

# Cervical and intra-amniotic markers of preterm birth and infection

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# Cervical and intra-amniotic markers of preterm birth and infection

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Göteborg 2009



**Finish what you start.....**

**The truth is never knowable with certainty.....**

*To Bonny and Gunnar*



## Abstract

**Background:** Preterm delivery (PTD; < 37 gestational weeks), is one of the greatest unsolved obstetrical problems worldwide. As much as 80% of the perinatal mortality and 50% of the long-term neurological handicaps are associated with PTD. Spontaneous preterm birth (SPTD), i.e. preterm labor (PTL) or preterm prelabor rupture of membranes (PPROM) is responsible for 55% of PTD. Clinical and experimental evidence suggest that maternal infection and/or inflammation are centre stages in SPTD and the major risk factors for fetal injury. Several cytokines and chemokines play a central role in SPTD. However, in most cases the precise mechanistic pathway leading to SPTD remains unknown and good markers of prediction and therapies are few.

**Aim:** To investigate if cervical and intra-amniotic proteins on their own and/or in combination with each other and/or with clinical characteristics could predict SPTD and intra-uterine infection/inflammation in women with singleton pregnancies in PTL. In particular the purpose was to investigate the predictive value of cervical markers (proteins or sonography) collected less invasively compared with amniotic fluid proteins collected via amniocentesis.

**Material and methods:** A cohort of 134 women in PTL and 30 with PPROM with singleton pregnancies and gestational age less than 34 weeks were studied. Amniotic fluid (AF) was retrieved transabdominally from 107 patients in PTL and in 30 patients with PPROM. Cervical fluid (CF) was sampled from the external cervical os in all PTL women, but from none of the PPROM cases. Transvaginal sonography (TVS) assessing cervical length (CL) was performed in all patients. Polymerase chain reaction analyses for *Ureaplasma urealyticum* and *Mycoplasma hominis* and culture for aerobic and anaerobic bacteria were performed. Interleukin (IL)-6, IL-8, IL-18, monocyte chemotactic protein (MCP)-1, MCP-2, and MCP-3 were analyzed with enzyme-linked immunosorbent assay. The multiplex sandwich immunoassay, flowmetric Luminex xMAP (multiple analyte profiling) technology analyzed 27 specific proteins, IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, IL-18, sIL-6 $\alpha$ , IFN- $\gamma$ , TNF- $\alpha$ , TNF- $\beta$ , MCP-1, TGF- $\beta$ , MIP-1 $\alpha$ , MIP-1 $\beta$ , MMP-9, TREM-1, BDNF, GM-CSF, NT-4, NT-3, sTNF RI, MIF and RANTES. Histological examinations of the placentas were performed in 42 cases in PTL and in 30 with PPROM. Maternal, antenatal and intrapartal variables were retrieved from medical records

**Results:** Non-lacto-bacillus dominated flora was detected in CF in 25% (22/89) and 17% had microbial invasion of the amniotic cavity (MIAC) and 45% had intra-amniotic inflammation. High levels of IL-6 and IL-8 were associated with PTD  $\leq$  7 days from assay and  $\leq$  34 weeks of gestation. Cervical length assessed by TVS predicted intra-amniotic inflammation as well as PTD. Intra-amniotic levels of IL-6, IL-8, IL-18, MCP-1 and MCP-3 were all significantly higher in PTL cases with histological chorioamnionitis (HCA) whereas such relationship was not found in the PPROM group. Cervical IL-6 and IL-8 in PTL were associated HCA and an IL-8

value of 10.0 ng/mL was a strong predictor of HCA with sensitivity 100%, specificity 67%, positive predictive value 63%, and negative predicted value 100%.

Several of the proteins analyzed in both AF and CF, by the xMAP technology, were associated with PTD  $\leq 7$  days from assay and with MIAC. Novel findings were that amniotic IL-17 and TREM1 and cervical IL-17, sIL-6 $\alpha$ , BDNF, NT4, NT3, IL-4, IL-5, and RANTES were significantly higher in the women delivering within 7 days of assay. We found that cervical IL-17, sIL-6 $\alpha$ , NT3, TNF- $\beta$ , IL-4, and TREM1 were significantly associated with MIAC which has not previously been reported. A multivariate model combining amniotic macrophage inflammatory protein (MIP)-1 $\beta$  with cervical interferon (INF)- $\gamma$  and MCP-1 predicted SPTD  $\leq 7$  days likelihood ratio (LR) 5.6 and area under the ROC-curve (AUC) 0.91 and a non-invasive multivariate model based on CL, cervical INF- $\gamma$ , IL-6 and MCP-1 predicted SPTD  $\leq 7$  days with LR 4.7 and AUC 0.91. The best multivariate model predicting MIAC based on cervical IL-17 and MCP-1 had LR 6.0 and AUC 0.87.

**Conclusions:** In the present studies, we have identified inflammatory markers in both cervical and amniotic fluid that together with cervical length as measured by transvaginal sonography can predict spontaneous preterm delivery, intraamniotic infection and/or inflammation and histological chorioamnionitis. It seems as the non-invasive route of sampling analytes can be used instead of the more commonly used invasive method of amniocentesis.

**Key words:** Spontaneous preterm delivery, preterm labor, preterm prelabor rupture of membranes, intra-amniotic infection/inflammation, inflammatory proteins, histological chorioamnionitis, cervical and amniotic markers.

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## List of publications

This thesis is based on the following articles and manuscripts, which will be referred to in the text by their Roman numerals:

- I. Holst R-M, Mattsby-Baltzer I, Wennerholm U-B, Hagberg H, Jacobsson B. Interleukin-6 and interleukin-8 in cervical fluid in a population of Swedish women in preterm labor: relationship to microbial invasion of the amniotic fluid, intra-amniotic inflammation, and preterm delivery. *Acta Obstet Gynecol Scand* 2005; 84: 551-557
- II. Holst R-M, Jacobsson B, Hagberg H, Wennerholm U-B. Cervical length in women in preterm labor with intact membranes: relationship to intra-amniotic inflammation/microbial invasion, cervical inflammation and preterm delivery. *Ultrasound Obstet Gynecol* 2006; 28: 768-774
- III. Holst R-M, Laurini R, Jacobsson B, Samuelsson E, Sävman K, Doverhag C, Wennerholm U-B, Hagberg H. Expression of cytokines and chemokines in cervical and amniotic fluid: Relationship to histological chorioamnionitis. *J Matern Fetal Neonatal Med* 2007; 20(12): 885-893
- IV. Holst R-M, Hagberg H, Wennerholm U-B, Skogstrand K, Thorsen P, Jacobsson B. Prediction of spontaneous preterm delivery in women with preterm labor – analysis of 27 proteins by Luminex xMAP technology in amniotic and cervical fluids. *Submitted for publication*
- V. Holst R-M, Hagberg H, Wennerholm U-B, Skogstrand K, Thorsen P, Jacobsson B. Prediction of microbial invasion of the amniotic cavity in women with preterm labor based on analysis of multiple proteins in amniotic and cervical fluids with Luminex xMAP technology. *Submitted for publication*



## **Abbreviations**

AUC – area under the curve  
AF – amniotic fluid  
BDNF – brain derived neurotrophic factor  
BV – bacterial vaginosis  
CCR – cell surface receptor  
CF – cervical fluid  
CGRP – calcitonin gene-related peptide  
CHR – corticotropin-releasing hormone  
CI – confidence interval  
CRP – C-reactive protein  
CS – caesarean section  
DAP – death associated protein  
ELISA – enzyme-linked immunosorbent assay  
FFN – fetal fibronectin  
FIRS – fetal inflammatory response  
GM-CSF – granulocyte-macrophage colony stimulating factor  
HCA – histological chorioamnionitis  
ICAM – intra cellular adhesion molecule  
IL – interleukin  
INF – interferon  
IS – inflammatory signs  
IUGR – intrauterine growth restriction  
LPS – lipopolysaccharide  
LR – likelihood ratio  
MCP – monocyte chemotactic protein  
MHC – major histocompatibility complex  
MIAC – microbial invasion of the amniotic cavity  
MIF – macrophage migration inhibitory factor  
MIP – macrophage inflammatory protein  
MMP – metalloproteinase  
NF- $\kappa$ B – nuclear factor-kappa B  
NK – natural killer  
NL-R – nodlike receptor  
NO – nitric oxide  
NOD – nucleotide-binding oligomerization protein  
NPV – negative predictive value  
NT – neurotrophin  
PCR – polymerase chain reaction  
PMN – polymorphonuclear  
PPV – positive predictive value  
PRR – pattern recognition receptor

PTD – preterm delivery  
PTL – preterm labor  
PPROM – preterm prelabor rupture of the membranes  
PRR – pattern recognition receptor  
RANTES – Regulated on Activation, Normal T- cell Expressed and Secreted  
ROC – receiver-operating characteristic  
SCFPMN – subchorionic fibrin polymorphonuclear  
sIL-6 $\alpha$  – soluble interleukin 6 receptor alpha  
SPTD – spontaneous preterm delivery  
SNPs – single nucleotide polymorphisms  
sTNF RI – soluble tumor necrosis factor receptor I  
TIMP – tissue inhibitor of metalloproteinases  
TLR – toll-like receptor  
TGF – transforming growth factor  
TNF – tumor necrosis factor  
TREM – triggering receptor expressed on myeloid cells  
Trk – tropomyosin-related kinase  
TVS – transvaginal sonography  
VCAM – vascular cell adhesion molecule  
VIP – vasoactive intestinal peptide  
xMAP – multiple analyte profiling

# Introduction

## 1. General introduction

Preterm delivery (PTD) is defined by the World Health Organisation (WHO) as delivery less than 37 completed weeks (WHO 1977). PTD continues to be one of the most profound clinical problems in obstetrics and perinatal medicine worldwide. Despite considerable efforts in maternal-fetal medicine during decades we still don't fully understand the biological pathways leading to PTD.

Preterm entry into the world places the neonate at an immediate disadvantage compared with a neonate born at term. The preterm born infant, especially the extremely preterm i.e. before 28 gestational weeks, (Hagberg et al. 2001; Slattery and Morrison 2002) will begin its life with a substantially higher risk of severe brain injury, chronic lung disease, respiratory distress syndrome, necrotizing enterocolitis, and a lifetime with increased medical, social and special educational needs (Hack and Fanaroff 1999; Marlow et al. 2005; Saigal and Doyle 2008; Stjernqvist and Svenningsen 1999).

Preterm labor (PTL) is now regarded to be a syndrome initiated by multiple mechanisms, including infection or inflammation, uteroplacental ischemia, haemorrhage, uterine overdistension, maternal stress, and other immunologically mediated processes (Romero et al. 2006c). However, irrespective of primary etiology a common inflammatory terminal pathway leads to increased uterine contractility, cervical ripening, and membrane/decidual activation (Romero et al. 2006c).

For many years the strategies to prevent PTD focused on the identification of risk factors (Crane and Hutchens 2008; Goldenberg et al. 2001; Herbst and Nilsson 2006; Iams 2003) and, when PTL was clinically manifest, on the treatment of uterine contractions using tocolytic drugs (Coomarasamy et al. 2003; Grimes and Nanda 2006; Thornton 2005). The efficacy of such drugs is however low and the administration of tocolytic treatment has only proven to delay PTD by 48 h and has not proven to improve neonatal outcome (Whitworth and Quenby 2008). However, this delay allows the clinician enough time to administer corticosteroids for fetal lung maturity and in utero transfer to a tertiary centre with neonatal intensive care unit facilities and these actions are associated with improved outcomes for preterm infants (Towers et al. 2000; Yeast et al. 1998).

