbuch 2016), and is recommended to commence clinical interventions (Avila *et al.* 2015).

The role of maternal CRP in the diagnosis of chorioamnionitis is controversial. Maternal plasma CRP value ≥20 mg/L and WBC value ≥15 x 10^9/L have served as diagnostic markers for chorioamnionitis (Keski-Nisula *et al.* 1995), but 10 to 12 hours pass before the inflammatory response is evident in CRP (Hofer *et al.* 2012). Furthermore, maternal CRP and WBC may be elevated during normal labor (Keski-Nisula *et al.* 1995), and CRP increases also in uneventful pregnancy when compared to the non-pregnant stage (Anderson *et al.* 2013). Close to delivery, the levels of CRP and WBC may show a wide range of variability, making a prediction of chorioamnionitis based on those values unreliable (Le Ray *et al.* 2015). Furthermore, CRP and WBC have shown poor diagnostic performance for HCA (Sereepapong *et al.* 2001), for clinical chorioamnionitis (van de Laar *et al.* 2009), or at least their clinical value is limited (Le Ray *et al.* 2015). In one study, however, CRP was a better predictor for HCA in cases without IAI than was AF MMP-9 or IL-6 (Oh *et al.* 2011).

Studies of CRP and chorioamnionitis in pregnancies with intact membranes are few (Cammu *et al.* 1989, Park *et al.* 2013b). In one such study, CRP in preterm pregnancies was associated with IAI (Park *et al.* 2013c). In systematic reviews of PPROM pregnancies, no clear consensus exists as to use of CRP in diagnosis of chorioamnionitis (Trochez-Martinez *et al.* 2007, van de Laar *et al.* 2009).

Nor is fetal cardiotocography a particularly sensitive tool in chorioamnionitis diagnostics, since only 16 to 38% of neonates having an infection had tachycardia during the delivery (Evers *et al.* 2012). Studies concerning fetal tachycardia prior to delivery in cases of chorioamnionitis are lacking, but one case report concerns identified tachycardia (Kelly *et al.* 2014).

Intra-amniotic infection / inflammation

Intra-amniotic infection / inflammation can be diagnosed through AF biomarkers obtained by AC or vaginally, through maternal serum biomarkers, through vaginal or cervical fluid biomarkers, or by means of risk-based systems.

Amniocentesis

Amniocentesis (AC) has been performed since 1877 (Woo 2006). It has been in use mostly for genetic karyotyping since 1956 (Fuchs, Riis 1956), and has served, though less frequently, for diagnosis of intra-amniotic infection since 1979 (Garite *et al.* 1979). Other common indications for this invasive procedure include testing for fetal lung maturation or chronic fetal hypoxia (erythropoietin).

Neonatal outcome was indeed improved when AC was incorporated into the protocol of management of PPROM pregnancies (Hitti *et al.* 2001, Porreco *et al.* 2008, Maki *et al.* 2015, Archabald *et al.* 2016) (Table 4).

Table 4. Amniocentesis and neonatal outcome.

Study	Year	n	Gestational age (weeks) at AC	Amniotic fluid markers studied	Setting	Outcome
Porreco	2008	AC 147, No AC 146. Neonates AC 154, No AC 167	24+0 - 35+6	Gram Stain, microbial culture, White cell count, glucose	PPROM pregnancies, compared retrospectively neonatal outcome in groups AC vs No AC. Also twins included.	Composite neonatal morbidity was increased in No-AC group OR 2.94 (95%CI 1.68-5.15); No difference in PVL, IVH, death, NEC, ROP. Neonatal sepsis in No-AC group similar to AC group with positive culture.
Hitti	2001	151	<34	Microbial culture, IL-6, TNF-α	Intact membranes, threatened preterm labor. Association of biomarkers / culture with adverse neonatal outcome, adjusted for gestational age.	AF infection and/or elevated TNF-α was associated with RDS aOR 1.7; Gr 3-4 IVH aOR 2.2; NEC aOR 1.8; Multiple organ dysfunction aOR 3.0
Maki	2015	AC 35, No AC 33	22-28	Gram Stain, microbial culture, White cell count, glucose	Intact membranes, threatened preterm labor. Comparison of neonatal outcome in AC vs No AC groups.	EONS n=5 vs n=13, p<0.03 Short-term outcome n=8 vs n=20, p<0.05 Long-term outcome n=7 vs n=21, p<0.01
Archabald	2016	Total 185, AC 123	24.8 - 33.1	Glucose, LD, WBC count, Gram stain, microbial culture	185 women with a pregnancy complicated with PPROM. AC for 123 women. Neonatal cord blood biomarkers and short term neonatal outcome was compared in the groups: AC+, proven infection, AC+, no infection, and no AC.	Antenatal exposion to IAI elevates risk for adverse neonatal outcome OR 3.0 (95% CI 1.15-7.59); higher gestational age and AC reduced risk for adverse neonatal outcome OR 0.61 (95%CI 0.52-0.7) and OR 0.37 (95% CI 0.14-0.95), respectively.

AC, amniocentesis; PPROM, preterm prelabor rupture of the membranes; OR, Odds ratio; aOR adjusted Odds Ratio; GW, gestational weeks; IL-6, interleukin-6; LD, lactate dehydrogenase; TNF-α, Tumor necrosis factor-α; RDS, respiratory distress syndrome; IVH, intraventricular hemorrhage; PVL, periventricular leucomalacia; ROP, retinopathy of prematurity; NEC, necrotizing enterocolitis; EONS, early-onset neonatal sepsis

Short-term outcome, presence of variables: IVH gradus 3-4, periventricular leucomalacia, hydrocephalus and brain atrophy.

Long-term outcome, presence of variables: death, cerebral palsy, epilepsy, mental retardation at age 1-2 years.

AC is usually performed transabdominally with ultrasound guidance and an aseptic technique. It is an invasive but a relatively safe procedure (Yeast *et al.* 1984, Gordon *et al.* 2002, Akolekar *et al.* 2015). The most common complications afterwards are miscarriage before 24 weeks of gestation (0.4-0.81%) (Enzensberger *et al.* 2012, Akolekar *et al.* 2015), or rupture of the amniotic membrane (0.9-4.2%) (Zalud,

Janas 2008, Lee *et al.* 2013b), as well as intra-amniotic infection (0.1-0.4%), and septic shock as a rare consequence (0.03-0.19%) (Wurster *et al.* 1982). The risk for any complication following AC that requires delivery is 0.7% (Stark *et al.* 2000). Notably, intra-amniotic inflammation / infection is the only variable associated with membrane rupture after AC (Lee *et al.* 2013b).

Amniotic fluid biomarkers:

AF WBC count, which is increased in inflammatory conditions, has for many years been the gold standard indicating AF inflammation (Buhimschi *et al.* 2013, Park *et al.* 2013a). WBC lysis in the presence of bacteria may yield an artificially lower result, and contamination with blood may yield false positive results. For the role of selected biomarkers in normal pregnancies as well as their sites of production, see Table 5.

Several other biomarkers studied in the diagnosis of IAI are not in clinical use. One of those is CA12-5, which has shown an association with intra-amniotic inflammation and preterm labor (Seong 2016). During recent years, biomarkers related to oxidative stress and inflammation as well as AF proteomics have been under investigation (Cobo *et al.* 2013, Chafer-Pericas *et al.* 2015).

Increased concentration of **AF-LD** reflects inflammation or neutrophil activation in the amniotic cavity. Lactate dehydrogenase (LD) is an enzyme which catalyzes the reversible conversation of lactate to pyruvate at the end of the glycolytic pathway. Leukocytes and macrophages at the site of inflammation are the major source of aerobic glycolysis and therefore of LD (Magloire *et al.* 2006a). In body fluids, for example in ascites fluid, LD acts as an inflammation marker (el-Touny *et al.* 1989, Kidokoro *et al.* 2006). The accuracies of AF-LD and other biomarkers for inflammation and infection in the setting of preterm labor with and without PPROM are presented in Table 6. One possible source of bias concerning AF-LD is the blood in AF producing falsely high AF-LD levels.

Table 5. Characteristics of amniotic fluid inflammatory biomarkers investigated.

Biomarker	Site of production	Role in normal pregnancy	Effect of infection	Effect of PPROM	References
LD	Neutrophils	Catalyzes oxidation of lactate to puryvate.	Raises	?	Garry et al 1996, Kidokoro et al 2006, Miura et al 2011
Glucose	Maternal liver and muscles	Reflects maternal serum glucose concentration.	Reduces	?	Hussey et al 1998, Rinala et al 2009
MMP-8	Neutrophils	Degrades extracellular matrix at parturition.	Raises	No effect	Maymon et al 2000, Romero et al 2014
Cathelicidin	Neutrophils and epithelial cells	Modulates adaptive immunity and takes part in innate immunity.	Raises	?	Tambor et al 2012
MMP-2	Fetal membranes	Cleave denatured collagen and basement membrane components. Major modulator of fetal membrane integrity throughout gestation.	Reduces*	Reduces	Maymon et al 2000, Locksmith et al 2001, Vincent et al 2015
MMP-9	Fetal membranes	Cleave denatured collagen and basement membrane components. Modulator of fetal membrane strength. Plays an important role in the activation of term labor.	Raises	Raises	Maymon et al 2000, Locksmith et al 2001, Weiss 2007, Vincent et al 2015
Timp-1	Fetal membranes	Regulates the concentration of matrix metalloproteinases.	Raises	Reduces	Vadillo-Ortega et al 1996, Locksmith et al 2001
IL-6	Amnion, choriodecidua, fetus	Pro- and anti-inflammatory role. Down-regulation of expression of pro-inflammatory cytokines.	Raises	Reduces	Lee SY et al 2011, Romero et al 2014
MPO	Neutrophils	Produces oxidative radicals, activates endothelial cells to produce cytokines, extra- and intracellular hypochlorus acid production.	Raises	?	Odobasic 2016
Elastase	Neutrophils	Hydrolyzes connective tissue components outside cells, such as elastin and collagen types I-IV. Plays a role in the pathogenesis of membrane rupture.	Raises	Raises	Kidokoro et al 2006, Miura et al 2011, Czajka 2009
Elafin	Amnion, chorion, decidua, placental syncytiotrophoblast	Prevents uterine infection. Regulation of inflammation. Inhibitory effect on neutrophil elastase. Chemotactic activity on neutrophils and macrophages.	Raises	Reduces	King et al 2007a, King et al 2007b, Stock 2007
CRP	Fetal liver	Not fully clarified	Raises	?	Malek et al 2006

*Only in PPROM pregnancies

Table 6. Test performances of selected amniotic fluid biomarkers for infection and inflammation.

Study	Year	N	GA at testing	Membrane status	Outcome	Biomarkers	cut-off	Sensit	Specif	PPV	NPV
Romero et al	1990	168	na	Intact	MIAC	Glucose	14 mg/dL	86.9	91.7	62.5	97.8
Garry et al	1996	131	23-35	Intact	MIAC	LD Glucose	419 IU/L 17 mg/dL*	75.0 81.0	90.0 93.0	50.0 62.0	96.0 97.0
Odibo et al	1999	88	22-36	Intact	HCA	Glucose	15 mg/dL**	28.3	94.6	88.2	47.9
Maymon et al	2001	371	22.4-33.2	Intact	MIAC	MMP-8 IL-6	30 ng/mL 17 ng/mL	82.4 62	78.0 90.0	36.0 30.5	97.7 94.3
Edwards et al	2001	44	22-35	Intact	MIAC	Glucose MMP-9 IL-6	15 mg/dL** 351 ng/mL 8.9 ng/mL	83 83 83	87 92 89	56 63 56	97 97 97
Harirah et al	2002	84	na	Intact and PPROM	MIAC	IL-6 MMP-9 IL-6+MMP-9	11.4 ng/mL 13.6 ng/mL	73 77 80	79 100 100	61 100 100	86 90 92
Kidokoro et al	2006	60	16-35	Intact and PPROM	HCA	LD HNE Glucose	250 IU/L 0.15ug/mL 30 mg/dL	84.1 88.9 82.2	66.7 73.3 66.7	88.1 90.9 88.1	58.8 68.8 55.6
Buhimschi et al	2007	169	17 - 36.1	Intact and PPROM	MIAC	LD Glucose IL-6 MMP-8	419 IU/L 15 mg/dL** 11.4 ng/dL 23.0 ng/dL	60.6 70.5 45.2 90.7	88.5 80.6 61.9 65.0	62.5 56.4 70.4 47.6	87.6 88.5 36.1 95.2
Miura et al	2011	56	15-35	Intact and PPROM	HCA	HNE IL-6	3563 ng/mL 11.3 pg/mL	72.0 95.7	90.3 54.8	85.7 63.0	80.0 94.1
Oh et al	2011	99	21-35	Intact and PPROM	HCA	IL-6 MMP-9	2.6ng/mL 15 ng/mL	68.2 65.9	81.8 80.0	75.0 72.5	76.3 74.6
Oh et al	2011	99	21-35	Intact and PPROM	IAI	IL-6 MMP-9	2.6ng/mL 15 ng/mL	89.3 96.4	78.9 81.7	62.5 67.5	94.9 98.3
Tambor et al	2012	72	24 - 34+5	PPROM	MIAC and HCA	Cathelicidin	4 ng/mL	47.0	95.0	na	na
Cobo et al	2012	47	22 - 33+6	PPROM	MIAC	IL-6	na	69.0	81.0	47.0	91.0
Romero et al	2014	100	22.3-33.4	Intact and PPROM	IA infl	IL-6 MMP-8	11.4 ng/mL 23ng/mL	95.0 97.5	91.7 70.0	na	na
Romero et al	2014	100	22.3-33.4	Intact and PPROM	MIAC	IL-6 MMP-8	11.4 ng/mL 23ng/mL	85.3 94.1	78.8 62.1	na	na
Chaemsaithong et al	2016	136	27-32.4	Intact	MIAC HCA or FS FS	IL-6	2.6 ng/mL	81.8 93.6 95.2	63.2 52.2 39.4	30.0 72.5 50.0	94.7 85.7 92.9

*17mg/dL = 0.94 mmol/L
**15mg/dl = 0.84 mmol/L

GA, gestational age; Sensit, sensitivity; Specif, specificity; PPV, positive predictive value; NPV, negative predictive value; PPROM, preterm prelabor rupture of the membranes; LD, lactate dehydrogenase, MMP-8, matrix metalloproteinase-8, IL-6, interleukin-6; HNE, neutrophil elastase; MMP-9, matrix metalloproteinase-9; HCA, histologic chorioamnionitis; IAI, intra-amniotic infection; FS, funisitis; IA infl, intra-amniotic inflammation

AF-glucose determination is a standard method for detecting IAI. AF-glucose levels show a negative correlation with AF inflammatory biomarkers, for example with LD (Garry *et al.* 1996). At the time of IAI, the pentose phosphate pathway of microbes fed by glucose is activated, leading to decreased concentrations of AF-glucose (Prince *et al.* 2016). AF-glucose concentrations are associated in high-risk pregnancies with maternal serum glucose concentrations (Rinala *et al.* 2009).

The family of **matrix metalloproteinases** (MMPs), consisting of 23 zinc- and calcium-dependent peptidases, represents genetically distinct but structurally related proteases with a potent ability to degrade almost all extracellular matrix components and modify immune responses by modulating cytokines and chemokines (Sorsa *et al.* 2006, Van Lint, Libert 2006, Alfakry *et al.* 2016). This action is necessary for recruitment of inflammatory cells to the inflammatory site (Alfakry *et al.* 2016). The main components of such extracellular matrix in the amnion-chorion are collagens (Vadillo-Ortega *et al.* 1996). MMPs, which can activate each other and form immunological cascades together with their counterparts (Alfakry *et al.* 2016), are produced by activated decidual neutrophils of maternal origin (Gomez-Lopez *et al.* 2014), and these degranulate MMPs and other proteolytic enzymes, *e.g.* neutrophil elastase (NE), as a response to inflammation and neoplasms (Weiss *et al.* 2007).

