INFECTIOUS AND INFLAMMATORY MECHANISMS IN PRETERM BIRTH AND CEREBRAL PALSY

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Para nacer he nacido
För att födas har jag fötts
Born to be born.

Pablo Neruda

To
Gunnar and Gun-Britt

ABSTRACT

Background: During the last two decades, several international studies of women in preterm labor (PTL) and preterm pre-labor rupture of the membranes (pPROM) have shown a significant association between microbial invasion of the amniotic cavity (MIAC), some cytokines and chemokines and preterm birth (PTB). These studies have been performed in countries with much higher incidence of PTB than that in Sweden. Cerebral palsy (CP) has also been shown to be associated with infectious and inflammatory mechanisms in several international epidemiological studies. Our aim was to examine the role of inflammatory mechanisms in PTB and CP in a setting with a low incidence of preterm birth and perinatal infections.

Material and Methods: In order to examine PTB mechanisms, amniotic fluid (AF) was retrieved transabdominally from 61 patients in PTL and 47 patients with pPROM, before 34 weeks of gestation in both groups. Forty-five women at term (≥ 37 weeks) were included. These women were scheduled for elective cesarean section after uncomplicated pregnancies. Cervical fluid was obtained from the external cervical os in all patients in PTL and in all term patients. Polymerase chain reaction analyses for *Ureaplasma urealyticum* and *Mycoplasma hominis* and culture for aerobic and anaerobic bacteria were performed. Interleukin (IL)-6, IL-8, IL-18 and monocyte chemotactic protein (MCP)-1 were analyzed with enzyme-linked immunosorbent assay.
In order to examine inflammatory mechanisms in CP, a population-based series of 148 preterm infants with spastic CP, born 1983-90, were included and matched with a control group (n=296). Subgroup analyses of patients with spastic diplegia and hemiplegia and those born at <32 and ≥32 weeks were performed. Maternal, antenatal and intrapartal variables were retrieved from obstetric records.

Results: MIAC was detected in 16% of women in PTL and 25 % of women with pPROM. Patients in PTL with MIAC had significantly elevated levels of IL-6, IL-8 and IL-18. The levels of IL-6, IL-8 and MCP-1 were elevated in MIAC cases in women with pPROM**.** There was also a significant association between elevated levels of IL-6, IL-8, IL-18 and MCP-1 and short amniocentesis-delivery interval (≤ 7 days) and preterm birth (< 34 weeks) in women in PTL, whereas this association was less evident in women with pPROM. A receiver-operator-characteristic curve was used to identify the best cut-off levels of IL-6 and IL-8 in AF for delivery within 7 days. This value was used to define an inflammatory response. The inflammatory response rate was 46 % in the PTL group and 51% in the pPROM group. Elevated IL-18 and MCP-1 were related to an inflammatory response in the women in PTL. MCP-1 was also related to an inflammatory response in women with pPROM.
There were higher levels of IL-18 and MCP-1 in the cervical fluid of women in PTL, compared with non-laboring women at term. There were elevated levels of MCP-1 in the cervical fluid of women in PTL who gave birth within 7 days or before 34 weeks of gestation, who had MIAC or had intra-amniotic inflammation.
In the epidemiological study of CP, clinical chorioamnionitis/pyelonephritis, long interval between rupture of membranes and birth, admission-delivery interval <4 hours and Apgar scores of <7 at 1 minute just significantly increased the risk of CP. Apgar scores of <7 at 5 and 10 minutes were strongly associated with an increased CP risk. Abruptio placentae, Apgar scores <7 at 1 minute and pathological non-stress test (reason for delivery) were significant risk factors for CP only in the moderately preterm and hemiplegic groups, whereas fever prior to delivery was a significant risk factor in the very preterm and spastic diplegic groups. Antibiotics administered during pregnancy only constituted a risk factor in the spastic diplegic CP group.

Conclusion: The occurrence of intra-amniotic microbial invasion and inflammation in this population of Swedish women in PTL and pPROM was similar to that reported in data from populations with a higher incidence of preterm delivery. In addition, our data support an association between antenatal infection/inflammation and CP.

Keywords: preterm birth, preterm labor, preterm prelabor rupture of membranes, interleukin-6, interleukin-8, interleukin-18, monocyte chemotachtic protein-1, cerebral palsy, antenatal riskfactors, chorioamninitis.

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CONTENT

LIST OF PUBLICATIONS

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

I. Jacobsson B, Mattsby-Baltzer I, Holst RM, Andersch B, Bokstrom H, Wennerholm UB, Hagberg H. Microbial invasion and cytokine response in amniotic fluid in a Swedish population of women in preterm labor. Acta Obstet Gynecol Scand 2003;182(2):120-8

II. Jacobsson B, Mattsby-Baltzer I, Holst RM, Andersch B, Bokstrom H, Nikolaitchouk N, Wennerholm UB, Hagberg H. Microbial invasion and cytokine response in amniotic fluid in a Swedish population of women with preterm prelabor rupture of membranes. Acta Obstet Gynecol Scand 2003;In press.

III. Jacobsson B, Mattsby-Baltzer I, Holst RM, Nikolaitchouk N, Wennerholm UB, Hagberg H. Interleukin-18 in cervical and amniotic fluid: relationship to microbial invasion of the amniotic cavity, intra-amniotic inflammation and preterm delivery. Br J Obstet Gynaecol 2003;In press.

IV. Jacobsson B, Holst RM, Wennerholm UB, Andersson B, Lilja H, Hagberg H. Monocyte chemotactic protein-1 in cervical and amniotic fluid: relationship to microbial invasion of the amniotic cavity, intra-amniotic inflammation and preterm delivery. Am J Obstet Gynecol 2003;In press.