PTL is a frequent cause of admission to hospital during pregnancy, but only a minority (8-38%) (Fuchs et al. 2004; Holst et al. 2006; Tsoi et al. 2003; Tsoi et al. 2004) of the patients admitted to hospital with PTL will deliver preterm. Separation of high- and low risk patients is important so that unnecessary hospital stays and potential harmful treatment in low risk patients can be avoided and attention can be focused on those women at truly high risk. This is, however, difficult since there are few clinical tools with high predictive ability to help the clinician to discriminate between harmless early contractions and true PTL. Research in biomedicine is, how-

ever, developing rapidly and new tools such as genomics and proteomics (Buhimschi et al. 2008; Romero et al. 2006b), are now available to address old medical problems like the preterm parturition syndrome. These new techniques give hope that in the future the preterm parturition syndrome will be fully understood so that clinical tools and therapies can be developed.

There is evidence that as much as 40-60% of the SPTDs are associated to infection/inflammation (Gardella et al. 2004; Goldenberg et al. 2000). This thesis is an attempt to find new cervical and intra-amniotic markers of preterm birth linked with intrauterine infection/inflammation and to enhance the understanding of the preterm parturition syndrome. This thesis does not provide a solution to the enigma of preterm birth, but it generates novel biological insights into the inflammatory process.

## **2. Preterm birth**

### **2.1 Frequency**

PTD is not evenly distributed among women. The frequency of PTD varies depending on geographical and demographic features of the population studied (Slattery and Morrison 2002). In many developing countries the burden of PTD is extremely high (Steer 2005a; b). An advancing knowledge of risk factors and mechanisms related to PTD has been highlighted over the last decades (Crane and Hutchens 2008; Goldenberg et al. 2001; Herbst and Nilsson 2006; Iams 2003) and several public health and medical interventions designed to reduce PTD have been introduced. So far all these efforts have fallen short of reducing PTD, and on the contrary, in most industrialised as well as in developing countries (Barros et al. 2005) the rates have increased (Slattery and Morrison 2002). In the USA the rate raised from 9.5 % in 1981 to 12.7 % in 2005. Part of the increase in singleton PTD rates is explained by the rising numbers of indicated PTDs (Ananth and Vintzileos 2006; Goldenberg et al. 2008). In both Denmark and Norway there were significant increases in total PTD rates from 1995 to 2004 5.3% to 6.1% and 6.0% to 6.4% respectively (Morken et al. 2008). In Sweden the frequency has been more or less unchanged, fluctuating around 5.6% over the last 20 years (Morken et al. 2005) (Figure 1). Comparing frequencies of PTD between different populations/countries is, however, complex (Morken et al. 2008). It is important to be aware of that the differences in PTD rates might be explained by differences in the methods of registration and calculation of gestational age and due to differences in the examined populations. One of the most obvious disparities is that the rate of PTD among African-American women in the USA is twice that of any other group of women (Goldenberg et al. 1996a; Morken et al. 2008).

### Frequency of Preterm Delivery in Sweden

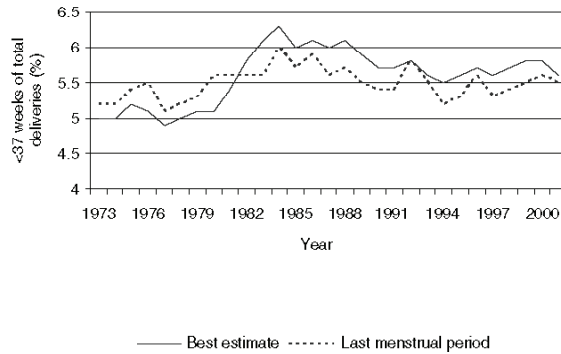


Figure 1: Incidence of preterm birth (< 37 weeks of gestation) in Sweden between 1973-2000 from Morken NH, et al *Acta Obstet Gynecol Scand* 2005; 84:558-65. Best estimate = determining gestational age estimate based on sonography before 20<sup>th</sup> week if available.

### 2.2 Subgroups

There are several ways of subgrouping PTD. Preterm infants can be subgrouped based on the gestational age at delivery into; **1.** extremely preterm delivery less than 28 weeks; **2.** very preterm 28 to 31+6 weeks; **3.** moderately preterm 32 to 36+6 weeks; **4.** late preterm or near term 34 to 36+6 weeks (Lumley 1993; Moutquin 2003). The majority of the preterm infants belong to the late preterm subgroup (Morken et al. 2008) (Figure 2).

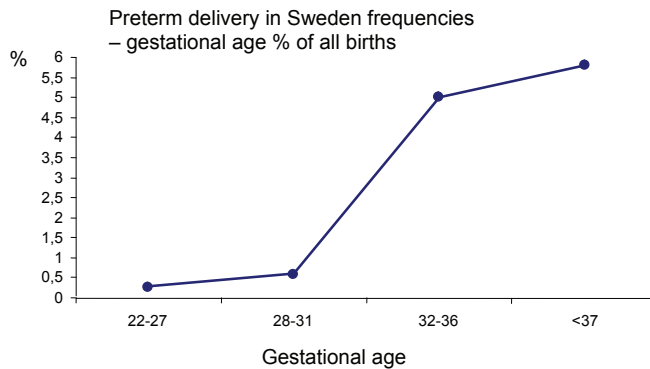


Figure 2: Incidence of preterm birth in Sweden according to gestational age. (constructed of data from Morken NH, et al *Acta Obstet Gynecol Scand* 2005; 84:558-65).



PTD is further categorized according to the mode of clinical presentation. In a recent register study of Swedish PTD (Morken et al. 2005), it was found that more than half (55%) of the deliveries were explained by spontaneous (idiopathic) preterm delivery (SPTD) in singletons. Other groups responsible for the total PTD were medically indicated for maternal or fetal reasons (e.g. severe preeclampsia, placental abruption, placenta praevia and severe IUGR), multiple birth, intrauterine fetal death, malformation, and unknown (Morken et al. 2005). SPTD is further divided into spontaneous onset of preterm labor (SPTL) and preterm prelabor rupture of membranes (PPROM) (Figure 3).

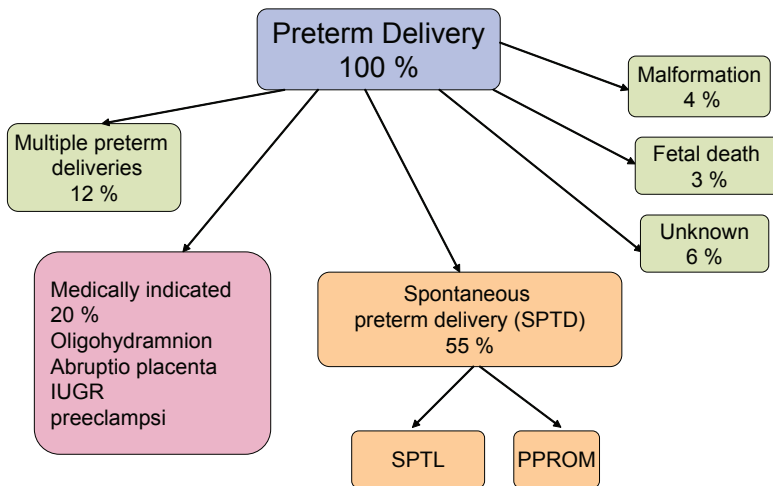
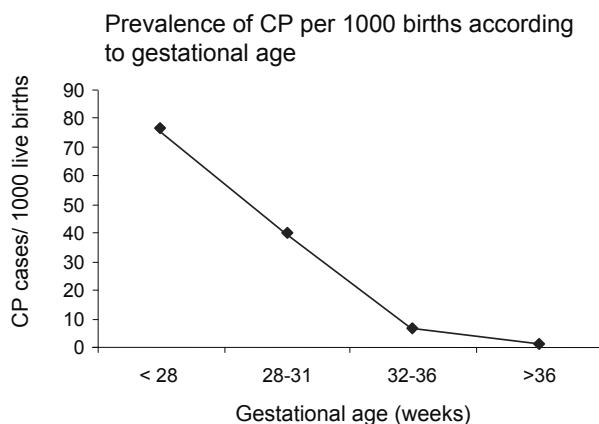


Figure 3: Subgroups of preterm birth in Sweden (data from Morken NH et al *Acta Obstet Gynecol Scand* 2005; 84:558-65).

### 2.3 Consequences of preterm birth

Short and long-term follow-up studies provide compelling evidence that prematurity is responsible for the majority (75-80%) of infant mortality (Ananth and Vintzileos 2006; Slattery and Morrison 2002) and 50% of long-term neurological handicaps, including blindness, deafness, developmental delay, cerebral palsy, and chronic lung disease (Bhandari and Bhandari 2003; Flores-Santos et al. 2007; Hack and Fanaroff 1999; Hintz et al. 2005; Marlow et al. 2007; Marlow et al. 2005; McCormick 1985; Patra et al. 2006; Vincer et al. 2006). About 40% of the infant mortality occurs in those delivered before 32 weeks of gestation and survivors in this group have the highest risk of morbidity (Ananth and Vintzileos 2006; Slattery and Morrison 2002). It is well-known that the prevalence of cerebral palsy (CP) is inversely related to gestational age (Hagberg et al. 2001; Himmelmann et al. 2005) (Figure 4).



*Figure 4: Prevalence of CP per 1000 births according to gestational age (modified from data in Himmelmann K et al Acta Paediatrica, 2005;94:287-294)*

There are compelling evidence that infection gives rise to an inflammatory response and that this inflammation contributes to neonatal brain injury (Goncalves et al. 2002; Wu 2002; Yoon et al. 2003; Yoon et al. 2000).

Over the last century, the limit of viability of newborn infants has changed substantially. Technological advances, earlier and more frequent use of antenatal corticosteroids, assisted ventilation, changing attitudes towards intensive care, and increased collaboration between obstetricians and neonatologists have greatly improved the survival rates for infants of borderline viability (Saigal and Doyle 2008). In the 1980s, the limit of viability was considered to be at a gestational age of 26 weeks, and now that limit is as low as 22 gestational weeks. Parallel to this increase in survival among infants in the lower gestational age groups, a rise in rates of long-term neurological and other health problems has occurred (Doyle et al. 2000). A Swedish study demonstrated an increase in the prevalence of cerebral palsy (CP) over the last decades (Hagberg et al. 2001), which might be an effect of the changing limit of viability.

Several studies of PTD have focused on the infants born before 28 gestational weeks, but there is clear evidence that infants born near term (34-36 gestational weeks), also have increased risk of mortality and morbidity compared with term infants (Escobar et al. 2006; Kramer et al. 2000). Experts have even argued that there is no such thing as a healthy preterm born infant (Raju 2006).

The economic costs associated with the effects of PTD are staggering for society (i.e. health care, social services, education etc) (Clements et al. 2007; Gilbert et al. 2003) and for the involved families. A disorder caused by PTD, independently of seriousness, affects every day of life for the affected individuals and puts great emotional burden on the families involved.

## 2.4 Risk factors

During the past decades numerous investigators have attempted to determine the risk for SPTD based on clinical information in order to find therapeutic agents. No single risk factor has proved to be highly predictive of SPTD (Creasy et al. 1980; Goldenberg et al. 1996c; Iams et al. 2001; Meis et al. 1998; Mercer et al. 1996). The majority of women delivering a preterm infant lacks an obvious risk factor and many of the women who carry risk factors deliver at term (Keirse 1989). Only few of the patients 8-40% (Fuchs et al. 2004; Holst et al. 2005; Tsoi et al. 2003; Tsoi et al. 2004) with symptoms of PTL deliver preterm.

