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**PATOPHYSIOLOGY OF POSTTERM
PREGNANCY
EPIDEMIOLOGY, RISK FACTORS AND CERVICAL
RIPENING**

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To my family

***“Cambia lo superficial
Cambia también lo profundo
Cambia el modo de pensar
Cambia todo en este mundo...”***

Julio Numhauser

ABSTRACT

Background: Postterm pregnancy, defined as a pregnancy of 42 or more completed weeks of gestation, occurs in approximately 5-10 % of all pregnancies. The condition is associated with increased maternal and fetal morbidity as well as an increased risk of perinatal mortality. The risk of postterm pregnancy is higher among women who are nulliparous and of advanced maternal age. Genetic predispositions may also contribute to an increased risk of postterm pregnancy. Certain characteristics, notably obesity, advanced maternal age and nulliparity are common in women with delayed parturition and are also common among women with polycystic ovary syndrome (PCOS). However, outcomes of pregnancy among women with PCOS have been difficult to investigate due to confounding variables. Normal and delayed parturition is a complex and poorly understood process. A key component of normal parturition is the inflammatory process in the cervix, termed “cervical ripening”, which precedes normal labor. The physiology of cervical ripening, the causes of absent cervical ripening postterm, and why some women do not respond with adequate cervical ripening after administration of prostaglandins, are not properly understood. This thesis aims to describe risk factors for postterm pregnancy and failed labor induction, to investigate expression of prostaglandin receptors and cytokines in postterm women with failed and successful labor induction, as well as investigating the association between polycystic ovary syndrome, postterm pregnancy and adverse pregnancy outcomes.

Methods: In paper I, risk factors for postterm pregnancy were determined using data from the Swedish Medical Birth Registry (MBR) where a cohort of term and postterm singleton births, taking place between 1992 to 2006 (total n=1,176,131), was identified. In paper II, a cohort of singleton births from 1995 to 2008 (n=1,191,336) was identified in the Swedish Medical Birth Registry, out of which 3,787 were born to a mother with a previous diagnosis of PCOS. In paper III and IV, transvaginal cervical biopsies were taken from non-pregnant, term pregnant and postpartal women as well as from postterm women with failed and successful labor induction. The biopsies were analyzed for mRNA expression with real-time PCR (RT-PCR). Immunohistochemistry was performed to analyze expression and distribution of cytokines (IL-1 β , IL-6, IL-8, IL-10 and IL-18), prostaglandin receptors (EP1-4 and FP) and stromal factors (CTGF, cathepsin B, furin and ALOX 15).

Results: We identified advanced maternal age, nulliparity and BMI > 30.0 kg/m² as risk factors for postterm pregnancy and cesarean section following labor induction postterm. We found that a previous diagnosis of PCOS was not associated with postterm pregnancy. However, we found that independently from assisted reproductive technology and BMI, infants born to women with PCOS were at increased risk of adverse pregnancy outcome. In paper III and IV, we found that impaired cervical ripening in postterm women with failed labor induction was associated with an elevated value of the ratio in the mRNA expression of EP3 and EP4. We also found an overall down-regulation of pro- and anti-inflammatory cytokines.

Conclusions: The results imply that pregnant women with PCOS should be considered as a high risk group of adverse pregnancy outcomes and the obstetric guidelines should be reviewed. Women with postterm pregnancy are obese, nulliparous and of advanced age as compared to others. Down-regulation of pro- and anti-inflammatory cytokines among women with impaired cervical ripening as well as differences between women in the expression of EP3 and EP4 provides important information for an improved understanding of the physiology of normal and delayed parturition.

Key words: *Postterm pregnancy, cervical ripening, cytokines, prostaglandin receptors, labor induction, polycystic ovary syndrome, obesity, cesarean section*
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1 LIST OF PUBLICATIONS

- I. Roos N, Sahlin L, Ekman-Ordeberg G, Kieler H, Stephansson O.
Maternal risk factors for postterm pregnancy and cesarean delivery following labor induction.
Acta Obstet Gynecol Scand. 2010 Aug;89(8):1003-10.
- II. Roos N, Kieler H, Sahlin L, Ekman-Ordeberg G, Falconer H, Stephansson O.
Risk of adverse pregnancy outcomes in women with polycystic ovary syndrome: populations based cohort study.
BMJ. 2011 Oct 13;343:d6309.
- III. Roos N*, Blesson CS*, Stephansson O, Masironi B, Reinert S, Vladic Stjernholm Y, Ekman-Ordeberg G, Sahlin L.
Prostaglandin receptors and stromal factors in human cervix at term and postterm pregnancy after failed and successful labor induction.
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- IV. Roos N, Andersson E, Vladic Stjernholm Y, Stephansson O, Sahlin L, Ekman-Ordeberg G.
Reduced gene expression of cytokines in postterm women with failed labor induction.
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Cover illustration: Hunter, William. *The Anatomy of the Gravid Uterus* (1774)

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2 ABBREVIATIONS AND DEFINITIONS

ABC	Avidin-biotinylated peroxidase complex
ANOVA	Analysis of variance
AFI	Amniotic fluid index
ART	Assisted reproductive technology
BMI	Body mass index
C	Control group (paper III and IV)
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
COX	Cyclooxygenase
CRH	Corticotropin-releasing hormone
C _T	Threshold cycles
DAB	Diaminobenzidine
ECM	Extracellular matrix
ICD	International Classification of Diseases
GAG	Glycosaminoglycan
Ig	Immunoglobulin
IL	Interleukin
LGA	Large for gestational age
LPS	Lipopolysaccharide
MBR	Swedish Medical Birth Registry
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NICU	Neonatal intensive care unit
NO	Nitric oxide
NOS	Nitric oxide synthase
NP	Non-pregnant group (paper III)
NR	Non-responder group (paper III and IV)
OR	Odds ratio
PCOS	Polycystic ovary syndrome
PGE ₂	Prostaglandin E ₂
PP	Postpartum group (paper III)
R	Responder group (paper III and IV)
RR	Relative risk
SD	Standard deviation
SGA	Small for gestational age
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TP	Term pregnant group (paper III)
Treg	Regulatory T-cells
WHO	World Health Organization

Low Apgar score	Apgar score of <7 at 5 minutes of age
Preterm birth	Infants born before 37 completed weeks of gestation
Moderately preterm birth	Infants born at 32 to 36 completed weeks of gestation
Very preterm birth	Infants born before 32 completed weeks of gestation
Stillbirth	Fetal death at 28 completed gestational weeks or later
Early neonatal death	Death before 7 days of life among live-born infants
Perinatal mortality	Still birth and early neonatal death
Neonatal death	Death before 27 days of life among live-born infants
Small for gestational age	Birth weight of less than 2 standard deviations below the mean birth weight adjusted for gestational age and sex

3 INTRODUCTION

The risk of perinatal death increases eightfold from 37 to 43 weeks of gestation (Hilder, Costeloe et al. 1998). In addition, postterm pregnancy increases both fetal and maternal morbidity (Lindstrom, Fernell et al. 2005; Norwitz, Snegovskikh et al. 2007). The risks faced by the mother and infant during progressing gestation can however be avoided with labor induction before the postterm period (Hilder, Costeloe et al. 1998; Gulmezoglu, Crowther et al. 2006). Hence, management of postterm pregnancy forms a major part of obstetric care in settings where mothers access good antenatal care and where labor induction therapy is available. In settings where women have poor access to proper dating of gestational age and where pregnancy monitoring and labor induction therapy is not available, postterm pregnancy is an important cause of adverse health outcomes for both the mother and the infant. Despite the importance of postterm pregnancy the area has received little attention in obstetric research.

The onset of labor is a complicated and not yet fully understood process. Normal term parturition is preceded by structural changes in the cervix starting during early pregnancy. This process of cervical ripening includes softening, dilation and effacement of the cervix during weeks to hours before the onset of labor. There is evidence that the final cervical ripening is characterized by an inflammatory reaction. This process of normal cervical ripening seems to be disturbed in many postterm women. Cervical ripening and labor induction can be induced in postterm pregnancy by administration of prostaglandins. There is however a group of postterm women where this therapy does not induce cervical ripening, preventing vaginal delivery (Sahlin, Stjernholm-Vladic et al. 2008).

The biological rationale behind failed labor induction is not well-known. Interestingly, both the types of risk factors and the fraction attributable to the risk factors vary in different populations (Zeitlin, Blondel et al. 2007).

In the work behind this thesis, I started together with colleagues to study maternal risk factors for postterm pregnancy and failed labor induction from an epidemiological perspective (paper I). Among other results from paper I, we found a higher risk for postterm pregnancy among women who were overweight and obese, nulliparous and of advanced maternal age. As these characteristics are frequently shared by pregnant women with polycystic ovary syndrome (PCOS), I hypothesized that there could be an important relationship between PCOS and postterm pregnancy. While I investigated the current knowledge base on this issue it became apparent that previous studies on adverse pregnancy outcomes among women with PCOS, including postterm pregnancy, had been small in size and had foremost included women undergoing assisted reproductive technology (ART) to become pregnant. The effect of PCOS on pregnancy outcome, irrespectively of ART and body mass index (BMI), had therefore not been properly investigated. This prompted me to investigate, together with colleagues, the risk of adverse pregnancy outcomes, including postterm pregnancy among women with a previous diagnosis of PCOS. Sweden offers a unique setting for epidemiological studies because of the personal identification number assigned to all

citizens at birth or immigration and which can be used to link different high-quality Swedish registers. Thus, using the comprehensive Swedish Patient, Education and Medical Birth registers, pregnancy outcomes of PCOS could be studied in-depth. The results from paper II are described in detail in section 8.2.

Epidemiological studies can provide key insights into the causes and prevention of adverse health outcomes. It is however often important to also understand the underlying pathophysiology of the adverse health outcomes in order to advance clinical practice. A number of genes and their corresponding proteins have been identified as participants in cervical ripening and normal labor, but knowledge about the detailed interactions between them is only beginning to take shape.

The second part of this thesis (paper III and IV) therefor focused on the pathophysiology of postterm pregnancy and failed labor induction and in particular on the influence of prostaglandin receptors and cytokines on the process of cervical ripening and labor induction. In this thesis we have not investigated the changes involved in the remodeling of the extracellular matrix, but the mediators involved in the remodeling at final cervical ripening.

In summary, in this thesis I investigate the consequences and mechanisms behind postterm pregnancy, labor induction and PCOS from both an epidemiological and physiological perspective. The thesis includes first a background on the present knowledge-base of the research area (section 4), then describes the specific aims of the thesis (section 5), the study methods (section 6) and the major results (section 7). I conclude with a discussion on the relationship between the results and earlier research (section 8), propose avenues for future research (section 9) and summarize the policy implications of the thesis (section 11).

4 BACKGROUND

4.1 POSTTERM PREGNANCY

4.1.1 Definition and prevalence of postterm pregnancy

Postterm pregnancy is defined by the WHO as pregnancy with gestational length of 42 completed weeks or 294 days or more from the last menstrual period or 14 days beyond the estimated day of delivery by obstetrical routine ultrasound (1977).

The estimated prevalence of postterm pregnancy is about 5 to 10 % (1977), but varies world-wide depending on the characteristics of the studied population. There are difficulties with the classification which makes it difficult to make an exact description of the proportion of postterm pregnancy in the population. Factors that may influence the prevalence are interventions, such as scheduled cesarean sections and routine labor induction, the prevalence of routine ultrasound pregnancy dating, the rate of spontaneous preterm birth as well as maternal age and number of nulliparous women in the population (Norwitz, Snegovskikh et al. 2007).

4.1.2 Misclassification of postterm pregnancy

The most common risk factor for postterm pregnancy is an error in pregnancy dating. It is not a risk factor in the true sense of the word but rather a misclassification of the condition. The proportion of postterm prevalence is lowered by using second trimester routine ultrasound pregnancy dating as compared to using last menstrual period (Blondel, Morin et al. 2002). Women are more likely to have delayed ovulations and hence last menstrual period will result in an overestimation of the postterm pregnancy rate (Saito, Yazawa et al. 1972; Savitz, Terry et al. 2002). Ultrasound dating is however not an unbiased method since it presumes that all fetuses have the same size at a certain gestational age. It has however been demonstrated that there are significant differences in biparietal diameter in respect to infant sex (Pedersen 1980; Wald, Cuckle et al. 1986; Tunon, Eik-Nes et al. 1998), maternal smoking (Persson, Grennert et al. 1978; Hanke, Sobala et al. 2004) and early growth restriction (Thorsell, Kaijser et al. 2008). Routine ultrasound assessment has been found to introduce systematic misclassification of gestational age by sex increasing perinatal mortality and morbidity among infant postterm girls as compared to their male equivalents (Skalkidou, Kieler et al.).

4.1.3 Risk factors for postterm pregnancy

It has been proposed that some postterm pregnancies are biologically determined, since women tend to repeat postterm pregnancy after a prior postterm pregnancy (Mogren, Stenlund et al. 1999; Olesen, Basso et al. 2003; Kistka, Palomar et al. 2007) and also have a higher risk of postterm pregnancy when being themselves a product of a postterm pregnancy (Mogren, Stenlund et al. 1999). Further, twin studies also support a genetic influence in the incidence of postterm pregnancies. The rate of postterm pregnancy is increased in monozygotic twins if a twin sister has had a previous postterm pregnancy (Laursen, Bille et al. 2004). An association to paternal genes, expressed through the fetus, has also been described influencing the length of gestation. The recurrence of postterm pregnancy dropped from 19.9% to 15.4% in women who

changed their partner between the first and the second pregnancy (Olesen, Basso et al. 2003). Apart from the genetic component, other risk factors for postterm pregnancy have been identified such as nulliparity and obesity (Stotland, Washington et al. 2007; Denison, Price et al. 2008). Male fetal sex has been mentioned in the literature as a risk factor for postterm pregnancy (Divon, Ferber et al. 2002). However, misclassification in gestational length as mentioned above could be a plausible explanation (Skalkidou, Kieler et al.). There seems not to be a consistent association between maternal age and postterm pregnancy in previous studies (Kistka, Palomar et al. 2007; Caughey, Stotland et al. 2009). More rare contributors to the incidence of postterm pregnancy are placental sulfatase deficiency, fetal adrenal insufficiency or hypoplasia and fetal anencephaly and will not be discussed further (Macdonald and Siteri 1965; Naeye 1978; Rabe, Hosch et al. 1983).

4.1.4 Fetal risks in postterm pregnancy

During postterm pregnancy the fetus continues to grow while placental function decreases past term (Nahum, Stanislaw et al. 1995; Jazayeri, Tsibris et al. 1998). This increases the risk for macrosomia and subsequent risk for shoulder dystocia as well as dystocia and fetal distress during labor, increasing the rates of cesarean deliveries (Alexander, McIntire et al. 2000). Stillbirth rates, calculated as the risk among ongoing pregnancies, increases up to six-fold at 43 weeks of gestation and neonatal seizures and deaths doubles at 42 weeks of gestation (Hilder, Costeloe et al. 1998; Alexander, McIntire et al. 2000). Figure 1

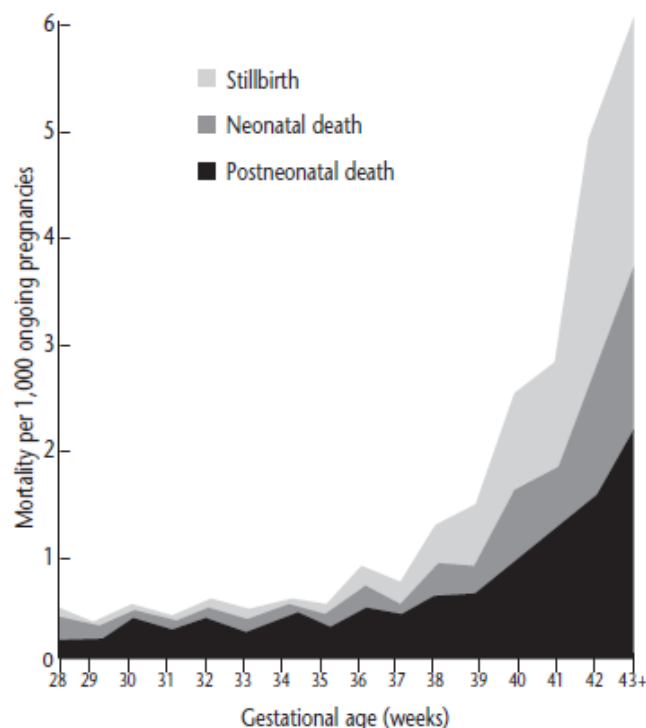


Figure 1 Illustration of perinatal mortality per 1000 ongoing pregnancies. From Hilder et al. *Br J Obstet Gynaecol.* 1998 Feb;105(2):169-73

With decreasing placental function, also the volume of amniotic fluid continues to decrease after term and may cause oligohydramnios (Link, Clark et al. 2007). Further, the rate of meconium release increases with advancing gestation after term and together with decreasing volume of amniotic fluid may increase the risk for meconium aspiration syndrome (Alexander, McIntire et al. 2000). It has also been shown that children born at 42 weeks of gestation or more have a higher rate of developmental abnormalities as compared with those born before 42 weeks of gestation, assessed by developmental tests at approximately 5 years of age (Lindstrom, Fernell et al. 2005).

4.1.5 Maternal risks in postterm pregnancy

postterm pregnancy is also associated with increased maternal risks such as protracted labor, third and fourth degree of perineal lacerations and cesarean section rates increase with advancing gestation as well as postpartum hemorrhage (Caughey, Stotland et al. 2007).

4.1.6 Management of postterm pregnancy

To prevent the risks associated with postterm pregnancy, routine labor induction is usually performed at 41 to 43 weeks of gestation depending on the setting and implemented guidelines. Antenatal fetal surveillance starting at 41 to 42 weeks of gestational is normally performed in low risk postterm pregnancies. There is however no consensus in the scientific community as to the optimal time of labor induction, nor the optimal way of surveillance. Commonly, measurements of amniotic fluid index (AFI) and non-stress testing are performed twice a week (Doherty and Norwitz 2008). Labor induction is usually performed using methods such as locally administered prostaglandin E₂ (PGE₂), oxytocin or by mechanical dilation using a Foley like catheter. In two systematic reviews exploring the benefits of routine labor induction as compared to expectant management there were fewer perinatal deaths in the group induced at 41 weeks of gestation as well as lower risk for mekoniumaspiration and cesarean section. The absolute risk for perinatal death is however very small (Gulmezoglu, Crowther et al. 2006; Caughey, Sundaram et al. 2009). The rate of labor induction irrespective of gestational age is increasing worldwide and in the United States the proportion has increased from 9.5 % to 22.5 % between 1990 and 2006. The rise in cesarean sections has been attributed to the increased rate of labor induction (Luthy, Malmgren et al. 2004; Martin JA 2009).

4.1.7 Prevention of postterm pregnancy

Membrane sweeping, also called stripping of membranes, starting at 38 weeks of gestation has been proven to reduce the rates of postterm pregnancy. The relative risk (RR) for continuing beyond 41 weeks of gestation is 0.59, with 95% confidence interval (CI) 0.46 to 0.74. Beyond 42 weeks of gestation the RR was calculated to 0.28, 95% CI 0.15 to 0.50. The procedure is performed by introducing the finger into the cervical os and by circular movements separating the inferior pole of the membranes from the lower uterine segment. The procedure is however associated with irregular contractions and bleeding and does not offer any other clinical benefits other

than reducing the risk of proceeding past term, and is hence not recommended (Boulvain, Stan et al. 2005).

