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Karolinska Institutet, Stockholm, Sweden

PRETERM AND TERM CERVICAL RIPENING

STUDIES ON CRH, HMGB1, TOLL-LIKE RECEPTORS, CYTOKINES AND MATRIX METALLOPROTEINASES

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To my family

Abstract

Objective: Preterm birth (PTB) is the leading cause of neonatal mortality and morbidity. Despite the existing treatment, the frequency of PTB has not changed in the past thirty years. Incomplete understanding of the biological and pathophysiological mechanisms underlying preterm delivery is the major obstacle to preventing PTB. Cervical ripening is necessary for vaginal delivery, for which reason understanding of preterm cervical ripening is required for developing new treatment strategies. The overall aim of the work presented in this thesis was therefore to determine possible differences between preterm and term cervical ripening.

Methods: Transvaginal cervical biopsies were obtained from women undergoing spontaneous delivery or elective caesarean section at preterm and term, and from non-pregnant women. Real-time RT-PCR was employed for analysis of mRNA, and immunohistochemistry, ELISA and Immulite for protein analysis. Corticotropin-releasing hormone (CRH), its binding protein (CRH-BP), its receptors (CRH-R1 and CRH-R2), matrix metalloproteinases (MMP) -1, -3, -8, -9, high-mobility group box protein 1 (HMGB1), receptor for advanced glycation end products (RAGE), Toll-like receptor 2 (TLR2), TLR4, interleukin (IL)-1α, IL1-β, IL-12, IL-18, IL-4, IL-10 and IL-13 were analyzed in cervical tissue. Preterm and term cervical fibroblast cultures were established and the secretion of IL-8, MMP-1 and MMP-3 was measured after stimulation with CRH.

Results: CRH, CRH-BP, CRH-R1, CRH-R2 and HMGB1 were identified in human cervical tissue for the first time. TLR2, TLR4, IL-10 and IL-12 were identified in the cervix for the first time in relation to pregnancy and labor. The distinct changes were determined in the cervix in labor irrespective of gestational age. There was downregulation of mRNA for CRH-BP, CRH-R2, RAGE, IL-12, IL-18, but upregulation of mRNA for TLR2, IL-10, IL-1β, MMP-1, MMP-3 and MMP-9. More extranuclear staining of HMGB1 in stroma and empty nuclei in squamous epithelium were observed in labor. TLR2 and TLR4 tissue expression was lower in labor. IL-4 and IL-12 concentrations were lower, but soluble RAGE, IL-18, MMP-8 and MMP-9 were higher in labor. Differences between preterm and term cervical ripening were found: mRNA expression of TLR2, TLR4 and IL12 was lower in preterm labor, while IL-10 protein expression was higher in the cervical epithelium in preterm labor. Furthermore, preterm and term cervical fibroblasts showed different secretion patterns with higher levels of IL-8 and MMP-1, but lower levels of MMP-3, at preterm. CRH significantly increased the secretion of IL-8 in cervical fibroblasts. Subgroup analysis revealed some differences in association with preterm premature rupture of membranes (PPROM) and positive vaginal and/or urinary cultures.

Conclusions: Preterm cervical ripening is an inflammatory process similar to cervical ripening at term. However, some differences still exist: these include downregulation of TLR2, TLR4 and IL-12 and higher levels of IL-10 in cervical epithelium. CRH and HMGB1 are probably involved in cervical ripening. Our results indicate that PPROM and PTL with infection could partly involve different mechanisms.

Key words: cervical fibroblasts, cervical ripening, cervix, chemokines, cytokines, CRH, HMGB1, interleukin, labor, preterm, preterm labor, matrix metalloproteinases, pregnancy, PPROM, Toll-like receptors

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Submitted

ABBREVIATIONS

ABC	Avidin-biotinylated peroxidase complex
AGE	Advanced glycation end products
BMI	Body mass index
cDNA	Complementary deoxyribonucleic acid
COX	Cyclooxygenase
CRF	Corticotropin releasing factor (=CRH)
CRH	Corticotropin-releasing hormone
CRH-BP	Corticotropin-releasing hormone binding protein
CRH-R	Corticotrophin-releasing hormone receptor
C_T	Threshold cycles
DAB	Diaminobenzidine
15d-PGJ_2	15-Deoxy-$\Delta^{12, 14}$- prostaglandin J_2
ECM	Extracellular matrix
ER	Estrogen receptor
esRAGE	Endogenous secretory receptor for advanced glycation end-products
FISH	Fluorescence in situ hybridization
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPCR	G protein-coupled receptors
HCG	Human chorionic gonadotropin
HMGB1	High-mobility group box protein 1
HPA	Hypothalamic-pituitary-adrenal
IFN	Interferon
Ig	Immunoglobulin
IGF-I	Insulin-like growth factor-I
IL	Interleukin
iTreg	Induced regulatory T cell
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor kappa B
NO	Nitric oxide

NOS	Nitric oxide synthase
NOx	Nitric oxide metabolites (nitrate and nitrite)
NP	Non-pregnant
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PGE_2	Prostaglandin E_2
PGN	Peptidoglycan
PPROM	Preterm premature rupture of membranes
PR	Progesterone receptor
PRR	Pattern recognition receptors
PTB	Preterm birth
PTD	Preterm delivery
PTL	Preterm labor
PTnotL	Preterm not in labor
RAGE	Receptor for advanced glycation end-products
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
sRAGE	Soluble receptor for advanced glycation end-products
Th	T-helper cell
TIMP	Tissue inhibitor of matrix metalloproteinases
TL	Term labor
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor-α
TnotL	Term not in labor
UCN	urocortin

BACKGROUND

Introduction

Preterm birth (PTB) is defined by the World Health Organization as one that occurs at less than 37 full weeks or 259 days of gestation. The global rate of preterm birth is 9.6% (personal communication, Mario Meraldi, World Health Organization). In the USA, the preterm delivery rate is 12-13%, in Europe and other developed countries the rates are 5-9% (Goldenberg et al. 2008). Preterm birth is the leading cause of neonatal mortality and morbidity, accounting for as much as 75% of perinatal deaths (Slattery and Morrison 2002). Preterm babies have an increased risk of neurological, metabolic and respiratory disorders (Saigal and Doyle 2008). The risk of severe medical disabilities increases sharply with decreasing gestational age at birth (Moster et al. 2008; Saigal and Doyle 2008). Infant survivors of very preterm birth (22-25 weeks of gestation) to approximately 50% can develop severe long-term physical or mental disability (Slattery and Morrison 2002). Follow-up of preterm born babies until adulthood show that they are more likely to have chronic health problems, lower IQ, subnormal weight, lower education, diminished long-term survival and lower reproduction (Slattery and Morrison 2002; Moster et al. 2008; Swamy et al. 2008). Care of preterm infants in US costs approximately $26 billion/year, $51 600 per infant, in medical and social expenses (Behrman and Butler 2006).

Preterm birth can be divided into induced deliveries or elective cesarean sections for maternal or fetal indications, and spontaneous preterm birth due to spontaneous preterm labor or preterm premature rupture of membranes (PPROM) (Goldenberg et al. 2008). Preterm birth is a multifactorial disorder. Multiple mechanisms such as infection, intrauterine over-distension, uteroplacental ischemia or hemorrhage, stress and endocrine factors, cervical disease and other immunologically mediated processes can lead to preterm birth (Challis et al. 2009). The possible pathways may involve corticotropin releasing hormone (CRH), Toll-like receptors, cytokines and prostaglandins and matrix metalloproteinases (Figure 1).

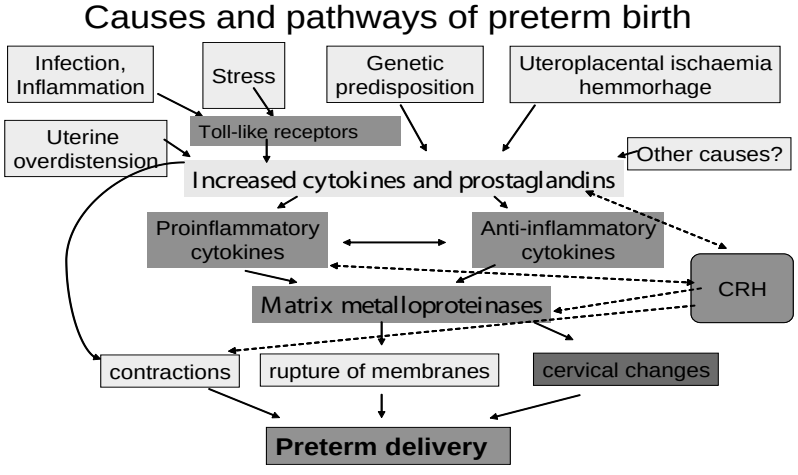

Figure 1. *Possible causes and pathways of preterm birth*

Despite existing treatment strategies, the spontaneous preterm birth rate has not diminished in the past thirty years. There is a need for new strategies for treatment and prevention of preterm birth. The treatment strategies used today concentrate on tocolytic agents and on diminishing the contractile state of the uterus. However, the actual preterm delivery first requires cervical softening, which then is followed by myometrial contractions. Indeed, even painfully strong contractions in combination with stiff and closed cervix do not result in preterm delivery. It is therefore of major importance to improve our understanding of the physiology of preterm cervical ripening. A pharmaceutical treatment inhibiting the softening process could be a new alternative to delaying a threatening preterm delivery.

Risk factors for preterm birth

The mechanisms starting preterm labor are still largely unknown. However, several risk factors, clinical diagnoses and pregnancy complications have been identified as associated with preterm birth. They can be divided into three main groups:

1. Maternal risk factors
2. Pregnancy history
3. Pregnancy characteristics

Maternal risk factors

In the USA, the rate of preterm birth is higher in African-American women than in Caucasian: preterm birth rates are 16-18% in black women compared to 5-9% in white women (Slattery and Morrison 2002; Goldenberg et al. 2008). The disparity of preterm birth continues even after adjustment for educational level and socioeconomic status. This indicates genetic predisposition. Other maternal risk factors are low socioeconomic and educational status, low and high maternal ages, single marital status and hard physical work under stressful conditions (Slattery and Morrison 2002; Goldenberg et al. 2008). There is a higher risk of PTB in pregnancies which arise in less than six months after previous delivery (Conde-Agudelo et al. 2006). Low and high maternal BMI is also associated with a higher risk of preterm birth (Goldenberg et al. 2008). Maternal dietary intake and low weight gain during pregnancy are associated with PPROM and PTB without PPROM (Gosselink et al. 1992).

Preterm birth is more common among women reporting stress and anxiety (Lobel et al. 1992). Although the mechanisms underlying the association between stress and increased preterm birth are unknown, the role of corticotropin releasing hormone has been proposed (Lockwood 1999).

Cervical procedures for cervical intraepithelial neoplasia have been associated with preterm delivery (Jakobsson et al. 2007).

Pregnancy history

The recurrence risk in women with previous preterm delivery ranges from 15% to more than 50% (Goldenberg et al. 2008). Women with previous preterm deliveries have a 2.5-fold increased risk of preterm delivery in their next pregnancy. An early (23-27 weeks of gestation) previous spontaneous delivery is more predictive of recurrence and it is very strongly associated with early spontaneous delivery in the next pregnancy (Mercer et al. 1999). Induced and spontaneous abortions are also associated with a higher risk of a subsequent preterm birth (Swingle et al. 2009).

Pregnancy characteristics

Multiple gestations carry a substantial risk of preterm delivery and result in 15-20% of all preterm births. Almost 60% of twins are born preterm (Goldenberg et al. 2008). Several pregnancy

complications, like polyhydramnios or oligohydramnios, placental abruption, placenta praevia, preeclampsia or eclampsia, lead to increased risk for indicated or spontaneous preterm birth. Maternal abdominal surgery or medical disorders, such as thyroid disease, asthma, diabetes, hypertension, are often associated with preterm delivery (Goldenberg et al. 2008).
Assisted reproductive treatment (ART) even in singleton pregnancies can predispose preterm birth (Perri et al. 2001; Slattery and Morrison 2002). This could be due to various infertility factors, such as uterine malformations, previous operative procedures or previous pelvic infection. This is supported by the finding that pregnancies derived in couples with strict male infertility, have no increased risk of preterm delivery (Perri et al. 2001).
Smoking during pregnancy, increases the risk of preterm birth. Both nicotine and carbon dioxide are powerful vasoconstrictors and can cause placental damage and decreased uteroplacental blood flow. The use of drugs, such as cocaine and heroine, has been associated with preterm birth in several studies (Slattery and Morrison 2002; Goldenberg et al. 2008).
The association between maternal infection, especially intrauterine infection, and preterm labor has been widely researched (Goldenberg et al. 2000; Romero et al. 2007). Intrauterine infection might occur in 25-40% of preterm births (Goldenberg et al. 2008). Infection causes preterm labor and PTB more frequently at lower gestational ages and at PPROM (Romero et al. 2002). Several non-genital tract infections, such as pyelonephritis, pneumonia and appendicitis, probably predispose to preterm birth (Goldenberg et al. 2008). Untreated asymptomatic bacteriuria is associated with preterm delivery/low birth weight and antibiotic treatment is effective in reducing low birth weight (Romero et al. 1989b). Periodontal disease also causes preterm birth (Jeffcoat et al. 2001). However, treatment of periodontal disease during pregnancy does not prevent preterm birth (Newnham et al. 2009).

Prediction of preterm birth

The early detection of preterm birth is difficult. Most women who deliver preterm have no obvious risk factors and more than half of preterm births occur in low-risk pregnancies (Iams et al. 2001). Only half of the women exhibiting the signs of preterm labor actually deliver preterm. There are still no good screening tests for asymptomatic, low-risk pregnancies (Iams et al. 2001).
Sonographic measurement of cervical length and fetal fibronectin in cervicovaginal secretions are established predictors of spontaneous preterm delivery in women with symptoms of preterm labor and in women with previous preterm delivery (Iams 2003; Spong 2007). In symptomatic women, the optimal threshold of cervical length to exclude preterm birth is 30 mm (Iams 2003). A presence of fetal fibronectin in cervicovaginal secretions after 22 weeks of gestation, suggests choriodecidual disruption (Spong 2007).
Many substances that can be found in body fluids (amniotic fluid, urine, cervical mucus, vaginal secretions, serum or plasma, saliva) have been assessed as possible biological markers of preterm birth, but few have shown clinical usefulness. The substances studied include cytokines, chemokines, estriol, and other substances especially related to inflammation (Goldenberg et al. 2005).
Genetic association studies have shown single-nucleotide polymorphism in several genes associated with preterm delivery and PPROM (Ferrand et al. 2002; Fujimoto et al. 2002; Lorenz et al. 2002; Annells et al. 2004; Hartel et al. 2004; Kalish et al. 2004; Engel et al. 2006; Murtha et al. 2006; Speer et al. 2006; Krediet et al. 2007; Rey et al. 2008). There is some evidence of gene-environment interaction in spontaneous preterm birth (Goldenberg et al. 2008).
Further genetic and proteomic studies are necessary to identify new possible predicting factors and biomarkers of preterm birth.

Prevention and treatment of preterm delivery

Current prevention and management of preterm labor is more symptomatic than causal, as the mechanisms underlying normal and preterm human parturition are still largely unknown, and prediction possibilities are limited. Interventions used to reduce morbidity and mortality related to preterm birth can be primary (directed to all women), secondary (intended to reduce the risk in women with known risk factors) or tertiary (after the parturitional process has begun, seeking to improve outcomes for preterm infants) (Iams et al. 2008).

Primary interventions include both preconceptional measures and measures during pregnancy. There are public educational programs, adjustment of pregnant women's working conditions, smoking cessation, increased access to prenatal care, screening and treatment of asymptomatic bacteriuria (Iams et al. 2008).

Secondary preconceptional interventions are: control of diabetes, seizures, asthma, hypertension and correction of Mullerian anomalies. Postconceptional secondary prevention include antibiotic treatment, progesterone supplementation and cervical cerclage (Iams et al. 2008). There is a disagreement on antibiotics treatment in women with previous preterm birth. The Cochrane review shows that antibiotics treatment of bacterial vaginosis in all pregnant women eradicates bacterial vaginosis, but there is little evidence that it prevents preterm birth or improves the outcome for the infants. In women with previous preterm birth, treatment does not affect the incidence of subsequent preterm birth; however, it decreases the risk of PPROM and low birth weight (McDonald et al. 2005). Conversely, another systematic review failed to show any benefits (Okun et al. 2005).

The role of progesterone for prevention of preterm birth is still uncertain. There is evidence that intramuscular 17-α-hydroxyprogesterone reduces the incidence of recurrent PTB. Vaginally administered progesterone diminishes preterm birth in women with a short cervix. However, the data is inconclusive on whether progesterone lowers perinatal mortality and morbidity (Dodd et al. 2008; Tita and Rouse 2009). Studies have failed to show any effect of progesterone on either PTB or perinatal morbidity and mortality in multiple pregnancies (Dodd et al. 2008; Tita and Rouse 2009).

Cervical cerclage is the intervention most commonly studied and used in clinical practice to prevent PTB when a short cervix is detected with ultrasound examination. However, it is one of the most controversial surgical interventions in obstetrics. Cervical cerclage in women with short cervices and without previous preterm birth does not reduce the risk of PTB. Cerclage might benefit women with short cervices who had previous PTB. However, the evidence is still inconclusive (Iams et al. 2008; Berghella 2009). Cerclage should not be used in twin pregnancies, as in these cases it is associated with a higher PTB rate and higher neonatal mortality (Berghella 2009). Screening for interleukins (IL), such as IL-8, could help better select the patients who can benefit from cervical cerclage (Sakai et al. 2006).

Treatment of preterm labor

Acute treatment of preterm labor includes administration of antibiotics, corticosteroids and tocolytics. Antibiotic treatment of all women with threatened preterm labor is recommended in the USA to prevent neonatal infection with group B streptococcus because infants run an increased risk of this infection. This strategy reportedly decreases mortality rates due to group B streptococcus infection (Iams et al. 2008). In the ORACLE I study, the use of erythromycin after PPROM was associated with pregnancy prolongation and health benefits for neonates (Kenyon et al. 2001a). However, ORACLE II found no benefits of antibiotics in spontaneous preterm labor (PTL) (Kenyon et al. 2001b).

