# Preterm delivery and selected biomarkers – phosphorylated insulin-like growth factor-binding protein-1 and matrix metalloproteinase-8 – in cervical fluid

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**University of Helsinki 2010**

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# Preterm delivery and selected biomarkers – phosphorylated insulin- like growth factor-binding protein-1 and matrix metalloproteinase-8 – in cervical fluid

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## Academic dissertation

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*To my family.....*

# CONTENTS

# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

I Rahkonen L, Unkila-Kallio L, Rutanen E-M, Paavonen J. Factors affecting decidual IGFBP-1 concentrations in the vagina and cervix in the first and mid-second trimester of pregnancy. BJOG 2009; 116: 45-54.

II Rahkonen L, Rutanen E-M, Nuutila M, Sainio S, Saisto T, Paavonen J. Elevated concentrations of decidual insulin-like growth factor binding protein-1 in cervical fluid in early and mid-pregnancy are associated with an increased risk of spontaneous preterm delivery. BJOG 2010; 117: 701-10.

III Rahkonen L, Unkila-Kallio L, Nuutila M, Sainio S, Saisto T, Rutanen E-M, Paavonen J. Cervical length measurement and cervical phosphorylated insulin-like growth factor binding protein-1 testing in prediction of preterm birth in patients reporting uterine contractions. Acta Obstet Gynecol Scand 2009; 88: 901-908.

IV Rahkonen L, Unkila-Kallio L, Rutanen E-M, Nuutila M, Nieminen P, Sorsa T, Paavonen J. Factors affecting matrix metalloproteinase 8 levels in the vaginal and cervical fluids in the first and second trimester of pregnancy. Hum Reprod 2009; 24: 2693-702.

V Rahkonen L, Rutanen E-M, Nuutila M, Sainio S, Sorsa T, Paavonen J. Matrix metalloproteinase -8 in cervical fluid in early and mid-pregnancy: Relation to spontaneous preterm delivery. Prenat Diagn 2010; in press.

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# ABBREVIATIONS

| | |
|---|---|
| ACOG | American College of Obstetricians and Gynecologists |
| ACTH | Adrenocorticotrophin |
| AF | Amniotic fluid |
| AFP | Alpha-fetoprotein |
| B19 | Parvovirus |
| β-hCG | Beta human chorionic gonadotrophin |
| BMI | Body mass index |
| BV | Bacterial vaginosis |
| CI | Confidence interval |
| CP | Cerebral palsy |
| CRH | Corticotrophin releasing hormone |
| CRP | C-reactive protein |
| E3 | Estriol |
| EMMPRIN | Extracellular matrix metalloproteinase inducer |
| FFN | Fetal fibronectin |
| HPA | Hypothalamic-pituitary-adrenal |
| IAI | Intra-amniotic inflammation without microbial invasion |
| IEMA | Immnoenzymometric assay |
| IFMA | Immunofluorometric assay |
| IGFBP-1 | Insulin-like growth factor-binding protein-1 |
| IL | Interleukin |
| IUGR | Intrauterine growth retardation |
| IVF | In vitro fertilization |
| LR+ | Positive likelihood ratio |
| LR- | Negative likelihood ratio |
| MAB | Monoclonal antibody |
| MIAC | Microbial invasion of amniotic cavity |
| MMP | Matrix metalloproteinase |
| MOM | Multiple of median |
| NPV | Negative predictive value |
| NT | Nuchal translucency |
| OR | Odds ratio |
| PAPP-A | Pregnancy-associated plasma protein A |
| PG | Prostaglandin |
| PhIGFBP-1 | Phosphorylated insulin like growth factor-binding protein-1 |
| PPROM | Preterm premature rupture of membranes |
| PPV | Positive predictive value |
| PROM | Premature rupture of membranes |
| PTD | Preterm delivery |
| PTL | Preterm labour |
| THL | National Institute for Health and Welfare |
| TNF-α | Tumour necrosis factor-alpha |
| WBC | White blood cell |

# ABSTRACT

Preterm delivery (PTD) is a major problem in obstetrics. Its incidence varies between 5% and 15% of all deliveries, depending on the geographic and demographic features of the population. Although many risk factors have been identified, early identification of pregnant women who will deliver before term remains an important goal. The main purpose of this study was to investigate whether measurement of phosphorylated insulin like growth factor-binding protein-1 (phIGFBP-1) and matrix metalloproteinase-8 (MMP-8) in cervical and vaginal fluids in early and mid-pregnancy can be used to identify women at high risk of spontaneous PTD, and to determine factors influencing phIGFBP-1 and MMP-8 concentrations in the lower genital tract. In one study the usefulness of rapid cervical phIGFBP-1 test in predicting PTD was examined among women with self-reported uterine contractions between 22 and 34 weeks of gestation.

The studies were carried out at the Maternity Clinic of the Department of Obstetrics and Gynecology, University of Helsinki, Finland. Vaginal and cervical swab samples for assay of phIGFBP-1 and MMP-8 were collected from 5180 unselected pregnant women in the first and second trimesters of pregnancy between April 2005 and December 2006. Data on pregnancy and delivery were collected from the hospital records. Concentrations of phIGFBP-1 were measured by immunoenzymometric assay and in one study by using a rapid strip test. Concentrations of MMP-8 were measured by immunofluorometric assay. A concentration of ≥10 μg/l was used as a cut-off for elevated cervical fluid phIGFBP-1. MMP-8 results were presented as percentiles. The main outcome measure was spontaneous PTD.

Median concentrations of phIGFBP-1 were over 10-fold higher in the cervix than in the vagina in both trimesters. Overall, 25–34% of the women had elevated cervical fluid phIGFBP-1 in the first trimester, and 20–28% in the second trimester. Nulliparity and vaginal bleeding during the current pregnancy were independently associated with elevated cervical fluid phIGFBP-1 levels in both trimesters. Sexual intercourse had no effect on cervical fluid phIGFBP-1 consentrations. MMP-8 was detected in almost all women, and the concentrations were 3-fold higher in the cervix than in the vagina. The distribution of MMP-8 concentrations in early pregnancy did not differ from that in mid-pregnancy. Multiparity, bacterial vaginosis and increased white blood cell counts in vaginal wet mounts were associated with increased MMP-8 in both trimesters. Recent sexual intercourse was associated with low MMP-8 concentrations in the cervix.

The rate of spontaneous PTD was 4%. An elevated cervical fluid phIGFBP-1 level in the first trimester was an independent predictor of PTD at < 32 and < 37 weeks' gestation, with odds ratios of 3.0 (95% CI 1.3–7.0) and 1.6 (95% CI 1.2–2.3), respectively. In women with uterine contractions between 22 and 34 weeks of gestation, a short cervix, a positive phIGFBP-1 strip test result (detection limit 10 μg/l), or a combination of both were associated with PTD at ≤ 34 weeks or within 14 days ($p < 0.01$). Cervical fluid phIGFBP-1 < 10 μg/l as well as a negative strip test result had high negative predictive values for PTD among asymptomatic and symptomatic women, respectively.

The overall distribution of MMP-8 concentrations in both trimesters was similar in women with term delivery and those with spontaneous PTD. However, elevated (> 90th percentile) cervical fluid MMP-8 concentrations in the first and mid-second trimester were associated with subsequent spontaneous PTD initiated by preterm labour (PTL). Decreased MMP-8 in the second trimester was associated with subsequent premature preterm rupture of membranes.

In conclusion, both markers are present in lower genital tract fluids, but the concentrations are more consistent in the cervix. Although cervical fluid phIGFBP-1 is not appropriate for use in the first and mid-second trimester for routine screening of asymptomatic women for PTD, it could play a role alone or in combination with other markers and risk factors, when assessing the risk of PTD in selected cases throughout pregnancy. The wide range of MMP-8 concentrations, and the fact that elevated as well as low concentrations are associated with an increased risk of PTD, indicate that cervicovaginal MMP-8 cannot be used for prediction of PTD, at least in early and mid-pregnancy.

# INTRODUCTION

Preterm delivery (PTD) is a multifactorial phenomenon having remarkable medical, health economic and human effects. Worldwide its frequency varies from 5.0% to 15.0% depending on the population (Slattery and Morrison 2002, Honest et al. 2009). Over half of PTDs are spontaneous. The molecular pathways leading to PTD are not sufficiently understood. Worldwide the rate of PTD has increased during the past few decades despite efforts to address the problem, and with medical progress. Preterm delivery accounts for approximately 75% of all neonatal deaths and one half of children`s long-term neurological disability. Although many risk factors have been identified, preterm birth is still difficult to predict (Romero et al. 2006b, Goldenberg et al. 2008).

Ascending infection, occult or clinical, from the vagina is believed to cause inflammation in the choriodecidual space by activating cells to produce pro-inflammatory cytokines (IL-1, IL-8, TNF-α). Cytokines increase the production of prostaglandins (PGs), endo- and exotoxins and proteases, which may lead to tissue disruption in the choriodecidual space. Tissue disruption results in leakage of chorionic and decidual products into the cervix and vagina (Lockwood et al 1999, Goldenberg et al. 2000, Goldenberg et al. 2002). Premature preterm rupture of the membranes (PPROM) and/or preterm labour (PTL) may be the first symptoms of ascending infection in the choriodecidual space.

During the past few decades many studies have been performed to discover new methods for prediction of PTD more effectively and earlier (Goldenberg et al. 2005b, Vogel et al. 2005, Honest et al. 2009). Most predictive markers are relevant when delivery is going to happen shortly (Vogel et al. 2005). However, we need markers to screen the general pregnant population as part of antenatal care in order to identify women at high risk of subsequent PTD and to focus preventive care on them.

For this study two potential biomarkers were selected: One of these is phosphorylated insulin like growth factor-binding protein-1 (phIGFBP-1), a major protein product of the decidua, which may leak into the lower genital tract as a result of inflammation-associated tissue disruption (Rutanen 2000). The other is matrix metalloproteinase-8 (MMP-8), a human neutrophil collagenase, regulated by pro-inflammatory mediators (Sorsa et al. 2006, Van Lint and Libert 2006). Our first aim was to determine the concentrations of both markers in cervical and vaginal fluids, and to investigate factors that may affect the concentrations in early and mid-pregnancy. Two studies were conducted to clarify whether or not assay of phIGFBP-1 and MMP-8 in samples from asymptomatic women in early and mid-pregnancy can be used to predict PTD. In one study, cervical fluid phIGFBP-1 levels and clinical examination in prediction of PTD among symptomatic pregnant women in later pregnancy were compared.

# REVIEW OF THE LITERATURE

## Preterm delivery

### Definition

Delivery occurring before 37 completed weeks of pregnancy is considered preterm, regardless of birth weight (World Health Organization 1970, Steer 2005, Goldenberg et al. 2008). Low birth weight, defined as less than 2500g, is often associated with preterm delivery (PTD). Although all births before 37 weeks of gestation are defined as preterm, most problems caused by PTD occur in infants delivered before 34 weeks. It is often convenient to divide PTD into subgroups according to gestational age. Extreme prematurity occurs at 23 weeks to 28 weeks, appearing in about 5% of preterm deliveries, about 15% represent severe prematurity at 28–31 weeks, about 20% represent moderate prematurity at 32–33 weeks and 60–70% represent near or late prematurity at 34–37 weeks (Goldenberg et al. 2008). There is no global lower limit for PTD, but 22+0 completed weeks of gestation is generally accepted, corresponding to an average fetal weight of 500 g (EURO-PERISTAT Project 2008, Honest et al. 2009, THL 2010). For an individual newborn delivered at these gestational weeks it is very difficult to predict the outcome. Parents' opinions regarding invasive intensive care should also be considered (Nuffield Council on Bioethics 2007). In Finland, intensive care of a preterm infant is started from 23–24 weeks of gestation, with some variation between hospitals. Expulsion at less than 22 weeks of gestation is called spontaneous abortion or miscarriage and resuscitation should not be started (Nuffield Council on Bioethics 2007).

Traditionally, determination of gestational age at delivery has been based on estimation of the first day of the last menstrual period (Steer 2005). The most accurate modern method is via assessment of fetal crown–rump length by vaginal ultrasonography in the first trimester (Goldstein and Wolfson 1994, Klebanoff 2007). In Finland, ultrasonographic estimation of gestational age is included in routine antenatal care.

## Incidence

The incidence of PTD varies, being an average 5% to 15% of all deliveries depending on the geographical and demographical features of the population. About 4% of pregnancies result in PTD before 34 weeks of gestation (Honest et al. 2009, Slattery and Morrison 2002,). Over the past several decades, for reasons that are not fully understood, the PTD rate has raised worldwide (Goldenberg and Culhane 2006, Goldenberg et al. 2008, Martin et al. 2008, Muglia et al. 2010, Slattery and Morrison 2002). However, in Finland and Sweden, the rate of preterm deliveries has stayed more stable than elsewhere (Jakobsson et al. 2008, Morken et al. 2008, THL 2010). In Finland the rate of PTD has not increased since 1987, being 5.2-5.4% among sigleton pregnancies in recent years (Jakobsson et al. 2008) and the incidence of PTD infants in Finland at 2008 was 5.7% (THL 2010). Sweden has reported even a decrease in PTD rate from 6.3% to 5.6% between1984-2001 (Morken et al. 2008). In Denmark the total rate of PTD has increased from 5.3% to 6.1% (1994 to 2004) and in Norway from 6.0 % to 6.4% (Morken et al. 2008). The rate of PTD has also increased in most part of Europe varying from about 5% to 11% (Vogel et al. 2005, EURO-PERISTAT Project 2008).

In the United States 13% of infants were born at less than 37 weeks of gestation in 2004-2008. Two decades earlier the figure was 9.4% (Goldenberg and Culhane 2006, Muglia et al. 2010, Martin et al. 2008). In Canada the increasing trend is similar being from 6.4 % to 7.1% (Vogel et al. 2005). During last decades the preterm rates in Oceania have been about 6.0 % (Tracy et al. 2007). The most impressive increase has been explained with medically indicated preterm deliveries. (Slattery and Morrison 2002, Ananth and Vintzileos 2006). However, also spontaneous PTD rates have increased (Goldenberg et al. 2008, Muglia et al 2010).

## Classification

### Spontaneous preterm delivery

Classically categorized, spontaneous PTD is preceded by either preterm labour or preterm premature rupture of the membranes. Spontaneous PTL is defined as labour with regular contractions and cervical ripening starting before 37 complete weeks of gestation, with intact fetal membranes. PPROM is defined as spontaneous rupture of fetal membranes at

least one hour before the onset of contractions prior to 37 weeks of gestation, irrespective of whether delivery is vaginal or by Caesarean section. About 40–45% of preterm deliveries are preceded by spontaneous PTL and 25–30% follow PPROM (Slattery and Morrison 2002, Goldenberg et al. 2008).

PPROM before or at 26 weeks of gestation complicates 0.6–0.7% of pregnancies, and has been defined as mid-trimester PPROM. The limit of fetal viability has progressively declined over the past few decades. Therefore, it is relevant to differentiate PPROM into subgroups. Pre-viable PROM occurs before the limit of viability, at less than 23 weeks of gestation. Treatments aimed at continuing the pregnancy may lead to extended latency and delivery of a potentially viable infant. The earlier that PPROM occurs, the higher the risk of fetal pulmonary hypoplasia (van Teeffelen et al. 2010). The risks of perinatal morbidity and mortality decrease with advancing gestational age at delivery (Mercer 2003). In a recent study infant survival seemed to be significantly lower when the onset of preterm birth was PPROM as compared with PTL or iatrogenic delivery (Johanzon et al. 2008).

### Iatrogenic preterm delivery

About 30–35% of all preterm deliveries are iatrogenic, i.e. labour is either medically induced or the infant is delivered by pre-labour Caesarean section (Goldenberg et al. 2008, Honest et al. 2009). Indications for induced PTD are serious maternal or fetal complications such as severe pre-eclampsia or intrauterine growth retardation (IUGR) (Goldenberg et al. 2008).

## Health consequences

Preterm delivery, particularly before 34 weeks of gestation, is associated with a significant risk of death and adverse health and developmental consequences in the newborn infant. It accounts for approximately three-quarters of cases of perinatal mortality and nearly one-half of cases of long-term neurological morbidity (Goldenberg et al. 2002, Slattery and Morrison 2002, Goldenberg et al. 2008). However, improvement in neonatal care in the last three decades has led to higher rates of survival of very premature infants. Nowadays 80% of 500–1000 g newborns are alive at one year of age (Goldenberg et al. 2002,EURO-PERISTAT Project 2008). Complications of PTD include acute health problems and long-lasting disabilities such as respiratory, gastrointestinal, immunological, hearing and vision

problems and cerebral palsy (CP), as well as long-term motor, cognitive, visual, hearing, behavioural, social-emotional, health and growth problems (Hack et al. 2002, Tommiska et al. 2007). However, because of better survival of very preterm infants, handicap rates have substantially increased (Goldenberg et al. 2002). The risks are greatest at the earliest gestational ages (≤ 32 weeks) and in low-birth-weight infants (< 1500 g) (Nuffield Council on Bioethics 2007, Slattery and Morrison 2002, Goldenberg et al. 2002). However, infants born just a few weeks too early are also six times more likely to die in their first week of life than full-term infants and three times more likely to die before their first birthday (Behrman and Butler 2007, Tomashek et al. 2007). In the long term, children born prematurely have increased risks of cardiovascular disease, hypertension and diabetes as adults and possibly an increased risk of cancer (Rich-Edwards et al. 1997, Spong 2007). Prematurity-associated medical complications also predict future educational and occupational impairments that extend into late childhood and beyond (Slattery and Morrison 2002, Lindstrom et al. 2007). The emotional impact on a family encountering this problem is enormous. In many cases the infant is in hospital for a long time and far from home, and parents suffer considerable anxiety, with doubts about the child's survival and full recovery.

## Economic consequences

The delivery of a preterm infant brings considerable health care costs which are strongly gestational age-dependent. The costs are not just those incurred while in the hospital's neonatal intensive care unit. Some health problems that develop at this time can persist for years, leading to long-lasting use of healthcare and social services, including special education and rehabilitation for those with physical handicaps (Escobar et al. 2006a, Escobar et al. 2006b, Honest et al. 2009). Maternal hospitalization before and after delivery and an increased number of Caesarean sections also increase costs.

It was recently estimated that PTD in the United States accounts for 85% of infant medical care costs, suggesting major cost savings as a result of prevention of preterm birth (Behrman and Butler 2007). Studies in the United Kingdom and Ireland show that the cumulative costs of hospital admissions incurred during the first 10 years of life are more than twice as high among preterm infants compared with children born at term (Petrou 2005). It has been predicted that even higher cost differences will occur at a later age, especially in the extremely preterm group.

The highest costs per case generally occur in the very preterm and extremely preterm groups, but the incremental cost for the large number of infants born between 28 and 32 weeks is approximately 80% of the cost for infants born at less than 28 weeks (Nuffield Council on Bioethics 2007).

# Risk factors of preterm delivery

PTD is a multifactorial syndrome. Although many risk factors for PTD are well known, the cause of this complication remains often unexplained. More than half of all preterm deliveries occur in apparently low risk pregnancies with no major risk factor and some women are more prone than others (Goldenberg et al. 2008, Romero et al. 2006b). Risks factors can be divided into three subgroups: Maternal risk factors, pregnancy history related risk factors and those associated with ongoing pregnancy.

## Maternal risk factors

In Finnish studies women with pregnancy outside marriage and not taking part in antenatal care are at higher risk for PTD (Raatikainen et al. 2005, Raatikainen et al. 2007). Both are often associated with low socioeconomic status, smoking, alcohol or drugs abuse and poor nutritional status all independent risk factors of PTD as well (Goldenberg et al. 2008, Slattery and Morrison 2002).

Smoking increases the risk of PTD 2-fold. The risk is highest in the extremely and moderately preterm groups. Cigarettes biochemical's, nicotine and carbon monoxide, are powerful vasoconstrictors, and are associated with placental damage and decreased uteroplacental blood flow. Both pathways lead to fetal growth restriction and induced PTD (Jakobsson et al. 2007, Tikkanen et al. 2006). Smoking is also associated with systemic host inflammatory response increasing the risk of PTD (Bermudez et al. 2002).

Heavy alcohol consumption during pregnancy leads to fetal alcohol syndrome and has also been associated with PTD. Further, drugs abuse, such as cocaine and heroin, has been associated with PTD in several studies (Bennett 1999, Little et al. 1990, Walton-Moss et al. 2009).

Maternal age younger than 20 years or extremes of maternal age increase rates of PTD (Morken et al. 2008, Slattery and Morrison 2002). In a Finnish study, advanced maternal age increased the risk of PTD by 2% for each additional year in age (Jakobsson et al. 2007).

Significant independent associations with PTD have been reported for low body mass index (BMI) at 20 weeks of gestation (Goldenberg 2003, Neggers and Goldenberg 2003). However, bigger problem nowadays is the increased prevalence of overweight and obesity, which has increased dramatically in developed countries. Overweight women are more likely to develop pre-eclampsia and diabetes, which increase the rate of induced PTD (Goldenberg et al. 2008). Being overweight is also associated with PPROM. Persistent inflammation may be an explanation for reported association between PPROM and overweight (Nohr et al. 2007, Rosenberg et al. 2005). However, in one study among obese women the rate of PTD after spontaneous PTL is lower than among normal weight women (Ehrenberg et al. 2009).

Psychological and social stress or depression increase the risk of PTD, whereas physical activity is not consistently related to the rate of PTD (Challis and Smith 2001, Goldenberg et al. 2008, Wadhwa et al. 2001a). One third of women have depressive symptoms during pregnancy and 16 % have clinical depression which both increased the risk of PTD 2-fold (Goldenberg et al. 2008).

Genetic factors seem to play an important role in spontaneous PTD. PTD rates are in the range of 16-18% among black women compared with 5-12 % for white women in the United States and United Kingdom (Goldenberg et al. 2008, Martin et al 2006, Slattery and Morrison 2002). Black women are also three to four times more likely to have a very early preterm birth than women from other racial or ethnic groups (Goldenberg et al. 1996). The lowest rates of PTD exist in East-Asians and Hispanics (Ananth and Vintzileos 2006, Goldenberg et al. 2008, Romero et al. 2006b, Slattery and Morrison 2002). The beginning of PTD also differs by ethnic groups. PTD is most commonly preceded by PTL in white women, while PPROM is more frequent among black women (Goldenberg et al. 1996, Goldenberg et al. 2008). Genetic polymorphisms of individual's genes may explain the greater risk of PTD among black women since environmental factors alone have not sufficiently explained this phenomenon (Holst and Garnier 2008, Warren and Silver 2009). Black women born in the United States are more likely to have PTD than black women born outside the United States. Those black women who have experience of racial discrimination are associated with the higher risk for PTD than other black women (Muglia et al. 2010).

In genetic studies women with PTD have higher risk to subsequent PTD (Porter et al. 1997). There is also a tendency for repeated PTD to occur at the same gestational age as the previous pregnancies with the same partner (Li 1999). Woman with a sister who has given birth to a preterm infant had an 80% higher risk to have a PTD (Goldenberg et al. 2008). Studies in twins have noted that genetics may account for about 17-36% of preterm deliveries (Holst and Garnier 2008).

Recent investigations have focused on inflammatory and immune response because of the evidence of involvement of inflammation in pregnancy and parturition (Holst and Garnier 2008, Macones et al. 2004, Romero et al. 2004). Genetic differences between individuals i. e. gene polymorphisms, can result in variation in production and activity of proinflammatory proteins, causing inadequate immunologic response to infectious stimuli (Holst and Garnier 2008).

Cervical insufficiency, a syndrome with early predominant cervical ripening is strongly associated with mid-trimester abortion and PTD. It can be primary cervical disease or due to uterine trauma or infection with activation of inflammatory cytokines (Lidegaard 1994, Lockwood and Kuczynski 1999, Romero et al. 2006b, Anum et al 2010). It has been estimated that infection is present in approximately 50% of women with acute cervical insufficiency. In addition to infections, cervical insufficiency may result from congenital hypoplasia or repeated cervical dilatation in connection with termination of pregnancy (Romero et al. 200b). One iatrogenic reason for cervical damage may be cervical loop electrosurgical excision procedure (LEEP) secondary to premalignant cervical disease (Jakobsson et al. 2007, Romero et al. 2006). In a Finnish study history of LEEP is associated with an increase in spontaneous PTD 3-fold (Jakobsson et al. 2007). In a more resent study this association was not found (Werner et al 2010). The rate of spontaneous PTD is also increased among women with uterine anomalies (Rossier et al. 2008).

Many maternal and fetal genetic and environmental factors may affect the risk of PTD independently or interactively. Some gene mutations increase the risk of PTD for one woman, but not for another. This fact has led to the hypothesis that gene mutations require the presence of certain environmental stimuli, such as infection or stress, to have clinical significance. For example, pregnant women with their fetuses who are genetically programmed to produce high levels of proinflammatory mediators would be more likely than those producing low concentrations, to exceed the threshold necessary to initiate PTL in response to environmental factors (Holst and Garnier 2008, Romero et al. 2004). On the other hand, genital tract immune hyporesponsiveness with low concentrations of

inflammatory cytokines are more likely to subsequently develop clinical chorionamnionis than those with high levels of cytokines (Simhan et al. 2003).

## Pregnancy history related risk factors

About 50 % of all PTD occur primiparous women (Morken et al. 2008, Jakobsson et al. 2007). However, history of previous PTD is actually the strongest single risk factor. It has been reported that risk of PTD ranges from 15% to more than 50 %, depending on the number of previous preterm deliveries. For each previous term delivery the risk of a subsequent PTD decreases (Goldenberg and Rouse 1998, Goldenberg et al. 2008, Kramer 2003, Mercer et al. 1999).

There is also an increased risk of PTD in pregnancies arising in close proximity to previous delivery. After adjustment for confounding factors, an interpregnancy interval of less than 6 months confers greater than 2-fold risk of PTD. A short interval decreases the opportunity to replenish the essential maternal nutrient stores which pregnancy consumes and to resolute the inflammatory status with the previous pregnancy (Conde-Agudelo et al. 2006, Smith et al. 2003).

In some studies the induced abortions increase the risk of PTD due to cervical damage during termination of pregnancy but the data are contradictory (Ancel et al. 2004, Henriet and Kaminski 2001, Thorp et al. 2003). In a recent Finnish study, previous induced abortion was not an independent risk factor for PTD (Raatikainen et al. 2006).

## Pregnancy associated risk factors

Multiple gestations, accounting for only 2-3% of pregnancies carry a substantial risk of PTD, and result in 12-27% of all preterm deliveries. Over the past decades there was a great increase in the incidence of multiple deliveries, largely as a result of the use of assisted conception technologies (Goldenberg and Culhane 2006, Goldenberg et al. 2008). The marked shift from double embryo transfer to single embryo transfer which has taken place during last decade in Finland and elsewhere has decreased the proportion of multiple deliveries. In Finland in 2008, 41.9% of all embryo transfers were performed as single

embryo transfer and 90.3 % of infants born after in vitro fertilization (IVF) were singletons (THL register 2010). Nearly 50-60 % of twins and nearby all higher order multiple gestations are born preterm (Goldenberg and Culhane 2006, Goldenberg et al. 2008). About 40% of twins will have spontaneous labour or PPROM before 37 weeks of gestation, with others having an indicated PTD because of maternal or fetal disorders (Goldenberg et al. 2008, Kramer 2003, Slattery and Morrison 2002).