4.1.8 Induction of cervical ripening and labor

Locally administrated PGE₂ (Dinoproston), vaginally or intracervically, is typically used for labor induction and cervical ripening before administration of oxytocin for labor augmentation. It has been found that PGE₂ shortens the time from induction to delivery when combined with oxytocin (Owen, Winkler et al. 1991).

Mechanical dilation is another mode for labor induction using a Foley like catheter introduced in the cervical os and with a downward tension it can lead to cervical ripening and contractions. It has been found that this method is effective for cervical ripening and shorter labors (Sherman, Frenkel et al. 1996). None of the above mentioned methods has been shown to be superior to the other (Chung, Huang et al. 2003).

In Sweden, standard practice for labor induction, differs between counties but encompasses the following methods: vaginal gel with prostaglandin E₂ (PGE₂) that is administered in the posterior fornix or intracervically, mechanical dilation with a Foley-like catheter or intravenous oxytocin infusion. The method of choice depends on the degree of cervical ripening assessed by Bishop's score (see table 1 for clarification).

4.2 PROGNOSTIC FACTORS FOR SUCCESSFUL LABOR INDUCTION

Cervical ripening is assessed by using the Bishop's score scheme which takes into account five characteristics of the cervix: cervical dilation, effacement, consistency, position and fetal station. The scoring system is used to predict labor induction outcome. It was first described in 1964 by EH Bishop (Bishop 1964). In Sweden a modified Bishop score is used (Westin score) taking into account the length of the cervix, the consistency, position, dilation and the position of the fetal head according to table 1. A modified Bishop's score of 9 or more predicts a successful labor induction. A Bishop's score of less than 5 identifies an unfavorable cervix with a need for labor induction.

Table 1 Schematic table of the parameters included in the modified Bishop's score (Westin score) used in cervical assessment before labor induction.

	0 points	1 point	2 points
Length of cervix	< 50 %	> 50 %	Effaced
Cervical consistency	Hard	Medium	Soft
Position of cervix	Sacral	Medium	Central
Dilation (cm)	<1	1-2	>2
Fetal head position	Above pelvic inlet	In pelvic inlet	Below pelvic inlet

Transvaginal sonography has been evaluated as an alternative method to Bishop's score, but has not been found to be superior in labor induction prediction (Hatfield, Sanchez-Ramos et al. 2007). However, both digital and transvaginal ultrasound cervical assessment appear to be poor predictors of vaginal delivery after labor induction in postterm pregnancy (Faltin-Traub, Boulvain et al. 2004; Hatfield, Sanchez-Ramos et al. 2007). Digital assessment of the cervix is nevertheless a widespread and easy method to

use and so far the best available instrument for prediction. Few studies have investigated maternal characteristics (age, BMI, parity) besides cervical assessment as independent predictors for failed labor induction (Faltin-Traub, Boulvain et al. 2004; Crane 2006; Hatfield, Sanchez-Ramos et al. 2007). In a recent study cervical length together with body mass index was shown to be superior to only using Bishop score in predicting labor induction success (Uyar, Erbay et al. 2009).

4.3 ANATOMY AND COMPOSITION OF THE NON-PREGNANT UTERUS

The uterus consists of two parts: the corpus uterus and the cervix uteri. The cervix undergoes changes during the ripening process to allow passage of the fetus and concomitantly the uterus changes from a quiescent state to a contractile organ to expulse the fetus through the birth canal. For illustration see figure 2.

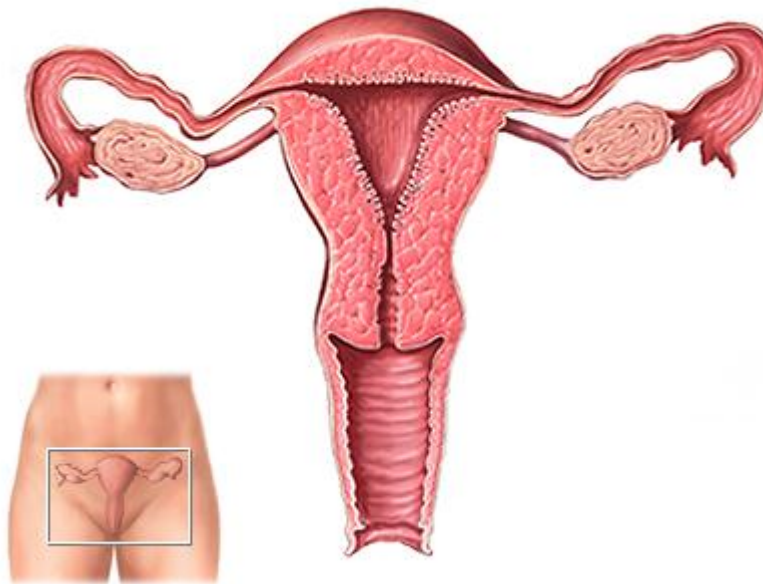


Figure 2 *Anatomy of the human cervix and uterus. Illustration published with license from ADAM images*

4.3.1 Cervix uteri

The major component of the non-pregnant cervix is extra cellular matrix (ECM), which accounts for about 85% of the tissue mass. Only 6-10% consists of muscle fiber and other cell types such as fibroblasts, epithelium and blood vessels (Danforth 1947; Schwalm and Dubrausky 1966; Rorie and Newton 1967). The ECM provides strength and stabilization to the cervical tissue. The main components of the ECM are fibrillar collagen, elastin, proteoglycans and polysaccharides (Uldbjerg, Ulmsten et al. 1983; Norman, Ekman et al. 1991). Collagen is the dominating component, from which type 1 (70%) and III (30%) are the major types (Uldbjerg, Ulmsten et al. 1983). The dominating proteoglycan is the dermatan sulfate decorin (Danforth 1947; Uldbjerg, Ekman et al. 1983; Uldbjerg, Malmstrom et al. 1983).

Decorin can form a glycosaminoglycan (GAG) chain and consolidates the collagen fibrils in the ECM together with a hydration of the tissue (Uldbjerg and Danielsen 1988; Norman, Ekman et al. 1991). Other important proteoglycans but in lower concentrations are versican, biglycan, fibromodulin, heparan sulphate and hyaluronic acid (Uldbjerg, Carlstedt et al. 1983; Westergren-Thorsson, Norman et al. 1998)

4.3.2 Corpus uteri

The uterus is divided into an upper and a lower part, the fundus and the isthmus respectively. In contrast to the cervix, 70% of the tissue weight of the corpus is muscular (Schwalm and Dubrausky 1966; Rorie and Newton 1967). The main contributors to the uterine ECM are collagen type I and III as well as proteoglycans and polysaccharides (Granstrom, Ekman et al. 1989; Hjelm, Barchan et al. 2002). For illustrative information on ECM content in the different parts of the uterus and cervix see figure 3.

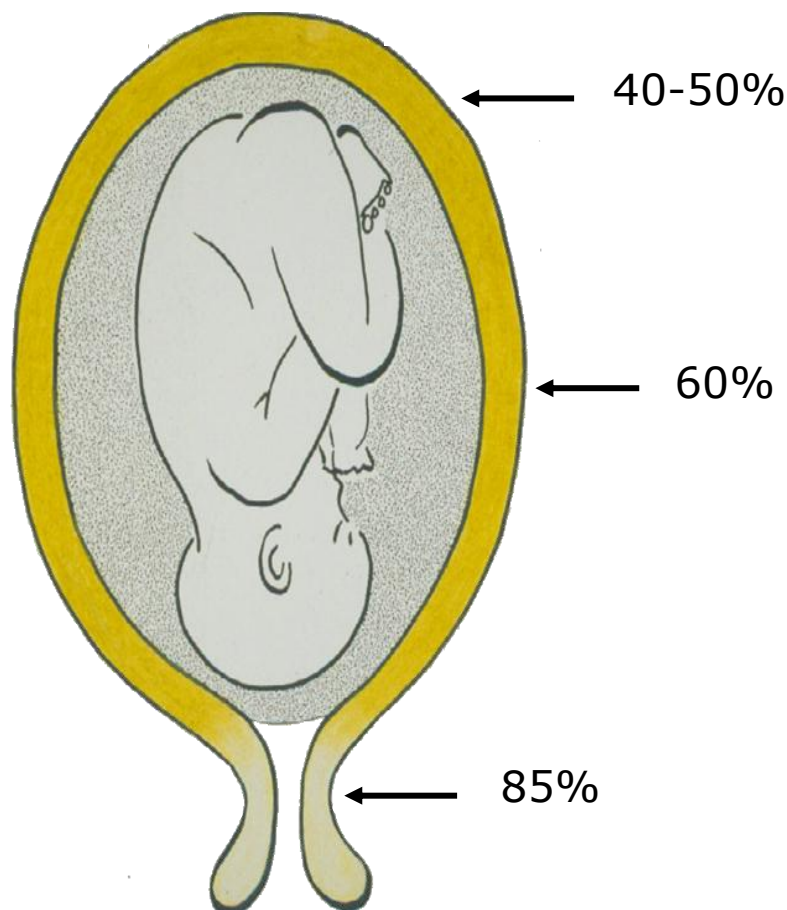


Figure 3 *The content of extracellular matrix (ECM) in different parts of the uterus and the cervix*

4.4 CHANGES TO THE CERVIX AND UTERUS DURING PREGNANCY AND PARTURITION

4.4.1 Cervical ripening

Radical morphological changes of the cervix are necessary to allow the fetus to be expelled from the uterus during labor. The structural changes taking place in the cervix include softening, dilation and effacement weeks before the onset of labor. Cervical ripening too early could result in preterm labor and the absence of cervical ripening can lead to postterm pregnancy or prolonged labor. During pregnancy, the cervix should undertake dramatic changes from being rich in fibrous connective tissue to become soft and dilated. This remodeling permits the passage of the infant through the birth canal as well as postpartum remodeling to allow a new future pregnancy cycle. See figure 4 for illustration of the remodeling process in the cervix.

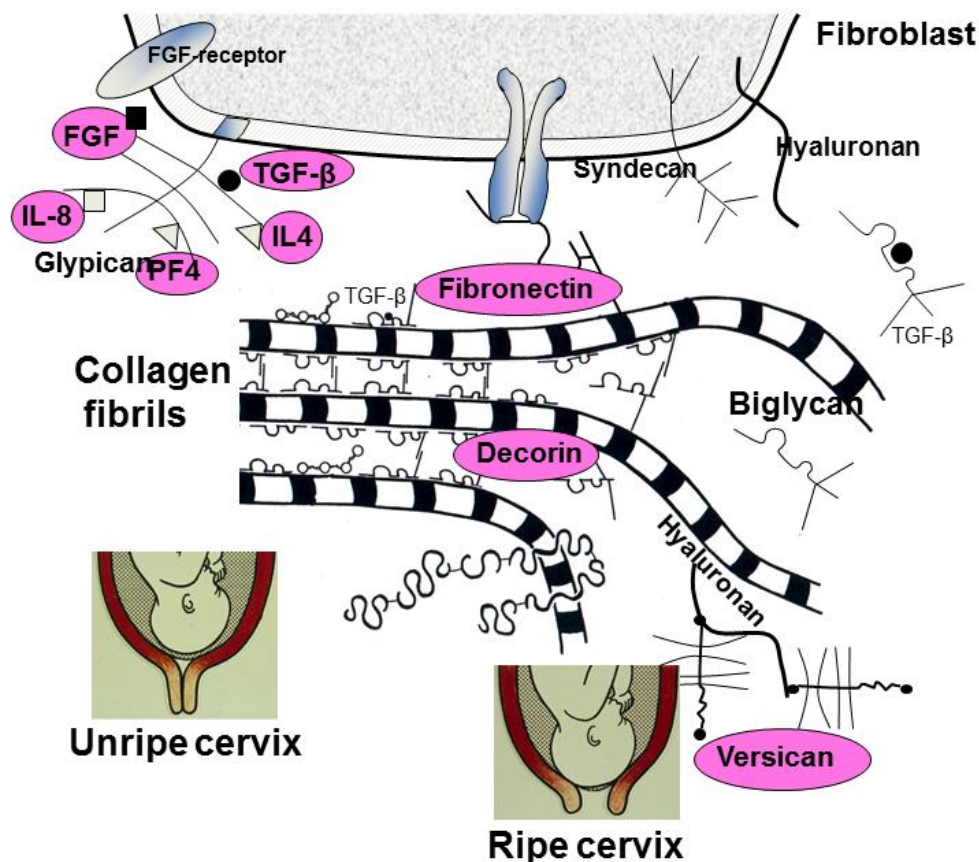


Figure 4 Schematic figure of the ripening process and changes in the extracellular matrix in the cervix. In the final ripening just before onset of labor the collagen fibrils are dispersed by an increase in large molecules i.e. large proteoglycan, versican and hyaluronan. (left: unripe, right: ripe).

The ripening process consists mainly of changes in the ECM. It is not affected by the frequency or the force of uterine contractions but can be induced by local application of PGE_2 (Ekman, Malmstrom et al. 1986).

Cervical ripening is partly an inflammatory process that can be divided into two phases: a slow phase occurring with a gradual decrease in collagen content as early as the first trimester without any signs of inflammation (Granstrom, Ekman et al. 1989) and a quick phase driven by an inflammatory reaction occurring hours before parturition (Uldbjerg, Ekman et al. 1983; Granstrom, Ekman et al. 1989) characterized by the presence of pro-inflammatory cytokines and leukocytes (Sennstrom, Ekman et al. 2000; Kelly 2002; Stygar, Wang et al. 2002; Keelan, Blumenstein et al. 2003). In genome wide studies, inflammatory genes have not been detected differentially expressed in cervical samples from term women with ripe and unripe cervixes (Huber, Hudelist et al. 2005; Hassan, Romero et al. 2006), indicating that cervical ripening inflammation characterizes the hours around onset of labor.

The collagen content decreases throughout pregnancy. As compared to the non-pregnant state the collagen content has decreased to 70% at 10 weeks of gestation and 30% at term (Uldbjerg, Ekman et al. 1983). Further, there are changes in the concentration and ratios of both small and large glycosaminoglycans together with decreased collagen content which explains the clinical changes known as softening and dilation. There is an increase in the large chondroitin sulphate proteoglycan versican which in turn can bind both water and hyaluronan and as a consequence disintegrates the collagen bundles. Parallel to this process there is a decrease in the content of the small proteoglycans biglycan and decorin (Norman, Ekman et al. 1993; Westergren-Thorsson, Norman et al. 1998; Wu, La Pierre et al. 2005)

The inflammatory reaction during the second stage of cervical ripening was suggested as early as 1978 (Liggins 1978) and is characterized by infiltration of neutrophils into the stroma as well as an increase in the protein and gene expression of the pro-inflammatory cytokines IL-6 and IL-8 (Sennstrom, Ekman et al. 2000; Young, Thomson et al. 2002; Osman, Young et al. 2003; Tornblom, Klimaviciute et al. 2005). The increase in cytokines recruit activated neutrophils to the cervical tissue. The invading neutrophils have been correlated to the increase in degradative enzymes such as matrix metalloproteinases (MMPs), specifically MMP-2, MMP-8 and MMP-9 (Barclay, Brennand et al. 1993; Stygar, Wang et al. 2002; Sennstrom, Brauner et al. 2003).

4.4.2 Changes to the myometrium during pregnancy and labor

During pregnancy the uterus, in contrast to the cervix, increases in size from 70 to 1100 grams. The cavity's volume concomitantly increases from less than 10 ml in the non-pregnant state to an average volume of five liters at term (F. Gary Cunningham 2010).

The myometrium is kept in a quiescent state throughout pregnancy by the influence of various substances and hormones such as nitric oxide (NO), corticotropin releasing hormone (CRH) and progesterone (Challis, Sloboda et al. 2002; Maul, Maner et al. 2003). During labor a pro-inflammatory remodeling of the ECM occurs with a decreased in collagen content together with an increased collagenase activity (Granstrom, Ekman et al. 1989). Further, the composition of proteoglycans in the uterus changes during parturition with a decrease in decorin and biglycan and an increase in

heparin sulphate proteoglycans and syndecan (Hjelm, Barchan et al. 2002; Hjelm Cluff, Malmstrom et al. 2005). There is also an increase in gap-junctions between the myometrial cells enabling communication between the myocytes and well-coordinated contractions in the uterus at the time of delivery (Garfield, Saade et al. 1998). Similar to the changes in the cervix, the uterus experiences also an infiltration of leukocytes and an increase in the mRNA and protein expression of pro-inflammatory cytokines IL-1 β , IL-6 and IL-8, in the uterus (Osmers, Blaser et al. 1995; Winkler, Fischer et al. 1998; Thomson, Telfer et al. 1999; Young, Thomson et al. 2002; Osman, Young et al. 2003).

4.5 MEDIATORS INVOLVED IN CERVICAL RIPENING

4.5.1 Cytokines

Cytokines encompass a variety of small proteins and glycoproteins that mediate immune- and inflammatory reactions, functioning as signaling molecules between immune cells and other cells as well as between immune cells. They can act in a paracrine (acting on adjacent cells), autocrine (acting on the cells producing the cytokines) or endocrine manner (acting on cells distant to their site of secretion) and can also act as chemoattractants, and are hence called chemokines. Many of the described cytokines were first detected in leukocytes, hence the name, interleukins, suggesting action and communication between leukocytes (Lichtman 2009). They can roughly be divided into two groups: pro-inflammatory and anti-inflammatory. As pregnancy advances the inflammatory responsiveness in women increases, involving a number of different cytokines (Brewster, Orsi et al. 2008). These cytokines are involved in the preparation of labor at three different levels: in cervical ripening, rupture/weakening of membranes and myometrial contractions (Orsi and Tribe 2008).

4.5.2 The immune system and cytokines

The immune system can be divided into an innate (natural or native immunity) and an adaptive part (specific or acquired immunity). The innate immune system encompasses the first line of the defense, mainly natural killer cells (NK-cells), dendritic cells, macrophages and monocytes and granulocytes. Their main function is to block the entry of pathogens and they are recruited to the site of injury/inflammation by chemokines. The adaptive response consists of a cell-mediated immunity and a humoral immunity. The humoral immunity is mediated by proteins called antibodies that are produced by B-lymphocytes and the T-lymphocytes, which comprise the cell-mediated immunity. The T-lymphocytes recognize antigens on the surface of pathogens as do the antibodies produced by the B-cells. The innate immune system communicates with the adaptive immune system by presenting pathogen antigens to T-lymphocytes and B-lymphocytes to initiate response.

T-lymphocytes are a major source of cytokines and they are divided into two major groups by the identification of cell surface molecules called CD4 and CD8.

Lymphocytes expressing CD4 are known as T-helper (Th) cells which can be divided further into Th1 and Th2 cells (Lichtman 2009).

T helper cells have a regulatory function and there is a subset of regulatory cells called Th3 cells with ability to secrete transforming growth factor β (TGF- β) which is a pluripotent cytokine but mainly with anti-inflammatory and remodeling properties in

the ECM (Commins, Borish et al. 2010). Further, regulatory T-cells (Treg), another subset of regulatory T-cells, have been described in pregnancy and their presence has been associated with successful pregnancy outcome while their absence is associated with pregnancy complications in murine models. They originate from the thymus but are soon released to the circulation as part of the peripheral tolerance (Ernerudh, Berg et al. 2011). The Treg cells are regulated by both estrogen and progesterone during pregnancy and are decreased in number during second trimester. They have the ability to produce IL-10 and IL-4, both anti-inflammatory cytokines, which explains their suppressive function during pregnancy (Mjosberg, Svensson et al. 2009).