Routine administration of antenatal corticosteroids to women presenting with threatening preterm delivery at less than 34 weeks of gestation reduces neonatal morbidity and mortality from respiratory distress syndrome and intra-ventricular hemorrhage (Crowley 2000).
Tocolytic drugs are used to prolong pregnancy in women with preterm labor with no clinical signs of infection. No studies have shown that tocolysis can improve infant outcomes (Olson et al. 2008). The main purpose of tocolytic drugs is to gain a 48h delay to be able to administer corticosteroids that may enhance fetal lung maturation and to transfer the women to a tertiary center with better care for a preterm infant available.
Tocolytic drugs presently available include β_2-agonistic drugs (ritodrine, terbutaline), oxytocin antagonist (atosiban), calcium-channel blockers (nifedipine), magnesium sulphate, nitric oxide donors (glyceryl trinitrate patches), indomethacin. Historically, ethanol was also used as tocolytic agent (Olson et al. 2008). β-mimetics have been used extensively in the past 20 years. They prolong pregnancy by at least 48h, but there is no evidence of improved perinatal outcome. They are associated with a high frequency of maternal and fetal side effects (de Heus et al. 2009). Atosiban is as effective as β-mimetics and it is the first-choice agent for tocolysis in Europe; but the evidence for its beneficial effects is still controversial. Calcium-channel blockers are as effective as β-mimetics with fewer side effects. Magnesium sulphate is reportedly ineffective in delaying preterm birth. Nitric oxide donors and indomethacin have been less studied and are used less in clinical practice (Tan et al. 2006; Olson et al. 2008).

Anatomy of the uterus

Cervix uteri

The uterus consists of two very different parts: corpus and cervix (Figure 2). Back in 1947 Danforth et al stated that the human cervix consists mainly of fibrous connective tissue (Danforth 1947), which stabilizes and strengthens the structure. The extracellular matrix (ECM) constitutes about 85% and muscle fibers only about 6-10% of non-pregnant cervix (Figure 3) (Danforth 1947; Schwalm and Dubrauszky 1966; Rorie and Newton 1967). The cervical extracellular matrix consists of fibrillar components, proteoglycans, the long polysaccharide hyaluronan, and glycoproteins. Fibrillar components are collagen and elastin. In the cervix, collagen arranged in fibrils makes up 80% of the fibrillar components of which collagen I and collagen III are the major types (Uldbjerg et al. 1983c). Non-pregnant cervix uteri proteoglycans make up 1 % of the dry weight (Uldbjerg et al. 1983c; Norman et al. 1991). The dominating proteoglycan in non-pregnant cervix uteri is the small dermatan sulfate decorin and its glucosaminoglycan chain influences collagen fibrillation and hence the stiffness of the non-pregnant cervix (Uldbjerg et al. 1983b; Uldbjerg et al. 1983c; Uldbjerg and Danielsen 1988; Norman et al. 1991). Fibromodulin and biglycan, and also large proteoglycans such as versican (Uldbjerg et al. 1983b; Norman et al. 1991; Westergren-Thorsson et al. 1998) and heparan sulfate proteoglycan (Cabrol et al. 1985) are present in small amounts.

Corpus uteri

The corpus uteri can be divided to an upper part, the fundus, and a lower part near the cervix, the isthmus. In contrast to the cervix, the uterus is a muscular organ (Figure 3). Muscle cells account for approximately 40-70% of tissue weight (Schwalm and Dubrauszky 1966; Rorie and Newton 1967). ECM surrounding the smooth muscles consists of collagen, proteoglycans and polysaccharides. The ECM helps to hold the muscle bundles together and provide tissue with strength. During pregnancy the smooth muscles of the corpus uteri must be relaxed to allow fetus to grow, but just before labor and during it, the uterus must be a contractile organ.

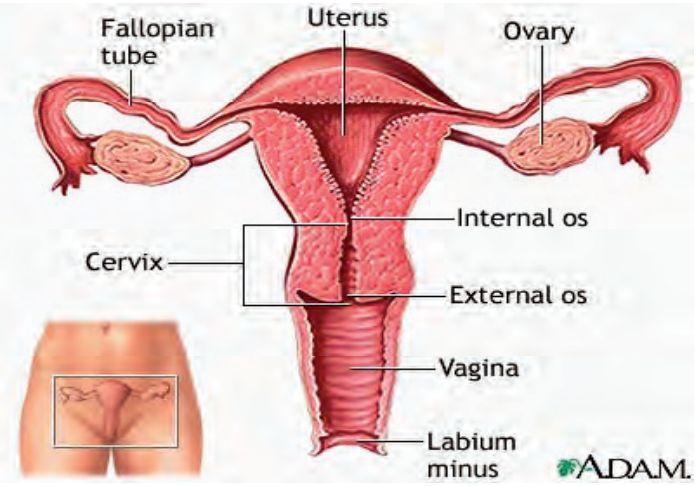

Figure 2. *Anatomy of the human uterus*

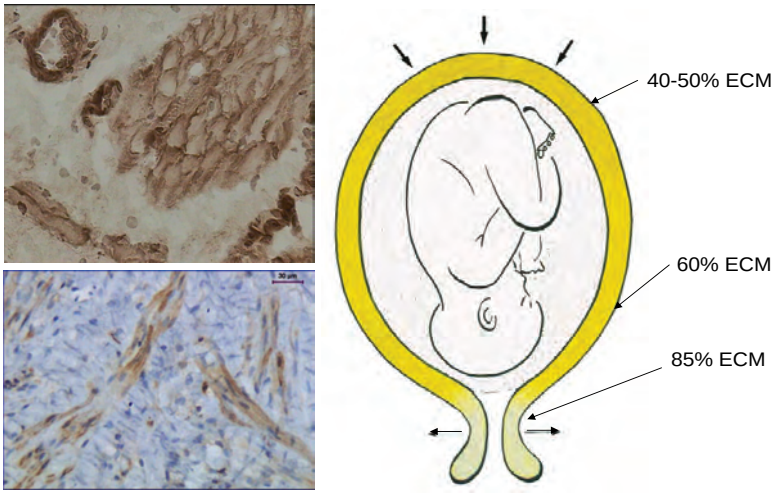

Figure 3. *Extracellular matrix (ECM) and smooth muscles in different parts of the uterus. On the left - immunohistochemical view of corpus uteri (upper picture) and cervix (lower picture).*

Fetal membranes

Fetal membranes are composed of amnion and chorion. The extracellular matrix components, including collagens, elastin, fibronectin and laminins, are important part of fetal membranes. The collagens are the major structural components of fetal membranes. The major tensile strength is provided by the interstitial collagens, types I and III, together with smaller amounts of collagens V, VI, VII in the amnion (Bryant-Greenwood 1998).

Changes during pregnancy and labor

Cervical ripening

The non-pregnant cervix uteri is stiff and non-distensible. Major changes occur during cervical ripening in pregnancy and labor (Figure 4). With the softening of the cervix, there is a gradual decrease in collagen during pregnancy and an increase in its extractability, suggesting the changes in organization of collagen fibrils (Uldbjerg et al. 1983a; Granstrom et al. 1989). Type I collagen shows a fibrillar pattern in the dense fibrous connective tissues of cervical stroma in the first trimester, while it is diffuse and dissociated in the edematous cervical stroma in the third trimester (Iwahashi et al. 2003). At gestational age of 10 weeks the collagen concentration is 70%, and at term 30%, of that in the non-pregnant cervix (Uldbjerg et al. 1983a). Cervical dilatation during labor correlates well to the concentration and physical state of cervical collagen (Ekman et al. 1986). Type I collagen mRNA expression decreases in the cervix during pregnancy - it is significantly lower in the third trimester than in the first (Iwahashi et al. 2003). Immediately after spontaneous delivery, mRNA levels for collagen I and III decrease by up to 60 % of those in the non- pregnant cervix, but during involution (2-4 days after delivery) the message is increased 2.5-fold and 3.5-fold respectively (Westergren-Thorsson et al. 1998).

There are significant changes in the concentration of proteoglycans during pregnancy. The main ones are decreased decorin concentration and an increase in the large chondroitin sulphate proteoglycan versican, in the small dermatan sulfate proteoglycan biglycan and in the heparan sulfate proteoglycans (Norman et al. 1993; Westergren-Thorsson et al. 1998). Versican can attract water and bind hyaluronan (Wu et al. 2005), resulting in disintegration of the collagen bundles and a change in the physical properties to produce a soft and elastic tissue, thus facilitating cervical dilatation.

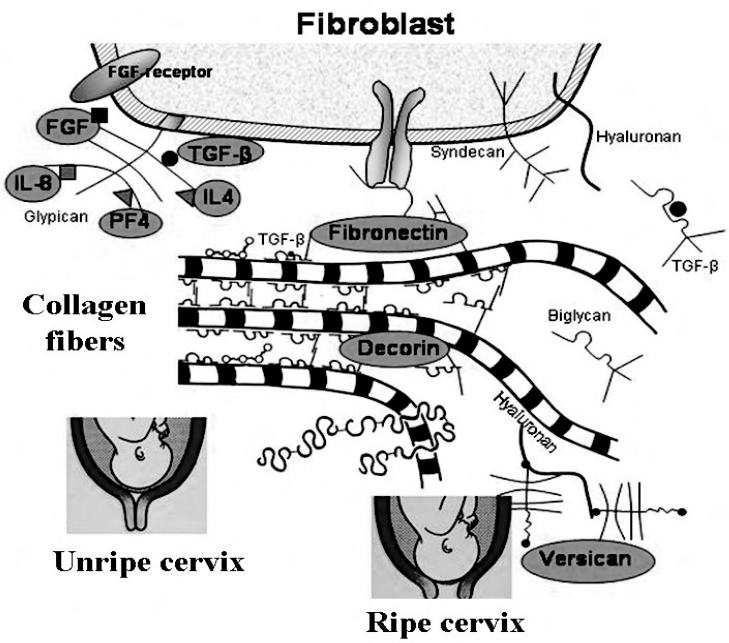

Figure 4. *Extracellular matrix in unripe (on the left) and ripe (on the right) cervix.*

During involution (2-4 days after delivery), the concentration of decorin protein and the expression of mRNA increase, while versican mRNA and protein concentrations decrease nearly to those in the non-pregnant cervix (Westergren-Thorsson et al. 1998).
The mechanisms that initiate and regulate the reorganization of ECM during human pregnancy are still largely unknown. Estrogen, progesterone and insulin-like growth factor-I (IGF-I) are involved in cervical ripening (Stjernholm et al. 1996; Stjernholm et al. 1997; Wang et al. 2001; Ekman-Ordeberg et al. 2003). During pregnancy the serum levels of estrogen and progesterone increase markedly, while estrogen (ER) and progesterone receptors (PR) decrease in cervix at term, with a further decrease in the maximally ripened cervix during parturition (Stjernholm et al. 1996; Stjernholm et al. 1997; Ekman-Ordeberg et al. 2003). ERα mRNA decreases in ripe cervix at delivery, while ERβ mRNA levels are increased in the term pregnant cervix not in labor (Wang et al. 2001). The ERβ antigen is also co-localized with leukocyte markers in cervix (Stygar et al. 2001). Levels of IGF-I mRNA has been shown to increase four-fold from the non-pregnant state till term and decline to half of it during parturition (Stjernholm et al. 1996; Stjernholm et al. 1997). All these findings indicate that cervical ripening at labor is related to significant hormonal changes.

Prostaglandins play an important role in parturition and cervical ripening. They are mainly synthesized in fetal membranes and decidua. Prostoglandins promote the process of cervical ripening, changes of membranes and stimulate contractions of myometrium (Gibb 1998). Local application of prostaglandin-E_2 (PGE_2) is considered to be the "golden standard" procedure for inducing cervical ripening and labor both at term and at preterm (Ekman et al. 1983; Ekman-Ordeberg et al. 1985; Abelin Tornblom et al. 2002). Preterm and term cervical ripening is also associated with decreased degradation of prostaglandins (Tornblom et al. 2004).
Further, the process of cervical ripening at labor can be regarded as an inflammatory reaction (Liggins 1978), since the levels of IL-6, IL-8, monocyte chemoattractant protein (MCP-1) increase both at preterm labor and at term labor (Sennstrom et al. 2000; Tornblom et al. 2005a). This process is also associated with cervical leukocyte invasion (Young et al. 2002; Osman et al. 2003). Cytokines recruit activated cells, in particular neutrophils, which in turn secrete degradative enzymes such as matrix metalloproteinases (MMPs). Thus, increased levels of MMP-1, MMP-2, MMP-3, MMP-8 and MMP-9 have been observed during pregnancy and/or the final cervical ripening at labor (Stygar et al. 2002; Sennstrom et al. 2003) .
Cervical nitric oxide (NO) release also plays important role in cervical ripening. NO has been suggested as an active mediator in cervical ripening (Chwalisz and Garfield 1998), and nitric oxide donors induce ripening of the human cervix (Thomson et al. 1997). The expression of nitric oxide synthase (NOS) isoforms rises in the cervix in late pregnancy and parturition (Ledingham et al. 2000), supporting the hypothesis that NO is involved in the process of cervical ripening. Further, preterm labor is associated with higher mRNA expression of NOS isoforms in the cervix (Tornblom et al. 2005b). Cervical fluid nitric oxide metabolite (NOx) levels rise during labor, nitric oxide donor administration, or cervical manipulation and are significantly related to cervical ripening. NOx levels are also higher in parous women than in nulliparous, which perhaps reflects the fact that the cervix, preceding labor is always more ripe in parous women than in nulliparous. (Vaisanen-Tommiska et al. 2003).
Fetal fibronectin is also a possible mediator of cervical ripening. Cervical fibronectin is increased in women with favorable cervices and fibronectin levels are even higher after PGE_2-induced ripening (Ekman et al. 1995; Sennstrom et al. 1998). Fetal fibronectin has been identified in the epithelial cells of the cervix (Sennstrom et al. 1998).
Relaxin is a polypeptide produced by the placenta during pregnancy. Results conflict regarding the effect of relaxin on cervical ripening in humans (MacLennan et al. 1980; Brennand et al. 1997). A

2001 review concluded that the place of relaxin as a cervical priming agent was still unclear (Kelly et al. 2001). However, relaxin has a potent widening effect in common marmoset monkeys, most prominently in combination with estrogen (Simon and Einspanier 2009).

Cervical fibroblasts

Fibroblasts play a crucial role in the remodeling of the extracellular matrix (Larsen et al. 2006). ECM remodeling occurs both under normal conditions such as labor and during many pathological processes, like asthma, myocardial infarction, and rheumatic diseases (WestergrenThorsson et al. 1996; Frangogiannis et al. 2002; Westergren-Thorsson et al. 2002). Highly active fibroblasts produce ECM components, cytokines and matrix metalloproteinases (Westergren-Thorsson et al. 2002; Malmstrom et al. 2007; Akerud et al. 2008). Altered proteoglycan production is seen in skin and bronchial fibroblasts in association with disease (WestergrenThorsson et al. 1996; Westergren-Thorsson et al. 2002). Cervical fibroblast cultures established from the biopsies from non-pregnant women, term pregnant women after elective caesarean section or partal women, present different and stable phenotypes (Malmstrom et al. 2007). There is a decrease in proteoglycan secretion and an increase in IL-6, IL-8, MMP-1, MMP-3 production in the cultures from partal donors (Malmstrom et al. 2007; Akerud et al. 2008).

Changes in myometrium during labor

In the myometrium, a pro-inflammatory remodeling of the extracellular matrix takes place which is similar to that in the cervix. In term pregnancy a significant decrease in collagen and an increase in collagenase activity are noted in the corpus (Granstrom et al. 1989). Major changes in proteoglycan composition and concentration occur during pregnancy and labor, with a decrease in decorin and biglycan, and a increase in heparan sulfate proteoglycans and syndecan-3 (Hjelm et al. 2002; Hjelm Cluff et al. 2005). There is an increase in protein and/or mRNA expression of IL-1β, IL-6 and IL-8 in association with labor (Osmers et al. 1995; Winkler et al. 1998; Young et al. 2002; Osman et al. 2003). There is an infiltration of inflammatory cells, predominantly neutrophils and macrophages, in human myometrium during spontaneous labor at term (Thomson et al. 1999). The above-mentioned cytokines can be immunolocalized to the leukocytes in myometrium (Young et al. 2002). There is up-regulation of MMP-9 in myometrium during labor and this process is cytokine-mediated in uterine smooth-muscle cells (Roh et al. 2000). NF-κB pathway activation and subsequent increase in cyclooxygenase-2 (COX-2) and MMP-9 have been observed in human term myocyte cultures (Choi et al. 2007).

Changes in fetal membranes during labor

An inflammatory process, similar to that described above for cervix and myometrium, occurs in fetal membranes. During labor, the production of IL-1β, IL-6, IL-8 and tumor necrosis factor-α (TNF-α) increases in fetal membranes (Dudley et al. 1996; Osman et al. 2003). The gene expression profile in chorioamniotic membranes during labor is associated with an inflammatory response (Haddad et al. 2006). Cytokines stimulate the production of MMP-9 by amnion (Arechavaleta-Velasco et al. 2002) and the production of prostaglandins in the fetal membranes (Romero et al. 1989a; Lundin-Schiller and Mitchell 1991). There is an increase in biglycan and hyaluronan in fetal membranes at labor. In cervical amnion there is also a 30% decrease in collagen and a 50% decrease in decorin concentration after delivery (Meinert et al. 2007).

Factors involved in the labor process

Corticotropin-releasing hormone (CRH)

Corticotropin-releasing hormone, CRH, also termed corticotropin-releasing factor (CRF), the 41-aminoacid hypothalamic peptide, was first isolated from ovine hypothalamic extracts in 1981 (Vale et al. 1981). It is the principal regulator of the hypothalamic-pituitary-adrenal (HPA) axis (Figure 5). It belongs to the mammalian "stress" peptides family, which includes the urocortins (UCN) – UCNI, UCNII and UCNIII (Bale and Vale 2004). CRH binding protein (CRH-BP), a 37kDa circulating protein capable of binding CRH and UCNI, can block the action of CRH (Hillhouse and Grammatopoulos 2006). CRH receptors (CRH-R1 and CRH-R2) belong to a class B subtype of G protein-coupled receptors (GPCR) which, on agonist binding, change their structural conformation and transduce signals across cells mainly through activation of heterotrimeric G proteins (Bale and Vale 2004; Hillhouse and Grammatopoulos 2006). CRH-R1 and CRH-R2 are encoded by different genes. In humans, CRH-R1 is located on chromosome 17 (17q12-q22) and CRH-R2 on chromosome 7 (7p21-p15) (Hillhouse and Grammatopoulos 2006). CRH-R1 and CRH-R2 share 70% homology at the amino-acid level, but are different at the N-terminus (Hillhouse and Grammatopoulos 2006). CRH-R1 has α and β isoforms in addition to c-h subtypes. CRH-R2 has three functional subtypes α, β and γ (Bale and Vale 2004). CRH –R1 variants are generated by various exon insertions or deletions, while CRH-R2 variants differ only in the N-terminal extracellular domains (Grammatopoulos 2008).