Several risk factors are thought to interact to cause a transition from uterine quiescence toward PTL or PPROM, but for most factors the relative risks are only approximately 1.5 to 2 (Mercer et al. 1996). Risk factors can be categorized into several groups; **1.** demographic information; **2.** previous pregnancy history; **3.** current pregnancy findings; **4.** nutritional (body size, weight gain); and **5.** associated biophysical markers (contractions and cervical length), (Goldenberg et al. 2001) ( Table 1).

*Table 1: Different risk factors for preterm birth in women with singleton pregnancies.*

### Risk factor

#### Demographic factors

African-American

Smoking and/or drug abuse

Low socioeconomic status

Maternal age (low or high)

#### Previous pregnancy history

Previous SPTD and/or late miscarriage

Short inter-pregnancy interval < 12 months

#### Current pregnancy findings

Extremes in amniotic fluid, oligo- and polyhydramnios

Stress

Vaginal haemorrhage

Infection/inflammation

Maternal abdominal surgery

Contractions

#### Nutritional and body size

Body mass index < 19.8 and > 29

Poor weight gain

Excess weight gain

#### Associated biophysical markers

Cervical insufficiency, Cervical length, fetal fibronectin (FFN)

Multiple pregnancies carry a high risk of PTD and approximately 50% of all twins and 80% of triplets are born preterm. A previous PTD is the strongest historic predictor among women with singleton pregnancies with reported relative risks (RR) in the range of 2 to 6 (Mercer et al. 1999). A short cervical length has been shown to be one of the best predictors of SPTD (Berghella et al. 2003). Screening for a short cervical length by sonography has been utilized in several populations, including asymptomatic women with singleton pregnancies at either low (Iams et al. 1996) or high risk (Owen et al. 2001) for preterm birth, multiple gestations (Goldenberg et al. 1996b) and symptomatic women with either PTL (Vendittelli et al. 2001) or PPROM (Carlan et al. 1997). In low risk populations there is not yet any clinical usefulness of sonography because of its poor predictive value and the absence of preventive therapy (Berghella et al. 2003; Iams et al. 1996). At the yearly meeting of the Society for Maternal and Fetal Medicine in San Diego, USA in 2009 it was reported of an ongoing randomised multicenter study involving 2000 low risk nulliparous women with singleton pregnancies. Inclusion criterium is a cervical length of less than 30 mm as measured in week 16-22 in women without risk factors. Half of the women will be randomised to receive weekly injections of 17-alpha-hydroxyprogesterone caproate and the other half placebo. Maybe this study will come out with new recommendations for risk evaluation of asymptomatic women with short cervical length. Studies of asymptomatic singletons with risk factors for SPTD (i.e. women with prior SPTD) have been published (Owen et al. 2001). In women with both historical risk factors and a short cervical length, interventions (i.e. cerclage and /or progesterone) may be beneficial in preventing SPTD (Berghella et al. 2005).

### **3. The role of the cervix in parturition**

Human parturition, whether term or preterm, demands a close coordination between the uterus and the cervix. A common final pathway of parturition involves three physiologically interdependent processes; **1.** remodelling of the cervix with softening and dilatation to allow the fetus to pass through the birth canal; **2.** weakening and rupture of the membranes in the region that overlies the cervix; **3.** the initiation of rhythmic contractions of increasing amplitude and frequency that ultimately expels the fetus out of the uterus (Romero et al. 2006c).

Cervical ripening precedes normal onset of labor and is necessary for uterine contractions to be effective. The cervix slowly undergoes this ripening process during several weeks before parturition (Chwalisz and Garfield 1998). During pregnancy, the uterine contractile function is suppressed while the cervix remains firm and closed. Uterine quiescence during pregnancy is essential for the normal development of the fetus. Progesterone, corticotrophin releasing hormone (CHR) and nitric oxide (NO) appear to have dominant roles in controlling both the uterus and the cervix (Chwalisz and Garfield 1997).

The fetus is under normal circumstances protected from invasion of microbes from the lower genital tract of the mother, by an outflow of cervical mucus which is bac-

tericidal (Hein et al. 2005) and an active innate defence system as well as adaptive immune responses that make wide use of IgA secretions from the epithelial surface (Kelly 2002). The mucus plug contains large amounts of antimicrobial factors, including defensins and secretory leukoprotease inhibitors (Hein et al. 2002).

Cervical ripening is clinically observed by changes in the consistency, effacement and dilatation of the cervix, as evaluated by Bishop's Score (Bishop 1964). This process can be divided into two phases, a slow ripening (preparatory) phase that takes place throughout pregnancy, reflected biochemically as a gradual decrease in collagen fibres, and the rapid phase that occur prior to or during early labor (Chwalisz et al. 1994; Granstrom et al. 1989). Cervical ripening precedes normal onset of labor and occurs independently of uterine contractions and the cervix shortens (Chwalisz and Garfield 1998). The slow ripening phase starts approximately at 32 weeks for term births, and as early as 16-24 weeks for preterm birth and can be visualised by transvaginal sonography (TVS) images of the cervix (Holst 1996; Iams 2003) (Figure 5). It has also been shown, by TVS, that the effacement begins at the internal cervical os through a process called funnelling with the membranes bulging into the cervical canal (Ziliani et al. 1995).

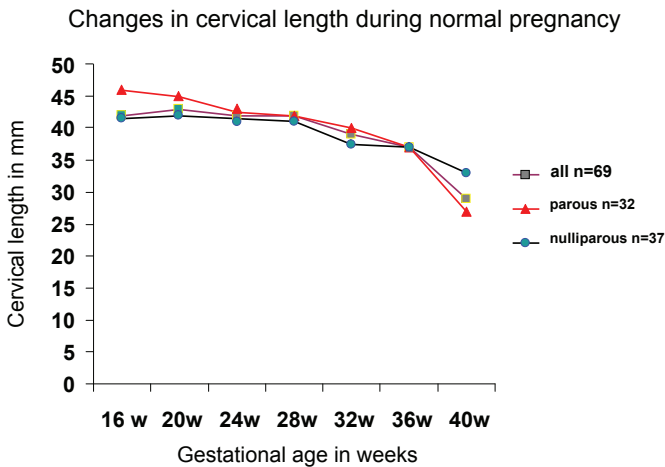


Figure 5: Cervical length in women with singleton pregnancy. A longitudinal study 1995-96 following 69 singleton (32 parous and 37 nulliparous) pregnancies with repeated measurement of cervical length, every 4 weeks, beginning at approximately 16 gestational weeks to term (Unpublished data Holst R-M, Mattsson L-Å, Hökegård K).

Cervical ripening represents an active inflammatory biochemical process accompanied by influx of neutrophils, leukocytes, and macrophages (Kelly 2002) regulated by cytokines and chemokines that increase several-fold in the cervical tissue (Junqueira et al. 1980; Sennstrom et al. 2000). Inflammation leads to cervical soften-

ing through activation of degrading matrix metalloproteinases (MMPs), disruption of tightly cross-linked collagen fibrils, changes in the proteoglycans, and increased extracellular fluid due to production of hyaluronan (Garfield et al. 1998). CHR, NO, estrogens, progesterone, and prostaglandins all contribute to cervical ripening as well as in the promotion of uterine quiescence before the onset of parturition (Sennstrom et al. 2000).

#### **4. Pathophysiology**

PTD is initiated by multiple mechanisms. Although the precise mechanism starting PTD cannot be established, four main pathological processes are involved leading to the common terminal pathway of uterine contractions, cervical remodelling and rupture of the membranes; **1.** infection and/or systemic inflammation in the decidua-chorion-amnionitic interface, approximately 40-50% of the PTD cases (Gardella et al. 2004; Hillier et al. 1993; Watts et al. 1992); **2.** maternal stress that activates the maternal or fetal hypothalamic-pituitary-adrenal axis with release of CRH and corticosteroid; **3.** decidual haemorrhage and placental abruption can also start the syndrome; **4.** overdistension of the uterus caused by polyhydramnios or multiple pregnancy leading to increased levels of prostaglandins and collagenases (Lockwood and Kuczynski 2001) (Figure 6).

The consequences of bacterial, viral or parasitic infections will depend on the virulence of the infecting microorganisms as well as its interaction with the host's immune system. The host has developed specific and non-specific factors for protection against invading pathogens, while the pathogens have evolved mechanisms to avoid these defences and cause disease. The endogenous vaginal microbiota is made up of mixed aerobic and anaerobic organisms of varying virulence potential. In pregnancy, host factors of relevance for outcome depend on the gestational age, previous maternal exposure and immunity, individual immune response variability in the mother and/or fetus, the effectiveness of the placental barrier, and development of fetal immunity (Garland et al. 2002; Peltier 2003).

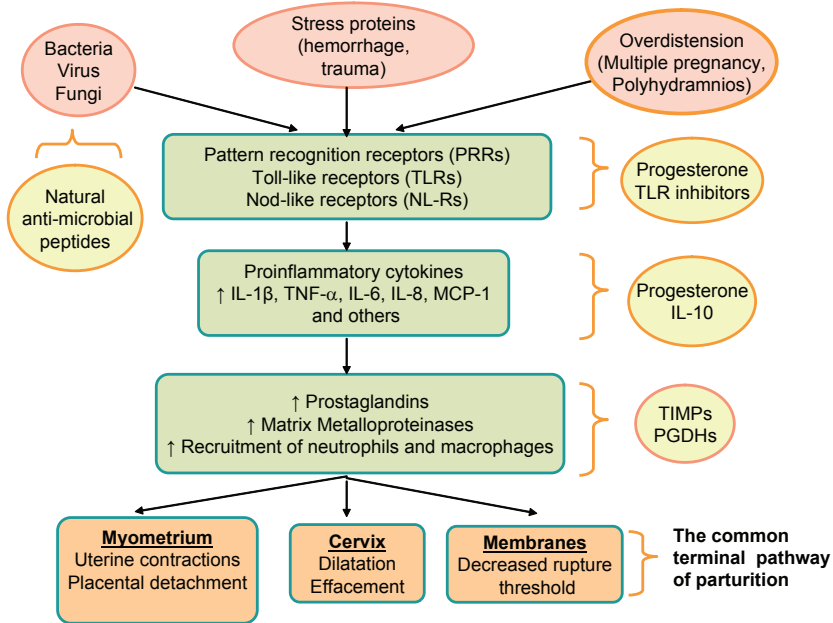


Figure 6: Model of the biochemical cascade involved in spontaneous preterm labor. TLRs recognise pathogens and activate cytokines and chemokines, which can recruit neutrophils and macrophages, induce prostaglandins and activate matrix metalloproteinases. It is not clear that progesterone, IL-10, TLR inhibitors, TIMPs are inhibitors of the different levels (modified after data from Peltier MR, *Reprod Biol Endocrin* 2003). Toll like receptor (TLR), tissue inhibitors of metalloproteinase (TIMP), prostaglandin dehydrogenase (PGDH).

#### 4.1. Basic immunology

The female reproductive tract is immunologically unique in its requirement for tolerance to the allogeneic sperm and to the conceptus. However, it must also be appropriately protected from an array of pathogens that may enter the vagina (Quayle 2002). During human pregnancy, a semi-allogeneic fetus implants in the uterus. At the fetomaternal interface, inflammatory processes can take place in case of invasion of microorganisms (Romero et al. 2004), but also due to a sterile maternal immune reaction against alloantigens on the fetus or trophoblast (Munn et al. 1998).