Structural similarities of MMPs indicate that they probably come from the same ancestor (Sorsa *et al.* 2006). They are subdivided based on their structure into collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, -11), matrilysins (MMP-7, -26), membrane-type MMPs (MMP-14, -15-17, -24, -25), and others (MMP-12, -19-21, -23, -27, -28).

During uncomplicated pregnancy MMP-1, -2, -3, -7, and -9 exist in AF and in the membranes, although during pregnancy until labor the amount of MMP-9 is low (Weiss *et al.* 2007).

MMP-8 (collagenase -2, neutrophil collagenase) is the most impressive initiator of destruction of the major collagen (collagen type-1) of the fetal membranes, the one providing their strength (Maymon *et al.* 2000b, Sorsa *et al.* 2011). MMP-8 is synthesized and stored mainly in polymorphonuclear neutrophils (PMNs) in secondary granules as a latent enzyme (Alfakry *et al.* 2016), and upon stimulation is released as a mature MMP-8. Several MMP-8 isoforms exist (Hanemaaijer *et al.* 1997). Notably, during inflammation, MMP-8 is expressed in other cell types as well, such as monocytes/macrophages, plasma cells, and epithelial cells and it participates in leucocyte recruitment.

MMP-8 levels are increased in preterm labor, in PPROM, and in IAI (Maymon *et al.* 2001a, 2001b), and MMP-8 participates in the fetal inflammatory response to IAI (Park *et al.* 2009, Lee *et al.* 2015) and has shown an association with neonatal morbidity with a cut-off of 30 ng/mL (Maymon *et al.* 2001a).

MMP-2 belongs to the gelatinases. It is expressed in endothelial cells and trophoblasts and is not produced by neutrophils (Seval *et al.* 2004). It is activated from proMMP-2 that exists on the cell surface (Sorsa *et al.* 2006, Vincent et al. 2015). At labor, MMP-2 is the major MMP in the decidua (Weiss *et al.* 2007) and responsible for its gelatinolytic activity. MMP-2, together with MMP-9, is responsible for the mechanism of membrane rupture (Maymon *et al.* 2000a), although MMP-2 is present in the fetal membranes throughout

the pregnancy; its concentration increases soon before oncoming labor (Vincent *et al.* 2015). In AF, MMP-2 levels are higher in midtrimester than at term, prior to labor (Maymon *et al.* 2000a). The effect of PPROM on MMP-2 levels has been contradictory among studies: Fortunato *et al.* (1999) reported an increase in MMP-2 levels, whereas Maymon *et al.* (2000a) reported a decrease in the PPROM setting. Levels of MMP-2 remain unchanged in preterm labor with intact membranes (Fortunato *et al.* 1999).

MMP-9 (gelatinase B) is produced mainly in neutrophils, but also in macrophages, eosinophils, and smooth muscle cells, as well as in endothelial and epithelial cells. It is stored in the secondary granules of neutrophils as a latent enzyme, as is MMP-8 (Alfakry *et al.* 2016). Unlike MMP-8, MMP-9 is released as an inactive form and is activated locally.

MMP-9 levels are increased during inflammation and neoplasm, and its expression can be induced by MMP-7 and by various cytokines (Sorsa *et al.* 2006, Alfakry *et al.* 2016). MMP-9 is not evident in fetal membranes without infection or labor (Weiss *et al.* 2007), but in AF its level remains almost constant during the pregnancy prior to labor (Maymon *et al.* 2000a).

Tissue inhibitor of metalloproteinases -1 (TIMP-1) is a glycosylated protein that can inhibit all MMPs except MMP-19 and membrane-type MMPs (Sorsa *et al.* 2006). Four TIMPs exist, each having its own targets (Visse, Nagase 2003). TIMPs are expressed in normal conditions as well as in inflammatory stages, and are produced mainly by endothelial cells, smooth muscle cells, monocytes, and macrophages (Alfakry *et al.* 2016); but in neutrophils it is scarce. Findings on TIMP-1 levels in cases of PPROM are contradictory. Fortunato reported them to be slightly increased, whereas the latter two studies reported them to be decreased in PPROM, reflecting the imbalance in MMP-8/TIMP-1 leading to rupture of the membranes (Vadillo-Ortega *et al.* 1996, Fortunato *et al.* 1999, Weiss *et al.* 2007).

Cathelicidin is an antimicrobial peptide participating in the innate host defence system (Ramanathan *et al.* 2002). The only cathelicidin in humans is hCAP-18/LL-37 (Tambor *et al.* 2012). Its active form is expressed mainly by neutrophils and epithelial cells including amnion epithelium (Tambor *et al.* 2012, Wang *et al.* 2014), but in a smaller amount it appears also in lymphocytes, monocytes, and mast cells (Williams *et al.* 2006). Cathelicidin has potent antimicrobial and anti-inflammatory activity (Wang *et al.* 2014), and also the capability to stimulate cytokines like IL-6 (Lim *et al.* 2015). It is actively passaged transplacentally during late pregnancy and delivery, but what is not fully clarified is whether AF cathelicidin is of maternal or fetal origin or both (Yoshio *et al.* 2003, Wang *et al.* 2014). Levels of cathelicidin are enhanced by stress such as normal labor (Mandic Havelka *et al.* 2010, Park *et al.* 2011).

Interleukin-6 (IL-6) is a pro-inflammatory cytokine (Burns *et al.* 2015) and also a potent activator of neutrophils and acute phase responses. It has the capacity to modulate the immune response from innate immunity to adaptive immunity (Buhimschi *et al.* 2009, Lee *et al.* 2011). One major function of IL-6 is recruitment of neutrophils to the inflammation site (McGeough *et al.* 2012). Its concentration increases along gestational age (Burns *et al.* 2015).

Elafin belongs to a family of antileukoproteinases (Stock *et al.* 2007) and is synthesized and secreted locally upon stimulation by cytokines (Williams *et al.* 2006). It is produced by neutrophils, chorion trophoblasts, decidua, and epithelial cells (Williams *et al.* 2006, King *et al.* 2007a, 2007b), for example in amnion epithelium (Stock *et al.* 2007). Among these sites, the chorion plays a massive role in elafin production (Stock *et al.* 2007). Elafin is a peptide with natural antimicrobial and anti-inflammatory properties as well as the capacity to modify immune responses (King *et al.* 2007b); it is an antiprotease of HNE (King *et al.* 2007b), protecting tissues from damage mediated by HNE (Williams *et al.* 2006). Its expression in amnion epithelium cells is reduced in cases of PPROM (King *et al.* 2007b).

Neutrophil elastase (HNE), a serine protease which fragments proteins ingested by leukocytes, is expressed and stored in the primary granules of activated neutrophils. It participates in a host response against bacteria (Alfakry *et al.* 2016), and can degrade many extracellular matrix components (Kidokoro *et al.* 2006) through MMP cascade activation (Alfakry *et al.* 2016). Elastase can affect the activation of MMPs and cytokines and the inactivation of TIMP-1 (Alfakry *et al.* 2016). In PPROM pregnancies, its expression is increased (Helmig *et al.* 2002).

Myeloperoxidase (MPO) is a cationic heme protein, which is stored in PMN, released mainly by neutrophils and monocytes, and upregulated with inflammation *(Kindzelskii et al. 2006, Leppilahti et al. 2014,* Alfakry *et al.* 2016). It has two kinds of properties: first, it degrades foreign particles oxidatively, and second, it suppresses the inflammatory reaction (Klebanoff *et al.* 2013). An important feature of MPO is its ability to generate oxidants like hypochlorus acid (HOCl), which can activate latent MMPs (Saari *et al.* 1992, Alfakry *et al.* 2016) and inactivate TIMP oxidatively. Another important feature is its capability of regulating neutrophil immigration to the site of inflammation (Alfakry *et al.* 2016). In sum: MPO can create an environment favorable for necrosis and for abscess formation (Klebanoff *et al.* 2013). Furthermore, MPO also has antimicrobial properties, and HOCl has microbicidal properties (Kindzelskii *et al.* 2006, Klebanoff *et al.* 2013).

CRP, an acute-phase reactant, is produced in the liver upon stimuli of pro-inflammatory cytokines (Genc, Ford 2010). AF CRP, of fetal origin (Malek *et al.* 2006), is a large-sized protein, incapable of crossing the placental barrier (Gutteberg *et al.* 1986).

Microbial invasion of the amniotic cavity

Microbial invasion of the amniotic cavity is determined as a positive microbial finding in the AF, detected either by 16S rRNA (PCR), bacterial culture, or Gram stain.

PCR is a molecular microbiologic technique used since 1983 (Mullis, Faloona 1987), and is based on cycles of heating and cooling DNA strips in order to melt and replicate DNA enzymatically. Primers, i.e. specific DNA fragments which are complementary to the target, will attach to the DNA strand. A cycle will be repeated many times. As the process continues, the generated DNA can serve as a template so the amount of DNA can be amplified.

PCR has allowed identification of more microbes than with cultivation techniques, and the rate of identified MIAC cases has increased 30 to 50% (DiGiulio 2012). PCR recognizes not only live microbes but also the footprints of pre-existing, non-viable microbes (DiGiulio 2012). PCR has also improved the detection of *Ureaplasma*, the most common microbe in AF (DiGiulio 2012). Furthermore, the method is quicker than the traditional cultivation technique.

Cultivation is a traditional method for identifying microbes. Because bacterial culture and PCR do not always identify the same microbes, the spectrum of microbes found has widened when both methods are used simultaneously (DiGiulio *et al.* 2010). With common cultivation techniques, *Candida* species can also be recognized. A negative culture result does not necessarily mean the absence of bacteria, but the impossibility of cultivating such a bacteria, because only 1% of bacteria are cultivable (Romero *et al.* 2006, DiGiulio 2012).

Gram stain is a rapid and cheap method for identifying MIAC. Its basis is to stain bacteria according to their cell structure. Gram-positive cells stain as purple and gram-negative as red. For a positive culture, Gram stain has a sensitivity of 60% and specificity of 99% (Romero *et al.* 1993b).

Vaginally obtained samples

After rupture of the fetal membranes, AF can be visible in the vagina during a speculum examination. In such situations, AC can be difficult or impossible to perform due the resulting oligohydramnion or anhydramnion. Investigation has therefore focused on the possibility of using vaginally obtained AF samples.

Increased AF-LD concentration, measured from vaginally obtained AF samples with a cut-off value of 1000 IU/L, has shown an association with MIAC detected by AC (Magloire *et al.* 2006b). Another study linked an AF-LD cut-off of 25 IU/L to HCA (Hendsch *et al.* 2001).

One study showed that vaginally obtained AF-Gluc correlates with glucose concentrations retrieved by transabdominal AC and is also associated with MIAC (Buhimschi *et al.* 2006). Vaginally obtained AF-Gluc concentrations less than 0.28 mmol/L predicted IAI (determined as low glucose concentration at AC with MIAC) in that same study with sensitivity of 47% and specificity of 100%.

IL-6 has failed to prove useful in vaginally obtained AF samples (Hendsch et al. 2001). However, a recent publication demonstrated its value in the diagnosis of MIAC, intra-amniotic inflammation, and IAI. It showed a strong correlation with IL-6 levels in AC samples, r_s=0.68, p<0.0001 (Musilova *et al.* 2016).

Recently, a new transcervical device has been introduced for AF collection after PPROM (Lee *et al.* 2015). This may help in sampling and in avoiding contamination with vaginal discharge.

Cervicovaginal fluid samples containing various biomarkers are under extensive study to discover the most suitable predictive biomarker for MIAC and inflammation. The most popular biomarker under investigation has been IL-6. An association of IL-6 and tumor necrosis factor –α (TNF- α) with fetal inflammatory response syndrome (FIRS) (determined as increased IL-6 in cord plasma) and funisitis has been reported in samples obtained by squeezing vaginal discharge from sanitary napkins (Kunze *et al.* 2016). IL-6 and IL-8 in cervical fluid samples have also shown an association with intra-amniotic infection /inflammation (Holst *et al.* 2005, Park *et al.* 2013b) and with MIAC and HCA (Kacerovsky *et al.* 2015a). Moreover, cervicovaginal fluid IL-6 has

also shown an association with MIAC in pregnancies with intact membranes (Combs *et al.* 2015), as has vaginal fluid IL-6 in pregnancies with PPROM (Kacerovsky *et al.* 2015b). One study combined gestational age with IL-6 values and found this to be best the predictor for IAI in PPROM pregnancies (Ryu *et al.* 2013).

A rapid point-of care test, based on IL-6 levels in vaginal secretions and developed for PPROM situations, has a positive predictive value (PPV) of 50% and a negative predictive value (NPV) of 97.4%, suggesting use of this test in inflammation exclusion.

Maternal serum samples

In pregnancies complicated with IAI, maternal serum IL-6 levels are increased (Dulay *et al.* 2015). Pregnancy itself does not raise IL-6 levels in uneventful pregnancy if levels are compared to those at the non-pregnant stage (Anderson *et al.* 2013). In fact, many conditions cause IL-6 overexpression. For instance, IL-6 is the only cytokine associated with MIAC in maternal serum samples in pregnancies with intact membranes, whereas in PPROM pregnancies, IL-18, monocyte chemoattractant protein -1, and IL-1β levels are slightly increased with MIAC. Moreover, the maternal serum response to MIAC is visible only at <32 weeks of gestation (Cobo *et al.* 2013). Other maternal serum markers have been investigated as well, but have not achieved the accuracy required for clinical use (Evers *et al.* 2012).

Others

IL-6, CRP, and procalcitonin tests of maternal urine to learn whether IAI can be diagnosed from urine samples have failed. Unfortunately, urine is not a useful biological product in the diagnosis of IAI with these biomarkers (Dulay *et al.* 2015).

Risk-based methods have also been developed. One such involves gestational age, cervical length, and maternal blood WBC count. This method predicts IAI, AUC 0.724 (Jung *et al.* 2011).

Histologic chorioamnionitis

HCA diagnosis is based on placental histopathologic examination after delivery. Several systems staging HCA severity exist. Currently, the most popular is the system of Redline (Redline *et al.* 2003, 2005). The definition and staging of acute HCA and funisitis according to the Amniotic Fluid Infection Nosology Committee of the Perinatal Section of the Society of Pediatric Pathology (Redline *et al.* 2003) are:

- “Stage 1 (acute subchorionitis/acute chorionitis): presence of neutrophils in the subchorionic zone or in the extraplacental membranes´ chorionic trophoblast layer.
- Stage 2 (acute chorioamnionitis): more than a few neutrophils accumulated in the chorionic plate and connective tissues or in the amniotic membrane.

- Stage 3 (necrotizing chorioamnionitis): robust neutrophilic infiltration with visible degenerating neutrophils, thickened amniotic basement membrane, and focal epithelial necrosis of the amniotic membranes."

Salafia and Blanc have also created classification systems (Blanc 1981, Salafia *et al.* 1989).

Maternal clinical symptoms and signs and infectious markers do not reliably predict HCA (Edwards 2005, Erdemir *et al.* 2013). However, maternal fever >38ºC, spontaneous onset of labor, and duration of labor >12 h are the best predictive factors among the symptoms and clinical signs (Roberts *et al.* 2012). Maternal CRP before delivery has been associated with funisitis (Lee *et al.* 2012), but according to one review and a recent study it has no relevant association with HCA in PPROM pregnancies (van de Laar *et al.* 2009, Stepan *et al.* 2016). On the other hand, in such pregnancies, HCA has been associated with oligohydramnios on admission, elevated maternal CRP level just before delivery, but not at admission, small gestational age at PPROM, and latency after PPROM (Wu *et al.* 2009, Xie *et al.* 2015), though controversial results also exist concerning the latency time (Kim *et al.* 2016).