The HPA axis has been of interest in parturition since Liggins induced premature parturition in sheep after corticotropin and cortisol infusion (Liggins 1968). CRH expression was identified in human placenta more than 20 years ago (Grino et al. 1987). Produced in fetomaternal tissues, CRH is secreted into the maternal circulation, and maternal plasma CRH levels rise during pregnancy, peaking at labor; while CRH-BP levels decrease during late pregnancy (Campbell et al. 1987; Linton et al. 1993; McLean et al. 1995; Hillhouse and Grammatopoulos 2002). After delivery,

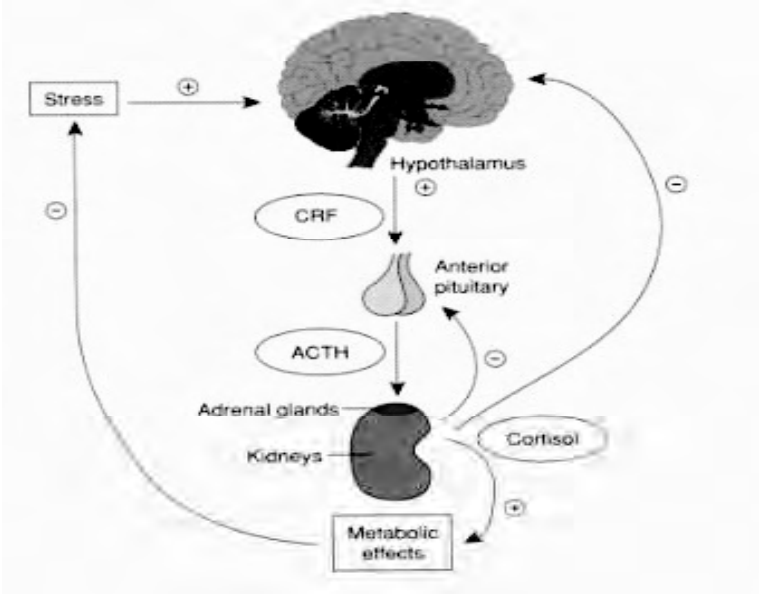

Figure 5. *Hypothalamic-pituitary-adrenal (HPA) axis.*

CRH levels fall to normal within 15 hours (Campbell et al. 1987). The "placental clock" model has been suggested, which determines the duration of gestation and the timing of parturition (McLean et al. 1995). Patients at risk of preterm birth have significantly elevated levels of CRH and lower CRH-BP levels (Hobel et al. 1999). The role of CRH as predicting factor of preterm birth has been studied extensively (Korebrits et al. 1998; Sibai et al. 2005; Hill et al. 2008). Furthermore, there is a substantial increase in maternal circulating CRH levels in other pregnancy complications (e.g. preeclampsia) (Perkins et al. 1995). The biological purpose of this increased CRH secretion during pregnancy complications is not yet known. It could be due to increased stress in the feto-maternal unit, CRH acting as adaptive hormone; or it might be involved in the mechanisms prompting labor (Grammatopoulos 2008).

During human pregnancy CRH appears to target multiple feto-maternal tissues, including fetal adrenals, fetal membranes, placenta and myometrial smooth muscle (Grammatopoulos 2008) (Figure 6). The action of CRH on human myometrium still remains enigmatic. It is considered that CRH has a dual action on myometrium: it is responsible for the quiescence of myometrium during pregnancy and stimulates contractility during labor. This dual action might be due to two receptors CRH-R1 and CRH-R2 and their different splicing variants (Grammatopoulos 2008).

CRH, CRH-BP, CRH-R1 and CRH-R2 have been identified at both mRNA and protein level in human placenta, deciduas, fetal membranes, endometrium and myometrium (Petraglia et al. 1992; Petraglia et al. 1993; Warren and Silverman 1995; Di Blasio et al. 1997; Rodriguez-Linares et al. 1998; Florio et al. 2002; Hillhouse and Grammatopoulos 2002; Wetzka et al. 2003; Sehringer et al. 2004). Furthermore, CRH increases MMP-9 protein secretion by cultured cells from placenta and fetal membranes (Li and Challis 2005). In addition, several studies have shown that CRH can stimulate the production of cytokines in different types of cells (Angioni et al. 1993; Yang et al. 2005; Wang et al. 2007).

However, no studies have been published on cervical tissue and possible role of CRH in cervical ripening.

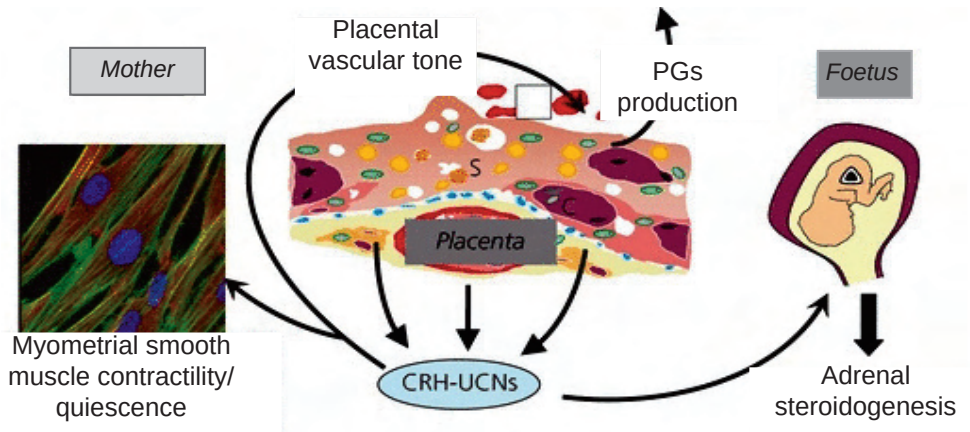

Figure 6. *Target tissues of placental corticotropin-releasing hormone (CRH) during pregnancy. Potential actions of CRH (and urocortins (UCNs)) involve modulation of myometrial activity, placental control of vascular tone, peptide and prostaglandin production and adrenal steroidogenesis and probably many more, yet unidentified processes (PG-prostaglandin, S-syncytiotrophoblast).*

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High-mobility group box protein 1 (HMGB1)

HMGB1 was first discovered 30 years ago as a DNA-binding protein and a transcription factor that migrates quickly during electrophoresis; it was named for this property. The human HMGB1 gene is located on chromosome 13q12 and encodes 215 amino acid peptide. It is highly conserved between the species with 99% identity between rodent, bovine and human proteins. HMGB1 is expressed by almost all cells, except those that have eliminated their nucleus (Lotze and Tracey 2005; Bianchi and Manfredi 2007). In 1999 it was discovered that activated macrophages secrete HMGB1 as a delayed mediator of inflammation (Wang et al. 1999). Later it was shown that HMGB1 has important extracellular cytokine-like functions mediating the late response to infection, injury and inflammation (Lotze and Tracey 2005). HMGB1 is nowadays also considered to be an alarmin (Harris and Raucci 2006). HMGB1 is either secreted actively by macrophages, dendritic cells and natural killer cells (Gardella et al. 2002; Dumitriu et al. 2005; Semino et al. 2005) or released passively from necrotic cells to signal tissue injury and initiate inflammation and repair. In contrast to necrotic cells, apoptotic cells retain nuclear components and do not activate inflammation (Scaffidi et al. 2002). HMGB1 induces NF-κB activation (Lotze and Tracey 2005) and stimulates pro-inflammatory cytokine synthesis (Andersson et al. 2000). It is released rather late during an inflammation reaction – after several hours (Lotze and Tracey 2005). It is involved in several conditions such as arthritis, cancer, diabetes and atherosclerosis (Lotze and Tracey 2005; van Beijnum et al. 2008).

Little is known regarding the involvement of HMGB1 in pregnancy and labor, even though parturition is an inflammatory process. Human term placenta expresses HMGB1. Labor does not influence its placental expression, although a tendency towards higher expression of extranuclear HMGB1 in placentas with preeclampsia has been observed (Holmlund et al. 2007). HMGB1 is expressed basally in human fetal membranes at term pregnancy, and human chorionic gonadotropin (HCG) can increase its expression (Ticconi et al. 2007).

A receptor for advanced glycation end-products (RAGE) and Toll-like receptor 2 (TLR2) and TLR4 are involved in HMGB1-mediated signaling (Bianchi and Manfredi 2007).

Receptor for advanced glycation end-products (RAGE)

The first proposed receptor for HMGB1 was RAGE, a multiligand receptor of the immunoglobulin family. RAGE is expressed on macrophages/monocytes, endothelial cells, neurons and on a variety of tumor cells. It interacts with various ligands, including HMGB1, advanced glycation end products (AGE), β-amyloids and S100 proteins (van Beijnum et al. 2008). There are three major spliced variants of RAGE: full-length, N-terminally-truncated, and C-terminally-truncated. The C-terminally-truncated form is secreted from the cell and is named endogenously-secreted RAGE (esRAGE). The other forms of soluble RAGE (sRAGE) are cleaved from cell-surface RAGE by matrix metalloproteinases (Koyama et al. 2007). sRAGE is considered to act as regulator/inhibitor of HMGB1 action (Lotze and Tracey 2005).

There are few studies on RAGE during pregnancy. RAGE is expressed in trophoblasts of first-trimester human chorionic villi from healthy women (Konishi et al. 2004). Human term placenta expresses RAGE, but labor does not influence this expression (Holmlund et al. 2007). RAGE is also expressed in human myometrium and its expression is increased during pregnancy. RAGE staining is especially increased in vasculature in myometrium in women with preeclampsia (Cooke et al. 2003). There are contradictory results concerning intra-amniotic infection/inflammation and amniotic fluid concentrations of sRAGE (Buhimschi et al. 2007; Romero et al. 2008). Romero et al. showed higher sRAGE and esRAGE levels in amniotic fluid in association with intra-amniotic infection/inflammation (Romero et al. 2008), while Buhimschi et al demonstrated no influence of intra-amniotic infection or inflammation on sRAGE concentration in amniotic fluid (Buhimschi et al.

2007). Women with threatening preterm birth had significantly higher serum sRAGE concentration than healthy pregnant women did. However, serum levels of sRAGE in healthy pregnant women were significantly lower than in non-pregnant (Hajek et al. 2008).

Toll-like receptors (TLRs)

"Toll" is derived from the German for "amazing" or "mad". It was first discovered in the fly Drosophila melanogaster as a plasma membrane protein with a role in developing of dorsoventral polarity during embriogenesis (Hashimoto et al. 1988). Later, it was shown that Toll has a role in the fly's immunity to fungal infections (Lemaitre et al. 1996). The first mammalian TLR4 was identified in 1997 and it was so called because of its sequence homology to the D.melanogaster gene Toll (Medzhitov et al. 1997). TLRs are pattern recognition receptors (PRR). They are expressed on different cell types belonging to innate, adaptive immune system and non-immunological cells. The PRRs recognize pathogen-associated molecular patterns (PAMPs) expressed by different microorganisms. Until now ten functional TLRs have been found in humans (Patni et al. 2007).

TLR2 binds gram-positive bacterial components such as bacterial lipoproteins, peptidoglycan (PGN) and also fungal zymosan and lipoteichoic acids present in mycoplasma. TLR4 interacts with bacterial gram-negative lipopolysaccharide (LPS) (Patni et al. 2007).

Recently, both TLR2 and TLR4 have been shown to be receptors for HMGB1 (Park et al. 2004), suggesting that TLRs can respond not only to microbial stimuli but also to endogenous ligands. Many cytokines, such as IL-6, IL-10, IL-12, TNF-α and interferons (IFNs), are secreted after TLR activation (Kanzler et al. 2007; Patni et al. 2007). Moreover, TLR2 and TLR4 polymorphisms are associated with preterm birth (Lorenz et al. 2002; Krediet et al. 2007; Rey et al. 2008).

TLR2 and TLR4 were the first TLRs to be demonstrated at the protein level in human term placenta (Holmlund et al. 2002). Expression of all ten TLRs has been described in human placenta and extensively studied in pregnancy and in labor (Patni et al. 2007; Koga and Mor 2008). Greater mRNA expression is seen of all TLR genes in the placenta in labor (Patni et al. 2009). However, no differences are seen in immunohistochemical expression of TLR2 and TLR4 in laboring human placenta compared to that from elective caesarean section (Holmlund et al. 2007). Higher protein levels of TLR2 and TLR4 are seen in fetal membranes (Kim et al. 2004), but lower TLR2 protein expression is seen in placentas from pregnancies with chorioamnionitis (Rindsjo et al. 2007).

TLRs in cervix have been less extensively studied. In pregnant mice, increased mRNA expression of TLR2, TLR3 and TLR4 is seen (Gonzalez et al. 2007). TLRs 1 to 6 mRNA and the protein of TLR2 and TLR4 have been found in human non-pregnant cervix (Pioli et al. 2004). TLR1-9 are expressed in human epithelial cells from endo- and/or ectocervix (Herbst-Kralovetz et al. 2008). Only one study has investigated these receptors in human cervix after vaginal delivery and caesarean section (Hassan et al. 2006). Here, upregulation of mRNA expression of TLR2 and TLR4, but downregulation of TLR3 and TLR5, were determined in labor with microarray analysis. The downregulation of TLR3 and TLR5, but not upregulation of TLR2 and TLR4, was confirmed with real-time RT-PCR (Hassan et al. 2006).

Despite all the evidence of a possible role of HMGB1 and its receptors in the labor process, no studies have been done on HMGB1 and its receptors in the human cervix at preterm and term labor.

Cytokines

Cytokines are small proteins (approximately 25 kDa) that are released by various nucleated cells. They are usually released in response to an activating stimulus and induce responses through binding to specific receptors. They can act in an autocrine, paracrine or endocrine manner. Chemokines are a class of cytokines that have chemoattractant properties, inducing cells with appropriate

receptors to migrate towards the source of chemokine. Since the first source of cytokine production identified was white blood cells, many cytokines are named interleukins, that means molecules that are secreted and mediating interactions between leukocytes (Janeway et al. 2001). The traditional role of cytokines is immunoregulatory, but they also have a range of mitogenic and proapaptotic functions on non-immune cells. They play three roles during parturition: they are involved in cervical ripening, promote fetal membrane weakening/rupture and enhance myometrial muscle contractility (Orsi and Tribe 2008).

Cytokines and the immune system

The immune system uses cytokines to communicate. Immune system can be divided to the adaptive (specific) and the innate (non-specific) response. These two responses influence and regulate each other. The innate immune system needs no prior activation and is the first defense against pathogens. Cells belonging to the innate immune system are natural killer cells, monocytes/macrophages, dendritic cells and granulocytes. They are the first to be recruited to the site of injury/inflammation and release chemokines and cytokines. They also signal to the adaptive immune system by releasing different factors. Further, antigen presenting cells, macrophages and dendritic cells, present antigen to adaptive cells through major histocompability complex (MHC) II, expressed on their surface. The adaptive immune system includes B lymphocytes (B cells) and T lymphocytes (T cells), which recognize specific epitopes on pathogens. B cells are important in the response to extracellular pathogens by secreting antibodies. T-cells are matured in the thymus and divided into two classes: T helper cells (Th cells) with expression of CD4 glycoprotein molecules and T cytotoxic cells (Tc-cells) with expression of CD8 glycoprotein molecules. T helper cells are divided into four subpopulations - Th1, Th2, Th17 and induced regulatory (iTreg) cells. They derive from the same precursor Th0 cells. These naïve Th0 cells are undifferentiated CD4+ helper T cells that become polarized and develop into Th1, Th2, Th17 or iTreg cells with the help of cytokines, stress hormones and antigen-presenting cells (Janeway et al. 2001; Plotnikoff et al. 2006; Zhu and Paul 2008). Th1 and Th2 cytokines gain their name from the T cells they are secreted from or from the response they regulate (Janeway et al. 2001; Plotnikoff et al. 2006). Th1 lymphocytes produce mainly IL-1, IL-2, IFN-γ and TNF-α and mediate immune responses against intracellular pathogens. Th2-cells are the source of IL-4, IL-5, IL-6, IL-10, IL-13 and granulocyte-macrophage colony stimulating factor (GM-CSF). They mediate host defense against extracellular parasites (Elenkov and Chrousos 2002; Wilczynski 2005; Raghupathy and Kalinka 2008; Zhu and Paul 2008; Challis et al. 2009). Th1 cytokines are considered to be pro-inflammatory, while some of the Th2 cytokines, like IL-4, IL-10, IL-13, are anti-inflammatory and inhibit Th1 responses (Elenkov and Chrousos 2002).

Th1 and Th2 cytokines in pregnancy

For many years, a healthy pregnancy was described as a Th2 phenomenon while Th1 cytokines were believed to be harmful to pregnancy outcome (Wegmann et al. 1993; Raghupathy 1997). This concept has now developed further and nowadays a complex and dynamic cytokine balance is considered to prevail during gestation (Chaouat 2007). Although Th1 type responses are associated with spontaneous abortions and reproductive failure, they also dominate early during the peri-implantation period as well as during labor (Wilczynski 2005; Raghupathy and Kalinka 2008). However, a pro-inflammatory cytokine bias with higher levels of intererleukin-2 (IL-2), interferon-γ and IL-12 and lower levels of IL-4 and IL-10 is seen in placentas in women with preterm delivery and PPROM than those at term (El-Shazly et al. 2004).

No studies have been done so far regarding the balance of pro-inflammatory and anti-inflammatory cytokines in cervical tissue during pregnancy and labor at term or preterm.

Pro-inflammatory cytokines

IL-1 and IL-8

IL-1 is one of the first cytokines to be described. In 1984, two cDNAs coding IL-1 were reported, which explained diverse biological effects of IL-1. In 1985 they were named IL-1α and IL-1β (Dinarello 1994).

IL-8 is a potent chemokine and an activator of neutrophils. These in turn secrete proteolytic enzymes such as MMP-8 and MMP-9 (Osmers et al. 1995; Stygar et al. 2002). IL-8 and IL-1β are produced by human fetal membranes, myometrium and cervix (Osman et al. 2003) and infiltrating leukocytes are the major source of these cytokines (Young et al. 2002). The concentration of IL-8 increases in the cervix at term labor and is involved in connective tissue remodeling (Sennstrom et al. 2000). It is also produced to a higher extent in the partal cervical fibroblast cultures *in vitro* (Malmstrom et al. 2007). IL-1α stimulates IL-8 production from fibroblasts in human dermal fibroblasts (Schroder et al. 1990). Furthermore, it induces production of proMMP-1 and proMMP-3 in human cervical fibroblasts (Imada et al. 1997a). Stimulation with IL-1α or IL-1β induces production of proMMP-9 in rabbit cervical fibroblasts (Imada et al. 1997b).