The human immune system is divided into innate and adaptive immunity. The phagocytes of the innate immune system provide a first line of defence against many common microorganisms and are essential for the control of common bacterial infections. The lymphocytes of the adaptive immune system have evolved to provide

more versatile means of defence that, in addition, provides an increased level of protection from a subsequent re-infection with the same pathogen. The cells of the innate immune system play a crucial part in the initiation and direction of the adaptive immune responses. It takes 4-7 days before the adaptive immune response takes effect (Janeway 1999). During this period the innate immune response has a critical role in controlling infections (Janeway 1999).

Epithelial surfaces (mucous membranes) represent the first physical barrier between the body and the microorganisms. Injuries to the epithelial surface provide a point of entry for microorganisms. These injuries can result from accidents or physiological processes (e.g. menstruation). However, microorganisms can cross intact epithelial barriers (Galask et al. 1984; Romero et al. 2006c). Most epithelia produce natural antimicrobial peptides (e.g. defensins) (King et al. 2003), which can kill the microorganisms by damaging their cell membranes. The major cell types included in innate immunity are macrophages, dendritic cells, natural killer cells (NK) and granulocytes (mast cells, neutrophils, eosinophils). These cells recognize microorganisms by their pattern recognition receptors (PRRs), which bind to structures on the surface of the microorganisms and respond immediately by phagocytosis of microorganisms and eradication of infected cells. The PRRs can be soluble like C-reactive protein (CRP), or bound to the membrane like most Toll-like receptors (TLRs) or intracellular like Nod1 and Nod2 (Hargreaves and Medzhitov 2005). When the cells in the innate immune system are activated they secrete cytokines and chemokines, which initiate the inflammatory response and facilitate the destruction of phagocytosed particles (Janeway 1999). They also initiate adaptive immune responses as well as aiding the adaptive immune response in the removal of pathogens.

B- and T- lymphocytes represent the key elements of adaptive immunity and are responsible for immunological memory. B-cells are involved in the humoral defence against extracellular antigens by producing specialized antibodies upon stimulation, furthermore, these cells have the capacity to present antigens to the T-cells. A “helping” T-cell response is needed to induce B-cell proliferation and differentiation. T-cells destroy intracellular pathogens by killing infected cells and by activating macrophages mediated in particular by the Th1 subset of CD4 positive T-cells. T-cells can also participate in the destruction of extracellular pathogens by activating B-cells, these cells are specialized Th2 cells. Only few antigens can activate naive B-cells on their own. Most antigens require an accompanying signal from helper T-cells before they can stimulate B-cells to proliferate and differentiate into plasma cells that secrete antibodies (Janeway 1999).

Thus the Th1 and Th2 cells have separate and counterbalancing actions depending on the cytokines they produce. Th1 cells produce mainly interleukins (IL) IL-1, IL-2, IL-12, IL-15, IL-18, interferon-gamma (INF- $\gamma$ ) and tumor necrosis factor alpha (TNF- $\alpha$ ) and Th2 cells are the source of IL-4, IL-5, IL-6, IL-10, IL-13 and granulocyte-macrophage colony stimulating factor (GM-CSF) (Wilczynski 2005). The main function of Th1 cells is phagocyte-mediated cytotoxic defence against



infections with intracellular microbes. The Th2 cells main function besides B-cell-plasma cell responses (above), is IgE- and eosinophil/mast cell mediated immune reactions, as well as inhibition of phagocytosis (Opal and DePalo 2000).

T-cells recognize their specific antigen by major histocompatibility complexes (MCH) which are molecules presenting the foreign peptides (derived from the protein of the pathogens) to the cell-surface of the T-cell resulting in T-cell activation. There are two major groups MHC-I and MHC-II (Janeway CA 1999; Mölne 2007). The cytokines, chemokines and other proteins analyzed in the present work are presented in Table 2.

*Table 2: Source, target and function of cytokines, chemokines and other proteins analyzed in the present work. Data from (Borish and Steinke 2003; Janeway CA 1999; Mölne 2007; Opal and DePalo 2000); <http://www.copewithcytokines.del>.*

Protein	Secreted by	Target cells/tissue - Function
<b>IL-1</b>		
pro-inflammatory	monocytes/macrophages, dendritic cells, endothelial cells, and others	<i>Th1 cells, B cells, NK cells, vascular endothelial cells, macrophages and neutrophils, hepatocytes, hypothalamus</i> co-stimulates activation of T-helper cells promotes inflammation, maturation and clonal expansion enhances activity of endothelial cells, fibroblasts, and muscle cells increases expression of ICAMs induces synthesis of acute phase proteins induces fever
<b>IL-2</b>		
immunoregulatory	T-cells	<i>antigen-primed T-cells</i> activates antigen-primed Th1 cells induces proliferation supports long-term growth enhances activity of naive T-cells
<b>IL-4</b>		
immunoregulatory	T-cells, NK cells and mast cells	<i>antigen primed B cells, resting B cells</i> facilitates production of antibodies stimulates Th2 cells down-regulates Th1 response
<b>IL-5</b>		
proliferative	T-cells, mast cells, eosinophils	<i>Eosinophils</i> stimulates eosinophil production activates eosinophils promotes adherence to VCAM-1 matures Th2 cells
<b>IL-6</b>		
mixed pro- and anti-inflammatory	many different cells mainly macrophages, monocytes	<i>proliferating B cells, multiple cell types</i> induces fever, acute-phase proteins, and cortisol production. inhibits synthesis of IL-1, TNF, IFN- $\gamma$ , GM-CSF stimulates production of antibodies
<b>IL-8 (CXCL8)</b>		
pro-inflammatory, chemoattractant	monocytes, macrophages, fibroblasts, endothelial and epithelial cells and others	<i>neutrophils and native T cells</i> chemotactic to polymorphonuclear cells stimulates neutrophil degranulation
<b>IL-10</b>		
anti-inflammatory	T- and B-cells, macrophages, dendritic cells	<i>macrophages, antigen presenting cells</i> inhibits production of INF- $\gamma$ and IL-2 from Th1 cells, and IL-4 and IL-5 from Th2 cells inhibits production of IL-1 $\beta$ , IL-6, IL-8, IL-12, TNF- $\alpha$ , GM-CSF, MIP-1 inhibits cytokines associated with cellular immunity and allergic inflammation stimulates humoral and cytotoxic immune responses
<b>IL-12</b>		
immunoregulatory	macrophages, dendritic cells, B-cells	<i>NK cells, T-cells</i> activates and induces NK cells induces INF- $\gamma$ production enhances the cytotoxic activity in CD8+ T and NK cells
<b>IL-17</b>		
pro-inflammatory and immunoregulatory	Th17-cells	<i>epithelial cells, macrophages</i> induces CXC chemokines and attracts neutrophils activates IL-6, IL-8 important mediator of chronic inflammation

<b>IL-18</b>		
pro-inflammatory and immunoregulatory	macrophages, monocytes, keratinocytes	<i>NK cells, T- cells, macrophages</i> enhances the inflammatory process by stimulating production of IFN- $\gamma$ , TNF- $\alpha$ , and IL- $\beta$ interacts with IL-12 stimulates cytotoxic activity of T-cells and NK cells
<b>sIL-6<math>\alpha</math></b>		
mixed pro-and anti-inflammatory	not known	<i>many cell types</i> mediates both local and systemic IL-6 mediated events (se IL-6 above)
<b>IFN-<math>\gamma</math></b>		
anti-viral and immunoregulatory	T helper cells, cytotoxic T-cells, NK cells, macrophages and dendritic cells	<i>NK cells, neutrophils</i> has antiviral and antiparasitic activity increases MHC I and II expression stimulates antigen presentation, cytokine production, and effector functions by monocytes stimulates killing by NK-cells and neutrophils inhibits IL-4 production and action
<b>TNF-<math>\alpha</math></b>		
pro-inflammatory	macrophages, dendritic cells, neutrophils, activated lymphocytes, NK-cells, endothelial cells, mast cells	<i>endothelial cells, neutrophils, leukocytes, lymphocytes, macrophages</i> increases adhesion molecules on endothelial cells (ICAM-1 VCAM-1) and chemoattractants activates neutrophils and phagocytosis induces oedema, vascular leakage, and coagulation mediator of apoptosis, tissue injury, toxic chock and sepsis induces production of IL- $\beta$ , IL-6 and IL-10
<b>TNF-<math>\beta</math></b>		
pro-inflammatory	T-cells, leukocytes, fibroblasts and others	<i>fibroblasts, monocytes, neutrophils and others</i> induces synthesis of GM-CSF, G-CSF, IL-1, collagenases, prostaglandins promotes proliferation of fibroblasts
<b>MCP-1 (CCL2)</b>		
chemotactic, pro-inflammatory	monocytes/macrophages, fibroblasts, B-cells, keratinocytes, smooth muscle cells	<i>monocytes, macrophages, NK, and T-cells</i> chemotactic for monocytes and T-cells activates macrophages induces basophil histamine release
<b>MCP-2 (CCL8)</b>		
chemotactic, pro-inflammatory	eosinophils	<i>monocytes</i> chemotactic
<b>MCP-3 (CCL7)</b>		
chemotactic, pro-inflammatory	monocytes, macrophages and a variety of tumor cell lines	<i>T-cells, monocytes</i> chemoattractant mainly for macrophages
<b>TGF-<math>\beta</math></b>		
immuno-regulatory and growth modulatory	macrophages, mast cells, platlets, fibroblasts, smooth muscle cells	<i>macrophages, granulocytes</i> inhibition of monocyte/macrophage MHC class expression and pro-inflammatory cytokine synthesis suppression of proliferation and cell growth but can also promote cell production e.g. neurogenesis stimulates formation of matrix proteins, stimulates TIMP and inhibits MMPs

<b>MIP-1<math>\alpha</math> (CCL3)</b>		
chemotactic, pro-inflammatory	T- and B-cells, mast cells, monocytes, fibroblasts, neutrophils	<i>monocytes, NK and T cells, basophils, dendritic cells</i> anti-viral defence chemotactic for monocytes, T-cells, neutrophils and eosinophils activates production of IL-1, IL-6 and TNF from monocytes and promotes Th1 immunity
<b>MIP-1<math>\beta</math> (CCL4)</b>		
chemotactic, pro-inflammatory	T- and B-cells, monocytes, mast cells, fibroblasts, neutrophils, endothelial cells	<i>monocytes, NK and T cells, dendritic cells</i> chemotactic for monocytes, T-cells activates production of IL-1, IL-6 and TNF from monocytes and expression of $\beta$ 1-integrins on endothelial cells enhances the cytolytic responses of cytotoxic T- and NK-cells
<b>TREM-1</b>		
pro-inflammatory	neutrophils	<i>neutrophils, monocytes</i> stimulates neutrophil and monocyte-mediated inflammatory responses through triggering and release of IL-8, TNF- $\alpha$ and IL-1 $\alpha$
<b>BDNF</b>		
trophic	neurons of CNS	<i>neurons</i> expressed in hippocampus, cortex and synapses of the basal forebrain supports survival of primary sensory neurons additive effect with NT-3
<b>GM-CSF</b>		
pro-inflammatory	T-cells (Th2), fibroblasts, endothelial cells, monocytes/macrophages, mast cells, neutrophils	<i>dendritic cells, neutrophils, macrophages</i> matures dendritic cells, neutrophils and macrophages activates mature neutrophils and mononuclear phagocytic cells prolongs survival and contributes to activity of eosinophils
<b>NT-3</b>		
trophic	neurons of CNS	<i>neurons</i> supports survival of neuronal cells additive effect with BDNF
<b>NT-4</b>		
trophic	neurons of CNS	<i>neurons</i> similar activity as NT-3
<b>sTNF RI</b>		
anti-inflammatory	multiple cell lines	binds to TNF trimers in the circulation, preventing membrane-bound TNF receptor-TNF ligand interaction
<b>MIF</b>		
immunoregulatory	T-cells, pituitary cells	Inhibits macrophage migration, stimulates macrophage activation
<b>RANTES (CCL5)</b>		
Chemotactic, pro-inflammatory	T-cells, platelets, endothelial cells	<i>eosinophils, monocytes, NK and T cells, basophils, dendritic cells</i> chemotactic for monocytes, eosinophils and basophils mediates histamine release from basophils activates T cells chronic inflammation

#### 4.2. Inflammatory mechanisms of SPTD

Inflammation and infection has emerged as important and frequent mechanisms of disease in preterm parturition (Gibbs et al. 1992; Goldenberg et al. 2000; Romero and Mazor 1988). The importance of infection as an etiological factor is most pronounced at low gestational age, i.e. intrauterine infection and inflammation are inversely related to gestational age (Yoon et al. 2001). Systemic maternal infection (i.e. pneumonia, pyelonephritis, malaria, etc) (Cunningham et al. 1973; Gilles et al. 1969; Madinger et al. 1989) and genital tract infections are known to predispose women to SPTD and these infections can begin early in pregnancy and even before conception (Andrews et al. 2000a; Andrews et al. 2000b). Most of the systemic maternal infections are not common in industrial countries today, but continue to burden the people in developing countries. Thus, the risk attributable to maternal systemic infection for prematurity is considered to be low in industrial countries.