Maternal serum IL-6 and IL-8 levels are increased in patients with HCA, but concerning IL-6 this can be seen only in PPROM pregnancies, and in term pregnancies with or without PROM (Murtha *et al.* 1996, Saji *et al.* 2000, Roberts *et al.* 2012).

Ultrasound

Reports state that in AF microbes can form biofilms (Romero *et al.* 2007). If such a biofilm exists near the internal os of the cervix, it can be visible during ultrasound as sludge (Figure 5) (Espinoza *et al.* 2005, Kusanovic *et al.* 2007, Kim *et al.* 2015a). Sludge occurs in 23% of preterm pregnancies with intact membranes, but in only 1% in term labors (Espinoza *et al.* 2005).

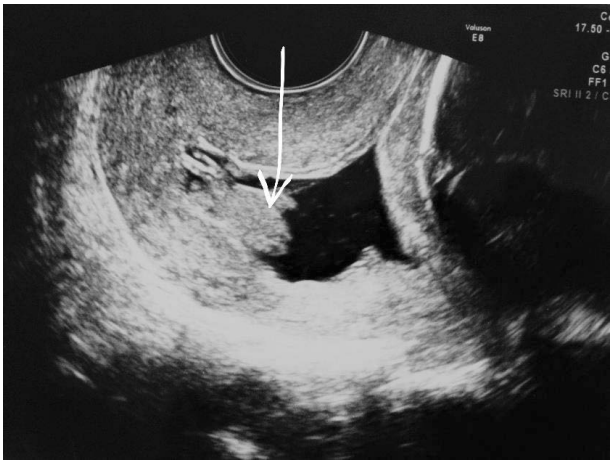


Figure 5. Sludge at ultrasound examination, an arrow indicating the location.

Ultrasound approaches to predicting fetal systemic inflammatory response (SIRS) do exist. For example, possible changes in the fetal spleen can be evaluated in response to HCA, and small size of the fetal thymus has been linked to IAI, HCA, and funisitis (Musilova *et al.* 2013, Mastrolia *et al.* 2016). Unfortunately, low specificity makes thymus size unusable as a non-invasive tool in the diagnosis of subclinical chorioamnionitis (Musilova *et al.* 2013). However, pulsation of the splenic vein can serve as such a tool, and it is linked to HCA and funisitis in PPROM pregnancies by the same investigators (Musilova *et al.* 2012). Incorporation of these ultrasound markers is not easily available, however, since they require expensive equipment and high expertise in fetal ultrasound.

A recent review on the utility of fetal ultrasonography in prediction of SIRS has summarized all relevant studies, with the conclusion that, currently, only invasive methods can be reliably useful for gathering information on fetal well-being in women with threatening preterm delivery (Mastrolia *et al.* 2016). The same authors, however, encourage the use of ultrasound in such pregnancies, to help identify those who may require invasive procedures.

Prevention and management

Prophylaxis

The major risk factor for developing clinical chorioamnionitis is expectant management after PPROM (Tita, Andrews 2010). Chorioamnionitis occurs in up to 70% of those developing contractions in such a situation, though antibiotic prophylaxis reduces the incidence (Tita, Andrews 2010). In a systematic Cochrane review by Kenyon *et al.* (2013), prophylactic antibiotics had no effect on the rates of respiratory distress syndrome (RDS), intraventricular hemorrhage (IVH), necrotizing enterocolitis (NEC), or perinatal death. Prophylactic antibiotics were, however, associated with prolongation of pregnancy after PPROM, reduced numbers with neonatal infection, reduced need for surfactant, and reduced consumption of oxygen (Kenyon *et al.* 2013).

The recommendation of induction of labor after 34 weeks of gestation in cases of PPROM (Tita, Andrews 2010) is still followed in modern obstetric practice worldwide, but recent studies have shown it unnecessary in the absence of intra-amniotic inflammation or infection (Kacerovsky *et al.* 2014, Morris *et al.* 2016). Two recently conducted randomized controlled trials have shown that in pregnancies complicated with PPROM between 34+0 and 36+6 weeks of gestation, induction of labor does not improve pregnancy outcomes more than does expectant management (van der Ham *et al.* 2012). Moreover, expectant management improves neonatal outcome without any increase in early-onset neonatal sepsis (EONS) (Morris *et al.* 2016). AC was not incorporated into the management of the patients in those 2014 and 2016 trials.

In pregnancies with intact membranes and the threat of preterm labor, antibiotic prophylaxis is not recommended in the absence of signs and symptoms of chorioamnionitis because of its possible further harm to the neonate (Flenady *et al.* 2013).

The antibiotic regimens and duration of prophylaxis vary among studies. In the 2010 review by Cousens et al. the most common prophylactic antibiotics were ampicillin and erythromycin. Clarithromycin has shown a better protective effect on progression of funisitis (Kwak *et al.* 2013). Co-amoxiclav, conversely, is associated with increased risk for NEC and the advice is therefore to avoid it (Kenyon *et al.* 2013).

Treatment

Treatment with broad-spectrum antibiotics immediately after diagnosis of chorioamnionitis reduces the rate of EONS cases (Fishman, Gelber 2012). Concerning maternal and neonatal complications associated with chorioamnionitis, according to a recent Cochrane review, no suggestions for antimicrobial regimens, for continuation of antibiotic use during the postpartum period, or for the length of antibiotic treatment are possible, due to limited evidence (Chapman *et al.* 2014).

The antibiotics chosen should be effective against the most common microbes causing neonatal sepsis: *Group B Streptococcus* and *E.coli*. With cesarean section, anaerobic coverage, for example with metronidazole, is the recommendation (Fishman, Gelber 2012). Antibiotic specimens vary among hospitals. In the Cochrane database, antibiotics for trials were ampicillin, ampicillin/sulbactam, gentamicin, clindamycin, and cefotetan (Chapman *et al.* 2014). No clear consensus exists as to whether antibiotic treatment for a mother with MIAC can eradicate microbes from the amniotic cavity and chorioamniotic space (Redline 2004, Gomez *et al.* 2007, Kim *et al.* 2015a, Lee *et al.* 2015). Ceftriaxone, clindamycin, and erythromycin have shown no ability to eradicate IAI (Gomez *et al.* 2007). However, eradication rates are better with ceftriaxone, clarithromycin, and metronidazole (Lee *et al.* 2015). Evidence of successful eradication of *Ureaplasma* exists in a primate model, but for humans, multiple doses of azithromycin allowing accumulation in AF may eradicate *Ureaplasma* (Acosta *et al.* 2014). Macrolides have a limited mean transplacental transfer, only 2.6 to 4.3%, thus affecting eradication results (Heikkinen *et al.* 2000). Of the macrolides, clarithromycin has the best mean transplacental transfer, of 6.1% (Witt *et al.* 2003).

Antipyretics can be administered for maternal fever (Higgins *et al.* 2016).

In cases of maternal chorioamnionitis, delivery should be completed. Any prolonged time-interval between diagnosis and delivery causes an increased risk for maternal sepsis (Johnson *et al.* 2014). Cesarean section should be performed only for obstetrical reasons, not only because of chorioamnionitis (Fishman, Gelber 2012). In pregnancies <33 weeks, administration of antenatal corticosteroids has been safe without negative effects on chorioamnionitis progression (Fishman, Gelber 2012).

Health consequences of chorioamnionitis for the mother

The main adverse outcome of clinical chorioamnionitis is increased risk for preterm delivery (Tita 2010). Other consequences to the mother during pregnancy and delivery include labor dystocia and cesarean section. Postpartum problems include increased blood loss, pelvic or uterine infection, uterine atony, and sepsis / septic shock (Tita, Andrews 2010, Fishman, Gelber 2012, Chapman *et al.* 2014).

Health consequences of chorioamnionitis for the fetus and newborn

Chorioamnionitis plays a key role in neonatal morbidity and mortality (Hitti *et al.* 2001, Yoon *et al.* 2001, Garcia-Munoz Rodrigo *et al.* 2014, Kacerovsky *et al.* 2014, Liu *et al.* 2014, Roescher *et al.* 2014, Kim *et al.* 2015a, Xie *et al.* 2015). Following clear symptoms of chorioamnionitis, prevention of adverse neonatal outcomes is difficult or impossible (Yoon *et al.* 2001, Kim *et al.* 2015b). Furthermore, intra-amniotic

inflammation causes similar adverse neonatal outcomes whether or not MIAC is present (Shim *et al.* 2004, Combs *et al.* 2014, Romero *et al.* 2014c). In fact, severity of inflammation is the variable correlating best with neonatal outcome (Combs *et al.* 2014, Lu *et al.* 2016). Notably, in PPROM pregnancies between 34 and 37 weeks of gestation in the absence of clinical chorioamnionitis, expectant management does not cause an increased rate of EONS (Vijgen *et al.* 2014, Morris *et al.* 2016, Ofman *et al.* 2016), but it does so in very low birth-weight infants (Klinger *et al.* 2009). Both risk for RDS and need for mechanical-ventilator support instead rise if labor is induced immediately (van der Ham *et al.* 2012, Morris *et al.* 2016). The risk for RDS and IVH in pregnancies of less than 34 weeks' of gestation can be reduced with administration of antenatal corticosteroids; this seems to be a safe procedure even if the mother has chorioamnionitis (Amiya *et al.* 2016).

The key element is whether a fetus develops FIRS, which is associated with short-term and long-term neonatal morbidity and mortality even after adjustment for gestational age (Mastrolia *et al.* 2016). FIRS is determined as elevation of circulating cytokines (IL-6) in the fetal circulation capable of causing damage to multiple fetal organs and threatening onset of preterm delivery (Redline 2004, Romero *et al.* 2014d). Fetal organs affected by FIRS include the skin, heart, lungs, kidneys, brain, intestine, thymus, adrenal gland, and blood cells (Gotsch *et al.* 2007). The fetal response is more severe in preterm pregnancies with intact membranes than in those with PPROM (Park *et al.* 2013d); FIRS is, however, more common in PPROM pregnancies (50%) compared to pregnancies with intact membranes (39%) (Mastrolia *et al.* 2016). Histologic indicators of FIRS include funisitis and vasculitis in the chorion, and funisitis is, by itself, associated with adverse neonatal outcomes (Romero *et al.* 2006).

If bacteria invade the uterine cavity, half the fetuses will be colonized by maternal microbes (Romano-Keeler, Weitkamp 2015), since microbes can reach the fetus by various routes, for example through the skin, ear, or gastrointestinal tract (Maxwell *et al.* 2006). Fetuses exposed to both MIAC and HCA in PPROM pregnancies develop more severe FIRS than do those fetuses exposed to either MIAC or HCA (Kacerovsky *et al.* 2014) (Table 7).

Table 7. Association of chorioamnionitis with neonatal outcome.

Study	Year	Setting	Inclusion criteria	n	Outcome
Soraisham	2013	HCA vs neurodevelopmental outcome	<29 GW	384	CP ↑
Suppiej	2009	HCA vs neurodevelopmental outcome	<32 GW	104	speech delay, hearing loss↑
Rovira	2011	HCA vs neurodevelopmental outcome	<32 GW or <1500 g	177	HCA: any grade motor abnormalities ↑ Funicitis: CP, moderate to severe neurological disability ↑ CC: disability of any grade, speech abnormalities ↑
Polam	2005	HCA vs neurodevelopmental outcome	22-29GW	177	IVH, ROP ↑ CP ↔ Mental and psychomotor index ↔
Horvath	2012	HCA vs cerebral palsy	<1500 g	141	CP ↑

Lu	2016	HCA and FIRS vs brain injury in PPROM pregnancies	<34 GW	103	IVH gr 3-4 , PVL↑
Gisslen	2016	HCA	32-36 GW	477	mechanical ventilation↑
Park	2015	HCA vs RDS	24.5-34 GW	378	mild to moderate HCA: RDS↓
Zanardo	2009	HCA vs respiratory outcome	<33 GW	287	Chronic lung disease ↑ Protection from RDS ↔
Plakkal	2009	HCA vs BPD	<29 GW	529	BPD ↓
Huetz	2016	HCA vs neonatal outcome	24 - 33+6	626	Early onset sepsis ↑ CP, death ↔
Lee	2013	HCA vs adverse neonatal outcome in PPROM pregnancies	34+0-36+6	244	Sepsis↑ RDS, ventilator, 5 min Apg score ↔
Hendson	2011	HCA vs adverse neonatal outcome	<32 GW and <1250 g	628	Early sepsis, BPD, ROP, mental delay ↑ RDS, CP ↔ After adjustment: any adverse outcome ↔
Xie	2015	Reprospective study; HCA vs adverse neonatal outcomes in PPROM pregnancies	PPROM <34 GW	371	Pneumonia, sepsis, BPD, mortality, abnormal us findings ↑
Lee JY	2016	CC and HCA vs NEC	<32 GW	354	NEC ↔
Pappas	2014	HCA and CC vs neurodevelopmental outcome at 18-22 months of age	<27 GW	2390	sepsis, IVH ↑ after adjustment for GA cognitive impairment ↑ if HCA and CC
Musilova	2015	Infection, inflammation, MIAC or negative in PPROM pregnancies	24+0-36+6 GW	166	No difference between the groups
Aziz	2009	CC vs no chorioamnionitis in PPROM pregnancies	24-34 GW	1153	low Apgar scores at 5 min of age ↑ RDS, NEC, IVH, pneumonia ↑
Ballard	2016	Chorioamnionitis vs BPD	<32 GW	1687	BPD ↔
Alexander	1999	Retrospective study; CC in term pregnancies	neonatal weight >2500 g	101 170	intubation↑ pneumonia↑ sepsis↑
Ylijoki	2016	Chorioamnionitis vs neurological outcome at 2 and 5 years of age	very low birth-weight and very low gestational age infants	197	CC: neurodevelopmental problems ↔ HCA: cognitive outcome at 5 year of age↓
Mu	2007	Chorioamnionitis vs neonatal outcome	<1500 g	95	BPD ↑ Mental and psychomotor index ↔
Wu	2000	Meta-analysis; CC and HCA vs CP	preterm and full term	26 studies	Preterm: both HCA and CC CP, cPVL↑ Term:CC CP↑
Shatrov	2010	Meta-analysis; chorioamnionitis vs CP	preterm and full term	15 studies	HCA: CP ↑ CC: CP↑

ACS, antenatal corticosteroids; BPD, broncopulmonary dysplasia; CC clinical chorioamnionitis; CP, cerebral palsy; cPVL, cystic periventricular leucomalacia; FIRS, fetal inflammatory response syndrome; GA, gestational age; HCA, histologic chorioamnionitis; IVH, intraventricular haemorrhage; MIAC, microbial invasion of the amniotic cavity; NEC, necrotizing enterocolitis; PPROM, preterm prelabor rupture of the membranes; RDS, respiratory distress syndrome; ROP, retinopathy of prematurity; US, ultrasound

Clinical chorioamnionitis raises risk for EONS OR 5.84 (95% CI 3.03-11.25) and for grade 3-4 IVH OR 1.60 (95% CI 1.16-2.21) when adjusted for birth weight and gestational age (Soraisham *et al.* 2009). In preterm pregnancies, risk for neonatal morbidity and mortality is the higher the smaller are gestational age and birth weight (Fishman, Gelber 2012). The rate of meningitis is 3%, NEC 4.5%, pneumonia 10-21%, sepsis 7-28%, IVH 22-24%, and RDS 62-63% in preterm pregnancies (Fishman, Gelber 2012). HCA and FIRS are more common in fetal survivors, suggesting that progression of fetal response may promote early survival (Lahra, Jeffery 2004). Although some studies question the profound impact of HCA on long-term neurodevelopmental outcome *(*Ylijoki *et al.* 2016), the majority of studies demonstrate this association (Rovira *et al.* 2011, Fishman, Gelber 2012); this was evident particularly in the increased incidence of CP (Tita, Andrews 2010, Kamyar *et al.* 2016).