IL-12 and IL-18

IL-12 is a pro-inflammatory cytokine, composed of two chains, a heavy chain or p40, and a light chain or p35, forming a disulfide-linked heterodimer, or p70 (Trinchieri 1998; Zhang and Wang 2008). IL-12, produced by activated monocytes, macrophages or other antigen presenting cells, is a major inducer of Th1 differentiation (Elenkov and Chrousos 2002; Zhang and Wang 2008).

IL-18 has structural similarities with IL-1 family proteins. It was initially discovered as an IFN-γ-inducing factor produced by macrophages stimulated with microbes or microbial products. Like IL-1β, it is synthesized as inactive precursor peptide, which is subsequently cleaved by caspase-1 (Arend et al. 2008; Carroll et al. 2008). IL-12 can act synergistically with IL-18 to provoke a Th1 response (Dinarello 1999). IL-18 induces both T and NK-cell maturation and potentiates cytotoxicity. It can induce IL-8 in isolated synovial tissue fibroblasts and endothelial cells (Carroll et al. 2008).

The role of IL-12 and IL-18 during pregnancy and parturition has attracted interest recently. IL-12 and IL-18 are important in regulating natural killer cell activities in early pregnancy, and are considered important for reproductive success. However, altered levels of theses cytokines can be hazardous, as higher circulating IL-12 levels in maternal blood have been described in preeclampsia (Bachmayer et al. 2006). In addition, higher IL-12 levels in mid-pregnancy are associated with preterm delivery (PTD) with chorioamnionitis before 35 weeks of gestation (Gargano et al. 2008). IL-18 levels in maternal blood are lower in women giving birth before 34 weeks of gestation. Patients having low IL-18 and high IL-12 had a twofold-increased risk of delivering before 34 weeks of gestation (Ekelund et al. 2008). When infection is present in PTL, higher levels of IL-18 are reportedly registered in amniotic fluid (Pacora et al. 2000; Jacobsson et al. 2003). The high levels of IL-18 in sera have been also observed in pregnancies complicated with PPROM, acute fatty liver of pregnancy and fetal growth restriction (Ida et al. 2000). In animal studies, the frequency of fetal loss was significantly higher in IL-18 knock-out mice and in mice receiving IL-18 binding protein than in wild-type controls. IL-18 knock-out mice also present with elevated IL-12 expression in uterine tissues (Wang et al. 2006).

IL-12 mRNA expression has been studied in the human cervical tissue in association with premalignant and malignant lesions (Giannini et al. 1998; de Gruijl et al. 1999). No studies are available on IL-18 in the human cervical tissue.

Anti-inflammatory cytokines

IL-4, IL-10 and IL-13

IL-10 is the most extensively studied of the anti-inflammatory cytokines. It decreases the production of pro-inflammatory cytokines such as IL-8, IL-6, TNF-α, IL-1β (Fortunato et al. 1996; Fortunato et al. 1997; Fortunato et al. 1998; Sato et al. 2003), matrix metalloproteinases (Fortunato et al. 2001) and prostaglandin E_2 (Brown et al. 2000) in LPS-stimulated fetal membranes. It reverses the effect of IL-1β and TNF in cultured human trophoblast cells (Pomini et al. 1999). IL- 10 can also cause selective inhibition of NF-κB in LPS-stimulated human monocytes (Wang et al. 1995). The ratio of IL-10/IL-8 decreases in cervical secretions with advancing gestational age (Mondestin-Sorrentino et al. 2007). The similar decrease of IL-10 expression is seen in term placental tissues (Hanna et al. 2000). IL-10 concentration does not change in amniotic fluid with advancing gestational age (Dudley et al. 1997; Gotsch et al. 2008), though controversial results exist (Greig et al. 1995). Contradictory findings also exist regarding amniotic levels of IL-10 during parturition. Gotsch et al show that spontaneous parturition at term and preterm is associated with increased intraamniotic levels of IL-10 (Gotsch et al. 2008). However, two other studies registered no changes at labor (Greig et al. 1995; Dudley et al. 1997). IL-10 was significantly reduced in placental tissues in chorioamnionitis-associated preterm labor as well as in term labor, compared with second-trimester normal pregnancy samples obtained from elective terminations (Hanna et al. 2006). However, patients who delivered preterm without intraamniotic infection, had a significantly higher median amniotic fluid IL-10 concentration than those who delivered at term (Gotsch et al. 2008).

Several animal studies show a possible role of IL-10 in preventing PTB. Administration of IL-10 to IL-$10^{-/-}$ mice attenuated LPS-induced pro-inflammatory cytokine synthesis and alleviated their increased susceptibility to preterm loss (Robertson et al. 2006). IL-10 treatment significantly reduced IL-1β-induced uterine contractility and amniotic fluid prostaglandins in pregnant rhesus monkeys (Sadowsky et al. 2003). Rats treated with both LPS and IL-10 delivered normal-weight pups at term with a litter size similar to that of saline-infused controls (Terrone et al. 2001).

IL-4 and IL-13 have been less studied in pregnancy and labor. IL-4 is higher in cervical secretions in women with normal pregnancies not in labor compared to PTL (Hollier et al. 2004). Interestingly, cigarette smoking in pregnancy is associated with increased levels of IL-4, IL-10 and IL-13 in cervical secretions (Simhan et al. 2005). Furthermore, higher anti-inflammatory/pro-inflammatory cytokine ratio in cervical secretions during early pregnancy is associated with a higher risk of subsequent spontaneous preterm birth (Simhan and Krohn 2009). In addition, IL-4, IL-10 and IL-13 gene polymorphism is associated with preterm delivery (Annells et al. 2004; Kerk et al. 2006; Heinzmann et al. 2009).

IL-10 and IL-4 have been studied in the non-pregnant cervix in association with human papilloma virus infection, human immunodeficiency virus infection, in premalignant lesions and in cancer (de Gruijl et al. 1999; Nicol et al. 2005; Kobayashi et al. 2008; Mindiola et al. 2008; Scott et al. 2009). There are no studies so far on IL-13 in cervical tissue, nor on these cytokines in pregnant and laboring cervical tissue.

Matrix metalloproteinases (MMPs)

The MMPs play a central role in the breakdown of ECM components. MMPs are a group of zinc-dependent proteinases, released in a latent zymogene form as proMMPs and activated by proteolytic cleavage. MMPs have broad and diverse substrate specificity: collagenases (MMP-1, -8 and -13) break down fibrillar and non-fibrillar collagens; stromelysins (MMP-3, -7 and -10) cleave proteoglycans, fibronectin, collagens IV, V and gelatins; gelatinases (MMP-2 and -9) target collagen IV, V, elastin, proteoglycan and fibronectin (Hulboy et al. 1997). Natural inhibitors of MMPs can

be divided into tissue inhibitors and plasma inhibitors. Four tissue inhibitors of MMPs (TIMPs) have been described: TIMP-1, TIMP-2, TIMP-3 and TIMP-4. The second group of MMP inhibitors are plasma α-macroglobulins (Hulboy et al. 1997). MMPs are involved in natural processes of tissue remodeling and repair in menstruation, embryogenesis, implantation and labor (Hulboy et al. 1997). The levels of MMPs in cervix, lower uterine segment, amniotic fluid, placenta, fetal membranes and maternal plasma increase at labor time (Osmers et al. 1995; Tu et al. 1998; Athayde et al. 1999; Winkler et al. 1999; Maymon et al. 2000a; Maymon et al. 2000b; Stygar et al. 2002; Xu et al. 2002; Park et al. 2003; Sennstrom et al. 2003). Also, polymorphism in MMP-1 and MMP-9 genes is associated with PPROM (Ferrand et al. 2002; Fujimoto et al. 2002).

Preterm birth, inflammation and infection

Although it is well established that intrauterine infection can lead to PTL, this does not appear to be the major cause of prematurity, since infection has been demonstrated in only 25-40% of preterm births (Slattery and Morrison 2002; Goldenberg et al. 2008). Parturition itself is an inflammatory process. Inflammatory events can be observed in the myometrium, cervix, fetal membranes and peripheral blood (Tornblom et al. 2005a; Norman et al. 2007; Challis et al. 2009). Recent studies from our group indicate that cervical ripening at both term and preterm is an inflammatory process even if no infection is present (Tornblom et al. 2004; Tornblom et al. 2005a).
Preterm birth is a complex disorder with multiple mechanisms and pathways involved (Figure 1). In this thesis we concentrate on the inflammatory changes in the cervix at preterm labor. Several factors important for labor and inflammation, such as CRH, HMGB1, Toll-like receptors, cytokines and matrix metalloproteinases are studied.

Aims of the study

The hypothesis of this work was that preterm cervical ripening presents different pathways compared to term. The overall aim of the work presented in this thesis was to determine the possible differences between preterm and term cervical ripening. For this, several factors involved in labor and inflammatory reaction, were analyzed in cervical tissue.

Specific aims were:

- to identify and localize CRH, CRH-BP, CRH-R1, CRH-R2, HMGB1, RAGE, TLR2, TLR4, IL-10 and IL-12 in the cervix during pregnancy, term and preterm labor
- to determine the changes in these substances and several other cytokines (IL-1α, IL-1β, IL-18, IL-4, IL-13) and MMP-1, -3, -8, -9 in the cervix during labor at preterm and term compared to the not in labor state
- to determine the possible differences in these molecules between preterm and term
- to find out possible disparity in cervical ripening in association with PPROM and positive vaginal and/or urinary cultures
- to determine the secretion patterns of IL-8, MMP-1 and MMP-3 in preterm and term cervical fibroblasts
- to investigate whether CRH affects the secretion of IL-8, MMP-1 and MMP-3 in preterm and term cervical fibroblasts

Material and Methods

Subjects

A total of 118 female subjects were included in this study. They formed five study groups: 36 women delivered vaginally or by acute caesarean section at preterm (PTL), 46 women delivered vaginally or by acute caesarean section at term (TL), 7 women delivered by elective caesarean section before the onset of labor at preterm (PTnotL) (studies I-II), 22 women delivered by elective caesarean section before the onset of labor at term (TnotL) and 7 non-pregnant women (NP) (studies I, III, IV). The number of women included in each group in every study is presented in Table I. Some of the biopsies were used in several studies (Table I). Forty-four of the biopsies used in studies I-II were also analyzed in earlier publications (Tornblom et al. 2004; Tornblom et al. 2005a; Tornblom et al. 2005b).

Preterm delivery was defined as delivery before the 37th week of gestation. The labor groups (PTL and TL) were in active labor and demonstrated a ripe cervix, with dilatation more than 4 cm. These patients were either delivered vaginally or by emergency caesarean section (4 women in PTL and 6 in TL) due to breech presentation (in PTL and TL) or due to protracted labor or threatening fetal asphyxia (in TL). In all patients delivered by caesarean section, the assessment of cervical dilatation was established immediately before surgery. Women in the PTnotL and TnotL groups had unripe cervices (with a Bishop score of <5 points) and were delivered by caesarean section prior to the onset of labor. The preterm indications were suspected placental abruption or intrauterine growth retardation. The term indications were breech presentation, humanitarian, earlier caesarean section or disproportion.

The non-pregnant women were used as references, as we identified several new substances in the cervical tissue. The non-pregnant women were having regular menstrual periods prior to undergoing hysterectomy for benign conditions such as myomas.

None of the subjects suffered from preeclampsia, diabetes or other systemic disease or intercurrent disease.

Vaginal and urine cultures were taken from the women in the PTL group. The women included in studies I and II showed no clinical signs of infection and had negative cultures. In studies III and IV nine (ten in study IV) of 20 (21 in study IV) women in the PTL group had negative vaginal and urinary cultures. Bacterial growth was demonstrated in six patients (three with ureaplasma urealyticum and three with group B streptococcus). In five patients the cultures were positive for candida.

Table I Subjects included in the study

Paper	Preterm in labor (PTL)	Term in labor (TL)	Preterm not in labor (PTnotL)	Term not in labor (TnotL)	Non-pregnant (NP)	Total	Number of subjects used in the subsequent papers		
							II	III	IV
I	14	18	7	21	7	67	48	14	14
II	18	18	7	17	-	60		12	12
III	20	24	-	10	4	58			58
IV	21	24	-	10	4	59			

In studies III and IV, ten of the PTL patients had PPROM, defined as a rupture of membranes at least one hour before contractions (Goldenberg et al. 2008).
There were no significant differences between the groups of pregnant women with respect to maternal age, parity, previous preterm births and previous caesarean sections. Clinical data on the women is presented in the tables in the respective papers.
The local Ethics Committee of Karolinska Institute approved the study and the subjects all gave their informed consent.

Sampling procedure

Immediately following vaginal delivery, caesarean section or hysterectomy a biopsy was taken transvaginally (at the 12 o'clock position) from the anterior cervical lip with scissors and tweezers. In study I, in the case of caesarian sections, biopsies were also taken from the upper edge of the lower segment incision. Biopsies from non-pregnant myometrium were obtained from the same location and, in addition, from the fundal region (study I). The samples for analysis of mRNA were immediately frozen in liquid nitrogen and stored thereafter at –70° C (studies I-II) or immediately immersed in RNA*later*® (Ambion Inc, Austin, TX, USA), kept at 4° C for 24 hours and thereafter frozen and stored at -70° (studies III-IV). The samples for protein analysis were immediately frozen and stored at –70° C (studies I-IV). Biopsies intended for immunohistochemical analysis were rinsed in a physiological saline solution and subsequently fixed in 4% formaldehyde solution for a maximum of 24 hours, followed by dehydration in 70% ethanol and embedment in paraffin (studies I, III-IV). In addition to preparation of the biopsies for immunohistochemistry as described above, biopsies from five women (1 NP, 2 TnotL, 2 TL), used in study I, were fixed somewhat differently (for details, see paper I).

The biopsies for the cultures of cervical fibroblasts (study II) were immersed in GIBCO™ RPMI 1640 Medium (Invitrogen Corporation, Paisley, Scotland, UK) and stored in a refrigerator for a maximum of 24 hours before cell cultures were established.

Not all the subsequent analyses could be performed on all women, due to the limited amount of tissue obtained from some of them.

Methods

Tissue homogenization (I-IV)
Frozen tissue was cut into small pieces on a block of dry ice and thereafter transferred to a capsule containing a Teflon-coated tungsten ball and maintained in liquid nitrogen for two minutes. These capsules were subsequently shaken repeatedly at full speed for two minutes in dismembranation apparatus (Retsch KG, Haan, Germany), with intermediate freezing, until the tissue had been pulverized. Thereafter followed either RNA extraction or protein extraction.

RNA extraction and reverse transcription (RT) (I-IV)
Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of total RNA obtained was determined employing either a DU64® spectrophotometer (Beckman, Palo Alto, CA, USA) (studies I-II) or a NanoDrop™ 1000 Spectrophotometer (Thermo scientific, Waltham, MA, USA) (studies III-IV). In studies I-II all samples had OD260/OD280 ratio higher than 1.5, in studies III-IV - higher than 1.8. One μg (studies III-IV) or two μg (studies I-II) of total RNA, pre-treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA), was used for RT reaction, which was performed using SuperScript™ RNase H^- Reverse Transcriptase (Invitrogen, Carlsbad, California, USA) as described previously (Tornblom et al. 2005a).

Real-Time RT-PCR (I-IV)

The levels of mRNA encoding CRH, CRH-BP, CRH-R1, CRH-R2, MMP-1, MMP-3, MMP-8, MMP-9, HMGB1, esRAGE, RAGE, TLR2, TLR4, IL-4, IL-10, IL-13, IL-1α, IL-1β, IL-12a (p35), IL-12b (p40) and IL-18 (studies I-IV) were quantified with real-time RT-PCR employing the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Appropriate primers and probes were purchased from commercially available Taqman® gene expression assays (Applied Biosystems). Assay IDs and Reference Sequence database accession numbers are presented in each paper. For each reaction, 5 µl of diluted cDNA (corresponding to 10 ng (studies III-IV) or 20 ng (studies I-II) total RNA) was used. The threshold cycles (C_T), at which an increase in reporter fluorescence above the baseline signal could first be detected, were determined. 18S (studies I-IV), β-actin (study II-IV) and cyclophilin A (studies III-IV) were used as endogenous controls. 18S (study I) or the geometric mean of two (study II) or three (studies III-IV) endogenous controls was used for normalizing the mRNA levels for the gene of interest (Vandesompele et al. 2002). In study I, the relative levels of the mRNA species of interest were determined employing serial dilutions of the placental cDNA (for CRH-BP and CRH-R1) or hippocampal cDNA (for CRH-R2) made from purchased total RNA (Ambion, Austin, TX, USA) and normalized against the levels of 18S rRNA detected. In study II, the geometric mean was subtracted from the respective gene, giving the ΔC_T as a reflection of relative mRNA expression. Since a higher ΔC_T corresponds to a lower mRNA expression, the ΔC_T values are presented inverted as $100/\Delta C_T$. In studies III-IV, relative gene expression was calculated using the $\Delta\Delta C_T$ method, where non-pregnant group was used as a control. The geometric mean of C_T of endogenous controls was subtracted from C_T of the respective gene, followed by subtraction of the median ΔC_T value of the control group, giving the $\Delta\Delta C_T$. The amount of products doubles in each cycle, so relative gene expression was calculated with the formula $2^{-\Delta\Delta C_T}$, given in the manufacturer's instructions. Serial dilutions of placental cDNA made from purchased total RNA (Ambion, Austin, TX, USA) were used for validating the experiment.

Protein extraction and concentration measurements (II-IV)

Following tissue homogenization, 1 ml of phosphate-buffered saline (PBS), including 0.01% Triton X-100, was added. After centrifugation at 10000g, 4°C for 10 min, the supernatant was retrieved and stored in aliquots at -70 °C until analyzed.

Total protein concentration was determined using a BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA) according to the manufacturer's instructions.

The concentrations of MMP-1, MMP-3, MMP-8, MMP-9, HMGB1, sRAGE, IL-4, IL-10, IL-12 and IL-18 (studies II-IV) in the supernatants or culture medium were determined employing respective ELISA kits. The concentrations of IL-8 in culture medium (study II) were measured with an IMMULITE Automated Analyzer. The concentrations of the proteins measured were normalized against the total protein concentration.