In vitro fertilization (IVF) is mainly associated to moderately preterm group (Jakobsson et al. 2007). The preterm rate for singleton pregnancies after IVF is approximately 12%, and this higher rate is due to various factors, including cervical trauma, disturbed implantation, infection, uterine malformation, and associated infertility factors (Poikkeus et al. 2007). For twin pregnancies conceived after IVF, there was no difference in the rate of PTD or mean gestational age at delivery compared with twins conceived spontaneously (Goldenberg et al. 2008, Poikkeus et al. 2007, Steer 2005).

Infection is the most common single risk factor of PTD. Genital tract infections account for about 25-40% of preterm deliveries. However, infection is difficult to detect due to the limitations of conventional microbial techniques and the difficulties in obtaining appropriate diagnostic samples during pregnancy (Goldenberg et al. 2002, Goldenberg et al. 2005a, Romero et al. 2006b, Goldenberg et al. 2008). It is known that the relation between infection and PTD is not consistent throughout gestation. Spontaneous preterm deliveries that occur before the 34th week of gestation, and particularly before the 30th week of gestation, have been strongly associated to intrauterine infection. The earlier the preterm birth the stronger the association. Infection is rare in late preterm deliveries (at 34-36 weeks) (Goldenberg et al. 2000, Goldenberg et al. 2002, Iams 2003, Redline 2004, Romero et al. 2006b).

In women with spontaneous PTL and intact membranes, the most commonly identified micro-organisms are *Ureaplasma urealyticum, Mycoplasma hominis, Gardnerella vaginalis, peptostreptococci,* and bacteroides species, all vaginal organisms of relatively low virulence. After membranes rupture the most often found microbes in the uterus are group *B streptococci* or *Escherichia coli* and less commonly *Chlamydia trachomatis* or *Neisseria gonorrhoeae* (Andrews et al. 2000c, Goldenberg et al. 2002, Goldenberg et al. 2005a, Romero et al. 2006b).

Bacterial vaginosis (BV), a condition characterized by a decrease in number of lactobacilli and overgrowth of several anaerobic or facultative bacteria, including *Garndenella vaginalis*, *Mycoplasma hominis* and *Ureaplasma urealyticum*, has been shown to be present in up to 15-20% of pregnant women. It is asymptomatic in approximately 50% of women and can resolve spontaneously. Despite substantial microbial overgrowth, BV is not associated with signs of inflammation (Goldenberg et al. 2000, Hay et al. 1994, Romero et al. 2004). However, BV has been shown to increase the risk for spontaneous PTD and PPROM 2- to 3- fold. (Carey et al. 2003, Flynn et al. 1999, Gibbs 1993, Goepfert and Goldenberg 1996, Goldenberg et al. 1996, Hauth et al. 2003, Kekki et al. 1999, Kimberlin and Andrews 1998, Meis et al. 1995) Also the risk of postpartum endometritis is increased (Goldenberg et al. 2000, Kekki et al. 2001). Randomized clinical trials have shown conflicting results in screening and treatment of BV to prevent PTD (Guise et al. 2001, Kekki et al. 2001, Klebanoff et al. 2003, Leitich et al. 2003, Mac Donald et al 2008, Nygren et al 2008).

Many other genital infections have been associated with PTD with a great variation. The reason for this is that women with genital tract infections usually have other risk factors as well (Goldenberg et al. 2005a). Syphilis and gonorrhoea have been associated with PTD with a 2-fold (Donders et al. 1993) and trichomoniasis with a 1.3-fold risk (Andrews et al. 2000c). *Chlamydia trachomatis* infection has been also associated with 2 to 3-fold risk of PTD, (Andrews et al. 2000c, Blas et al. 2007, Cheney and Wray 2008). Still the treatment of Chlamydia has not been associated with a decreased frequency of PTD (Andrews et al. 2006). However, routine screening and treatment for *Chlamydia trachomatis* is important due to high incidence especially among young women. This is a necessary part of prenatal care to reduce adverse pregnancy outcomes, post-partum endometritis and to reduce the risk of the newborn for conjunctivitis and pneumonitis (Blas et al. 2007, Cheney and Wray 2008). Vaginal group *B streptococcus* colonisation is not associated with increased risk of PTD, but can cause neonatal sepsis and that is why the antibiotic treatment is indicated in cases of PPROM (Goldenberg et al. 2005a).

In addition, any systemic infections, such as asymptomatic bacteriuria, pyelonephfritis, appendicitis and pneumonia can trigger uteroplacental response leading to PTL and PTD (Goldenberg et al. 2008, Slattery and Morrison 2002). Even periodontitis has been linked to PTD (Agueda et al. 2008, Boggess 2005, Offenbacher et al. 1996). Malaria is the most widely distributed infectious disease associated with spontaneous PTD. Worldwide, 40% of pregnant women are exposed to malaria during pregnancy and annually more than 200,000 neonatal deaths are due to malaria. Also HIV and tuberculosis infections globally predispose women to spontaneous PTD and PPROM (Steer 2005).

Viral infections, such as cytomegalovirus, parvovirus (B19), varicella, enterovirus, Coxsakie virus, Echovirus and hepatis E virus, can transfer the placenta. The viruses attract directly the fetal tissues causing fetal diseases and later by disseminated infection possible stillbirth but in some cases they also associate to PTD (McClure and Goldenberg 2009). Herpex simplex viruses do not or very rarely cross the placenta and have not been associated with PTD (McClure and Goldenberg 2009). As a whole, it seems unlikely that maternal viral infection plays an important part in PTD, but further studies are needed (Srinivas et al. 2006).

The history of vaginal bleeding at any time of pregnancy is associated with PTD and also other adverse perinatal outcomes (Harlev et al. 2008, McCormack et al. 2008). Vaginal bleeding is a manifestation of decidual damage, but can also be idiopathic (McCormack et al. 2008, Romero et al. 2006b). The risk factors for vaginal bleeding include maternal cigarette smoking and cocaine use, chronic hypertension and pre-eclampsia, maternal trauma, and hereditary coagulopathies (Tikkanen et al. 2006). It may be a sign of retroplacental haematoma detected by ultrasound examination in the first trimester. In late pregnancy vaginal bleeding is associated with placental abruption which is a major obstetrical emergency (Romero et al. 2006b). The incidence of placental abruption in Finland is 0. 42% and it is strongly associated with PTD (Tikkanen et al. 2006). General risk factors for placental abruption are maternal smoking, use of alcohol, placenta preavia, pre-eclampsia and chorionamnionitis (Oyelese and Ananth 2006, Tikkanen et al. 2006).

# Pathophysiological pathways leading to preterm delivery

Four different main pathways have been described which may lead to PTD: infection/ inflammation, activation of the maternal fetal hypothalamic-pituitary-adrenal (HPA) axis, decidual haemorrhage, and uterine over-distension (Figure 1). Many pathways can be involved simultaneously (Slattery and Morrison 2002, Vogel et al. 2005, Romero et al. 2006b, Goldenberg et al. 2008).

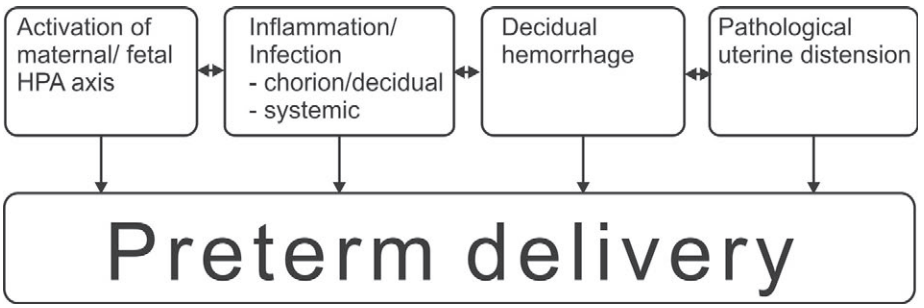


Figure 1. Main pathways leading to preterm delivery

## Infection and inflammation

Infection and inflammation are the most important mechanisms leading to PTD by acting on the innate immune system (Goldenberg et al. 2000, Romero et al. 2006b). Inflammation is the response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants, while infection is caused by an exogenous pathogen. Inflammation, especially chronic inflammation, can be simultaneously destructive and protective (Robbins et al 2010). Histologically confirmed bacterial infections within the uterus can occur between the maternal tissues and fetal membranes (chorioamnionitis), within the amniotic fluid (amnionitis) or within the umbilical cord or the fetus (funisitis). Infection within the placenta (villitis) is rare (Figure 2) (Goldenberg et al. 2000).

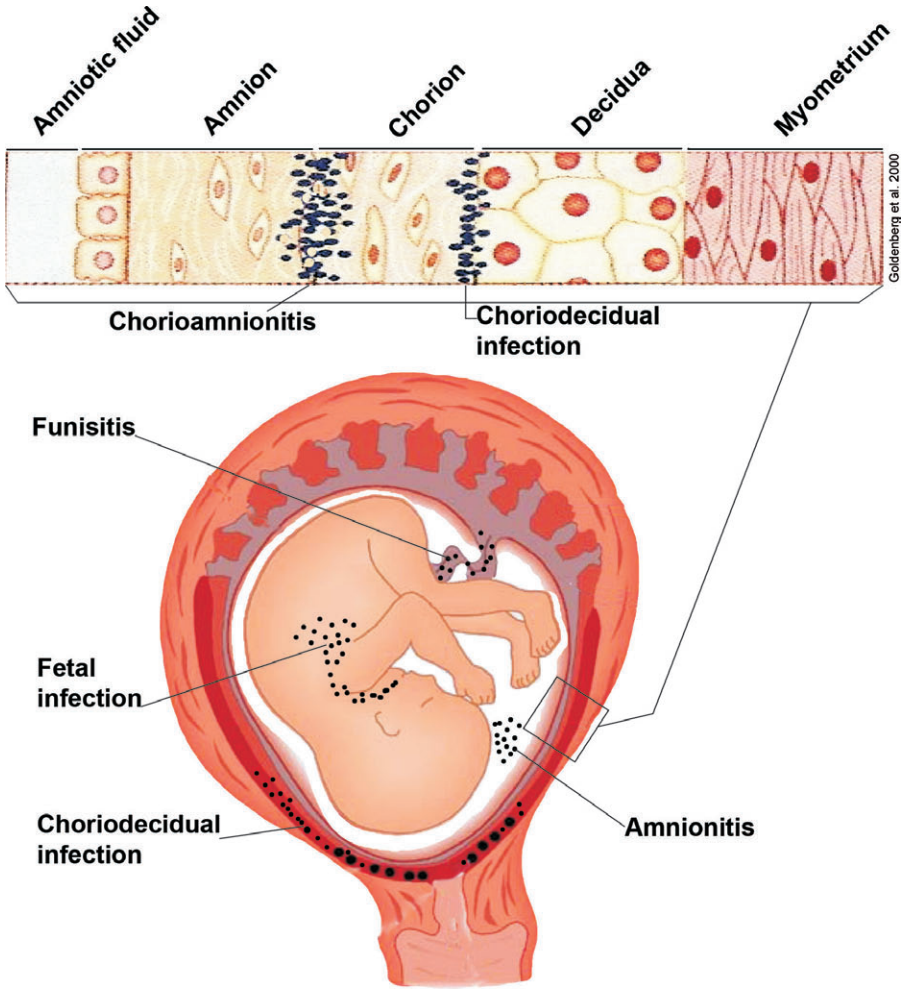


Figure 2. Potential sites of bacterial infection within the uterus

In most cases micro-organisms invade the uterine cavity by ascending from the vagina and cervix. Micro-organisms may also invade the uterus by migration from the abdominal cavity through the Fallopian tubes, haematogenously through the placenta, or by needle contamination at the time of amniocentesis or chorionic-villus sampling (Figure 3) (Goldenberg et al. 2000, Slattery and Morrison 2002, Romero et al. 2004, Goldenberg et al. 2008).

The timing of intrauterine infection is not sufficiently understood. Microbial colonization may precede conception, as is the case in chronic endometritis (Korn et al. 1995), or micro-organisms may invade the uterus during pregnancy (Romero et al. 2004, Goldenberg et

al. 2008). During the first trimester, the placenta and embryo are relatively well isolated from infection (Goldenberg et al. 2000, Redline 2004). At 18 to 20 weeks of gestation, the gestational sac fuses with the uterine lining and organisms from the decidua parietalis access the fetal membranes (Redline 2004, Goldenberg et al. 2008). After the fetal membranes seal the uterine cavity, the organisms usually no longer ascend from the vagina to the uterus (Goldenberg et al. 2000). However, in cases with PPROM the uterine cavity is exposed to vaginal bacterial flora even later in pregnancy.

In most cases, especially in early pregnancy, infection and inflammation in the uterus are asymptomatic. If ascending micro-organisms are not destroyed by the mother's immune system after the expanding membranes have sealed the uterine cavity, the infection may lead to infection and inflammation in the chorion, amnion and fetal membranes, before becoming symptomatic. Premature preterm rupture of the membranes and PTL may be the first symptoms of infection (Goldenberg et al. 2000, Goldenberg et al. 2008).

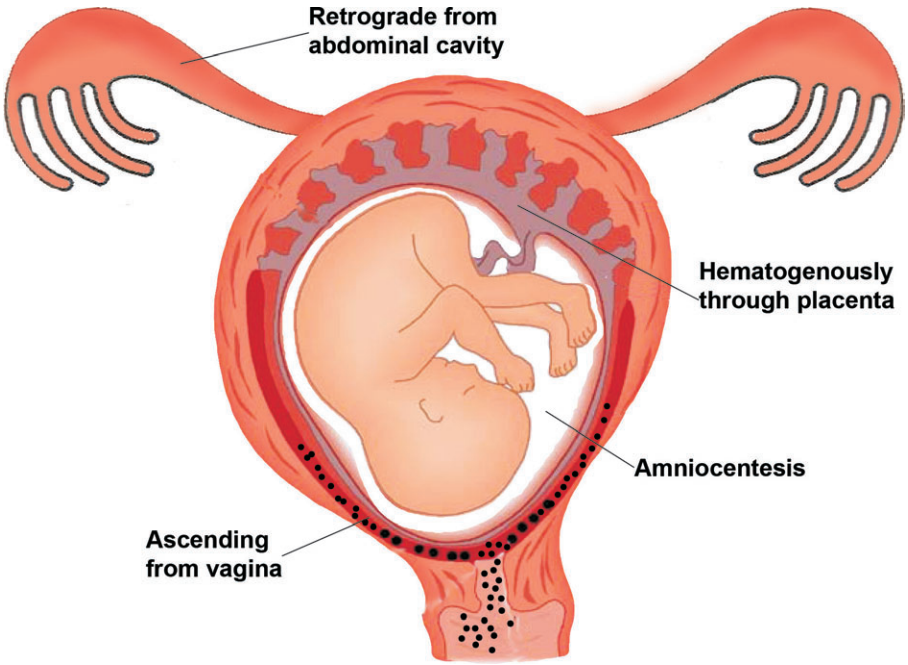


Figure 3. Potential pathways of microorganisms into the uterus

Micro-organisms in the vagina produce enzymes such as proteases and mucinases, which allow penetration of the cervical mucus plug, favouring the ascent of bacteria. Cervical mucus, containing antimicrobial proteins and peptides such us lysozyme, lactoferrin, defensin and immunoglobulins, acts like a mechanical and immunological barrier to ascending infection from the vagina (Hein et al. 2001). After penetration of the cervical mucus, micro-organisms spread into the uterine cavity, the decidua and fetal membranes, the so-called choriodecidual space. Choriodecidual cells are activated by micro-organisms to produce pro-inflammatory cytokines, such as interleukins-1 (IL-1α and IL-1β), interleukin-6 and 8 (IL-6 IL-8) and tumour necrosis factor-α (TNF-α). These cytokines act with exotoxins and endotoxins to stimulate prostaglandin synthesis from the arachidonic acid reservoir of the fetal membranes (Lockwood et al. 1999, Goldenberg et al. 2000, Goldenberg et al. 2002, Romero et al. 2006b). Chorionic infection also directly decreases the activity of prostaglandin dehydrogenases, allowing increasing quantities of prostaglandins to reach the myometrium, while without infections the prostaglandin dehydrogenases in chorionic tissue inactivate prostaglandin production (Goldenberg et al. 2000). Prostaglandins have a pivotal role in contractions of the smooth muscle of the uterus and the biophysical changes associated with cervical ripening (Lockwood et al. 1999, Goldenberg et al. 2000, Goldenberg et al. 2002, Romero et al. 2006b).

Cytokines also initiate neutrophil chemotaxis, infiltration and activation, leading to synthesis and release of matrix metalloproteinases, causing proteolysis in the extracellular matrix (Goldenberg et al. 2002, Romero et al. 2006b). While prostaglandins stimulate uterine contractions, matrix metalloproteinases attack the chorioamniotic membranes by weakening them, leading to rupture of the fetal membranes. In addition, matrix metalloproteinases cause tissue disruption in the choriodecidual space and facilitate separation of the chorion from the decidual layer in the lower uterine segment as well as ripening of the cervix by remodelling the collagen fibres (Lockwood et al. 1991, Rath et al. 1998, Weiss et al. 2007). Tissue disruption in the lower segment of the uterus due to either uterine contractions or infection-induced proteolysis, may cause leakage of choriodecidual products, such as insulin-like growth factor-binding protein -1 and fetal fibronectin, into the cervix and vagina, where they become detectable (Figure 4) (Lockwood et al. 1991, Rutanen 2000).

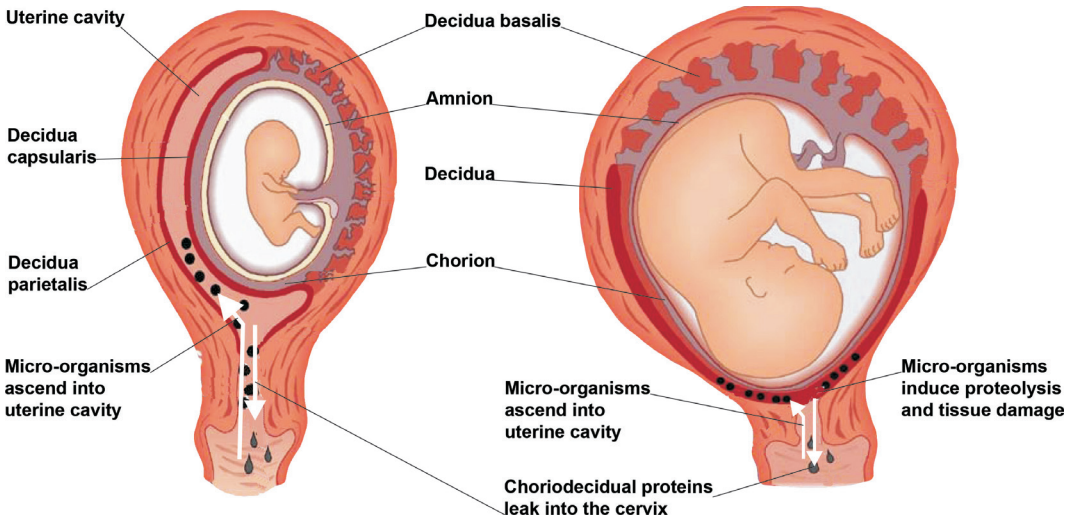


Figure 4. Ascending infection during early and late pregnancy

When infection reaches the fetus, an increase in both fetal hypothalamic and placental production of corticotrophin-releasing hormone causes an increase in fetal corticotrophin secretion leading to increasing fetal adrenal production of cortisol. The increased cortisol secretion in turn increases the production of prostaglandins, leading to myometrial contractions (Yoon et al. 1998, Goldenberg et al. 2000, Goldenberg et al. 2008). The pathways leading from choriodecidual infection to PTD are shown in Figure 5.

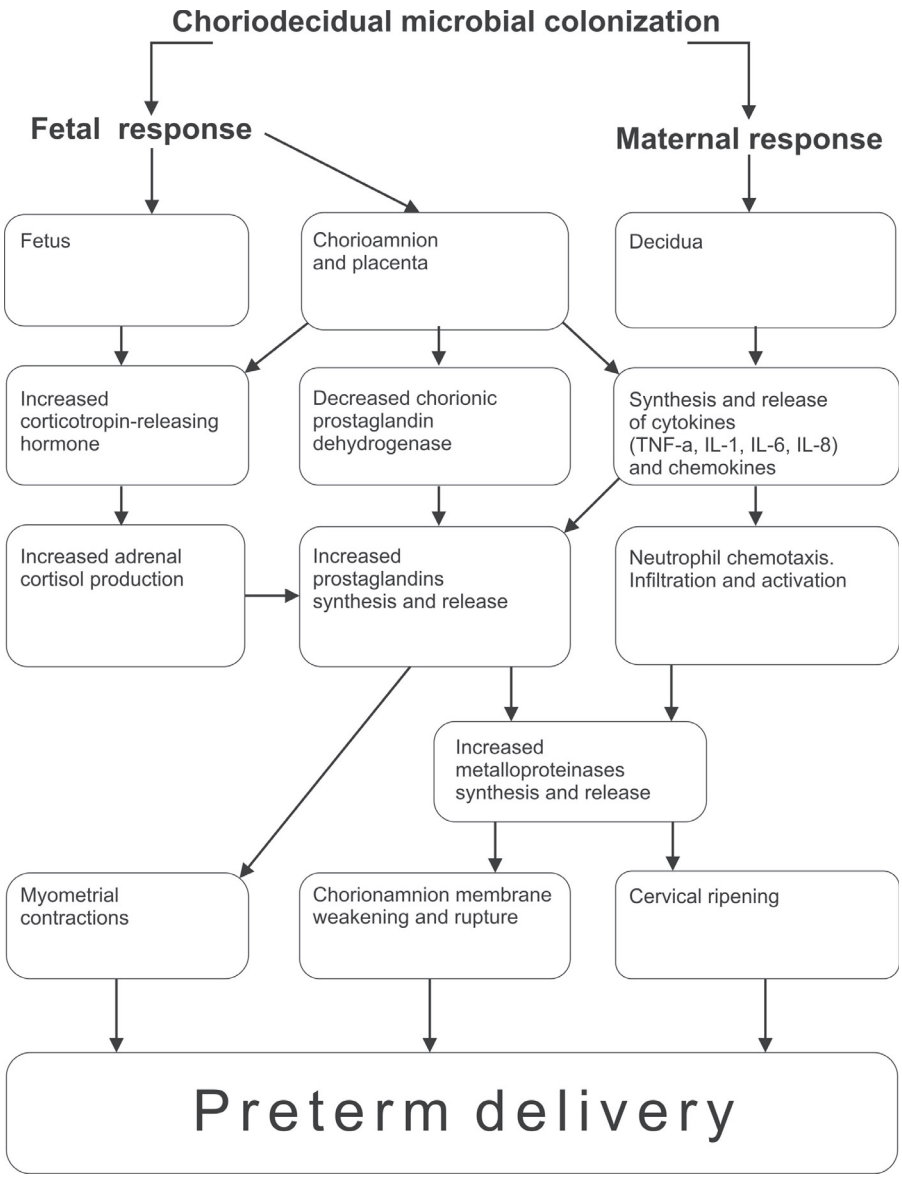


Figure 5. Potential pathways from choriodecidual bacterial colonization to PTD

Systemic infection may activate an inflammatory process that triggers an uteroplacental response leading to PTD (Goldenberg et al. 2005a). Micro-organisms serve as a source of endotoxins which increase local inflammatory mediators, including prostaglandins and cytokines. This damages host inflammatory and immune responses, leading to an increase of systemic inflammatory mediators that invade the bloodstream and organs including the placenta and uterus (Boggess 2005, Goldenberg et al. 2005a, Goldenberg et al. 2008).

## Activation of the maternal-fetal hypothalamic-pituitary-adrenal axis

Stress, psychological, social or physical, is one of the main independent risk factors leading to PTD, accounting for about 30% of cases. This pathway is typically associated with PTL after 32–34 weeks (Iams 2003).

Maternal stress may activate the maternal- fetal hypothalamic-pituitary-adrenal axis system, the neuroendocrine stress response, leading to increased concentrations of corticotrophin-releasing hormone (CHR) (Campbell et al. 1987, Hobel et al. 1999, Wadhwa et al. 2001b). CRH mediates pituitary adrenocorticotrophin (ACTH) secretion through maternal and fetal pathways, increasing maternal and fetal adrenal cortisol secretion. Cortisol contributes to the increased production of prostaglandins by fetal membranes and the decidua through the up-regulation of PG synthase and the down-regulation of PG dehydrogenase enzymes (Challis and Smith 2001). Prostaglandins cause contractions and bring about cervical ripening and they sensitize the myometrium to oxytocin (Campbell et al. 1987).

## Decidual haemorrhage

The pathway of PTD by way of decidual haemorrhage, which can occur at any time, involves about 20% of preterm births, especially those related to PPROM (Iams 2003). Vaginal bleeding caused by decidual haemorrhage or placental abruption is associated with a 3- to 4-fold increased risk of subsequent PTD, with damage to the uterine spiral arteries (Romero et al. 2006b , Goldenberg et al. 2008). Maternal vascular lesions lead to uteroplacental ischaemia. This can manifest as visible or occult vaginal bleeding at any time of pregnancy. The precise mechanism leading to onset of PTD with uteroplacental ischaemia is not known, but it is thought to be closely related to thrombin generation.

Thrombin stimulates coagulation and clot formation, leading to tissue disruption in the choriodecidual space (Elovitz et al. 2001, Romero et al. 2006b).

### Uterine over-distension

Intra-amniotic pressure remains relatively constant throughout gestation despite the growth of the fetus and placenta. It is the result of progressive myometrial relaxation due to the effects of progesterone and endogenous myometrial relaxants such as nitric oxide. Premature delivery may be triggered by mechanical stretching of the myometrium caused by an increase in uterine size that exceeds the ability of the uterus to compensate. Uterine over-distension is caused by multi-fetal pregnancy, overload of amniotic fluid (AF), so-called polyhydramnion, or uterine anomaly (Iams 2003, Romero et al. 2006b). Stretching of the fetal membranes caused by uterine over-distension triggers the increased production of cytokines and prostaglandins, and activates oxytocin receptors, leading to an increase in myometrial contractility (Romero et al. 2006b).

## Prediction of preterm delivery

### Rationale

Accurate prediction of PTD among asymptomatic pregnant women and those with threatened PTL might offer an opportunity to target more intensive antenatal surveillance and prophylactic measures to those most likely to benefit from primary, secondary or tertiary prevention. Primary prevention is prevention of the onset of spontaneous PTD in asymptomatic women by cessation of smoking and/or alcohol use, by maintaining a healthy genitourinary tract and periodontal status or by administration of maternal progestational agents by injection, or use of cervical cerclage in special cases with previous PTD (Vogel et al. 2005, Iams et al. 2008, Holst et al. 2009). Secondary prevention involves steps that can be taken to attenuate, stop or reverse the progress of spontaneous PTL in its early stages, before advanced cervical dilatation by using tocolytic agents. Tertiary prevention means measures aimed at preventing neonatal complications associated with prematurity by using antenatal corticosteroids to accelerate fetal lung maturity.