Aims of the study

The present study was carried out to evaluate whether vaginally obtained AF biomarkers could prove clinically valuable in the diagnosis of HCA, and to find the association of selected AF biomarkers obtained by AC in women with suspected IAI with MIAC, HCA, and neonatal outcome.

The detailed aims were to study:

1. Whether vaginally obtained AF can serve for IAI diagnostics based on AF-LD and AF-Glucose measurements and to evaluate their association with HCA.

2. The association of AF-LD and AF-Glucose in AC samples with MIAC and HCA and to determine cut-off values for clinical purposes.

3. The association of the novel AF biomarker cathelicidin and AF-MMP-8 in AC samples with MIAC, and to determine cut-off values for clinical purposes.

4. The difference in selected novel biomarkers between IAI-suspected cases and controls without IAI suspicion, and among IAI-suspected cases to study their association with MIAC.

Subjects and methods

Subjects

A description of the women enrolled in the studies (I-IV) is presented in Table 8.

Table 8. Patients and methods in Studies I-IV.

	Study I	Study II	Study III	Study IV
Study Group	PPROM patients GW 22+0-h36+6	PPROM and no PPROM patients with suspected IAI GW 22+0-h36+6	PPROM and no PPROM patients with suspected IAI GW 22+0-h35+0	patients with intact membranes and IAI suspicion GW 22+0-32+0
Design	prospective, observational	prospective, observational	prospective, observational	prospective, case-control
Main outcome	LD and glucose vs HCA	LD and glucose vs MIAC and HCA	MMP-8 and cathelicidin vs MIAC	new biomarkers in cases and controls; within cases new biomarkers vs MIAC
Methods	vaginally obtained AF samples	AC samples of AF	AC samples of AF	AC samples of AF
Outcome	HCA	MIAC and HCA	MIAC	MIAC and adverse neonatal outcome
Number of patients	53	70	54	27
		3 from Study I 67 new patients	3 from Study I 27 from Study II (includes 3 from Study I) 27 new patients	8 from Study II 19 from Study III (includes 8 from Study II) 8 new patients
Controls	-	-	-	46

PPROM= preterm prelabor rupture of membranes; GW, gestational weeks; LD= lactate dehydrogenase; HCA= histologic chorioamnionitis; MIAC, microbial invasion of the amniotic cavity; AC, amniocentesis; AF, amniotic fluid

A total of 232 women with suspected IAI or PPROM were recruited as cases and 80 women as controls in the period between March 2012 and October 2015. AC was performed in 139 cases with suspected IAI and in the 80 controls. Vaginal AF samples were from 96 women with PPROM. After exclusions, the final study group comprised 155 women and the control group 46 women, for whom AC was performed in 105 cases and in 46 controls (Figure 6).

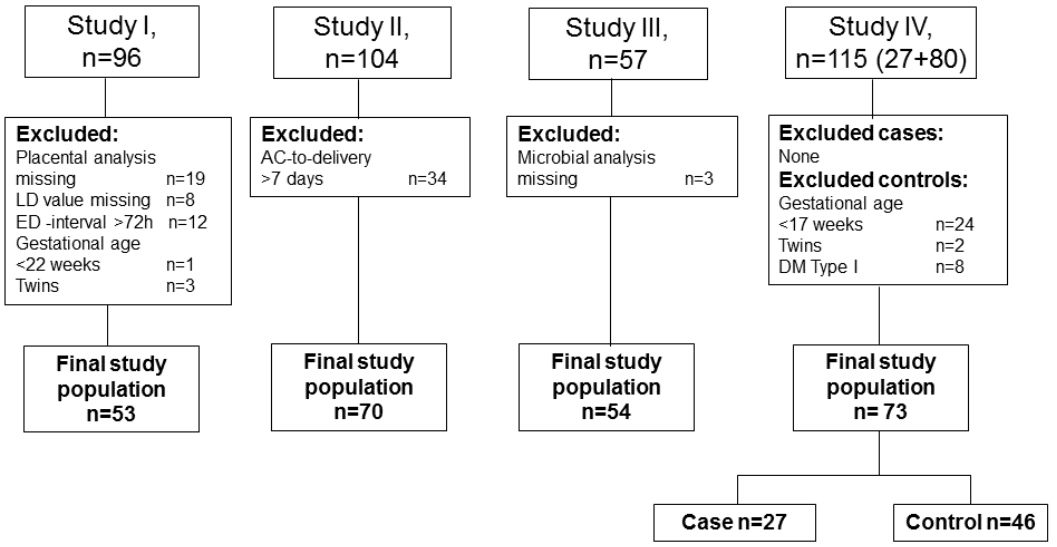


Figure 6. Enrolled women and exclusion criteria.

All studies were conducted at the University Hospital of Helsinki, Finland, Department of Obstetrics and Gynecology. PPROM was diagnosed by clinical examination or by a positive vaginal point-of-care test (ActimProm®, Medix Biochemica, Espoo, Finland). Determination of gestational age was based on the first-trimester ($12+^{0}$ - $13+^{6}$ weeks of gestation) ultrasound screening. IAI suspicion was evoked by at least one of the following criteria of chorioamnionitis: uterine tenderness, maternal fever, fetal tachycardia, infectious discharge from uterine cervix, increased maternal plasma CRP >10 mg/L, total maternal plasma WBC count > 20 x 10^{9}/L, or sludge visible at ultrasound examination (Table 9).

Table 9. Signs and symptoms of intra-amniotic infection (IAI) in IAI suspected cases.

	PPROM	No PPROM	p-value
n	100	55	
Uterine contractions n (%)	55 (55)	40 (73)	0.03
Uterine tenderness n (%)	11 (11)	25 (45)	<0.001
Maternal fever >38°C n (%)	5 (5)	2 (4)	1.0
Malodorous discharge n (%)	13 (13)	11 (20)	0.25
Maternal CRP >10 mg/L n (%)	71 (71)	50 (91)	0.004
Maternal WBC count >20 x 109 /L n (%)	18 (18)	5 (9)	0.129
Sludge n (%)	13 (13)	11 (20)	0.25

CRP, C-reactive protein; WBC, white blood cell; PPROM, preterm prelabor rupture of the membranes

Prophylactic intravenous antibiotics (azithromycin 500 mg daily and cefuroxime 1.5 g three times daily) for three consecutive days after PPROM and two subsequent doses of intramuscular betamethasone for fetal lung maturation (12 mg twice 24 h apart) were administered to all women with PPROM. Antenatal corticosteroids, but not routine antibiotics, were administered also to women with intact fetal membranes and imminent preterm labor before 35 weeks of gestation. Tocolysis was administered to women by decision of the obstetrician in charge if no signs of clinical chorioamnionitis were present. If possible, the women with PPROM were managed expectantly until 34 weeks of gestation, when labor was induced or caesarean section was performed. Immediate delivery was performed earlier if symptoms and signs consistent with clinical chorioamnionitis occurred or at any sign of maternal and fetal compromise. The results of AF-LD, AF-Gluc, AF PCR, and AF microbial culture were available to obstetricians during the study period. If AF-LD was less than 419 IU/L and AF-Gluc more than 0.7 mmol/L without MIAC at AC, pregnancy was considered normal and could continue with follow-ups.

Study I

The study population comprised 96 pregnant women with PPROM. This prospective study was conducted between March 2012 and March 2015. Gestational age on admission was between the 22^{+0} and 36^{+6} weeks. All had vaginally collected AF samples on admission and then repeated samples every third day if possible if undelivered. The placental histopathologic result after delivery was recorded. The main outcome measurement was association of AF-LD and AF-Gluc with HCA in vaginally collected AF samples. We excluded the 19 women without placental histopathology and eight lacking an AF-LD value, those 12 with their last examination–to-delivery-interval beyond 72 hours, the one with gestational age less than 22 weeks at examination, and three with multiple pregnancies. Thus, the final study group comprised 53 women.

No microbial analysis of vaginally obtained AF samples took place. Classical criteria were those that determined clinical chorioamnionitis (Fishman 2012), but this occurred in no women upon admission.

Study II

The study population comprised 104 women with singleton pregnancies between 22^{+0} and 36^{+6} weeks of gestation with or without PPROM. The study was conducted between March 2012 and March 2015. AC was performed for any suspected IAI. The 34 women with an AC-to-delivery interval of over 7 days were excluded. The final study group comprised 70 women. The main outcome measures were the association of AF-LD and AF-Gluc with MIAC and HCA.

The following laboratory tests were performed: AF-LD and AF-Gluc concentrations, AF PCR and AF microbial culture. Microbial results were available for 62 cases. The results of bacterial culture were available for 46 and of AF-PCR for 59, while results for both were available in 43 cases.

Study III

The study population comprised 57 women with singleton pregnancies between 22^{+0} and 35^{+0} weeks of gestation and suspected IAI with or without PPROM. The study was conducted between June 2012 and March 2015. Exclusion criteria for recruitment were multiple gestations, pregnancies with structural fetal anomaly, proven or suspected fetal aneuploidy. Those pregnancies lacking any microbial analysis were excluded from the final analysis, which comprised 54 women. The main outcome measures were the association of AF-MMP-8 and AF-cathelicidin with MIAC and with HCA.
The following laboratory tests were performed: AF-MMP-8 and AF-cathelicidin concentrations, AF microbial culture, and AF PCR.
Controls were 32 healthy women with singleton pregnancy between 15^{+0} and 36^{+6} weeks of gestation and uneventful pregnancy outcome who underwent measurements of AF-MMP-8 and AF-cathelicidin concentrations. Indications for AC in these pregnancies were karyotyping in 24 or evaluation of fetal lung maturity in eight.

Study IV

This cohort included 73 women with a singleton pregnancy and intact fetal membranes. The study group comprised 27 women with suspected IAI who were recruited in the study group and 80 women with AC performed for other indication than IAI who were recruited as controls. We excluded the 24 controls with gestational age less than 17 weeks, eight with diabetes mellitus type I, and two with twin pregnancies. The final study group, comprising 27 cases and 46 controls, was gathered between June 2013 and October 2015. Membranes were considered intact in the absence of any clinical signs of membrane rupture.

The cases underwent AC between 22^{+0} and 32^{+0} weeks of gestation and the controls between 17^{+0} and 37^{+5} weeks of gestation. Indications for AC in controls were mid-trimester chromosomal analysis in 28, determination of fetal lung maturity in one, and evaluation of fetal chronic hypoxia by erythropoietin measurement in 17. Of controls, five had pre-eclampsia, and four had insulin-treated gestational diabetes. Fetal growth restriction (fetal growth below -2 standard deviation) was noticeable in four pregnancies. Exclusion criteria were fetal structural anomaly, proven or suspected fetal aneuploidy, and diabetes type I. The selected biomarkers were AF-MMP-8, AF-MMP-9, AF-MPO, AF-IL-6, AF-HNE, AF-Elafin, AF-MMP-2, AF-TIMP-1, AF- MMP-8/TIMP-1 molar ratio, and AF-CRP. AF microbial culture and AF PCR, AF-LD, and AF-Gluc concentrations were also determined.
The main outcome measures were the difference in selected AF biomarkers between cases and controls, and the association of those biomarkers with MIAC in cases. Neonatal short-term outcome was also recorded.

Methods

Collection of the clinical data

Data for the study population such as maternal age, body mass index (kg/m^2), parity, smoking, gestational diabetes, delivery mode, and neonatal outcome came from the hospital database and laboratory results from analyses performed for clinical purposes, not for the study.

Samples and assays

Vaginally obtained amniotic fluid samples (I)

AF samples were obtained either during a speculum examination for 19 with a syringe in cases of AF pooling in the vagina, or by self-collection by 34 in a favorable situation with a plastic cup if no AF was visible during clinical examination. AF samples were collected on admission and afterwards on every third day if possible.

AF-LD and AF-Gluc were analyzed in HUSLAB by the Modular P System (Roche Diagnostics, Penzberg, Germany) and according to International Federation of Clinical Chemistry (IFCC) recommendations. AF-LD measurement was by a quantitative assay for total enzymatic activity of lactate dehydrogenase (including the activity of all LD-isoenzymes, LD1-LD5), according to IFCC recommendations. This method was generated for automated clinical chemistry analyzers of Roche. The intra-assay coefficient of variation (CV) for AF-LD was < 2.3%, and the detection limit was 5 IU/L (I, II). The detection limit of AF-Gluc during the study period was 0.50 mmol/L (I, II). In the low concentration levels (under 4 mmol/L), the intra-assay CV was 4.7%, while at higher concentration levels (up to 42 mmol/L), the intra-assay CV was 1.9%. Values of AF-Gluc < 0.5 mmol/L were recorded as 0 in statistics.

Amniotic fluid samples by amniocentesis (II-IV)

AF samples were obtained by transabdominal AC under ultrasound guidance and an aseptic technique from women with suspected IAI and from healthy controls undergoing AC for chromosomal analysis or evaluation of fetal lung maturity.

AF-LD and AF-Gluc were analyzed in HUSLAB by the methods described under “Vaginally obtained amniotic fluid samples”.

AF samples were divided into aliquots, frozen, and stored at -20 °C until analyzed in a single laboratory (Medix Biochemica, Espoo, Finland).

Immunoenzymometric assay (IEMA) of MMP-8 (III, IV)

MMP-8 IEMA is a quantitative enzyme immunoassay for the determination of human MMP-8. This sandwich assay uses two monoclonal antibodies against human MMP-8 (Medix Biochemica, Espoo, Finland). By that method, microplate wells were coated with one monoclonal antibody against MMP-8. To run the assay, 80 µl of Assay Buffer and 20 µl of standards, controls and samples were added to the microplate wells. Next, the plate was left for incubation for one hour at room temperature on a shaker. At that time, MMP-8 was bound to the microplate wells. Thereafter, unbound substances were washed out. In the next step, 100 µl of the enzyme conjugate was added to each well. The plate was then incubated again, 100 µl of enzyme substrate was added, and the plate was shaken again for 15 minutes. The reaction was stopped by addition of 50 µl of an acidic stopping solution. The absorbance of the solutions in the wells was measured at 414 nm with a microplate reader (Multiskan, Thermo Fisher Scientific, Vantaa, Finland). The concentrations of controls and samples were obtained from the standard curve created.

Other biomarkers (III, IV)

Commercial enzyme-linked immunosorbent assays (ELISA) allowed analysis of other biomarkers according to the manufacturer´s instructions: AF-cathelicidin [Human LL-37 [HK321] ELISA kit, Hycult biotech, PB Uden, The Netherlands], AF-CRP [Human C-Reactive Protein (CRP) ELISA kit, R&D Systems, Minneapolis, MN, USA], AF-Elafin [Human Trappin-2 (Elafin) ELISA kit, RayBiotech, Norcross, GA, USA], AF-HNE [Polymorphonuclear (Human PMN Elastase) Sandwich ELISA kit, eBioscience, Vienna, Austria], AF-IL-6 [Interleukin-6 (IL-6) ELISA kit, R&D Systems], AF-MMP-2 [Matrix Metalloproteinase 2 (MMP-2) ELISA kit, R&D Systems], AF-MMP-9 [Matrix Metalloproteinase 9 (MMP-9) ELISA kit, R&D Systems], AF-MPO [Myeloperoxidase (MPO) ELISA kit, Immunodiagnostik AG, Bensheim, Hesse, Germany], and AF-TIMP-1 [Tissue inhibitor of metalloproteinases 1 (TIMP-1) ELISA kit, GE Healthcare, Buckinghamshire, UK].

Microbiological analyses (II-IV)

MIAC was determined as either positive AF microbial culture or positive microbial PCR. Cultivation of AF samples for aerobic and anaerobic bacteria was performed on chocolate blood agar in 5% CO2 and on Fastidious Anaerobe Agar, enriched with a thioglycolate broth, in anaerobic conditions at 35±1 °C. The cultivation took seven days, and the specimens were inspected after one, two, and seven days. Detection of common *Candida* spp. and *Mycoplasma hominis*, but not *Ureaplasma* spp. was achievable.