V. Jacobsson B, Hagberg G, Hagberg B, Ladfors L, Niklasson A, Hagberg H. Cerebral palsy in preterm infants: a population-based case-control study of antenatal and intrapartal risk factors. Acta Paediatr 2002;91(8):946-51.

ABBREVIATIONS

AF	amniotic fluid
c-AMP	adenosine 3 ,5 -cyclic phosphate
CI	confidence interval
CoNS	coagulase negative *Staphylococcus*
COX	cyclooxygenase
CP	cerebral palsy
CRH	corticotropin releasing hormone
CS	cesarean section
ELISA	enzyme-linked immunosorbent assay
FIRS	fetal inflammatory response syndrome
IAI	intra-amniotic inflammation
IL	interleukin
INF	interferon
LPS	lipopolysaccharide
MBR	Swedish Medical Birth Register
MCP-1	monocyte chemotactic protein-1
MIAC	microbial invasion of the amniotic cavity
NF-κB	nuclear factor kappa B
NO	nitric oxide
OR	odds ratio
PCR	polymerase chain reaction
pPROM	preterm prelabor rupture of membranes
PTB	preterm birth
PTL	preterm labor
PVL	periventricular leukomalacia
RR	relative risk
ROC	receiver-operation-characteristic
SIRS	systemic inflammatory response syndrome
TNF	tumor necrosis factor

INTRODUCTION

1. General introduction

Preterm birth (PTB) remains one of the main causes of perinatal mortality and long-term morbidity (1). The US incidence of PTB in 2000, 11.6 % (2), was double that in Sweden, 5.6% (3). There has been a slight increase during the last decades in the USA (1, 2) and Canada (4). In the USA, the PTB rate has risen steadily from 9.4 % in 1981, to 10.6 % in 1990 and 11.6 % in 2000 (2). Among the non-Hispanic white population, the PTB rate has risen from 8.5 % in 1990 to 10.4 % in 2000, but there has been a slight reduction in the black population from 18.9 % in 1991 to 17.3 % in 2000 (2). In Sweden, the incidence has been stable since 1973 with a slight increasing tendency in the beginning of the 1980s and subsequently then stabilizing (Fig. 1) (3). Two exceptions from the stable or increased PTB rate are France (5, 6) and Finland (7) that have reported decreasing incidence of PTB.

Although all births before 37 weeks of gestation are considered preterm according to the World Health Organization's definition, births before 32 weeks of gestation account for most neonatal deaths and disorders (1). Sweden and the USA also differ when it comes to the rate of births occurring < 32 weeks of gestation, 0.85 % and 1.93 % respectively (2, 3). PTB accounts for 75 % of the total perinatal mortality and over two thirds of this emanates from the 30-40% of preterm infants who are born before 32 weeks of gestation (8). Although major efforts have been made to reduce the incidence of PTB in industrialized countries, they have so far not yielded any tangible effect (1).

PTB is also a leading cause of long-term neurological morbidity (1, 9, 10). An increasing prevalence of cerebral palsy (CP) has been observed since the mid-seventies, especially among very and extremely preterm children (9). During the last four decades, there has been a successive decrease in perinatal mortality and

Fig. 1

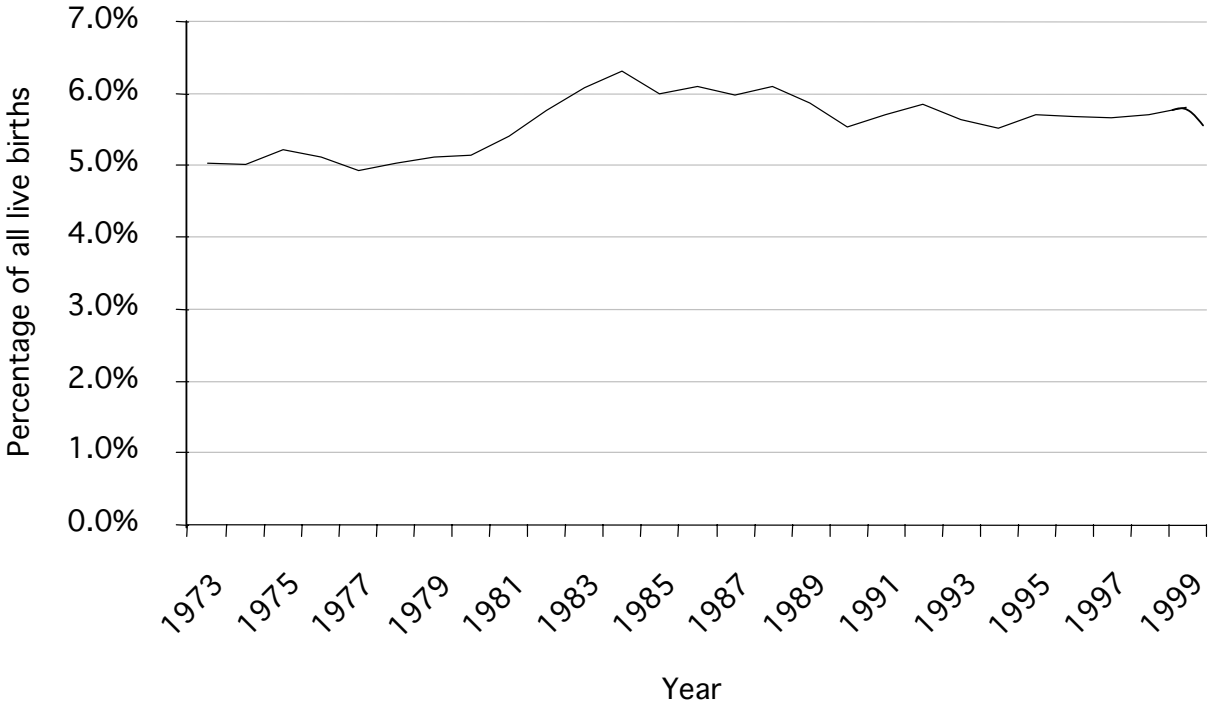

Fig. 1. Incidence of preterm birth (< 37 weeks of gestation) in Sweden between 1973 – 2000. (From Medical Birth Registration in 2000: Swedish National Board of Health and Welfare; 2002).