Immunohistochemical analysis of the tissue (I, III-IV)

The biopsies were sectioned, mounted on glasses and stained. In studies I and III, the avidin-biotinylated (ABC) peroxidase complex method was used; while MACH3™ Mouse–Probe HRP Polymer Kit (Biocare Medical, CA, USA) was employed in study IV. Stainings were developed using DAB and counterstained with Mayer's hematoxylin. Control sections were stained in the same manner, but omitting the primary antibody in study I. In study III, controls for specificity of the staining were based on parallel stainings with the primary antibodies pre-incubated with blocking peptides specific for each antibody. Stainings with primary isotype-matched immunoglobulin of irrelevant antigen-specificity IgG_{2B} (for IL-10) or IgG_1 (for IL-12) (R&D systems) were used as negative controls in study IV. (For further methodological details and information about antibodies, see papers I, III, IV).

For all immunohistochemical examinations, the immunoreactivity was checked in the squamous epithelium, the glandular epithelium, the vascular endothelium and in the stroma. Positive staining 0-3+ was scored manually and blindly by 2-3 independent observers in studies III-IV. The immunoreactivity was classified and observations were made by four different observers in study I.

Cell cultures (II)
Cervical fibroblasts were established from the cervical biopsies obtained directly after vaginal delivery, as described in paper II.
Phenotype of the fibroblasts was identified both morphologically and employing three fibroblast-markers: vimentin, anti-fibroblast surface protein antibody 1B10 and prolyl-4-hydroxylase beta (for details, see paper II).

Stimulation experiments (II)
Cervical fibroblasts were seeded on 12-well cell culture plates and grown until confluence, usually after three days. Cells were stimulated for 18 hours with CRH (Sigma, Saint Louis, Missouri, USA) at concentrations ranging between 10^{-6} and 10^{-13} M and non-stimulated control cells were cultured in parallel. All concentrations were performed in duplicate. Media samples were collected and frozen until analysis.

Statistical analysis

Two independent groups were compared using the Mann-Whitney *U* test. When more than two groups were compared, the Kruskal-Wallis test was applied, followed by multiple comparison with Bonferroni/Dunn correction. For comparison of cervical and isthmic biopsies from the same patient, the Wilcoxon matched pair test was employed (study I). For comparing the results in the stimulation experiments, the Friedman Anova & Kendall's concordance and the Wilcoxon matched pair test were employed (study II). Spearman's rho was used for analyzing non-parametric correlations. Fisher's exact test was used to test whether associations were nonrandom (studies III-IV). In all cases a p-value of <0.05 was considered to be statistically significant. All calculations were performed with the STATISTICA 6.0 (study I), 8.0 (studies II-IV) software (StatSoft Inc, Tulsa, OK, USA) and GraphPad Prism 5.01 (GraphPad Software Inc, CA, USA) (studies III-IV).

Results

New substances identified in the human cervical tissue (I, III-IV)

During the present work, several new substances were identified in the cervical stroma and epithelium. We demonstrated CRH (Figure 7A-C), CRH-BP (Figure 7D-F), CRH-R1 (Figure 7G-I) and CRH-R2 (Figure 7J-L) in cervical tissue in study I. In study III we demonstrated HMGB1 (Figure 8A-J). TLR2, TLR4 (Figure 9A-J) (study III), IL-10 (Figure 10A-E) and IL-12 (Figure 11A-E) (study IV) proteins were demonstrated for the first time in the cervix in relation to pregnancy, vaginal delivery and caesarean section.

Changes in the laboring cervix (I-IV)

Major differences were detected in the laboring cervix irrespective of gestational age compared to the not in labor state.

Changes at mRNA level (I-IV)
The mRNA expression of CRH-BP ($p=0.0001$) and CRH-R2 ($p<0.01$) was lower in the laboring cervix (Figure 12A, C) (study I).
There was a tendency towards lower mRNA expression of HMGB1 in labor (Figure 13A). The mRNA expression of RAGE was lower ($p<0.05$), but mRNA of TLR2 was higher ($p<0.01$) in the laboring groups (Figure 13B-C) (study III).
We registered a significant upregulation of mRNA of IL-10, IL-1β, but downregulation of IL-12a and IL-18 in labor (Figure 14A-D) (study IV).
MMP-1, MMP-3 and MMP-9 mRNA expression was upregulated in labor (Figure 15A-C) (study II).

Changes at protein level (II-IV)
We observed more extranuclear staining of HMGB1 in the cervical stroma (Figure 8F-I) and empty nuclei in epithelium in labor (Figure 8A-E). Additionally, the staining of HMGB1 was lower in the laboring groups ($p<0.05$) (Figure 8K). Similarly, significantly lower tissue expression of TLR2 and TLR4 was seen in labor (Figure 9A-L) (study III). However, the sRAGE concentration in the cervical tissue was higher in labor ($p<0.05$) (see paper III).
There was a tendency towards lower protein concentrations of IL-4 and IL-12 in labor; this was significant for the PTL group (Figure 16A-B). However, the concentration of IL-18 was higher in labor (Figure 16 C). IL-10 was not affected by labor (see paper IV).
We found a tendency towards higher protein levels of MMP-8 and MMP-9 in labor. This difference was significant for the TL group (Figure 17A-B) (study II).

Differences between preterm and term cervical ripening

Although major changes were detected in the laboring groups irrespective of gestational age, some differences were still identified between preterm and term cervix.

mRNA expression (III-IV)
TLR2, TLR4 (study III) and IL-12a (study IV) mRNA expression was lower in preterm labor ($p<0.05$) (Figure 18A-C).

Protein levels (II, IV)
IL-10 protein expression was higher in the cervical epithelium in preterm labor (Figure 10A-B, F) (study IV).
In the cervical fibroblast study, we detected different secretion patterns at preterm and term. The secretion of IL-8 and MMP-1 was significantly higher ($p<0.001$ and $p<0.05$, respectively), but MMP-3 secretion significantly lower in preterm cervical fibroblasts ($p<0.001$) (Figure 19A-C) (study II).

Differences between PPROM and PTL (III-IV)

HMGB1 mRNA expression was lower in PPROM than in the rest of PTL group, which was higher than TL (Figure 20A) (study III).
There was a tendency towards higher tissue expression of TLR4 in cervical epithelium in PPROM than in PTL ($p=0.06$) (Figure 20B) (study III).
We noted a tendency towards lower protein levels of IL-4 and IL-18 in PPROM (Figure 20C-D) (study IV).

Differences in association with infection (III-IV)

There was a tendency towards lower TLR2 and TLR4 mRNA expression in the preterm subgroup with bacterial infection compared to the non-infected subgroup (Figure 21A-B) (study III).
We found a strong tendency towards higher TLR4 protein expression ($p=0.05$) in cervical epithelium in the subgroup with bacterial infection (Figure 21C) (study III).
The concentration of IL-4 was significantly higher in the subgroup with bacterial infection ($p<0.05$) (Figure 21D) (study IV).

CRH increases IL-8, but not MMP-1 and MMP-3 secretion in cervical fibroblasts (II)

CRH significantly increased the secretion of IL-8 from the term and preterm cervical fibroblasts at concentrations of 10^{-7}-10^{-6} and 10^{-8}, 10^{-6} M respectively, compared with controls (Figure 19A). The levels of MMP-1 and MMP-3 were not significantly changed by CRH (Figure 19B-C).

Changes in pregnant cervix (I, III-IV)

We had a non-pregnant group as a reference since several new substances were analyzed in the cervix. We saw changes in the cervix in pregnant state compared to non-pregnant.

mRNA expression (I, IV)
The mRNA expression of CRH-BP, CRH-R1 and CRHR2 was significantly higher in non-pregnant cervix compared to pregnant cervix not in labor and in labor (Figure 12A-C) (study I).
The mRNA expression of IL-18 was lower in non-pregnant cervix than in term-pregnant cervix (Figure 14D) (study IV).

Protein expression (III-IV)

HMGB1 protein concentration was significantly higher in non-pregnant cervix than in term pregnant cervix and laboring cervix (see paper III).

sRAGE concentration was significantly higher in non-pregnant cervical tissue than in term pregnant not in labor tissue (see paper III).

IL-12 protein concentration was below the detection limit in all the non-pregnant cervical tissue homogenates (Figure 16B). IL-12 also showed lower immunohistochemical staining in the stroma and vascular endothelium of the non-pregnant cervix, but this was significant only for PTL group (Figure 11F-G) (study IV).

The protein levels of IL-18 were higher in the non-pregnant cervix than in the term-pregnant and in the term laboring cervix (Figure 16C) (study IV).

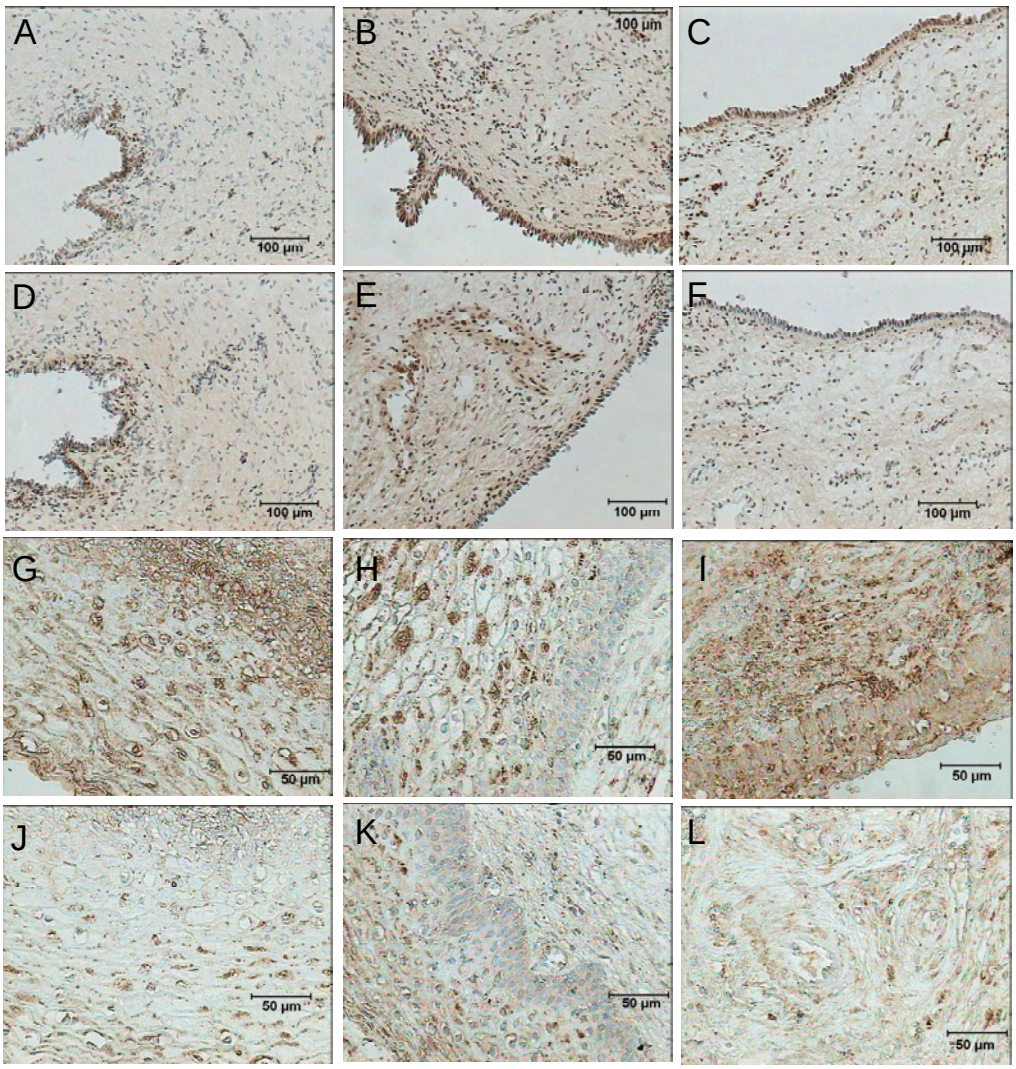


Figure 7. *Immunohistochemical localization of CRH (A-C), CRH-BP (D-F), CRH-R1 (G-I) and CRH-R2 (J-L) in the non-pregnant (left column), term pregnant not in labor (middle column) and term in labor (right column) human cervix. Magnification x100 (A-F) and x200 (G-L).*

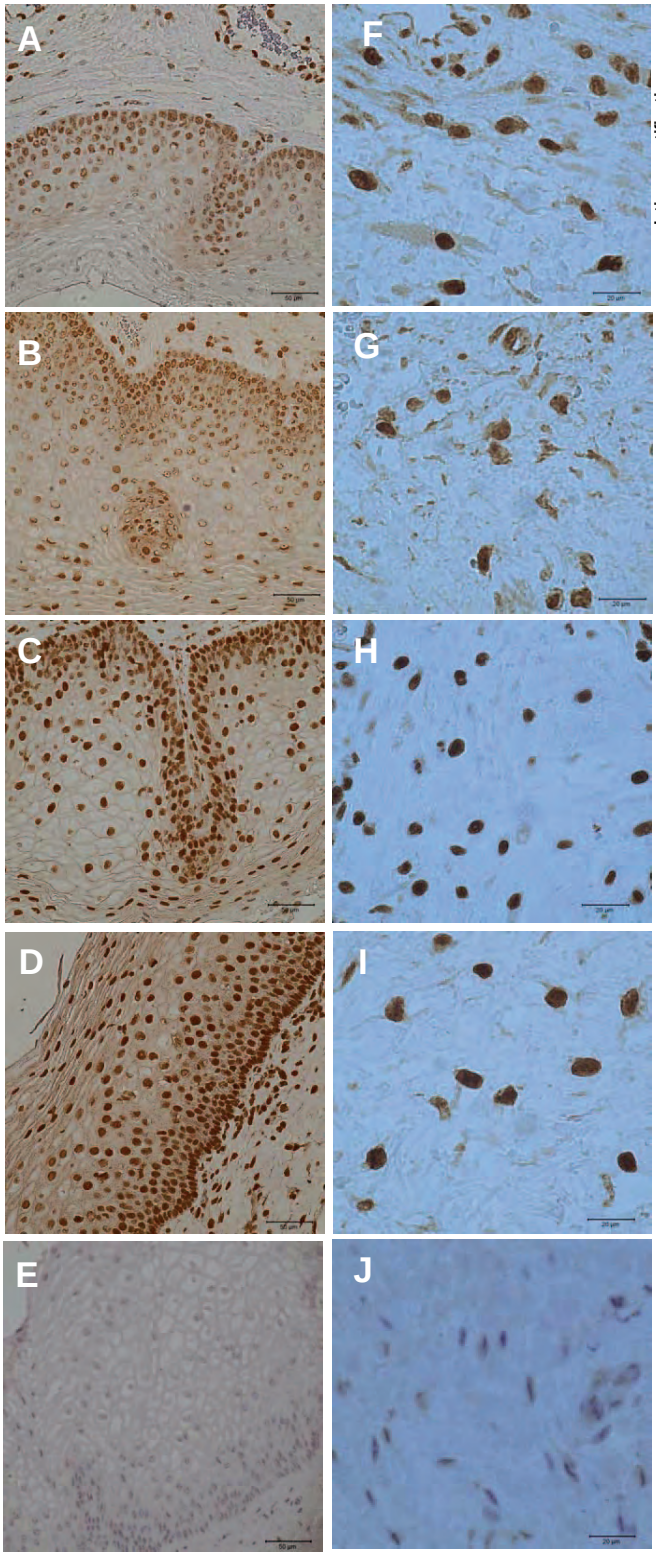


Figure 8. *Immunohistochemical staining of HMGB1 in the squamous epithelium (left column) and in the stroma (middle column) in the cervical tissue at preterm labor (A, F), term labor (B, G), term not in labor (C, H) and non-pregnant state (D, I). Negative controls (E, J). Magnification x400 (A-E) and x1000 (F-J).*
*Quantification of HMGB1 staining in stroma (K). Preterm labor (PTL), term labor (TL), term not in labor (TnotL) and non-pregnant (NP). Semiquantitative scale from 0 to + + +. * $p<0.05$.*

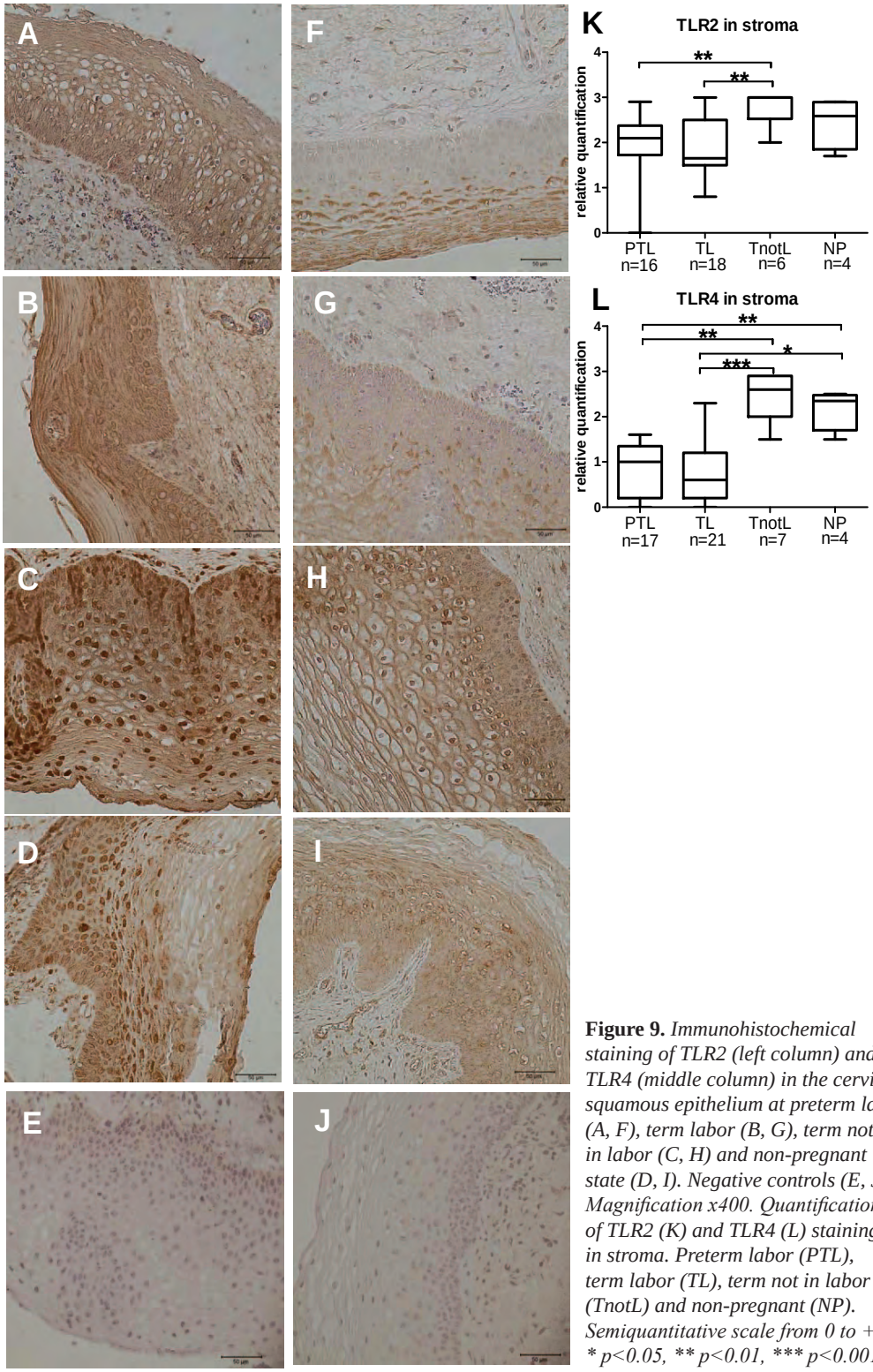


Figure 9. *Immunohistochemical staining of TLR2 (left column) and TLR4 (middle column) in the cervical squamous epithelium at preterm labor (A, F), term labor (B, G), term not in labor (C, H) and non-pregnant state (D, I). Negative controls (E, J). Magnification x400. Quantification of TLR2 (K) and TLR4 (L) staining in stroma. Preterm labor (PTL), term labor (TL), term not in labor (TnotL) and non-pregnant (NP). Semiquantitative scale from 0 to + + +. * p<0.05, ** p<0.01, *** p<0.001.*