Predictive markers, in addition to known risk factors, can be specific findings in physical examination, such as short cervical length in ultrasonographic examination, or the presence of a particular substance in body fluids (Goldenberg et al. 2005b, Vogel et al. 2005, Holst et al. 2009). The markers can be used among asymptomatic and symptomatic women. Thus, prediction of PTD is useful among asymptomatic women to prevent PTD, and in symptomatic women to delay threatened delivery (Holst et al. 2009).

It is likely that in the majority of cases PTD reflects a long-standing process dating back to the first trimester. Therefore, it would be of clinical importance if this condition could be predicted before it becomes manifest clinically, as spontaneous PTL or PPROM (Iams 2003, Goldenberg et al. 2005b, Vogel et al. 2005). Before systematic screening, effective prophylactic intervention or treatment for individuals with positive test results should be available. In addition, the test should be efficient, with high sensitivity, specificity and a high positive predictive value, and be of low cost. At present, despite all efforts, there are no accurate screening methods. Primary or secondary interventions to prevent PTD have also been largely unsuccessful. If an accurate marker of threatened PTD were to be available, it could be used to tailor intervention among individual women at risk, and management might be more successful (Vogel et al. 2005, Holst et al. 2009). In addition, definition of high-risk women among asymptomatic women would give better understanding of the pathophysiological pathways leading to PTD. This may provide an opportunity to develop methods to prevent PTD.

In women presenting with symptoms the cascade resulting in delivery has already started, and there may be no way to stop it, although delay may be possible. Among symptomatic women there are situations where prediction of PTD could be important and clinically helpful. The goal of early diagnosis of PTD in symptomatic women is the appropriate application of antenatal interventions that reduce perinatal morbidity and mortality: First, transfer of high-risk women with PTL to a facility with a neonatal intensive care unit. Second, administration of glucocorticoids to the mother at the right time for fetal lung maturation. Timing is desirable because the benefit of glucocorticoids does not last beyond seven days (Murphy et al. 2001, Crowther and Harding 2007). Third, the use of tocolytic drugs to prolong pregnancy for a few days. The delay allows sufficient time for maternal transfer and treatment with antenatal steroids. Because tocolytic drugs have significant side effects, accurate diagnosis is important to avoid the risks and costs of unnecessary treatment. In cases with PPROM, antibiotic treatment may delay the delivery and reduce the incidence of chorioamnionitis and improve neonatal morbidity (Kenyon et al 2009). However, for symptomatic women with intact membranes antibiotic treatment is not recommended

(King et al. 2009). Similarly, in women with group B β-haemolytic streptococci in the vagina, antibiotic treatment during labour reduces the rate of neonatal group B streptococcal sepsis, but not the rate of spontaneous delivery (Valkenburg-van den Berg et al. 2009). In addition, prediction of PTD among symptomatic women may enable physicians to avoid unnecessary hospitalization of women who have a low risk of premature delivery.

## Predictive methods

### Traditional methods

Traditionally, prediction of PTD is based on obstetric history, symptoms and epidemiological risk factors, with limited accuracy. Nevertheless, obtaining an accurate history is the first step in identification of high-risk women (Goldenberg et al. 1998, Iams et al. 1998, Goldenberg et al. 2003, Chandiramani and Shennan 2006). Classic digital examination, including assessment of position, effacement, softness and dilation of the cervix is used to create a Bishop score. Low predictive value and considerable intra- and inter-observer variability have limited the utility of the Bishop score (Copper et al. 1990, Onderoglu 1997, Iams et al. 2002, Owen et al. 2003). Furthermore, cervical effacement begins at the internal os and it is not possible to assess by digital palpation (Zilianti et al. 1995).

Several other risk-scoring systems have been developed, with poor sensitivity and specificity (Creasy et al. 1980, Goldenberg et al. 1998). Thus most women who deliver preterm are not identified by risk-scoring systems and most of those identified as high risk do not deliver preterm. About 50% of women who experience PTD have no obvious risk factors. Furthermore, because traditional risk factor scores are based largely on previous obstetric history their accuracy is particularly low among women carrying their first child; such women constitute approximately 50% of those affected by PTD (Goldenberg et al. 1998, Iams 2003, Goldenberg et al. 2008). More sensitive and specific markers for the identification of women at high risk of PTD are therefore needed.

**Cervical assessment by ultrasonography**

The human cervix consists of smooth muscle cells (10–15%) and connective tissue (85–90%) (Danforth 1983). Collagen bundles are at the highest ratio in the area of the internal os and provide the rigidity of the cervix (Leppert 1995, Kelly 2002). During pregnancy the cervix undergoes intensive remodelling of the extracellular matrix (ECM), including extensive changes in the concentration and composition of collagens, in two phases: ripening and dilatation (Leppert 1995). Cervical effacement and ripening begin, weeks before delivery, at about 32–36 weeks as regards term delivery and as early as at 16–24 weeks in cases of PTD; the cervix softens, shortens, rotates anteriorly, and dilates. The pattern of changes varies between individuals (Rath et al. 1998, Bergelin and Valentin 2002, Iams 2003). As the cervix effaces, the upper part (the internal os) opens and becomes indistinguishable from the lower segment of the myometrium (Kelly 2002).

The most accurate and reproducible method of cervical evaluation is transvaginal ultrasonography, with higher positive predictive value than digital palpation (Iams et al. 2002, Owen et al. 2003). Three different ultrasonographic signs are considered. Signs of cervical incompetence are dilatation of the internal os, funnelling with protrusion of the amniotic membranes into the cervical canal and a short cervix in the absence of uterine contractions (Rozenberg et al. 2002, Owen et al. 2003). Dilatation of the internal os of > 5 mm before 30 weeks of gestation is associated with premature delivery (Rozenberg et al. 2002, Owen et al. 2003). The presence of funnelling shortens functional cervical length and increases the risk of spontaneous PTD (Rozenberg et al. 2002). Funnelling can be observed by ultrasonography even though digital cervical examination may indicate a long and closed cervix. In early and mid-pregnancy serial examinations of the cervix can be used to find women at a high risk of PTD and those who might benefit from intervention (Guzman et al. 1997). It has been reported that in asymptomatic women, at 22–24 weeks of gestation, cervical length of less than 25 mm is associated with a 5- to 6-fold increased risk of PTD before 34 weeks of gestation (Guzman et al. 1997, Andrews et al. 2000a, Iams 2003). The shorter the cervical length is, the greater the risk of PTD (Iams et al. 1996, Guzman et al. 1997, Iams et al. 1998, Andrews et al. 2000a). However, there is no agreement for the definition of a (sonographically) short cervix. Some authors have proposed a cut-off of 15 mm and some 10 mm at mid-pregnancy in a subgroup of women at high risk of early PTD (Guzman et al. 1997, Macdonald et al. 1997, Norman 2007). However, the most common threshold used in asymptomatic women has been 25 mm between 20 and 24 weeks of gestation (Iams et al. 1996, Andrews et al. 2000a, Owen et al. 2003). Among symptomatic women, the most common threshold has been 15 mm, predicting spontaneous PTD within 7 days of testing (Gomez et al. 1994, Tsoi et al. 2005). Altogether, the evidence supports the

idea that cervical competence is not a dichotomous variable with a specific cut-off point, but more likely a functional variable that can be evaluated by using a reproducible method during pregnancy with no apparent risks (Iams et al. 1996, Owen et al. 2003).

**Biomarkers**

A biomarker is defined as a biochemical substance in a body fluid that by decreasing or increasing in concentration is likely to identify people at risk of a particular event (e.g. PTD). Biomarkers should be easy to collect at low cost and cause minimal risk and discomfort (Goldenberg et al. 2005b, Vogel et al. 2005). The sources of biomarkers for PTD include AF, urine, secretions in the vagina and cervical canal, serum and plasma. In addition, saliva and even fluid taken from dental pockets of women with periodontal disease are used to predict PTD. Many biomarkers reflect responses to inflammation and some are infectious organisms (Vogel et al. 2005). All biomarkers can be measured in both asymptomatic and symptomatic individuals.

Obtaining cervical and vaginal secretions is easy and safe, with minimal discomfort for the woman. However, many substances in vaginal and cervical fluid are affected by various micro-organisms, which may limit their use. On the other hand many of these infective agents and their relationships to PTD have been evaluated in order to predict PTD (Goldenberg et al. 2005b). The most commonly studied predictors are BV and proteins such as fetal fibronectin (FFN), insulin-like growth factor-binding protein-1 (IGFBP-1), and cytokines in cervical and vaginal fluid.

Many studies have been focused on infection in the amniotic fluid. This contains substances of both maternal and fetal origin. The amniotic cavity is usually sterile as regards bacteria, and it does not contain white blood cells. Fewer than 1% of women not in labour at term have bacteria in the AF. The cell type most frequently recruited into the amniotic cavity during the course of an inflammatory process is the neutrophil. They are thought to be of fetal rather than of maternal origin, reflecting a fetal inflammatory response (Romero et al. 1991, Sampson et al. 1997, Romero et al. 2006b, Goldenberg et al. 2008). As well as containing bacteria, AF in women with intrauterine infections has lower glucose concentrations, higher white cell counts and higher concentrations of various cytokines than AF of uninfected women. However, using substances in AF as markers of PTD requires an invasive examination, amniocentesis. This procedure carries a 0.5–1% risk of fetal loss, which minimizes its clinical use (Vogel et al. 2005). For this reason AF is

generally not obtained from asymptomatic women, except at the time of amniocentesis for genetic indications. This makes studies difficult to interpret and compare with other studies among symptomatic women (Goldenberg et al. 2005b). The use of AF is also limited by cost implications, and it causes discomfort to the patient.

Biomarkers in serum or plasma are easy to collect, with minimal discomfort to the patient. Alpha fetoprotein, ferritin, C-reactive protein, various cytokines and relaxin are examples of biomarkers that can be measured in serum and plasma and are used for predicting spontaneous PTD (Goldenberg et al. 2005b).

Saliva is an ultrafiltrate of plasma and among other biological fluids it is the easiest to collect. Various hormones in saliva have been evaluated to predict subsequent PTD. The concentrations of steroid hormones in saliva are close approximates of the levels of unbound hormone in plasma, because unconjugated steroid hormones enter the saliva through diffusion. A limitation of the use of saliva for marker assessments is that various substances in the mouth can be confounding factors (Goldenberg et al. 2005b). In research work urine is used for measurements of some hormones and organisms predictive of PTD. Sexually transmitted diseases such as Chlamydia infection and gonorrhoea can be identified by urine DNA examination (Andrews et al. 2000b).

Timing has to be considered when biological markers are used. It can be assessed by hour, day, week, or month before PTD and also in relation to gestational age. Some markers, for example fetal fibronectin in cervicovaginal fluid, can be powerful predictors in late pregnancy but less predictive in early pregnancy (Goldenberg et al. 1997, Goldenberg et al. 2000). It is also important to understand when a marker turns positive in relation to PTD.

***Inflammation-associated biomarkers***

Inflammation-associated proteins are produced in response to inflammation in the choriodecidual space and also in extra-uterine tissues. Proteins of the choriodecidua are thought to leak into the AF, plasma or into cervical and vaginal fluid from the placenta or choriodecidual space as a result of tissue disruption. Some proteins, IGFBP-1, FFN and prolactin, may have no actual role in the pathway leading to PTD but may serve as predictors, while substances such as cytokines and matrix metalloproteinases (MMP-8 in particular) are involved in pathophysiological mechanisms of PTD.

**Insulin like growth factor-binding protein-1**

Insulin-like growth factor-binding protein-1 is synthesized and secreted by fetal and adult liver cells and by decidualized endometrial cells during pregnancy. Progesterone and other factors that enhance decidualization induce IGFBP-1 production in the endometrium (Rutanen 2000). The physiological role of IGFBP-1 in pregnancy may be essential for proper endometrial/decidual function and endometrial-trophoblast interaction (Giudice and Irwin 1999). IGFBP-1 can either inhibit or enhance the effect of insulin like growth factors (IGFs). In maternal circulation, the concentration of IGFBP-1 increases during pregnancy, and in amniotic fluid IGFBP-1 is a major protein from the second trimester onwards (Rutanen 2000).

The phosphorylation status of IGFBP-1 varies in different body fluids and tissues (Koistinen et al. 1993, Westwood et al. 1994, Martina et al. 1997). In AF, a non-phosphorylated isoform of IGFBP-1 predominates, but in mid and late gestation phosphorylated isoforms, except for the most highly phosphorylated form are also present in AF. The concentration of IGFBP-1 is 100–1000 times higher in AF than in maternal serum (Rutanen 2000). Human decidual cells secrete predominantly phosphorylated IGFBP-1(phIGFBP-1) isoforms, including the highly phosphorylated one, and decidual tissue is a source of phIGFBP-1 throughout pregnancy (Westwood et al. 1994, Martina et al. 1997, Rutanen 2000). Different patterns of IGFBP-1 phosphoisoforms, originating either from AF or the decidua, can be distinguished using specific monoclonal antibodies (Figure 6) (Rutanen 2000).

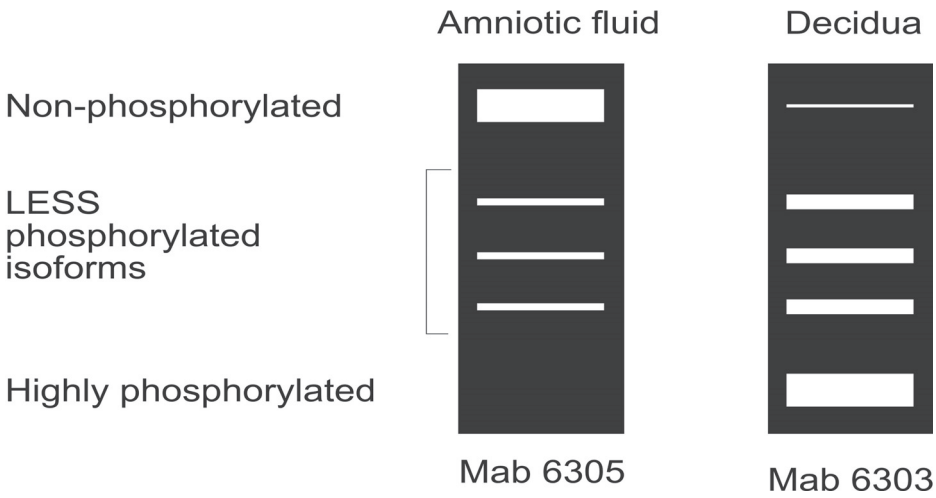


Figure 6. Immunoblot (schematic) of IGFBP-1 with two different antibodies

Non-phosphorylated and less phosphorylated isoforms of IGFBP-1 in cervical and vaginal samples can be quantified by immunoenzymometric assay using the monoclonal antibody 6305 (Medix Biochemica, Kauniainen, Finland) (Figure 6) (Rutanen et al. 1996). Detection of AF isoforms in vaginal fluid is used as a marker of ruptured fetal membranes (Rutanen et al. 1993, Lockwood et al. 1994) and a rapid bedside test (Actim PROM test, Medix Biochemica) is available for this purpose. The detection limit of this test is 25-50μg of AF isoforms per litre in a swab sample, (Rutanen et al. 1996). Urine and seminal plasma do not interfere in the test (Rutanen et al. 1993).

The phIGFBP-1 isoforms, including the highly phosphorylated isoform, can be detected in lower genital tract secretions by using the monoclonal antibody 6303 (Medix Biochemica) (Figure 6) (Nuutila et al. 1999). With tissue disruption in the choriodecidual space, due either to uterine contractions or infection-induced proteolysis, fetal membranes begin to detach from the decidua and cause leakage of decidual IGFBP-1 phosphoisoforms into the cervical and vaginal secretions (Rutanen 2000). In late pregnancy the increased concentrations of phIGFBP-1 (> 10 μg/l) in cervical secretions predict cervical ripening (Nuutila et al. 1999). The presence of phIGFBP-1 in the lower genital tract in early and mid-gestation has been poorly studied During medical termination of pregnancy, the concentration of phIGFBP-1 in cervicovaginal fluid was found to increase, returning to undetectable in two weeks (Honkanen et al. 2004). In women with asyptomatic BV, elevated cervical fluid concentrations of phIGFBP-1 in early pregnancy (10–17 weeks of gestation) have been associated with postpartum infectious complications (Kekki et al. 1999). Elevated concentrations of phIGFBP-1 also predict neonatal infectious morbidity (Kurkinen-Raty et al. 2001).

Kekki et al. (2001) first showed that a phIGFBP-1 concentration of at least 10 μg/l in cervical fluid in the later half of pregnancy in symptomatic women indicated a 10-fold increased risk of PTD. An immunochromatographic rapid bed-side test for detection of phIGFBP-1 (≥10 μg/l) has been developed (Actim Partus test, Medix Biochemica). The test result is not affected by urine or seminal plasma (Rutanen 2000), but maternal blood may interfere, since the same phosphorylated isoforms of IGFBP-1 predominate in decidua and maternal blood (Martina et al. 1997).

The sensitivity of the rapid phIGFBP-1 test in the prediction of PTD among symptomatic women with singleton pregnancies varies between 66.7 and 100%, with a negative predictive value (NPV) between 88.0 and 100% (Table 1) (Lembet et al. 2002, Akercan et

al. 2004, Kwek et al. 2004, Elizur et al. 2005, Eroglu et al. 2007, Paternoster et al. 2007, Ting et al. 2007, Altinkaya et al. 2009 Paternoster et al. 2009, Tanir et al. 2009, Brik et al. 2010). Only one study included multifetal pregnancies, with no significant difference between positive and negative results (Elizur et al. 2005). The sensitivity of the phIGFBP-1 test has been shown to be highest in prediction of PTD within 7days (Lembet et al. 2002, Kwek et al. 2004, Ting et al. 2007).

**Table 1.** Accuracy of rapid cervical fluid phIGFBP-1 bedside test in prediction of spontaneous preterm delivery among symptomatic women

| Study/Outcome | Year | N | Testing wks | Sensitivity % | Spesifity % | PPV % | NPV % | LR+ | LR- |
|---|---|---|---|---|---|---|---|---|---|
| **< 48 hours** | | | | | | | | | |
| Lembet et al | 2002 | 54 | 20-36 | 93.3 | 81.0 | 77.8 | 94.4 | 4.6 (1.9-11.3) | 0.2 (0.04-0.6) |
| Kwek et al | 2004 | 47 | 23-33 | 66.7 | 61.7 | 22.2 | 91.7 | 1.6 (0.8-3.2) | 0.6 (0.2-1.8) |
| Ting et al | 2007 | 94 | 24-34 | 100.0 | 74.0 | 18.0 | 100.0 | | |
| Brik et al | 2010 | 276 | 24-34 | 73.7 | 64.9 | 16.1 | 96.4 | 2.1 (1.5-2.9) | 0.4 (0.2-0.9) |
| **< 7 days** | | | | | | | | | |
| Lembet et al | 2002 | 54 | 20-36 | 93.8 | 85.0 | 83.3 | 94.1 | 6.3 (2.2-17.9) | 0.1 (0.01-0.5) |
| Kwek et al | 2004 | 47 | 23-33 | 83.3 | 73.3 | 55.6 | 91.7 | 2.9 (1.5-5.5) | 0.2 (0.1-0.8) |
| Ting et al | 2007 | 94 | 24-34 | 69.0 | 78.0 | 39.0 | 92.0 | | |
| Tanir | 2009 | 68 | 24-37 | 93.3 | 79.2 | 56.0 | 97.6 | 4.4 (2.1-5.2) | 0.8 (0.4-0.9) |
| Brik et al | 2010 | 276 | 24-34 | 73.1 | 66.2 | 21.8 | 95.0 | 2.2 (1.6-3.0) | 0.4 (0.2-0.8) |
| **< 14 days** | | | | | | | | | |
| Ting et al | 2007 | 94 | 24-34 | 72.0 | 80.0 | 46.0 | 92.0 | 3.6 | 0.4 |
| Tanir | 2009 | 68 | 24-37 | 60.7 | 80.0 | 68.0 | 74.4 | 2.8 (1.1-4.3) | 0.4 (0.1-0.6) |
| **< 32 wks** | | | | | | | | | |
| Brik et al | 2010 | 276 | 24-34 | 76.2 | 65.5 | 18.4 | 96.4 | 2.2 (1.6-3.0) | 0.3 (0.2-0.8) |
| **< 34 wks** | | | | | | | | | |
| Tanir et al | 2009 | 68 | 24-37 | 70.5 | 74.5 | 48.0 | 88.8 | 2.8 (1.1-3.8) | 0.3 (0.1-0.9) |
| Brik et al | 2010 | 276 | 24-34 | 59.0 | 66.0 | 23.4 | 88.6 | 1.8 (1.2-2.4) | 0.6 (0.4-0.9) |
| **< 35 wks** | | | | | | | | | |
| Elizur et al | 2005 | 64 | 24-35 | 81.8 | 64.1 | 32.1 | 94.4 | 4.2 (1.4-12.0) | 0.3 (0.03-3.4) |
| Erogu et al | 2007 | 51 | 24-35 | 70.0 | 87.8 | 58.3 | 92.3 | 5.7 | 0.3 |
| **< 36 wks** | | | | | | | | | |
| Kwek et al | 2004 | 47 | 23-33 | 73.7 | 82.7 | 77.8 | 79.2 | 3.9 (1.5-9.7) | 0.3 (0.2-0.7) |
| **< 37 wks** | | | | | | | | | |
| Kekki et al | 2001 | 63 | 22-36 | 70.0 | 81.1 | 41.2 | 93.5 | 3.7 | 0.4 |
| Lembet et al | 2002 | 54 | 20-36 | 89.5 | 94.1 | 94.4 | 88.9 | 15.2 (2.3-102.5) | 0.1 (0.03-0.4) |
| Akercan et al | 2004 | 77 | 24-36 | 78.0 | 87.0 | 73.0 | 90.0 | 6.1 (2.3-15.8) | 0.3 (0.1-0.7) |
| Elizur et al | 2005 | 64 | 24-35 | 69.6 | 70.7 | 57.1 | 80.5 | 2.0 (0.7-5.9) | 0.5 (0.2-1.7) |
| Pasternoster et al | 2007 | 109 | 22-34 | 69.2 | 90.5 | 50.0 | 95.6 | 7.3 (3.6-15.0) | 0.3 (0.2-0.8) |
| Altinkaya et al | 2009 | 105 | 24-34 | 70.0 | 87.0 | 56.0 | 92.5 | 5.4 | 0.3 |
| Pasternoster et al | 2009 | 210 | 24-34 | 52.9 | 89.2 | 48.7 | 90.8 | 4.9 | 0.5 |

In one study involving cervical ultrasonography and phIGFBP-1 in symptomatic women, both cervical length ≤ 26 mm (OR 7.37; 95% CI 2.27–23.96) and a positive phIGFBP-1 test result (OR 15.7; 95% CI 3.95–58.18) were independent predictors of PTD (Paternoster et al. 2009). In another study, both short cervical length and phIGFBP-1 test results had similar negative predictive values, of 91.1 and 92.3% respectively (Table 2) (Eroglu et al. 2007). One study in asymptomatic women with a history of PTD showed that cervical length should be measured between 22 and 24 weeks of gestation, and the phIGFBP-1 test was most accurate when used at 30 weeks of gestation (Bittar et al. 2007).

**Table 2.** Accuracy of combinations of rapid cervical phIGFBP-1 bedside test with either cervicovaginal fetal fibronectin (FFN) or cervical length in prediction of spontaneous PTD among symptomatic women

| Study | Year | N | Testing wks | Test | Outcome | Sensitivity % | Spesifity % | PPV % | NPV % | LR + | LR - |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Ting et al | 2007 | 94 | 24-34 | phIGFBP-1 | < 48 hours | 100 | 74.0 | 18.0 | 100 | 3.8 | |
| | | | | | < 7 days | 69.0 | 78.0 | 39.0 | 92.0 | 3.2 | 0.4 |
| | | | | | < 14 days | 72.0 | 80.0 | 46.0 | 92.0 | 3.6 | 0.4 |
| | | | | FFN | < 48 hours | 60.0 | 72.0 | 11.0 | 97.0 | 2.1 | 0.6 |
| | | | | | < 7 days | 56.0 | 76.0 | 32.0 | 89.0 | 2.3 | 0.6 |
| | | | | | < 14 days | 61.0 | 78.0 | 39.0 | 89.0 | 2.8 | 0.5 |
| Erogu et al | 2007 | 51 | 24-35 | phIGFBP-1 | < 7 days | 83.3 | 84.4 | 41.7 | 97.4 | 5.4 | 0.2 |
| | | | | FFN | | 83.3 | 80.0 | 35.7 | 97.3 | 4.2 | 0.2 |
| | | | | CX < 20mm | | 66.7 | 95.6 | 66.7 | 95.6 | 15.0 | 0.4 |
| | | | | CX < 25 mm | | 66.7 | 88.9 | 44.4 | 95.2 | 6.0 | 0.4 |
| | | | | Cx <25mm + phIGFP-1 | | 80.0 | 97.1 | 80.0 | 97.1 | 27.6 | 0.2 |
| | | | | Cx <25mm + FFN | | 80.0 | 97.0 | 80.0 | 97.0 | 26.7 | 0.2 |
| | | | | phIGFBP-1 | < 35 wks | 70.0 | 87.8 | 58.3 | 92.3 | 5.7 | 0.3 |
| | | | | FFN | | 70.0 | 82.9 | 50.0 | 91.9 | 4.1 | 0.7 |
| | | | | CX < 20mm | | 60.0 | 100.0 | 100.0 | 91.1 | | 0.4 |
| | | | | CX < 25 mm | | 60.0 | 92.7 | 66.7 | 90.5 | 8.2 | 0.4 |
| Paternoster et al | 2009 | 210 | 24-34 | phIGFBP-1 | < 37 wks | 52.9 | 89.2 | 48.7 | 90.8 | 4.9 | 0.5 |
| | | | | CX < 26mm | | 86.4 | 71.9 | 34.5 | 96.8 | 3.1 | 0.2 |
| | | | | CX < 26mm+phIGFBP-1 | | 40.1 | 96.1 | 64.3 | 90.4 | 10.3 | 0.6 |

In low-risk asymptomatic pregnant women, both the sensitivity and the positive predictive value (PPV) of the phIGFBP-1 test have been found to be lower, but NPV was as high as in symptomatic women when tested between 20 and 37 weeks of gestation (Table 3) (Kekki et al. 2001, Bittar et al. 2007, Paternoster et al. 2007, Balik et al. 2008, Altinkaya et al. 2009).