Fig. 2

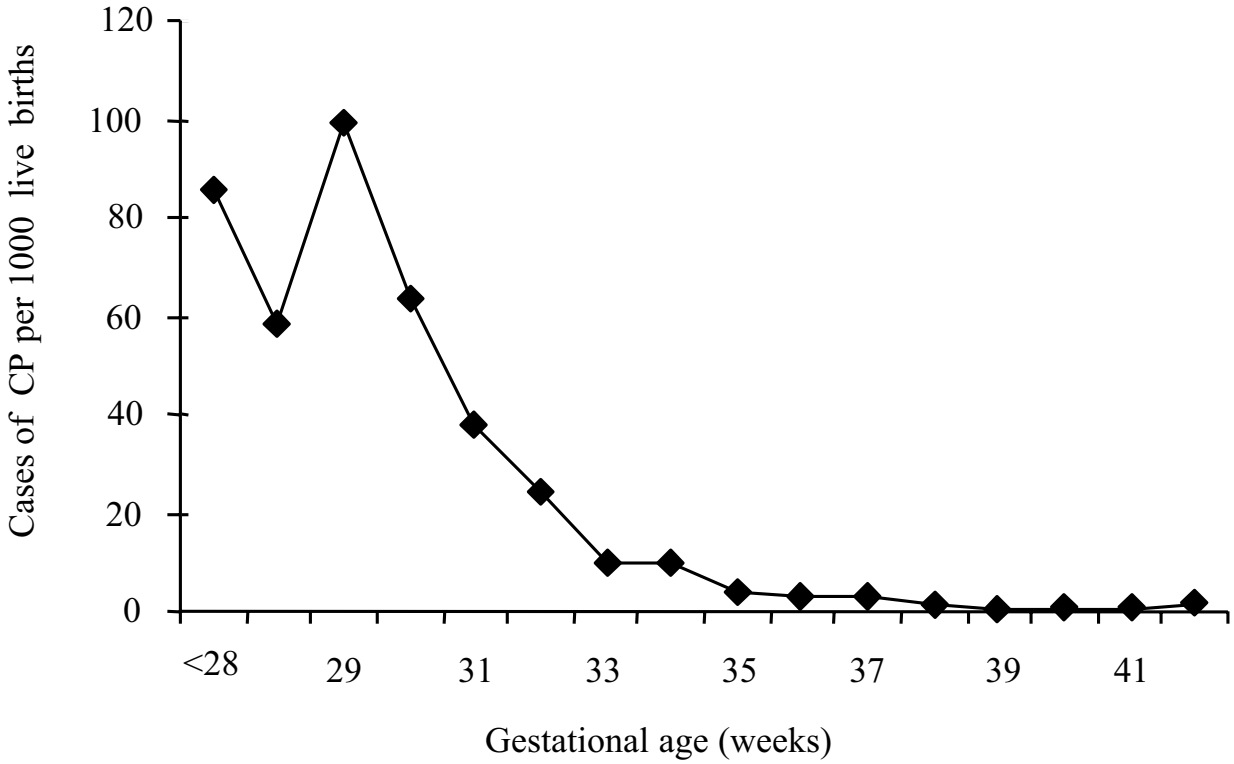

Fig. 2. Prevalence of CP per 1000 live births according to gestational age. (From data in Hagberg B, Hagberg G, Beckung E, Uvebrant P. Changing panorama of cerebral palsy in Sweden. VIII. Prevalence and origin in the birth year period 1991-94. Acta Paediatr 2001;90(3):271-7).

a parallel increase in the potential for survival at ever lower gestational ages, which might explain the increased prevalence of CP (9, 11). The live birth prevalence in Sweden of CP during the period 1991-94 was 2.12 per 1000 and is inversely correlated with gestational age, with a 60-70 times higher rate at a gestational age less than 28 weeks, compared to term delivery (9). The risk of developing CP related to gestational age is shown in Fig. 2 (9). The prevalence of CP was 8.6 % in children born before 28 weeks, 6 % between 28 and 31 weeks, 0.6% between 32 and 36 weeks and 0.13% in those born at term (9).

Preterm children are also at increased risk of developing cognitive and behavioral abnormalities and of achieving poorly in educational situations (12), problems which persist until adulthood (13, 14). Non-retinopathy-related visual abnormalities are also seen in extremely preterm children (15, 16).

Against this background, it is understandable that the economic costs for PTB are considerable, related both to the initial hospitalization in the neonatal intensive care unit and to the long-term consequences of PTB. However, comparatively little research has been devoted to measuring the economic costs of preterm delivery, especially the combination of the short-term and long-term costs (17). One study has estimated the magnitude of the additional costs attributable to low birth weight at 35 % of all health care costs for infants (< 1 year) and 10 % of all health care costs for children (18). This is equivalent to the cost of unintentional injuries, the leading cause of death for children after infancy (17). It has been shown that a collaborative quality improvement of the neonatal care of very preterm infants can quickly and substantially reduce the costs of care (19). This implies that preventive measures aimed at reducing the incidence of PTB will immediately release economic resources, and

in the long run, reduce human suffering from life-long disabilities.