For molecular microbiologic technique analyses, a minimum of 500 µL of AF was subjected to ceramic bead-beating cell lysis (Precellys®24 tissue homogenizer, Bertin Technologies, Montigny-le-Bretonneux, France) and then to a magnetic-bead-based DNA extraction method (NucliSENS kit with easyMAG automatic nucleic acid purification platform, bioMérieux, Marcy l'Etoile, France) according to the manufacturer´s instructions. The extracted DNA was extended in duplicates by PCR using the following primers: 5´- TTG GAG AGT TTG ATC MTG GCT C -3´ (forward) and 5´- GTA TTA CCG CGG CTG CTG -3´ (reverse). Inhibition control in the PCR reaction was based on DNA of λ-phage. Gel-electrophoresis served for verification of a positive PCR product, 5 µl of the PCR product was sequenced in a core facility, and the sequence acquired was compared to a NCBI BLAST sequence database (www.ncbi.nlm.nih.gov/blast). The Ripseq mixed analysis tool (https://www.ripseq.com/) served for mixed sequences analyses, when appropriate.

Placental samples (I, II)

A pathologist examined all placentas histopathologically, one with experience in perinatal pathology. Gross examination of the placenta, fetal membranes, and umbilical cord preceded accompanied recording of placental weight. Sampling sites were cord insertion, placental margin, chorionic plate, cord, and extraplacental membranes. Sections of tissue blocks were stained by standard hematoxylin and eosin techniques. HCA was defined as a visible diffuse infiltration of polymorphonuclear leukocytes - which are not normally present in the chorioamniotic membranes - in any sample associated with edema and congestion of the vessels. Inflammation of the umbilical cord (funisitis) was separately recorded, but not analyzed as an independent variable in this study. HCA was categorized as present or absent.

Maternal and neonatal blood samples (I, IV)

Maternal and neonatal plasma CRP was measured by an immunoturbidometric method (Modular System, Roche Diagnostics). Maternal and neonatal blood samples were obtained according to standard clinical protocol and analyzed for clinical purposes by HUSLAB (HUSLAB, Helsinki, Finland).

Statistical analyses

Comparisons of categorical variables of baseline clinical data were analyzed by Chi-square test and by the Fisher exact test if the number of cases was under five. Data with continuous variables were analyzed by T-test when the data followed a normal distribution (I). Data with continuous variables not following a normal distribution were analyzed by Mann-Whitney U-test (I-IV). Comparisons of continuous variables in three groups were calculated by Kruskall-Wallis test (unpublished data). Non-parametric correlations were calculated by Spearman´s rank correlation coefficient test (III-IV). Bivariate correlation analysis served to test the association of biomarkers with neonatal outcome (IV). AF biomarker concentration values were regressed on gestational age at sampling and on gestational age at delivery, and the residual served as a dependent variable for computing adjusted p-values (I-IV). Receiver operating characteristic (ROC) curves were derived to evaluate the diagnostic performances of AF biomarkers in prediction of MIAC or HCA and area under the curve (AUC), with 95% confidence interval (95% CI) determined (I, II, III). The sensitivity, specificity, PPV, and NPV were calculated (I, II, III). All tests were two-sided and processed by the Microsoft Statistical Package for the Social Sciences (SPSS) for Windows (Chicago, IL, USA), version 21.0 (I), and version 22.0 (II, III, and IV) software. P-values < 0.05 were considered statistically significant.

Ethics

Each patient signed a written informed consent. The study was approved by the Helsinki University Hospital Ethics Committee for gynecology and obstetrics, pediatrics, and psychiatry (75/13/03/03/2013). Patients were advised that the risk for any undesirable consequences requiring immediate delivery after AC (fetal injury, placental abruption, large fetal-vessel laceration, or PPROM in cases of intact membranes) is approximately 0.7% (Stark *et al.* 2000) and that the data obtained by analyzing samples might provide more information on the intrauterine status. All women were counselled by a specialist in perinatal medicine and a neonatologist.

Results

Of all the women, 87 (43%) were primiparous, and 49 had a body mass index (BMI) >30 (BMI missing for 3) and were considered obese. All AC samples in cases were obtained between 22+0 and 36+5 weeks of gestation. Selected variables of demographic data of the study population are in Table 10.

Table 10. Demographic data of the study population.

	Study I		Study II		Study III		Study IV				
n	53		70		54		cases n=27		controls n=46		
Maternal age; median (range)	30	(18-46)	31	(18-44)	29	(18-44)	31	(20-43)	32.5	(17-48)	
Nulliparity; n (%)	20	(37.7)	32	(45.7)	24	(44.4)	10	(37)	21	(46)	
BMI; median (range)	23*	(17-41)	24**	(17-49)	26*	(18-49)	27*	(20-49)	25.5	(18-48)	
Smoking; n (%)	9	(17)	9	(12.9)	11	(20.4)	5	(18.5)	9	(20)	
In vitro fertilization; n (%)	4	(7.5)	5	(7.1)	2	(3.7)	1	(3.7)	1	(2.2)	
Gestational diabetes; n (%)	9	(17)	9	(12.9)	7	(13)	5	(18.5)	18	(39)	
Gestation weeks at examination; median (range)	30.2	(23+3 - 34+4)	27.5	(22+5 - 36+5)	27.5	(22-34+2)	27.6	(22-32)	23.1	(17-38)	
Gestation weeks at delivery; median (range)	30.4	(23+4 - 34+5)	27.5	(23+2 - 36+6)	30.56	(23+2 - 41+4)	37.9	(24-42)	38.3	(28-42)	
Examination-delivery- interval weeks; median (range)	0.14	(0 - 0.42)	0.14	(0 - 0.84)	0.42	(0 - 17.7)	7.4	(0-17+5)	8.2	(0-24+2)	

*missing n=1
**missing n=2
BMI, body mass index

Vaginally obtained amniotic fluid samples (I)

HCA occurred in 37 (70%) women. Mean (SD) gestational age at PPROM was 28.5 (±3.9) weeks of gestation. PPROM occurred earlier in women with HCA than in those without HCA (p=0.04). The median concentrations of AF-LD were higher in patients with HCA than in those without HCA (Table 11), but this difference disappeared when the concentrations were adjusted for gestational age at sampling (unpublished data). AF-Gluc concentrations between women with or without HCA did not differ.

Table 11. Median concentrations of AF-LD and AF-Gluc in women with and without histologic chorioamnionitis (HCA).

	HCA n=37	**No HCA n=16**	**p-value**	**Adjusted p-value***
LD IU/L median (range)	1400 (128-23000)	784.5 (135-3542)	0.005	0.59
Gluc mmol/L median (range)	0.0 (0.0-3.5)	0.65 (0.0-3.0)	0.20	0.41

LD, lactate dehydrogenase; Gluc, Glucose

*Adjusted for gestational age at sampling; unpublished data

The cut-off value of AF-LD based on the ROC curve was 1029 IU/L (AUC 0.74; 95% CI 0.61-0.88) for HCA. AF-LD concentration 1029 IU/L predicted HCA with a sensitivity of 65%, specificity 69%, PPV 83%, and NPV 46%. Positive likelihood ratio (LR+) was 2.1 for HCA. A total of 30 (57%) women had AF-Gluc concentration below 0.5 mmol/L. When AF-LD and AF-Gluc were used in a combination with cut-off values for AF-LD 1029 IU/L and for AF-Gluc 0.5 mmol/L, they showed a performance for HCA with a sensitivity of 53%, specificity 81%, PPV 86%, and NPV 43%.

The examination-to delivery interval for vaginally collected amniotic fluid samples before delivery was less than 72 hours. Furthermore, nine women had three repeatedly obtained AF-LD samples during a 10-day period. Eight (89%) of those women had HCA. AF-LD showed marked fluctuation in repeatedly obtained samples (Figure 7). No difference in AF-LD (p=0.15) or in AF-Gluc (p=0.42) concentrations occurred based on sampling method, self-sampling or speculum samples.

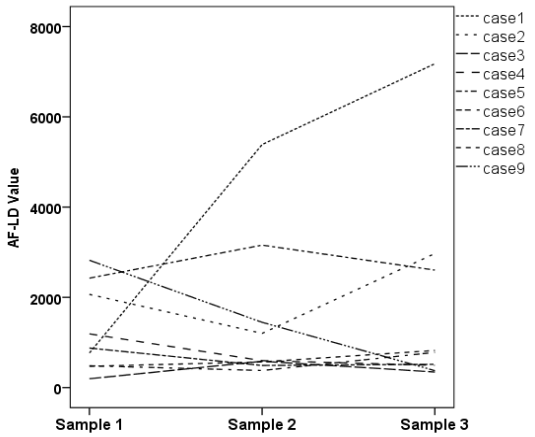


Figure 7. Fluctuation of AF-LD in vaginally collected samples in nine cases during a 10-day period. Sample 3 (the last) was obtained <72 h before delivery. All except case number 9 had HCA.

AF, amniotic fluid; LD, lactate dehydrogenase; HCA, histologic chorioamnionitis.

Amniotic fluid samples obtained with amniocentesis (II-IV)

Association of biomarkers with MIAC (II, III, IV)

MIAC was detectable in 30 (48%) women in Study II, in 18 (33%) women in Study III, and in 7 (26%) IAI-suspected cases in Study IV. All biomarkers except AF-MMP-2 and AF-CRP were associated with MIAC, and the association with MIAC concerning biomarkers of Studies III and IV appeared also when biomarker concentrations were adjusted by gestational age at sampling (unpublished data concerning Studies II and III). AF-cathelicidin and AF-MMP-8 were associated with MIAC both in pregnancies with intact membranes and in pregnancies with PPROM. When biomarker concentrations were adjusted by gestational age at AC, AF-cathelicidin, AF-MMP-8, AF-MMP-9, AF-MPO, AF-IL-6, AF-HNE, AF-Elafin, AF-TIMP-1, and AF-MMP-8/TIMP-1 molar ratio were associated with MIAC (Table 12).

Table 12. Association of biomarkers studied with MIAC in Studies II to IV.

		MIAC	No MIAC	p-value	Adjusted p-value*
Study II	**n**	**30**	**32**		
	LD IU/L	1514.5 (91-9698)	694 (62-3882)	0.012	0.317
	Gluc mmol/L	0.05 (0.0-3.4)	1.1 (0.0-6.0)	0.002	0.063
Study III	**n**	**18**	**36**		
	MMP-8 ng/mL	4907.5 (45-13292)	13.5 (3-6591)	<0.001	<0.001
	cathelicidin ng/mL	25.6 (3.2-42.9)	1.3 (0.5-28.1)	<0.001	<0.001
Study IV	**n**	**7**	**20**		
	MMP-8 ng/mL	3019 (69-5431)	7.5 (3-2372)	<0.001	<0.001
	MMP-9 ng/mL	2981 (84-9678)	3.4 (0.7-730)	<0.001	<0.001
	MPO ng/mL	4866 (267-79095)	299 (75.6-55185)	0.002	0.001
	IL-6 ng/mL	94.9 (5.6-540)	0.6 (0.03-1021)	<0.001	0.002
	HNE ng/mL	11280 (315-16380)	12.3 (2.8-9862)	<0.001	<0.001
	Elafin ng/mL	1533 (58-10491)	191 (7-4642)	0.031	0.016
	MMP-2 ng/mL	311 (266-938)	231.5 (153-696)	0.081	0.055
	TIMP-1 ng/mL	3958 (1456-9752)	1178 (633-9317)	0.001	0.001
	MMP-8 /TIMP-1 molar ratio	0.167 (0.011-0.973)	0.004 (0.001-0.155)	<0.001	<0.001
	CRP ng/mL	133 (23-1783)	42.5 (5-985)	0.092	0.092

median (range)
MIAC, microbial invasion of the amniotic cavity

MMP-8, matrix metalloproteinase -8; MMP-9, matrix metalloproteinase -9; MPO, myeloperoxidase; IL-6, interleukin -6; HNE, neutrophil elastase; MMP-2, matrix metalloproteinase -2; TIMP-1, Tissue inhibitor of matrix metalloproteinase -1; CRP, C-reactive protein; LD, lactate dehydrogenase; Gluc, glucose

The most optimal cut-off values based on the ROC curve for MIAC was determined for both AF-LD and AF-Glucose (Study II), and for AF-MMP-8 and AF-cathelicidin (Study III). The performances and accuracies of these biomarkers in relation to MIAC are in Table 13. Concomitant use of AF-LD together with AF-Gluc (Study II) or AF-MMP-8 with AF-cathelicidin (Study III) did not improve the accuracies.

Table 13. Cut-off values based on ROC curve and accuracies of amniotic fluid biomarkers in amniocentesis samples.

Biomarker	n	Outcome	Prevalence %	Cut-off	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	LR+
LD IU/L	62	MIAC	48	429	0.69 (0.55-0.82)	87	38	57	75	1.4
Glucose mmol/L	62	MIAC	48	0.7	0.72 (0.59-0.85)	67	66	65	66	2.0
LD and Glucose	62	MIAC	48			67	66	65	66	2.0
MMP-8 ng/mL	54	MIAC	33	41.5	0.90 (0.82-0.98)	100	69	62	100	3.2
cathelicidin ng/mL	54	MIAC	33	11.6	0.90 (0.82-0.98)	89	81	70	94	4.7
MMP-8 and cathelicidin	54	MIAC	33			89	81	70	94	4.7

LD, lactate dehydrogenase; MMP-8, matrix metalloproteinase-8; MIAC, microbial invasion of the amniotic cavity; HCA, histologic chorioamnionitis

The most optimal cut-off values are based on ROC curve.

MIAC in pregnancies with PPROM and intact membranes (II, III)

Of all 97 women in Studies II to III, PPROM occurred in 50 (52%). In Study II, PPROM occurred in 41 (59%) cases and in Study III in 25 (46%), but 16 of the women served in both studies. Microbial results were available in 90 women of Studies II and III, and MIAC occurred in a total of 35 (39%) of those; 13 women with microbial result served in both studies. MIAC occurred equally often in women with PPROM and in those with intact fetal membranes (Figure 8).

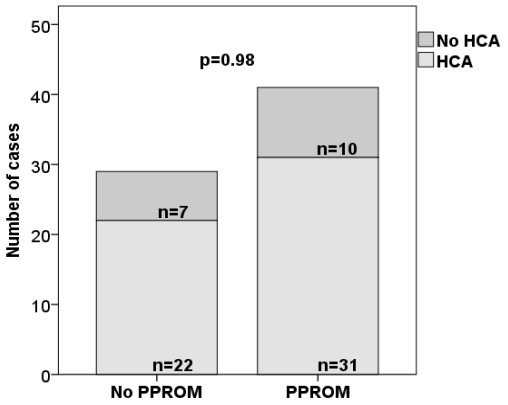


Figure 8. The frequency of MIAC in pregnancies with or without PPROM. In the No PPROM 4/43 were missing microbial analysis results and in the PPROM 3/47.

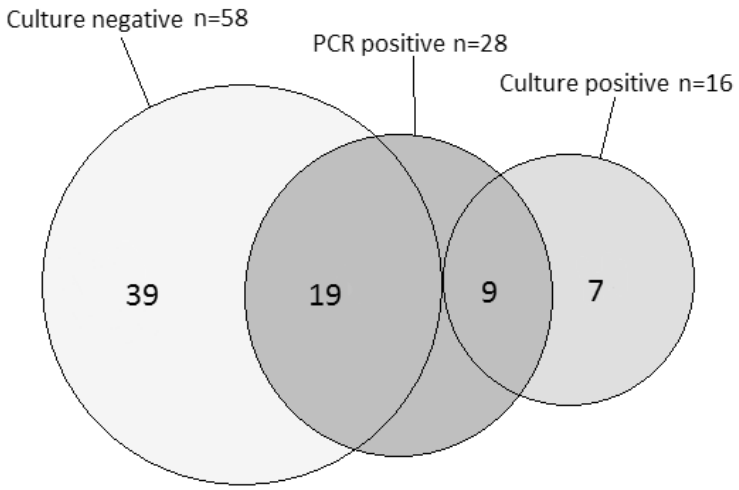
Microbial findings (II-IV)

Table 14. Microbiologic findings in amniocentesis samples, Studies II to IV.