**Table 3.** Accuracy of rapid cervical fluid phIGFBP-1 bedside test in prediction of spontaneous PTD among asymptomatic women

| Study | Year | N | Testing wks | Outcome (wks) | Sensitivity % | Spesifity % | PPV % | NPV % | LR + | LR - |
|---|---|---|---|---|---|---|---|---|---|---|
| Kekki et al. * | 2001 | 58 | 22-37 | < 37 | | 94.7 | | 98.1 | | |
| Pasternoster et al | 2007 | 223 | 22-34 | < 37 | 22.2 | 91.8 | 11.8 | 96.0 | 2.7 | 0.8 |
| Balic et al. | 2008 | 80 | 24-34 | < 37 | 80.0 | 93.3 | 44.4 | 98.6 | 11.9 | 0.2 |
| Altinkaya et al | 2009 | 73 | 24-34 | < 37 | 40.0 | 82.3 | 14.3 | 94.9 | 2.3 | 0.7 |

* All delivered > 37 wks

**Fetal fibronectin**

Fibronectins are a family of ubiquitous glycoproteins found in the plasma and extracellular matrix. An oncofetal isoform of fibronectin, fetal fibronectin (FFN), synthesised by chorionic cells, is found in AF, placental tissue and extracellular matrix of decidua basalis next to the placental intervillous space (Lockwood et al. 1991, Honest et al. 2002). It is thought to adhere to the chorionic membrane of the blastocyst and the decidua (Lockwood et al. 1991, Goldenberg et al. 1996, Goldenberg et al. 2000). It is thought to be released into the cervix and vagina from the choriodecidual interface through mechanical- or inflammation-mediated damage to the membranes or placenta before delivery. Fetal fibronectin is present in high quantities in AF and in lesser quantities in maternal serum and cervicovaginal secretions (Vogel et al. 2005). It can be detected in cervicovaginal secretions before 20 weeks of gestation (Goldenberg et al. 1997, Goldenberg et al. 2000). In an early study by Lockwood et al. (1991), 24% of cervical samples and 17% of vaginal samples had detectable FFN concentrations before 22 weeks of gestation. In another study, only about 4% of pregnant women had detectable FFN concentrations in cervicovaginal secretions after 20 weeks of gestation (Goldenberg et al. 2000, Vogel et al. 2005). After 23 weeks of gestation, the presence of FFN ($\geq$ 50 μg/l) in cervicovaginal samples from women with PTL predicts PTD (Lockwood et al. 1991, Goldenberg et al. 1997, Peaceman et al. 1997, Leitich et al. 1999, Honest et al. 2002). Fetal fibronectin can be measured in samples obtained from the ectocervix or posterior vaginal fornix using an immunochromatographic test with a detection limit of $\geq$ 50 μg/l (Honest et al. 2002, Stafford et al. 2008). The accuracy of the FFN test in predicting spontaneous PTD varies (Honest et al. 2002). It is most accurate in predicting spontaneous PTD within 7–10 days after testing among symptomatic women (Honest et al. 2002, Goldenberg et al. 2003). A negative FFN test result rules out delivery within the next two weeks (NPV of 85–94%) (Rizzo et al. 1996, Goldenberg et al. 1997, Hincz et al. 2002, Honest et al. 2002, Goldenberg et al. 2003, Ting et al. 2007). A positive FFN test result at < 37 weeks of gestation in asymptomatic women increases the risk of spontaneous PTD 3- to 4-fold and in symptomatic women the risk is 6- to 7-fold increased (Honest et al. 2002). A repeat positive test result further increases the risk and a negative test result after a positive result lowers the risk to baseline (Goldenberg et al. 1997).

The poor positive predictive value of the FFN test is the main reason why it is not recommended by the American College of Obstetricians and Gynecologists (ACOG) for routine screening, but only for screening of symptomatic women (ACOG 2001). The FFN test result may be affected by urine, seminal plasma and maternal blood, and it is not useful if the membranes have ruptured or if the woman has had sexual intercourse in the previous

24 hours (Shimoya et al. 1998, Honest et al. 2002). The concentrations of fetal fibronectin in maternal serum are high in pre-eclampsia, perhaps as a result of endothelial damage (Madazli et al. 2005).

The accuracies of the phIGFBP-1 and FFN tests for predicting PTD are similar (Table 2) (Erogu et al. 2007, Ting et al. 2007).

**Cytokines**

Cytokines have been studied in maternal serum, AF and cervicovaginal secretions as protein biomarkers of PTD. They are low-molecular-weight proteins that mediate inflammatory reactions by activating immune cells. In addition, cytokines coordinate production and secretion of antibodies, and other cytokines. Cytokines also participate in cell-to-cell communication and cellular activation (Vogel et al. 2005, Honest et al. 2009). They are released by many cells, including epithelial cells, endothelial cells and cells of the immune system. Cytokines are generally classified as either pro-inflammatory (TNF-α, IL-1, IL-2, IL-3, IL-6, IL-8) or anti-inflammatory (interleukin-4, -10 and -13) and they have a wide spectrum of biological activity in coordinating immune responses to infection (Goldenberg et al. 2005b, Vogel et al. 2005, Goldenberg et al. 2008).

During pregnancy cytokines stimulate uterine contractions by production of prostaglandins and they are involved in preterm cervical ripening and PPROM by stimulation of metalloproteinases (Goldenberg et al. 2000, Slattery and Morrison 2002, Vogel et al. 2005, Goldenberg et al. 2008). Levels of interleukin (IL)-6 and IL-8 increase 10-fold in cervical tissue at term (Sennstrom et al. 2000). IL-8 and IL-1 promote recruitment and activation of neutrophils. Degranulation of neutrophils produces proteolytic enzymes which have a crucial role in cervical ripening. IL-8 and IL-1β induce hyaluronic acid production by human cervical fibroblasts (Osmers et al. 1995, Rath et al. 1998, Stygar et al. 2002). In addition, pro-inflammatory cytokines such as IL-1β, IL-6, IL-8 and TNF-α protect the host against invading micro-organisms. This response can be both protective and harmful to the host (Keelan et al. 1999, Keelan et al. 2003). The pro-inflammatory immune cascade is tightly regulated. Resolution of the inflammatory process and healing occur through the activity of anti-inflammatory cytokines such as interleukin-receptor antagonist. Imbalance between pro- and anti-inflammatory responses has been implicated in the pathogenesis of infection-related premature delivery (Genc et al. 2004). PTD has been associated with elevated concentrations of IL-1, IL-6 and IL-8 even in amniotic fluid in the absence of signs

of intrauterine infection (Arntzen et al. 1998).

The role of pro-inflammatory cytokines in maternal serum and AF during infection and before parturition has been extensively studied. There are also studies which indicate that measuring cytokines in cervicovaginal fluid could be of value in the prediction of intrauterine infection and PTD, especially close to delivery. High concentrations of IL-1, IL-6, IL-8 and TNF-α in maternal serum, AF and cervicovaginal secretions predict PTD with variable accuracy. Comparison of these studies is difficult because of different definitions and thresholds of elevated concentrations (Lockwood et al. 1994, Foulon et al. 1995, Ghidini et al. 1997, Wenstrom et al. 1998 Goepfert et al. 2001, Kurkinen-Raty et al. 2001, Vogel et al. 2005, Honest et al. 2009).

Recently the genetic polymorphism of cytokines has attracted attention (Holst and Garnier 2008, Kalinka and Bitner 2009, Sata et al. 2009, Warren and Silver 2009). Genital tract immune hyporesponsiveness, as reflected in low cervical concentrations of cytokines, may increase the risk of clinical chorioamnionitis (Simhan et al. 2003).

**Matrix metalloproteinases**

Matrix metalloproteinases (MMPs) are genetically distinct but structurally related zinc-dependent metallopeptidases which can be classified on the basis of their primary structures and substrate specificities into subgroups: collagenases (MMPs-1,-8–13), gelatinases (MMPs-2,-9), stromelysins (MMPs-3,7–10,-11) and membrane-type MMPs. MMPs can collectively degrade almost all extracellular matrix and basement membrane components, and process serpins, growth factors, pro- and anti-inflammatory cytokines and chemokines, as well as modify apoptotic signals to regulate immune responses. They have been regarded as being important in various tissues with destructive, inflammatory and malignant pathologies. MMPs are secreted in inactive forms and are inhibited by MMP-specific tissue inhibitors. The expression of MMPs is attenuated through the expression of relaxins, integrins and extracellular matrix metalloproteinase inducer (EMMPRIN) (Sorsa et al. 2006, Weiss et al. 2007).

Matrix metalloproteinases are important mediators in the activation of labour at term but they also play an important role in pathological processes leading to PTD as well as in pre-eclampsia and intrauterine growth restriction (Weiss et al. 2007). MMPs are also involved

as proteolytic enzymes in the perinatal complications of prematurity; chronic lung disease, necrotizing enterocolitis, intraventricular haemorrhage, cystic periventricular leukomalacia and retinopathy of prematurity (Cockle et al. 2007).

Matrix metalloproteinases are believed to have two different roles in pregnancy. They are responsible for turnover and degradation of connective tissue proteins in the extracellular matrix (ECM) and they also affect the activity of various cytokines, consistent with their dual role in activation and inactivation of the inflammatory system. During normal gestation MMPs-1,-2,-3,-7 and -9 are found in AF and fetal membranes. MMP-3, MMP-7 and MMP-8 appear to be important in inflammatory processes (Rath et al. 1998, Weiss et al. 2007).

Collagenase 1 (MMP-1) is a physiological constituent of the AF. Parturition, whether term or preterm, has not been shown to be associated with an increase of MMP-1 in the AF. However, premature rupture of membranes (PROM) at term has been shown to be associated with lower concentrations of MMP-1 in the AF. In contrast, PPROM is associated with increased concentrations of MMP-1 (Maymon et al. 2000a, Weiss et al. 2007). Preterm cervical fibroblasts secrete more MMP-1 than term fibroblasts (Dubicke et al. 2008).

The concentration of MMP-3 in AF is constant during pregnancy, but it increases during labour, whether preterm or term, or if infection is present. Spontaneous PROM (term or preterm) has no effect on MMP-3 concentrations (Weiss et al. 2007). In vitro, MMP-3 secretion from preterm cervical fibroblasts is lower compared with term fibroblasts, suggesting that preterm and term cervical fibroblasts may have different phenotypes (Dubicke et al. 2008). MMP-7 in AF increases during gestation, but does not appear to play a major role in normal labour (Weiss et al. 2007). The results of one study indicated MMP-7 expression in fetal membranes (amnion, chorion) and decidua with increasing activity in the amnion in cases with PROM at term, suggesting possible involvement of MMP-7 in PROM (Nishihara et al. 2008).

Gelatinases, both MMP-9 and MMP-2, are detectable in the AF in mid and the third trimester of pregnancy, suggesting that these MMPs have a physiological role in pregnancy (Maymon et al. 2000b). At labour, MMP-2 is dominant in the decidua while MPP-9 is responsible for gelatinolytic activity in membranes by degradation of the basement membrane and other extracellular matrix components (Weiss et al. 2007). The active form of MMP-9 increases in AF at the time of parturition (both term and preterm), while AF MMP-2 decreases at term but not in cases of PTD. The reason for this inverse relationship

between the two gelatinases is not known, but it suggests that these MMPs have different regulatory mechanisms (Maymon et al. 2000b). Maternal serum MMP-9 concentrations rise 24 h before the initiation of labour. Such late prediction is of little value in allowing initiation of preventive steps, but it can aid in understanding the mechanism of PTD (Tu et al. 1998).

Except for MMP-8 and MMP-9, the roles of MMPs in the prediction of delivery have not been studied. Recent findings have indicated that there may be a genetic predisposition to PPROM mediated though polymorphisms of the MMP genes and their promoters (Romero et al. 2002, Crider et al. 2005, Srinivas and Macones 2005).

**Matrix metalloproteinase-8**

Matrix metalloproteinase-8, a human neutrophil collagenase, or collagenase-2, was previously thought to be synthesized as a latent proenzyme only by specific granules of polymorphonuclear leukocytes, neutrophil precursors, during late myeloid maturation (Weiss 1989). It has recently become evident that a wide range of inflammatory, mesenchymal, epithelial and malignant cells can also express MMP-8 (Van Lint and Libert 2006). Furthermore, recent generation of MMP-8-deficient mice has confirmed that MMP-8 is a central mediator in both acute and chronic inflammation (Owen et al. 2004, Gueders et al. 2005, Kuula et al. 2009). MMP-8 is released from the cells on chemotactic stimulation during inflammatory or infectious conditions. Neutrophil degranulation induced by pro-inflammatory or microbial factors is the key step in the regulation of MMP-8 concentrations. MMP-8 is activated by other proteases and oxidants in the extracellular milieu or at cell surfaces. Increased MMP-8 concentrations may reflect either increased synthesis or release (Hanemaaijer et al. 1997, Maymon et al. 2000c, Owen et al. 2004, Sorsa et al. 2006, Van Lint and Libert 2006,).

During pregnancy MMP-8 has been implicated in cervical ripening, rupture of membranes and intra-amniotic infection or inflammation (Maymon et al. 2000c, Maymon et al. 2001b, Sennstrom et al. 2003, Nien et al. 2006, Kim et al. 2007). Cervical ripening is associated with influx of neutrophils. Neutrophils are a source of collagenase, and the cervix is dependent on collagen for its rigidity (Sennstrom et al. 2003). During cervical ripening, increased type I collagen turnover and processing result in a 30% reduction in collagen content compared with the non-pregnant state (Uldbjerg et al. 1983a, Uldbjerg et al. 1983b). Concentrations of MMP-8 correlate closely with cervical ripening and which it is primarily localized in

stromal tissue (Osmers et al. 1995a, Osmers et al. 1995b, Sennstrom et al. 2003, Dubicke et al. 2008). One study indicated concentrations of selected MMPs in the cervical mucus plug at term (Becher et al. 2004). MMP-8 turned out to be the most abundant MMP in the cervical mucus plug, with a mean concentration of 4.8µg/g. The role of MMP-8 in cervical fluid has not been evaluated in the context of PTD.

**Table 4.** MMP-8 concentrations in amniotic fluid

| Study | Year | N | Testing weeks | | Patient characteristics | MMP-8 µg/l | p-value |
|---|---|---|---|---|---|---|---|
| Maymon et al | 1999 | 25 | 15-17* | I | **Intact membranes** | 2.1 (<0.06-32.7) | |
| | | | 20-36 | II | **PTL + intact membranes** | | |
| | | 19 | | II a | PTL+ PTD < 37 wks + MIAC | 208.1 (4.2-14600) | *** |
| | | 34 | | II b | PTL+ term delivery | 3.1 (<0.06-415.1) | ** / **** |
| | | 33 | | II c | PTL+PTD < 37 wks | 32.5 (<0.06-6006.6) | |
| | | | 20-36 | III | **PPROM** | | |
| | | 29 | | III a | PPROM + MIAC | 317.9 (2.16-14500) | ° |
| | | 22 | | III b | PPROM without MIAC | 35.1 (0.71-1184.1) | |
| | | | ≥ 37 | IV | **Term delivery** | | |
| | | 25 | | IV a | In labour | 16.8 (0.33-1650) | °° |
| | | 25 | | IV b | Not in labour | 3.3 (< 0.06-38.6) | °°° |
| | | 20 | ≥ 37 | IV c | Term PROM, Not in labour | 5.6 (0.22-19.8) | |
| Maymon et al | 2001 | 371 | 20-36 | | **Intact membranes** | | |
| | | 200 | | | PTD < 36 wks | 19.5 (<0.06-16600) | <0.001 |
| | | 171 | | | Delivery ≥ 36 wks | 2.1 (<0.06-500.0) | |
| | | 34 | | | Microbial invasion | 605.6 (0.65-15000) | <0.001 |
| | | 337 | | | No microbial invasion | 10.6 (<0.06-16600) | |
| | | 20 | | | Neonatal death | 1048.3 (10.65-16600.0) | |
| | | 351 | | | No neonatal death | 12.5 (<0.06-14750.0) | <0.001 |
| Maymon el al | 2001 | 101 | 24-36 | | **PPROM** | | |
| | | 37 | | | Adverse neonatal outcome | 54.4 (0.82-14500) | <0.05 |
| | | 64 | | | No adverse neonatal outcome | 28.9 (0.78-2451.8) | |
| | | 46 | | | Microbial incasion | 143.8 (1.83-14500) | 0.003 |
| | | 55 | | | No microbial incasion | 23.2 (0.28-6006.7) | |
| Yoon et al | 2001 | 114 | 16-23.3 | | **Intact membranes** | | |
| | | 19 | | | PTD < 32 wks | 3.1 (0.3-1954.9) | <0.01 |
| | | 95 | | | Delivery > 37 wks | 1.3 (<0.3-45.2) | |

PTL = Preterm labour
PTD= Preterm delivery
MIAC= Microbial inavasion of the amniotic cavity
* All delivered at term
** II b /II c, p< 0.05
*** II a / II c, p <0.001
**** II b / II c, p =0.003
° III a /III b, p< 0.01
°° IV a / IV b, p= 0.06
°°° IV b /IV c, p= 0.9

Matrix metalloproteinase-8 in AF has been studied extensively in recent years. Its concentrations are elevated in AF in women who experience spontaneous PPROM, microbial invasion of the amniotic cavity (MIAC), and spontaneous PTL, suggesting that increased AF-specific MMP-8 concentrations could be used to identify women who are at risk of PTD and adverse neonatal outcome (Maymon et al. 2000c, Angus et al. 2001). In addition, correlations between MMP-8 and leukocytes in AF as well as between MMP-8 and MMP-9 have been found (Maymon et al. 2000c). Amniotic fluid MMP-8 concentrations are elevated not only in women with intra-amniotic infection and intact membranes, but also in those who deliver preterm with negative AF cultures (Table 4) (Maymon et al. 2001a, Nien et al. 2006). Elevated MMP-8 concentrations ($\geq$ 23–30 μg/l) are also associated with adverse neonatal outcome after adjustment for gestational age at birth and other confounding variables, including microbial invasion in the AF (Maymon et al. 2001a, Maymon et al. 2001b, Nien et al. 2006, Kim et al. 2007). In addition, recent evidence demonstrates that patients with intra-amniotic inflammation but negative microbiological culture results have outcomes similar to those among patients with positive culture results, which may reflect adverse effects of the fetal inflammatory response (Maymon et al. 2001b, Yoon et al. 2001, Shim et al. 2004). Therefore, it has been thought that in patient management, detection of signs of inflammation in the AF may be more practical than the detection of infection (Shim et al. 2004). Moymon et al. have demonstrated that a combination of three tests, white blood cell count (> 30 cells/mm³), Gram staining and assay of AF MMP-8 (> 30 μg/l), represents the most sensitive method for prediction of a positive AF culture in patients with PTD and intact membranes (Maymon et al. 2001a). In cases with PPROM the combination of white blood cell count (> 30 cells/mm³), assay of interleukin-6 (> 17 μg/l), Gram staining and assay of MMP-8( > 30 μg/l) showed the highest performance in the prediction of PTD (sensitivity 88.4%, specificity 48.4%, PPV 63.3% and NPV 80.8%) (Maymon et al. 2001b). In these two studies a concentration of MMP-8 < 30 μg/l alone had the highest negative predictive value (97.7% and 75.6% respectively) for microbial invasio of the amniotic cavity (Table 5) (Maymon et al. 2001a, Maymon et al. 2001b).

**Table 5.** Test performance and odds ratios of selected biomarkers of intra-amniotic infection, inflammation and preterm delivery

| Study | Year | N | Testing weeks | Membranes status | Outcome | Test results | Sensiv | Spesif | PPV | NPV | LR+ | LR- |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Maymon et al | 2001 | 371 | 20-36 | Intact | | | | | | | | |
| | | 34 | | | MIAC | | | | | | | |
| | | | | | | MMP-8 > 30 µg/l | 82.4 | 78.0 | 36.0 | 97.7 | 3.7 | 0.2 |
| | | | | | | Gram stain | 32.4 | 97.6 | 57.9 | 93.3 | 13.5 | 0.7 |
| | | | | | | WBC > 30 cells/mm³ | 52.9 | 91.1 | 38.3 | 94.9 | 5.9 | 0.5 |
| | | | | | | IL-6 > 17 µg/l | 62.0 | 90.0 | 30.5 | 94.3 | 6.2 | 0.4 |
| Maymon et al | 2001 | 101 | 24-36 | Ruptured | | | | | | | | |
| | | 46 | | | MIAC | | | | | | | |
| | | | | | | MMP-8 > 30 µg/l | 76.1 | 61.8 | 62.0 | 75.6 | 2.0 | 0.4 |
| | | | | | | WBC > 30 cells/mm³ | 44.4 | 83.6 | 69.0 | 64.8 | 2.7 | 0.7 |
| | | | | | | IL-6 > 17 µg/l | 40.6 | 92.3 | 81.3 | 65.5 | 5.3 | 0.6 |
| | | 37 | | | ANO | | | | | | | |
| | | | | | | MMP-8 > 30 µg/l | 65.8 | 50.8 | 44.6 | 71.1 | 1.3 | 0.7 |
| | | | | | | IL-6 > 17 µg/l | 34.6 | 84.4 | 56.3 | 69.1 | 2.2 | 0.8 |
| | | | | | | WBC > 30 cells/mm³ | 34.2 | 74.2 | 44.8 | 64.8 | 1.3 | 0.9 |
| Yoon et al | 2001 | 114 | 17-22.3 | Intact | PTD < 32 wks | | | | | | | |
| | | 19 | | | | MMP-8 > 23 µg/l * | 42.0 | 99.0 | 88.9 | 89.5 | 42.0 | 0.6 |
| | | | | | | IL-6 > 0.6 µg/l ** | 42.0 | 92.0 | 50.0 | 88.8 | 5.3 | 0.6 |
| | | | | | | Angiogenin > 13.85 µg/l *** | 37.0 | 87.0 | 36.8 | 87.4 | 2.8 | 0.7 |
| Nien et al. | 2006 | 327 | 22-35 | Intact | | | | | | | | |
| | | | | | PTD | | | | | | | |
| | | 38 | | | < 48h | MMP-8 > 23 µg/l | 61 | 97.00 | 70.0 | 95.0 | 20.3 | 0.4 |
| | | 66 | | | <7 d | | 47 | 99.0 | 94.0 | 88.0 | 47.0 | 0.5 |
| | | 80 | | | <14 d | | 39.0 | 99.0 | 94.0 | 83.0 | 39.0 | 0.6 |
| | | 32 | | | < 32 wks | | 56.0 | 98.0 | 86.0 | 89.0 | 28.0 | 0.5 |
| | | 61 | | | < 34 wks | | 44.0 | 98.0 | 87.0 | 86.0 | 22.0 | 0.6 |
| | | 24 | | | MIAC | | 83.0 | 95.0 | 56.0 | 99.0 | 16.6 | 0.2 |
| | | 38 | | | IAI IL- 6 > 2.6 µg/l | | 84.0 | 99.0 | 89.0 | 98.0 | 84.0 | 0.2 |
| Lee et al | 2008 | 155 | | Variable | | | | | | | | |
| | | 29 | | | MIAC | | | | | | | |
| | | | | | | Positive MMP-8 test ( ≥ 10ng/ml) [a] | 86.2 | 74.6 | 43.9 | 95.9 | 3.4 | 0.2 |
| | | | | | | WBC >19cells/mm³ [aa] | 75.9 | 80.2 | 46.8 | 93.5 | 3.8 | 0.3 |
| | | | | | | Glugose < 17mg/dl [aaa] | 58.6 | 76.2 | 36.2 | 88.9 | 2.5 | 0.5 |
| Kim et al | 2007 | 141 | 20.4-34.9 | Ruptured | | | | | | | | |
| | | 60 | | | IAI / I IL-6 > 2.6 µg/l | Positive MMP-8 test ( ≥ 10µg/l) | 90.0 | 80.2 | 77.1 | 91.5 | 4.6 | 0.1 |
| | | 25 | | | IA infection | | 92.0 | 59.5 | 32.9 | 97.2 | 2.3 | 0.1 |

MIAC= Microbial invasio of the amniotic cavity
MMP-8 = Matrix metalloproteinase 8
WBC= White blood cells
IL = interleukin
PTD= Preterm delivery
IAI = intraamniotic inflammation without microbial invasion
ANO = Adverse neonatal outcome
IA = intraamniotic

* OR 68.4 (95% CI 7.8-599.0)
** OR 7.9 (95% CI 2.5- 25.3)
*** OR 4.0 (95% CI 1.3-12.3)

[a] OR 18.4 (95% CI 5.9-56.8)
[aa] OR 12.7 (95% CI 4.9-33.0)
[aaa] OR 4.5 (95% CI 1.9-10.6)

Recently, a bedside test has been developed for the diagnosis of intra-amniotic inflammation based on the detection of elevated concentrations of MMP-8 in AF (SK Pharma Co. Ltd, Kyunggi-do, Korea) (Nien et al. 2006). The prevalence of a positive MMP-8 test result ( ≥ 23 µg/l) in AF among women with PPROM has been found to be 50%, but it was only 11% in women with PTL and intact membranes (Nien et al. 2006). This rapid bedside test for MMP-8 has a sensitivity of 56% and a specificity of 98% for predicting PTD at < 32 weeks of gestation. The test allows identification of patients with intra-amniotic infection with a sensitivity of 83% and a specificity of 95% and identification of inflammation

with a sensitivity of 84% and a specificity of 99% among patients with spontaneous PTL and intact membranes (Nien et al. 2006). Lee et al. showed that the MMP-8 rapid test is more sensitive (86.2%), with a high negative predictive value (95.9%) in the diagnosis of microbial invasion of the amniotic cavity than the commonly used glucose test and white blood cell count in AF (Lee et al. 2008b). Moreover, an elevated MMP-8 concentration in AF with or without microbial invasion is associated with a shorter amniocentesis–delivery interval in patients with intact membranes or with PPROM (Maymon et al. 2001a, Maymon et al. 2001b, Shim et al. 2004, Kim et al. 2007, Kim et al. 2007).

Biggio et al. analysed AF MMP-8 at the time of genetic amniocentesis between 14 to 21 weeks of gestation. An elevated level of MMP-8 (above the 90th percentile) was highly associated with subsequent PPROM compared with normal term delivery, suggesting that the physiological processes that contribute to PPROM may begin in early pregnancy (Biggio et al. 2005).

**Other biomarkers**

Several biomarker have been studied for prediction of PTD, but none of them are in clincal use for that purpose.

Prolactin is produced by the decidua, maternal adenohypophysis and fetal pituitary during pregnancy. Tissue disruption in the choriodecidual space, mechanical or due to infection or inflammation, may lead to leakage of prolactin into the cervix and vagina (Honest et al. 2009). Threshold values for prolactin vary, making it difficult to compare data. In two studies among symptomatic women the threshold was 2.0ng/ml in cervicovaginal secretion. The positive predictive value was 36–80% and the negative predictive value 65–94% for prediction of PTD at < 34 weeks of gestation (Jotterand et al. 1997, O'Brien et al. 1994).