2. Preterm birth

2.1 Sub-grouping of preterm birth

The possibility that the clinical consequences of prematurity for the infant could vary according to causal mechanism has led to a subdivision of PTB into sub-groups (20). One classification of PTB is based on the manner in which the patients presents, i.e. spontaneous preterm labor (PTL), preterm prelabor rupture of membranes (pPROM) and elective induction or cesarean section (CS) (usually due to a complication of pregnancy), respectively (20). The first two groups are often combined and called spontaneous PTB, in contrast to indicated PTB. This subdivision has been questioned but is still the one in most common use (21). All studies related to this issue are hospital-based.
The proportion of preterm deliveries resulting from PTL varies in different studies from 18 to 64 % (22-24), while that resulting from pPROM is reported at between 7 and 51% (25-27). The proportion of preterm deliveries classified as indicated varies between 18 and 38% (23, 25, 28).

2.2 Spontaneous preterm birth

Since the beginning of the eighties a compelling amount of evidence has been presented, suggesting a strong association between microbial invasion of the amniotic cavity (MIAC), PTL and pPROM. MIAC and the presence of pro-inflammatory cytokines in the amniotic fluid (AF), the intra-amniotic inflammatory response, are known to induce the release of uterotonic substances such as prostaglandins from gestational tissue, thereby causing uterine contractions and labor (29). Infection may cause up to 30% of PTL cases and may either be clinically evident or, more often, subclinical (30). At any rate, the etiology of PTB is multifactorial and the exact cause in each case can rarely be identified.

Evidence of activation of the fetal hypothalamic-pituitary-adrenal axis in relation to preterm delivery has also been presented (31). Spontaneous parturition in primates is characterized by fetal adrenal synthesis of C19 androgens which, in turn are aromatized by the placenta into estrogens, including estrone, estradiol and estriol. Among non-human primates, a rise in AF concentration of estrone precedes or coincides with increases in AF prostaglandin concentrations, which begin to rise several days before parturition (32). In humans, placental corticotropin releasing hormone (CRH) (33, 34), maternal salivary estradiol (35) and maternal plasma estradiol concentrations all increase before spontaneous preterm parturition (31). In contrast to infection-associated parturition, spontaneous parturition at term is associated either with no increase or minor increases in AF concentrations of cytokines (36, 37).

2.3 Animal models for studying spontaneous preterm birth associated with infection and inflammation

Several animal models have been developed in order to study further the role of infection and inflammation in PTB. Some investigators have used a mouse model for studies of infection- and inflammation-associated PTB (38-41). Lipopolysaccharide (LPS) and pro-inflammatory cytokines have been injected in pregnant mice (day 12-14 of 19-day pregnancy) and preterm delivery has been observed within 24 hours.
Others have used a rabbit model (42-47); Dombroski et al have, for instance, shown that bacterial inoculation via the vagina results in PTB (46). There is also a primate model; pregnant rhesus monkeys (36, 48) can be infected (day 130 of 167 day pregnancy) which will cause PTB (36).

3. Cerebral palsy in preterm infants

CP is defined as a group of non-progressive, but often changing, motor impairment syndromes, secondary to lesions or abnormalities of the brain, and arising in the

early stages of development (49). CP diagnosed in the first two years of life may resolve during early childhood, especially when functional impairment is mild (50-52). Mild CP had resolved in 72% of the cases on reexamination at age seven in one study (50). Nonetheless, children with resolved CP were almost 10 times more likely to be mentally retarded, which indicates that the neurological abnormalities observed were a valid indication of antecedent brain damage rather than a variation of the normal (50, 53). This indicates that the child must be at least 4 or 5 years old before a reliable diagnosis can be made (9).

CP is associated to cerebral white matter damage. The classical neuropathological white matter damage findings associated to CP include periventricular leukomalacia (PVL) and periventricular hemorrhagic infarction (54-56). A diffuse white matter injury with widespread gliosis and hypomyelination is also commonly seen (57, 58). The motor function impairment may be explained by the observed white matter damage (59). Cranial ultrasonography is therefore one of the most important examinations for predicting the development of CP (60, 61). Periventricular echodensities are the cranial ultrasound finding that most often predicts CP, followed by echolucency and ventricular enlargement (due to loss of white matter). Echolucencies have been the most studied and presumably the most specific cranial ultrasound finding (62). Very preterm neonates with this finding have a CP risk of 59 %, about 20 times higher than very preterm neonates without these findings (53, 62). Conversely, only 3 % of infants with normal cranial ultrasound scans developed CP (62). The diffuse white matter injury is more easily seen with magnetic resonance imaging (63, 64). The strong association between cranial ultrasound findings indicative of white matter damage and CP suggest that etiologic studies of white matter damage may provide information about the etiology of CP.

3.1 Epidemiological studies (different etiologies)

As an effect of the success of newborn intensive care during the last three decades ensuring an increasing survival of children born very and extremely preterm, the prevalence of CP among preterm children has risen (9, 65). The etiology of CP is still poorly understood. Since CP is not a disease but a symptom complex, clinically defined at 4-5 years of age, it is not surprising that there are considerable problems associated with epidemiological studies of CP etiology: the long time-lag between recognition of CP and the presumed brain damage, disagreements among examiners about clinical findings in patients and changes in clinical findings over time and, due to the lack of a definitive test for CP, multiple and different possible causes (53). Some risk factors have been repeatedly observed to be related CP: low gestational age (9, 53), low Apgar scores (66), male gender (53), multiple gestation (67), intrauterine viral infections (e.g. rubella, cytomegalovirus) (68-70), iodine deficiency (71), exposure to methyl mercury during pregnancy (69, 72) and maternal thyroid abnormalities (69, 73). The higher risk of CP in multiple births is apparently related to prematurity or to antenatal death of a co-twin or co-triplet (67, 74, 75) .