4.5.3 Th1 and Th2 cytokines during pregnancy

Pregnancy is a state of immunologic tolerance towards the semiallogenic fetus without compromising the maternal defense against infections. The current view has however switched from pregnancy being a “Th2 phenomenon” with an inhibition of a Th1 response for normal pregnancy to develop to a “Th1 and Th2 cooperation” view with a dynamic balance between the Th1 and Th2 response during pregnancy (Wilczynski 2005). A Th1 response is crucial for the onset of parturition and is also important during the time frame of implantation (Wilczynski 2005; Mjosberg, Berg et al. 2010). The Th1 cells produce pro-inflammatory cytokines such as IL-1, IL-2, IFN- γ TNF- α and IL-18 while Th2 cells produce anti-inflammatory cytokines such as IL-4, IL-5 and IL-10. A disturbance in cytokine balance has been described in recurrent miscarriage (Kruse, Varming et al. 2003), pre-eclampsia (Borzychowski, Croy et al. 2005) and preterm birth (Romero, Espinoza et al. 2002).

4.5.4 Pro-inflammatory cytokines

IL-1 β has been shown to be an important inducer of IL-6, IL-8 and IL-10 (Simpson, Keelan et al. 1998; Osman, Young et al. 2003). Stimulation in vitro of fibroblasts from lower uterine segment by IL-1 β has been shown to yield IL-8 (Winkler, Fischer et al. 1998) and is produced in vitro from fibroblasts isolated from postpartal women (Malmstrom, Sennstrom et al. 2007).

IL-6 is a 26 kDa molecule that can be produced by various cell types such as T-cells, B-cells, monocytes, fibroblasts and endothelial cells. It is a multifunctional cytokine and overproduction has been described in various inflammatory diseases but is also induced by microbial pathogens and IL-1 (Kishimoto 2005). During the final step of cervical ripening, the mRNA and protein expressions for IL-6 and IL-8 in the cervix increases significantly during the ripening process.

IL-8 is a small 8.4 kDa but potent chemokine and activator of neutrophils that can be produced by cervical epithelial cells and attracts neutrophils from the vessels to migrate into the tissue and release granules containing collagenase and elastase (Peveri, Walz et al. 1988; Barclay, Brennand et al. 1993).

IL-18 is like IL-1 β secreted as a precursor protein of 24 kDa and becomes active after cleavage. Both cytokines are related to IL-1 family in relation to function and structure (Dinarello, Novick et al. 1998). It was first described in 1989 and was named interferon- γ (IFN- γ) stimulating factor for its capability to induce IFN- γ (Dinarello 1999). The capability to induce IFN- γ is only in the context of a second stimulus such as IL-12 or pathogens. IL-18 cannot alone induce a IFN- γ response (Dinarello, Novick

et al. 1998). IL-18 plays a regulatory role in the innate immune system and has the ability to promote both a Th1 and Th2 response depending on the cytokine micro milieu (Nakanishi, Yoshimoto et al. 2001). It is a potent pro-inflammatory cytokine in companionship with IL-12, with the ability to induce IL-8 and IL-1 β as well as nitric oxide (Puren, Fantuzzi et al. 1998). IL-18 has been previously described to increase in the cervix from both term and preterm parturients as compared to non-laboring groups irrespective of gestational age (Dubicke, Fransson et al. 2010).

4.5.5 Anti-inflammatory cytokines

The most potent anti-inflammatory cytokine is IL-10. From the group of anti-inflammatory cytokines IL-10 is the only one being handled in this thesis. IL-10 is not strictly a Th2 cytokine but rather a general immune modulating cytokine. It has been shown not only to inhibit Th1 but also Th2 immune response (Commins, Borish et al. 2010). IL-10 has been shown to decrease the production of pro-inflammatory cytokines such as IL-1 β , IL-6 and IL-8 as well as endogenous PGE₂ (Fortunato, Menon et al. 1996; Fortunato, Menon et al. 1998; Sato, Keelan et al. 2003). During pregnancy, IL-10 secretion has been shown to be higher in normal pregnancies as compared to non-pregnant and in women with recurrent abortions (Marzi, Vigano et al. 1996; Hanna, Hanna et al. 2000) suggesting that IL-10 is the main facilitator for pregnancy. IL-10 is mainly derived from antigen presenting cells such as dendritic cells, macrophages and B-cells but is also produced in small amounts by T-cells (Commins, Borish et al. 2010). There is also evidence that IL-10 is up regulated in the cervix and amniotic fluid during labor irrespective of gestational age suggesting a mechanism quenching an exaggerated pro-inflammatory response during labor (Gotsch, Romero et al. 2008; Dubicke, Fransson et al. 2010). With advancing gestation there is a gradual decrease of IL-10 in the placenta and also the ratio of pro-inflammatory (IL-8) and anti-inflammatory (IL-10) cytokines in cervical secretions increases with advancing gestation (Hanna, Hanna et al. 2000; Mondestin-Sorrentino, Smulian et al. 2007). Cells from choriodecidual cells in vitro have been shown to produce IL-10 upon stimulation with bacterial lipopolysaccharide (LPS) (Dudley, Edwin et al. 1997). Up to date, no anti-inflammatory cytokines have been described in postterm women.

4.5.6 Prostaglandins and prostaglandin receptors

4.5.6.1 Prostaglandins

Prostaglandins play an important role throughout the whole pregnancy. They are mainly produced in fetal membranes and decidual cells. Levels of prostaglandins in amniotic fluid have been shown to increase during gestation and even higher levels have been seen during parturition (Mitchell, Romero et al. 1995), hence having a critical role in the onset of labor and its progression. There is evidence that the cervical tissue can produce large amounts during cervical ripening and early stages of labor (Ellwood, Mitchell et al. 1980). It is well established that locally administered PGE₂ is effective for labor induction (Wingerup, Ekman et al. 1983; Hertelendy and Zakar 2004) and is used routinely both in preterm, term and postterm labor induction (Ekman-Ordeberg, Uldbjerg et al. 1985; Ekman, Persson et al. 1986; Abelin Tornblom, Ostlund et al. 2002). The exact mechanism of action is still unknown, however the

collagenolytic activity increases and the composition of different proteoglycans are changed (Uldbjerg, Ekman et al. 1983).

4.5.6.2 Prostaglandin receptors

There is compelling evidence of the importance of prostaglandins in the female reproductive tract during menstrual cycle, implantation, and quiescence in the uterus during pregnancy to allow a growing fetus and at term the uterus to change to a contractile organ to expulse the fetus (Grigsby, Sooranna et al. 2006; Catalano, Wilson et al. 2011). PGE₂ is the natural ligand for the prostaglandin (PG) receptor subtypes EP1-4 (also denoted PTGER1-4) whereas prostaglandin F₂ α (PGF₂ α) is the ligand for FP (also denoted PTGFR) (Breyer, Bagdassarian et al. 2001). The prostaglandin receptors are G-protein coupled receptors mediating smooth muscle cell contractility and relaxation through different signaling pathways (Woodward, Jones et al. 2011). Contractility in smooth muscle cells are mediated by EP1, EP3 and FP whereas EP2 and EP4 mediate relaxation (Breyer, Bagdassarian et al. 2001). Cervical ripening and the expression of prostaglandin receptors and the mechanisms by which prostaglandins act on the cervix are well described in animal studies but available human studies are nonexistent.

4.5.7 Stromal factors

Connective tissue growth factor (CTGF) is involved in the regulation of extracellular matrix production, tissue remodeling, cell migration and differentiation. In porcine endometrium, CTGF was highly expressed in the epithelium during the first days of implantation suggesting the epithelium as a source for CTGF and extracellular matrix remodeling in the reproductive tract (Moussad, Rageh et al. 2002). Calgranulin B is a calcium binding protein expressed mainly in neutrophils (Hessian, Edgeworth et al. 1993; Guignard, Mauel et al. 1995). It is involved in a series of inflammatory diseases (Johne, Fagerhol et al. 1997) by recruitment of neutrophils to inflammatory sites (Ryckman, Vandal et al. 2003). Calgranulin B is an important factor during parturition exhibiting increased mRNA levels in the uterus and cervix of women in labor (Havelock, Keller et al. 2005). Calgranulin B could also be involved in the cervical ripening process through remodeling of the extracellular matrix by being a substrate for MMP-2 and MMP-9 (Greenlee, Corry et al. 2006).

Furin, a calcium dependent serine protease mainly located in the Golgi network, may be an important factor regulating the inflammatory response and a possible mediator in cervical ripening catalyzing the activation of a variety of proteins such as MMPs (Dubois, Laprise et al. 1995; Sato, Kinoshita et al. 1996; Verma and Hansch 2007). Arachidonate 15-lipoxygenase (ALOX15) is involved in arachidonic acid metabolism and is secreted by leukocytes, among other cell types. ALOX15 is a mediator of inflammation and thus a possible participant in the parturition process (Narumiya, Salmon et al. 1981; Wen, Gu et al. 2007). In arteriosclerotic lesions ALOX15 is expressed by macrophages and monocytes inducing the production of pro-inflammatory molecules (Hulten, Olson et al. 2009). It has also been detected in human myometrium during pregnancy with a lower expression at term and at labor (Lei and Rao 1992). Thus, via these cells ALOX15 could play a role regulating the onset of labor.

4.5.8 Nitric oxide (NO)

Nitric oxide is highly reactive free radical, and therefore with a short half-life. It is a relatively novel molecule as a mediator of several biological functions, among them relaxation of smooth muscles (Moncada, Palmer et al. 1988). The relaxation of smooth muscles in vessels results in increased blood flow. NO is synthesized by nitric oxide synthase (NOS) by converting L-arginine and oxygen into citrulline and NO (Moncada, Palmer et al. 1988). Three different isomers of NOS exist; neuronal nNOS, endothelial eNOS and inducible iNOS, all three have been described in cervical tissue at early pregnancy, term and preterm (Ledingham, Thomson et al. 2000; Tornblom, Maul et al. 2005).

NO plays an essential role during pregnancy by maintaining the quiescence in the uterus by inducing muscle relaxation (Maul, Maner et al. 2003). NO is also a key player in final cervical ripening by inducing local vascular permeability enabling leukocytes to infiltrate and activating matrix metalloproteinases (MMPs) involved in the cervical remodeling (Chwalisz and Garfield 1997). NO acts in synergy with cytokines by activating their synthesis (Ianaro, O'Donnell et al. 1994). NO-donors, such as isosorbide mononitrate, have been shown to be efficient inducers of cervical ripening (Bullarbo, Orrskog et al. 2007). Lower concentrations of nitric oxide metabolites in cervical fluid is associated with postterm pregnancy and failed to progress in labor (Vaisanen-Tommiska, Nuutila et al. 2004).

4.6 POLYCYSTIC OVARY SYNDROME (PCOS)

4.6.1 Definition and prevalence of PCOS

Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting 5-15 % of women of reproductive age (Knochenhauer, Key et al. 1998; Asuncion, Calvo et al. 2000; Archer and Chang 2004). The condition was first described by Stein and Leventhal in 1935, from whom the original name Stein-Leventhal syndrome was adopted (ML 1935). According to the 2003 Rotterdam consensus the PCOS diagnosis requires two of the three criteria: presence of oligo- or amenorrhea, clinical or biochemical hyperandrogenism and polycystic ovaries determined by ultrasound (2004) (see figure 5). Before the Rotterdam consensus was adopted for diagnosis of PCOS, the National Institute of Health (NIH) criteria were used. The NIH criteria included having both oligo- / amenorrhea and clinical or biochemical signs of hyperandrogenism. Comparing these two diagnostic criteria, the Rotterdam criteria classified twice as many cases as having PCOS as compared to the NIH criteria (March, Moore et al. 2009).

4.6.2 Etiology and comorbidities

The syndrome is strongly associated with insulin resistance, accumulation of abdominal fat and hypertension. These characteristics are also hallmarks of the metabolic syndrome, which predisposes the women to diabetes and cardiovascular disease later in life (Ehrmann 2005). Obesity ($\text{BMI} \geq 30.0 \text{ kg/m}^2$) is present in about 30% and up to 75% of the women with PCOS (Ehrmann 2005; Pasquali, Gambineri et al. 2006).

The major cause of the syndrome is not known but there is strong evidence that a genetic component is involved which has been recognized in both family and twin studies (Legro, Driscoll et al. 1998). The condition is probably of a polygenetic origin. However, in a recent case-control study in a Sardinian population, a genetic variant has been identified, which is strongly correlated to PCOS (Capalbo, Sagnella et al. 2012).

4.6.3 Infertility in polycystic ovary syndrome

Anovulatory infertility with associated oligo- or amenorrhea among women with PCOS is a major cause of infertility, accounting for almost 70% of anovulatory infertility registered at infertility centers (Brassard, AinMelk et al. 2008). Women with PCOS often need ART or ovulation induction to become pregnant (Rajashekar, Krishna et al. 2008). Lifestyle changes aiming at achieving weight reduction can however improve the metabolic consequences of having PCOS and also reduce the anovulatory infertility among these women (Moran, Hutchison et al. 2011).

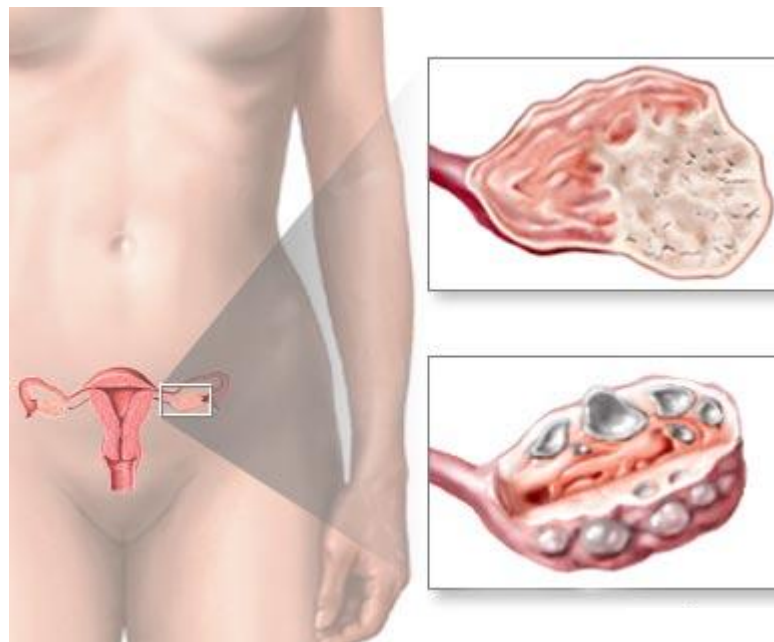


Figure 5 Normal ovary and polycystic ovary in a schematic drawing. Illustration published with license from ADAM images

4.6.4 Polycystic ovary syndrome and pregnancy outcome

There is evidence suggesting that PCOS may be associated to adverse pregnancy outcomes both in early and late gestation as well as perinatal complications. There is an increased risk of spontaneous and recurrent abortions as well as implantation failure in women with PCOS (Glueck, Wang et al. 2002; Cocksedge, Li et al. 2008), suggesting that endometrial factors may be of importance as well. There are no studies large enough to determine whether the increased risk of miscarriage is attributed to having PCOS or whether obesity or other underlying causes of infertility produce the increased risk. Populations based studies are needed to address this question.

In concurrence with the association between PCOS and characteristics of the metabolic syndrome, there is evidence that PCOS during pregnancy is associated with an increased risk of gestational diabetes, hypertensive disorders, preterm birth and intrauterine growth restriction (Boomsma, Eijkemans et al. 2006). The available studies have however been small in size and have mainly included women undergoing PCOS (Boomsma, Eijkemans et al. 2006; Heijnen, Eijkemans et al. 2006).

5 AIMS OF THE THESIS

The overall aim of this thesis is to increase knowledge about the epidemiology, risk factors and pathophysiology of postterm pregnancy and failed labor induction.

The specific research questions in the thesis are the following:

- What are the risk factors for postterm pregnancy and cesarean section after labor induction postterm (failed labor induction)?
- What are the associations between polycystic ovary syndrome, postterm pregnancy and adverse pregnancy outcomes?
- Is postterm pregnancy and successful labor induction postterm associated with the expression of prostaglandin receptors and stromal factors in the cervix?
- Is postterm pregnancy and successful labor induction postterm associated with the expression of pro- and anti-inflammatory cytokines in the cervix?

6 MATERIALS AND METHODS

The thesis includes research methods from epidemiology (paper I and II) and molecular biology (paper III and IV). These methods are described in separate sections below.

Table 2 Characteristics of the study cohorts in paper I and II

	PAPER I	PAPER II
Years	1992-2006	1995-2008
Setting	National data	National data
Subjects (cases)	1,176,131 (105,197)	1,191,336 (3,787)
Exposure variables	Maternal age, body mass index, parity, smoking, cohabiting status, educational level	Maternal previous diagnosis of polycystic ovary syndrome (ICD-7-10)
Outcome measures	Postterm pregnancy, cesarean section after labor induction postterm	Gestational diabetes, pre-eclampsia, antepartum bleeding, cesarean section, preterm birth, postterm birth, stillbirth, Apgar score < 7 at 5 min of age, neonatal death, meconium aspiration, large for gestational age, macrosomia, small for gestational age
Covariates	Maternal age	Maternal age, parity, body mass index, years of education, cigarette consumption, assisted reproductive technology, year of delivery

6.1 PAPER I AND II

6.1.1 The Swedish health care system

The Swedish health care system offers free health care for pregnant women. Among these women, 98% adhere to the routine antenatal care program, which starts in early pregnancy and more than 99% of Swedish deliveries take place at hospitals (Odlind, Haglund et al. 2003). Routine ultrasound by trained midwives and physicians has been offered to all pregnant women since 1990, at 17-18 gestational weeks counted from last menstrual period. Approximately 95% of the women agree to ultrasound for gestational dating (Hogberg and Larsson 1997).

6.1.2 Population based registers used in paper I and II

6.1.2.1 The Swedish Medical Birth Registry

The Swedish Medical Birth Registry (MBR) is the primary source of information for paper I and II. Data from the Swedish Medical Birth Registry contains information on about 98% of all births in Sweden since 1973 (Petra Otterblad Olausson and Pakkanen 2003). The data is collected prospectively starting in early pregnancy at each woman's first antenatal visit (Cnattingius, Ericson et al. 1990) and includes information on the mother as well as the pregnancy, delivery and neonatal period.

6.1.2.2 The Swedish Patient Registry

The Swedish Patient Registry was established in 1964 and has been implemented nationwide since 1987. It includes information on dates of hospital admissions, discharges and diagnoses classified according to the ICD codes (International Classification of Diseases, 7-10th revisions) (Ludvigsson, Andersson et al. 2011). Since 2001 this registry has also included information on out-patient hospital visits. For paper II, we retrieved the diagnosis codes for PCOS according to the International classification of disease (ICD) codes. See figure 6 for illustration of the timeline and use of the different ICD revisions.