Case no	PPROM +/-	Microbe in PCR	Microbe in culture
		PCR+ and Culture+	
1	-	*Streptococcus viridans*	*Streptococcus viridans*
2	-	*Streptococcus pneumoniae*	*Streptococcus pneumoniae*
3	-	*Fusobacterium nucleatum*	*Candida*
4	+	*Hemophilus influenzae*	*Hemophilus influenzae*
5	+	*Peptostreptococcus anaerobius*	*Peptostreptococcus anaerobius*
6	-	*Peptostreptococcus anaerobius*	*Peptostreptococcus anaerobius*
7	-	*Streptococcus agalactiae*	*Streptococcus agalactiae*
8	+	*Ureaplasma urealyticum / parvum* *Bacteroides ureolyticus*	*Candida*
9	+	*Ureaplasma urealyticum / parvum*	*Candida*
		PCR+ and Culture -	
10	+	*Ureaplasma urealyticum / parvum*	Neg
11	+	*Ureaplasma urealyticum / parvum*	Neg
12	-	*Ureaplasma urealyticum / parvum*	Neg
13	-	*Ureaplasma urealyticum / parvum*	Neg
14	+	*Ureaplasma urealyticum / parvum*	Neg
15	-	*Ureaplasma urealyticum / parvum*	Neg
16	+	*Ureaplasma urealyticum / parvum*	Neg
17	-	*Ureaplasma urealyticum / parvum*	Neg
18	-	*Ureaplasma urealyticum / parvum*	Neg
19	-	*Bacteroides ureolyticus*	Neg
20	+	*Ureaplasma urealyticum / parvum*	Neg
21	+	*Fusobacterium nucleatum*	Neg
22	+	*Mycoplasma hominis*	Neg
23	+	*Ureaplasma urealyticum / parvum*	Neg
24	-	*Bacteroides ureolyticus*	Neg
25	+	*Streptococcus viridans*	NA
26	+	*Ureaplasma urealyticum / parvum*	Neg
27	-	*Mycoplasma hominis*	Neg
28	-	*Fusobacterium nucleatum* *Bacteroides ureolyticus*	Neg
		PCR- and Culture+	
29	-	Neg	*Coagulase negative Staphylococcus*
30	+	Neg	*Gardnerella vaginalis*
31	-	Neg	*Candida*
32	+	Neg	*Gardnerella vaginalis*
33	+	Neg	*Candida*
34	-	Neg	*Gardnerella vaginalis*
35	+	Neg	*Candida*

PPROM, preterm prelabor rupture of the membranes; PCR, polymerase chain reaction, implemented for 16Sr RNA detection; NA, not available

Microbial results were available for 97 cases, of which 35 (36%) had MIAC (Table 14). The most common microorganism was *Ureaplasma* species (n=14) followed by *Candida* species (n=6). Of MIAC cases, 4 (11%) were polymicrobial. Only by PCR were 19 (54%) cases detectable and 7 (20%) only by bacterial culture (Figure 9). Of the MIAC cases in the whole study population, 18 (51%) occurred in PPROM pregnancies.



Culture result was available in 74 (missing n=23) and PCR result in 92 women (missing n=5).

Figure 9. Distribution of microbiology results according to method.

Infection and inflammation (III)

Infection and inflammation were more common at a lower gestational age when either AF-MMP-8 or AF-cathelicidin was a marker of inflammation and infection was determined as an increased value of inflammation marker in the presence of MIAC (Figures 10 and 11). Membrane status had no an effect on infection rate. Rate of colonization was affected neither by gestational age nor by membrane status.

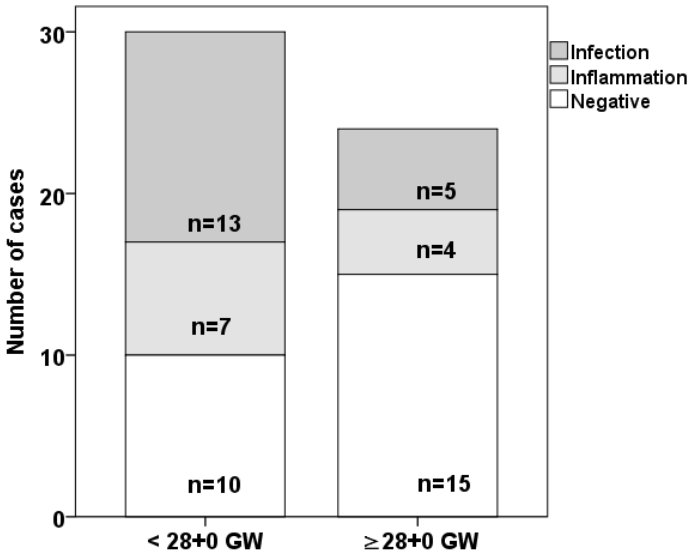


Figure 10. Number of patients at amniocentesis with infection (microbial invasion of the amniotic cavity+ (MIAC+), MMP-8 >41.5 ng/mL), inflammation (MIAC-, MMP-8 >41.5 ng/mL), colonization (MIAC+, MMP-8 <41.5 ng/mL), and negative (MIAC-, MMP-8 <41.5 ng/mL) in AF by MMP-8. None had colonization (MIAC+, MMP-8 <41.5 ng/mL). GW, gestational weeks.

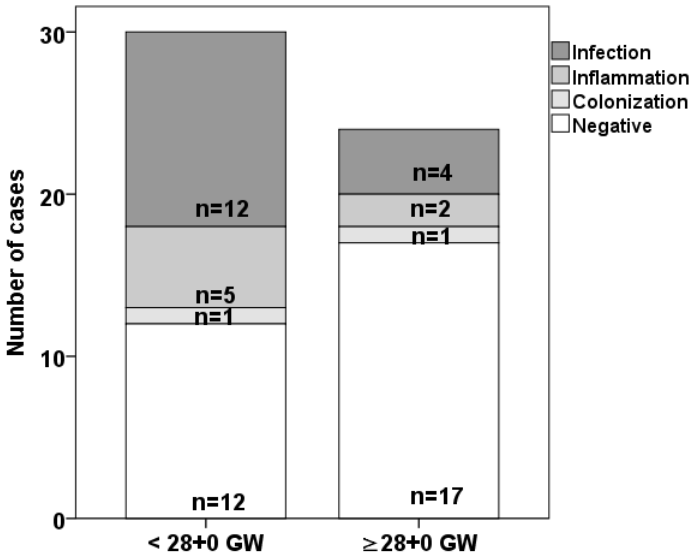


Figure 11. Number of patients at amniocentesis with infection (microbial invasion of the amniotic cavity+ (MIAC+), cathelicidin >11.6 ng/mL), inflammation (MIAC-, cathelicidin >11.6 ng/mL), colonization (MIAC+, cathelicidin <11.6 ng/mL), and negative (MIAC-, cathelicidin <11.6 ng/mL) in AF by cathelicidin. GW, gestational weeks.

Association of biomarkers with HCA (II, and unpublished data)

In Study II, HCA occurred in 53 (76%) of the women, and funisitis in 19 (36%). The most optimal cut-off values based on the ROC curve for HCA were for AF-LD and AF-Glucose. The performances and accuracies of these biomarkers in relation to HCA are in Table 15.

Table 15. Cut-offs of AF-LD and AF-Glucose for the prediction of HCA.

Biomarker	n	Outcome	Prevalence %	Cut-off	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	LR+
LD IU/L	70	HCA	76 %	429	0.76 (0.62-0.90)	83	65	88	55	2.4
Glucose mmol/L	70	HCA	76 %	0.7	0.70 (0.58-0.83)	56	82	91	38	3.1

AF, amniotic fluid; LD, lactate dehydrogenase; HCA, histologic chorioamnionitis

PPROM was diagnosed in 41 (59%) women. No difference appeared either in rate of HCA between pregnancies with PPROM and with intact membranes (Figure 12), or in rate of symptoms and signs of IAI except for uterine tenderness, which occurred more frequently in pregnancies with intact membranes.

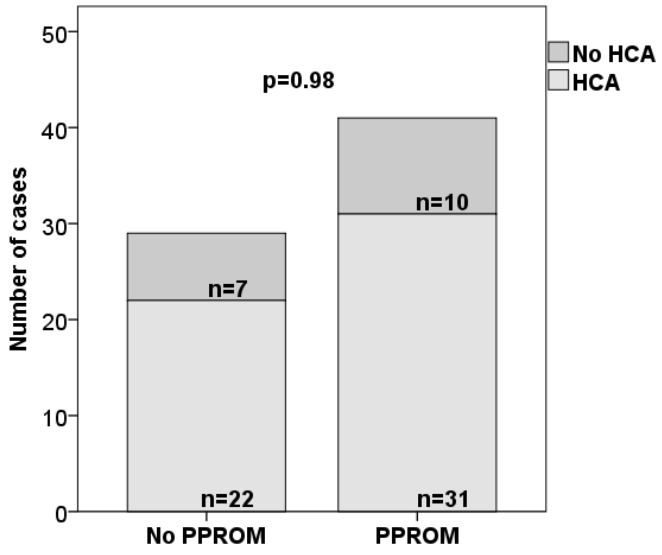


Figure 12. Frequency of HCA-cases in pregnancies with and without PPROM.

Association of MMP-8 with HCA with or without MIAC (unpublished data)

The study population comprised 34 women, of whom 18 (53%) had PPROM. MIAC occurred in 18 (53%). In comparing AF-MMP-8 concentrations between groups with both HCA and MIAC (n=18), HCA without MIAC (n=8), and with neither HCA nor MIAC (n=8), AF-MMP-8 had the highest median concentration in cases with both HCA and MIAC [4219.5 ng/mL (45-13292)], followed by those with HCA but no MIAC [1550.5 ng/mL (90-6145)], while the lowest concentrations were in those with neither HCA nor MIAC [53.5 ng/mL (3-6591); p=0.021 between the three groups] (Myntti *et al.*, unpublished data).

Neonatal outcome (IV)

Neonatal short-term outcome was evaluated of 27 neonates, of whom, 11 (41%) were born before 32 weeks of gestation. All nine cases of adverse neonatal outcome occurred among neonates born before 29 weeks of gestation. Six (67%) of them were born from a pregnancy complicated with MIAC. EONS occurred in two (7%) neonates. Table 16 demonstrates that inflammation and MIAC are more common in neonates born <32 weeks of gestation than in those born ≥ 32 weeks.

Table 16. Inflammation and MIAC by gestational age at delivery.

	<32+0 GW	**>32 GW**	**p-value**
n	11	16	
GW at AC	29 (27-31)	30 (22-31)	0.8
AF Inflammation, n (%)	8 (73%)	1 (6%)	0.031
AF MIAC, n (%)	6 (55%)	1 (6%)	0.10

median (range)
Inflammation, MMP-8 >41.5ng/mL

MIAC, microbial invasion of the amniotic cavity; GW, gestational weeks;
AF, amniotic fluid; AC, amniocentesis; MMP-8, matrix metalloproteinase-8

Discussion

Preterm delivery is the major cause of neonatal death and an important causative factor of neonatal morbidity (Slattery, Morrison 2002, Rovira *et al.* 2011). Intra-amniotic infection or inflammation should be diagnosed appropriately for optimal timing of delivery, steroid administration, and magnesium neuroprophylaxis, as well as for the decisions regarding tocolysis or antibiotics therapy. However, regarding massive cytokine release in AF as a consequence of MIAC or endogenous mediators, preterm birth or PPROM in many cases can no longer be prevented (Kim *et al.* 2015a). Unfortunately, no accurate method exists to diagnose IAI with reliability noninvasively (Buhimschi *et al.* 2013, Dulay *et al.* 2015), which we also noticed in our study of vaginally obtained amniotic fluid samples.

Romero *et al.* (2016a) have reported on increased levels of pyrogenic cytokines in maternal plasma in term pregnancies with symptoms of clinical chorioamnionitis despite intra-amniotic infection or inflammation. These results reflect the fact that pyrogenic cytokines are produced separately from IAI or clinical chorioamnionitis, which fact makes fever an unreliable sign for chorioamnionitis. In our studies, fever was rarely present despite the more frequent presence of IAI. Furthermore, these results of the Romero group confirm that symptoms and signs of clinical chorioamnionitis are only nonsensitive and nonspecific subjective findings.

Vaginally obtained amniotic fluid samples (I)

We demonstrated that in vaginally obtained AF samples, AF-Gluc was useless as a biomarker for HCA prediction. The cut-off for AF-Gluc in vaginal samples in the Buhimschi study was 0.28 mmol/L (Buhimschi *et al.* 2006), but during the study period our laboratory could not determine any exact values below 0.5 mmol/L. Over half of the AF-Gluc results were below our detection limit, without any difference exerted by either the presence or the absence of HCA. One reason for this finding may be the difficulty of obtaining AF vaginally without contamination by vaginal discharge, leading to possible bias in the analysis of results; vaginal discharge and serum have almost identical glucose concentrations (Ehrstrom *et al.* 2006).

In our study, AF-LD was associated with histologic chorioamnionitis, although that association disappeared with data adjustment by gestational age. Small sample size may affect the results after adjustment. Although the PPV was quite high, this marker is insufficiently accurate for use in clinical practice due to fluctuation of its concentration in samples repeatedly obtained. Our cut-off value for AF-LD based on the ROC curve in relation to HCA was in line with others' findings, where vaginally obtained AF-LD concentration was associated with MIAC determined by AC sample (Magloire *et al.* 2006b). We found no difference between AF-LD and AF-Gluc concentrations in self-sampling and speculum samples, indicating that no special device for AF collection vaginally is necessary, at least concerning these biomarkers, although Lee *et al.* (2015) have introduced a new transcervical device for AF collection in order to obtain samples uncontaminated with vaginal discharge. Their study, however, reports only the correlation of biomarker concentrations in vaginally obtained samples with AC samples, not their association with MIAC or HCA.

Association of biomarkers with MIAC (II-IV)

The benefits of AF-Gluc and AF-LD determination in IAI diagnostics are these markers' wide availability, rapidity, and low costs. The Romero group in 1990 was first to observe an association between AF-Gluc and MIAC with a cut-off of 0.8 mmol/L, which was in line with our study findings, though our method for MIAC detection differs from theirs. Although, in several reports, decreased glucose levels are associated with MIAC (Romero *et al.* 1990, Garry *et al.* 1996, Edwards *et al.* 2001, Ford, Genc 2011), no such association exists with sterile inflammation (Romero *et al.* 2014). This may be explained by the fact that glucose metabolism is not altered in the absence of microbes. Thus, AF-Gluc is not a marker of inflammation, but instead, a marker of MIAC.