Intrapartal factors producing asphyxia have traditionally been assumed to be the principal cause of CP, but this assumption has been reconsidered during the 80- and 90-ties. Today, intrapartal asphyxia is still believed to account for a relatively large proportion of CP in term and near-term born infants, around 10 % (53); some researchers feel that this figures is too low (9). There is consensus that intrapartal asphyxia seems to be of less importance for the development of CP in very preterm infants. Birth asphyxia is a poorly defined term relating to a sequence initiated by hypoxia (76). There is no definitive test for asphyxia and the clinical signs are not specific (53). Using indirect signs of birth asphyxia, recent studies suggest that 1) birth asphyxia may not be such an important cause of CP as was previously assumed, but it may sometimes constitute one element of a multifactorial cause, 2) neonatal signs

associated with birth asphyxia may be early manifestations of CP from a variety of causes, of which birth asphyxia is only one, 3) the majority of pathways to CP commence pre-delivery (53, 73, 76-78).
The proportion of CP acquired post-neonatally varies from 8 to 18 %; the causes are mainly infectious, anoxic and traumatic (53).

4. Infectious and inflammatory mechanisms in preterm birth and cerebral palsy

4.1 General inflammatory mechanisms

4.1.1 Inflammatory cells

Maternal and fetal inflammatory responses include the involvement of cytokines, chemokines, adhesion molecules, extracellular matrix proteins with adhesive properties and matrix metalloproteinases. All five of these groups facilitates the transendothelial migration of cells out of the circulation and into adjacent organ parenchyma, which is essential for the inflammatory process (79). When it comes to PTB, nearly all of the disparate maternal and fetal cell types in the uteroplacental unit are integrated into the cytokine network. The highly versatile macrophage, abundant in uteroplacental tissues, has turned out to be a potential pivotal cell type in this context (80). The polymorphonuclear leukocytes found in AF are of fetal origin in the majority of cases (81). These data have raised the question of how the fetal leukocytes might gain access to the AF; via extravasation from the umbilical cord, from chorionic vessels, from the chorionic plate of the placenta or from fetal endothelium in vessels in the alveolar space of the lung (81). A primate model has been developed to study this (82) but results have not yet been published. The brain was previously thought to be protected by the blood-brain-barrier, but it has been established that the brain can no longer be described as an immune-privileged organ devoid of the capacity to initiate an inflammatory response (83). The blood-brain barrier is relatively ineffective in preterm infants (84). It has been hypothesized that cytokines and chemokines activate endothelial cells and leukocytes in the circulation, diminish the effectiveness of the blood-brain barrier (85) and gain access to and activate microglia and astrocytes, which in turn produce chemokines and more cytokines (79). The chemokines function as chemo-attractants for activated inflammatory cells, including those in the circulation (79).
The immediate response of the brain to a variety of insults is characterized by the proliferation and hypertrophy of microglia cells (the resident inflammatory cells). The process of gliosis is accompanied by the infiltration of activated inflammatory cells derived from the periphery, the inflammatory products of which act in consort with centrally derived mediators in eliciting a response to the injury (83). Although some monocytes, macrophages and microglia in the brain are derived from resident cells, others are derived from the circulation (86).

4.1.2 Pro-inflammatory cytokines

The concept of pro-inflammation is based on the genes coding for the synthesis of small mediator molecules that are up-regulated during inflammation. Type II phospholipase A2, cyclooxygenase (COX)-2 and inducible nitric oxide (NO) synthase are examples of these genes. These genes code for enzymes that increase the synthesis of platelet activating factor and leukotrienes, prostanoids and NO. Pro-inflammatory cytokine-mediated inflammation does not normally occur in healthy individuals (87). Interleukin (IL)-1 and tumor necrosis factor (TNF)-α are considered to be the prototypic pro-inflammatory cytokines. Although inflammatory products such as endotoxins act as triggers, the cytokines IL-1 and TNF-α are particularly effective and act synergistically in the process of activating the inflammatory genes (87). Interferon (IFN)-γ, IL-6 and IL-18 are other cytokines considered to possess pro-inflammatory properties. Genes coding for chemokines are also considered to be pro-inflammatory (87).

IL-6

IL-6 is a protein consisting of 185 amino acids and glycosylated at positions 73 and 172. It is synthesized as a precursor protein consisting of 212 amino acids. Monocytes express at least five different molecular forms of IL-6 with molecular masses of 19-26 kDa. It maps to human chromosome 7p21-p14.

The IL-6 gene promoter contains many different regulatory elements allowing the induction of gene expression by various stimuli, including glucocorticoids and cAMP. The nuclear factor kappa B (NF-κB) binding site is responsible for the induction of IL-6 production in non-lymphoid cells, so NF-κB is considered to be an important transcription factor for IL-6 (88).

IL-6 is a cytokine with pro-inflammatory and immunomodulatory properties, produced by many cell types including activated phagocytes, macrophages, monocytes and endothelial cells. IL-6 functions in both innate and specific immunity and stimulates the synthesis of acute phase proteins by hepatocytes as well as the growth of antibody-producing B lymphocytes (88). TNF-α and IL-1 stimulate the transcription of IL-6 which acts synergistically with these two cytokines in many situations (87). IL-6 is known to be a multifunctional cytokine that regulates immune response, hematopoesis, the acute phase response and inflammation (88).

IL-18

IL-18 is a recently described member of the IL-1 cytokine family (due to its structure, receptor family and signal transduction pathways) and was initially defined as an IFN-γ inducing factor (89, 90). IL-18 is synthesized as a precursor, requiring caspase-1 for cleavage into the active form (90). Human pro-IL-18 contains 193 amino acids and has a molecular mass of 24 kDa (91). Activated IL-18 cytokine has a molecular weight of 18kDa (92). It maps to human chromosome 11q22 (90). The intracellular signal pathway includes the transcription factor NF-κB which up-regulates transcription of the IL-18 gene (92).