Pregnancy-associated plasma protein-A (PAPP-A) is a glycoprotein which is secreted from the trophoblastic cells of the placenta. It is generally assayed in conjunction with ultrasonographic measurement of nuchal translucency (NT) and assay of free β-human chorionic gonadotrophin (β-hCG) as part of screening programmes for trisomy 21 and other forms of aneuploidy in the first trimester (Dugoff et al. 2004, Malone et al. 2005). Low concentrations of maternal serum PAPP-A are associated, in the absence of an abnormal karyotype, with an increased (twofold) risk of PTD (Smith et al. 2006, Spencer et al. 2008)

and when there was a low level of PAPP-A together with a high level of alpha fetoprotein the risk increased to 9.9-fold (95% CI 4.4–22.0)(Smith et al. 2006). A similar predictive value has not been found by using a combination PAPP-A and C-reactive protein (CRP) in maternal serum or AF (Ozer et al. 2005). Low maternal serum PAPP-A concentrations during the first trimester may reflect a trophoblast invasion defect at the maternal-fetal interface, resulting in subsequent PTD, particularly in cases with PPROM (She et al. 2007).

Corticotrophin-releasing hormone (CRH) is a principal regulator of the hypothalamic-pituitary-adrenal (HPA) axis. During pregnancy CRH is produced in the syncytiotrophoblast, decidua and fetal membranes and secreted into the maternal circulation. CRH drives the pituitary-adrenal axis to produce increased amounts of cortisol during the latter half of normal pregnancy, so that its concentrations increase more than 20-fold during the 5 weeks before delivery (Campbell et al. 1987, Hillhouse and Grammatopoulos 2002, Linton et al. 1993). Elevated plasma CRH concentrations as early as at 18 to 20 weeks of gestation are causally related to PTD, reflecting exposure to stress, hypoxaemia or inflammation (Hobel et al. 1999, Majzoub and Karalis 1999, Ellis et al. 2002). CRH is difficult to quantify, with large inter-individual variation. For that reason it is not in clinical use as a single marker of PTD (Vogel et al. 2005).

Alpha- fetoprotein (AFP) in maternal circulation during pregnancy is believed to be mainly of fetal origin. Elevated concentrations of AFP are commonly associated with structural fetal anomalies including congenital nephrosis and neural tube and abdominal defects (Chandra et al. 2003). Elevated concentrations of maternal serum AFP ($\geq$ 2 MOM) at 14–24 weeks of gestation among asymptomatic women (without fetal neural tube defects) have been associated with PTD, with a 3- to 5-fold risk of delivery at less than 37 weeks (Heinonen et al. 1996, Tikkanen et al. 2007, Yuan et al. 2009). AFP is not useful as a single marker, but it is in the context of other markers of abnormal pregnancy (Vogel et al. 2005, Yuan et al. 2009).

Beta human chorionic gonadotrophin (β-hCG), produced by the fetal-placental unit, is present in maternal serum and AF, but concentrations in AF are much lower than in maternal serum during pregnancy. Commonly, serum concentrations are measured in the second trimester to screen for chromosomal abnormalities (Dugoff et al. 2005). In cases of normal chromosomes, elevated β-hCG may be associated with adverse pregnancy outcomes, such as early fetal loss, PTD, and pre-eclampsia. In addition to AF and maternal serum, β-hCG is measurable in cervicovaginal secretion, where it leaks after disruption

in the choriodecidual space (Honest et al. 2009). After exclusion of fetal chromosomal abnormalities and pregnancy complications such as pre-eclampsia, placental abruption and rupture of fetal membranes, elevated levels of cervical β-hCG in symptomatic women predict spontaneous PTD (Guvenal et al. 2001, Sanchez-Ramos et al. 2003, Gurbuz et al. 2004). The β-hCG cut-off level in maternal serum for adverse perinatal outcome is usually ≥ 2 MOM, but the value as regards predicting PTD is lower in asymptomatic women (Morssink et al. 1998, Spencer 2000, Yaron et al. 2002, Chandra et al. 2003, Tikkanen et al. 2007, Spencer et al. 2008). Measurement of β-hCG in any fluid cannot be used as a single marker of PTD, but it can perhaps be used in combination with other markers.

Relaxin in human pregnancy is both a systemic hormone from the corpus luteum and an autocrine/paracrine hormone at the maternal-fetal interface formed by the decidua, placenta and fetal membranes. Measurement of maternal serum relaxin in the first half of pregnancy among asymptomatic women can predict spontaneous PTD (1.5-fold increase) (Weiss et al. 1993, Vogel et al. 2002, Vogel et al. 2006). In one study among symptomatic women serum relaxin was shown to predict PTL and spontaneous PTD (Vogel et al. 2002). However, relaxin has not turned out to be a clinically useful predictor of PTD.

C-reactive protein (CRP), produced by hepatocytes, is an acute-phase reactant associated with the presence of systemic infections and may indicate a risk of spontaneous PTD. It is an easily detectable and reliable marker. CRP concentrations in serum in the first trimester, including highly sensitive CRP, has not been found to increase the estimated risk of PTD (Karinen et al. 2005, Tikkanen et al. 2008). Among symptomatic women, CRP concentrations in serum have been found to have low sensitivity (38%) but high specificity (94%) in predicting PTD at < 34 weeks of gestation (Foulon et al. 1995).

Thrombin is a coagulation factor which is a multifunctional protease capable of inducing myometrial contractions. This enzyme has been implicated in PTL. A second trimester plasma thrombin-antithrombin concentration of > 3.9 μg/l predicts subsequent PPROM, with a sensitivity of 88% and specificity of 68%. Thrombin may become a new predictor of adverse pregnancy outcome (Rosen et al. 2001, Chaiworapongsa et al. 2002). So far, thrombin has not been used in a clinical context for prediction of PTD.

# AIMS OF THE STUDY

The present study was undertaken to analyse whether or not the biochemical markers phIGFBP-1 and MMP-8, measured in vaginal and cervical fluid in early and mid-pregnancy, can be used to identify women at high risk of subsequent spontaneous PTD.

The specific aims were to:

1. Measure the concentrations of phIGFBP-1 in vaginal and cervical fluid, and examine factors which may affect the concentrations of this biomarker in the lower genital tract during the first and mid-second trimester of pregnancy

2. Evaluate whether elevated cervical fluid phIGFBP-1 concentrations in asymptomatic women in the first or mid-second trimester are associated with subsequent spontaneous PTD

3. Assess the accuracy of ultrasonographic cervical length measurement and the rapid cervical phIGFBP-1 test separately and in combination in the prediction of PTD among women with self-reported uterine contractions in later pregnancy

4. Measure MMP-8 concentrations in vaginal and cervical fluid, and examine factors that affect MMP-8 concentrations in the lower genital tract during the first and mid-second trimester of pregnancy

5. Evaluate whether cervical fluid MMP-8 concentrations in asymptomatic women in the first and second trimester are associated with subsequent spontaneous PTD

# SUBJECTS AND METHODS

## Subjects

The studies were conducted with the approval of the Ethics Committee of the Department of Obstetrics and Gynaecology, Helsinki University Hospital (Dnro 49/E8/05). The Department of Obstetrics and Gynaecology includes three different hospitals (the Maternity Hospital, the Women's Clinic and Jorvi Hospital) and each of them has a Maternity Clinic. The study population was enrolled and data collected between April 2005 and December 2006. Overall information concerning the prospective study was mailed to 15,641 consecutive pregnant women who had registered for routine ultrasonographic screening as part of routine antenatal care in the first trimester (between 12+0 and 13+6 weeks of gestation, based on the last menstrual period) and in the second trimester (between 18+0 and 20+6 weeks of gestation). Gestational age was confirmed or corrected at the first ultrasonographic examination and used accordingly at the second screening. A total of 5180 (33.0 %) volunteers participated in the study and signed an informed consent document (Figure 7). Subjects and outcomes of each study are shown in table 6. In every study all women had intact fetal membranes, based on history and speculum examination and none had vaginal bleeding at the time of examination.

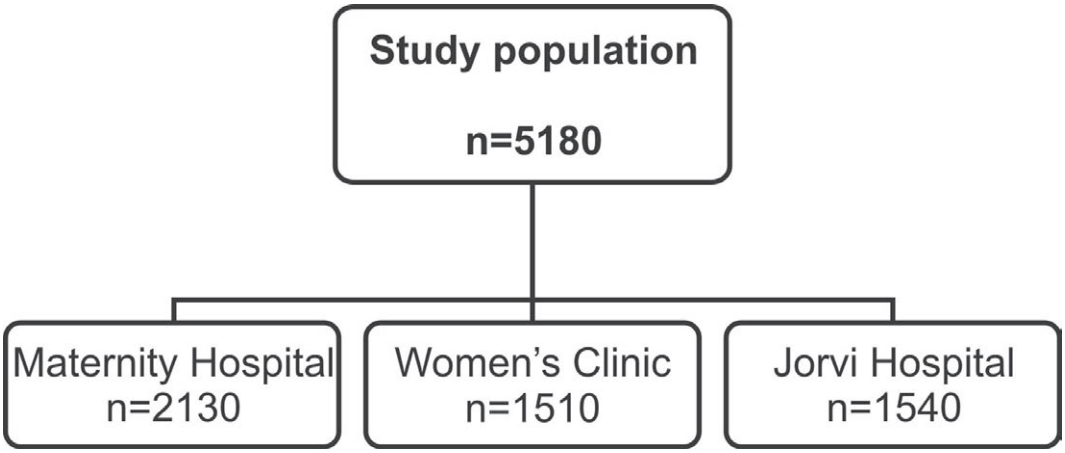


Figure 7. Study population

**Table 6.** Subjects and outcomes in each study

| Study | Year | Stage of pregnancy | Subjets | Outcomes | PTD (%) | Term delivery |
|---|---|---|---|---|---|---|
| I | 2005-2006 | First trimester | 1690 | | | |
| | | Second trimester | 1607 | | | |
| II | 2005-2006 | First trimester | 4984 | < 32 wks | 26 (0.5) | 4958 |
| | | | 4984 | < 37 wks | 189 (3.8) | 4795 |
| | | Second trimester | 4630 | < 32 wks | 21 (0.5) | 4609 |
| | | | 4630 | < 37 wks | 167 (3.6 | 4463 |
| III | 2005-2006 | 22-34 wks | 246 | ≤ 34wks | 10 (4.1) | 236 |
| IV | 2005-2006 | First trimester | 1979 | | | |
| | | Second trimester | 1950 | | | |
| V | 2005-2006 | First trimester | 4855 | < 37 wks | 184 (3.9) | 4671 |
| | | Second trimester | 4671 | < 37 wks | 164 (3.5) | 4407 |

**Study I**

Study I dealt with phIGFBP-1. It included participants from the Maternity Hospital. The study population is shown in figure 8.

The outcome measures were phIGFBP-1 concentrations in the vaginal and cervical fluids separately and the associations between phIGFBP-1 concentrations of ≥ 10 µg/l and selected factors. The selected factors were age, parity, gestational age at examination time, vaginal pH, cervical length, history of sexual intercourse less than 48 hours before the examination, history of vaginal bleeding during the current pregnancy, history of the use of antibiotics during the current pregnancy and the type of pregnancy (single or abnormal). Women with multiple gestation were excluded.

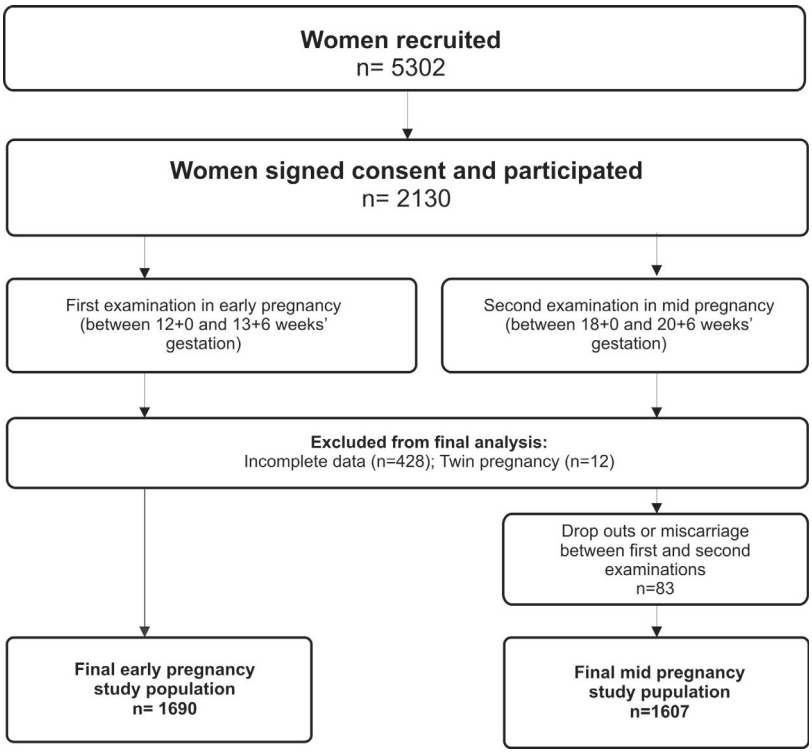


Figure 8. Study I population

**Study II and Study V**

Study II dealt with phIGFBP-1 and Study V with MMP-8. A total of 5180 pregnant women participated in Study II and V. The study populations are shown in figure 9. The outcome measure was spontaneous PTD at < 32 weeks of gestation and at < 37 weeks of gestation in

Study II and spontaneous PTD at < 37 weeks of gestation in Study V (Table 6). Spontaneous PTD was defined as PTD after the spontaneous onset of contractions or PPROM, regardless of whether the delivery was vaginal or by Caesarean section, or in the case of membrane rupture, induced.

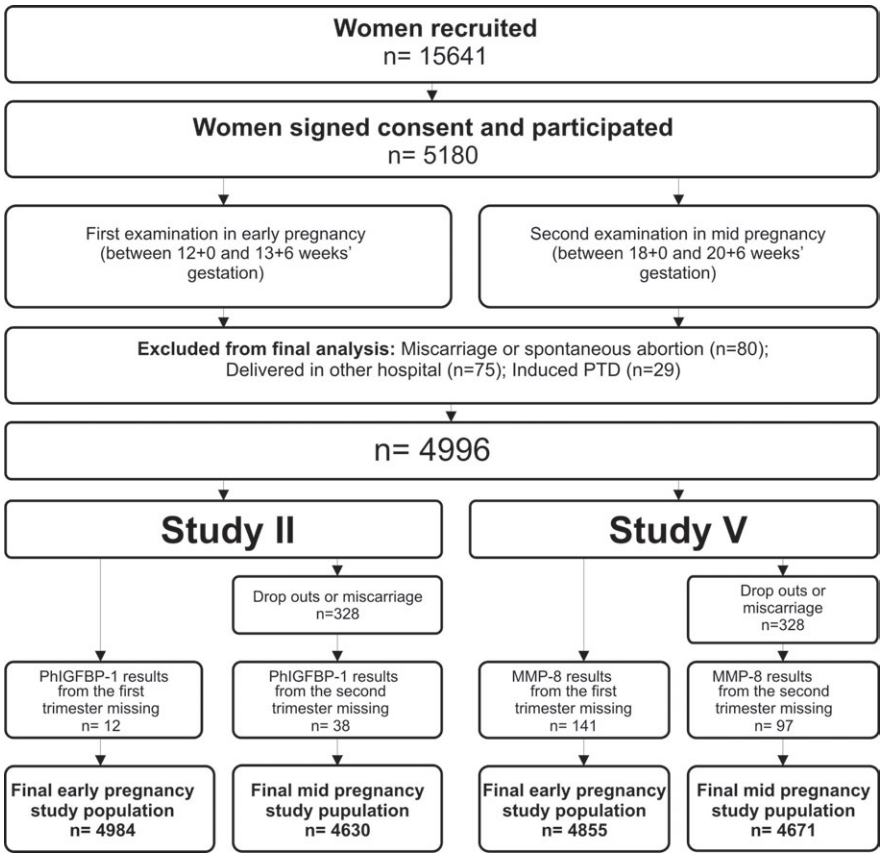


Figure 9. Study II and V populations

**Study III**

The study group consisted of a total of 249 women with singleton pregnancies who presented at an emergency antenatal clinic with self-reported uterine contractions and intact membranes at any time between 22 and 34 weeks of gestation. The study population was collected from all three participating hospitals of the Department of Obstetrics and Gynaecology. The presence of uterine contractions was confirmed by external tocography, but no strict definition for the frequency or intensity of the contractions was set. Other inclusion criteria were a) no known major fetal anomalies, b) no vaginal bleeding at

presentation, c) no placenta praevia, and d) signed informed consent. Three patients were excluded because they delivered prematurely as a result of severe pre-eclampsia. Thus, the final study sample included 246 women (Table 6). All women had intact fetal membranes, based on history and speculum examination.

The outcome measures were spontaneous PTD (after spontaneous onset of contractions or spontaneous rupture of the membranes, regardless of whether delivery was vaginal or by Caesarean section) at ≤ 34 weeks of gestation and delivery within 14 days of examination. The median time interval from initial evaluation to delivery was also calculated.

**Study IV**

Study IV dealt with MMP-8. The study included all participants in the Maternity Hospital. The study population is shown in figure 10. The outcome measures were the MMP-8 concentrations in vaginal and cervical fluids and the associations between MMP-8 and selected factors. The selected factors were age, gravidity, parity, cervical length, type of pregnancy (single, twin or abnormal), gestational age at examination time, bacterial vaginosis, vaginal leukocytosis and candidiasis.

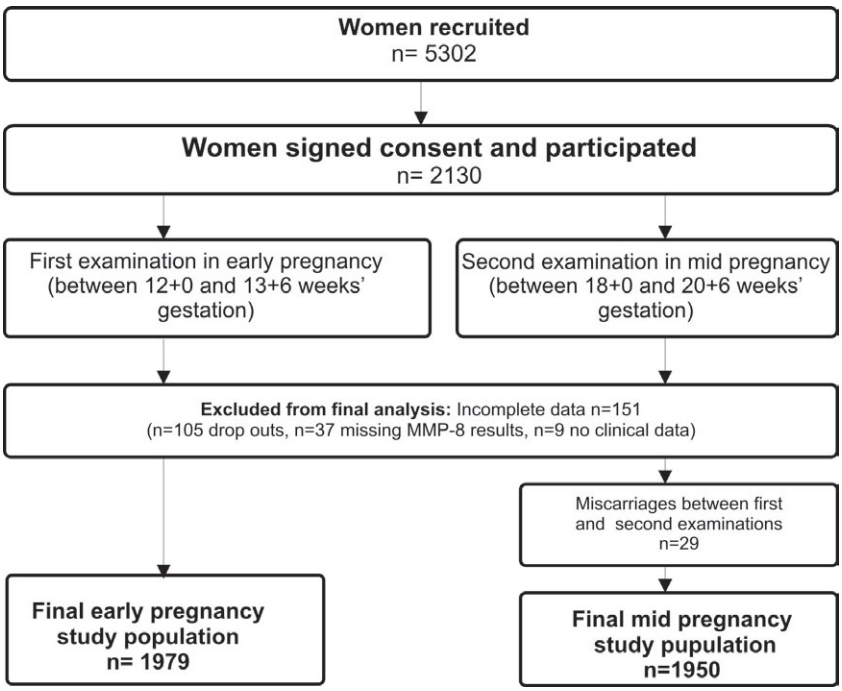


Figure 10. Study IV population

# Methods

## Collection of clinical data

Relevant clinical data were recorded from hospital charts and by interviewing. The same person interviewed and examined all participants in Studies I and IV. In Studies II and V ultrasonographic screening was carried out by midwives who also interviewed and examined all study participants. Before the first ultrasonographic screening, between 12+0 and 13+6 weeks of gestation, gestational age was assessed on the basis of the time of the last menstrual period. Final gestational age was confirmed at the first ultrasonographic examination and used accordingly at the second screening, between 18+0 and 20+6 weeks of gestation.

The type of pregnancy was diagnosed by ultrasonography at the first screening. Types were recorded as single, multiple and abnormal pregnancies. Women with multiple gestations were included in Studies II, IV and V.

In Studies I, II, IV and V a history of obvious vaginal bleeding before the first examination or between the first and the second examination, use of oral antibiotics during the last four weeks and sexual intercourse during the last 48 hours before examination were asked about and recorded. The physician who examined the patient in the emergency obstetric clinic in Study III took the study samples and asked about a history of previous bleeding, but information on sexual intercourse was asked about via questions enclosed in envelopes.

Measurement of pH in vaginal smears (Study I) was performed by using indicator sticks (Macherey-Nagel Gmb&Co, phFIX 3.6-6.1) at both screenings. For statistical analysis, the pH readings were divided into categories: $< 4.7$ and $\geq 4.7$.

Cervical length (Studies I, III, IV) was measured by using a Hitachi EUB-5500 ultrasound machine (EUP-V53W) (Hitachi Medical Corp., Tokyo, Japan) equipped with a 5–9 MHz transvaginal probe, which was inserted into the vagina and placed in the anterior fornix after the woman had emptied her bladder. Cervical length was measured as the length of the cervical canal from the external os to the internal os in a sagittal plane view. Three measurements were performed and the shortest measurement associated with the best

image was recorded as the length of the cervix. Cervical lengths of < 25 mm (Study III) and < 30 mm (Studies I, IV) were used to define a short cervix. The presence or absence of funnelling, defined as a protrusion of the amniotic membranes into the cervical canal, was considered. In the first study a print was taken at each measurement and the technically best, shortest distance was recorded. All scans were carried out by one midwife (Study I and IV), and the first 100 cervical measurements were performed under the guidance of a physician. Intra-observer variation was found to be 4–10%. In addition, an experienced sonographer checked the first 150 prints to confirm the validity of the imaging and measurement technique. In Study III, the physicians at the emergency obstetric clinic examined the patient and measured cervical length.

Deliveries before 32 and 37 completed gestational weeks were defined as preterm in Study II. In Study V we used 37 gestational weeks as a limit for PTD. In Study III the outcome measure was delivery at ≤ 34 weeks, which is the upper limit for tocolysis in our hospital, and within 14 days of examination. Spontaneous PTD was defined as delivery preceded by spontaneous onset of uterine contractions with intact membranes, or spontaneous PPROM in the absence of contractions, regardless of whether the delivery was vaginal or by Caesarean section, or in the case of membrane rupture, induced. Iatrogenic preterm deliveries induced by physicians for maternal or fetal indications were excluded.

Body mass index (kg/m²), smoking status and history of previous PTD and in vitro fertilisation (IVF) (Studies II and V) were obtained from the forms filled for each pregnancy by the National Institute for Health and Welfare, Finland. BMI > 30 kg/m² was used as a cut-off for a markedly overweight condition. All women who smoked in the first and second trimester were defined as smokers. Women who had been subjects in assisted reproductive technology were placed in the IVF category. Assisted conception referred to women who had undergone ovulation induction, intrauterine insemination, standard in vitro fertilisation or intracytoplasmic sperm injection. Women of ≥ 37 years (Study V) were defined as “old” when pregnant.

Information regarding the current pregnancy, such as use of corticosteroids, tocolysis, hospital admission and puerperal infections during the hospital stay were obtained from the patient’s hospital records (Study III). The examining physician made the decision concerning admission and management based on the patient’s obstetric history, presence of other risk factors, symptoms and clinical findings, including ultrasonographic findings. Tocolytic agents (intramuscular or intravenous β-sympathomimetic agents, oral calcium-

channel blockers or an intravenous oxytocin receptor antagonist) were given when clinically indicated. Corticosteroids were given for fetal lung maturation. Puerperal infection was defined by standard clinical and laboratory criteria, including fever of > 38 °C, tenderness of the uterus and purulent discharge, elevated C-reactive protein concentration and leukocyte count. Cultures for Chlamydia trachomatis and Streptococcus agalactiae were performed when clinically indicated (Study III) and recorded.

Data on pregnancy complications and delivery were collected from the patients' hospital records (Studies I–V).

**Samples and assays**

**Immunoenzymometric assay (IEMA) of phIGFBP-1 (I, II, III)**

Swab samples of cervical and vaginal fluids for phIGFBP-1 measurement were obtained before any other procedure. One swab was kept in the external part of the cervix, and one in the side- or posterior fornix of the vagina simultaneously for about 20 seconds. Thereafter, each swab was placed in a test tube containing 0.5 ml of extraction buffer and it was rinsed in the buffer for 10–15 seconds. The polyester swabs absorb approximately 150 µl of fluid when saturated, and the average dilution of the vaginal/cervical sample in the buffer is approximately 1:5. The specimens were frozen and stored at -20 °C until phIGFBP-1 concentrations were measured by immunoenzymometric assay, using monoclonal antibody 6303 as the detecting antibody (Medix Biochemica, Kauniainen, Finland). This antibody detects all phosphorylated isoforms, including the highly phosphorylated isoform, which is produced by the decidua and is not present in AF (Martina et al. 1997, Rutanen 2000). The detection limit of the assay was 0.3µg/l. All samples were measured in duplicate. When exploring factors that may influence phIGFBP-1 concentrations in the vagina and cervix, a concentration of phIGFBP-1 $\geq$ 10 µg/l (Studies I and II) was considered elevated, as in previous studies (Nuutila et al. 1999, Kekki et al. 2001). A cut-off of 10µg/l is also the detection limit of the bedside test (Actim Partus test, Medix Biochemica, Kauniainen, Finland). The rapid bedside test of phIGFBP-1 was used in Study III. The laboratory staff were blinded to the clinical status of the patients, and the phIGFBP-1 test results were not available to the physician.

**Immunofluorometric assay (IFMA) of MMP-8 (IV, V)**

For MMP-8 measurement, one swab was kept in the external part of the cervix and one in the posterior vaginal fornix for about 20 seconds. Thereafter, each swab was placed in a test tube containing 0.5 ml of extraction buffer and rinsed in the buffer for 10–15 seconds. The polyester swabs absorb approximately 150 µl of fluid when saturated, and the average dilution of the vaginal/cervical sample in the buffer is approximately 1:5. The specimens were frozen and stored at -20 °C until MMP-8 concentrations were measured by time-resolved immunofluorometric assay (IFMA) in a single laboratory (Wallac,Turku, Finland) (Hanemaaijer et al. 1997, Tuomainen et al. 2007). This assay detects pro- and active MMP-8 as well as MMP-8 complexed with tissue inhibitors or α-2-macroglobulin. Vaginal/cervical samples were further diluted to 1:10 prior to assay. First, standards and samples diluted in DELFIA® buffer were incubated in anti-MMP-8-coated wells of a plate for 1 hour. After washing, Eu-labelled anti-MMP-8 antibody diluted in DELFIA buffer was added to the wells and the plate was incubated for 1 hour. After washing again, DELFIA Enhancement solution was added and fluorescence was measured using VICTOR™ 2D equipment. The sensitivity of the assay was at least 0.08µg/l.

**Bacterial vaginosis (III, IV)**

Vaginal smears for detection of BV and leukocyte counts were obtained at speculum examinations. A large cotton-tipped swab, moistened with distilled water, was placed in the posterior fornix and swirled for 5 seconds. A thin smear of the secretion was applied to a dry slide and allowed to dry. The slides were processed by using Gram stain and read by LR (Study III) or a laboratory technician (Study IV). Both were blinded to any patient information at the time of reading. The presence of BV was determined on the basis of a Nugent Gram stain score of 7–10 (Nugent et al. 1991).