Inflammation occurs as response to injury from exposure to microorganisms or from non-microbial related insults. Inflammation accomplishes three main goals;

1. delivery of neutrophils, macrophages, lymphocytes, and molecules for suppressing infection;
2. generation of physical barriers to spread infections, which often is accomplished by activation of the coagulation system and the formation of thrombi;
3. The promotion of repairs to the injured tissue (Romero et al. 2006a).

Intrauterine infection, which often is subclinical, is common (Goldenberg et al. 2000) and can be classified into two broad categories, intra-amniotic and extra-amniotic, according to the location of the microorganisms (Goncalves et al. 2002). Amniocentesis is the gold standard method for diagnosis of intra-amniotic infection and inflammation. Samples of amniotic fluid (AF) for the diagnosis of infection is most commonly obtained for isolation of microorganisms with standard culture techniques. Extra-amniotic infection can only be diagnosed after delivery by culturing between the chorioamniotic membranes. Only a fraction of the microbes can be detected by standard culture techniques (Romero et al. 2006a) which suggests that the frequency of microbial invasion of the amniotic cavity (MIAC) reported represents an underestimation. The possibility to use molecular microbiologic techniques enhances the detection of infection but is yet not widely used.

Most studies conducted to determine the presence of infection in patients with PTL have focused on MIAC, defined as a positive culture of amniotic fluid (AF) retrieved by trans-abdominal amniocentesis. The amniotic cavity is considered sterile since less than 1% of the AF at term contains bacteria (Romero et al. 2006a), therefore, the isolation of any microorganism constitutes evidence of microbial invasion. This condition often exists in the absence of clinical signs and symptoms of infection. Clinical chorioamnionitis (defined as fever  $\geq 37.8^{\circ}\text{C}$  at two occasions at least 4 h apart and if more than two of the following criteria are present; uterine tenderness, malodorous vaginal discharge, fetal tachycardia ( $<160$  beats/min), maternal tachycardia ( $>100$  beats/min) and maternal leukocytosis ( $>15000$  cells/ $\text{mm}^3$ ) (Gibbs et al. 1982) appears only in a fraction of women with proven intra-amniotic infection.

Romero et al showed that only 12.5% of women with PTL and intact membranes with a positive amniotic fluid culture had clinical chorioamnionitis (Romero et al. 1989b). Histological chorioamnionitis, defined by the infiltration by polymorphonuclear cells in the tissue (see paper III) is the gold standard of postnatal diagnosis of chorioamnionitis.

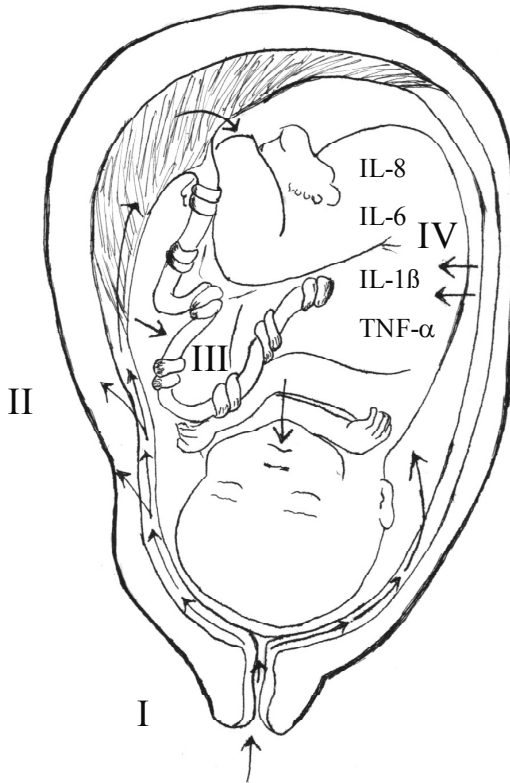
By standard culturing methods MIAC is present in approximately 16% of Swedish patients with PTL (Jacobsson et al. 2003b) and in 25% of patients with PPROM (Jacobsson et al. 2003a) and none of these had clinical chorioamnionitis. Using microbiologic technique, bacterial footprints can be detected in AF of as many as 60% of patients with PTL (Markenson et al. 1997).

Bacterial vaginosis (BV) is an imbalance of the normal vaginal flora with an overgrowth of anaerobic bacteria and a lack of the normal lactobacillary flora. It is the most common genital tract infection among women of reproductive age, ranging in frequencies from 9% to 23% (Nygren et al. 2008). BV during pregnancy has been associated with poor perinatal outcome and, in particular PTD (Hillier et al. 1995). The most common microorganisms found in the amniotic cavity and chorioamnion are genital *Ureaplasma urealyticum*, *Mycoplasma hominis*, and *Gardnerella vaginalis* all associated with BV. Other bacteria found are *Streptococcus agalactiae*, *Escherichia coli*, and *Fusobacterium* species (Romero et al. 2006c). There has been continued debate about the value of screening and treating asymptomatic pregnant women for BV. No benefit has so far been found in treating women with low- or average risk pregnancies for asymptomatic BV (Nygren et al. 2008). However, it is appropriate to treat pregnant women with symptoms.

The microorganisms may gain access to the amniotic cavity by the following pathways; **1.** Ascending from the vagina and cervix; **2.** Haematogenous dissemination through the placenta (transplacental infection); **3.** Retrograde seeding from the peritoneal cavity through the fallopian tubes; **4.** Accidental introduction at the time of invasive procedures such as amniocentesis, chorionic villous sampling, or chordocentesis (Goncalves et al. 2002).

The ascending route is considered the most common pathway of intrauterine infection (Romero et al. 2006c). Romero and Mazor proposed a four-stage process leading to intrauterine infection. First stage (**I**) consists of a change in the vaginal/cervical microbial flora and/or the presence of pathological organisms in the cervix. Bacterial vaginosis (BV) may be an early manifestation of stage I. In stage **II** the microorganisms gain access to the decidua where a localized inflammatory reaction leads to deciduitis. Microorganisms may then reside in the chorionic and amniotic membranes. From here the infection may invade the fetal vessels (chorionvasculitis) or continue through the amnion (amnionitis) into the amniotic cavity, leading to MIAC, stage **III**. Rupture of the membranes is not necessary for intra-amniotic infection, since microorganisms can cross intact membranes (Galask et al. 1984). Once in the amniotic cavity, the bacteria can gain access to the fetus by different

ports of entry, stage **IV**. The fetus can aspirate the infected amniotic fluid which can lead to congenital pneumonia or direct spreading of bacteria can lead to otitis, conjunctivitis and omphalitis that may result in bacteremia and sepsis (Goncalves et al. 2002) (Figure 7)



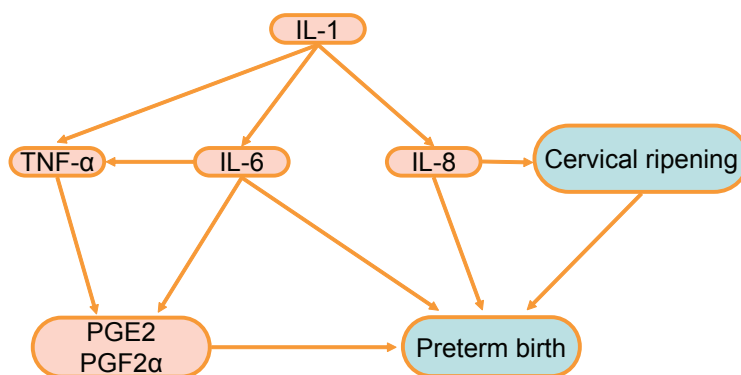
*Figure 7: Four stages of ascending genital tract infection that cause histological chorioamnionitis and activation of pro-inflammatory cytokines in the fetal-maternal unit Interleukin (IL), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). (Illustration: G-M Holst)*

There are compelling evidence that infection gives rise to an inflammatory response and that this inflammation contributes to neonatal tissue injury including brain injury (Goncalves et al. 2002; Wu 2002; Yoon et al. 2003; Yoon et al. 2000).

The mechanisms responsible for the initiation of preterm labor in the setting of infection have been the subject of intensive investigation. It has been proposed that the host, fetus and/or mother, signal the onset of labor by activation of pro-inflammatory cytokines such as IL-1, TNF- $\alpha$ , IL-6, and IL-8. These cytokines are capable of inducing a complex set of biological actions including triggering of prostaglandin biosynthesis, degradation of extracellular matrix and neuroendocrine activation leading to uterine contractions and cervical ripening, described above, culminating

in preterm birth (Romero et al. 1989a; Romero et al. 1991; Romero et al. 1989b) (Figure 8). The inflammatory response of the mother eliminates infected tissues (membranes, decidua and/or fetus) to maintain reproductive health (Romero et al. 2006c), and when the fetus is mature the fetal inflammatory response (FIRS) may start the onset of PTL which has survival value if initiated when the intrauterine environment is hostile and threatens the survival of the maternal-fetal pair (Gomez et al. 1998).

Current evidence suggests that amniotic fluid cytokine concentrations may be better markers of onset of PTL than microbial presence, since the former reflects the host reaction to microbial presence rather than the microbial presence itself (Greci et al. 1998).



*Figure 8: Pro-inflammatory cytokines interact and induce synthesis of prostaglandins (PGE2 and PGF2α which start uterine contractions. Interleukin-8 (IL-8) attracts leucocytes to cervix and hereby induces cervical ripening. The contractions and cervical ripening lead to preterm birth.*

#### 4.3. Biomarkers of SPTD

For most strategies aimed at lowering PTD in the general population, identification of pregnant women at risk and enhancement of the understanding of the pathways leading to SPTD is essential. Biomarkers are objective measurements of biological processes that can substantially improve the precision with which we evaluate disease risk, diagnosis, and progression, and guide treatment. They also promise to revolutionize both the development and use of therapeutics.