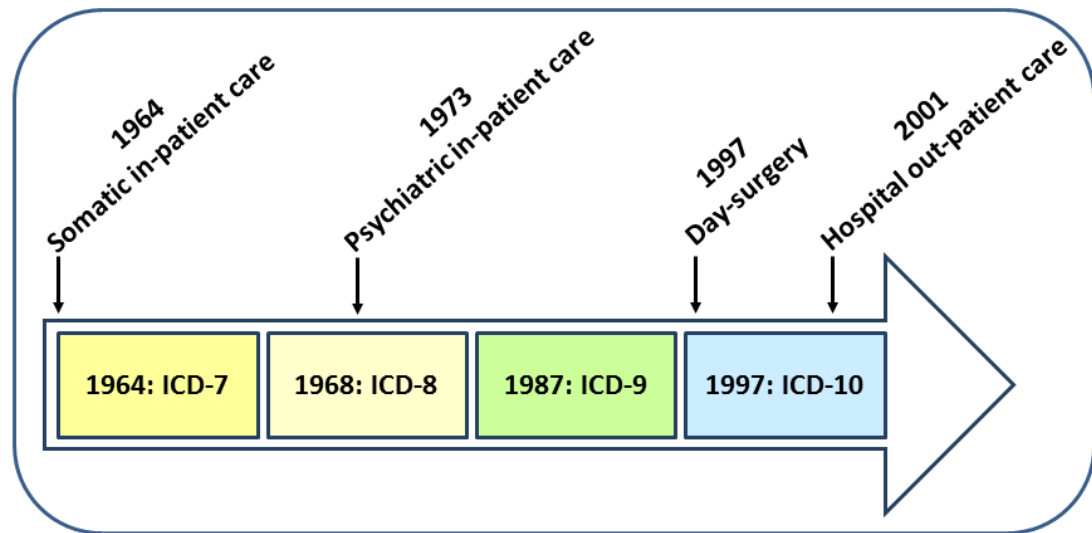


Figure 6 Time line of the Swedish Patient Registry. Years inside the arrow indicate the first year that the different ICD revisions were used.

6.1.2.3 The Swedish Education Registry

The Swedish Education Registry was established in 1985 and comprises the population between 16 and 74 years of age registered as residents in Sweden at 1st January each year. The Swedish Education Registry is updated yearly with graduation and examination data from regular educational institutions in Sweden, such as primary and secondary schools and universities. The register records the highest completed educational level for each individual (Larsson 2006).

6.1.2.4 The Swedish Personal Identity Number

The Swedish personal identity number was introduced in 1947 and consisted of a nine-digit number that represented the year, month and day of birth together with a three-digit number making the personal identification number unique. In 1967 a check digit was added to the three-digit number. The check digit verifies that data of birth and the three-digit number are correct (Ludvigsson, Otterblad-Olausson et al. 2009). Using the unique personal identification number assigned to each citizen at birth or immigration the data from the Swedish medical birth registry was linked with the Swedish Patient Registry as well as with the Swedish Education Registry.

6.1.3 Paper I

6.1.3.1 *Study population and design*

From the Swedish Medical Birth Registry we retrieved all singleton births between 1992 and 2006, in total 1,442,675 births. Multiple birth pregnancies were excluded because of the increased risk for cesarean section and differences in duration of gestation of these births compared to singletons births. From the study base of 1,442,675 births we excluded 1,868 births with unknown gestational age, 73,919 preterm births (gestational age before 37 weeks of gestation), 74,778 elective cesarean births before 42 gestational weeks, 90,047 births with elective induction of labor before 42 gestational weeks, 25,932 births with unknown mode of initiation of labor before 42 gestational weeks. After these procedures the final dataset consisted of 1,176,131 singleton births from which 105,197 were postterm and 1,070,934 were term. These groups were then compared in order to investigate risk factors for postterm pregnancy. In paper I we also investigated risk factors for failed labor induction postterm (cesarean section following labor induction). This second part of the study was composed of all women that were postterm and comprised in total 36,498 women. Among these women receiving labor induction 28,435 women delivered vaginally and 8,063 women were delivered with cesarean section. The latter two groups were compared in relation to investigate maternal risk factors for failed labor induction with regard to maternal risk factors. In a sub-analysis we included 8,983 parous women undergoing labor induction postterm from 1999 to 2006. A total of 7,908 women with successful labor induction were compared to 1,075 women with failed labor induction (undergoing cesarean section after labor induction). Data on a previous cesarean section was introduced into the Swedish Medical Birth Registry in 1999. See figure 7 for graphic illustration of the study groups.

6.1.3.2 *Risk factors*

Maternal age was categorized as less than 20, 20-24, 25-29, 30-34 and 35 years or older. Body mass index (kg/m^2) was calculated from height and weight recorded at the first antenatal visit and categorized into four groups: lean (BMI less than 20.0), normal weight (BMI 20.0-24.9), overweight (BMI 25.0-29.9) and obese (BMI 30.0 or more). Smoking habits were categorized as non-smokers, moderate smokers (1-9 daily cigarettes) or heavy smokers (10 daily cigarettes or more). Parity was categorized into nulliparous and parous women.

6.1.3.3 *Outcome measures*

Outcome measures were maternal age, body mass index, parity, cigarette consumption, educational level, cohabiting status and country of origin. Gestational age was principally based on prenatal ultrasound measurement if present and otherwise recorded date of the first day of the last menstrual period (data on ultrasound pregnancy dating is available in 95% of all pregnancies as described in section 8.1.1). We defined term pregnancy as delivery at 37-41 completed gestational weeks and postterm pregnancy as labor at 42 weeks of gestation

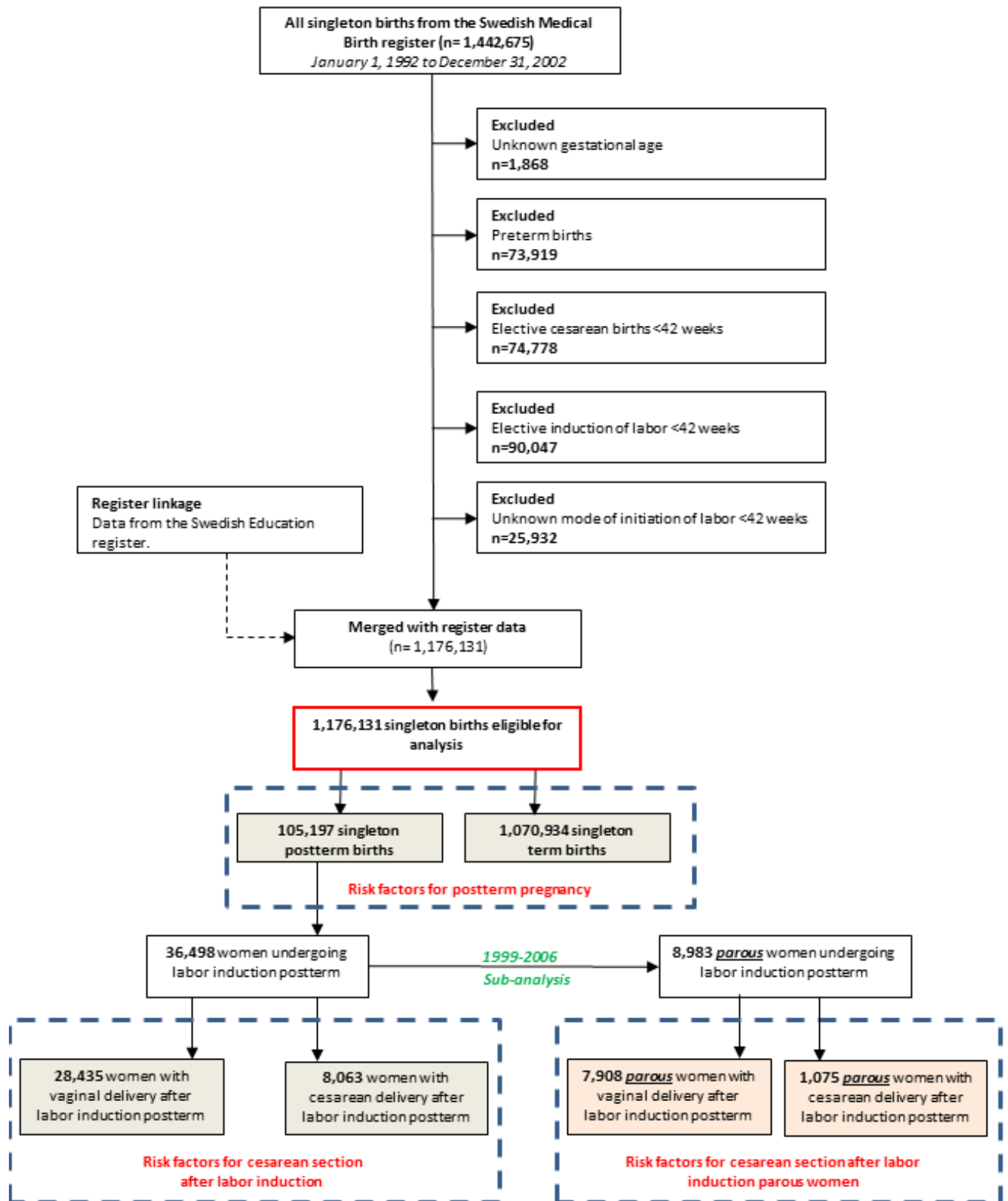


Figure 7 Study design for paper I

6.1.3.4 Statistical analysis

We compared postterm pregnant women with term pregnant women with spontaneous onset of labor in relation to maternal risk factors (maternal age, body mass index, parity, cigarette consumption, educational level, cohabiting status and country of origin). We used unconditional logistic regression analysis with adjusted odds ratios and 95% confidence intervals to analyze the risk of postterm pregnancy and failed labor induction in association to maternal characteristics.

In the second part of the study we included women with labor induction postterm and compared women with vaginal deliveries with women with cesarean section following labor induction in adjusted for maternal age, body mass index, parity, cigarette consumption, years of formal education, cohabiting with the infant's father and country of origin in the logistic regression analysis. In a sub analysis we compared parous women undergoing labor induction postterm between 1999 and 2006 and compared women with vaginal deliveries with women with a failed labor induction (i.e. delivery by cesarean section) with regard to maternal risk factors as previously mentioned, including a previous cesarean section. All analyses were performed using the statistical software SAS version 9.1 (SAS Institute Inc., Cary, NC, USA)

6.1.4 Paper II

6.1.4.1 Study population and design

From the Swedish Medical Birth Registry we created a cohort of women with singleton pregnancies who gave birth between 1995 and 2007. Because women with PCOS are more likely to undergo ART to become pregnant, they are more likely to have multiple births. Since multiple birth pregnancies differ in fetal growth and duration of gestational length and have a higher occurrence of complications, it would have been difficult to clarify the effect of PCOS on pregnancy outcome. Hence, we excluded such pregnancies from the study population.

6.1.4.2 Exposure data

We classified maternal age (in years) at delivery into four categories; 13-24, 25-29, 30-34 and ≥ 35 years. BMI was calculated according to the weight and height measured at the first antenatal visit at the beginning of pregnancy ($\text{weight (kg)} / \text{height}^2 (\text{m}^2)$) and the women were categorized as lean (body mass index ≤ 19.9), normal weight (20.0-24.9), overweight (25.0-29.9) or obese (≥ 30.0). Parity was classified into nulliparous and parous women. Cigarette smoking was registered as none, 1-9 daily cigarettes or ≥ 10 daily cigarettes recorded at the first antenatal visit at the beginning of pregnancy. By linkage with the Swedish Education Registry we obtained information on the number of years of formal education completed as of 1 January 2008. Educational level was categorized as 11 or fewer or 12 or more years. The Swedish Medical Birth Registry also contains information on whether the pregnancy was conceived by ART or not. Information on concurrent diseases such as diabetes mellitus and essential hypertension is also available by checkbox as well as International Classification of Disease codes (ICD)-9 and ICD-10 codes. Diagnosis of PCOS was acquired from the Swedish Patient

Registry according to the corresponding codes: ICD-7, 275.20; ICD-8, 256.90 (Stein-Leventhals syndrome); ICD-9, 256E and ICD-10 E28.2. In the Swedish version of ICD-7 and ICD-8 PCOS is included under its former name, Stein-Leventhals syndrome. For ICD-9 and ICD-10 the code is specified as PCOS and Stein-Leventhals syndrome.

6.1.4.3 Outcome measures

Gestational diabetes was defined as plasma glucose levels 12.2 mmol/L or more after oral glucose tolerance test (75 g glucose orally administered and plasma glucose measured after 2 hours) or fasting blood glucose levels 7.0 mmol/L or more. Pre-eclampsia was defined as a blood pressure reading of 140/90 mmHg or more with proteinuria of more than 0.3 g over 24 hours. Preterm birth was defined as delivery at less than 37 weeks of gestation, classified as moderately (32+0 to 36+6 week) and very preterm birth (<32 weeks). Postterm pregnancy was defined as delivery at 42 gestational weeks or more. Stillbirth was defined as intrauterine fetal death after 28 weeks of gestation. Neonatal death was defined as death of the infant from 0 to 27 days after birth. Small for gestational age was defined as an infant with birth weight of less than two standard deviations below the mean for its gestational age and infant sex. Large for gestational age was defined as an infant with birth weight of more than two standard deviations above the mean for its gestational age and infant sex. A low Apgar score was defined as a score of less than 7 at five minutes of age. Presence of meconium aspiration was obtained by diagnosis at discharge from the hospital.

6.1.4.4 Statistical analysis

The main outcome measures were gestational diabetes, pre-eclampsia, preterm birth, still birth, neonatal death, low Apgar score, meconium aspiration, large and small for gestational age. Logistic regression analysis was used to estimate the risk of adverse pregnancy outcomes in relation to PCOS by crude and adjusted odds ratios with 95% confidence intervals. We compared women with a diagnosis of PCOS with women without such a diagnosis adjusting for possible confounders such as maternal age, body mass index, parity, educational level (years of formal education), cigarette consumption, ART and calendar year of delivery. ART only refers to in vitro fertilization including intracytoplasmic sperm injection. It does not refer to other forms of ART such as ovulation induction or insemination.

Absolute rates were standardized for difference in characteristics between women with and without PCOS. In additional analyses residual confounding caused by maternal age, body mass index and parity was tested for using finer categorization and by analyzing continuous variables in a linear or linear quadratic model.

Women in general tend to repeat pregnancy outcomes in successive births. Among women with PCOS, 74.1% delivered one infant during the study period, 23.2% two infants and 2.7% delivered three infants or more. The corresponding figures for women without PCOS were 53%, 38.1% and 8.9%. Because observations are not independent in women who delivered more than once during the study period we calculated

estimates using clustered data in the generalized estimation equation method (PROC GENMOD). We used a formal interaction test in the logistic regression model to estimate the possible effect modification of ART and body mass index on the association between PCOS and preterm birth.

Statistical software SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) was used for all analyses and for data management.

6.2 PAPER III AND IV

6.2.1 Subjects in paper III and IV

In paper III and IV cervical biopsies were taken from a number of female study subjects. All the recruited women to the studies were healthy, non-smoking and did not suffer from any intercurrent diseases. The pregnant women had uncomplicated pregnancies and were not taking any regular medication. Gestational length was estimated according to routine ultrasound dating in the second trimester of gestation.

6.2.2 Sampling of biopsies in paper III and IV

Cervical biopsies were taken immediately after vaginal delivery or cesarean section (paper III-IV) and after hysterectomy (paper III) at the anterior lip of the cervix (12 o'clock position) at a depth of 10-20 mm. The biopsies were divided into two separate pieces. The first one was prepared for mRNA analysis in one of the following ways: i) immediately immersed in *RNAlater*® (Ambion Inc. Austin, TX, USA) and then stored at -70°C until RNA extraction (paper IV), ii) one piece frozen and then stored at -70°C until transferred to *RNAlaterIce*® before RNA extraction (paper III; part 2 and IV), iii) frozen and then stored at -70°C until RNA extraction (paper III; part 1). The second piece was prepared for immunohistochemistry. It was fixed in a 4% formaldehyde solution during a maximum of 24 hours at 4°C. It was then stored over night for dehydration in 70% ethanol and subsequently embedded in paraffin. When the biopsy was too small to be divided, it was fixed and embedded in paraffin. For this reason, RNA preparations are not available from all samples.

We could not obtain large enough biopsies for both paraffin embedding and RNA preparation from all women included in these two studies. Therefore the *n* values of the specific analyses differ from the total *n*. The correct figures are given in legends to figures. Some of the RNA samples were finished during the experiments, hence some groups turned out very small.

6.2.3 Paper III

A total of 35 women were included in paper III. In part one of paper III the women were divided into three groups (Term pregnant group (TP), Postpartal group (PP) and non-pregnant group (NP)). In part two 45 women were included and divided into three groups (controls (C), responders (R) and non-responders (NR)). Details about the study subjects are included in table 3.

6.2.3.1 *Part one*

Women in the term pregnant (TP) group (n=12) had unripe cervixes with a Bishop's Score of less or equal to 5 points and none of them were in labor. They had a mean age (\pm SD) of 33.2 ± 3.8 years, and a median gestational length of 38+3 (38 completed gestational weeks and three days) with a range 37+2 to 39+6 weeks. All women in this group were primiparous, except for four women who had undergone one previous caesarean section. Cervical biopsies were obtained at elective caesarean sections before the onset of labor.

Women in the postpartum (PP) group (n=15) were all at term and primiparous. They had a mean age of 30.3 ± 4.8 years, and a median gestational age of 39+6 (range 39+1 to 41+6) weeks. Cervical biopsies from the term pregnant group with vaginal delivery were obtained immediately after parturition.

The non-pregnant (NP) group consisted of women who were undergoing hysterectomy due to benign uterine disorders not affecting the cervix and without any cervical pathology. They were all healthy, menstruating regularly and without any kind of regular medication. This group (n=8) had a mean age of 43.4 ± 5.5 years, and a median parity of II (range I-III). Clinical characteristics are summarized in table 3.

6.2.3.2 *Part two*

In part two of paper III three groups of women were included. The two postterm groups consisted of women with an unripe cervix, defined as a Bishop's score ≤ 5 points. Labor induction was initialized by locally administrated PGE₂ in a vaginal gel preparation (Minprostin® Pharmacia, Sweden) in all but one woman who received PGE₂ in a vaginal slow-release delivery system in pessary formulation (Propess® Ferring, Sweden). Women who responded to PGE₂ treatment were classified as responders (R, n=15), i.e. underwent vaginal parturition after labor induction. They had a mean age (\pm SD) of 29.6 ± 5.2 years, a median gestational length of 42+4 weeks (range 42+1 to 42+5) and a median cervical score of 3 out of maximum 10 points (range 0-5) before treatment. Two women were delivered by caesarean section at 6 and 7 centimeters of dilation due to fetal distress. This responder group received a median number of one (range one to five) PGE₂ applications. Cervical biopsies were obtained immediately after birth.

The non-responders (NR, n=11) who experienced a failed labor induction, underwent caesarean section. All women that were classified as non-responders showed no response in cervical ripening hence they received repeated PGE₂ applications during 24 hour or more induction process. No caesarean section was performed for other reasons than failed labor induction. Non-responders had a mean age of 30.6 ± 3.0 years, a median gestational length of 42+4 weeks (range 42+3 to 42+6) and a median cervical score of 2 points (range 0-4) before treatment. These women received a median number of three (range one to four) PGE₂ applications. Cervical biopsies were taken during caesarean sections.

Women with spontaneous onset of labor and vaginal delivery at term were included as controls (C, n=19). These women had a mean age of 29.8 years \pm 4.5 and a median gestational age of 40+2 weeks (range 37+0 to 41+1).

Oxytocin infusion (Syntocinon® 10 Units/500 milliliters 5.5% glucose) for augmentation of labor was administered to all women in the R group 5 hours after the last prostaglandin application. In the NR group, five out of eleven women received oxytocin infusion 5 hours after the last prostaglandin application. The remaining six women offered still a very unripe cervix after 3 or more vaginal prostaglandin applications and were therefore not given oxytocin. All women in the C group received oxytocin infusion during the active phase of labor.