**Leukocyte counts (IV)**

Leukocytes counts from vaginal smears or dry slides were assessed in a semi-quantitative manner (0 = none, 1+ = few, 2++ = moderate, 3+++ = heavy). Groups 1–3 were markedly positive and 0 was markedly negative.

**Statistical analysis**

All data were analysed by using Microsoft's Statistical Package for the Social Sciences (SPSS) for Windows, version 15.0 (I, II) and version 16.0 (III, IV and V). Because the phIGFBP-1 (I, II) and MMP-8 data (IV, V) did not follow normal distributions, not even after logarithmic transformation, nonparametric tests were used. A p-value less than 0.05 was considered significant.

**Study I**

Paired comparisons of continuous variables were carried out by using the Wilcoxon signed ranks test and unpaired comparisons with the Mann–Whitney test. Comparisons of categorial data were carried out by using the Chi-Square test and McNemar's test when appropriate. Multivariate analysis of factors affecting cervical phIGFBP-1 concentrations ($< 10$ μg/l = 0, $\geq 10$ μg/l = 1) was performed by multivariate logistic regression. All studied covariates were categorial and were entered in the model in one step. Adjusted ORs are given, with 95% confidence intervals (95% CIs).

**Study II**

Unpaired comparisons were analysed by using the Mann–Whitney test. Concentrations of phIGFBP-1 of $\geq$ 10μg/l and $<$ 10 μg/l were categorial variables and compared by the $\chi^2$ test, and Fisher's exact test when appropriate. Logistic regression was used for multivariate analysis with parity, twin pregnancy, IVF, history of PTD, smoking, BMI, history of vaginal bleeding and phIGFBP-1 concentrations $\geq$10 μg/l as confounding factors for prediction of PTD at $<$ 34 and $<$ 37 weeks of gestation. It was also used to evaluate the possible contribution of previous vaginal bleeding and nulliparity, factors shown to affect cervical fluid phIGFBP-1 concentrations. Receiver operating characteristic (ROC) curves were constructed to evaluate the diagnostic performance of cervical fluid phIGFBP-1 concentrations in prediction of PTD.

**Study III**

Categorial variables were compared by Fisher's exact test and continuous data with the Mann–Whitney test. Logistic regression was used for multivariate analysis (preterm birth at $\leq$ 34 weeks and delivery within 14 days as dependent factors, and cervical length, phIGFBP-1 test result and history of previous preterm birth as independent factors). Likelihood ratios for both outcomes were calculated for each factor.

**Study IV**

Paired comparisons of continuous variables were performed by using the Wilcoxon signed-ranks test and unpaired comparisons with the Mann–Whitney test (two groups) or the Kruskal–Wallis test (more than two groups). Correlations were calculated by using Spearman's rank correlation coefficient test.

**Study V**

Unpaired comparisons of continuous variables were analysed by using the Mann–Whitney test. Categorial variables were compared by using the $\chi^2$ test. Correlations between early and mid-pregnancy MMP-8 concentrations were examined by using Pearson's correlation coefficient. Multiple logistic regression was carried out, with maternal age, parity, twin pregnancy, IVF, history of PTD, smoking, BMI > 30 kg/m², sexual intercourse < 48 hours earlier, history of vaginal bleeding during the current pregnancy and > the 90th percentile and < the median of MMP-8 concentrations taken into account to control for possible confounding factors and to calculate adjusted ORs and their 95% confidence intervals for the risk of PTD initiated by PPROM < 37 weeks of gestation.

# RESULTS

### PhIGFBP-1 concentrations in the first and mid-second trimester (I, II)

The characteristics of the study I population are shown in Table 7. Data concerning first trimester sampling and mid-second trimester sampling were analysed separately in study I and II.

**Table 7.** Characteristics of the Study I population

| | First trimester | Second trimester |
|---|---|---|
| Participants | 1690 | 1607 |
| Drop outs | | 83 (4.9) |
| Maternal age, yrs | 29.8 (4.6) | 29.7 (4.6) |
| Parity | | |
| Nullipara | 912 (54.0) | 874 (54.4) |
| Primigravida | 687 (75.3) | 657 (75.2) |
| Non-primigravida | 225 (24.7) | 217 (24.8) |
| Multipara | 778 (46.0) | 733 (45.6) |
| Gestational age, wks | 12.8 (0.6) | 19.2 (0.8) |
| pH ≥ 4.7 | 164 (9.6) | 110 (6.8) |
| Cervical length < 30 mm | 11 (0.7) | 6 (0.4) |
| History of sexual intercourse (<48h) | 362 (21.4) | 329 (20.1) |
| History of vaginal bleeding | 147 (8.7) | 38 (2.4) |
| History of use of antibiotics | 101 (6.0) | 83 (5.2) |
| Pregnancy | | |
| Singleton | 1673 (99.0) | 1607 (100) |
| Abnormal*** | 17 (1.0) | 0 |

***Blighted ovum or fetal death
Numbers are n (%) or mean (SD)

***First trimester samples***

PhIGFBP-1 was detected in 40.7% of the vaginal samples (I) and in 87.2% (I) and 69.9% (II) of the cervical samples (Table 8). The concentrations ranged from 0.3µg/l to 176.0µg/l (I) and from 0.3 µg/l to 174.0 µg/l respectively (I, II). Concentrations of phIGFBP-1 were significantly higher in cervical than in vaginal samples (I) ($p < 0.001$) (Table 8).

In the vaginal samples, the frequency of phIGFBP-1 concentrations of ≥ 10 µg/l was 5.8%. In the cervical samples, the corresponding rates were 34.3% (I) and 24.5% (II) (Table 8). Distributions and concentrations of phIGFBP-1 are shown in table 8 (I).

***Mid-second trimester samples***

PhIGFBP-1 was detected in 34.8% of the vaginal samples. In cervical samples the corresponding rate wash 86.8% (I) (Table 8) and 70.6% (II). The concentrations ranged from 0.3µg/l to 55.0µg/l and from 0.3 µg/l to 126.0µg/l (I) and to 204.0µg/l respectively (II). Concentrations of phIGFBP-1 were also significantly higher in cervical than in vaginal samples ($p < 0.001$) (I) (Table 8).

In the vaginal samples, the frequency of phIGFBP-1 concentrations of ≥ 10 µg/l was 1.5% (I). In the cervical samples, the corresponding rates were 28.3% (I) and 20.2% (II) (Table 8).

**Table 8.** Distribution and concentrations of phIGFBP-1 in vaginal and cervical samples in the first and the second trimesters of pregnancy

| phIGFBP-1 | n | Undetectable n (%) | > 0.3- 9.9 µg/l n (%) | ≥ 10 µg/l n (%) | Median (range) µg/l |
|---|---|---|---|---|---|
| **First trimester** | | | | | |
| Vagina | 1687 | 1001 (59.3) | 588 (34.9) | 98 (5.8) | <0.3 (<0.3-176) |
| Cx | 1690 | 216 (12.8) | 896 (53.0) | 578 (34.2) | 4.8 (<0.3-174) |
| **Second trimester** | | | | | |
| Vagina | 1606 | 1047 (65.2) | 534 (33.3) | 25 (1.5) | <0.3 (<0.3-55) |
| Cx | 1607 | 212 (13.2) | 940 (58.5) | 455 (28.3) | 3.6 (<0.3-126) |

***Comparison of phIGFBP-1 in the first and mid-second trimester***

The concentrations in both vaginal (I) and cervical samples (I, II) were higher in the first trimester than in the mid-second trimester (vagina, $p < 0.001$, cervix, $p < 0.001$) (Table 8). Of the women with cervical phIGFBP-1 concentration of ≥ 10 µg/l in the first trimester, 54.9% had values of ≥ 10 µg/l in the mid-second trimester as well. In contrast, 85.5% of the women with cervical phIGFBP-1 concentration of < 10 µg/l in the first trimester had a value of < 10 µg/l in the mid-second trimester sample as well (I). The corresponding rates in vaginal samples were 7.0% and 98.7%, respectively (I).

**Factors affecting phIGFBP-1 concentrations (I, II, III)**

***First trimester samples***

Of the factors studied in multivariate analyses, nulliparity ($p < 0.001$) and a history of vaginal bleeding ($p < 0.001$) were independently associated with cervical phIGFBP-1 concentrations of ≥ 10 µg/l (I,II) (Table 9); nulliparity versus multiparity, OR 1.4, 95% CI 1.1–1.7, and history of vaginal bleeding versus no bleeding, OR 2.5, 95% CI 1.7–3.5 (I). Similar associations were found in study II

Among nulliparous women, those who had had previous pregnancies (miscarriage or termination of pregnancy), showed a higher rate of phIGFBP-1 concentrations of ≥10µg/l compared with nulliparous primigravid women (OR 1.5; 95% CI 1.1–1.2) (I). Sexual intercourse during the past 48 hours, or use of antibiotics within 4 weeks before sampling had no effect on either vaginal (I) or cervical phIGFBP-1 concentrations (I,II) (Table 9).

***Mid-second trimester samples***

Nulliparity and vaginal bleeding prior to sampling were significantly associated with phIGFBP-1 concentrations ≥10µg/l both in univariate and multivariate analyses ( nulliparity versus multiparity, OR 3.2; 95% CI 2.3–5.5; vaginal bleeding vs. no bleeding OR 2.6; 95% CI 1.3–5.2) . The similar associations were found in study II. Sexual intercourse or use of antibiotics had no effect on either vaginal (I) or cervical phIGFBP-1 concentrations (I, II) (Table 9).

**Table 9.** Associations (p-values) between phIGFBP-1 concentrations of ≥ 10 µg/l and selected factors in vaginal and cervical samples

| | First trimester | | Second trimester | |
|---|---|---|---|---|
| Patient characteristics | Vagina (n=1687) | Cervix (n=1690) | Vagina (n= 1606) | Cervix (n=1607) |
| Age, < 20 / 20-39 / > 40 years | 0.023 | Ns | Ns | Ns |
| Gestational age, < 12 / 12 to 14 / > 14 wks | Ns | Ns | Ns | Ns |
| Multiparity / nulliparity | < 0.001 | 0.007 | Ns | <0.001 |
| Vaginal pH ≥ 4,7 / < 4.7 | 0.047 | Ns | Ns | Ns |
| Cervical length , < 30 mm / ≥ 30 mm | Ns | Ns | Ns | Ns |
| History of sexual intercourse (<48h) | Ns | Ns | Ns | Ns |
| History of vaginal bleeding | < 0.001 | < 0.001 | < 0.001 | 0.003 |
| History of use of antibiotics | Ns | Ns | Ns | Ns |

***Late-second trimester and third trimester samples (III)***

Vaginal bleeding during the current pregnancy was significantly associated with a positive phIGFBP-1 test result (p = 0.003). Bacterial vaginosis, subsequent puerperal infection and sexual intercourse within 48 hours had no association with phIGFBP-1 test results.

**Association of phIGFBP-1 with PTD (II)**

In the first trimester study population the rates of spontaneous PTD at < 32 and < 37 weeks of gestation were 0.5% and 3.8%, respectively. In the second trimester study population the corresponding rates were 0.5 % and 3.6 %, respectively. Characteristics and obstetric outcomes of the study populations are shown in Table 10.

**Table 10.** Characteristics and obstetric outcomes of study populations in the first and second trimesters

| | First trimester | | | | Second trimester | | | |
|---|---|---|---|---|---|---|---|---|
| | < 32 weeks | ≥ 32 weeks | < 37 weeks | ≥ 37 weeks | < 32 weeks | ≥ 32 weeks | < 37 weeks | ≥ 37 weeks |
| n | 26 | 4958 | 189 | 4795 | 21 | 4609 | 167 | 4463 |
| Maternal age (years) | 31.4 ( 4.9) | 30.0 (4.5) | 29.8 (4.8) | 30.1 (4.5) | 30.7 (5.0) | 30 (4.5) | 29.8 (4.8) | 30.0 (4.6) |
| Nulliparity, % | 42.3 | 49.3 | 55.0 | 49.0 | 42.9 | 49.6 | 55.7 | 49.3 |
| Twin pregnancy,% | 3.8 | 0.6* | 6.3 | 0.4* | 4.8 | 0.6* | 6.6 | 0.4* |
| History of preterm delivery,% | 19.2 | 3.1* | 10.1 | 2.9* | 19.0 | 3.1* | 10.2 | 2.9* |
| IVF,% | 7.7 | 3.3 | 6.3 | 3.2* | 4.8 | 3.3 | 6.6 | 3.1* |
| Smoking,% | 20.8 | 11.2 | 21.0 | 10.9* | 26.3 | 11.1* | 21.7 | 10.7* |
| History of vaginal bleeding during | 23.1 | 9.4* | 17.6 | 9.1* | 35.0 | 11.7* | 20.9 | 11.4* |
| BMI kg/m² | 23.2 (5.2) | 23.5 (4.1) | 23.7 (4.8) | 24.5 (4.1) | 24.6 (4.3) | 23.5 (4.2) | 23.8 (4.2) | 23.5 (4.8) |
| BMI > 30 kg/m², % | 17.4 | 7.1 | 8.8 | 7.1 | 11.1 | 7.4 | 8.6 | 7.3 |
| Gestational age at examination | 12.9 (0.6) | 12.8 (0.7) | 12.7 (0.9) | 12.8 (0.7) | 19.3 (0.4) | 19.3 (0.7) | 19.2 (0.9) | 19.3 (0.9) |
| Gestational age at delivery (weeks) | 27.9 (3.0) | 40.1 (1.4)* | 34.5 (3.0) | 40.3 (1.2)* | 24.6 (3.1) | 40.1 (3.1)* | 35.6 (3.0) | 40.2 (1.9)* |
| Birth weight (g) | 1196 (649) | 3564 (429)* | 2575 (727) | 3575 (464)* | 1100 (692) | 3563 (491)* | 2472 (700) | 3591 (464)* |

Mean (SD) or %
* p< 0.05

***First trimester samples***

The median phIGFBP-1 concentration was higher in women with spontaneous delivery at < 32 weeks of gestation compared with women who delivered at > 32 weeks (12.0 vs. 2.5 μg/l, p = 0.004). The median phIGFBP-1 concentration in women who delivered at < 37 weeks of gestation was also higher compared with women who had term delivery (3.7 vs. 2.5μg/l, p <0.001).

The rates of spontaneous PTD at < 32 weeks and < 37 weeks of gestation were higher in women with elevated (≥10μg/l) cervical fluid phIGFBP-1 compared with women who had cervical phIGFBP-1 levels of < 10 μg/l (1.1% vs. 0.3%; p < 0.001 and 5.7% vs. 3.2%; p < 0.001, respectively).

Elevated cervical fluid phIGFBP-1 levels in the first trimester increased the risk of subsequent spontaneous PTD at < 32 weeks of gestation (OR 3.6; 95% CI 1.7–7.9) and at < 37 weeks of gestation (OR 1.9; 95% CI 1.3–2.5). Multiple logistic regression analysis confirmed that, even when other risk factors were accounted for, an elevated level of cervical fluid phIGFBP-1 was an independent predictor of PTD at < 32 weeks of gestation, with an OR of 3.0 (95% CI 1.3–7.0) and at < 37 weeks of gestation, with an OR of 1.6 (95% CI 1.2–2.3) (Table 11).

**Table 11.** Multivariate analysis of risk factors of preterm delivery at < 32 weeks' gestation and < 37 weeks' gestation in the first trimester and second trimester (II)

| | First trimester | | Second trimester | |
|---|---|---|---|---|
| | < 32 weeks | < 37 weeks | < 32 weeks (n=21) | < 37 weeks |
| Nulliparity | 1.1 (0.4-2.9) | 1.6 (1.1-2.2) | 1.5 (0.5-4.7) | 1.6 (1.1-2.4) |
| Twin | 7.1 (0.9-59.1) | 13.5 (6.0-30.3) | 13.6 (1.6-115.2) | 18.9 (8.2-43.6) |
| IVF | 1.6 (0.3-7.9) | 1.5 (0.8-3.0) | 1.3 (0.2-10.9) | 1.6 (0.8-3.2) |
| History of preterm delivery | 7.6 (2.4-24.1) | 4.7 (2.6-8.3) | 10.5 (2.7-40.7) | 5.2 (2.8-9.3) |
| Smoking | 2.2 (0.8-5.9) | 2.0 (1.4-3.0) | 3.1 (1.1-9.1) | 2.2 (1.5-3.4) |
| BMI > 30 kg/m² | 2.6 (0.8-7.8) | 1.2 (0.7-2.1) | 1.7 (0.4-7.6) | 1.1 (0.6-2.0) |
| History of vaginal bleeding | 2.7 (1.0-7.3) | 2.1 (1.4-3.1) | 5.2 (1.9-14.1) | 2.0 (1.3-3.1) |
| Cervical phIGFBP-1 ≥ 10 micrograms/l | 3.0 (1.3-7.0) | 1.6 (1.2-2.3) | 1.9 (0.7-5.3) | 1.4 (1.0-2.1) |

Adjusted OR (95% CI)

The ROC curve illustrating the accuracy of cervical fluid phIGFBP-1 is shown in Figure 11 A. The area under the curve (AUC) as regards predicting spontaneous PTD at < 32 weeks was 0.66 (95% CI 0.55–0.76) and at < 37 weeks of gestation, 0.59 (0.54–0.63). A cut-off value of 10µg/l predicted PTD at < 32 and < 37 weeks of gestation with sensitivities of 53.8% and 37.0%, and false-positive rates of 24.3% and 24.0%, respectively. Negative predictive values were 99.7% and 96.8%, respectively.

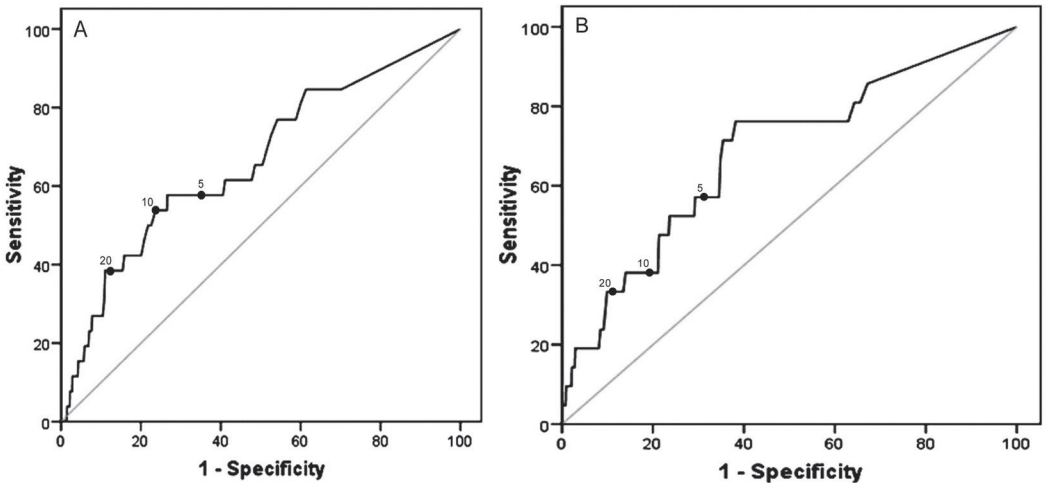


Figure 11. ROC curves of cervical fluid phIGFBP-1 levels in the first (A) and second (B) trimesters for predicting preterm delivery before 32 weeks of gestation. The sensitivity and 100-specificity points for phIGFBP-1 values of 5, 10 and 20 µg/l are marked

***Mid-second trimester samples***

The median phIGFBP-1 concentration was higher in women with spontaneous delivery at < 32 weeks of gestation compared with women who delivered at ≥ 32 weeks (7.5 vs. 2.1 μg/l, p = 0.003). No difference was found in cervical fluid phIGFBP-1 concentrations between women with PTD at < 37 weeks of gestation and those with term delivery (2.5 vs. 2.1 μg/l, p = 0.059).

The rates of spontaneous PTD at < 32 weeks and at < 37 weeks of gestation were higher in women with elevated cervical fluid phIGFBP-1 concentrations compared with women who had cervical fluid phIGFBP-1 levels of < 10 μg/l (0.9% vs.0.4%; p = 0.041 and 5.1% vs. 3.2%; p = 0.005, respectively).

An elevated cervical fluid phIGFBP-1 level increased the risk of subsequent spontaneous PTD at < 32 and <37 weeks of gestation (OR 2.4; 95% CI 1. 0–5.9 and OR of 1.6; 95% CI 1.2–2.3). In multiple logistic regression analysis an elevated cervical fluid phIGFBP-1 level was not an independent predictor of PTD (Table 11).

The ROC curves illustrating the accuracy of cervical fluid phIGFBP-1 concentrations are shown in Figure 11 B. The AUC (95% CI) as regards the prediction of spontaneous PTD at < 32 weeks was 0.68 (0.56–0.80) and at < 37 weeks of gestation, 0.54 (0.50–0.59). A cervical fluid phIGFBP- 1 concentration of ≥ 10 μg/l predicted subsequent spontaneous PTD at < 32 and < 37 weeks of gestation with sensitivities of 38.1% and 28.7%, and with false-positive rates of 20.1% and 19.9%, respectively. Negative predictive values were 99.6% and 96.8%, respectively.

**PhIGFBP-1 strip test and cervical length measurement in prediction of PTD (III)**

The overall rate of spontaneous PTD at ≤ 34 weeks of gestation was 4.1% (10/246). Seven of ten cases (70.0%) occurred within 14 days of examination. Of the studied historical risk factors, a history of previous PTD in parous women, and puerperal infection were associated with PTD at ≤ 34 weeks of gestation (p = 0.003 and p = 0.037, respectively) (Table 12).

**Table 12.** Demographic and clinical characteristics and obstetric outcomes of the study population in Study III

| | Delivery ≤ 34 weeks n = 10 | | Delivery > 34 weeks n = 236 | | |
|---|---|---|---|---|---|
| Maternal age, years | 29.1 | (21-36) | 29.9 | (18-40) | |
| Parity | | | | | |
| Nullipara | 6 | (60.0) | 97 | (41.1) | |
| Multipara | 4 | (40.0) | 139 | (58.9) | |
| History of preterm birth | 3 | (75.0) | 11 | (7.9) | * |
| Gestational age at examination, weeks | 28.2 | (25.4-32.8) | 28.1 | (22.0-34.0) | |
| BMI before pregnancy | 21.6 | (18-38) | 22.4 | (17-47) | |
| Smoking | | | | | |
| Yes | 1 | (10.0) | 5 | (2.1) | |
| Stop 1. trimester | 2 | (20.0) | 27 | (11.6) | |
| No | 7 | (70.0) | 201 | (86.3) | |
| Sexual intercourse within 48 hours | 3/7 | (42.9) | 34/179 | (19.0) | |
| Bacterial vaginosis | 0 | (0.0) | 7 | (3.0) | |
| *Chlamydia trachomatis* | 0 | (0.0) | 0 | (0.0) | |
| *Streptococcus agalactiae* | 1/7 | (14.2) | 35/142 | (24.6) | |
| Previous vaginal bleeding | 2 | (20.0) | 34 | (14.4) | |
| Hospital admission | 7 | (70.0) | 11 | (4.7) | * |
| Use of tocolysis | 9 | (90.0) | 14 | (5.9) | * |
| Use of corticosteroids | 8 | (80.0) | 11 | (4.7) | * |
| Gestational age at delivery, weeks | 31.9 | (27.1-33.8) | 39.8 | (35.0-42.4) | * |
| Puerperal infection** | 2 | (20.0) | 6 | (2.5) | * |
| Time from examination to delivery, weeks | 1.2 | (0- 8.4) | 11.7 | (2.8-19.8) | * |
| Birth weight, g | 1790 | (1000-2355) | 3484 | (1930-4830) | * |

*Note* : Data expressed as *n* (%) or *median*
* $p < 0.05$
** Puerperal infections: one sepsis,one infection after cesarean section, three amnionitis, three endometritis

A short cervix was found in 7.3% (18/246) of the patients, with funnelling in four of them (22.0%). Rapid phIGFBP-1 test result was positive in 14.6% (36/246) of the patients. The median gestational age as regards both positive and negative phIGFBP-1 results was 28.1 weeks (range 22.0–33.7 weeks and 22.1–34.0 weeks, respectively). The frequency of a positive phIGFBP-1 test result was 22.2% (4/18) among patients with a cervical length of < 25 mm, and 14.0 % (32/228) among patients with a cervical length of > 25 mm. Using

this cut-off, the agreement between cervical length and cervical phIGFBP-1 test results was poor (kappa = 0.056).

Eighteen women were admitted on the basis of the physician's judgment, including knowledge of cervical changes. Eight of the admitted patients had a short cervix and six had a positive phIGFBP-1 test result. Tocolytic treatment was given to 16 of the 18 hospitalized patients and to 7 of the 228 discharged women.

A short cervix (p = 0.003) and a positive phIGFBP-1 test result (p = 0.007) were both associated with PTD at ≤ 34 weeks or within 14 days (p < 0.001) (Table 13). The relevance of a short cervix, a positive phIGFBP-1 test result, their combination, and the likelihood ratios (LRs) for spontaneous PTD at ≤ 34 weeks of gestation and within 14 days of examination are shown in Table 14. In multiple regression analysis, short cervical length (OR 8.6; 95% CI 2.2–33.4) and a positive phIGFBP-1 test result (OR 6.6; 95% CI 1.8–24.2) remained significant independent predictors of PTD at ≤ 34 weeks and within 14 days of examination (OR 9.4; 95% CI 1.9-46.9 and OR 9.2; 95% CI 1.9-44.4, respectively).