Biological fluids that have been used as sources to define markers for PTD include serum, plasma, amniotic fluid, urine, vaginal and cervical secretions, saliva, and periodontal fluid. Cytokines, chemokines, oestriol, and several other analytes have been assessed, and many, especially those related to inflammation, are associated



with preterm birth. Studies on biomarkers have improved the understanding of the mechanisms of disease leading to SPTD, but few biomarkers have shown clinical significance (Goldenberg et al. 2005). The understanding of timing related to gestational age of collecting the analyte and at delivery is also necessary. For example, the concentrations of matrix metalloproteinase-9 in serum rise about 24 h before the initiation of labor. Such a late prediction is of little value in prevention.

Fetal fibronectin is a glycoprotein produced by fetal membranes and trophoblasts which forms a biological glue that adheres the fetal membranes and placenta to the decidua. Before approximately 20 gestational weeks it is normally found in secretions of the cervix and vagina. Thereafter it is a pathological finding and a marker of chorio-decidual disruption (Goldenberg et al. 1996c). To date, cervical or vaginal fetal fibronectin (values > 50 ng/mL) obtained after 24 gestational weeks are the most powerful biochemical prediction marker of SPTD due to the high negative predictive values (Berghella et al. 2008; Goldenberg et al. 2008). When a positive value of fetal fibronectin, sampled at 24 weeks, is combined with cervical length < 25 mm and a history of preterm birth the risk of SPTD is 60 % (Iams 2003).

Goldenberg et al studied several different markers of SPTD in a nested case-control study, consisting of asymptomatic women at a gestational age 23 to 24 weeks. The majority of the markers were analysed in maternal serum and some from vaginal-cervical fluids. They found that there was very little overlap of the markers suggesting that a multi-marker test would enhance the ability to predict SPTD (Goldenberg et al. 2001).

Biomarkers predicting intrauterine infection and/or inflammation are equally important since the infection can contribute to fetal brain damage. It has been shown (Watts et al. 1992) that infection is inversely related to gestational age and that these infections give rise to fetal brain injury. The studies on which this thesis is based have been examining several possible markers of intra-amniotic infection/inflammation.

## **Aims of the study**

The general aim of this thesis was to investigate if cervical and intra-amniotic proteins on their own and/or in combination with each other and/or with clinical characteristics could predict spontaneous preterm birth, and intra-uterine infection/inflammation in women with singleton pregnancies in preterm labor. In particular the purpose was to investigate the predictive value of cervical markers (proteins or sonography), collected less invasively, compared with amniotic fluid proteins collected via amniocentesis.

The specific aims were:

1. To examine the relationship between cervical IL-6/IL-8 and MIAC, IAI and PTD (delivery  $\leq 7$  days and  $< 34$  weeks of gestation).
2. To evaluate if cervical length measured by transvaginal sonography could identify women with high risk of IAI.
3. To correlate cervical and amniotic fluid cytokines and chemokines to histological chorioamnionitis (HCA) in women with PTL and PPROM
4. To analyze the predictive ability of an array of specific proteins and their ability to predict PTD  $\leq 7$  days and MIAC.



## Material and Methods

### Patients (I, II, III, IV, V)

A cohort of 164 women with singleton pregnancies and with symptoms of spontaneous preterm delivery, 134 with PTL and 30 with PPROM, were recruited prospectively at the Department of Obstetrics and Gynecology at Sahlgrenska University Hospital in Göteborg, Sweden, between 1996 and 2005. The women were enrolled at a gestational age of 22+0 to 33+6 weeks.

**PTL** in women with intact membranes was defined as regular uterine contractions in combination with cervical changes documented by digital examination and cervical length < 30 mm measured by transvaginal sonography (TVS) (see Paper I-V for details).

**PPROM** was defined as amniorrhexis (diagnosed by a speculum examination confirming pooling of AF in the vagina) before the onset of spontaneous labor. Digital examination was not performed.

Women with known uterine abnormalities, cervical cerclage, fetal malformations, significant vaginal bleeding, imminent delivery, or fetal distress were not included. Gestational age was determined by routine ultrasound in the second trimester in all women except in three. In these women date of last menstruation was used as a proxy for gestational age determination. Tocolytic therapy (intravenous terbutaline and/or indomethacin, the latter if the gestational age was < 28 weeks) was administered according to department protocol.

### Sampling of CF and AF

Samples of **CF** were obtained from the external os with a Cytobrush (Cytobrush Plus GT, Medscan Medical AB, Malmö, Sweden). The CF was weighed and kept in a refrigerator (+ 4°C) until it was processed, which occurred within 5 h of sampling. The Cytobrush with the mucus was submerged in 1.0 mL NaCl and shaken for 30 min at + 4°C, followed by centrifugation at 855 g at +4°C for 10 min and storage at -80°C until analysis. These samples were analyzed for proteins and *Ureaplasma urealyticum* and *Mycoplasma hominis* by polymerase chain reaction (PCR). An additional sample of cervical mucus was collected by a cotton-tipped swab and immediately placed in Ames-modified Stuart medium and sent to the microbiological laboratory for culturing.

Samples of **AF** were obtained by ultrasound-guided transabdominal amniocentesis which was performed under antiseptic conditions in 89 women in PTL and in 30 women with PPROM. A needle with diameter of 0.7 mm was used and 30 - 50 ml AF was aspirated. The aspirate was immediately placed in a refrigerator (+4°C) and centrifuged within 5 h of sampling at 855 g at +4°C for 10 min. The supernatant was stored at -80°C until analysis. A sample of uncentrifuged AF was transported to the microbiological laboratory for PCR analysis of *Ureaplasma urealyticum* and *Mycoplasma hominis* and for aerobic and anaerobic culture. Microbial invasion of the amniotic cavity (MIAC) was defined as positive PCR and/or growth of any bacteria in the amniotic fluid, except for coagulase-negative *Staphylococcus* in AF which

was considered a skin contamination. However, coagulase-negative *Staphylococcus* in AF from patients with intra-amniotic inflammation (IAI defined as IL-6  $\geq$  1.5 ng/mL and/or IL-8  $\geq$  1.3 ng/mL in AF (Jacobsson et al. 2003b)) was considered to be MIAC. Four cases were culture-positive for coagulase-negative *Staphylococcus* and three of these also had IAI. The patients who were sampled of both AF and CF had the samples taken at the same occasion.

### **Culturing procedures**

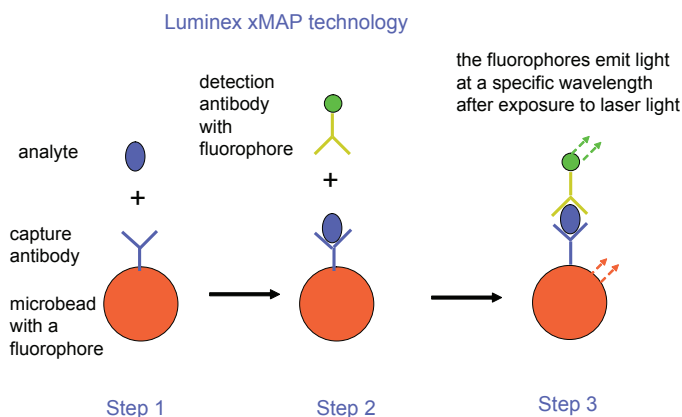
Culture of CF and AF were performed by the Department of Clinical Bacteriology, Sahlgrenska University Hospital, Göteborg (see paper I-V for details). The persons handling the cultures did not have clinical information of the patients.

### **Analysis of cytokines and chemokines**

For **paper I, II and III** the levels of the cytokines IL-6 and IL-18 and the chemokines IL-8 (CXCL8), MCP-1 (CCL2), MCP-2 (CCL8) and MCP-3 (CCL7) were analyzed in CF and AF by enzyme-linked immunosorbent assay (ELISA: Commercially available paired antibodies and standards (from R&D Systems, Minneapolis, MN, USA)) were used in a sandwich set-up. The procedures are described in paper I-III. Concentrations of the 27 proteins in CF and AF presented in **paper IV and V** were analyzed by a multiplex sandwich immunoassay based on flowmetric Luminex technology (Skogstrand et al. 2005).

The xMAP (multiple analyte profiling) technology is based on microbeads, microspheres, which have been colour-coded to generate about 100 distinct sets of microspheres. Each bead can be coated with 1000s of antibodies (capture antibody) allowing the capture and sensitive detection of the specific analyte of interest from a sample. Another antibody (reporter-antibody) marked with a fluorophore is then added and bounds to the capturing antibody in a complex with unique spectral quality. Within the Luminex 100 analyzer, which can read 50-100 beads per analyte, a red laser identifies the bead type while a green laser excites the fluorophore on the reporter antibody and quantifies the fluorescent signal corresponding to the concentration of the analyte in the test sample (Figure 9).

The amniotic and cervical samples were measured undiluted in duplicates. 50  $\mu$ l of sample was added to each filter plate well and 50  $\mu$ l of a suspension of capture-antibody-conjugated beads, 1500 beads per analyte. After 1.5 h of incubation, beads were washed twice and subsequently reacted for 1.5 h with a mixture (50  $\mu$ l) of relevant biotinylated detection antibodies, each diluted 1:1000; next 50  $\mu$ l streptavidin-phycoerythrin, 20  $\mu$ g/mL, were added to the wells. Incubation was then continued for an additional 30 minutes. Finally, the beads were washed twice and re-suspended in 125  $\mu$ l buffer and analyzed on the Luminex 100 platform. The intra-assay coefficient of variation (CV), working range and detection levels are specified in paper IV-V.



*Figure 9: xMAP technology. Step 1: a fluorescent microbead attached to a specific capture antibody against the particular analyte of interest is mixed with the sample. Step 2: a biotinylated detection antibody marked with a fluorophore is added. Step 3: the complex is read by Luminex 100 instrument. Red laser light identifies the microbeads and the green laser light quantifies the detection antibody which corresponds to the concentrations of the analyte in the tested sample.*

The levels of IL-8 and MMP-9 in CF and AF (**paper IV and V**) were excluded from analyses due to methodological problems. The levels were in the upper range of the standard curve and a limited amount of sample volume did not permit further testing.

### Stability of proteins over time

It is known that cytokines are fragile and that plasma and serum samples should promptly be frozen for long-term storage and that repeated freeze-thaw cycles should be avoided (Flower et al. 2000). Some evidence indicates that there exists a partial degradation of the proteins over time even when stored at  $-80^{\circ}\text{C}$  (Spong et al. 1998). A recent study (Skogstrand et al. 2008) demonstrates that trustworthy measurement of various inflammatory proteins in serum, plasma and whole blood relies on the handling of the analytes directly after sampling and on the time until preparation before long-term storage. The conclusions were that the best temperature to preserve blood before separation into plasma and serum was  $4^{\circ}\text{C}$ . It should, however, be noted that even at this low temperature there occurred significant increases in measurable concentrations of several of the inflammatory markers during hours of storage. The handling effects on concentrations of inflammatory markers in AF and CF have to our knowledge not been thoroughly studied. All our samples were divided into several tubes and immediately stored at  $-80^{\circ}\text{C}$ . Aliquots were not thawed until analysis and none were re-frozen and re-thawed for later analysis. IL-6 and IL-8 in paper I, II and III were analyzed at the same occasion. MCP-1, MCP-2, MCP-3 and IL-18 in paper III were analyzed later, and all the inflammatory proteins in paper IV and V were also analyzed at a later occasion.

### **Evaluation of placenta**

Placentas from 42 women in PTL and 30 women with PPRM were analyzed in **paper III**, by a perinatal pathologist (RL) who did not have clinical information on the patients. Tissue samples were obtained from umbilical cord (proximal and distal samples), roll of membranes, and umbilical cord insertion, and a minimum of six standard (routine) samples including two full thickness samples were taken from central areas of the placenta. In addition, a varying number of extra samples were obtained depending on the findings during gross examination. Histological chorioamnionitis (HCA) was defined as described in Paper III.