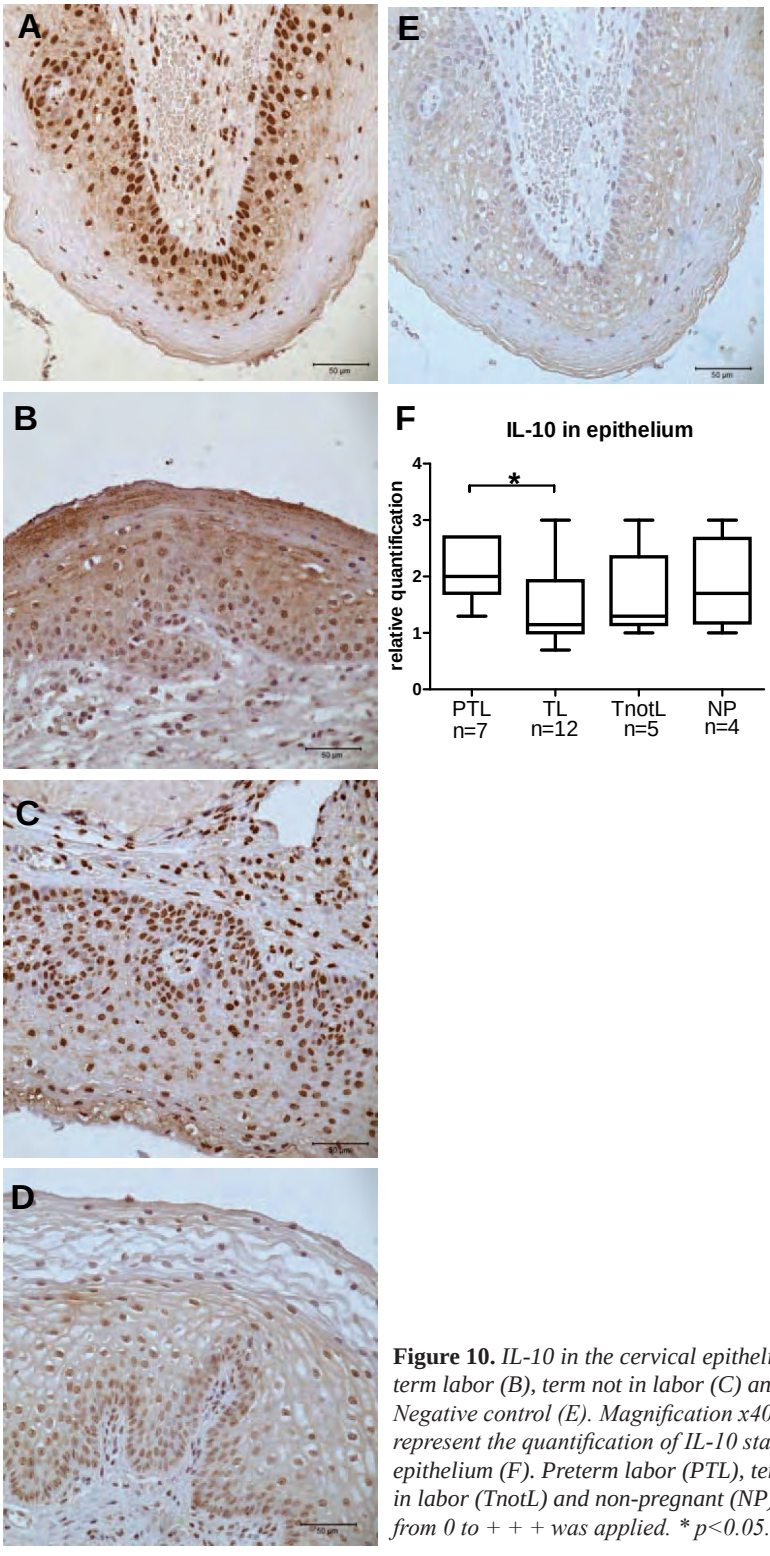


Figure 10. *IL-10 in the cervical epithelium at preterm labor (A), term labor (B), term not in labor (C) and non-pregnant state (D). Negative control (E). Magnification x400. Box and whisker plots represent the quantification of IL-10 staining in the squamous epithelium (F). Preterm labor (PTL), term labor (TL), term not in labor (TnotL) and non-pregnant (NP). Semiquantitative scale from 0 to + + + was applied. * $p<0.05$.*

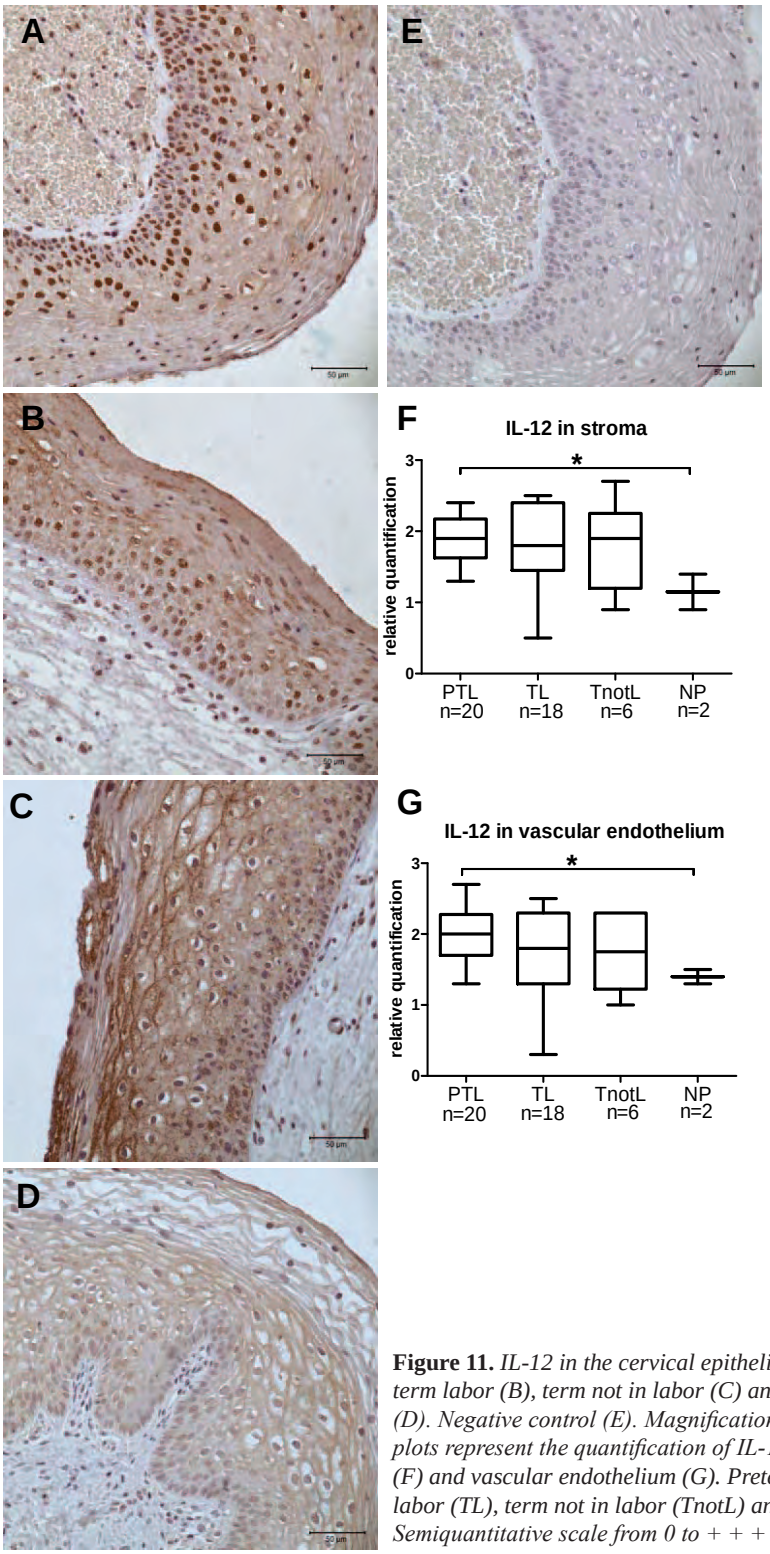
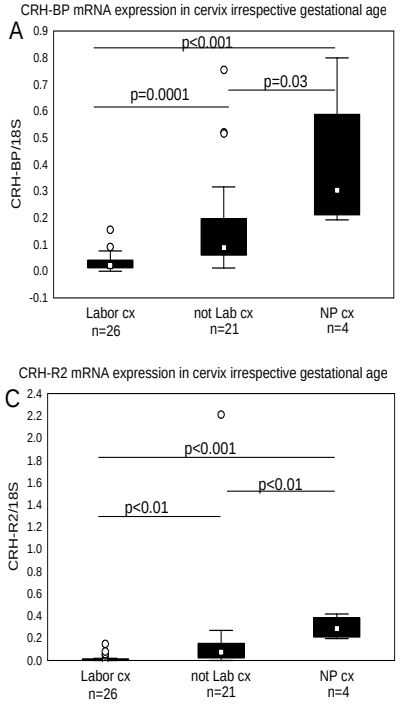


Figure 11. *IL-12 in the cervical epithelium at preterm labor (A), term labor (B), term not in labor (C) and non-pregnant state (D). Negative control (E). Magnification x400. Box and whisker plots represent the quantification of IL-12 staining in stroma (F) and vascular endothelium (G). Preterm labor (PTL), term labor (TL), term not in labor (TnotL) and non-pregnant (NP). Semiquantitative scale from 0 to + + + was applied. * p<0.05.*



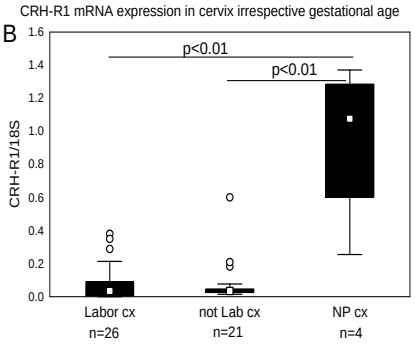


Figure 12. *CRH-BP (A), CRH-R1 (B) and CRH-R2 (C) mRNA expression in cervical tissue irrespective of gestational age. Labor cx – in labor group both at term and preterm, not Lab cx- term and preterm pregnant group not in labor, NP cx – non-pregnant group.*

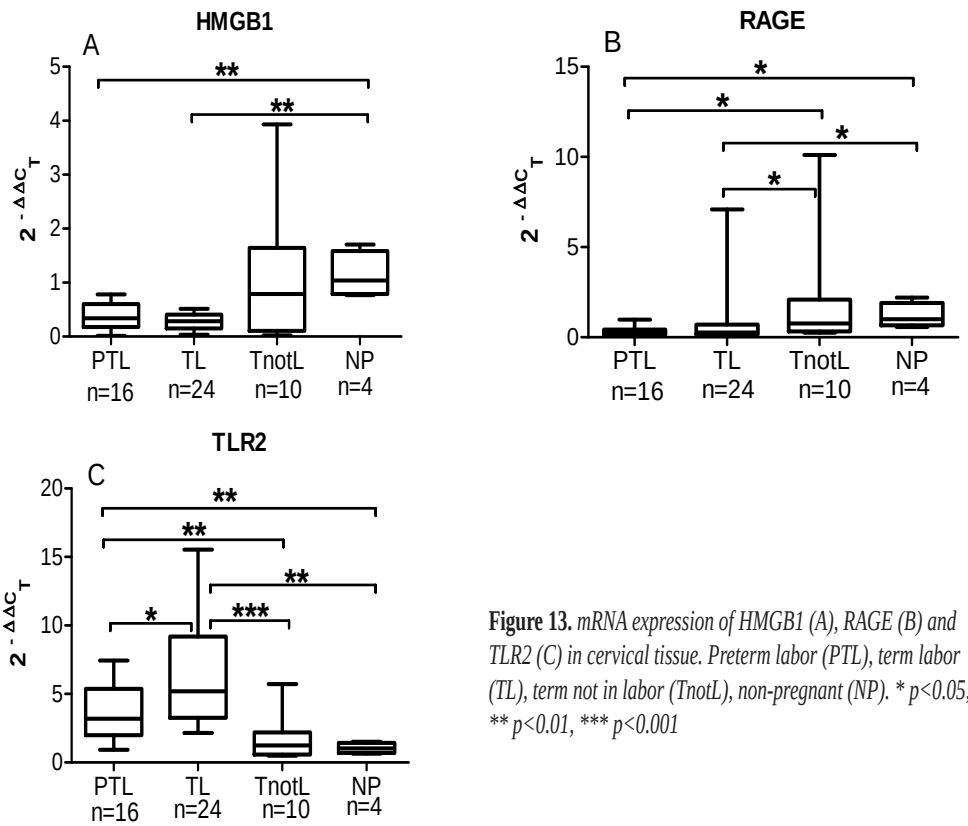


Figure 13. *mRNA expression of HMGB1 (A), RAGE (B) and TLR2 (C) in cervical tissue. Preterm labor (PTL), term labor (TL), term not in labor (TnotL), non-pregnant (NP). * p<0.05, ** p<0.01, *** p<0.001*

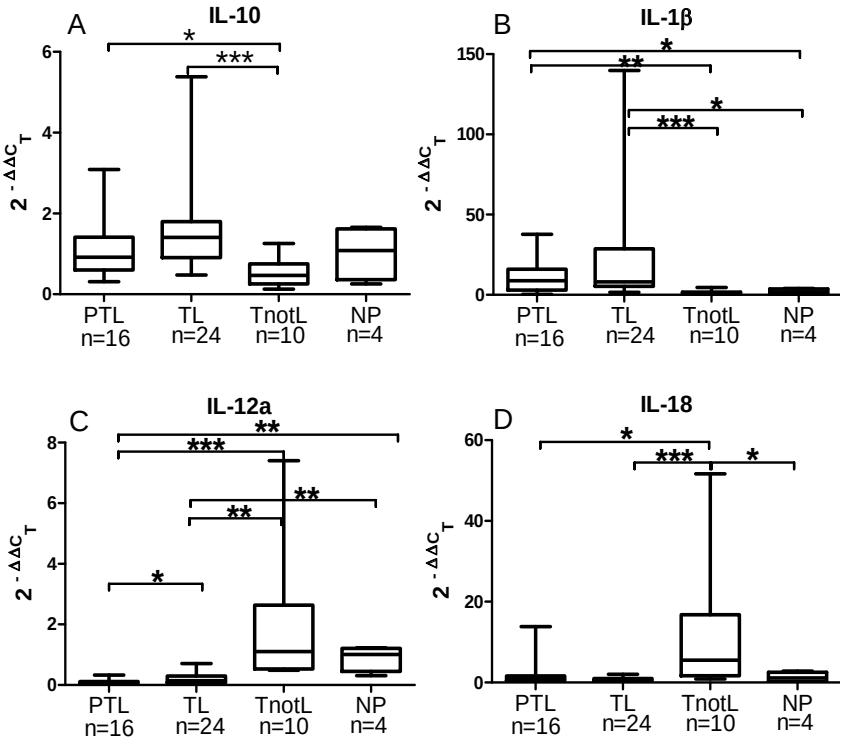


Figure 14. *mRNA expression of IL-10 (A), IL-1β (B), IL-12a (C) and IL-18 (D) in cervical tissue. Preterm labor (PTL), term labor (TL), term not in labor (TnotL), non-pregnant (NP). * p<0.05, ** p<0.01, *** p<0.001*

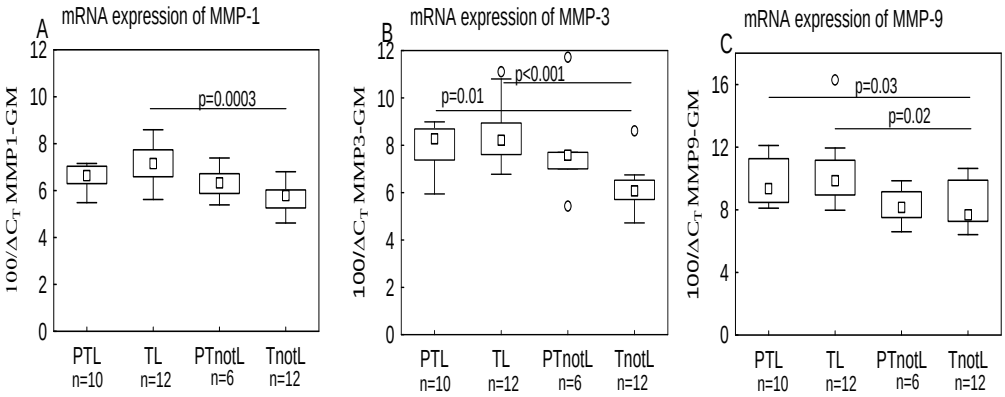


Figure 15. *mRNA expression of MMP1 (A), MMP-3 (B) and MMP-9 (C). Preterm labor (PTL), preterm not in labor (PTnotL), term labor (TL), term not in labor (TnotL).*