6.2.3.3 RNA preparation and reverse transcription (RT)

Total RNA from frozen cervical tissue samples was purified with the RNeasy® Mini kit (Qiagen GmbH, Hilden, Germany) according to a procedure for RNA isolation from fibrous tissues, including a DNase step, as recommended by the manufacturer. Two µg of total RNA from each sample was reverse transcribed at 37°C for 60 min in a final volume of 30 µl with a reaction mixture (Qiagen) containing 1×RT buffer, dNTP mix (0.5 mM each dNTP), 600 ng random primers (Invitrogen, Paisley, UK), 30 units RNase inhibitor (5 Prime GmbH, Hamburg, Germany), and 4 U of Omniscript™ reverse transcriptase (Qiagen).

6.2.3.4 Quantification of mRNA

To standardize the quantification method, cyclophilin A was selected as the housekeeping gene. Several genes were tested and cyclophilin was chosen since it showed no variation between the groups. The PCR amplification rate and the cycle threshold (Ct) values were analyzed using iCycler™ iQ 3.1 software (Bio-Rad). The values of relative expression of genes of interest were normalized against the cyclophilin A product. Due to the limited amount of samples, mRNA determinations were only performed for EP1-4 and FP.

6.2.3.5 Real time PCR analysis

The oligonucleotide primers for EP1, EP2, EP3, EP4, FP and cyclophilin A are presented in table 3 in paper III, as well as their predicted sizes. Real time polymerase chain reaction (RT-PCR) was performed in an iCycler™ iQ Real Time PCR System (Bio-Rad Laboratories, Inc). For PCR, cDNAs corresponding to 66 or 100 ng RNA (table 4 in paper III) were added to 12.5 µl of iQ™ SYBR® Green Supermix (Bio-Rad) and 0.3 µM of each oligonucleotide primer in a final volume of 25 µl. After initial incubation for 3 min at 95°C, the samples were subjected to 40 cycles of ten seconds (s) at 95°C, followed by 45 s at 56 or 59°C (Table 3 in paper III). All PCR assays were performed in replicates twice. The purity of each PCR product was confirmed by a melting curve analysis. Further, the PCR products were checked on agarose gels and all exhibited single bands of the expected sizes. Each PCR assay included a negative control containing an RNA sample without reverse transcription. The primers were

based on the sequences of the human genes. The primer pairs (see table 3 in paper III) were designed using the NCBI/Primer-BLAST program.

6.2.3.6 Immunohistochemistry

Immunostaining was performed using the avidin-biotin peroxidase complex (ABC) method. Paraffin sections (5µm thick) of cervical biopsies were dewaxed in Bioclear (Bio-Optica, Milan, Italy) and rehydrated. Antigen retrieval, when performed, was executed by microwaving the sections in 0.01 M sodium citrate buffer (pH 6.0) for 10 min followed by allowing the sections to cool for 20 min. The sections were treated with 3 % H₂O₂ in methanol for 10 min to quench the endogenous peroxidase activity, followed by blocking non-specific binding of the primary antibody by incubation as shown in Table 4 (in paper III), at room temperature (RT) for 30 min (15 min for CTGF). The sections were then incubated with the primary antibodies overnight at concentrations as shown in Table 4 (in paper III). For negative controls the primary antibody was replaced by the corresponding immunoglobulin G (IgG) in the same concentration. Secondary biotinylated antibodies were incubated as shown in table 5 (in paper III), followed by incubation with an avidin-biotin horseradish peroxidase complex (Vectastatin Elite, Cat# PK-6100) for 30 min at RT. The site of the bound enzyme was visualized by the application of 3,3'-diaminobenzidine (DAB kit, DAKO, CA), a chromogen which produces a brown, insoluble precipitate when incubated with enzyme. The sections were counterstained with haematoxylin and dehydrated before they were mounted with Pertex (Histolab, Gothenburg).

6.2.3.7 Manual scoring

All assessments were performed independently by two observers blinded to the identity of the slides. The staining was evaluated semi-quantitatively using a grading system. The staining intensity and amount of positive cells were graded on a scale of: (0) no staining, (1) faint staining/few positive cells, (2) moderate staining/many positive cells and (3) strong staining/majority of cells positive. This scoring method has been used before by us (Vladic-Stjernholm, Vladic et al. 2009; Hulchiy, Zhang et al. 2011) and others (Altmae, Salumets et al. 2011).

Table 3 Clinical characteristics of the women in paper III

	Part 1			Part 2		
	NP	TP	PP	C	R	NR
<i>n</i>	8	12	15	19	15	11
Maternal age (years)	43.4 ± 5.5 ^b (38-49)	33.2 ± 3.8 (28 to 38)	30.3 ± 4.8 (22 to 37)	29.8 ± 4.5 (21 to 37)	29.6 ± 5.2 (21 to 39)	30.6 ± 3.0 (26 to 37)
BMI (kg/m²)^d	Missing	23.8 ± 3.6 (18.9 to 29.8)	21.7 ± 2.8 (16.9 to 26.8)	23.9 ± 5.8 (18.4 to 42.5)	24.6 ± 3.7 (18.9 to 30.5)	25.0 ± 3.4 (20.2 to 31.2)
Gestational length (weeks)^c	N.A.	38+3 (37+2 to 39+6) ^g	39+6 (39+1 to 41+6) ^{ag}	40+2 (37+0 to 41+1) ^e	42+4 (42+1 to 42+5) ^{eg}	42+4 (42+3 to 42+6) ^{eg}
Birth weight (grams)	N.A.	3 734 ± 292 (3 350 to 4 285)	3 616 ± 449 (2 990 to 4 420)	3 462 ± 452 (2595 to 4175)	3 785 ± 441 (870 to 4540)	3 918 ± 843 (2616 to 5130)
Cervical score	N.A.	N.A.	N.A.	N.A.	3 (0 to 5) ^e	2 (0 to 4) ^e
<i>n</i> of vaginal PGE₂ applications	N.A.	N.A.	N.A.	N.A.	1 (1 to 5) ^e	3 (1 to 4) ^{eg}
<i>n</i> receiving oxytocin	-	0	11	19	15	5

Note: Means with (±) standard deviations and (range) are presented unless other is stated.

^a significantly different compared to TP

^b significantly different compared to TP and P

^c completed gestational weeks and days

^d Weight and length recorded at the first antenatal visit presented as Body Mass Index (BMI) (kg/m²)

^f significantly different compared to C group

^g significantly different compared to R

6.2.4 Paper IV

The women included in paper IV did not differ from each other concerning age, body mass index (BMI) or parity. For a summary of clinical characteristics see table 4.

Table 4 Clinical characteristics of the women in paper IV

	C	R	NR
N	22	18	14
Maternal age (years)	30.7 ± 4.6 (21 to 38)	24.9 ± 3.5 (19 to 31)	24.8 ± 3.4 (20 to 31)
BMI (kg/m²)^a	23.8 ± 5.5 (18.8 to 42.5)	42+3 ± 2 (42+1 to 42+6) ^c	42+4 ± 2 (42+1 to 42+6) ^c
Gestational length (weeks)^b	39+5 ± 8 (37+0 to 41+1)	29.1 ± 4.9 (21.0 to 39.0)	29.6 ± 3.7 (23.0 to 37.0)
Birth weight (grams)	3 489 ± 407 (2 595 to 4 175)	3 844 ± 445 (2 870 to 4 540)	3 852 ± 802 (2 616 to 5 130)
Cervical score	N.A	3 (0 to 5) ^d	2 (0 to 4) ^d
n of vaginal PGE₂ applications	N.A	2 (1 to 5) ^d	3 (1 to 4) ^d
n receiving oxytocin	20	17	8

Note: Means with (±) standard deviations and (range) are presented unless other is stated.

^a Weight and length recorded at the first antenatal visit presented as Body Mass Index (BMI) (kg/m²)

^b completed gestational weeks and days

^c significantly different compared to C group

^d Median (range)

A total of 54 women were included and divided into three different groups (controls (C), postterm responders (R) and non-responders (NR)). Women in the control group were at term (range of 37+0 to 41+1 completed gestational weeks and days), with spontaneous onset of labor and vaginal delivery (C, n=22). Postterm pregnancy was defined as a gestational length of 42 completed weeks or more (≥ 42+0 completed gestational weeks and days). Postterm women were classified as either responders (R, n= 18) with vaginal delivery after labor induction with prostaglandin E₂ (PGE₂) and non-responders (NR, n=14) who failed to respond to PGE₂. All women in the NR group had to be delivered by cesarean section.

Labor induction among the postterm women was implemented by locally administered PGE₂ in a gel preparation applied either intracervically or in the posterior fornix of the vagina (Minprostin®, Pharmacia, Sweden). The postterm groups had an unripe cervix, defined as cervical score ≤ 5 points. Women were classified as responders if they underwent vaginal delivery after PGE₂ treatment and non-responders if they, in spite of three or more PGE₂ applications had to be delivered with cesarean section due to a unripe cervix. One patient received PGE₂ in a vaginal slow-release delivery system in pessary formulation (Propess® Ferring, Sweden). Oxytocin infusion (Syntocinon® 10 Units/500 milliliters 5% glucose solution) was administered to all women in the R group 5 hours after the last prostaglandin application and all women in the C group received oxytocin during the active phase of labor. In the NR group eight out of 14

women received oxytocin which was administered if the cervix had dilated to 2.5-3.0 centimeters (cm).

6.2.4.1 Tissue homogenization and RNA extraction

The biopsies, previously stored in RNAlater in -70°C, were divided into smaller pieces on a block of dry ice and transferred to a small container with a Teflon coated tungsten ball that was immersed for 2 minutes in liquid nitrogen. The container was shaken for 2 minutes at full speed in a dismembration apparatus (Reutsch KG, Haan, Germany) and intermittently submerged in liquid nitrogen in repeated cycles until the tissue was pulverized. Total RNA was then extracted with TRIzol® reagent (Invitrogen, Carlsbad, CA USA) in accordance to the instructions from the manufacturer. The total RNA concentration was measured by photometer at 260/280 nm (NanoDrop™1000 Spectrophotometer) (Thermo Scientific, Waltham, MA, USA). All the samples had an OD260/OD280 proportion higher than 1.7. To assess the integrity of the total RNA, an electrophoresis on a 1.5% agarose gel was performed. The RNA was, after staining with ethidium bromide, visualized with ultraviolet light.

6.2.4.2 Reverse transcription

One µg of total RNA was taken from each sample and added 1 µl (200 ng) of Random hexamer primers pd(N)6 (Amersham Pharmacia), 1 µl (10 mM) dNTP (Amersham Pharmacia) and sterile water to a total volume of 12 µl and incubated for 5 minutes at 65 °C followed by rapid cooling and centrifugation. The reaction mixture (consisting of 4 µl of 5 x buffer, 2 µl (0.1 mM) DTT (Invitrogen, Carlsbad, CA, USA) and 1 µl (40U/µl) of RNase Inhibitor (Roche, Mannheim, Germany) was added to each sample and incubated at room temperature. One µl (200U) of Superscript™ II RNase H Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) was added to each sample and mixed followed by incubation for 10 minutes at room temperature. The reverse transcription step was carried out at 42°C for 50 minutes and followed by heating at 70°C for 15 minutes to inactivate the enzyme. The cDNA was then stored at -70°C until used.

6.2.4.3 Real-time PCR analysis

Appropriate primers and probes encoding for IL-1β, IL-6, IL-8, IL-10 and IL-18 were purchased from commercially available Taqman® gene expression assays (Applied Biosystems). The levels of mRNA were quantified using Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Assay IDs and gene bank sequence accession numbers are presented in table 6. Each measurement was performed in triplicate in Taqman Universal PCR Master Mix (Applied Biosystems) on 96-well optical PCR plates. For each reaction 5µl of diluted cDNA (equivalent to approximately 10 ng of total RNA), 12.5 µl of Universal Master Mix, 1.25 µl of assay mixture (Applied Biosystems) and 6.25 µl of sterile water was used. The real-time PCR reaction was carried out according to the manufacturer's standard protocol. The threshold cycles (C_t), at which an increase in the fluorescence from the reporter could first be detected, were determined. Ribosomal 18S RNA and cyclophilin A, were used

as housekeeping genes (endogenous controls) (Applied Biosystems). The mean was used for normalizing the mRNA levels of the gene of interest. The geometric mean of the two endogenous controls was subtracted from the respective gene, generating a ΔC_t value reflecting the relative mRNA expression. The calculations of relative gene expression were carried out using the $\Delta\Delta C_t$ where the C_t values from the C group (term women with spontaneous onset of labor) were used as controls and subtracted from the ΔC_t . The amount of the products doubles in each cycle, so the relative gene expression was calculated with the formula $2^{-\Delta\Delta C_t}$ according to the manufacturer's instructions (figure 1 in paper IV). A higher ΔC_t value corresponds to a lower mRNA expression why the values are presented inverted as $10/\Delta C_t$ in (figure 2 in paper IV). Serial dilutions with the transcribed cDNA were performed to verify the quality of the RNA.

6.2.4.4 Immunohistochemistry

Immunostaining was performed using the MACH 3TM Mouse-Probe and Goat-Probe HRP Polymer Kit (Biocare Medical, CA, USA). Paraffin sections (5 μ m thick) of cervical biopsies were mounted on glass slides and pre-heated for one hour at 60°C. Thereafter, the sections were dewaxed and antigen retrieval was effected in a 2100 autoclave (Prestige medical, Minworth, England) using Diva DecloakerTM (Biocare Medical) and Hot RinseTM (Biocare Medical) according to the manufacturer's instructions. The peroxidase activity was eliminated by undiluted PeroxidazedTM (Biocare Medical) for 5 minutes and non-specific binding was blocked using Background SniperTM (Biocare Medical). All the sections were incubated for 60 minutes with the primary mouse or goat antibodies. Antibodies and their concentrations are shown in table 7 (in paper IV). Successively, the sections were incubated with mouse-probe or goat-probe MACH3 (Biocare Medical) followed by incubation for 15 minutes with M-Polymer HRP (Biocare Medical) according to the manufacturer's instructions. TBS (Tris buffered saline)-buffer was used in between all steps for rinsing. Betazoid DAB (Biocare Medical) was used to detect antibody reactivity and followed by rinsing with distilled water and counterstaining with Mayer's hematoxylin. As final point, the sections were dehydrated using increasing concentrations of ethanol and finally in xylene before they were mounted with Pertex (Histolab, Gothenburg, Sweden). Universal Negative Control Mouse IgG₁, IgG_{2B}, IgG₃ and IgM (Dako, CA, USA) and Normal Goat IgG (for IL-10) (R&D Systems, MN, USA) were used to verify the specificity of the antibodies.

6.2.4.5 Manual scoring and evaluation of immunohistochemical staining

The assessment of the immunostaining of the slides was performed by two independent observers blinded to the identity of the slides (NR and EA). To evaluate both the intensity (staining intensity=SI) and the distribution patterns (percent positive cells) of the immunohistochemical staining we used the semi-quantitative method (IRS-score) previously described by Remmele and Stegne (Remmele and Stegner 1987) as follows: The semi-quantitative scale was used grading the optical staining intensity (0= no staining, 1=weak staining, 2= moderate staining, 3= strong staining) and the percentage of positive cells (0= no positive cells, 1= <10% positive cells, 2= 11-50% positive cells,

3= 51-80% positive cells, 4= >80% positive cells). The two scores were multiplied resulting in a final score (SI x PP=IRS). The highest of the scores from the two independent observers was used for statistical analysis.

The immunohistochemical staining intensity was evaluated in vascular endothelium, vascular smooth muscle, glandular epithelium, in squamous epithelium and three fields in the stroma by conventional light microscopy at a magnification x200.

6.2.5 Statistical analyses in paper III and IV

Statistical analysis was performed by ANOVA on ranks (Kruskal-Wallis test) or one way ANOVA. Significances were evaluated by Dunn's test. If two independent groups were compared, the Mann-Whitney U test was used. Values were considered significantly different when $P < 0.05$.

6.3 ETHICAL CONSIDERATIONS PAPER I-IV

Ethical approval was obtained from the regional ethical board prior to the initiation of all the studies. For paper I and II (reference number 2008/1182-31/4) the research committee did not require the women to provide informed consent. For paper III and IV (reference number 04-637/4) informed consent was obtained from all women before the biopsies were collected.

7 RESULTS

7.1 PAPER I

The annual rate of postterm pregnancy remained relatively unchanged during the study period (mean 8.94%). The rate of postterm pregnancy increased with increasing maternal age and body mass index. Rates were higher among primiparous compared with parous women. Cigarette smokers experienced lower rates of postterm pregnancy compared with nonsmokers (table 5). Among women with a higher educational level compared with lower educational level. Women who were not living with the baby's father had higher rates when compared with cohabiting women (see table 1 in paper I).

Table 5 Adjusted odds ratios for delivery of postterm birth associated with maternal characteristics between 1992 and 2006

Characteristic	No. of Births (N=1,176,131)	No. of postterm births (N=105,197)	Rate % (8.94 %)	Adjusted Odds Ratio 95% CI	p-value ^a
Age (yrs)					< 0.0001
≤ 19	24,377	1,861	7.72	0.90 (0.84-0.95)	
20-24 ^b	190,683	14,669	7.80	Ref	
25-29	415,674	34,854	8.55	1.20 (1.18-1.23)	
30-34	372,426	34,223	9.40	1.44 (1.40-1.47)	
≥ 35	172,225	17,361	10.36	1.67 (1.63-1.72)	
Data missing	746	64	-	-	
Parity					< 0.0001
0	507,610	54,042	10.65	1.65 (1.63-1.68)	
≥ 1 ^b	668,502	51,153	7.65	Ref	
Data missing	19	2	-	-	
Body-mass index (BMI)					< 0.0001
≤ 19.9	105,643	6,486	6.22	0.74 (0.72-0.76)	
20.0-24.9 ^b	560,677	46,323	8.40	Ref	
25.0-29.9	224,550	22,834	10.42	1.31 (1.29-1.33)	
≥ 30.0	82,013	9,845	12.42	1.63 (1.59-1.67)	
Data missing	203,248	17,943	-	-	
Cigarette smoking					< 0.0001
None ^b	958,222	86,931	9.07	Ref	
1-9 cigarettes/day	101,243	8,905	8.80	0.99 (0.97-1.02)	
≥ 10 cigarettes/day	49,309	4,002	8.12	0.90 (0.87-0.94)	
Data missing	67,357	5,359	-	-	

^a Wald test of the overall effect (test of general heterogeneity).

^b The births with this characteristic served as the reference group. Adjusted for maternal age, parity, BMI, years of education, living with the baby's father, cigarette smoking and country of birth.

In the multivariate analysis, a more than 50% increase in risk of postterm pregnancy was found among women with advanced maternal age and among primiparous women (Table 5). Compared to normal weight women, the risk of postterm pregnancy was about 60% higher among obese women (adjusted OR: 1.63, 95% CI 1.59 to 1.67). Lean women had an approximately 25% lower risk of postterm pregnancy (adjusted OR: 0.74, 95% CI 0.72 to 0.76) than normal weight women. A slight risk reduction of postterm delivery was observed in heavy smokers compared with non-smokers. Being of non-Nordic origin marginally

decreased the risk of postterm pregnancy (see table 1 in paper I). Excluding women with pre-eclampsia, gestational and prior diabetes did not alter the results for the estimates for risk of postterm pregnancy (data not shown).