### **Management of patient data**

Epidemiological data including maternal demographic characteristics (age, profession, matrimonial status, race, smoking habits, alcohol use, length, and weight), reproductive history (parity, abortions, previous preterm gestations, previous genitally and sexually transmitted diseases), and medical complications (urinary tract infections and treatment with antibiotics) were extracted from the medical records. These data and the perinatal data were entered into a database. This database was protected by a user ID and a personalized password. The database was depersonalized before the analysis.

### **Statistics**

Non-parametric statistical tests were used in all papers, due to the uneven distribution of parameters obtained from the relatively small numbers of women recruited for the studies. The Mann-Whitney U-test was used for unpaired comparisons of continuous variables in all papers. In paper III comparison between three groups were analyzed with the Kruskal-Wallis test combined with Dunn's multiple comparisons test. Fisher's exact test compared dichotomous variables in paper I, II, IV, V and Chi-square test was used in paper III. A p value of < 0.05 was considered significant. Continuous variables in I, II, IV and V were dichotomized from ROC curve analysis to find optimal prediction and area under the curve was calculated. Based on a stepwise logistic regression on the dichotomized variables (paper IV and V) prediction models were constructed. Kaplan Meier survival curve is presented in paper III. Spearman's rank correlation test was used in paper II, IV and V. A p value of < 0.05 or a confidence interval (CI) not including 1.0 was considered significant.

Calculations were made by StatView 5.01(SAS Institute INC., Cary, NC, USA) in paper I, II, III and in paper III by Graph Pad Prism and InStat (Graph Pad Software, San Diego, CA, USA). In paper IV and V calculations were done by SAS 9.1 or 9.2 (SAS Institute INC., Cary, NC 27 513, USA).

### **Ethics**

All studies were approved by the local ethics committee at the University of Gothenburg (349-95 and 476-05) and all patients gave informed consent before enrolment in the studies.

## Results

### General

A total of 164 women with singleton pregnancies were examined. 134 had symptoms of PTL (I n=91; II n=87; III n=42; IV and V n=89) and 30 of PPRM (III).

### PTL (paper I, II, III, IV, V)

The majority of the study population in PTL consisted of Swedish women, 14% (19/134) were of non-Swedish origin, Hispanics n=5, black Africans n= 4, from the Middle East n=9, and Asia n=1.

Cervical samples were collected in all women for analysis of the different marker proteins and culture of microbes, some (n=46) were also analysed by PCR for *Ureaplasma urealyticum* and *Mycoplasma hominis* (Paper I).

107 of the women underwent amniocentesis. In five of the women the amount of AF was not sufficient for analysis of proteins and in one patient the procedure was interrupted because the woman complained of pain. The remaining 21 patients declined to undergo amniocentesis. The amniotic fluid was analysed of the different marker proteins and culture and PCR analyses were performed in all samples. The samples of amniotic and cervical fluids were collected at the same occasion with only a few minutes between the procedures.

Corticosteroids were administered to 24 of the women before sampling (paper IV and V). Seventeen of the patients were delivered by caesarean section (CS) and the indications were; fetal stress n =8 (week 25, 26, 33, 34, 36, 37, 39, 40), breech presentation n=3 (week 29, 30, 33), preeclampsia n=1 (week 32), obstetric history n=2 (week 36, 37), dystocia n=1 (week 39), unknown n=2 (week 35, 37). Ten patients were induced and the indications were; post term n=3 (week 42, 43, 42), preeclampsia n=2 (week 35, 36), PPRM n=1 (week 35), oligohydramnios n=1 (week 41), painful contractions n=2 (week 35, 36), psychological reasons n=1 (week 38).

### PPROM (paper III)

In the PPRM group the median gestational age at sampling was 32+0 weeks and at delivery 33w+1d. 63% delivered within 7 days from sampling and 77% were delivered before 34 weeks. 13% (4/30) of the women were of non Swedish origin, black African n=1, Hispanic n=1, from the Middle East n=2.

All of the 30 women underwent amniocentesis before uterine contractions started and the fluid was analysed of protein concentrations and cultured for microbes and PCR analysis for *Ureaplasma urealyticum* and *Mycoplasma hominis*. Samples from CF were not collected in this group of women.



Five women were induced, 2 because of PPRM and 34 weeks of gestation, 3 due to prolonged PPRM. The indications for the five women who underwent CS were transverse lie n=1 (week 34), suspected placental abruption n=1 (week 32), breech presentation n=1 (week 31), fetal stress n=1 (week 38), suspected chorioamnionitis n=1 (week 33).

### **Paper I**

Non-lactobacillus-dominating biota was detected in CF in 25% (22/89) and the presence of microorganisms in the AF in 16% (9/59) of the patients. The presence of *Ureaplasma urealyticum* in CF (21/46) was associated with significantly higher levels of IL-6. Women with intraamniotic infection/inflammation and who delivered  $\leq 7$  and/or before 34 weeks of gestation had significantly higher concentrations in CF of IL-6 and IL-8. Levels of cervical IL 6  $\geq 1.7$  ng/mL was related to IAI (RR 2.67; range 1.50-4.74) with sensitivity, specificity, PPV, NPV of 58, 83, 75 and 69% respectively. Similar data were obtained for concentrations of IL-8  $\geq 6.7$  ng/mL.

### **Conclusion**

The concentrations of IL-6 and IL-8 in CF were moderately predictive of intraamniotic infection/inflammation and preterm birth within 7 days and  $< 34$  weeks of gestation..

### **Paper II**

IAI was present in 45% (25/55) of the women and they had a significantly shorter cervical length (median 10 (range 0-34) mm) than those without IAI (median 21 (range 11-43) mm)  $p < 0.0001$ . ROC-curve analysis showed that a cervical length of 15 mm predicted IAI (RR 3.6; CI, 1.9-10.0) with sensitivity, specificity, PPV, and NPV of 72, 83, 78, and 78% respectively. Cervical length was also significantly associated with preterm birth  $\leq 7$  days from sampling and before 34 weeks.

### **Conclusion**

Cervical length assessed by transvaginal sonography predicts IAI as well as preterm birth and can be used as a clinical tool in the management of women in preterm labor.

### **Paper III**

Intraamniotic levels of IL-6, IL-8, IL-18, MCP-1 and MCP-3 were significantly higher in women with PTL who also had HCA compared to non-HCA controls, whereas no such relationship was obtained in women with PPRM. Cervical levels of IL-8 and IL-6 (but not IL-18, MCP-1, MCP-2, or MCP3) in PTL women were associated with HCA. At a cut off level of 10.0 ng/mL IL-8 was a strong predictor of HCA in the PTL cases with sensitivity, specificity, PPV, and NPV of 100, 67, 63, and 100% respectively. The cytokine and chemokine levels in the group with inflammatory signs were generally higher than in controls but lower compared to the concentrations in the HCA group.

## Conclusion

The amniotic levels of IL-6, IL-8, IL-18, MCP-1, and MCP-3 in PTL patients all predicted HCA, whereas only IL-8 was a clinically useful marker in CF. In addition there is indication that the levels of inflammatory proteins are related to the degree of inflammatory infiltration in placental tissue samples.

## Paper IV and V

The patients were included at a median age of 30w+5d and the median gestational age at delivery was 34w+3d. 38 % (34/89) of the women delivered within 7 days of sampling, 47% (42/89) before 34 weeks, and 71% (63/89) delivered before 37 weeks. All women had samples taken from AF and CF for culture and PCR (only in AF) for *Ureaplasma urealyticum* and *Mycoplasma hominis*. An array of proteins were analysed by the xMAP technology in the two fluids (See paper IV and V).

## Paper IV

The concentrations of several of the individual proteins in both CF and AF were significantly higher in the women delivering  $\leq 7$  days compared to the women delivering later (see table 2-3 in paper IV). Novel findings were that some of the proteins, that to our knowledge, previously not have been studied in the CF and/or AF showed significantly higher levels in the cases delivering within 7 days as compared to those who did not. In AF the concentrations of IL-17 and TREM1 were significantly higher. In CF the proteins IL-17, sIL-6 $\alpha$ , BDNF, NT4, NT3, IL-4, IL-5 and RANTES showed significantly higher levels.

In the multivariate analysis cervical length was the only background variable which showed a significant association to delivery  $\leq 7$  days. The women who delivered  $\leq 7$  days had a significantly shorter cervical length 5.5 mm versus 20 mm in the women who delivered later.

Three prediction models were applied. One based on proteins collected only in AF consisted of high levels of IL-6 and MIP-1 $\beta$  and predicted delivery  $\leq 7$  days (LR 2.6 and AUC 0.89). Using proteins collected in CF it was the combination of high concentrations of IFN- $\gamma$ , IL-6 and MCP-1 which together with cervical length ( $< 10$  mm) predicted PTD before 7 days (LR 4.7 and AUC 0.91). Finally when CF and AF were combined amniotic MIP-1 $\beta$  combined with cervical INF- $\gamma$ , and MCP-1 predicted SPTD  $\leq 7$  days (LR 5.6 and AUC 0.91).

## Conclusions

Several proteins in both AF (IL-17 and TREM1) and CF (IL-17, sIL-6 $\alpha$ , BDNF, NT4, NT3, IL-4, IL-5, and RANTES) not previously studied, were associated with birth  $\leq 7$  days and were thus shown to be part of the immuno-inflammatory response leading to PTD. A combination of proteins in AF and CF or proteins in CF combined with cervical length can more accurately predict preterm delivery than any individual protein.

### **Paper V**

The concentrations of several of the individual proteins in both CF and AF were significantly higher in the women with MIAC compared to the women who did not have MIAC (see table 3-4 in paper V). Novel findings were that some of the proteins that to our knowledge not have been studied previously in the CF in connection to MIAC had significantly higher levels in the MIAC group than in the cases without MIAC. The following proteins had all significantly higher cervical concentrations in the MIAC cases IL-17, sIL-6 $\alpha$ , NT3, TNF- $\beta$ , IL-4, and TREM1 than in the no MIAC.

None of the background variables (cervical length, previous PTD and/or late abortion or maternal smoking) qualified to be included in the multivariate prediction models for MIAC. In the samples collected via amniocentesis it was high concentrations of MIF, TNF- $\alpha$  and IL-6 which contributed to the prediction of MIAC (LR 3.8 and AUC 0.91). The non-invasive prediction model was based on proteins sampled from CF. High cervical levels of IL-17 and MCP-1 predicted MIAC (LR 6.0 and AUC 0.87). When proteins from both the amniotic and cervical compartments were combined, amniotic TNF- $\alpha$  and cervical IL-17 predicted MIAC (LR 3.0 and AUC 0.82).

### **Conclusions**

We found that cervical IL-17, sIL-6 $\alpha$ , NT3, TNF- $\beta$ , IL-4, and TREM1 were associated with MIAC in cases with PTL which has not previously been reported. The model based on proteins sampled from the CF was as valid in the prediction of MIAC as the model based on proteins collected via amniocentesis or the combination of proteins obtained from the two compartments.

## Discussion

The importance to enhance the understanding of SPTD and find markers with high predictive abilities and therapies is obvious, since the perinatal mortality and morbidity is so high in infants born preterm. We now know that genital tract infection can occur in a subset of pregnant women and persist in a chronic and subclinical state, eventually resulting in SPTD (Andrews et al. 2000b). We also know that such infections give rise to inflammatory responses in the host (fetus/mother) which can contribute to neonatal brain injury (Dammann and Leviton 1997; Yoon et al. 2003; Yoon et al. 2000).