IL-18 is a cytokine with pro-inflammatory and pro-apoptotic properties. It is mainly synthesized by macrophages, monocytes and keratinocytes, but can also be produced by epithelial cells (93-97). IL-18 enhances the inflammatory process by stimulating the production of INF-γ and TNF-α and IL-1β (91). IL-12 can act synergistically with IL-18 to provoke a T helper 1 response (97, 98).

4.1.3 Chemokines

Chemokines (short for chemoattractive cytokines) are small (8-10 kDa) inducible proteins (99, 100). Chemokines have the capacity to activate leukocytes and mediate inflammatory reactions. These proteins are involved in diverse immune responses to which they recruit various leukocytes (99). Four classes of chemokines have been described, based on the conserved cysteine residues on the mature protein: α-chemokines, β-chemokines, γ-chemokines and fractalkine or neurotactin (101). The two major sub-families are: C-X-C chemokines (α-chemokines) which have an amino acid between two cysteines, and C-C chemokines (β-chemokines), which have two adjacent cysteines. α-chemokines possess potent chemoattractive properties, mostly for neutrophils; β-chemokines exert their effect on monocytes, lymphocytes, eosinophils and mast cells, but not on neutrophils (88, 99, 100).

IL-8

IL-8 is the prototypic α-chemokine, previously called neutrophil attractant/activating peptide-1. IL-8 is produced by the processing of a precursor protein consisting of 99 amino acids. Processing of this precursor by specific proteases yields N-terminal variants of IL-8. IL-8 contains 72-77 amino acids (no glycosylated sites) and has a molecular weight of 8-11 kDa. The IL-8 gene maps to human chromosome 4q12-q21. NF-κB is an important transcription factor for IL-8 as well (88, 99).

IL-8 is produced by monocytes, macrophages, fibroblasts, endothelial cells, keratinocytes, melanocytes, hepatocytes, chondrocytes, and a number of tumor cell lines in many different cell types (88, 99). The synthesis of IL-8 is strongly stimulated by IL-1 and TNF-α in many cell types (88, 99). IL-8 is thought to be the

primary regulatory molecule of acute inflammatory states, because of its potent neutrophil chemotactic effect (99) . Neutrophil migration into the inflamed peritoneal cavity is severely inhibited in IL-8 transgenic mice (102).

Monocyte chemotactic protein - 1

Monocyte chemotactic protein (MCP)- 1 is the prototypic β-chemokine. MCP-1 is produced by the processing of a precursor protein consisting of 99 amino acids. MCP-1 contains 76 amino acids (one glycosylated site) and has a molecular weight of 11-15 kDa. The MCP-1 gene maps to human chromosome 17(q12-q21) (88, 99). NF-κB is also an important transcription factor for MCP-1 (99).

A great deal of research has identified the chemoattractants and activators responsible for neutrophil and lymphocyte traffic but less is known about the molecules regulating monocyte migration (103). Certain chemokines, such as MCP-1, have been demonstrated to recruit monocytes into foci of active inflammation (103). MCP-1 is produced in a great variety of cells, including monocytes and macrophages. There are indications that MCP-1 is uniquely essential for monocyte recruitment in several inflammatory models in vivo (104).

4.2 Infectious and inflammatory mechanisms in preterm delivery

One reason for the lack of progress in reducing PTB is the poor understanding of the pathophysiological process related to PTL and pPROM (30). Pathologic processes implicated in the etiology of the preterm labor syndrome include infection, uteroplacental ischemia, uterine overdistension, abnormal allograft recognition, allergic phenomena and cervical disease (105, 106). Over the past two decades, the important concept has emerged that PTL most likely represents a heterogeneous syndrome characterized by uterine contractility, cervical ripening, and/or membrane rupture (105). Inflammatory responses can be initiated by any foreign stimulus, from foreign bodies to specific bacterial pathogens (105). As only a minority of PTL cases can be attributed to a clinically evident intra-uterine infection, most PTL cases secondary to infection are likely to be the result of an inflammatory response in the maternal and fetal gestational tissue generated in response to these pathogens. This inflammatory process is mediated by cytokine production and ultimately results in prostaglandin production, an important cascade in the onset and propagation of myometrial contractility and subsequent labor and birth (107).

4.2.1 Microbial invasion of the amniotic cavity

Microorganisms may gain access to the amniotic cavity by the following pathways: 1) ascending from the vagina and cervix; 2) hematogenic dissemination through the placenta (trans-placental); 3) retrograde seeding from the peritoneal cavity through the fallopian tubes; 4) accidental introduction at the time of invasive procedures such as amniocentesis, percutaneous fetal blood sampling or chorionic villous sampling (107). Several lines of evidence support the idea of the ascending route as the most common (29, 106, 107): histological chorioamnionitis is more common and severe at the membranes rupture sites than in other parts of the membranes (108), bacteria identified in cases of congenital infections are similar to those found in the lower genital tract (106, 107, 109), inflammation of the chorionic membranes is present in most cases of congenital pneumonia (stillbirth or neonatal), the membranes of the first twin more often has signs of inflammation in twin gestations and signs of inflammation have not been observed exclusively in the second twin in the absence of such signs in the first twin (106, 107, 110).