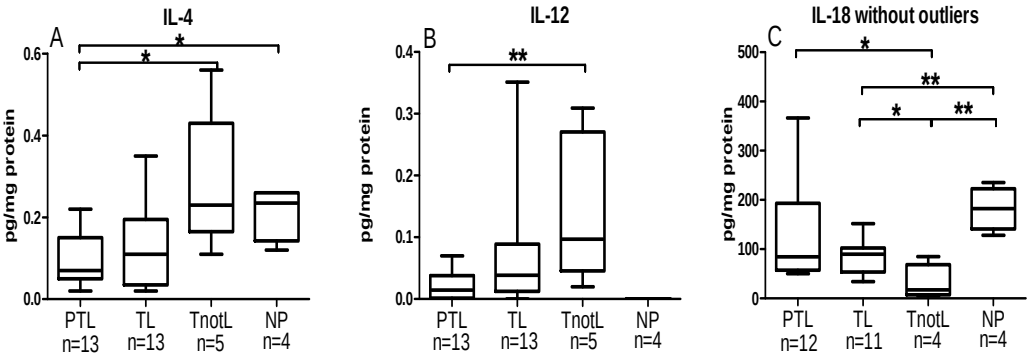


Figure 16. *Protein levels of IL-4 (A), IL-12 (B) and IL-18 (C) in cervical tissue. IL-18 levels in the groups are shown when extreme outliers (1 from PTL, 2 from TL, 1 from TnotL) are excluded. Groups studied: preterm labor (PTL), term labor (TL), term not in labor (TnotL) and non-pregnant (NP). * p<0.05, ** p<0.01.*

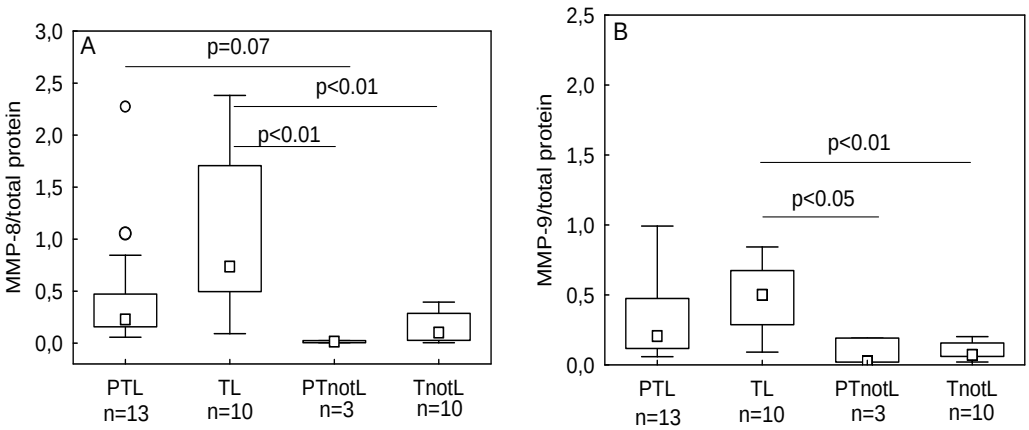


Figure 17. *Protein levels of MMP-8 (A) and MMP-9 (B) in cervical tissue. Preterm labor (PTL), preterm not in labor (PTnotL), term labor (TL), term not in labor (TnotL).*

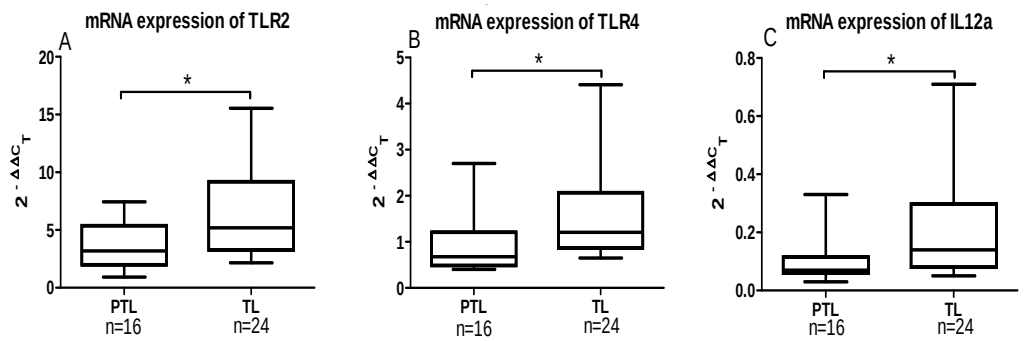


Figure 18. *mRNA expression of TLR2 (A), TLR4 (B) and IL-12a (C) in cervical tissue in preterm (PTL) and term (TL) labor. * p<0.05*

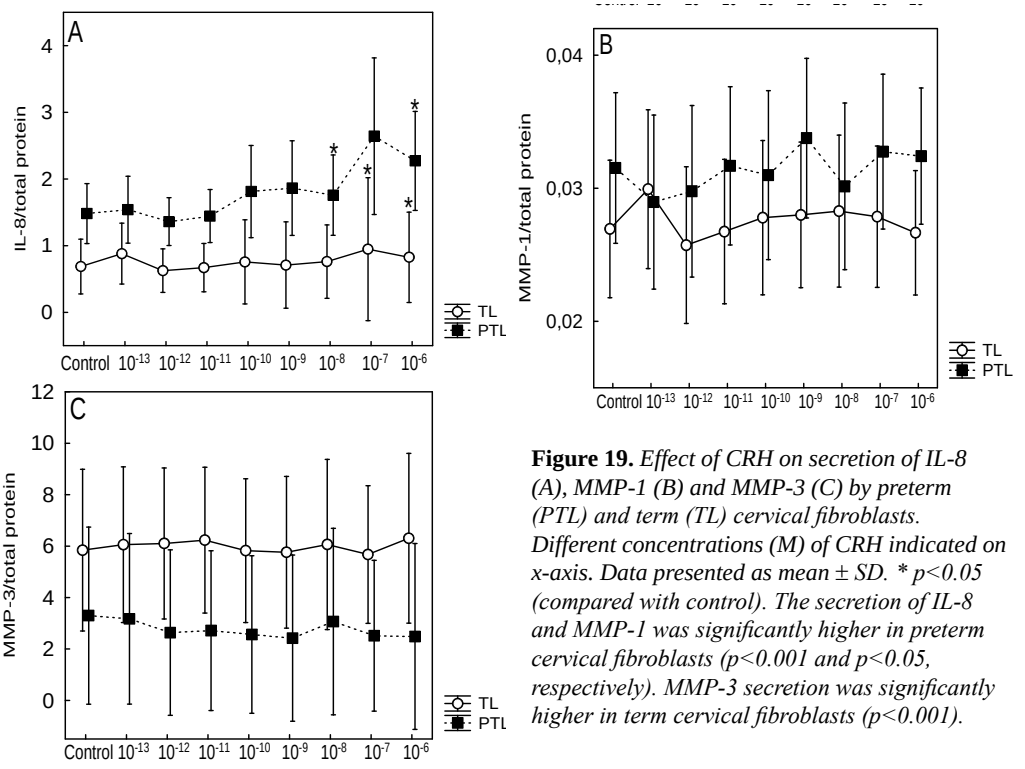


Figure 19. *Effect of CRH on secretion of IL-8 (A), MMP-1 (B) and MMP-3 (C) by preterm (PTL) and term (TL) cervical fibroblasts. Different concentrations (M) of CRH indicated on x-axis. Data presented as mean ± SD. * p<0.05 (compared with control). The secretion of IL-8 and MMP-1 was significantly higher in preterm cervical fibroblasts (p<0.001 and p<0.05, respectively). MMP-3 secretion was significantly higher in term cervical fibroblasts (p<0.001).*

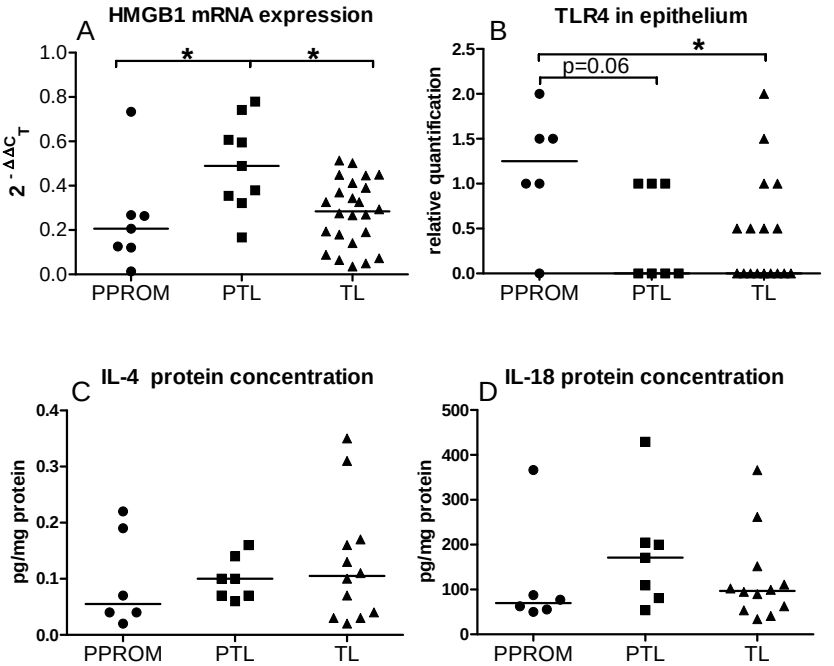


Figure 20. *HMGB1 mRNA expression(A), TLR4 in epithelium (B), IL-4 (C) and IL-18 (D) protein levels in cervical tissue in women with preterm premature rupture of membranes (PPROM), in women with preterm labor (PTL) and women delivered at term (TL). * p<0.05*

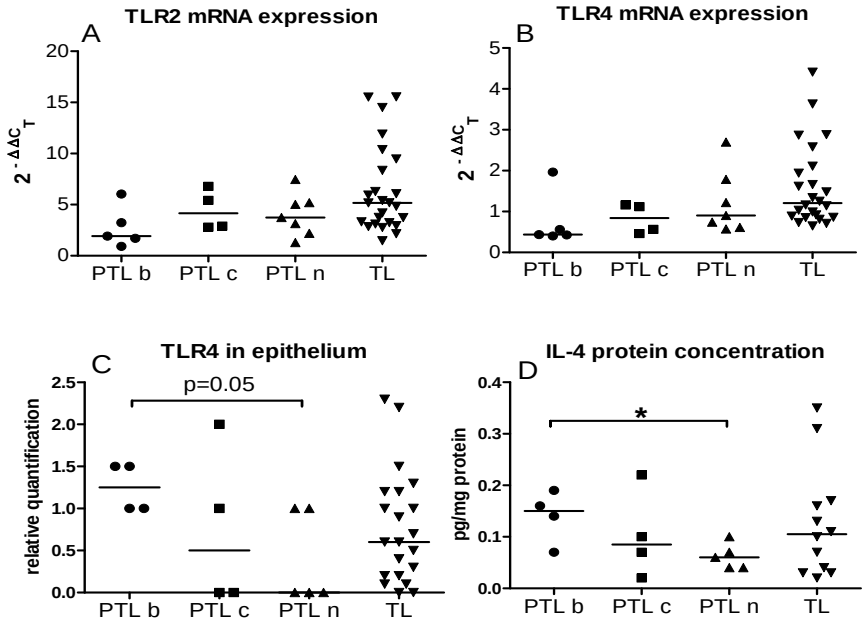


Figure 21. *TLR2 (A) and TLR4 (B) mRNA expression, TLR4 in epithelium (C) and IL-4 protein levels (D) in the preterm subgroup with bacterial infection (PTL b), with candida infection (PTL c), and with negative cultures (PTL n) and in term in labor group (TL). * p<0.05*

Discussion

Preterm birth is a complex disorder. Incomplete understanding of the biological and pathophysiological mechanisms underlying preterm delivery is the major obstacle to preventing PTB. Better understanding of the process of cervical ripening both at term and at preterm could help to develop new strategies for PTB prevention. Therefore, the work presented in this thesis focused on the changes of the cervix during preterm and term labor.

During this work several novel factors, which are important in labor and inflammation, were identified in the cervix. One of the most interesting findings is HMGB1 in the cervix. HMGB1 has pro-inflammatory properties when it is released to cytoplasm and extracellular milieu; and it is a mediator of chronic inflammatory diseases (Kokkola et al. 2002; Barkauskaite et al. 2007; Palmblad et al. 2007). However, knowledge of the role of HMGB1 in the labor process is still very limited. Holmlund et al. recently demonstrated HMGB1 in placenta. No changes were registered during active labor, although there was a tendency towards higher cytoplasmic HMGB1 expression in the decidua obtained from preeclamptic pregnancies (Holmlund et al. 2007). In this thesis, we show that HMGB1 is expressed at both mRNA and protein levels in the cervix. We also show both nuclear and cytoplasmic localization of HMGB1, with distinct changes during labor. We show release of HMGB1 from the nuclei in the squamous epithelium as well as more cytoplasmic staining in the stroma in the cervix during preterm and term labor. This indicates that HMGB1 could be a novel pro-inflammatory mediator of cervical ripening at labor. However, the opposite is also possible - that the tissue damage in the cervix during vaginal delivery causes the release of HMGB1 from the nuclei, since HMGB1 can mediate reaction to injury (Lotze and Tracey 2005). On the other hand, we observed empty nuclei even in those three cervical biopsies taken from acute caesarean sections.

One more novel substance identified in the cervix for the first time is CRH. CRH has been of interest in pregnancy and labor for over 20 years, when it was identified in placenta; and it was shown that CRH plasma levels increase progressively through pregnancy and decline within hours after delivery (Grino et al. 1987; Sasaki et al. 1987). The role of CRH in pregnancy and labor has been discussed extensively (Grammatopoulos 2008). CRH stimulates production of pro-inflammatory cytokines in different cells (Angioni et al. 1993; Yang et al. 2005; Wang et al. 2007), although some studies present contradicting results (Angioni et al. 1993; Sehringer et al. 2000). CRH stimulates MMP-9 secretion by cultured cells from placenta and fetal membranes (Li and Challis 2005). It can upregulate the expression of NOS (Aggelidou et al. 2002) and enhance production of prostaglandins (Jones and Challis 1989). Thus, CRH could be involved in the inflammatory process of labor. In this work, we show that CRH is localized in the cervical tissue. CRH-BP and CRH receptors are expressed at mRNA and protein levels in cervical tissue with changes in the laboring cervix. Furthermore, CRH stimulates IL-8 secretion in cervical fibroblast cultures established from biopsies from both preterm and term deliveries. IL-8 promotes recruitment and activation of neutrophils, which in turn secrete matrix metalloproteinases. IL-8 is an important mediator of cervical ripening (Sennstrom et al. 1997; Sennstrom et al. 2000). All this data supports the role of CRH in the process of cervical ripening at labor.

During this project, we identified several substances in the cervical epithelium: CRH, CRH-BP, CRH-R1, CRH-R2, HMGB1, TLR2, TLR4, IL-10 and IL-12. These findings together with earlier findings from our group, where IL-8 (Sennstrom et al. 2000), MMP-8 (Sennstrom et al. 2003), fetal fibronectin (Sennstrom et al. 1998) and syndecan-1 (Sahlin et al. 2008) were localized in the cervical epithelium, confirm the possible role of cervical epithelium in the cervical ripening process. This is supported by the finding of the components of the mitogen-activated protein kinase (MAPK) pathway in the glandular and squamous epithelium in the cervix (Wang and Stjernholm 2007). The MAPK pathway is activated by estrogen and pro-inflammatory cytokines (Kato et al. 1995; Herlaar and Brown 1999). Epithelial surface present the first-line physical and biochemical barrier against microorganisms (Romero et al. 2007). Furthermore, epithelium plays an important role in homeostasis and diseases in other organs, for example lungs, intestines, urinary bladder (Moore and Goldman 2006; McCole and Barrett 2007; Holgate 2008). The role of cervical epithelium in the labor process still needs to be elucidated.

We detected major differences in the mRNA and protein expression of several substances at labor time irrespective of gestational age. We observed a release of HMGB1 to extranuclear space, lower levels of TLR2 and TLR4 and a higher concentration of sRAGE. Through its signaling receptors, HMGB1 can mediate inflammatory reactions (Lotze and Tracey 2005). Furthermore, IL-18 protein levels are higher at labor, IL-4 and IL-12 levels tend to be lower and IL-10 does not change. Together with our earlier findings of higher IL-6, IL-8 and MCP-1 in the laboring cervix (Tornblom et al. 2005a), there is a shift towards a pro-inflammatory state in both the term and the preterm cervix at labor time. Thus, cervical ripening at preterm seems to be an inflammatory reaction similar to that at term; it is just that it takes place earlier in the pregnancy.

However, we still saw some differences in the preterm cervix compared to term. We detected lower mRNA expression of TLR2, TLR4 and IL-12 at preterm labor. The physiological role of this decrease is unclear, as we did not see the same disparity at protein level. Real-time RT-PCR is a very sensitive technique, whereas immunohistochemistry is not a quantitative technique, so it may be difficult to detect minor differences in TLR2 and TLR4 protein expression between preterm and term labor. Even IL-12 quantification with ELISA technique is less sensitive than mRNA quantification with real-time RT-PCR. The other possible explanations could be temporal differences between mRNA synthesis and protein expression, shorter half-life of mRNA or post-translational regulation of these proteins.

There were also some differences at protein level, with higher protein expression of IL-10 in cervical squamous epithelium at preterm labor than at term. This is a very interesting finding, as we saw no differences in protein level in homogenized cervical tissue. Here again, we can see the important role of cervical epithelium. Higher IL-10 levels in epithelium at preterm labor could be a protective mechanism against too early pro-inflammatory changes in the cervix, since pro-inflammatory cytokines like IL-β can upregulate IL-10 expression (Trautman et al. 1997). Moreover, IL-10 can inhibit COX-2 expression and diminish prostaglandin release in preterm human placenta (Hanna et al. 2006). IL-10 is also able to inhibit NF-κB activation in LPS-stimulated human monocytes (Wang et al. 1995). This supports a possible protective role of IL-10 during preterm labor. Higher levels of IL-10 in amniotic fluid of women with preterm labor delivering at preterm could also reflect a mechanism that counter-regulates the pro-inflammatory cervical ripening and the delivery process (Gotsch et al. 2008). However, there is another explanation of higher IL-10 in cervical epithelium: that higher IL-10 levels in epithelium may initially create a hyporesponsiveness and permissive environment for ascending infection, since women with a higher anti-inflammatory/pro-inflammatory cytokine ratio in cervical secretions during early pregnancy are at higher risk of subsequent spontaneous preterm birth (Simhan and Krohn 2009). However, there is also evidence that IL-10 can have a paradoxical pro-inflammatory effect in amnion (Mitchell et al. 2004).