Labor induction in postterm pregnancy increased from 28 to 46%, and the proportion of CS following labor induction increased from 14 to 26% 1992 to 2006 (figure 2 in paper I). The rate of cesarean section following induction of labor in postterm pregnancy increased with maternal age and increasing BMI (table 6). The risk of cesarean section following labor induction was more than doubled among women 35 years and older compared to women between 20 and 24 years of age (OR 2.28, 95% CI 2.04 to 2.56). The rate of cesarean section following labor induction among parous women was 10.49% compared with 31.76% among nulliparous women with an OR of 5.05 (95% CI 4.71 to 5.42). Overweight and obese women had higher risks of cesarean section than normal weight women accounting for about 50% of all cesarean section following labor induction. Cigarette smokers compared to non-smokers were at increased risk for cesarean section following labor induction in a dose-dependent manner (table 6).

Table 6 Adjusted Odds Ratios for risk of cesarean section following labor induction in postterm pregnancies associated with maternal characteristics, in Sweden between 1992 and 2006.

Characteristic	No. of labor inductions (N=36,498)	No. of cesarean sections** (N=8,063)	Rate % (22.09%)	Adjusted Odds Ratio 95% CI	p-value ^b
Age (yr)					< 0.0001
≤ 19	584	115	19.69	0.90 (0.69-1.16)	
20-24 ^b	4,764	954	20.02	Ref	
25-29	11,903	2,519	21.16	1.38 (1.25-1.52)	
30-34	12,492	2,894	23.17	1.88 (1.70-2.08)	
≥ 35	6,726	1,574	23.40	2.28 (2.04-2.56)	
Data missing	29	7	-	-	
Parity					< 0.0001
0	19,912	6,324	31.76	5.05 (4.71-5.42)	
≥ 1 ^b	16,585	1,739	10.49	Ref	
Data missing	1	0	-	-	
Body-mass index (BMI)					< 0.0001
≤ 19.9	1,945	296	15.22	0.66 (0.58-0.76)	
20.0-24.9 ^b	15,537	3,087	19.87	Ref	
25.0-29.9	8,874	2,172	24.48	1.45 (1.36-1.55)	
≥ 30.0	4,194	1,141	27.21	1.87 (1.71-2.04)	
Data missing	5,948	1,367	-	-	
Cigarette smoking					0.057
None ^b	30,087	6,624	22.02	Ref	
1-9 cigarettes/day	3,140	653	20.80	1.05 (0.94-1.17)	
≥ 10 cigarettes/day	1,341	277	20.66	1.21 (1.03-1.42)	
Data missing	7,784	1,462	-	-	

^a Wald test of the overall effect (test of general heterogeneity).

^bThe births with this characteristic served as the reference group. Adjusted for maternal age, parity, BMI, years of education, living with the baby's father, cigarette smoking and country of birth.

^cCesarean section following labor induction in postterm pregnancy.

There was no association between socioeconomic status measured as years of formal education and the mother cohabiting with the baby's father or not and cesarean section following labor induction. Being of non-Nordic origin (adjusted OR 1.33, 95% CI 1.22 to 1.45) was associated with an increased risk of cesarean section following labor induction (see table 2 in paper I).

In a sub-analysis of parous women (n = 8,983) from 1999 onwards undergoing labor induction for postterm pregnancy, the rate of cesarean section was 37.5%. Parous women with a previous cesarean section had a sevenfold increased risk of cesarean section following labor induction (adjusted OR 7.19, 95% CI 5.93 to 8.71) compared with parous women with no previous cesarean section. Because the cohort included women with repeated pregnancies, we performed additional analyses restricted to primiparous and parous women, respectively. The results from the analyses did not influence the findings based on the full model.

7.2 PAPER II

A total of 1,195,123 singleton births between 1995 and 2007 were included in the cohort, with 3,787 births among mothers with a diagnosis of PCOS.

Women with PCOS were more likely to be nulliparous than women with no such diagnosis (53.0%, n=1,990 v 43.8%, n=520 106, P<0.001). Undergoing ART was more common in women with PCOS (13.7%, n=510 v 1.5%, n=18,058, P<0.001). Women with PCOS had an almost doubled prevalence of a body mass index greater than 25.0 (60.6%, n=1,980 and 34.8%, n=348,340, P<0.001). Giving birth at advanced maternal age (>35 years) was more common in women with than without PCOS (19.9%, n=753 and 17.6%, n=209,125, P<0.001). Women with PCOS were more likely to have hypertensive disease and diabetes mellitus than women without PCOS (table 7). The absolute rates, absolute risk differences standardized for the difference in characteristics between women with and without PCOS, and crude and adjusted relative risks for adverse pregnancy and birth outcomes are presented in table 8.

In the adjusted analysis, women with a previous diagnosis of PCOS had a higher risk of developing gestational diabetes than women with no such diagnosis (adjusted odds ratio 2.32, 95% confidence interval 1.88 to 2.88), whereas the adjusted risk difference was 1.81%. There was also a strong association between PCOS and pre-eclampsia (1.45, 1.24 to 1.69) and very preterm birth (2.21, 1.69 to 2.90). Women with PCOS had an 18% higher risk of undergoing caesarean section (both emergency and elective) compared with women without PCOS (table 8).

Infants born to mothers with a previous diagnosis of PCOS were more often large for gestational age (1.39, 1.19 to 1.62) and were also at increased risk of meconium aspiration (2.02, 1.13 to 3.61) and a low Apgar score at five minutes (1.41, 1.09 to 1.83). The risk of neonatal death was not significantly increased (1.58, 0.81 to 3.07)

(See table 8). No residual confounding for maternal age, body mass index, and parity was found in sensitivity analyses (data not shown).

In a stratified analysis the adjusted odds ratio for preterm birth (<37+0 weeks) among women with PCOS undergoing ART was 1.08 (95% confidence interval 0.76 to 1.53) and among women with PCOS conceiving spontaneously was 1.58 (1.33 to 1.79) (P=0.055 for interaction, see table 9). The interaction between body mass index and PCOS and the association with preterm birth was not significant.

Table 7 Characteristics of women with and without polycystic ovary syndrome (PCOS) giving birth to singleton pregnancies (1 195 123 total births) in Sweden 1995 to 2007

Maternal characteristics	No of births (rate %)		P value*
	Women with PCOS (n=3,787)	Women without PCOS (n=1,191,336)	
Age (years):			
13-24	339 (8.95)	188,795 (15.85)	<0.001
25-29†	1,180 (31.16)	390,088 (32.74)	
30-34	1,515 (40.01)	403,303 (33.85)	
≥35	753 (19.88)	209,125 (17.55)	
Data missing	0	25	
Body mass index:			
≤19.9	148 (4.53)	99,360 (9.91)	<0.001
20.0-24.9†	1,140 (34.88)	554,456 (55.33)	
25.0-29.9	932 (28.52)	245,606 (24.51)	
≥30.0	1,048 (32.07)	102,734 (10.25)	
Data missing	519	189,180	
Parity:			
0	1,990 (52.55)	520,106 (43.66)	<0.001
≥1†	1,797 (47.45)	671,206 (56.34)	
Data missing	0	24	
Education level (years):			
≤11	2,139 (56.69)	663,449 (56.11)	0.47
≥12†	1,634 (43.31)	518,974 (43.89)	
Data missing	14	8,913	
Cigarette consumption (daily):			
None†	3,192 (90.42)	988,375 (88.60)	0.003
1-9	232 (6.57)	87,909 (7.88)	
≥10	106 (3.00)	39,291 (3.52)	
Data missing	257	75,761	
Assisted reproductive technology:			
Yes	515 (13.60)	18,020 (1.51)	<0.001
No	3,272 (86.40)	1,173,316 (98.49)	
Essential hypertension:			
Yes	26 (0.69)	3,345 (0.28)	<0.001
No	3,761 (99.31)	1,187,991 (99.72)	
Diabetes mellitus:			
Yes	76 (2.01)	4,596 (0.38)	<0.001
No	3,711 (97.99)	1,186,767 (99.62)	

*Wald test of overall effect (test of general heterogeneity).

†Reference group.

Table 8 Pregnancy and perinatal outcomes in women with/without polycystic ovary syndrome (PCOS) giving birth to singleton pregnancies in Sweden 1995 to 2007

Pregnancy outcomes	No of births (rate %)		Standardized absolute risk difference* (%) in women with PCOS	Crude odds ratio (95% CI)	Adjusted odds ratio† (95% CI)	P value
	Women with PCOS (n=3,787)	Women without PCOS (n=1,191,336)				
Gestational diabetes:						
Yes	125 (3.30)	1,672 (0.90)	1.85	3.78 (3.16 to 4.52)	2.32 (1.88 to 2.88)	<0.0001
No	3,662 (96.70)	1,180,664 (99.10)	—	1.00	1.00	
Pre-eclampsia:						
Yes	221 (5.84)	35,129 (2.95)	1.74	2.04 (1.78 to 2.34)	1.45 (1.24 to 1.69)	<0.0001
No	3,566 (94.16)	1,156,207 (97.05)	—	1.00	1.00	
Very preterm birth ($\leq 31+6$)‡:						
Yes	65 (1.73)	7,999 (0.67)	0.94	2.59 (2.02 to 3.31)	2.21 (1.69 to 2.90)	<0.0001
No	3,701 (98.27)	1,178,296 (99.33)	—	1.00	1.00	
Data missing	4	1,145	—	—	—	
Moderately preterm birth (32+0 to 36+6)‡§:						
Yes	226 (6.11)	50,352 (4.27)	1.38	1.46 (1.28 to 1.67)	1.31 (1.13 to 1.53)	0.0004
No	3,475 (93.89)	1,127,944 (95.73)	—	1.00	1.00	
Data missing	4	1,145	—	—	—	
Postterm birth ($\geq 42+0$)‡:						
Yes	252 (6.69)	86,771 (7.31)	-1.39	0.91 (0.80 to 1.03)	0.82 (0.71 to 0.95)	0.0069
No	3,514 (93.31)	1,099,524 (92.69)	—	1.00	1.00	
Data missing	4	1,145	—	—	—	
Apgar score <7 at 5 minutes‡:						
Yes	71 (1.89)	12,909 (1.10)	0.54	1.74 (1.38 to 2.21)	1.41 (1.09 to 1.83)	0.0095
No	3,680 (98.11)	1,165,776 (98.90)	—	1.00	1.00	
Data missing	19	8,755	—	—	—	
Meconium aspiration‡:						
Yes	13 (0.34)	1,738 (0.15)	0.17	2.36 (1.37 to 4.07)	2.02 (1.13 to 3.61)	0.018
No	3,757 (99.66)	1,185,702 (99.85)	—	1.00	1.00	
Large for gestational age:						
Yes	238 (6.32)	45,421 (3.84)	1.69	1.69 (1.48 to 1.93)	1.39 (1.19 to 1.62)	<0.0001
No	3,526 (93.68)	1,138,532 (96.69)	—	1.00	1.00	
Data missing	23	7,383	—	—	—	

*Estimated for significantly associated outcomes.

†Adjusted for maternal age, parity, BMI, years of education, cigarette consumption, assisted reproductive technology, and year of delivery.

‡Live births only.

§Risk compared with deliveries at 37 weeks and later.

Table 9 Adjusted odds ratios of women with and without polycystic ovary syndrome (PCOS) and singleton preterm births (<37+0 gestational weeks) undergoing assisted reproductive technology in Sweden 1995 to 2007

Preterm birth	Assisted reproductive technology*			Adjusted odds ratio† (95% CI)	No assisted reproductive technology*			Adjusted odds ratio† (95% CI)
	Women with PCOS	Women without PCOS	Rate		Yes	No	Rate	
Yes	41	1 400	7.96	1.08 (0.76 to 1.53)	256	58 723	7.83	1.54 (1.33 to 1.79)
No	474	16 618	7.77	1.00	3012	1 113 426	5.01	1.00
Data missing	2	—	—	—	4	1167	—	—

*Interaction analysis for assisted reproductive technology and PCOS in relation to preterm birth, p-value = 0.055.

†Adjusted for maternal age, parity, BMI, years of education, cigarette consumption and assisted reproductive technology.

7.3 PAPER III

7.3.1 Clinical data for the women in paper III and IV

In part 1 of paper III, the women in the NP group were significantly older than the women in the TP and PP groups. This could be anticipated, since hysterectomies are usually performed only in women of more advanced age. Further, for part 1, there were no statistically significant differences in maternal age, BMI or birth weight between the TP and PP groups, but the gestational age was significantly lower in the TP than in the PP group. This difference was expected since the TP group consists of women at term having planned caesarean sections, normally 2 weeks prior to expected due date. For part 2 there were no statistical significant differences between the controls (C) and the postterm responders (R) and non-responders (NR) concerning maternal age, BMI, birth weight or cervical score. No statistical significant difference was found between the postterm groups (R and NR) concerning gestational length, but for both groups it was significantly longer than for the control group. The number of vaginal PGE₂ applications administered to the NR group, and the total amount of PGE₂, was significantly higher (p=0.003 and p<0.001 respectively) as compared to the R group.

7.3.2 Prostaglandin receptor EP1

EP1 mRNA levels were very low and close to detection limit and hence could not be reliably quantified. The immunoreactivity for EP1 was moderate to high in squamous epithelium (SQ) and endothelium, low to strong in glandular epithelium (GE) and low to moderate in smooth muscle cells (smc) of arterial walls and stroma (data not shown). No significant changes were found between the groups in part 1 or 2 of paper III.

7.3.3 Prostaglandin receptor EP2

The EP2 mRNA level was increased in the PP group as compared to the TP group (Figure 3, paper III). EP2 immunostaining was low to moderate in stroma and SQ, low to high in GE and low or absent in smc and endothelium (data not shown) in both part 1 and 2. Staining for EP2 in stroma was significantly higher in the TP than in the NP

group (Figure 4; Figure 1 a-d, paper III) but no differences were found among the postterm women in part 2 (Figure 4: Figure 2 e-h, paper III).

7.3.4 Prostaglandin receptor EP3

The EP3 mRNA level was higher in the NR group, as compared to the R and C groups, and reached statistical significance towards the R group (Figure 3, paper III). EP3 immunoreactivity was moderate to high in stroma and endothelium in both studies, whereas it was low to strong in SQ, smc and GE (Figure 1 e-h, Figure 2 i-l in paper III, scoring data not shown). The immunostaining was significantly higher in the SQ of the TP group as compared to the NP group (Figure 4, paper III). No differences in the other compartments were found in any of the two studies.

7.3.5 Prostaglandin receptor EP4

The EP4 mRNA level exhibited a large variation in the NP group and was significantly lower in the TP group as compared to NP group in part 1 (Figure 3, paper III). In part 2, the EP4 mRNA level was lower in the NR group as compared to the R and C groups (Figure 3, paper III). EP4 protein was absent or faintly expressed in stroma, smc and endothelium, and low to strong in SQ and GE in both studies. Immunostaining was mostly below detection limit in the TP group, although faintly present in SQ and endothelium (Figure 1 i-k, Figure 2 m-o in paper III, scoring data not shown). There was more EP4 immunostaining in the stroma of the NR group as compared to C and R groups (Figure 4, paper III). There was higher immunostaining in GE of the PP group as compared to the TP group (Figure 4, paper III).

7.3.6 Prostaglandin receptor FP

No differences in FP mRNA levels were found between the groups (Figure 3, paper III). Immunostaining of FP was low to moderate in stroma and smc, while it was low to high in endothelium, SQ and GE in both part 1 and part 2 (Figure 2q-s in paper III, data not shown for part 1). The FP immunoreactivity was significantly higher in the NR group as compared to the R and C groups in stroma and endothelium, respectively (Figure 4, paper III).

7.3.7 Ratio between EP3 and EP4 mRNA

To determine if the difference in EP3 mRNA and EP4mRNA levels between the NR group and the R and C groups also resulted in a changed balance within each subject, the ratio between the expression of the contracting EP3 and relaxing EP4 was determined for each sample. We found that there is indeed a change in balance within each subject, the ratio of EP3 was approximately five-fold higher in the NR group as compared to the C and R groups as depicted in table 10.

Table 10 Ratio between EP3 and EP4 mRNA in the individual subjects in paper III

	Groups	EP3/EP4 ratio	P-value
Part 1	NP	1.03 (0.76-2.54)	<i>0.158</i>
	TP	2.99 (0.86-5.21)	
	PP	1.44 (0.35-2.56)	
Part 2	C	0.99 (0.65-1.41) ^a	<i>0.003</i>
	R	1.05 (0.75-2.22) ^a	
	NP	5.20 (3.88-11.2) ^b	

Results are shown as median and range.

Results with different letter designations are significantly different.

7.3.8 Stromal factors

Stromal factors were only investigated for part 2 in paper III. Results will only be presented for ALOX15.

A strong immunostaining of ALOX15 was found in stroma, smc and endothelium, whereas it was absent in GE and low to absent in SQ (Figure 2 u-w in paper III, scoring data not shown). The level of immunostaining was higher in the NR group as compared to the C group (Figure 5, paper III), with a similar trend towards the R group.

7.3.9 Leukocytes

Manual scoring of immunostaining in leukocytes, as recognized by morphology, within the cervical tissue showed a relation to the amount of leukocytes (supplementary figure 1 and 2 in paper III). Consequently, the immunostaining results mimic the amount of leukocyte influx. The immunostaining of EP1, EP2 and EP3 was increased in the PP group as compared to the NP group. For EP1 and EP2 in the PP group there was also a significant difference compared to the TP group. The immunostaining for EP2 was lower in the NR group as compared to controls, whereas EP1 and EP3 showed similar trends which did not reach statistical significance. We found very sparse, if any, EP4 immunostaining in the leukocytes. Compared to the NP group, there was a tendency towards an increase in FP immunoreactivity in the PP group, as well as a decrease in the NR group compared to controls. ALOX15 exhibited very low immunoreactivity in leukocytes, possibly because the enzyme was secreted from the leukocytes and could therefore be present in the surrounding tissue.

7.4 PAPER IV

7.4.1 Clinical data

There were no differences between the women concerning age, BMI or birth weight. Neither was there any statistical difference in gestational length between the postterm groups, but a significantly lower gestational age in the C group. The NR group received significantly more PGE₂ applications than the R group ($p=0.032$).

7.4.2 mRNA expression

Expression of mRNA for all the investigated cytokines was detected in all groups. The expression of the pro-inflammatory cytokine IL-1 β was 20-fold lower in the NR group as compared to both the R and C group ($p < 0.001$) (figure 1 and figure 2A, paper IV). Correspondingly to IL-1 β , IL-6 expression was lower in the NR group exhibiting a 25-fold lower expression ($p < 0.001$) (figure 1 and figure 2B, paper IV). Also IL-8 revealed a similar pattern displaying a lower expression in the NR group, 50 times lower, as compared to the C and R groups ($p < 0.01$) (figure 1 and figure 2C, paper IV). There was no statistical significant difference between the C and R groups for the cytokines IL-1 β , IL-6 and IL-8.

In contrast to the other pro-inflammatory cytokines, there were no statistical significant differences between the groups in relation to mRNA expression for IL-18 (figure 1 and figure 2E, paper IV). For IL-10 the expression was 4 times lower in the NR group as compared to the C and R groups ($p < 0.05$). There was no statistical significant difference between the C and R groups for cytokine IL-10 (figure 1 and figure 2D, paper IV).