None of the already existing risk factors or biomarkers associated with SPTD and/or MIAC has high enough predictive values enabling the clinician to identify all the pregnant women in PTL who will give birth to a preterm neonate, and to separate them from the women who will continue their pregnancies to term. However, it has been shown that the best marker of SPTD is FFN. When FFN is present at  $>50$  ng/mL in vaginal-cervical fluids from approximately 22 to 24 weeks of gestation and onwards, it is a strong predictor of SPTD  $< 32$  weeks with odds ratio (OR) of 27.6 compared with other biomarkers varying between 0.4-5.5 (Goldenberg et al. 2005; Goldenberg et al. 2001; Berghella et al. 2008). A short cervical length defined as  $\leq 25$  mm had 5.5 OR and previous SPTD 4.3 OR. A combination of these non-invasive markers has so far been the best prediction model of SPTD.

Today the diagnostic ability of biomarkers exceeds by far the therapeutic ability. However, markers with negative predictive values of SPTD could make it possible to prevent low risk women from hospitalization, bed rest and repeated monitoring, and high positive predictive values could assist in the timing of corticosteroid administration to high risk cases. The study of new potential markers that could participate in the process of SPTD will enhance the understanding of the SPTD syndrome and possibly generate not only improved predictive tests but also find targets for novel treatment and define risk groups apt for treatment.

There is an international ongoing debate concerning progesterone administration and the use of cerclage in high risk women (i.e. with a history of previous preterm birth). The administration of progesterone either as a weekly intramuscular injection of 17-alpha-hydroxyprogesterone caproate or a daily vaginal administration of natural progesterone are used in several countries. In the randomised controlled study by Meis and colleagues intramuscular progesterone was associated with a reduction in the risk of preterm birth less than 37 weeks' gestation, and infant birthweight less than 2500 grams in women with a history of previous preterm birth (Meis et al, 2003). A randomized controlled study by Fonseca et al (Fonseca et al, 2007) showed that SPTD less than 34 weeks of gestation was less frequent in the progesterone group than in the placebo group in women with a short cervix. The women who presented with a short cervix i.e.  $\leq 15$  mm measured by TVS at approximately 22

gestational weeks were randomized to either daily vaginal progesterone or placebo. However, the study did not show any improvement in neonatal morbidity. A recent Cochrane review on prenatal administration of progesterone for preventing preterm birth concluded that it is still unclear if the prolongation of gestation will improve maternal and longer-term infant health outcomes (Dodd et al, 2006).

Berghella (Berghella 2009) suggests in a recent review article that high risk pregnant women with prior PTD should be screened with TVS at gestational age between 16-24 weeks and women with cervical length less 25 mm should be offered cerclage. Neither of progesterone or cerclage is widely used in Sweden today.

In creation of a test or prediction model of SPTD and/or MIAC, issues such as patients' convenience, risks involved in sampling the analyte, ease of testing, test performance, reliability, and costs are all important once acceptable levels of prediction are achieved.

We have in our study population, consisting of a majority of socially and economically well situated Swedish women, found that MIAC is present in approximately 17%, IAI in 45%, and HCA and IS in 48% of the women with PTL. None of these women had clinical signs of chorioamnionitis. The gold standard to detect MIAC and IAI during pregnancy has so far been by the invasive method of amniocentesis, and analyses of the AF for microorganisms and/or inflammatory proteins. There are only few studies available on the safety of third-trimester amniocentesis. In a recent study (Zalud and Janas 2008) 111 women at a gestational age of 36 weeks underwent amniocentesis for control of lung maturity or MIAC. The patients were followed up within 24 h of amniocentesis and a complication rate of 3.6 % was found. Although all complications were self-limited, and there was no perinatal or maternal morbidity directly linked to the procedure and no patients with complications needed urgent delivery, it is reasonable to believe that the patients suffered of some anxiety and discomfort. It is therefore of special interest to find markers collected more non-invasively and that can predict intra-amniotic infection/inflammation and SPTD with at least the same precision as markers collected from AF.

A speculum examination and collection of analytes from the cervical os is a method that offers a simple and less costly way of assessing the cervix and is associated with few, if any (Stillson et al. 1997), risks and with high acceptance by the women. Transvaginal sonography evaluating the cervix is also a method that offers a reliable and simple way of an objective assessment with high acceptance by the women (Clement et al. 2003; Dutta and Economides 2003).

Using non-invasive methods we found that cervical IL-8 was a strong predictor of HCA. Like many others we found that cervical length measured by TVS predicted SPTD. A novel finding was that cervical length also can predict IAI. By using the multiplex flow cytometric assay system Luminex xMAP technology our group could investigate an array of inflammatory proteins simultaneous in CF and AF. Many of

the proteins detected in both CF and AF were significantly associated to both SPTD and to MIAC and cervical length. Several of the proteins (e.g. IL-6, TNF- $\alpha$ , IL-1 $\beta$ ) that were analyzed in both AF and CF have, been studied previously in relation to MIAC or SPTD, while others (TGF- $\beta$ , IL-17, sIL-6 $\alpha$ , neurotrophins, IL-4, IL-5, TREM-1, IL-12, and INF- $\gamma$ ) to our knowledge, have not. Examples of the functions of some of the studied proteins are given below. Table 2 (pages 25-27) describes in short source, target and function of all cytokines, chemokines and other proteins analyzed in the present work.

**IL-17** in both CF and AF was significantly associated with delivery  $\leq 7$  days and cervical IL-17 showed a strong relationship to MIAC. Furthermore, levels of IL-17 together with levels of MCP-1 in CF contributed to prediction of MIAC in the non-invasively model with a reasonably high proportion of correctly predicted cases (85%), likelihood ratio (6.0) and a negative predictive value of 94%. This is a novel finding that may be important as IL-17 could play a critical role in the pathophysiology of PTL.

IL-17 is produced by a novel type of CD4 positive T cell, TH17 cells, under the influence of IL-23 production by macrophages (Schmidt-Weber et al. 2007; Schmidt-Weber and Blaser 2002). IL-17 promotes expansion and recruitment of innate immune cells such as neutrophils (Matsuzaki and Umemura 2007), and also cooperates with Toll-like ligands, IL-1 $\beta$ , and TNF- $\alpha$  to enhance inflammatory reactions (Yu JJ). Its receptor, IL-17RA, is ubiquitously expressed and shares many features with classical innate immune receptors. IL-17 seems to be of particular importance not only in autoimmunity but also in chronic inflammation and suggestedly this cytokine could contribute in prolonging and enhancing the inflammatory response in connection with preterm birth (Kramer and Gaffen 2007; Matsuzaki and Umemura 2007; Schmidt-Weber et al. 2007). Secondly, IL-17 expression indicates that both innate and adaptive immunity is activated in preterm labor as Th17 cells do not respond to IL-23 unless they have been activated by antigen-presenting cells in secondary lymphoid organs (Stockinger et al. 2007; Yu and Gaffen 2008). Thirdly, the data imply that not only the Th1/Th2 balance is of importance in preterm labor (Wilczynski 2005) but also Th17 (Yu and Gaffen 2008). Hopefully, future experimental and clinical data will clarify to what extent IL-17 is causally involved in the immune response leading to PTD.

Another new finding is that cervical **NT-3, NT-4 and BDNF** were all significantly higher in women subsequently delivering  $\leq 7$  days versus  $>7$  days and NT-3 was also significantly higher in cases with MIAC. This is the first report to suggest that neurotrophins participate in preterm labor and in our view the most likely explanation is that neurotrophins participate in the local immuno-inflammatory response. BDNF and NT4 activates the TrkB receptor, NT3 activates primarily the TrkC receptor but also TrkA and TrkB to some extent. In a study that attracted considerable attention, Nelson et al. (Nelson et al. 2001) found much higher levels of BDNF, NT-4, NT-5 (and also VIP, CGRP) in neonatal blood from infants that

subsequently developed autism and/or mental retardation. It was hypothesized that systemic levels of these trophic factors being essential for CNS development somehow served as markers of brain impairment. However, these interesting results could not be confirmed when proteins were re-analysed with a different technique (ELISA instead of recycling chromatography) (Nelson et al. 2006) making the initial observations questionable and the link between blood neurotrophins and brain disorders uncertain.

Recent studies do suggest, however, that immune cells produce neurotrophins and express neurotrophin receptors. Neurotrophins have been shown to increase both in response to local and systemic inflammation in connection with allergy, pulmonary infection and skin disease (Correale and Villa 2004; Nockher and Renz 2005). It has also been suggested that neurotrophins may be of particular importance in the delayed reparative phase in inflammation.

There is very little information available on **TNF- $\beta$**  in fetal-placental tissue or in cervical fluid. We found that TNF- $\beta$  was significantly higher in cases with MIAC supporting a role of this mediator in host-defence. TNF- $\beta$  is also called lymphotoxin or TNF ligand superfamily member 1 (TNFSF1) according to the new classification. Cytokines in this family, including TNF- $\beta$  have profound pleiotrophic roles involving inflammation, cell proliferation and stimulation of the immune system (Aggarwal 2003; Goetz et al. 2004; Spahn et al. 2005). The levels of TNF- $\beta$  in amniotic fluid and cervix were however not different in women delivering within 7 days compared to those with longer latencies which agree with a previous report showing limited response of this cytokine to labor (Laham et al. 1997).

**TREM-1** (triggering receptor expressed on myeloid cells) is a cell surface molecule expressed on neutrophils and monocytes implicated in the propagation of the inflammatory response. It is a transmembrane glycoprotein that consists of a single extracellular immunoglobulin-like domain. TREM-1 activates myeloid cells by signalling through the adaptor protein DAP 12. TREM-1 triggers phagocyte secretion of pro-inflammatory chemokines and cytokines, amplifying the inflammation that is induced by bacteria and fungi (Bouchon et al. 2000). TREM1 has been shown to stimulate the production of pro-inflammatory chemokines and cytokines. IL-8 (CXCL8) is strongly induced by the engagement of TREM1, followed by the production of monocyte chemoattractant protein 1 (MCP-1, CCL2), MCP-3 (CCL7) and macrophage inflammatory protein 1 $\alpha$  (MIP1 $\alpha$ , CCL3). Triggering of TREM1 also induces granulocytes to release myeloperoxidase but does not induce phagocytosis. A marked up-regulation of the production of tumor-necrosis-factor (TNF) and IL-1 $\alpha$  by monocytes in response to monoclonal antibodies specific for TREM1 is observed when LPS is used as co-stimulus showing that TREM1 can amplify inflammatory responses that are initiated by toll-like receptors (TLRs).

In a study (Vogel et al. 2007) of pregnant women with  $\geq 1$  previous early SPTD serum samples were collected. This study showed a relative risk RR of 4.6 for preterm birth less than 35 weeks in women with high maternal serum levels of TREM1.

We found that TREM 1 in AF and CF were not significantly different in relation to preterm birth within 7 days, but TREM1 in relation to MIAC was significantly higher in CF in the patients with MIAC than in those without ( $p= 0.05$ ). The levels in AF were not different in the MIAC versus no MIAC groups.





## **Conclusions**

We have identified several novel immuno-inflammatory proteins in amniotic and cervical fluid that may have an important role in the pathophysiology of preterm labor and these findings could contribute to development of novel therapies in the future. Furthermore, based on multiple cytokine analyses, we propose novel non-invasive prediction models that could help the clinician to separate cases with high risk of PTB and infection that require action from those at low risk that should not be hospitalized and exposed to unnecessary intervention.



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