AF-LD has also shown an association with MIAC, as first described almost 40 years ago (Bobitt, Ledger 1978). They had a cut-off level of 400 IU/L, although their samples were collected during delivery. Garry was the first, in 1996, to describe an AF-LD cut-off 419 IU/L for MIAC, which was later clinically evaluated by other authors (Buhimschi *et al.* 2007a, 2007b, Dulay *et al.* 2009). Although the NPV of AF-LD in the Garry study was high, its PPV was markedly low (Garry *et al.* 1996). The results of our research are also in line with these, and our cut-off for the presence of MIAC is close to theirs, although the methods of detecting MIAC differed. Our association of AF-LD and AF-Gluc with MIAC was lost after adjustment for gestational age, which may, at least in part, be explained by our small study population. Moreover, and importantly, the earlier studies did not adjust their concentrations of AF-LD and AF-Gluc for gestational age.
It is of importance that contrary to many earlier studies of AF-LD and AF-Gluc, we used both AF cultures and a molecular microbiology technique for the determination of MIAC, which enabled us to find more MIAC cases than by AF culture only.
Interestingly, although our cut-off value for AF-LD in relation to MIAC was almost identical to that determined by Garry *et al.* in 1996, Buhimschi *et al.* in 2007b, and Dulay *et al.* in 2009, our cut-off value for AF-MMP-8 concentration was set at 41.5 ng/mL, higher than the cut-off chosen in the previous studies (Park *et al.* 2001, Shim *et al.* 2004, Lee *et al.* 2007, Park *et al.* 2013a, Lee *et al.* 2015a). Importantly, we determined MMP-8 concentrations by the IEMA method, whereas they used Amersham ELISA or ELISA by R&D Systems (Buhimschi *et al.* 2007b, Lee *et al.* 2016). MMP-8 antibodies vary in their affinities to different MMP-8 isoforms, which leads to divergence in MMP-8 levels measured. Studies show that due to the higher affinity for the active form of MMP-8 of antibodies that they used, as well as in our study, higher overall levels of MMP-8 will result (Hanemaaijer *et al.* 1997, Sorsa *et al.* 2010, Buduneli *et al.* 2011). A correlation of MMP-8 measurement by immunofluorometric assay (IFMA) with measurement by Amersham ELISA exists (Sorsa *et al.* 2010), as well as a strong correlation between the IEMA and IFMA methods of Medix Biochemica (Sorsa T., unpublished data). Taken these into account, comparison of MMP-8 levels between studies becomes irrelevant if the antibodies used differ.

MMP-8 has been associated with inflammation (Nien *et al.* 2006, Lee *et al.* 2010, Kim *et al.* 2016), and with MIAC in pregnancies with or without PPROM (Maymon *et al.* 2000b). A rapid MMP-8 bedside test does exist for prediction of intra-amniotic infection and inflammation (Nien *et al.* 2006) and of MIAC (Lee *et al.* 2008). It has been able to predict MIAC and IAI in pregnancies with or without PPROM with high NPV, making it a valuable additional tool in ruling out IAI (Nien *et al.* 2006, Kim *et al.* 2007, Park *et al.* 2008, Lee *et al.* 2008). Interestingly, test performances for IAI and MIAC were almost similar in the study of Nien *et al.* (2006) to those of Lee *et al.* (2008), although in the study of Nien 2006, MIAC was determined only with cultivation, and Lee in 2008 also used PCR, as did we.

Cathelicidin is expressed mostly in fetal membranes and on other epithelial surfaces –ones that are naturally in contact with environmental microbes (Tambor *et al.* 2012). Because spontaneous delivery is an inflammatory process, it is unsurprising that after normal delivery cathelicidin is expressed in myometrium (Lim *et al.* 2015). Cathelicidin is also produced by fetal vernix caseosa (Yoshio *et al.* 2003), which may be an additional source of AF-cathelicidin. The association of AF-cathelicidin with MIAC has been evident in PPROM pregnancies (Tambor *et al.* 2012). However, studies evaluating the association of AF-cathelicidin with MIAC in pregnancies with intact membranes were lacking, until we first demonstrated that AF-cathelicidin levels are higher in MIAC cases despite the membrane status. This observation reflects the potential up-regulation of cathelicidin expression in MIAC and furthermore the antimicrobial properties concerning the host defence system described earlier (Ramanathan *et al.* 2002, Yoshio *et al.* 2003, Lim *et al.* 2015).

It is of importance that both MMP-8 and cathelicidin retained their association with MIAC also when the data was adjusted by gestational age at AC. That strengthens the value of these biomarkers in the diagnosis of IAI.

On study showed AF-MMP-2 levels to be decreased in MIAC, but this is visible only in PPROM pregnancies, where AF-MMP-2 overall levels are lower than in pregnancies with intact membranes (Maymon *et al.* 2000a). In our study of pregnancies with intact membranes, AF-MMP-2 levels did not differ by MIAC. AF-MMP-9 levels, instead, are increased in the presence of MIAC regardless of fetal membrane status (Fortunato *et al.* 1997, Maymon *et al.* 2000a) and also during labor (Vadillo-Ortega *et al.* 1996, Weiss *et al.* 2007). We studied only pregnancies with intact membranes, where we could confirm the association of AF-MMP-9 with MIAC. The difference between the present study and that of Maymon is that we used a molecular microbiology technique and cultivation for our determination of MIAC instead of Maymon´s cultivation only.

AF-TIMP-1 has also been linked to MIAC in pregnancies with intact fetal membranes, but unlike ours, only bacterial cultivation has been used to determine MIAC (Athayde *et al.* 1998).

Several studies have shown the association of AF-IL-6 with MIAC (Greig *et al.* 1993, Romero *et al.* 1993a, 1993b, 1993c, 2014d, Cobo *et al.* 2013, Dulay *et al.* 2015), which we could confirm, although our determination of MIAC differed from that in studies that used cultivation and Gram stain. When compared to AF-Gluc, WBC count, and Gram stain, AF IL-6 has been a superior predictor of MIAC in pregnancies with preterm labor or PPROM, though the levels of IL-6 were lower in women with PPROM and IAI than in women with intact membranes and IAI (Lee *et al.* 2011). Our study population was too small to allow comparison of the accuracies of various biomarkers, and furthermore, we studied only pregnancies with intact membranes. Contrary to earlier results, Cobo *et al.* (2011) found AF-IL-6 to be only a weak marker for inflammation in PPROM pregnancies, though these studies used similar methods for MIAC determination.

One report states that AF-elafin levels are increased in chorioamnionitis, and AF-HNE levels are increased in MIAC regardless of membrane status (Rivero-Marcotegui *et al.* 1997, Helmig *et al.* 2002, King *et al.* 2007a). Contrary to our findings, these studies determined MIAC with only cultivation. We also demonstrated increased AF-Elafin and AF-HNE concentrations in pregnancies with MIAC, although we studied only preterm pregnancies with intact membranes.

Studies concerning AF-MPO and AF-CRP regarding IAI are few. Kacerovsky *et al.* (2013) have reported an association of AF-MPO with MIAC and HCA in PPROM pregnancies. We could extend that association also to preterm pregnancies with intact fetal membranes.

AF-CRP levels have been higher in women with MIAC than in those without MIAC (Dulay *et al.* 2015), although AF-CRP concentrations have not been associated with maternal serum CRP concentrations (Malek *et al.* 2006). The Dulay study, unlike ours, included pregnancies with PPROM and with intact membranes. We observed that AF-CRP levels did not differ based on the presence or absence of MIAC. The Dulay group determined MIAC by cultivation, Gram stain, and mass-spectrometry score, as we did not. Their sample size was larger than ours, which may affect results; however, they published no exact AF-CRP values, but observed some overlapping between groups.

AF-CRP is produced in the fetal liver (Malek *et al.* 2006), and its concentration is not supposed to rise until the fetus is infected and CRP production has begun. Furthermore, as a large protein, CRP probably is incapable in a substantial amount of transference through the fetal kidneys into urine and AF, at least no longer in later pregnancy, though it has been detectable in fetal urine at the time of genetic AC (Raio *et al.* 2003). CRP´s large molecular size makes it also incapable of crossing the placenta, a fact that can explain the lack of association between AF and maternal serum CRP levels (Gutteberg *et al.* 1986).

Overall, we found that, in MIAC cases, AF biomarkers reflecting neutrophil activation and degranulation showed increased concentrations. In contrast, biomarkers that are not neutrophil-based, *i.e.* AF-MMP-2 and AF-CRP, did not react with MIAC.

Microbial findings (II-IV)

The rate of MIAC ranges in preterm pregnancies with intact membranes between 8.7% and 34% and in PPROM pregnancies between 17% and 57.7% (Review by Kim *et al.* 2015a). Our rate of MIAC was in line with those values. *Ureaplasma* spp. and *Mycoplasma* spp. are reported to comprise about half the microbes detectable in AF (Keelan *et al.* 2016). We discovered *Ureaplasma* spp. in 40% of our MIAC cases. Other microbes commonly found in AF in IAI cases are *Fusobacterium nucleatum*, *Mycoplasma hominis*, *Gardnerella vaginalis*, *Streptococcus* species, *Bacteroides* spp., and *Escherichia coli* (Mysorekar, Cao 2014, Fox, Eichelberger 2015, Kim *et al.* 2015a). Less frequent is *Listeria monocytogens* (Buhimschi *et al.* 2013), and a gastrointestinal bacteria *Coprobacillus* spp., first described by DiGiulio *et al.* 2010. We observed similarities in diversity of AF microbes, as in previous studies. In our study, *Candida* was the second most prevalent microbe, in one-fifth of the cases, though in the literature it has been a markedly less frequent AF microbe (DiGiulio *et al.* 2010). MIAC has been polymicrobial in the recent literature in 11% to 22% of pregnancies with PPROM (Musilova *et al.* 2015, Stepan *et al.* 2016) and, when PCR or PRC and cultivation methods are used in the detection of MIAC, in 9% of pregnancies with intact membranes (Romero *et al.* 2014a). Our rate of polymicrobial MIAC was in line with rates in those studies. With only cultivation used, one could speculate whether antibiotic treatment before AC had wiped out some MIAC cases, but we detected MIAC by PCR, which can also recognize the footprints of pre-existent microbes.

The role of *Ureaplasma* spp. as a pathogen of the upper genital tract has been under discussion *(*Dando *et al.* 2012). Modern microbiologic techniques provide the possibility to detect *Ureaplasmas* more often than with earlier cultivation techniques. *Ureaplasma* can also be cultivated by special methods, but not in media suitable for cultivation of common bacterial species. We detected *Ureaplasma* spp. exclusively by reliable PCR techniques. Most studies have used no molecular microbiology techniques (Park *et al.* 2013a, Combs *et al.* 2014), or PCR has been done only for *Ureaplasma* and *Mycoplasma* detection (Tambor *et al.* 2012). Our

AF samples have been analyzed by both broad spectrum 16S rRNA and general bacteria cultivation in order to achieve the maximum MIAC detection. Oyarzun *et al.* 1998 demonstrated an increase in detection rate by adding PCR as a diagnostic tool to identify MIAC, observing a threefold increase.

The theory had been that ascending infection is the most common route for bacteria to reach the amniotic cavity and the amnio-choriotic membranes, which leads to higher prevalence of MIAC in pregnancies with PPROM than in pregnancies with intact membranes (Soto *et al.* 2007). However, microbes can indeed pass the intact amniotic membranes; therefore MIAC also occurs in pregnancies with intact membranes (Galask *et al.* 1984, Ramos Bde *et al.* 2015). We observed that the MIAC prevalence was similar in the presence or absence of PPROM. This finding differs from ones of Soto *et al.* 2007, where prevalence of MIAC in PPROM pregnancies was over twofold that in pregnancies with intact membranes. One explanation may be the differing methods of MIAC detection between studies.

During recent years, some have demonstrated that the taxonomic profile of the placental microbiome resembles that of the oral flora more than of vaginal or fecal flora (Fox, Eichelberger 2015, Vinturache *et al.* 2016), which favors hematogenous spread as being one of the crucial pathways of microbial invasion. Increased vascular permeability in the gingival tissue during pregnancy concomitantly with periodontal disease allows recurrent bacteremia moving towards the placenta (Redline 2012, Madianos *et al.* 2013, Parthiban, Mahendra 2015). We found in AF samples the typical oral microbes *Fusobacterium nucleatum* and *Streptococcus viridans*. *Fusobacterium* plays the role of a door-opener for other bacteria species. That property may explain the common polymicrobial nature of MIAC (Buhimschi *et al.* 2013); in our study, *Fusobacterium* was present in half the polymicrobial cases.

Microbes tend to form biofilms, termed “sludge”. If such is observed, eradication by antibiotics is more unlikely than in pregnancies with free-floating AF microbes. Microbes from sludge are also more difficult to cultivate (Stewart, Costerton 2001, Donlan, Costerton 2002). This may result in the poor success rate of attempts to eradicate IAI, and, at least in part, explain sterile intra-amniotic infection. Overall, because only 1% of bacteria are cultivable (Romero *et al.* 2006, DiGiulio 2012), sterile intra-amniotic inflammation may reflect only the low sensitivity of detection methods or rapid and effective elimination of microbes by the host response (Redline 2012). However, by PCR, microbes eliminated by the host response should be recognizable, because PCR does not need living organisms for detection.

Infection and inflammation (III)

The rate of intra-amniotic infection and inflammation is higher at lower gestational ages (Hitti *et al.* 2001, Yoon *et al.* 2001, Combs *et al.* 2014), in line with our observations. In addition, our observation that rate of AF colonization remained stable across gestational ages support views in other publications (Combs *et al.* 2014).

Evoking of the host defence mechanism is the crucial factor affecting some microbes in some women causing chorioamnionitis and sometimes just existing as harmless colonization. The intensity of the inflammatory reaction depends on gestational age at the time of the exposure, the virulence factor of a microbe, the amount of invading microbes (Kacerovsky *et al.* 2011, 2012), and the individual properties of the host defence system (Genc, Ford 2010). Unfortunately, our analyses did not include quantitative PCR.

Controversial reports exist as to the origin of the AF neutrophils: fetal (Sampson *et al.* 1997, Redline 2012, Park *et al.* 2016) or maternal (Kim *et al.* 2015a). If they are considered as of maternal origin, they reflect the maternal inflammatory response to endogenous mediators or MIAC. The maternal host response is crucial in continuation of pregnancy, and on the other hand in limiting the infectious site in the uterus. The uterine inflammatory process in its first stage is usually circumscribed, is limited to a certain place until the host defence mechanisms fail, yielding to more general infection. In the first stage of infection, inflammatory changes are visible only at the site of infection, and only later in a more generalized stage in other places. In clinical work, we remain unaware of the current stage of the infection, which clarifies why sampling of maternal serum, urine, or of cervical secretions is unreliable in clinical use.

Antibiotics have anti-inflammatory properties and have the capacity to modify the immune system, in addition to their ability to eradicate microbes, and the (Bode *et al.* 2015). The effect on the immune system is transmitted by Toll-like receptors and cytokines, and the spectrum and amplitude of effects on the lymphocytes and neutrophils varies among antibiotics (Bode *et al.* 2014). Doxycycline and macrolides, for example azithromycin (Culic *et al.* 2001), seem effective in immune modulation (Bode *et al.* 2014). On the basis of the available literature (Lee *et al.* 2016), *Ureaplasma* as a common pathogen in IAI (Keelan *et al.* 2016), and in the preliminary results of our study, we have already modified our antibiotics policy in everyday clinical practice by adding azithromycin in addition to the cephalosporines in the management of PPROM pregnancies. Macrolides have been included in the antibiotic protocol in another study, as well, although they found clarithromycin to be the drug of choice (Lee *et al.* 2016). One advantage favoring azithromycin is its oral administration.

A new macrolide antibiotic, solithromycin, seems to be valuable in prevention and management of intra-amniotic infections due to its broad microbial spectrum, high tissue uptake, easy oral administration, anti-inflammatory properties, and effective placental transfer (Keelan *et al.* 2016). Antenatal corticosteroids also have anti-inflammatory properties, which can be seen in reductions in maternal serum IL-6 and CRP levels (Nayeri *et al.* 2014). Non-steroidal anti-inflammatory drugs do pose a risk for narrowing or closing the fetal ductus arteriosus (Bermas 2014), and, more specifically, the use of indomethacin poses a risk for adverse neonatal outcome (Hammers *et al.* 2015); these limit their use in pregnancy. Some reports, however, demonstrate that NSAID use during pregnancy does not affect infant survival, neonatal complications, or congenital malformations (Nezvalova-Henriksen *et al.* 2013, Damase-Michel, Hurault-Delarue 2014).