A four-stage process leading to intrauterine infection has been proposed (111). Stage I: an overgrowth of facultative organisms or the presence of pathological organisms in the vagina. Bacterial vaginosis may be one of the manifestations at this stage. Stage II: the microorganisms ascend, gain access to the uterine cavity and then reside in the decidua. Stage III: a localized inflammatory reaction

leads to deciduitis which extends into chorionitis, leading to choriovasculitis (fetal vessels inflammation) and, via the amnion membrane (amnionitis), into the amniotic cavity resulting in intra-amniotic infection. Stage IV: once in the amniotic cavity, the bacteria may gain access to the fetus by different routes of entry: aspiration, otitis, conjunctivitis and omphalitis. Seeding from any of these sites to the fetal circulation leads to bacteremia and sepsis (111, 112).

The bacteria isolated from the upper genital tract among women who have had PTB are all microorganisms generally believed to be of low virulence, typically representative of the normal microbial flora in the cervix and vagina (113, 114). The timing of the upper genital tract infection is not completely understood but most evidence indicates that bacterial ascension from the lower to the upper genital tract occurs earlier rather than later in gestation (114, 115). Regardless of when or how they arrive in the upper genital tract, these bacteria may reside in the chorio-decidual interface, where they are associated with chronic low-grade inflammation for many weeks before resulting in spontaneous labor or rupture of the membranes (114). Activation of the host response as early as mid-trimester by elevated levels of AF IL-6 at amniocentesis in a subset of women that later had a late pregnancy loss or an early spontaneous preterm delivery serves as evidence of this process (116, 117). There is also data available to support the possibility that upper genital tract microbial colonization and inflammation may be present prior to conception (118). If *Ureaplasma urealyticum* is detected at a mid-trimester amniocentesis, the patient is likely to give birth at around 24 weeks of gestation (119, 120). Viral infections seem to be of less importance related to late fetal loss or early preterm delivery (116).

In summery, it is an established fact that clinically silent upper genital tract infection and inflammation are strongly associated with an increased risk of spontaneous PTB (29, 106, 107, 111, 114, 120). The proportion of women with MIAC is much greater in women whose pregnancies have been the object of sampling and delivery at earlier gestational age (115, 121, 122). However, although infection may only account for perhaps 20–25% of all PTB and although intra-amniotic inflammation (IAI) may account for a similar proportion. Infection and inflammation may account for the majority of the early spontaneous PTB and thus the majority of infant morbidity and mortality (115, 122).

4.2.2 The inflammatory process within the uterine cavity

The main hypothesis on how ascending bacteria can lead to spontaneous PTB is as follows: bacterial invasion of the chorio-decidual space activates monocytes both in the decidua and the fetal membranes to produce a number of pro-inflammatory cytokines. Some combinations of these cytokines stimulate prostaglandin synthesis and release and initiate a sequence of neutrophil chemotaxis, infiltration, and activation, culminating in the synthesis and release of a number of metalloproteases. The cytokines and prostaglandins stimulate contractions, while the proteases attack the chorioamniotic membranes, leading to rupture of membranes. Various proteases also remodel cervical collagen, resulting in cervical ripening (120). An overview is presented in Fig. 3.

Macrophages seem to play a pivotal role in the defense against microbial invasion of the uterine cavity by phagocytosis and by synthesis and secretion of large amounts of cytokines and other regulatory molecules. Macrophages are a major cell type in both the maternal and fetal compartments of the uteroplacental unit. They are abundant in the decidua and in fibrous tissues near the placenta. In the placenta, macrophages can be isolated in the mesenchymal stroma. In the extraplacental membranes, fetal macrophages populate the mesenchymal stroma between the amnion and chorion layers (80).

The bacteria invading the choriodecidual interface release endotoxins and exotoxins that activate the decidua and the fetal membranes. Several studies have shown that phospholipase A2 and C and LPS are capable of stimulating production of prostaglandins by the amnion and