We found one more interesting difference at protein level, that preterm and term cervical fibroblasts presented different secretion patterns of IL-8, MMP-1 and MMP-3. Preterm cervical fibroblasts secrete higher levels of IL-8 and MMP-1, but lower levels of MMP-3 than term cervical fibroblasts. In our recent study, we also showed the difference in proteoglycan production in preterm and term cervical fibroblasts (Akerud et al. 2008). These findings indicate that cervical fibroblasts at preterm delivery and term delivery may have different phenotypes.

Taken together, our findings indicate that even though preterm cervical ripening resembles cervical ripening at term, there still could be some differences in the pathways involved. These differences might be a target for a new treatment for threatening PTB.

Our study is also consistent with earlier research showing that preterm birth is a multifactorial disorder. We found differences between PPROM and PTL subgroups, with lower HMGB1 mRNA expression in PPROM than in the rest of the PTL group, which was higher than TL. This finding is striking since here the preterm labor group, not the PPROM group, is the one that differs most from the term labor group. We also found a tendency towards higher tissue expression of TLR4 in cervical epithelium and a tendency towards lower protein levels of IL-4 and IL-18 in PPROM. This suggests that PPROM and PTL could partly involve different mechanisms, as seen in previous studies of fetal membranes, amniotic fluid and maternal blood (Fortunato et al. 1999; Fortunato et al. 2000; Menon et al. 2001; Hajek et al. 2008).

There was a 30% frequency of bacterial infection in randomly-selected women in our study (bacterial growth in cultures in six women of 20 or 21), which is consistent with the literature (Romero et al. 2006; Goldenberg et al. 2008). Despite few samples, we saw some differences between the infected and non-infected subgroups. The concentration of IL-4 was significantly higher in the subgroup with bacterial infection. There were also tendencies towards lower mRNA expression of TLR2 and TLR4 in cervical tissue, but higher expression of TLR4 protein in the cervical epithelium, in the subgroup with bacterial infection. These findings confirm that infection is not the only cause of PTB. Infection was long considered the most important cause of PTB, the only pathological process for which a firm causal link with preterm birth has been established (Romero et al. 2001; Romero et al. 2006). Subclinical infection and its role in PTB has been discussed a lot. Chorioamnionitis has been even used as diagnostic of intrauterine infection. (Romero et al. 2001; Romero et al. 2006). Here, we saw some differences in cervical ripening between the subgroup with infection confirmed by cultures and the subgroup with negative cultures. We have no data about the possible subclinical infection or microbial invasion of the amniotic cavity. Nevertheless, infection in pregnancy does not always end up with PTB. Although the amniotic cavity is considered to be sterile for bacteria, microbial growth in amniotic fluid cultures is present in approximately 19% of patients in labor at term with intact membranes. No differences have been observed in the chorioamnionitis rates and neonatal outcome between culture-positive and culture-negative patients (Romero et al. 1993). Furthermore, fluorescence in situ hybridization (FISH) with DNA probe, specific for conserved regions of bacterial 16S ribosomal RNA sequence, has detected bacteria in the fetal membranes of up to 70% of women undergoing elective caesarean section at term (Steel et al. 2005). These findings suggest that not infection itself but an inflammatory reaction of the host could be the cause of preterm delivery. This is supported by racial differences in inflammatory response, gene polymorphism and susceptibility for preterm delivery (Menon et al. 2006; Menon et al. 2007).

The present findings give more information on the inflammatory process of cervical ripening at both preterm and term labor. This inflammatory reaction also takes place in myometrium, fetal membranes and is even resembled in peripheral blood (Thomson et al. 1999; Young et al. 2002; Osman et al. 2003; Tornblom et al. 2005a; Norman et al. 2007; Challis et al. 2009). Since preterm labor is a heterogenous condition, it is unlikely that one treatment will prevent all cases of preterm birth in patients at risk (Romero et al. 2006). But the substances that could stop and reverse this

inflammatory process in the reproductive organs could be candidates for the treatment of PTL.
One possible future treatment method for PTL could be to target TLR. There is evidence that TLRs detect not only infection but also sterile tissue injury. TLR4 can be activated by HMGB1 (Lotze and Tracey 2005), fibronectin (Okamura et al. 2001), hyaluronan (Taylor et al. 2007) and proteoglycan biglycan (Schaefer et al. 2005). These substances play a role in sterile inflammation, suggesting that antagonizing TLR4 could be one possible treatment of inflammatory reaction and PTD. Animal models are used for testing various hypotheses and treatments of preterm birth (Elovitz and Mrinalini 2004). TLR4-deficient mice are protected against heat-killed E.coli and LPS-induced preterm labor (Elovitz et al. 2003; Wang and Hirsch 2003). Further, pretreatment with TLR4 antagonists inhibits LPS-induced preterm uterine contractility and is associated with lower levels of cytokines and prostaglandins in amniotic fluid in a non-human primate model (Adams Waldorf et al. 2008). In addition, the inhibition of several aspects of the TLR4 signaling pathway with anti-inflammatory prostaglandin 15-Deoxy-$\Delta^{12,14}$- prostaglandin J_2 (15d-PGJ_2) delays LPS-induced preterm delivery and protects from LPS-induced fetal mortality in a mouse model (Pirianov et al. 2009). All these results indicate that by targeting TLR4 receptors, preterm delivery could be prevented. Interestingly, we see downregulation of TLR2 and TLR4 mRNA in the cervix during preterm labor. There was a tendency towards further downregulation in our subgroup with bacterial infection. TLR2 and TLR4 could be downregulated in responses to stimuli secreted during preterm pro-inflammatory labor and this could be a protective mechanism. In vitro studies show downregulation of TLR4 in monocytes after stimulation with LPS (Amoudruz et al. 2005), which could be a possible mechanism of LPS tolerance. Furthermore, lower TLR2 levels have been detected in placentas with chorioamnionitis (Rindsjo et al. 2007).
However, further studies are necessary to elucidate the benefits and side-effects of antagonizing TLR to prevent PTB. It is also essential to study whether TLR4 antagonists are helpful when administered after the onset of infection/inflammation and preterm labor, since this is the situation in humans.
One possible method to prevent PTB could be treatment with IL-10. IL-10 is considered to be a key cytokine for the maintenance of pregnancy, since its levels in placenta are decreased at term not in labor compared to first and second trimester (Hanna et al. 2000). In this study, we do not have data on the cervical IL-10 in the first and second trimesters. We saw no changes in the protein levels in homogenized tissue in the labor state, but we did see an upregulation of IL-10 mRNA levels. IL-10 has been used in several animal models for preterm labor treatment. Dexamethasone and IL-10 decreased IL-1β-induced uterine activity and amniotic prostaglandins in pregnant rhesus monkeys (Sadowsky et al. 2003). The administration of IL-10 has also been associated with improved pregnancy outcomes in rat and mouse models (Terrone et al. 2001; Rodts-Palenik et al. 2004; Robertson et al. 2006). Importantly, IL-10 helped to prevent preterm birth and fetal wastage in rats even when it was administered 24h later than bacterial endotoxin (Terrone et al. 2001), which makes it more clinically applicable in humans. Also, IL-10 can prevent white matter injury in neonatal rats born to infected dams. This suggests that maternal IL-10 therapy could provide neuroprotection for infants born to mothers with intrauterine infection (Rodts-Palenik et al. 2004).
Although progesterone was not studied in the present work, it is also one of the most important candidate substances for preventing PTB, and it has been tested in humans. Progesterone is essential in pregnancy. Systemic progesterone withdrawal occurs before parturition in rodents, but not in humans. There is evidence that functional progesterone withdrawal is involved in parturition in humans. Complex molecular mechanisms including the expression and function of several progesterone receptor isoforms, co-regulators and chromatin remodeling factors, underlie functional progesterone withdrawal (Dong et al. 2005; Condon et al. 2006; Merlino et al. 2007; Zakar and Hertelendy 2007). The mechanisms by which progesterone could help to maintain

pregnancy include downregulation of pro-inflammatory cytokines, like MCP-1 (Shynlova et al. 2008), suppression of LPS-induced TLR2 and TLR4 upregulation (Elovitz and Mrinalini 2005), modulation of gene expression in cervix (Xu et al. 2008), downregulation of mRNA expression of gap junction component connexin-43 in myometrium (Anderson et al. 2009) or upregulation of caspase-3 levels, which cleaves uterine myocyte contractile proteins (Jeyasuria et al. 2009). Although progesterone prevents preterm delivery in women with earlier spontaneous preterm birth or in women with short cervices, the benefits of progesterone on perinatal outcome and in twin pregnancies are still unclear (Dodd et al. 2008; Tita and Rouse 2009). Two studies (OPPTIMUM and STOPPIT) addressing these issues are in progress in the UK (www.opptimum.org.uk, www.charttrials.abdn.ac.uk/stoppit/).

In summary, in the work reported in this thesis several new substances, such as HMGB1 and CRH, which are probably important for cervical ripening, were identified in cervical tissue. Our results indicate that cervical epithelium might play a significant role in cervical ripening. We show that preterm and term cervical ripening are similar inflammatory processes, but diverge at some points. The differences in Toll-like receptor mRNA expression and IL-10 expression in cervical epithelium support the possible targeting of these proteins for prevention of preterm birth.

Conclusions

- HMGB1 and its receptors are localized and produced in the cervix with distinct changes in labor
- CRH may be involved in cervical ripening, since CRH, CRH-BP, CRH-R1 and CRH-R2 are localized in cervical tissue, and CRH stimulates IL-8 secretion from both preterm and term cervical fibroblasts
- Several important molecules, such as CRH, CRH-BP, CRH-R1, CRH-R2, HMGB1, TLR2, TLR4, IL-10, IL-12, are localized in the cervical epithelium indicating its role in the process of cervical ripening during labor
- Major inflammatory changes occur in the cervix at labor irrespective of gestational age. However, preterm cervical ripening could still present some differences from term in the downregulation of mRNA expression of Toll-like receptors and IL-12, higher levels of IL-10 in cervical epithelium, and may present different secretion patterns of cervical fibroblasts
- Preterm cervical ripening, like preterm delivery itself, is a multifactorial disorder with partly differing pathways involved for PPROM and infected preterm labor

FUTURE PERSPECTIVES

Further research in the field of PTB and preterm cervical ripening is still necessary to understand the molecular mechanisms involved in it. Thus, our further aim is to investigate the whole phenomenon with an approach of system biology. We are planning to perform both genomics and proteomics on the maternal blood and serum respectively along with transcriptomics on the cervical biopsies in collaboration with the research groups in Australia and USA. Currently, we are studying the differential gene expression in cervix of women with PTL (n=3), PPROM (n=3) and comparing it with the controls in the TL group (n=3). For this, Affymetrix GeneChip® Human gene 1.0 ST arrays containing 28000 genes were used. Preliminary results show 1214 and 340 genes respectively with 1.5 and 2.0 fold change comparing PTL and TL groups (Table II). On further analysis using Ingenuity Pathways Analysis (Ingenuity Systems®, www.ingenuity.com) we observed that most of the differentially regulated genes belonged to the functional pathways related to inflammatory or immunological disease, antigen presentation, cell-mediated and humoral immune response (Figure 22A).. The analysis of canonical pathways between PTL and TL shows changes in gene expression related to IL-10 and pattern recognition receptors pathways, which is in line with the present findings reported in this thesis (Figure 22B).

These results will be analyzed further and the mRNA expression of the most interesting genes will be confirmed with real-time RT-PCR. Further on, the results will be compared with those from proteomics and genomics. Hopefully, this systemic analysis will help to identify several new genes and proteins involved in PTB, which could be used as biomarkers of PTB or targeted for its prevention and treatment.

Table II. Number of differentially expressed genes comparing between different study groups observed by microarray analysis

	Number of genes		
Fold changes	PPROM/PTL	PTL/TL	PPROM/TL
1.5	812	1214	1792
2	174	340	556

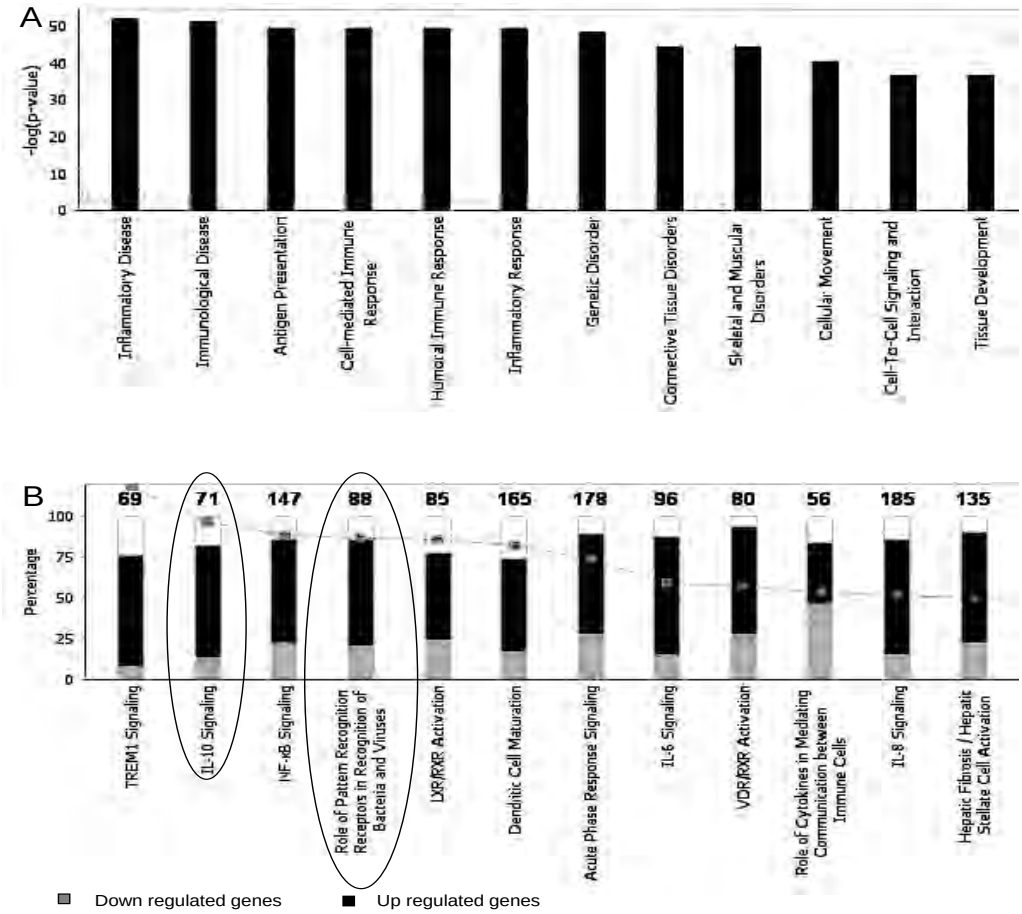


Figure 22. *Preliminary results of microarray analysis comparing PTL and TL group. Twelve the most represented functional (A) and canonical (B) pathways.*

Sammanfattning på svenska

Prematur förlossningen inträffar 4 veckor för tidigt och 6-15 % av alla gravida kvinnor föder för tidigt. Prematur förlossning orsakar 75 % av dödlighet hos nyfödda barn. Överlevande barn födda för tidigt kan få komplikationer som andningsproblem, neurologiska och metaboliska skador, som hos några även ger sämre hälsa i vuxen ålder, lägre IQ och kortare livslängd. Tidigare forskning kring prematur förlossning har mest varit fokuserat på ett för tidigt värkarbete. Behandlingen har utgjorts av värkhämmande medel. Men livmodern består av två olika delar; livmoderskroppen (corpus uteri) som till största delen består av glatt muskulatur och livmoderhalsen (cervix) som domineras av bindväv (85 %). Under graviditeten måste livmodern vara avslappnad och livmoderhalsen fast och sluten. Men under förlossningen blir livmodern ett mycket aktivt organ med täta kraftiga kontraktioner och livmoderhalsen blir mjuk och kan då öppnas 10 cm. Om livmoderhalsen är fast (omogen) blir det ingen etablerad förlossning. Utmognad av cervix är därför helt nödvändig för att det skall etableras en prematur förlossning.
I detta projekt jämförs utmognad vid prematur och normal förlossning. Cervixutmognaden är en inflammatorisk reaktion med förhöjda nivåer av inflammatoriska molekyler ex. interleukin-6 (IL-6), IL-8 och IL-1β. Den inflammatoriska processen leder till att bindväven (proteoglykaner, kollagen) bryts ned och dess sammansättning förändras och ger en mjuk och ”sladdrig” konsistens, vilket är förutsättningen för att cervix skall kunna öppna sig och låta fostret passera. Flera hormoner (prostaglandiner,östrogen, progesteron) är involverade i utmognaden av cervix. Corticoptropin Releasing Hormone (CRH) kan möjligen spela en roll för förlossningsstarten.
Det är inte klarlagt om cervix utmognad vid förtidig förlossning är densamma som vid förlossning i normal tid. Mer kunskap om förändringarna vid cervixutmognad båda vid för tidig och normal förlossning är nödvändig för att nya terapeutiska alternativ skall etableras för att förhindra för tidig förlossning.

Min avhandling fokuserar på molekylära förändringar i livmoderhalsen vid normal och för tidig förlossning. För att kunna studera detta, har vävnadsbiopsier tagits från livmodershalsen hos kvinnor efter vaginal förlossning och vid kejsarsnitt vid både normal och prematur förlossning. Eftersom flera nya substanser analyserats, så har även vävnadsbiopsier från icke-gravida kvinnor analyserats.
Med olika metoder har koncentrationen av olika ämnen och deras genetiska aktivitet analyserats.

Avhandlingen har visat:

- Flera nya substanser (corticotropin-releasing hormone, high- mobility group box protein 1 och deras receptorer, cytokiner IL-10 och IL-12), som kan vara av betydelse utmognad under förlossningen, har identifierats i cervix.
- Cervix utmognad vid förtidig förlossning liknar den process som sker i normal tid.
- Några skillnader i molekylerna i livmodershalsen under för tidig förlossning (lägre genuttryck av toll-like receptorer 2 och 4, IL-12; högre uttryck av IL-10 i epitelet i cervix; högre utsöndring av IL-8 och MMP-1, men lägre utsöndring av MMP-3 från cervix fibroblaster) har registrerats.
- Livmoderhalsens förändringar kan vara olika under förtidig förlossning i samband med infektion och för tidig vattenavgång.

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.
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