In an ovine model, Ireland *et al.* (2015) demonstrated that intra-amniotic inflammation can be suppressed by administration of a cytokine-suppressive anti-inflammatory drug intra-amnially as a single bolus. In another animal model, the rate of murine preterm labor and fetal demise was reduced if intra-amniotic inflammation induced with lipopolysaccharide was treated with N-acethylcysteine (an agent with both antioxidant and anti-inflammatory properties) (Buhimschi *et al.* 2003, Paintlia *et al.* 2008). Importantly, a recent randomized controlled trial on human newborns exposed to chorioamnionitis shows that treatment with N-acethylcysteine is safe (Jenkins *et al.* 2016). Finally, Buhimschi *et al*. in 2003 demonstrated in a murine model that release of free radicals and a shift in oxidative balance as a consequence of inflammation can be normalized with administration of N-acetylcysteine, and the rate of preterm birth thereby reduced. Oxidative stress in a human intra-amniotic infection is clearly established, as well (Chafer-Pericas *et al.* 2015). However, the applicability of such anti-inflammatory drugs for treatment of intra-amniotic infection and inflammation in humans needs further investigation of optimal dosage, duration, and the route of administration. Moreover, safety of the treatment for the mother should be ensured.

Association of biomarkers with histologic chorioamnionitis (II)

AF-Gluc has been associated with HCA with cut-off values of 1.1 mmol/L, though the accuracies in our study were reported for a cut-off value of 0.8 mmol/L. AF-Gluc predicted HCA with a sensitivity of 28% and a specificity of 95% (Odibo *et al.* 1999). In another study concerning AF-Gluc levels and HCA in pregnancies with intact membranes, different cut-offs for AF-Gluc (0.3 mmol/L- 0.9 mmol/L) were calculated, yielding sensitivities of 41% to 55% and specificities of 94% to 100% (Greig *et al.* 1994, Odibo *et al.* 1999). AF-LD has also shown an association with HCA (Kidokoro *et al.* 2006). We could confirm the association of AF-Gluc and AF-LD with HCA. Our cut-off value for AF-Gluc based on its ROC curve was in line with those in other studies. Additionally, we found the same cut-off value to be suitable for both MIAC and HCA. Another difference between these studies and ours is the method of MIAC determination, which in those studies was based on cultivation.

Our cut-off value for AF-LD based on the ROC curve was higher than the value in a study of the Kidokoro group, 250 IU/L. Its setting differed from ours, since that group, reporting in 2006, accepted women between 16 and 35 weeks of gestation eligible for the study. AF-LD has also shown increased levels in cases of funisitis (Buhimschi *et al.* 2007a), which rarely is observed without HCA, because it is considered an advanced HCA stage. Their median concentration of AF-LD in funisitis cases was, however, only 414.5 IU/L, which was lower than our cut-off level for HCA.

AF-MMP-8 in our study was higher in women with HCA than in those without, which is in line with previous findings (Park *et al.* 2013a, Kim *et al.* 2015b). Our AF-MMP-8 levels were increased also in cases with sterile HCA, i.e. HCA without MIAC, which have been also demonstrated earlier (Park *et al.* 2013a), though in that study, MIAC was determined with cultivation only, unlike our procedures. We demonstrated additionally that AF-MMP-8 concentrations rose more in cases with concomitant HCA and MIAC than in pregnancies with one of these alone; this supports use of invasive procedures for diagnosing MIAC, which often leads to HCA.

HCA exists in over half of the cases with MIAC (Romero *et al.* 2014c, Vajrychova *et al.* 2016) and in about half the cases with sterile inflammation (Romero *et al.* 2014c), indicating that placental pathology does not always correlate with IAI (Pettker *et al.* 2007). We observed a similar rate of sterile HCA, although we had pregnancies both with intact membranes and with PPROM included, but the Romero group studied only pregnancies with intact membranes.

Neonatal outcome (IV)

Intra-amniotic inflammation has reportedly caused a more severe fetal response in preterm labor with intact membranes than in PPROM pregnancies (Park *et al.* 2013d). Additionally, the intra-amniotic inflammation may cause an adverse neonatal outcome regardless of the presence or absence of MIAC (Shim *et al.* 2004, Combs *et al.* 2014, Romero *et al.* 2014c). These findings should warrant a more serious view and acute awareness of the complex issue of subclinical chorioamnionitis in pregnancies with or without PPROM; in such cases the inflammation, even in the absence of MIAC, can cause an adverse neonatal outcomes.

The single most important risk factor for neurological disabilities is preterm birth (Salmeen *et al.* 2014). We observed that all neonatal adverse-outcome cases occurred in neonates born at <29 weeks of gestation, one-third of them without exposure to MIAC. Preterm birth, *per se*, even late preterm birth occurring at 34+0 to 36+6 weeks of gestation, carries a lifelong risk for some neurocognitive disorder, a risk which may be slightly alleviated by a high level of education (Heinonen *et al.* 2015). However, gestational age at delivery plays a crucial role in short-term neonatal outcome, one more important than the presence or absence of MIAC, sterile inflammation, or IAI (Musilova *et al.* 2015).

Principal findings on new biomarkers and IAI (IV)

We demonstrated that neutrophil-based proinflammatory cascades are present in AF similar to those in periodontal tissues (Alfakry *et al.* 2016). Concentrations of the neutrophil-based biomarkers AF-MMP-8, AF-MMP-9, AF-MPO, AF-IL-6, and AF-HNE in IAI and MIAC cases were increased. MMP-8 is a potent protease capable of degrading extracellular matrix in many human tissues. The process causing a tooth to detach is similar to the process leading to membrane rupture or cervical shortening and opening. IL-6 is a locally, and upon-stimuli-secreted, multifunctional cytokine, being an inducer of the neutrophil-based inflammatory cascade and causing PMN extravasation at the sites of inflammation (McGeough *et al.* 2012). Infection and inflammation can induce IL-6 and the degranulation of proteases, i.e. MMP-8, MMP-9, HNE, and MPO, from activated neutrophils. IL-6, by itself, can act as a PMNs irritating chemoattractant and induce the degranulation of MMP-8, MMP-9, and MPO, which form a PMN-derived proteolytic and proinflammatory cascade. Furthermore, MPO is an oxidative activator of MMP-8 and MMP-9 as well as an oxidative inactivator of TIMP-1 by producing hypochlorus acid (HOCl) (Alfakry *et al.* 2016). Additionally, HNE and MPO, individually or together, can proteolytically and oxidatively inactivate TIMP-1, which leads to reduction in the anti-proteolytic shield in the AF.

HNE can act as a proteolytic activator of MMP-9 but not of MMP-8; thus HNE and MMP-9 form another PMN-derived proteolytic cascade, with which elafin associates while being an antiprotease of HNE. That role of elafin can explain the increased elafin concentrations in IAI and MIAC cases, though it is only in part produced by PMNs and also in epithelial cells. MMP-2 and TIMP-1 are not produced by PMN, but instead by resident epithelial cells. TIMP-1 concentrations were also increased in IAI and MIAC cases, thus reflecting TIMP-1´s role as a coordinator of MMP-8 concentrations.

We found that the neutrophilic proinflammatory profile was associated with the general inflammatory marker AF-CRP, suggesting activated neutrophils to be the major source of AF-MMP-8, AF-MMP-9, AF-MPO, and AF-HNE. Our study demonstrates this by the association of these biomarkers with MIAC and IAI. These biomarkers retained the association with MIAC also when the data were adjusted for gestational age at AC, which strengthens the potential of these biomarkers in the diagnosis of IAI. In this regard, it is important that no corresponding associations were detectable in AF-MMP-2 and AF-TIMP-1, which are not produced by neutrophils and therefore do not react in neutrophil activation; this observation fortifies the theory of neutrophil activation being behind IAI.

Clinical implications and future prospects

Our results confirm the known association of IAI in patients with preterm labor both in PPROM pregnancies and in those with intact fetal membranes. Unlike earlier publications, we used cultures together with molecular microbiology techniques. According to the preliminary results of MIAC etiology here and in the literature, we have modified our prophylactic antibiotic policy and decided to administer azithromycin to all patients with PPROM.

Our forthcoming study regarding comparison of neonatal and maternal outcomes in pregnancies with suspected IAI undergoing AC and those without AC is under way. Certainly, the only way to achieve an answer concerning the impact of AC on long-term outcome is an adequately powered multicenter randomized control trial.

We suggest that in daily clinical practice, AC should be a routine procedure in IAI diagnosis. The results of the currently widely used biomarkers AF-LD and AF-Gluc are available to clinicians, but the delay in receiving results of microbial analysis may be several days, resulting in anxiety. We have demonstrated, however, that the current biomarkers AF-LD and AF-Gluc are unfortunately not very accurate in MIAC or HCA prediction. We were, to our knowledge, the first to study the concomitant use of these biomarkers, so that MIAC was detectable by PCR and microbial culture both.

In vaginally collected AF samples, we demonstrated that AF-LD and AF-Gluc are not useful biomarkers, even if used concomitantly. Notably, we also demonstrated that sampling method had no effect on biomarker concentrations. Based on these results, vaginal AF sampling has been abandoned thus far in our clinic, until we can introduce for vaginal sampling a better biomarker.

In the present study, we determined cut-off values for the newer biomarkers AF-MMP-8 and AF-cathelicidin and observed better accuracies than with AF-LD and AF-Gluc. This may help in clinical decision-making in pregnancies with suspected IAI. Our method to measure MMP-8 concentrations differs from the method of earlier IAI studies, as it recognizes better the active form of MMP-8 and yields higher overall concentrations of MMP-8. We seem to be the first to demonstrate the association of AF-cathelicidin with MIAC in pregnancies with intact membranes.

A rapid bedside test with the novel markers would be of great value for clinicians, helping ease further management decisions and the follow-up of such pregnancies (administration of antenatal corticosteroids, magnesium-neuroprophylaxis, and tocolysis) and decisions on optimal delivery timing. Qualitative point-of care tests of AF-MMP-8 and AF-IL-6 already exist (Kim *et al.* 2007, Chaemsaithong *et al.* 2016a, 2016b). IAI exists, however, not just as present or absent; one must bear in mind that intensity of inflammation correlates with neonatal outcome, so the magnitude of inflammation should also have an influence on clinical decisions. A simple test of just a positive or negative result does not provide information on inflammation severity, and therefore we hope to have a rapid test which shows biomarker concentrations.

Based on our results, in which MIAC was equally common in pregnancies with intact membranes as in PPROM pregnancies, we must change our conception of pregnancies with intact membranes and mild symptoms as being harmless to being seen instead as potential sites of inflammation. Administration of AC as added to the clinical protocol would be of great value in IAI-suspicious cases (Figure 13), at least in PPROM pregnancies, because the result of AC can influence our management of such pregnancies. Furthermore, AC is justified regardless of gestational week in PPROM pregnancies, because IAI and adverse neonatal outcomes are independent of gestational age at PPROM (Cobo *et al.* 2011).

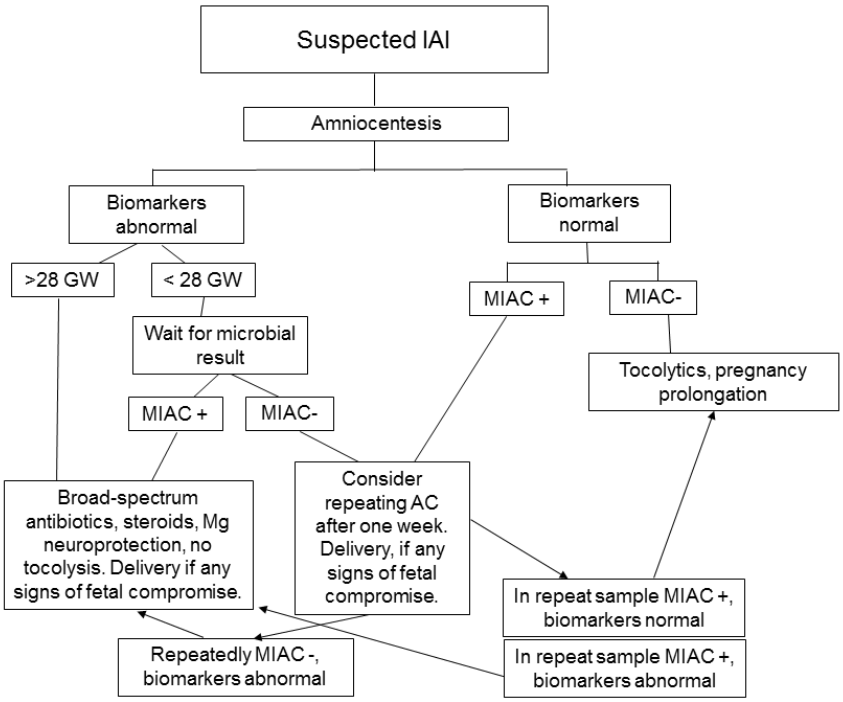


Figure 13. Flowchart of suggested protocol of treatment of IAI-suspected women (By author). IAI, intra-amniotic infection; GW, gestational weeks; MIAC, microbial invasion of the amniotic cavity; AC, amniocentesis; Mg, magnesium.

We found that IAI seems to be associated with activation and degranulation of PMN in the same manner as in inflammatory conditions in other body systems. Based on that finding, we speculate that neutrophil-based biomarkers have the best accuracy in the diagnosis of IAI. In the future, neutrophil-based biomarker investigation in a larger material and in different panels would be of interest in order to confirm and extend further the existing findings.

Strengths and limitations of the studies

Strengths

We studied a single tertiary center, which can be assumed to be both a strength and a limitation. Its strength: every patient was treated in the same manner, and a single laboratory analyzed our samples, so no interobserver variation occurred. Its limitations: the study population was relatively small in every study (I-IV), and a multicenter setting could have yield a larger study population.

We used both PCR and cultivation for determination of MIAC, yielding a higher number of MIAC cases identified.

Limitations

No multivariate analyses were made, but because the small study population, such analyses would have been meaningless.

Power-analysis is lacking. While studying biomarkers as linear variables, it is difficult to specify a sufficient difference between positive and negative results to calculate an adequate number for the study population.

Some patients underwent antibiotic administration before AC, which may lead to some false-negative microbial results. PCR, however, recognizes the fingerprints of microbes and requires no living microbes to provide a positive result.

Some patients underwent administration of antenatal corticosteroids before AC, which may lead to some misleadingly low biomarker concentrations.

Racial variabilities in biomarker concentrations were not taken into account. IL-6 concentrations are, overall, higher in races of African origin, and there they also show a more robust stress-induced response (Christian et al. 2013). Whether the same cut-offs are suitable for all races is unknown. In Finland it is impossible to register ethnicity. Our study population consisted mostly of white individuals, which is not the case in studies from, for example the United-States or Korea. Comparing such results may be misleading.

Conclusions

On the basis of this study, the following conclusions can be drawn:

1. In vaginally obtained AF, the lack of association between AF-Gluc and HCA, and an existing association, but marked fluctuation in AF-LD makes both of these unreliable for diagnosis of IAI. These results indicate that better biomarkers for vaginal AF sampling are essential (I).

2. In AC samples, AF-LD and AF-Glucose are associated with MIAC and HCA, and the same cut-off value is suitable for both MIAC and HCA, which do not necessarily coexist. Although the accuracies of these biomarkers are not very high, they can serve as a directional, additive diagnostic tool in diagnosis of IAI (II).

3. The novel biomarker AF-cathelicidin and the biomarker AF-MMP-8 are associated with MIAC in AC samples both in pregnancies with intact membranes and in those with PPROM, reflecting the capability of MIAC to upregulate concentrations of cathelicidin. Cut-off values were determined, and the cut-off for AF-MMP-8 was higher than in earlier studies (III).

4. AF neutrophil-based biomarkers are associated with MIAC in preterm pregnancies with intact membranes, and are capable of separating IAI cases from healthy controls. These findings suggest that neutrophil-derived proinflammatory cascades are associated with IAI, as they are with other inflammatory conditions in various body systems. The further extension of these results in PPROM pregnancies remains to be studied, as well as the benefit of using several biomarkers as a panel (IV).

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