7.4.3 Immunohistochemical staining

IL-1 β was detected in all groups and in all tissue components (vascular endothelium, vascular smooth muscle, glandular epithelium, squamous epithelium and stroma). However, no significant differences were detected between the groups in any of the tissue compartments (data not shown, representative images of IL-1 β immunostaining in squamous epithelium are shown in figure 5 a-c, paper IV). In contrast, IL-6 was detected in all examined compartments of the biopsy. The immunohistochemical staining for IL-6 was significantly stronger in vessel endothelium, vascular smooth muscle, glandular epithelium, squamous epithelium and stroma in both postterm (R and NR) groups, irrespective of response to PGE₂ treatment, as compared to the controls (C) (figure 3A-E and figure 5 d-f, paper IV). For IL-8 the immunostaining in squamous epithelium differed between the groups with a significantly higher score in both postterm groups as compared to the C group. In stroma IL-8 was significantly stronger in the R group as compared to the NR and C groups (figure 4 A-B and 5 g-I, paper IV). IL-10 immunostaining in the vascular the smooth muscle was stronger in the postterm R and NR groups in comparison with the C group. Further, IL-10 was significantly stronger expressed in squamous epithelium in the R and NR groups as compared to the controls (figure 4 C-D and figure 5 j-l, paper IV). IL-18 immunostaining was more prominent in squamous epithelium in the R and NR groups as compared to the C group. In vascular smooth muscle IL-18 had a higher score in the NR group as compared to the C and R groups (figure 4 E-F and figure 5 m-o, paper IV).

The corresponding IgGs for the monoclonal mouse derived antibodies (figure 5p, paper IV) (Dako, CA, USA) and the polyclonal goat antibody (figure 5q, paper IV)(R&D Systems, MN, USA) were used for negative controls and did not exhibit any staining.

8 DISCUSSION

8.1 MAIN FINDINGS OF PAPER I AND II

In paper I, we found that Swedish women who were overweight, obese, nulliparous and of advanced age, had an increased risk of postterm pregnancy and cesarean section following labor induction. In addition, women with at least one previous cesarean section were at highly increased risk of cesarean section following labor induction postterm. We also showed that over the past two decades, the rate of labor inductions postterm and the rate of cesarean section following labor induction have almost doubled in Sweden (figure 2, paper I).

In paper II, we showed that pregnant women with a previous diagnosis of polycystic ovary syndrome had a strongly increased risk of giving birth preterm and to have an infant with meconium aspiration and low Apgar score at 5 minutes of age. Furthermore the women with a previous diagnosis of PCOS were at increased risk of experiencing pre-eclampsia and gestational diabetes. These increases in risks existed both for women with PCOS who had undergone assisted reproductive treatment and those who had conceived naturally. The increased risks remained after adjusting for maternal characteristics such as maternal age, body mass index, parity, cigarette consumption, cohabiting status, educational level and undergoing assisted reproductive technology.

8.2 COMPARISON WITH OTHER STUDIES (PAPER I)

8.2.1 Maternal age

The average age in both nulliparous and parous women have increased in Sweden over the last three decades (Bennis 2009). This is also true for many other high-income countries. In the US the birthrate for women 35-39 years of age has risen nearly 50% (Martin JA 2009).

We showed that among Swedish women maternal age was a strong and dose-dependent risk factor for postterm pregnancy adjusting for maternal age, parity, BMI, years of education, living with the infant's father, cigarette consumption and country of birth. This finding has been supported by several previous studies such as a US study and a study using the Swedish Medical Birth Registry (Kistka, Palomar et al. 2007; Denison, Price et al. 2008). In the study by Denison et al. the presented estimates were unadjusted for possible confounding factors, which was improved upon paper I. Interestingly, a Danish study (Olesen, Westergaard et al. 2006) did not find the same result. The gestational length of the women included in the Danish study was however estimated using the last menstrual period (LMP) and not by second trimester ultrasound. The latter method was used in our study and is a more accurate method for dating gestational age. There may hence have been misclassification bias in the Danish study. Such misclassification would underestimate the association between the dependent and independent variables in the study, and thus decrease the chances of finding a true association.

We also showed that the risk of cesarean section after postterm labor induction was more than doubled in women > 35 years of age. Earlier research has found that advanced maternal age (>35 years) is an independent risk factor for cesarean section independently of whether labor starts spontaneously or by induction (Vrouenraets, Roumen et al. 2005). In studies concerning risk of cesarean section after labor induction irrespective of gestational age, advanced maternal age appeared as a risk factor (Ennen, Bofill et al. 2009). In addition, older parturients, as compared to younger, were in a US study found to more often have non-progressive labor and require oxytocin more often and for longer periods of time to achieve vaginal delivery (Adashek, Peaceman et al. 1993). However, in an earlier Swedish case-control study, maternal age was not found to be a risk factor for cesarean section but could be attributed lack of power (Cnattingius, Hoglund et al. 2005).

8.2.2 Overweight and obesity

An increase in mean maternal age in both nulliparous and parous women, also the mean BMI has increased in Sweden during the last 15 years (Bennis 2009). In paper I, overweight and obesity accounted for almost 20 % of all postterm pregnancy and 45 % of all cesarean sections following labor induction at postterm, which is in line with previous studies (Denison, Price et al. 2008; Ennen, Bofill et al. 2009). The risk for both postterm pregnancy and cesarean section following labor induction increased in a dose-dependent manner.

8.2.3 Parity

Being nulliparous was strongly associated with an increased risk for postterm pregnancy. This is in line with several earlier research findings (Olesen, Westergaard et al. 2006; Stotland, Washington et al. 2007). Nulliparity was also the strongest risk factor for cesarean section following labor induction with a five-fold risk increase as compared to parous women. Among women with term pregnancies, nulliparity has also been shown to be a risk factor for cesarean section after labor induction (Maslow and Sweeny 2000; Ziadeh 2002; Ennen, Bofill et al. 2009).

8.2.4 Socioeconomic factors and smoking

In the present study there was no association between postterm pregnancy and registered socioeconomic factors, e.g. years of formal education, cohabiting status and country of origin. Low socioeconomic status has been associated to postterm pregnancy in a US study (Kistka, Palomar et al. 2007). However in the study setting, good health care during pregnancy is not available to all women of all socioeconomic strata. The women in our study in the Swedish setting had access to antenatal and obstetrical care, free of charge. Furthermore, second trimester ultrasound is performed in almost all pregnancies (95 %) in Sweden which make errors in pregnancy dating less likely to bias the results. The classification “non-Nordic” for the variable country of birth is however a heterogeneous group in terms of socioeconomic belonging and hence makes it difficult to draw any conclusions from the estimates.

Smoking was in the present study associated with a lower risk for postterm pregnancy which has been confirmed by one study using Swedish population based data (Denison, Price et al. 2008). Further, in a US population based study no association was found between smoking and postterm pregnancy (Kistka, Palomar et al. 2007).

8.3 COMPARISON WITH OTHER STUDIES (PAPER II)

8.3.1 Preterm birth

Previous studies have correlated preterm birth to obesity, ART and PCOS (Cnattingius, Bergstrom et al. 1998; Schieve, Meikle et al. 2002; Boomsma, Eijkemans et al. 2006). However, comparing mean length of gestation in women with PCOS and controls, no difference was found (Boomsma, Eijkemans et al. 2006). Furthermore, previous studies have foremost included women undergoing ART or ovulation induction, which is associated to multiple births. In previous reports the strongest predictor for preterm birth in women with PCOS was multiple birth (Mikola, Hiilesmaa et al. 2001). A meta-analysis found a 75% increased risk of preterm birth in women with PCOS. However, when comparing mean gestational length the controls did not differ from the women with PCOS (Boomsma, Eijkemans et al. 2006). We found in paper II that the risk of very preterm birth was more than doubled. In contrast to previous studies we were able to exclude multiple births and control for possible confounders such as maternal age, BMI, parity, years of education, cigarette consumption, year of delivery and most of all undergoing ART.

8.3.2 Fetal growth

In paper II we found that a higher proportion of macrosomia and being large for gestational age (LGA) among infants born to a mother with PCOS. This finding differs from other studies which have not established this association (Vollenhoven, Clark et al. 2000; Mikola, Hiilesmaa et al. 2001; Haakova, Cibula et al. 2003). It is well known that conditions such as being overweight, having gestational diabetes or glucose intolerance is associated with an increased birth weight in the off spring (Baeten, Bukusi et al. 2001). We have however controlled for BMI in the multivariate analyses and our study therefor provides stronger evidence for PCOS as an independent risk factor for accelerated fetal growth, irrespective of BMI.

In paper II, there was further no association between an earlier diagnosis of PCOS and being small for gestational age, which has been found in some studies (Vollenhoven, Clark et al. 2000; Mikola, Hiilesmaa et al. 2001; Haakova, Cibula et al. 2003) but disconfirmed by others (Sir-Petermann, Hitchensfeld et al. 2005). In contrast to these earlier results, our study controlled for maternal age, BMI, parity, years of education, cigarette consumption, year of delivery and undergoing ART. Our results could not be correlated to an increased risk of being small for gestational age suggesting that PCOS is not associated with an increased risk for growth restriction.

8.3.3 Maternal pregnancy complications

It was supported by our study that women with a previous diagnosis of PCOS are at increased risk of gestational diabetes (Radon, McMahon et al. 1999; Boomsma, Eijkemans et al. 2006). Further, we found a strong association between PCOS and pre-eclampsia. Some studies have reported this finding (Fridstrom, Nisell et al. 1999;

Boomsma, Eijkemans et al. 2006), while others have not (Mikola, Hiilesmaa et al. 2001). It is well supported that women undergoing ART have a higher risk of hypertensive disease during pregnancy, which has been attributed to the underlying cause of infertility rather than to the technique itself (Jackson, Gibson et al. 2004; Kallen, Finnstrom et al. 2005).

8.3.4 Perinatal mortality and asphyxia

In spite of the large sample size we could not find an association between stillbirth and neonatal death and a previous diagnosis of PCOS. Infants born to mothers with a previous diagnosis of PCOS were more likely to have low Apgar score at five minutes of age and to experience meconium aspiration. In a meta-analysis there was an increased risk for perinatal mortality as well as admission to the neonatal intensive care unit (NICU) (Boomsma, Eijkemans et al. 2006). Previous studies have included twin pregnancies which are more common in women undergoing ovulation induction and ART, as in the case of anovulatory women with PCOS. Twin pregnancies are further at increased risk of admission to the NICU as well as an increased mortality which can be attributed to increased rate of premature birth in this group. Previous studies have been small in size and have not been able to exclude multiple births reducing the possibility to elucidate whether the increased risk is associated to PCOS or to multiple births. In paper II we excluded all multiple births to be able to study the effect of having a previous diagnosis of PCOS on pregnancy outcomes. Multiple pregnancies differ in gestational length and growth and the associated risks are hence difficult to differentiate from the risks associated to having PCOS.

8.4 METHODOLOGICAL CONSIDERATIONS AND LIMITATIONS (PAPER I AND II)

8.4.1 Internal and external validity

The major threat to the validity of a study is systematic and random errors. The internal validity refers to the lack of systematic errors in a study while external validity refers to the generalizability of the findings to a larger population.

Systematic errors are also called bias. Random errors can be accounted for by increasing sample size. Systematic errors on the other hand have to be accounted for in the design of the study and cannot be corrected by increasing the sample size. Bias can be divided into three categories: selection bias, information bias and confounding that will be discussed further in this chapter. To increase the internal validity of the study, one should try to prevent bias already in the design of the study and take into account possible confounders.

8.4.1.1 Selection bias

Selection bias occurs when the procedure for selection of subjects to the study is associated to the incidence or prevalence of the exposure or the outcome. Selection bias also exists when the characteristics of the subjects included in the study does not represent the characteristics of the source population. This will lead to an over- or

underestimation of the association between the exposure and the outcome. Information in the Swedish Medical Birth Registry covers almost all births in Sweden with a frequency of missing in only 1.4% of all births (Petra Otterblad Olausson and Pakkanen 2003). If the lack of information is randomly distributed among people in the study, the impact on the estimates will be small and selection bias is hence not an important systematic error. However, if the characteristics of the missing population differ in characteristics from the sampled population, selection bias may be an important issue, especially in rare outcomes.

8.4.1.2 *Information bias*

Information bias refers to when information from study subjects is collected systematically incorrect, misclassified. Misclassification bias can either be *differential* or *non-differential*: Differential misclassification is when the misclassification differs in the exposed and the non-exposed group leading to under- or overestimation of the true association. If misclassification bias is non-differential the misclassification occurs equally in both exposed and non-exposed groups leading to underestimation of a true association.

In paper I, the major strength, is the large population size and the prospective recording of the maternal risk factors such as smoking, height and weight and consequently the risk of misclassification bias might be small. Further Sweden is an exceptional country for this kind of study with a large proportion of pregnancies dated by routine ultrasound in second trimester (98%), hence misclassification of gestational age is consequently low and is unlikely to have substantial impact on the results in both paper I and II.

In paper II, women with PCOS according to the Rotterdam criteria but not seeking medical assistance for irregular menstrual periods, infertility problems or hirsutism might have been incorrectly classified as not having the syndrome. Such misclassification would lead to an underestimation of the association between PCOS and adverse pregnancy outcome. Further, when the National Institute of Health and the Rotterdam criteria for PCOS were compared the prevalence was doubled using the Rotterdam criteria from 2003 (Tehrani, Simbar et al. ; March, Moore et al. 2009). This implies that there is an underestimation of women with PCOS at the beginning of the study period when the National Institute of Health criteria were used.

8.4.1.3 *Confounding*

Confounding is a form of bias and an essential concern in epidemiological studies. On a simple level it may be considered as a confusion of effects. A confounder is a factor that is associated with both the exposure and the outcome without being an intermediate step in the causal pathway. Confounding occurs when the association between the exposure and the outcome is influenced by one or more other factors which can distort the association between the exposure and the outcome.

When designing a study different measures can be taken to control for confounding, either in the design of the study or at the phase of the analysis. For example, age and BMI are often considered as confounders in perinatal epidemiology. One way to control for confounding is by stratification and regression analysis.

In paper I and II we have through unconditional multivariate regression analysis controlled for a number of possible confounders such as maternal age, parity, BMI, smoking, education level and cohabiting status. In paper II we also included ART in the regression model. However, in paper I and II we did not have information on lifestyles and environmental factors such as drug use, alcohol consumption and caffeine use during pregnancy which may constitute confounding factors for the studied outcomes in both papers.

In paper II, it was evident that women with PCOS are older than their counterparts. Advanced maternal age is a well known mediator for adverse pregnancy outcomes (Usta and Nassar 2008). Even though we adjusted for maternal age in the multivariate analysis, residual confounding could still be present.

8.4.1.4 Internal validity

The internal validity of paper I and paper II depends mainly on the quality of the parameters in Swedish Medical Birth Registry (Petra Otterblad Olausson and Pakkanen 2003). Regarding the exposure variables, data on smoking is relatively good with missing information in 4.2 to 9.0 % of cases (Petra Otterblad Olausson and Pakkanen 2003). As we only have data on smoking habits registered at the first antenatal visit there is a risk that we have misclassified some smokers as non-smokers, due to smokers who stopped smoking at their first antenatal visit later on in pregnancy took up smoking again. Further, there may be misclassification if smokers report themselves as non-smokers. Hence this would induce an underestimation of smoking as an exposure to postterm pregnancy in paper I and as an underestimation of smoking as a confounder in paper II.

BMI was calculated from weight and height at the first antenatal visit. Data is available in about 65 % of the cases but with a reasonable high validity and accuracy as investigated in earlier research (Petra Otterblad Olausson and Pakkanen 2003). Since exposure data was recorded prospectively before the outcome was known it will have little effect on the risk estimates.

8.4.1.5 External validity

The Swedish Medical Birth Registry registers 98.6% of all deliveries taking place at registered health care institutions in Sweden. The remaining births are not recorded in the Swedish Medical Birth Registry because the documents about the delivery of the infant have not been sent to the registry. If mothers and infants with missing data have characteristics which are very different from the mothers and infants with recorded data, certain rare descriptive statistics of pregnancy outcomes may be biased and not generalizable to all births in Sweden. One could for example imagine unregistered births taking place among marginalized paperless immigrants with substantially higher perinatal mortality rates than at public health institutions. Results on the association between the exposure and outcome variables in paper I and paper II are however unlikely to be affected to any relevant extent of this particular selection bias. Further, in paper II we may however have included women with a more severe manifestation of PCOS and the findings may hence not be generalizable to all women with PCOS.

8.4.2 Cohort study design

The cohort design is adequate to study the effect of a certain exposure on certain outcomes. For paper I and II, we used data from the Swedish Medical Birth Registry which is prospectively collected in a systematic manner. The usual shortcomings of cohort studies, such as time and money consuming, has therefor been eliminated. Another advantage of the Swedish Medical Birth Registry is the large size of the cohort allowing us to study rare outcomes such as stillbirth, neonatal death and meconium aspiration as well as several numbers of different outcomes as in paper II. It is also possible to study the effect of an exposure on the outcome in different strata, such as maternal age and BMI like in paper I.

In paper II, with a large cohort study design it was also possible for us to include both spontaneously conceived pregnancies and pregnancies conceived by ART in women with and without PCOS. It was further also possible to test for interaction between PCOS and BMI as well as PCOS and ART. Our results do not support the notion that ART mediates adverse pregnancy outcomes in women with PCOS. This finding is supported by a Norwegian study, stating that adverse pregnancy outcome is mediated by the cause of infertility rather than by the reproductive technology (Romundstad, Romundstad et al. 2008).

8.4.3 Hypotheses and possible mechanisms

In relation to paper I, overweight and obesity seems to be the only modifiable risk factor why intervention strategies for reducing obesity would be of interest in reducing the rate of postterm pregnancy and cesarean section after labor induction but also in preventing other adverse pregnancy outcomes that are associated to increased body mass index (Cnattingius, Cnattingius et al. 1998). Also, nulliparity is a risk factor for postterm pregnancy and cesarean section after labor induction. The biological rationale behind this finding could be that a previous pregnancy may have given rise to a higher number of gap junctions between the myocytes in the uterus, resulting in a shorter second stage of labor in parous women.

There was a reduced risk of postterm pregnancy in smoking women in paper I. In studies concerning risk factors for preterm birth, smoking is a well-established risk factor but the biological underlying principle is not completely understood (Cnattingius 2004). A possible explanation may be that smoking inhibits release of progesterone from granulosa-, luteal- and trophoblast-cells in vitro (Miceli, Minici et al. 2005) and consequently triggers the onset of labor.

The results from paper II do not support the belief that ART mediates adverse pregnancy outcomes among women with PCOS. This finding is supported by a Norwegian study, which reports that adverse pregnancy outcomes is mediated by the cause of infertility rather than to the factors related to reproductive technology (Romundstad, Romundstad et al. 2008). Women with PCOS have increased levels of androgens which has been associated with development of pre-eclampsia. Treatment with metformin has been related to improved severe pregnancy and postpartum

outcomes. The mechanism may be mediated by reduced uterine artery impedance, since the androgen levels do not improve during metformin treatment (Troisi, Potischman et al. 2003; Vanky, Salvesen et al. 2004; Salvesen, Vanky et al. 2007).

8.5 MAIN FINDINGS OF PAPER III AND IV

In paper III, we found a higher expression of the mRNA for the contractile EP3 and a lower expression of the relaxatory EP4 in the postterm non-responder group. We found a more than five-fold increase in the EP3/EP4 ratio comparing the non-responder group (NR) to the controls (C) and responder (R) groups.

We found in paper IV that the cytokine expression, both pro- and anti-inflammatory, was down regulated in the postterm non-responder group as compared to the control group and the postterm responder group.