**Table 13.** Rate of spontaneous PTD (less than 34 weeks or within 14 days) among 246 women reporting uterine contractions in relation to cervical length and cervical phIGFBP-1 results (Study III)

| Examination | Rate | Delivery ≤ 34 weeks | Delivery > 34 weeks | *P* -value | Delivery ≤ 14 days | Delivery > 14 days | *P* -value |
|---|---|---|---|---|---|---|---|
| n | | 10 | 236 | | 7 | 239 | |
| Cervical length | | | | p=0.003 | | | p=0.001 |
| < 25 mm | 18 (7.3) | 4 (22.2) | 14 (77.8) | | 4 (22.2) | 14 (77.8) | |
| ≥ 25 mm | 228 (92.7) | 6 (2.6) | 222 (97.4) | | 3 (1.3) | 225 (98.7) | |
| phIGFBP-1-test | | | | p=0.007 | | | p=0.001 |
| Positive | 36 (14.6) | 5 (13.9) | 31 (86.1) | | 5 (13.9) | 31 (86.1) | |
| Negative | 210 (85.4) | 5 (2.4) | 205 (97.6) | | 2 (1.0) | 208 (99.0) | |

Data expressed as *n* (%)

**Table 14.** Test performance of short cervix, phIGFBP-1 or their combination, and clinician's judgment with likelihood ratios (LRs) of spontaneous preterm delivery at less than 34 weeks' gestation and within 14 days of examination

| | *n* | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | LR+ | LR- |
|---|---|---|---|---|---|---|---|
| ***Delivery at ≤ 34 weeks*** | 10 | | | | | | |
| Cervix < 25mm | 4 | 40.0 | 94.1 | 22.2 | 97.4 | 6.8 | 0.6 |
| phIGFBP-1 + | 5 | 50.0 | 86.9 | 13.9 | 97.6 | 3.8 | 0.6 |
| Cervix < 25 mm and phIGFBP-1 + | 3 | 30.0 | 99.6 | 75.0 | 97.1 | 75.0 | 0.7 |
| Cervix < 25 mm or phIGFBP-1 + | 6 | 60.0 | 81.4 | 12.0 | 98.0 | 3.2 | 0.5 |
| ***Delivery within 14 days*** | 7 | | | | | | |
| Cervix < 25mm | 4 | 57.1 | 94.1 | 22.2 | 98.7 | 9.7 | 0.5 |
| phIGFBP-1 + | 5 | 71.4 | 87.0 | 13.9 | 99.0 | 5.5 | 0.3 |
| Cervix < 25 mm and phIGFBP-1 + | 3 | 42.9 | 99.6 | 75.0 | 98.3 | 107.3 | 0.6 |
| Cervix < 25 mm or phIGFBP-1 + | 6 | 85.7 | 81.6 | 12.0 | 98.0 | 4.7 | 0.2 |

**MMP-8 concentrations in first and mid-second trimester (IV, V)**

Characteristics of the study population IV are shown in Table 15. MMP-8 was detectable in almost 100% of vaginal (IV) and cervical swab samples (IV,V) in the first and mid-second trimesters. The concentrations ranged from 0.08μg/l to about 2000 μg/l (Table 16 and 19). The overall ranges of cervical fluid MMP-8 concentrations and median values were similar in the first trimester and in the second trimester (IV,V). Significant correlations were found between first and second trimester vaginal samples ($r = 0.67$, $p < 0.01$) and between first and second trimester cervical samples ($r = 0.54$, $p < 0.01$) (IV).

**Table 15.** Characteristics of the study populations (Study IV)

| | First trimester n (%) | Second trimester n (%) |
|---|---|---|
| Total | 1979 | 1950 |
| Pregnancy, n (%) | | |
| Singleton | 1944 (98.2) | 1932 (99.1) |
| Twin | 18 (0.9) | 18 (0.9) |
| Abnormal* | 17 (0.9) | |
| Miscarriage between | 12 (0.6) | |
| Parity | | |
| Nullipara | 1055 (53.3) | 1041 (53.4) |
| Primigravida | 799 (75.7) | 787 (75.6) |
| Non-primigravida | 256 (24.3) | 254 (24.4) |
| Multipara | 924 (46.7) | 909 (46.6) |
| History of vaginal bleeding | 177 (8.9) | 47 (2.4) |
| History of sexual intercourse (<48h) | 452 (22.8) | 422 (21.7) |
| History of use of antibiotics | 117 (5.9) | 104 (5.3) |
| Vaginal Gram-stain findings** | | |
| Bacterial vaginosis | 81 (4.1) | 76 (3.8) |
| Leukocytes in vagina | 598 (30.5) | 550 (28.4) |
| 1+ | 542 (27.7) | 448 (23.1) |
| 2+ | 54 (2.8) | 87 (4.5) |
| 3+++ | 2 (0.1) | 15 (0.8) |
| Candidiasis | 62 (3.1) | 37 (1.9) |
| Cervical length < 30 mm | 5 (0.3) | 6 (0.3) |

* Blighted ovum or fetal death
** Missing values: 20 in the first trimester, 13 in the second trimester

***First trimester samples***

Median MMP-8 concentrations were significantly higher in cervical than in vaginal samples in the first trimester ($p < 0.001$) (Table 16). Significant correlations were found between vaginal and cervical MMP-8 concentrations ($r = 0.54$, $p < 0.01$).

**Table 16.** MMP-8 concentrations in vaginal and cervical samples (Study IV)

| | First trimester (n = 1979) MMP-8 (µg/l) | | Second trimester (n=1950) MMP-8 (µg/l) | |
|---|---|---|---|---|
| | Vagina | Cervix | Vagina | Cervix |
| Median | 107.4 | 318.3 | 112.5 | 344.8 |
| Range | <0.08° - 2406.6 | 0.1 - 2074.6 | <0.08°° - 2093.4 | 0.4 - 1783.5 |
| Percentiles | | | | |
| 10 | 3.1 | 47.2 | 4.6 | 55.2 |
| 25 | 16.4 | 118.3 | 23.5 | 132.2 |
| 50 | 107.4 | 318.3 | 112.5 | 344.8 |
| 75 | 379.6 | 620.0 | 416.2 | 657.5 |
| 90 | 762.0 | 901.7 | 842.1 | 918.2 |

°Four
°°One

***Second trimester samples***

Median MMP-8 concentrations were significantly higher in cervical than in vaginal samples in the (IV,V)($p < 0.001$) (Table 16). Significant correlations were found between vaginal and cervical MMP-8 concentrations ($r = 0.53$, $p < 0.01$).

## Factors affecting MMP-8 concentrations

Associations between selected factors and MMP-8 concentrations in vaginal and cervical samples are shown in Table 17.

***First trimester samples***

Multiparity, bacterial vaginosis, and vaginal leukocytosis were associated with increased MMP-8 concentrations in vaginal and cervical samples. Vaginal and cervical fluid MMP-8 concentrations were significantly higher in women who had both BV and an increased number of vaginal leukocytes than in women with either BV or an increased number of vaginal leukocytes, or negative findings (IV). Sexual intercourse within the previous 48 hours was associated with lower cervical fluid MMP-8 concentrations (IV,V). BMI $\geq 30$ kg/m² was associated with MMP-8 concentrations greater than the 90th percentile with OR 1.6; 95% CI 1.2–2.2 (V).

***Second trimester***

Multiparity and vaginal leukocytosis were associated with increased MMP-8 concentrations in vaginal and cervical samples. Bacterial vaginosis was associated with increased vaginal MMP-8 but not with cervical MMP-8. Vaginal and cervical fluid MMP-8 concentrations were significantly higher in women who had both BV and an increased number of vaginal leukocytes than in women with either BV or an increased number of vaginal leukocytes, or negative findings (IV). Sexual intercourse was associated with lower cervical fluid MMP-8 concentrations as in first trimesters (IV,V). BMI ≥ 30 kg/m² was associated with increased MMP-8 concentrations with OR 1.9; 95% CI 1.4–2.6) (V).

**Table 17.** Associations (p-values) between MMP-8 concentrations and selected factors in vaginal and cervical samples in the first and second trimesters

| Selected factor | First trimester | | Second trimester | |
|---|---|---|---|---|
| MMP-8 (µg/l) | Vagina | Cervix | Vagina | Cervix |
| n | 1979 | | 1950 | |
| Age, < <20 / 20-39 / >40 years | Ns | Ns | Ns | Ns |
| Gestational age, < 12 / 12 to 14 / > 14 wks | Ns | Ns | Ns | Ns |
| Gravidity, nulligravida/ nullipara multigravi | Ns | Ns | Ns | 0.051 |
| Multiparity / nulliparity | <0.001 | <0.001 | <0.001 | <0.001 |
| Bacterial vaginosis | <0.001 | <0.001 | <0.001 | Ns |
| Vaginal leukocytosis | 0.001 | 0.001 | <0.001 | 0.008 |
| Vaginal candidiasis | Ns | Ns | Ns | Ns |
| Cervical length , < 30 mm / ≥ 30 mm | Ns | Ns | Ns | Ns |
| History of sexual intercourse (<48h) | Ns | <0.001 | 0.004 | <0.001 |
| History of vaginal bleeding | Ns | 0.037 | Ns | Ns |
| History of use of antibiotics | Ns | Ns | Ns | Ns |
| Type of pregnancy | | | | |
| Single / Twin | Ns | Ns | Ns | Ns |
| Normal / Abnormal *** | Ns | Ns | Ns | Ns |

*** Blighted ovum or fetal death

**Association of cervical fluid MMP-8 with PTD (V)**

Demographic and clinical characteristics of the first and second trimester study populations are shown in Table 18. The mean gestational age at examination was 12.8 weeks in the first trimester and 19.3 weeks in the second trimester. The overall rate of spontaneous PTD at < 37 weeks of gestation was 3.8%. The mean gestational age among preterm deliveries was 34.6 weeks (range 22.8 to 36.8), and in the term delivery cohort it was 40.3 weeks (range 37.0 to 42.8).

**Table 18.** Characteristics of the first and second trimester study populations in relation to the time of delivery

| | First trimester | | | | | Second trimester | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | < 37 wks | | > 37 wks | | | < 37 wks | | > 37 wks | | |
| | n= 184 | | n = 4671 | | | n= 164 | | n = 4407 | | |
| Age ≥ 37 years | 14 | (7.6) | 379 | (8.1) | | 11 | (6.7) | 342 | (7.8) | |
| Multipara | 84 | (45.7) | 2395 | (51.3) | | 73 | (44.5) | 2241 | (50.9) | |
| Twin pregnancy | 12 | (6.5) | 19 | (0.4) | * | 11 | (6.7) | 16 | (0.4) | * |
| History of preterm delivery | 19 | (10.3) | 138 | (3.0) | * | 16 | (9.8) | 127 | (2.9) | * |
| IVF | 12 | (6.5) | 153 | (3.3) | * | 11 | (6.7) | 140 | (3.2) | * |
| Smoking | 36 | (21.1) | 484 | (11.0) | * | 34 | (22.1) | 446 | (10.7) | * |
| BMI > 30 kg/m² | 15 | (9.1) | 317 | (7.2) | | 13 | (8.8) | 307 | (7.4) | |
| Sexual intercourse < 48h | 38 | (20.8) | 981 | (21.0) | | 32 | (20.0) | 811 | (18.6) | |
| History of vaginal bleeding | 32 | (17.6) | 422 | (9.1) | * | 13 | (8.1) | 146 | (3.4) | * |

* $p < 0.05$
*n (%)*

There was no difference in median MMP-8 concentrations between women with spontaneous PTD (< 37) and at term delivery (> 37 weeks of gestation) (Table 19). Cervical fluid MMP-8 concentrations in women with spontaneous PTD preceded by PPROM and in those with PTD initiated by spontaneous labour were also assessed in relation to MMP-8 concentrations in women with term delivery (Figure 12). No differences were detected in median MMP-8 concentrations, in either the first or in the second trimester, between the women who had PTD after spontaneous onset of labour versus those with spontaneous delivery at term. Cervical fluid MMP-8 concentrations were lower among women with subsequent PPROM at < 37 weeks of gestation compared with those with subsequent term delivery and those with PTD initiated with preterm contractions with intact membranes, the differences being significant in the second trimester only ($p = 0.016$ and 0.023, respectively) (Table 19).

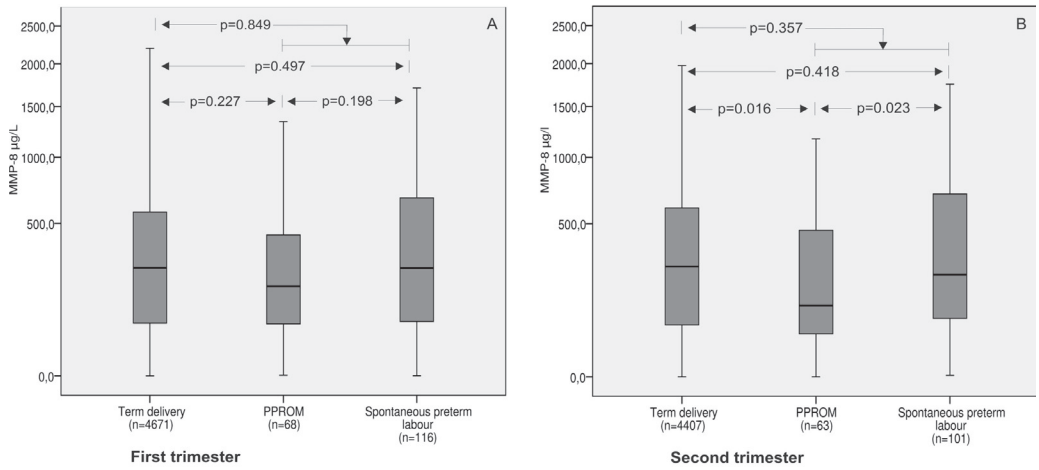


Figure 12. A and B Boxplots of cervical fluid MMP-8 concentrations in women with term delivery and in preterm subgroups (preterm labour and PPROM) in the first (A) and second (B) trimesters.

**Table 19.** Cervical fluid MMP-8 concentrations in women with spontaneous preterm delivery and term delivery

| MMP-8 (µg/l) | Spontaneous preterm delivery | | | Term delivery | p-value |
|---|---|---|---|---|---|
| | Total | Preterm labour | PPROM | Total | |
| First trimester (No) | 184 | 116 | 68 | 4671 | |
| Median (range) (10th-90th percentile) | 233.6 (< 0.08-1708.6)* (13.9-923.6) | 258.6 (< 0.08- 1708.6)° (11.5-1079.0) | 183.2 (0.2- 1341.0)°° (17.2-817.9) | 258.9 (< 0.08-2197.5) (11.3-915.2) | 0.849* |
| Second trimester (No) | 164 | 101 | 63 | 4407 | |
| Median (range) (10th-90th percentile) | 185.6 (< 0.08-1750.9)* (11.0-1050.5) | 231.7 (0.4-1750.9)° (14.1-1156.0) | 119.4 (< 0.08-1169.9)°° (4.0-738.5) | 268.0 (< 0.08-1973.3) (9.9-918.0) | 0.357* |

* Spontaneous preterm delivery vs.term delivery
° Preterm labour vs. term delivery (p=0.497 in the first trimester; p=0.418 in the second trimester)
°° PPROM vs. term delivery (p=0.227 in the first trimester; p=0.016 in the second trimester)

For further analysis, the MMP-8 concentrations were dichotomized, and the 90th percentile value among cases ending with term delivery was used as the upper limit of normal. Among the women with PTD initiated by spontaneous labour, 16.9% had cervical fluid MPP-8 concentrations greater than the 90th percentile (≥ 918. 0 µg/l) among term controls in the mid-second trimester (OR 1.8, 95% CI 1.1–3.1). This cut-off level showed a sensitivity of 16.8% and a specificity of 90.0%. After adjusting for maternal age, parity, twin pregnancy, history of PTD, IVF, smoking, body mass index, sexual intercourse in the previous 48 hours and previous vaginal bleeding, MMP-8 concentration in the second trimester greater

than this 90th percentile value remained as an independent factor associated with PTD initiated by spontaneous labour (OR 2.0; 95% CI 1.1–3.5) (Table 20)

**Table 20.** Multivariate analysis of risk factors of preterm delivery at < 37 weeks' of gestation among women with subsequent preterm labour

| | Adjusted OR (95% CI) | |
|---|---|---|
| | First trimester | Second trimester |
| Maternal age, >37 years | 0.9 (0.4-1.8) | 0.8 (0.3-1.8) |
| Multipara | 0.6 (0.4-0.9) | 0.6 (0.3-0.9) |
| Twin pregnancy | 14.6 (5.4-39.6) | 17.4 (6.3-48.2) |
| History of preterm delivery | 6.9 (3.6-13.3) | 6.6 (3.2-13.6) |
| IVF | 1.8 (0.9-3.9) | 1.9 (0.8-4.5) |
| Smoking | 2.0 (1.2-3.3) | 2.5 (1.5-4.2) |
| BMI ≥ 30 kg/m² | 1.8 (1.0-3.3) | 1.4 (0.7-2.8) |
| Sexual intercourse (< 48hours) | 1.0 (0.6-1.6) | 0.7 (0.4-1.3) |
| History of vaginal bleeding | 3.1 (1.9-5.1) | 2.7 (1.2-6.1) |
| Cervical fluid MMP-8 > 90 percentile* | 1.3 (0.7-2.3) | 2.0 (1.1-3.5) |

* MMP-8 concentration (90 percentile in term controls 915.2 µg/l in the first trimester and 918.0 µg/l in the second trimester)

Of the women with subsequent PPROM at < 37 weeks of gestation, 66.7% had cervical fluid MPP-8 concentrations under the median (268.0 µg/l) of term controls in the second trimester. MMP-8 concentrations below the median were associated with an increased risk of PPROM, with an OR of 2.0 (95% CI 1.2–3.4).

However, after adjusting for affected factors cervical fluid MMP-8 concentrations under the median value remained associated with subsequent PPROM (OR 2.0; 95% CI 1.1–3.2) in the second trimester, but not in the first trimester (OR 1.3; 95% CI 0.7-2.7) (Table 21).

**Table 21.** Multivariate analysis of risk factors of preterm delivery at < 37 weeks' of gestation among women with subsequent PPROM

| | Adjusted OR (95% CI) | |
|---|---|---|
| | First trimester | Second trimester |
| Maternal age, >37 years | 1.0 (0.4-2.6) | 0.6 (0.2-2.0) |
| Multipara | 0.7 (0.4-1.3) | 0.7 (0.4-1.3) |
| Twin pregnancy | 18.0 (5.7-57.0) | 22.6 (6.9-73.5) |
| History of preterm delivery | 2.1 (0.6-7.2) | 2.6 (0.7-9.0) |
| IVF | 1.0 (0.3-3.6) | 1.3 (0.4-4.5) |
| Smoking | 1.9 (1.0-3.6) | 1.9 (1.0-3.8) |
| BMI ≥ 30 kg/m² | 0.4 (0.1-1.7) | 0.5 (0.1-1.9) |
| Sexual intercourse (< 48hours) | 0.5 (0.3-1.1) | 1.6 (0.9-2.8) |
| History of vaginal bleeding | 0.9 (0.4-2.4) | 3.3 (1.3-8.6) |
| Cervical fluid MMP-8 < median * | 1.3 (0.7-2.2) | 2.0 (1.1-3.7) |

* MMP-8 concentration (median 258.9 µg/l in first trimester and 268.0 µg/l in second trimester)

# DISCUSSION

Worldwide preterm delivery(PTD) complicates about 3% of pregnancies before 34 weeks of gestation and 5% - 15 % before 37 weeks of gestation (Goldenberg et al. 2008, Slattery and Morrison 2002). In Finland the incedence of PTD has staied stabile and is about 5-6% (Jakobsson et al 2008) while it has increased in alsewere in the world (Goldenberg et al. 2008, Muglia et al. 2010). Two thirds of PTD occur spontaneously. In our study, spontaneous PTD rate < 32 weeks of gestation was 0.5% and < 37 weeks of gestation 3.8%. Prematurity is still a major cause of perinatal morbidity and mortality and has serious effects on society, making PTD an important issue to public health worldwide. Therefore, prediction and prevention of PTD remains a big challenge in obstetrics. If a woman could be identified to be at high risk for PTD in early pregnancy, she could be targeted for more intensive antenatal surveillance and prophylactic interventions. Furthermore, when a woman presents with symptoms of threatened PTD and, the likelihood of having a spontaneous PTD can be determined, interventions can be developed to prevent or delay delivery, and to improve subsequent neonatal mortality/morbidity.

Many biomarkers, including phosphorylated insulin-like growth factor binding protein-1 (phIGFBP-1) and matrix metalloproteinase-8 (MMP-8), have recently been evaluated for prediction of PTD among symptomatic women but so far none of them has appeared to be superior in clinical practice (Honest et al. 2009), and their performance among asymptomatic women in early pregnancy has remained unclear. Before our study the knowledge of the presence, concentrations, and factors associating with phIGFBP-1 and MMP-8 in the lower genital tract in early- and mid-gestation was limited (Honkanen et al. 2004, Kekki et al. 1999).

The main purpose of this study was to evaluate whether phIGFBP-1 and MMP-8, as measured in lower genital tract fluids in early and mid-second pregnancy are associated with subsequent PTD, and whether they can predict this detrimental event in asymptomatic women.

We first measured the concentrations of phIGFBP-1 and MMP-8 in cervical fluids and examined factors influencing the concentrations in early and mid-pregnancy. Secondly, associations between elevated cervical fluid phIGFBP-1 and spontaneous PTD < 32 weeks of gestation and < 37 weeks of gestation as well as the associations between cervical fluid MMP-8 and PTD < 37 weeks of gestation were analyzed. Thirdly, the performance of the rapid phIGFBP-1 strip test in prediction of PTD among women with self-reported uterine contractions during the second half of pregnancy was evaluated.

**Concentrations of phIGFBP-1 in the lower genital tract in the first and mid- second trimester (I, II)**

IGFBP-1 is produced by human decidua, in which the phosphorylated isoforms, phIGFBP-1, predominate (Giudice and Irwin 1999, Westwood et al. 1994). These are the first reports (I, II) on phIGFBP-1 concentrations in vaginal and cervical secretions of unselected, asymptomatic pregnant women during the first and mid-second trimester. Mechanism(s) accounting for the absence and presence of phIGFBP-1 in the lower genital tract fluid in early pregnancy have not been well defined. The most likely explanation is that small amounts of chorionic/decidual products leak into the cervical canal before the fusion of capsular and parietal decidua. This supposed to be completed during the early second trimester (Lockwood et al 1991).

Although the range of phIGFBP-1 concentrations, as measured by immunoenzymometric assay, was roughly similar in the vaginal and cervical samples in the first and mid-second trimester, the protein was detectable in cervical samples more than twice as often as in the vaginal samples in both trimesters, and the median concentrations were significantly higher in cervical samples than in vaginal samples. Thus, our data clearly show that the site of sampling has to be defined and considered when phIGFBP-1 is used as decidual marker, and data interpreted for research or clinical purposes. For comparison, the diagnosis of ruptured fetal membranes was based on the detection of amniotic fluid isoforms of IGFBP-1 in vaginal swab samples (Actim PROM test; Medix Biochemica).

In Studies I and II, we used the same cut-off level for elevated phIGFBP-1 ($\geq$ 10 μg/l) as used previously by us (Kekki et al. 1999, Kekki et al. 2001, Nuutila et al. 1999) and an other study (Paternoster et al. 2007) to enable comparisons between early and late pregnancy. This is also the detection limit of the rapid strip test for phIGFBP-1 (Actim Partus test, Medix Biochemica) that has been used in many recent studies for prediction of spontaneous PTD (Kwek et al. 2004, Lembet et al. 2002, Paternoster et al. 2009, Tanir et al. 2009, Ting et al. 2007). However, in some studies a strong positive rapid phIGFP-1 test result has been interpreted to be equal to 30 μg/l instead 10 μg/l (Altinkaya et al. 2009, Brik et al. 2010, Elizur et al. 2005).

In study I, the rate of the phIGFBP-1 values $\geq$10 μg/l was about six times higher in the cervical samples than in the vaginal samples in the first trimester, and during the second trimester the ratio was 18. The instability in vaginal fluid phIGFBP-1 concentrations let us

to use cervical samples in later studies (II, III). In Study I, one third of women and in study II one fourth of women had an elevated cervical fluid phIGFBP-1 level in the first trimester. In the second trimester, the rates were one fourth to one fifth, respectively. The high rate of phIGFBP-1 values above this cut-off in cervical samples among asymptomatic pregnant women suggests that a concentration ≥10 μg/l in cervical fluid may also be physiological during the first half of pregnancy.

**Factors affecting phIGFBP-1 concentrations in the lower genital tract in the first and mid-second trimester (I II)**

In study I, only half of the women who had cervical phIGFBP-1 level ≥ 10μg/l in the first trimester had the same result in the second trimester, whereas most (85.6%) of the women with cervical phIGFBP-1 < 10μg/l in the first trimester showed no change in the second trimester. These data are in line with the hypothesis that the disappearance of phIGFBP-1 from the lower genital tract occurs concurrently with the fusion of capsular and parietal decidua. The change from the level < 10μg/l to ≥ 10μg/l between the first and the second trimester in 14.5% of women requires another explanation. One possibility could be subclinical ascending infection and tissue disruption at the choriodecidual interface between the first and second sampling. In support of this, a previous study by Kekki et al (1999) showed that women with BV in early pregnancy concurrently with a cervical fluid phIGFBP-1 ≥ 10μg/l had s significantly increased risk of peripartum infections as compared to women with BV and phIGFBP-1 < 10μg/l (Kekki et al. 1999). The similar association between BV and elevated FFN has been described by Goldenberg (Goldenberg et al. 2000). Unfortunately we did not have data on BV in Study I and II.

It has been shown previously that IGFBP-1 concentration in seminal plasma varies from undetectable to very low (Rutanen et al. 1993), suggesting that phIGFBP-1 could be used as a decidual marker even after sexual intercourse. However, the effect of sexual intercourse itself on phIGFBP-1 in the lower genital has not been reported previously. In our study I and study II, one fifth of women expressed having had sexual intercourse during the last 48 h before sampling. Sexual intercourse appeared to have no effect on cervical fluid phIGFBP-1 concentrations, confirming that phIGFBP-1-measurement in the lower genital tract remains reliable even after intercourse. In comparison, the FFN test result may be affected seminal plasma, and is not useful if the woman has had sexual intercourse during 24 hours (Shimoya et al. 1998, Honest et al. 2002).

In Study I and II we show that history of vaginal bleeding in current pregnancy is associated with elevated cervical fluid phIGFBP-1. An occult bleeding might account for this association, since the same phosphoisoforms of IGFBP-1 predominate in the decidua and in the maternal serum (Martina et al. 1997). Another possibility is that previous decidual haemorrhage may have caused an injury at the choriodecidual interface and, consequently, leakage of phIGFBP-1 into the cervix.

Nulliparous women had significantly higher rate of phIGFBP-1 concentrations ≥ 10µg/l in cervical fluid than parous women in the first and mid-second trimester. The reason for this remains unclear, and is in contrasts with the data of fetal fibronectin (Goldenberg et al. 2000). It is of interest that the association with elevated phIGFBP-1 was strongest in nulliparous women with previous pregnancies, i.e miscarriage or termination of pregnancy. This suggests that women with previous miscarriage(s) may have reasons, still unknown to us, which increase phIGFBP-1 leakage from choriodecidual interface and may predict complications in ongoing pregnancy as well.

**PhIGFBP-1 as a predictor of spontaneous PTD among asymptomatic women in the first and mid-second trimester (II)**

The overall PTD rate at < 37 weeks of gestation in our study (4.4%) was lower than the national figure 5.2% in Finland (Jakobsson et al. 2008), and also lower compared with that at our university hospital area (5.5 %) during the same time period (THL, unpublished data). This indicates that our study population represented low risk women.

Because spontaneous PTD is a long-standing process, dating back to the first trimester, we examined whether elevated concentrations of phIGFBP-1 in cervical fluid among asymptomatic women in early- or mid-pregnancy are associated with an increased risk of subsequent spontaneous PTD.