8.6 COMPARISON WITH OTHER RESEARCH (PAPER III AND IV)

There are very few studies in relation to several prostaglandin receptors in human gestational tissues and to the best of our knowledge no studies have been presented concerning expression of prostaglandin receptors during human cervical ripening. The results will hence be discussed mostly in relation to animal studies and human non-pregnant tissue from the reproductive tract.

In paper III, EP1 was present in very low amounts in cervical tissue. EP1 mRNA has been identified in myometrium and endometrium in the non-pregnant state (Catalano, Wilson et al. 2011) and in human myometrium at term together with EP3. In a previous study it was found that the expression of EP1 and EP3 did not change with the onset of labor (Arulkumaran, Kandola et al. 2011). In a recent study EP1 has been found to be down-regulated by estradiol in the rodent uterus (Blesson, Buttner et al. 2012). Women at term have high estradiol levels which could explain the low amounts of EP1 in paper III.

EP2 mRNA was found to be up-regulated immediately after parturition in the term postpartal groups as compared to term pregnant and non-pregnant. This finding is consistent with the changes seen in caprine cervix during labor (Gu, Gao et al. 2012). Expression of EP2 mRNA in endometrial tissue is increased during the secretory phase when progesterone and estrogen levels are high (Catalano, Roman-Drago et al. 1998). In earlier studies, estrogen and progesterone levels have been shown to be increased postpartum as compared to term pregnant not in labor (Stjernholm, Sahlin et al. 1996; Stjernholm, Sahlin et al. 1997).

EP3 is the primary subtype that mediates myometrial contractility (Arulkumaran, Kandola et al. 2011) as well as vascular smooth muscle. EP3 mRNA was increased in the cervix in the postterm non-responder group implicating a decreased blood flow in the cervix. The mRNA for EP4 was low in the term pregnant group before labor and increased after labor. Similarly, EP4 was low in the postterm non-responder group as compared to the laboring women. These findings may indicate that mRNA for EP4 is

up-regulated during cervical ripening and is consistent with findings from caprine cervixes during labor (Gu, Gao et al. 2012).

In paper IV, IL-1 β mRNA was significantly decreased in the postterm non-responder group. In previous studies on cytokines in gestational tissues, IL-1 β has been shown to be an important inducer of IL-8, IL-6 and IL-10 production in the lower uterine segment and in the choriodecidua (Simpson, Keelan et al. 1998; Winkler, Fischer et al. 1998). Further, IL-1 β mRNA and protein has been demonstrated to increase during dilation to a certain degree during the dilation process and then to decrease in the latter phase of cervical dilation (Winkler, Fischer et al. 1998; Osman, Young et al. 2003). In the same study, IL-8 protein was shown to increase during the whole dilation process. This is in agreement with the results in paper IV, with a higher mRNA for IL-8 in the laboring groups. The postterm non-responder group had low expression of IL-8 as well. It has been identified that IL-8 promotes recruitment of neutrophils which in turn stimulate IL-6 and IL-8 production from cervical fibroblasts (King, Kelly et al. 2001). Further, cultured cervical epithelial cells from non-pregnant women have a basal secretion of IL-1 β and IL-8 (Barclay, Brennand et al. 1993; Woodworth and Simpson 1993; Fichorova and Anderson 1999).

IL-10 is a potent anti-inflammatory agent, which acts through decreasing production of the pro-inflammatory cytokines IL-1 β , IL-6, IL-8 and endogenous PGE₂ (Fortunato, Menon et al. 1996; Fortunato, Menon et al. 1998; Sato, Keelan et al. 2003). IL-10 mRNA has been shown to increase in cervical tissue at the time of parturition irrespective of gestational age (Dubicke, Fransson et al. 2010). However, in contrary IL-10 expression decreases in the placenta with advancing gestation (Hanna, Hanna et al. 2000) in order to benefit pro-inflammatory actions associated with term parturition. The different actions by IL-10 suggest that the mechanism through which IL-10 acts is different at early and late gestation. In paper IV, the mRNA expression of IL-10 as well as the mRNA of the pro-inflammatory cytokines IL-1 β , IL-6, IL-8 and IL-18 were down regulated in the postterm non-responder as compared to the controls and postterm responder groups suggesting that IL-10 might have a pro-inflammatory mode of action at term and onwards. IL-10 expression can be induced by IL-1 β in the decidua (Trautman, Collmer et al. 1997) and it may be possible that a down regulated pro-inflammatory cytokine network also affects the expression of IL-10.

Progesterone plays a key role in the regulation of cervical ripening and onset of labor. In humans progesterone levels remain elevated but a functional withdrawal of progesterone is seen by progesterone receptor withdrawal (Stjernholm-Vladic, Wang et al. 2004). Postterm women not responding to PGE₂ treatment have an increased progesterone receptor expression, at mRNA and protein level, as compared to postterm women responding to PGE₂ and term women with spontaneous onset of labor (Vladic-Stjernholm, Vladic et al. 2009). In conclusion PGE₂ treatment is associated with a progesterone receptor withdrawal which has been confirmed in in vitro studies with myometrial cells (Madsen, Zakar et al. 2004). Women not responding to prostaglandin treatment also have a decreased cytokine expression. In cervical fibroblasts, IL-1 β has been associated with a progesterone receptor

withdrawal. The finding of decreased pro-inflammatory cytokine expression in postterm women not responding to PGE₂ can hence be coupled with an absence of progesterone receptor withdrawal in the cervix (Stjernholm-Vladic, Wang et al. 2004).

Prostaglandins are some of the main mediators in the control of parturition, and their production by intrauterine tissues can be up-regulated by pro-inflammatory cytokines (Keelan, Blumenstein et al. 2003). Without an increased cytokine expression the prostaglandin expression may not be initiated. An overall quenched inflammatory response is seen in the non-responder group. It has been shown that pro-inflammatory cytokines such as IL-1 β and IL-4 increase the expression of the contractile prostaglandin receptors EP1 and EP3 in the uterus (Spaziani, Tsibris et al. 1997). It has also been shown that cultured human cervical fibroblasts respond to IL-1 β stimulation by an increase in the expression of EP4 and a decrease in EP1.

Looking at the immunohistochemical analyses in paper IV, the epithelium was shown to express all studied cytokines stronger than any other tissue compartment. It can hence be hypothesized that the epithelium may be an important participant in cervical ripening by containing cytokines and is described in other studies for other agents involved in cervical ripening e.g. CRH (Dubicke, Akerud et al. 2008), fibronectin (Sennstrom, Granstrom et al. 1998), IL-8 (Sennstrom, Ekman et al. 2000) and MMP-8 (matrix metalloproteinase 8) (Sennstrom, Brauner et al. 2003).

8.7 METHODOLOGICAL CONSIDERATIONS AND LIMITATIONS (PAPER III AND IV)

A weakness of paper III and paper IV was the limited amount of cervical tissue, permitting only analysis by RT-PCR and immunohistochemistry. For mRNA expression of the prostaglandin receptors the available tissue was very limited and therefor, in some of the groups, mRNA was only analyzed in a few samples. The groups are relatively small since it is difficult to obtain cervical biopsies from women in labor or about to be induced for labor. However, significant differences are still detected in both mRNA- and protein expressions for the cytokines and prostaglandin receptors.

Immunohistochemistry is a semi-quantitative method with which one can identify specific parts of the cervical tissue as possible sources for the studied prostaglandin receptors and cytokines. An ideal complement to the performed mRNA and protein analyses would have been a western blot to quantify the protein levels. The mRNA results are obtained in a homogenate of cervical tissue, while the protein is detected in separate tissue compartments.

The biopsies were taken at defined time points; at term before labor and after delivery, after vaginal delivery in postterm responding to PGE₂ application and after cesarean section in those not responding to PGE₂. We were unable to temporally follow the changes seen in an individual's cervix as labor progresses or during cervical ripening

after PGE₂ administration due to obvious limitations in tissue sampling during parturition. It has been discussed that the cervical ripening as an inflammatory reaction is a result of the mechanical influence during cervical dilation. However, no significant differences on the cytokine expressions in cervical biopsies from women with different degrees of dilation (four, nine and ten cm of dilation) have been registered (Tornblom, Klimaviciute et al. 2005).

8.8 POSSIBLE MECHANISMS (PAPER III AND IV)

The unfavorable change in EP expression could be the reason for the absent ripening after prostaglandin priming in postterm women not responding to PGE₂. This changed balance may influence the vascular function (vascular smooth muscle) and the attraction of activated cells into ECM. It may be plausible that a quenched cytokine response in the postterm non-responder group may influence the finding of increased EP3/EP4 ratio causing the lack of response to PGE₂ labor induction. Hence, we cannot exclude that the repeated PGE₂ applications to this group may have influenced the results.

Recruitment of leukocytes to the cervical tissue is an important step in the changes seen during cervical ripening (Osman, Young et al. 2003; Sahlin, Stjernholm-Vladic et al. 2008). Postterm women not responding to prostaglandin treatment have been identified to have impaired leukocyte influx into the cervical tissue (Sahlin, Stjernholm-Vladic et al. 2008). Migration of leukocytes is important for releasing degradative enzymes for the remodeling of the extracellular matrix (Osman, Young et al. 2003; Sahlin, Stjernholm-Vladic et al. 2008). It might not only be impairment in leukocyte function but secretion of chemoattractants such as cytokines in the cervix that is impaired that could explain findings.

The high mRNA expression for all cytokines seen in the laboring groups as compared to the postterm non-responder groups may be a consequence to the mechanical influence of the infant passing the birth canal. Further, there was a higher expression of the cytokine proteins seen in the postterm groups may be explained by prostaglandin application. A step towards elucidating the reason for the discrepancies in mRNA and protein expression in the different groups could be to prospectively take brush swabs or fine needle biopsies from the cervix and sample epithelial cells that could be analyzed concerning both pro- and anti-inflammatory cytokines. Since the sampling is non-invasive and would be prospectively gathered, the cytokine expression could be followed over time. Exposures such as prostaglandins, oxytocin and mechanical influence on the tissue that may influence the cytokine and prostaglandin receptor expression could then be monitored.

In paper IV, the expression of both pro- and anti-inflammatory cytokines were down regulated with a concomitant up regulation of proteins in the postterm group not responding to PGE₂ for labor. The term control group and the postterm responder group in contrast had up regulated cytokines and the proteins in the control group were down regulated in contrast to the post term groups. The regulation at gene and protein

level may be dysfunctional in the non-responder group. Potential regulatory mechanisms that may affect gene expression could be explained by microRNAs. MicroRNAs consist of few nucleotides as compared to other RNA molecules. They function as transcriptional regulators by preventing translation of mRNA or activating degradation of mRNA and gene silencing (Kusenda, Mraz et al. 2006; Bartel 2009). Although there are few studies, existing research suggest that microRNAs play a critical role in multiple processes of human physiology such as embryonic development and granulocyte differentiation (Fazi, Rosa et al. 2005; Yang, Yang et al. 2005) and explaining disease onset in different fields such as cancer (Lu, Getz et al. 2005), pre-eclampsia (Montenegro, Romero et al. 2007) and chorioamnionitis (Pineles, Romero et al. 2007). There is also involvement of microRNAs in embryonic development. For future studies, it would be of interest to investigate whether the difference in cytokine gene expression between term and postterm women responding and not responding to PGE₂ treatment may be regulated by microRNAs. Recent research indicates gestational age dependent gene expression patterns of microRNAs in choriomniotic membranes (Montenegro, Romero et al. 2009). Further, differential expression of microRNAs has been described in cervical tissue at term after labor and vaginal delivery and not in labor (Hassan, Romero et al. 2010), suggesting participation in transcriptional regulation of genes involved in cervical remodeling.

Toll-like receptors (TLR) are key components of the innate immune response and are pattern recognition receptors. They have the ability to recognize bacterial structures as well as single and double-stranded RNA in virus and bacteria. Apart from microbial structures, endogenous ligands can also induce TLR response (Patni, Flynn et al. 2007). Activation through TLR has been shown to induce secretion of a number of pro-inflammatory cytokines, TNF- α and interferons (Parker, Prince et al. 2007) also seen in the physiological inflammatory response during labor. TLR have been described in several gestational tissues such as placenta, fetal membranes and cervical tissue (Patni, Flynn et al. 2007). In relation to the process of cervical ripening, TLR2 and TLR4 increase in cervical tissue both in term and preterm labor (Hassan, Romero et al. 2006; Dubicke, Andersson et al. 2010) and a decrease in cervical TLR3 and TLR5 with microarray analysis in term labor as compared to not in labor (Hassan, Romero et al. 2006). Given the poor cytokine response in postterm women not responding to PGE₂, TLR may play a role in this disabled inflammatory pathway.

9 FUTURE RESEARCH

The results from this thesis give rise to a number of further research questions, which could be addressed in future research.

In paper II there was no correlation between postterm pregnancy and PCOS. However, the findings in the study concerning PCOS and adverse pregnancy outcome are of clinical interest and further research is warranted on several research issues. PCOS is a heterogeneous group with a broad range of clinical presentations. Women with PCOS range from those presenting with light or no symptoms to those who have severe manifestations with amenorrhea and hyperandrogenism. Given this situation, it would be of interest to identify if there is a specific phenotype in women with PCOS that is associated with increased risks of adverse pregnancy outcomes and whether there are components in the syndrome that may be predictive of specific complications such as gestational diabetes and pre-eclampsia. It would also be of interest to know if specific interventions could improve outcomes. The association between improved pregnancy outcome and insulin sensitizers such as metformin has been contradictory because of power issues in the current studies. Larger prospective studies are needed with the possibility to clarify whether Metformin could be a possible treatment for improving certain adverse pregnancy outcomes in women with PCOS.

In paper IV, the expression of both pro- and anti-inflammatory cytokines were down regulated with a concomitant up regulation of proteins in the postterm group not responding to PGE₂ for labor. The term control group and the postterm responder group in contrast had up regulated cytokines and the proteins in the control group were down regulated in contrast to the post term groups. For future studies, it would be of interest to investigate whether the difference in cytokine gene expression between the postterm women may be regulated by miRNAs.

In normal cervical ripening the remodeling in ECM is synchronized with infiltration of leukocytes and increased vascular permeability. In the light of the finding of scarce influx of leukocytes into the cervix in postterm women not responding to prostaglandin treatment, it would be of interest to examine whether there is an abnormal angiogenesis in the cervix during pregnancy in these women. The overall down regulated cytokine expression may be associated to decreased angiogenesis already in early pregnancy.

10 CONCLUSIONS

Advanced maternal age, nulliparity and BMI > 30.0 kg/m² are risk factors for postterm pregnancy and cesarean section following labor induction postterm. For accurate risk estimation of failed labor induction, bishop score should be included.

Women with PCOS are at increased risk of adverse pregnancy and perinatal outcomes that could not be attributed to ART, or obesity.

Prostaglandin receptors in postterm women not responding to prostaglandin treatment exhibit an increased expression of the contractility inducing EP3 and a down-regulation of the relaxation-inducing EP4 in the cervix. This unfavorable balance in the expression of these prostaglandin receptors may be of importance in disturbed cervical ripening among postterm women.

An overall down-regulation in the mRNA expression of both pro- and anti-inflammatory cytokines may exist among postterm women who do not respond to prostaglandin treatment. Future research on the causes of postterm pregnancy could thus benefit from investigating biochemical pathways with the capacity of global cytokine down-regulation.

11 POLICY IMPLICATIONS

We found that women with a previous diagnosis of polycystic ovary syndrome were at increased risk of adverse pregnancy and perinatal outcomes. The risk increase could not be attributed to assisted reproductive technology or obesity. These women should be considered a high-risk group in obstetric care. Local obstetric guidelines should ensure that the group is appropriately monitored during pregnancy and parturition.

12 SVENSK SAMMANFATTNING

Bakgrund: Överburenhet inträffar när en graviditet varat 42 veckor eller mer. Överburenhet drabbar ungefär 5-10 procent av alla fullgångna graviditeter. Tillståndet är associerat med ökad maternell och fetal sjuklighet såväl som med ökad perinatal dödlighet. Kvinnor som är äldre och förstföderskor löper en ökad risk för överburenhet. Genetiska anlag kan också bidra till en ökad risk för överburenhet. Vissa egenskaper som är vanligare hos kvinnor med överburenhet, i synnerhet obesitas, hög maternell ålder och att vara förstföderska är också vanliga bland kvinnor med polycystiskt ovarie syndrom (PCOS). En normal och fördröjd förlossningsstart är en komplex och inte helt klarlagd process. En nyckelkomponent vid normal förlossning är en inflammatorisk process i livmoderhalsen, som benämns ”cervixmognad”, och som föregår den normala förlossningen. Hos en del överburna kvinnor sker ingen cervixmognad, trots försök till förlossningsinduktion med prostaglandiner. Denna avhandling syftar till att beskriva risk faktorer för överburenhet och misslyckad induktion, att undersöka uttrycket av prostaglandin receptorer och cytokiner i cervixvävnad hos överburna kvinnor som genomgått lyckad respektive misslyckad induktion samt att undersöka sambanden mellan PCOS, överburenhet och graviditetsutfall.

Metod: För studie I, identifierades en kohort med fullgångna och överburna enkelbördsgraviditeter födda mellan 1992 och 2006 (totalt n=1,176,131) från Medicinska Födelseregistret. Flerbördsförlossningar uteslöts. Riskfaktorer för överburenhet och kejsarsnitt efter induktion analyserades. I studie II identifierades på samma sätt en kohort av enkelbördsgraviditeter födda mellan 1995 och 2008 (total n= 1,191,336) varav 3,787 var barn födda av mödrar med en tidigare PCOS diagnos. För studie III och IV, togs transvaginala cervix biopsier från icke-gravida, fullgångna gravida och postpartala kvinnor samt från överburna gravida kvinnor med lyckad respektive misslyckad induktion. Biopsierna analyserades med realtids PCR med avseende på mRNA uttryck. Immunhistokemi utfördes för att analysera uttrycket och distributionen av cytokiner (IL-1 β , IL-6, IL-8, IL-10 och IL-18), prostaglandin receptorer (EP1-4 och FP) samt stromafaktorer (CTGF, Calgranulin B, furin och ALOX15).

Resultat: Vi fann att hög maternell ålder, att vara förstföderska och att ha ett BMI över 30 kg/m² var riskfaktorer för överburenhet och kejsarsnitt efter induktion. Vi fann att en tidigare PCOS-diagnos inte var associerat till överburenhet. Däremot, och oberoende av assisterad befruktning och BMI, hade barn till mödrar med tidigare PCOS-diagnos ökad risk för graviditets-komplikationer. I studie III och IV fann vi att överburna kvinnor med utebliven cervixmognad hade ett högt ratio mellan prostaglandinreceptorerna EP3 och EP4 samt en generell nedreglering av både pro- och antiinflammatoriska cytokiner i livmoderhalsen.

Slutsats: Resultaten talar för att gravida kvinnor med PCOS bör ses som en högriskgrupp för att drabbas av komplikationer under graviditet och förlossning och därmed bör de obstetriska riktlinjerna ses över. Överburenhet är associerat med obesitas, att vara förstföderska och hög maternell ålder. Nedregleringen av pro- och anti-inflammatoriska cytokiner och ett högre ratio mellan prostaglandinreceptorerna EP3 och EP4 bland kvinnor med utebliven cervixmognad ger viktig information för att förstå normal och patologisk förlossningsfysiologi.

Nyckelord: Överburenhet, cervix utmognad, cytokiner, prostaglandinreceptorer, förlossningsinduktion, polycystiskt ovariesyndrom, obesitas, kejsarsnitt.
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