Our data (II) demonstrate that women with subsequent PTD at < 32 weeks of gestation had significantly higher cervical fluid phIGFBP-l concentrations in both trimesters compared with women who delivered at ≥ 32 weeks of gestation. Secondly, our study showed that the frequencies of PTD among women with an elevated cervical fluid phIGFBP-1 level either in the first trimester or in the mid-second trimester were significantly higher compared with

women with cervical phIGFBP-1 < 10 µg/l. The risk of PTD < 32 weeks of gestation was almost 4-fold-increased among women who had cervical phIGFBP-1 concentration ≥ 10 µg/l compared with those with cervical phIGFBP-1 < 10 µg/l in the first trimester. The risk of PTD among the women with cervical fluid phIGFBP-1 ≥ 10 µg/l in the second trimester was almost 2-fold.

After multivariate analysis including selected historical risk factors for PTD, an elevated cervical fluid phIGFBP-1 in the first trimester remained an independent predictor of PTD < 32 weeks of gestation as well as < 37 weeks of gestation. Of the historical risk factors only previous PTD carried higher OR for PTD than elevated phIGFBP-1. This observation is in line with FFN. High values of FFN from 13 to 22 weeks of gestation have been shown to be associated with 2 to 3-fold increased risk of subsequent spontaneous PTD (Goldenberg et al. 2000). There are several explanations for our data. First, some women could have had subclinical intrauterine infection already before pregnancy or in early pregnancy, known to increase the risk of PTD, and an elevated cervical fluid phIGFBP-1 reflected infection-induced tissue disruption at choriodecidual interface. This hypothesis is supported by Kekki's study (1999), in which the women who had concurrently an asymptomatic BV and elevated phIGFBP-1 in cervical secretion in early second trimester had increased risk of peripartum infections (Kekki et al. 1999). Considering the high frequency of elevated cervical fluid phIGFBP-1 concentrations in our study population, this is probably not the only explanation. One reason for a higher rate of preterm deliveries among women with elevated cervical fluid phIGFBP-1 in the first trimester than in the second trimester could be the following: Before the fusion of capsular and parietal decidua there is an "open route" for the leakage of phIGFBP-1 from the decidua down to the cervix. Infections ascending from the vagina during that stage of pregnancy, indicated by the leakage of phIGFBP-1, may more often become chronic, resulting finally in PTD. This theory is in line with observations that the earlier in pregnancy the abnormal microbial intrauterine invasion has been detected the greater the risk for adverse outcome (Goldenberg et al. 2002, Iams 2003, Lamont and Sawant 2005, Redline 2004, Romero et al. 2006b).

Yet, another explanation for an increased cervical fluid phIGFBP-1 level as well as for an increased rate of PTD among women with elevated phIGFBP-1 could be past decidual haemorrhage. Decidual haemorrhage, appearing as vaginal bleeding is a well known etiologic factor of PTD (Goldenberg et al. 2008, Romero et al. 2006b). Although women with visible bleeding at the time of sampling were excluded, those with the history of vaginal bleeding were included. Among those an elevated cervical fluid phIGFBP-1 could be a marker of previous (occult or clinical) decidual haemorrhage and associated tissue

disruption in decidua. Significant association between previous vaginal bleeding and elevated cervical fluid phIGFBP-1, as described in Study I and II supports this hypothesis. Previous vaginal bleeding in current pregnancy and elevated cervical fluid phIGFBP-1 in the first trimester also independently increased the risk for PTD.

Elevated cervical fluid phIGFBP-1 in the first trimester was stronger predictor of PTD at < 32 weeks of gestation than at < 37 weeks. This is an interesting observation considering that subclinical infection at choriodecidual interface, particularly in early pregnancy, has been associated especially with early PTD (Goldenberg et al. 2000, Iams 2003). According to the ROC curve analysis 10μg/l appeared to be optimal cut-off for cervical fluid phIGFBP-1 in predicting PTD < 32 weeks of gestation. The sensitivity (53.8%) of the first trimester cervical fluid phIGFBP-1 in the prediction of subsequent PTD < 32 weeks of gestation was strikingly high considering the multifactorial etiology and multiple pathophysiological pathways of PTD as well as timing of cervical fluid sampling among asymptomatic women. In the mid-second trimester, the sensitivity of cervical phIGFBP-1 was lower. One reason for the low positive predictive values of phIGFBP-1 in both trimesters depends on the low rate of endpoints especially < 32 weeks of gestation. The negative predictive value of in this study was high, being in line with other studies among asymptomatic women at later stage of pregnancy (Altinkaya et al. 2009, Kekki et al. 2001, Paternoster et al. 2007). However, the high rate of false positive (physiologic) phIGFBP-1 values in early pregnancy limits the use of cervical phIGFBP-1 as a screening test for subsequent spontaneous PTD among asymptomatic women. Still, cervical fluid phIGFBP-1 testing may be of value as an additional test in the assessment of preterm risk in selected cases also in early pregnancy. Of course, we need to consider that any testing adds little or nothing to outcome if we do not have right interventions to prevent spontaneous PTD (Honest et al. 2009). Thus, to develop new predictive and preventive methods more information is needed on physiology and pathophysiology of PTD in early pregnancy.

**PhIGFBP-1 as a predictor of spontaneous PTD among symptomatic women (III)**

Preterm contractions are very subjective symptom but a cause of major anxiety for pregnant women and strain heltcare services. It is known that 30-40% of women with preterm contractions will end to PTD (Herbst and Nilsson 2006). In our study, 4.1% of women who presented unscheduled with self reported contractions between 22 and 34 weeks of gestation delivered < 34 weeks of gestation. This suggests that the study population represented

predominantly low-risk women. Secondly, this indicates that uterine contractions alone are poor predictor of PTD. Therefore, more sensitive and more specific tests to be used in combination with clinical judgment for identifying women who would benefit of admission and preventive interventions are needed. Also unnecessary admissions and treatments should be avoided.

In study III, we assessed the diagnostic performance of the rapid strip test for cervical fluid phIGFBP-1 (actim partus test, Medix Biochemica) with the detection limit of 10 μg/l, and cervical length measurement as single tests and in combination with physician's clinical judgment in the prediction of PTD ≤ 34 weeks of gestation in patients with self reported uterine contractions and intact membranes during the second half of pregnancy. Cervical length measurement was a part of clinical evaluation, whereas phIGFBP-1 test results were not available to the managing physician.

The presence of phIGFBP-1 in cervical/vaginal fluids during the second half of pregnancy is thought to be an indicator of infection/inflammation associated tissue disruption at the choriodecidual interface (Rutanen 2000, Kekki et al. 2001). Also cervical ripening is associated with an increase in cervical fluid phIGFBP-1 concentrations (Nuutila et al. 1999).

In our data (III) the rapid phIGFBP-1 test was positive in 14.6%. In a previous study by Kekki et al. (2001) about 5% of asymptomatic women and 27 % of women with preterm labor had phIGFBP-1 values ≥ 10μ/L in the lower genital tract in the late second and third trimester (Kekki et al. 2001). Similar results were reported by Eroglu et al 2007 (Eroglu et al. 2007). Two studies have reported three times higher rates of elevated phIGFBP-1 concentrations in asymptomatic women between 24 to 34 weeks. In one of these studies women had a history of previous PTD (Bittar et al. 2007), whereas in another study women with a history of previous PTD were excluded (Altinkaya et al. 2009). In women with monitored regular contractions or PTL the reported rates of elevated phIGFBP-1 vary between 24-50 % (Altinkaya et al. 2009, Eroglu et al. 2007, Kekki et al. 2001, Kwek et al. 2004, Lembet et al. 2002, Paternoster et al. 2009, Spinelli et al. 2009, Tanir et al. 2009, Ting et al. 2007). Thus, the low rate of positive phIGFBP-1 test results in our data in women with self-reported contractions also indicates that these women represented more low than high risk patients for PTD.

The phIGFBP-1 test identified 50% of patients who delivered ≤ 34 weeks, and 71.4% of those who had PTD within 14 days. The positive predictive values of the rapid phIGFBP-1 test were lower in our study than in many other studies among symptomatic patients (Altinkaya et al. 2009, Akercan et al. 2004, Elizur et al. 2005, Eroglu et al. 2007, Kwek et al. 2004, Lembet et al. 2002, Paternoster et al. 2007, Paternoster et al. 2009, Ting et al. 2007), but in line with two studies among asymptomatic women (Altinkaya et al. 2009, Paternoster et al. 2007), also supporting that our study population was mainly low risk. One reason accounting for the difference may be that patients in our study had self reported contractions, while in other studies patients have been in labour with monitored contractions.

In our study short cervix (< 25 mm) was detected in 7.3% of women. Short cervix alone had lower sensitivity but higher positive predictive value than phIGFBP-1 alone, while clinician's judgment (admission), including cervical length measurement predicted 7/10 preterm deliveries < 34 weeks of gestation. This reveals the value of careful clinical assessment which includes sonographic cervical length measurement. Since a negative phIGFBP-1 test has a high negative predictive value for PTD, comparable to that of cervical length measurement in this and other studies (Eroglu et al. 2007, Paternoster et al. 2009), it may provide a valuable alternative to ultrasonographic cervical length measurement to be used in combination with clinical evaluation in prediction of PTD, especially if equipments or skills for ultrasonographic cervical length measurement are missing.

Unfortunately, we did not have repeated tests from the study population so we do not know whether phIGFBP-1 persisted in some women or whether it was only associated with contractions. It is possible that increased uterine activity may cause tissue disruption or decidual bleeding in choriodecidual interface which subsequently heals and pregnancy continues uncomplicated. This was supported by an association between previous vaginal bleeding and a positive phIGFBP-1 test result. Further studies with sequential sampling are needed to clarify this issue. As informed by the manufacturer (Medix Biochemica), overt bleeding is a confounder for phIGFBP-1 bed-side testing since similar phosphoisoforms of IGFBP-1 predominate in decidua and maternal blood (Martina et al. 1997).

In line with our study, previous studies with combinations of phIGFBP-1 rapid test and cervical length measurements, using the cutoff of 25mm, have lower sensitivity but higher specificity suggesting better likelihoods ratios for PTD (Bittar et al. 2007, Eroglu et al. 2007, Paternoster et al. 2009). Two studies have compared phIGFBP-1 and FFN bedside

tests for predicting PTD within 7 days. Both test showed to have equal efficiency in patients with signs and symptoms of PTD (Eroglu et al. 2007, Ting et al. 2007).

**MMP-8 concentrations and factors affecting them in cervical and vaginal fluids in the first and mid-second trimester (IV,V)**

Matrix metalloproteinase 8 (MMP-8), a collagenase, is released from the secondary granules of polymorphonuclear cells in response to chemotactic stimulation during inflammatory or infectious conditions. Furthermore, evidence is emerging that a wide range of inflammatory, mesenchymal, epithelial and malignant cells can express MMP-8 (Hanemaaijer et al. 1997, Maymon et al. 2000c, Sorsa et al. 2006, Van Lint and Libert 2006). We report for the first time the presence, concentrations, and factors associating with MMP-8 in the lower genital tract in first and mid-second trimester. To our knowledge there is only one previous report on MMP-8 concentrations in vaginal fluid (Diaz-Cueto et al. 2006). Increased MMP-8 in cervical tissue with cervical ripening has been described in several studies (Dubicke et al. 2008, Sennstrom et al. 2003, Winkler et al. 1999). MMP-8 concentrations and their associations with infections and PTD have mainly been studied in AF (Biggio et al. 2005, Kim et al. 2007, Lee et al. 2008a, Maymon et al. 2000c, Maymon et al. 2001a, Maymon et al. 2001b, Nien et al. 2006, Park et al. 2008).

In our study IV almost 100% of women had detectable MMP-8 concentrations in lower genital tract. The distribution of MMP-8 was markedly skewed in both trimesters, reflecting that most pregnant women typically have low levels of this substance in the lower genital tract. The overall distribution was similar in early and mid second trimester. However, the median MMP-8 concentration was about three times higher in the cervix than in the vagina in both trimesters.

Possible explanations for the detected differences between vaginal and cervical MMP-8 concentrations can be for example individual variations in the amount of vaginal or cervical fluids as well as variations in the time the swab is allowed to absorb those fluids. Elevation of MMP-8 concentrations in the cervical canal also indicates enhanced neutrophil activation and may eventually reflect the variety of pathophysiologic pathways that are involved in ascending infection into the decidua. Other cellular origins and biological functions for MMP-8 are also possible. However, these variables do not account for the wide range of MMP-8 values in our study. In a previous study, MMP-8 concentration was consistently

higher in amniotic fluid obtained from the lower uterine compartment than in the fluid from the upper compartment, suggesting that MMP-8 may act locally and play a role in tissue remodelling before rupture of the membranes (Maymon et al. 2000c). This and our present findings suggest that MMP-8 in the lower uterine compartment and in cervical canal is involved in the physiologic inflammation process during pregnancy.

In agreement with reports on MMP-8 in amniotic fluid and fetal membranes, MMP-8 concentrations in cervical fluid were highly variable (Biggio et al. 2005, Lee et al. 2007, Maymon et al. 2000). This and the only moderate correlation between the first trimester and second trimester MMP-8 values, as shown in our study, suggest that factors or events that regulate local MMP-8 concentrations in the cervical fluid, such as neutrophil degranulation induced by proinflamatory mediators or microbial factors, are inconstant at least in early and mid-pregnancy.

In our study IV and V, MMP-8 concentrations in cervical fluids were 10-100 times higher than those reported in amniotic fluid without microbial invasion, but similar to those in amniotic fluid with microbial invasion (Biggio et al. 2005, Lee et al. 2007, Maymon et al. 2000).

BV, with incidence only of 4 % in our study IV, was associated with increased MMP-8 concentrations in the cervical and vaginal samples. In previous studies BV has been reported in 15 % - 30 % of non-pregnant women, and in up to 50 % of pregnant women (Goldenberg et al. 1996, Goldenberg et al. 1998, McGregor and French 2000, Meis et al. 1995, Nelson et al. 2009). In another study with BV incidence of 47 %, BV was associated with increased MMP-8 concentration in vagina (Diaz-Cueto et al. 2006). In two previous Finnish studies among nulliparous women, the BV incidence has been 10% to 21%, indicating some biologic fluctuation in Finnish population over time in the incidence of BV (Kekki et al. 1999, Kekki et al. 2001, Kurki et al. 1992). The same diagnostic criteria of BV were used as in our study. According the previous studies BV increases the risk for spontaneous PTD and PPROM 2- to 3-fold (Goldenberg et al. 2000, Romero et al. 2004). Thus, low incidence of BV may partly explain the low incidence of PTD (4%) in our study.

One third of women (IV) had elevated amount of leukocytes, quantified from vaginal smears, with significant association with increased MMP-8 concentrations in lower genital tract in both trimesters. This association was strongest in the women with increased vaginal leukocytes together with BV. Elevated amount of leukocytes may be a marker of vaginal

infection or inflammation and may reflect the host defence mechanisms, while BV alone is not associated with signs of inflammation despite substantial microbial overgrowth (Goldenberg et al. 2000, Hay et al. 1994, Romero et al. 2004). The most likely explanation for elevated MMP-8 concentration is the recruitment and activation of neutrophils by micro-organisms in the vagina and cervical canal which may further ascend into the choriodecidual space. This hypothesis is supported by an in vitro study, where the incubation of membranes with microbial proteases reduced their strength and elasticity (McGregor et al. 1986). In addition, an elevated MMP-8 level cervicovaginal fluid could be also a potential marker for infection in the lower genital tract and, therefore, an indicator of an increased risk of PTD. However, it is not clear whether MMP-8 acts only locally.

MMP-8 has been described to correlate with cervical ripening (Dubicke et al. 2008, Sennstrom et al. 2003). In our study IV and V, cervical and vaginal fluid MMP-8 concentrations were three times higher in multiparous than in nulliparous women in the first and the second trimester. This suggests that the cervical connective tissue, mainly type I collagen, is more exposed to proteolytic modulation due to the previous deliveries. In a murine model the enhanced proteolytic burden involving MMP-8 in post partum cervical involution, associated with earlier deliveries, can eventually modify the cervical connective tissue by revealing cryptic (extracellular matrix) neoepitopic fragments in extracellular matrix, possessing thus chemotactic activities for neutrohilic leukocytes (Van Lint and Libert 2006, Uldbjerg et al. 1983a). Consequently, the triggered neutrophils may degranulate their MMP-8, or alternatively, neutrophil-derived proinflammatory mediators may induce de-novo expression of MMP-8 by resident cervical non-neutrophil-lineage cells (fibroblasts, epithelial cells etc). In this regard the cervical connective tissue compartment of multiparous women eventually differs from nulliparous women whose cervical connective tissue has not experienced the delivery-associated enhanced proteolytic burden. Another explanation may be that the volume and quality of cervical mucus plug differ between multiparous and nulliparous women. This smear separates uterine cavity from potentially harmful bacteria colonizing the vagina. The neutrophils are expected to invade the plug from the surrounding endocervix, a permanent site for inflammation during pregnancy. The plug is bactericidal and contains large amounts of antimicrobial factors, including secretory leukoprotease inhibitors, suggesting local defence mechanism, a physical but also a biochemical barrier which protects the fetomaternal unit against ascending intrauterine infection during pregnancy (Hein et al. 2001).

In addition to it's classical tissue destructive activity, MMP-8 can also process and activate anti-inflammatory cytokines, chemokines and defensins involved in the defensive

processes, and thus exert protective action against infection induced inflammations (Balbin et al. 1998, Gueders et al. 2005, Kuula et al. 2009, Owen et al. 2004, Sorsa et al. 2006, Van Lint and Libert 2006). Therefore, elevated MMP-8 in vagina and in cervix of multiparous relative to the nulliparous women may reflect, at least in part, host protection against infections and BV.

In our studies IV and V previous vaginal bleeding during pregnancy had no effect on vaginal and cervical MMP-8 concentrations, suggesting that MMP-8 level in vaginal or cervical secretion does not reveal the reason of vaginal bleeding.

In our study IV and study V, one third to one forth women expressed having had sexual intercourse during the last 48 hours before sampling. Both our data showed that sexual intercourse was associated with decreased MMP-8 concentration in the lower genital in both trimesters. This finding is of a great interest but difficult to explain, since no reports have been published on the relationship between MMP-8 and seminal plasma in female genital tract. Our results suggest that seminal plasma may decrease MMP-8 release from leukocytes, or seminal plasma may contain factors which can consume MMP-8.

In study V remarkable overweight (BMI > 30 kg/m$^2$) was associated higher MMP-8 concentrations, which may reflect persistent inflammatory process in obese women. In previous studies, obesity has been associated with an increased risk of PPROM (Nohr et al. 2007, Rosenberg et al. 2005), but this association was not found in our study.

**MMP-8 as a predictor of spontaneous PTD among asymptomatic women in the first and mid-second trimester (V)**

In our study V when all preterm deliveries were analysed as a group and compared with term deliveries, no difference was found in the median cervical fluid MMP-8 concentrations either in the first or mid-second trimester. However, low MMP-8 concentrations in the cervical fluid were associated with PTD initiated by PPROM. Our data suggests that distinct molecular pathways may result in PTD initiated by PPROM and PTL.

The increase of MMP-8 concentrations in cervical canal eventually may reflect enhanced neutrophil activation and maternal inflammatory response to micro-organisms ascending from the vagina. Other cellular origins and biological functions for cervical MMP-8 are also possible. It is well known that the cervix becomes softer in pregnancy due to increased vascularity and a loosening of the connective tissue. This process, beginning weeks before delivery, involves intensive remodelling of the extracellular matrix with extensive changes in concentration and composition of collagens and other matrix components. The changes increase as pregnancy advances and peak as the cervix effaces and dilates during delivery (Bergelin and Valentin 2002, Iams 2003, Leppert 1995, Rath et al. 1998, Weiss et al. 2007). Considering its specific collagenolytic activity, MMP-8 may play an important role in this process. In keeping with this theory, MMP-8 is increased in cervical tissue during cervical ripening at term as compared with cervical tissue of non-pregnant women (Sennstrom et al. 2003). In our study V, high cervical fluid MMP-8 concentrations in some of the women ending to PTD with intact membranes may reflect increased collagenolytic activity in the cervix due to MMP-8.

It has been thought that neutrophils and MMP-8 in amniotic fluid represent fetal inflammatory response to infections ascending from the vagina into choriodecidual space (Goldenberg et al. 2008, Mercer 2003, Iams 2003, Nien et al. 2006, Romero et al. 2006b). Microbial invasion of the amniotic cavity has been associated with a significant increase in amniotic fluid MMP-8 concentrations in patients with PTL and intact membranes as well as in patients with PPROM (Maymon et al. 2000). The association between low cervical fluid MMP-8 concentrations and subsequent PPROM, as seen in our study V, differs from the amniotic fluid findings by Biggio et al (2005). They demonstrated that the overall distribution of mid-trimester amniotic fluid MMP-8 concentrations did not differ between women with term and subsequent PTD, but marked elevation (> the 90 percentile) of MMP-8 was highly associated with subsequent PPROM (Biggio et al. 2005). These findings suggest that distinct functions of MMP-8 are involved in amniotic fluid and in cervical fluid in cases with PPROM during pregnancy. In addition, Lee at al (2008) recently demonstrated that intraamniotic inflammation, regardless of amniotic fluid culture result, is present in approximately 80% of patients with acute cervical insufficiency (Lee et al. 2008a). It is thought that the detection of inflammation in amniotic fluid may be more practical than the detection of infection in patient management (Shim et al. 2004). Furthermore, recent evidence has demonstrated that patients with intraamniotic inflammation but a negative microbiologic culture have a similar adverse pregnancy outcome than those with positive culture, which may reflect the fetal inflammatory response (Maymon et al. 2001b, Shim et al. 2004, Yoon et al. 2001). Still, the source of neutrophils in amniotic fluid and cervical fluid may differ. Further studies are needed to clarify reason(s) for this diversity in cervical fluid MMP-8 (maternal response) and amniotic fluid MMP-8 (fetal response) concentrations in relation to PPROM.

Recent genetic epidemiological PTD studies suggest that certain women are hyperresponders and some hyporesponders to microorganisms (Romero et al. 2004, Simhan et al. 2003). In support of this hypothesis Simham et al recently reported that women with lower concentrations of cytokines in the vaginal fluid in early pregnancy more likely develop clinical chorionamnionitis than those without low concentration of these cytokines (Simhan et al. 2003). Hyporesponding patient with low concentration of cytokines, and consequently possibly MMP-8, then would be predisposed to overgrowth of microorganisms and ascending intrauterine infection (Romero et al. 2004). This suggestion is further supported and extended by our findings revealing decreased cervical fluid MMP-8 concentrations in mid pregnancy in some women with subsequent PPROM. Low level of MMP-8 in cervical fluid reflects reduced release of MMP-8 from neutrophils in the lower genital tract. This might reflect an impaired defensive immunoresponse to microbial invasion into the decidua and membranes in certain women. In this regard, MMP-8 seems to induce a protective and anti-infammatory action against infection as demonstrated by MMP-8 knock out mice studies (Gueders et al. 2005, Kuula et al. 2009). Indeed, low MMP-8 concentrations are associated with delayed or impaired neutrophil function (Van Lint and Libert 2006).

Altogether data of recent MMP-8 studies suggest dual roles in the lower genital tract, destructive and defensive. The clinical significance of these observations regarding PTD remains to be clarify.

**Strengths and limitations of the studies**

The strength of this study is that it gives us baseline knowledge of phIGFBP-1 and MMP-8 with large size of sample. Another, strength was it was population based with a low drop out rate. All phIGFBP-1 and MMP-8 measurements were performed each in a single laboratory and in study IV the vaginal smear slides were processed and examined by one laboratory technician without the knowledge of any clinical characteristics of the study subjects. In study II and V the rate of preterm deliveries in our study was comparable to that in general population. In addition, known risk factors and their contributions for the risk of PTD were considered.

One of the limitation is that vaginal bacterial flora was analyzed only in study III and IV. Furthermore we did not test specific vaginal infections, such as Clamydia trachomatis, Trichomoniasis, Mycoplasma or group B β- streptococcus, considering that vaginal infections with neutrophil activation may play a crucial role in cervical canal and

choriodecidual space. Unfortunately in study III, we had not repeated tests from the study population so we do not know whether phIGFBP-1 persisted in some women or whether it was only associated with contractions. And we did not have FFN test at the same time for comparison with phIGFBP-1 test performances. In addition, in study V we analyzed only one matrix metalloproteinase, MMP-8, although other metalloproteinases and their regulators are likely to play a role in pregnancy and some of them evidently also contribute to the inflammatory mileu of the lower genital tract. Furthermore, we unfortunately did not have the results of leukocytes in vaginal smear and BV together with MMP-8 concentrations for prediction to PTD in study V. In the future, further studies for diversity in cervical fluid MMP-8 with bacterial vaginosis and specific infections in cases of PPROM and spontaneous labour are required to draw conclusions.

# CONCLUSIONS

The following conclusions can be drawn:

- PhIGFBP-1 and MMP-8 are present in lower genital tract fluids in the first and mid-second trimester. Both phIGFBP-1 and MMP-8 concentrations were higher in cervical fluid than in vaginal fluid. These data indicate that the site of sampling is important when these biomarkers are measured in the lower genital tract.

- The levels and the occurrence of cervical fluid phIGFBP-1 concentrations of $\geq$ 10 µg/l decreased significantly from the first to the second trimester. Nulliparity, especially in women with previous pregnancies and previous vaginal bleeding in the current pregnancy, was were associated with phIGFBP-1 values of $\geq$ 10 µg/l in cervical fluid samples.

- An elevated concentration ($\geq$ 10 µg/l) of cervical fluid phIGFBP-1 in the first trimester was an independent predictor of PTD (OR 3.0) with a high NPV for PTD among asymptomatic women in early and mid-pregnancy. Because of its relatively low sensitivity and specificity, assay of cervical fluid phIGFBP-1 is not appropriate for screening for PTD risk in early pregnancy, but it may provide an additional tool when assessing the risk of PTD in selected cases in early pregnancy.

- In women with self-reported uterine contractions between 22 and 34 weeks of gestation, a positive rapid phIGFBP-1 bedside test result was associated with a 7-fold increased risk of PTD, and a negative test result had a negative predictive value comparable to that of ultrasonographic cervical length measurement. Thus, the rapid phIGFBP-1 strip test may provide a valuable alternative or addition to ultrasonographic cervical length measurement to be used in combination with clinical assessment in prediction of PTD in symptomatic women presenting with contractions.

- MMP-8 was present in the lower genital tract in almost 100% of the women, at concentrations ranging from 0.08 to 2000 µg/l. No difference was found in the values between the first and second trimester. Bacterial vaginosis, vaginal leukocytosis, multiparity and an overweight condition are significantly associated with increased levels of MMP-8 in cervical fluids.

- Cervical fluid MMP-8 concentrations in early and mid-pregnancy are not related to subsequent PTD. However, low concentrations of cervical fluid MMP-8 were associated with PTD initiated by PPROM. These data suggest that the molecular mechanisms and pathophysiological pathways resulting in PTD after PTL and after PPROM differ, and MMP-8 concentrations in the low and upper ranges possibly reflect dual roles of MMP-8, i.e. destructive and defensive. The clinical significance of these findings in relation to obstetric outcomes remains to be studied.

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