Fig. 3

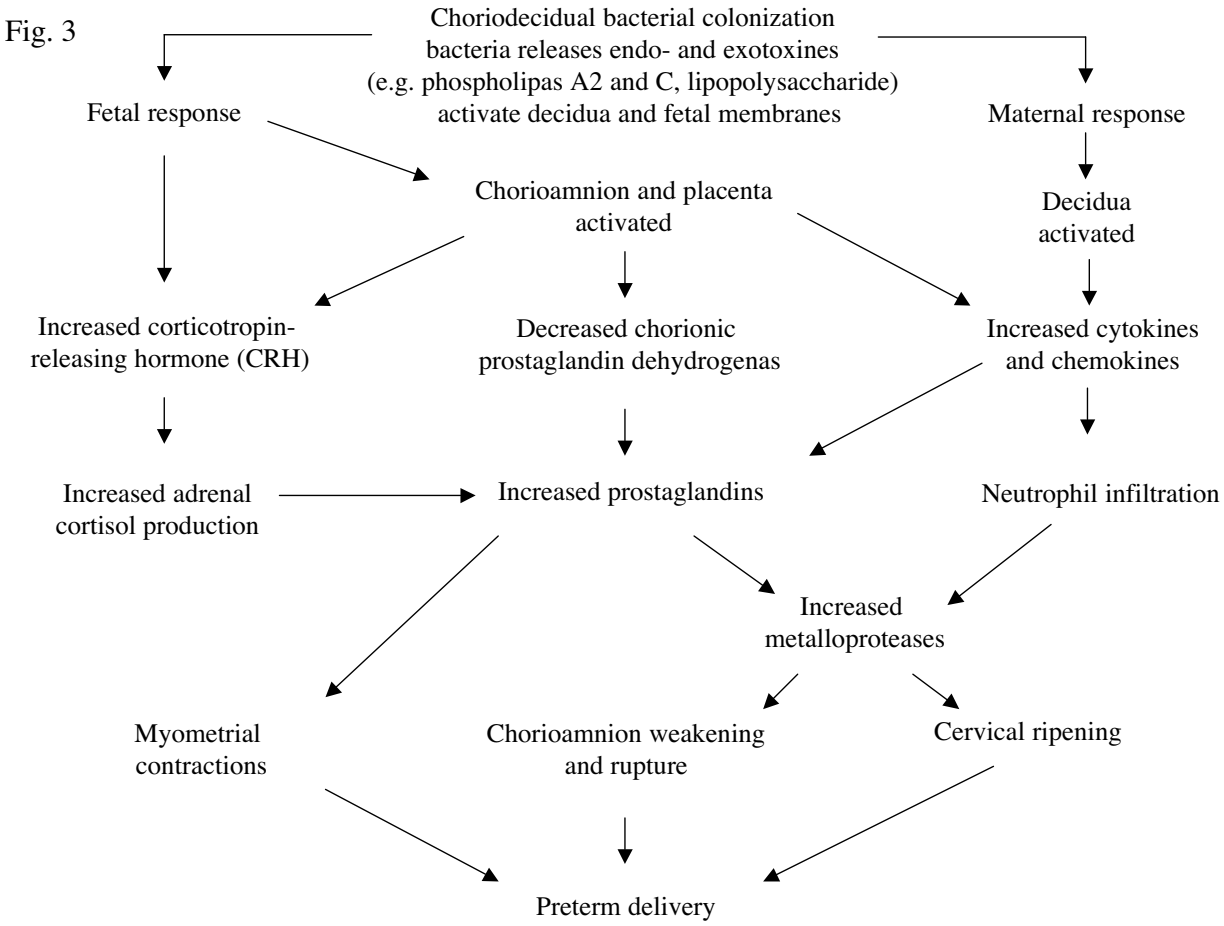

Fig. 3. Mechanisms of preterm labor due to infection (Modified from Goldenberg RL, Hauth JC, Andrews WW. Intrauterine infection and preterm delivery. N Engl J Med 2000;342(20):1500-7).

decidua in vitro (123). Phospholipase A2 can cleave and release arachidonic acid, which can be metabolized to prostaglandins, leukotrienes and epoxides. Substantial evidence supports the critical role of prostaglandins in the preterm and term labor process by inducing myometrical contractility, as well as, changes in the extracellular matrix metabolism associated with cervical ripening; they are also presumed to be involved in decidual and fetal membrane activation (114, 123).

Until recently it was thought that bacteria alone were responsible for various clinical and metabolic derangements seen with infection, but today we know that host immune responses and endogenous products precipitated by bacterial endotoxins are, to a large extent responsible for the deleterious effects of the infection (123). There is a growing body of evidence that PTB may, in the presence of bacterial invasion or infection, be caused by the host-mediated response through activation of the macrophage-monocyte system by bacterial products and tissue injury. Pro-inflammatory cytokines and chemokines (TNF-α, IL-1α, IL-1β, IL-6, IL-8 and granulocyte-colony stimulating factor (G-CSF)) are released on activation of these cells by microbial products (114, 123). The release of these pro-inflammatory cytokines and exotoxins create a positive feedback loop which recruits more monocytes and macrophages to the infected area, sustaining the host-mediated response (123).

Other pathways may play a role as well. In chorionic tissue, prostaglandin dehydrogenases normally inactivate the prostaglandins produced in the amnion, preventing them from reaching the myometrium and causing contractions. If the chorionic membrane is infected the dehydrogenase activity decreases, allowing increasing quantities of prostaglandins to reach the myometrium (124, 125) and uterine contractions.

Recent evidence suggests that infection may, in many cases, involve the fetus. A fetal host-response is induced with increased levels of plasma cytokines. This is the background of the concept of fetal inflammatory response syndrome (FIRS) (123, 126, 127). In fetuses with infection, increases in both the fetal hypothalamic and placental production of corticotropin-releasing hormone cause an increase in fetal corticotropin secretion. This results in fetal adrenal production of cortisol, which in turn increases the production of prostaglandins (114).

An alternative hypothesis has recently been suggested, i.e. that the main event actually happens in the choriodecidual interface and that localized inflammation here is actually a sufficient cause of preterm delivery. Choriodecidual inflammatory syndrome implies that this inflammatory process and its effects, rather than a silent infection in the amniotic cavity, are the main issues in the pathogenesis of PTL (128). This hypothesis does not explain the strong association between intra-amniotic cytokines and preterm delivery.

4.2.3 Fetal inflammatory response syndrome

FIRS is thought to represent an acute-phase response resembling that seen in adult patients with septic chock, systemic inflammatory response syndrome (SIRS). This syndrome apparently includes aberrations in multiple organ systems (e.g. hematological, endocrine, connective tissue and cardiovascular) (129). FIRS was launched as a concept based on studies of cytokines from cordocentesis and umbilical cord blood from neonates. Proof has accumulated of fetal capability to initiate an inflammatory response to microorganisms that have gained access to fetal circulation. FIRS was originally defined in 1998 by relating IL-6 levels in cord plasma to severe neonatal morbidity (respiratory distress syndrome, proven or suspected sepsis, intraventricular hemorrhage, PVL, necrotizing enterocolitis, bronchopulmonary dysplasia) (126, 127). An IL-6 fetal plasma level above 11 pg/mL is a major independent risk factor for developing severe neonatal morbidity. These data suggest that the pathophysiological derangements responsible for some neonatal complications may begin before birth rather than merely being attributable to immaturity.

MIAC has been associated with a fetal plasma IL-6 level > 11 pg/mL, suggesting that fetal exposure to bacteria may result in elevation of plasma IL-6 (127). Fetal bacteremia has been demonstrated, by cordocentesis, in 30 % of patients with MIAC and pPROM (130). However, the mode of bacterial entry, into the fetus from the AF is unknown. It has been suggested that the fetal lung represents the route of entry into the fetal compartment after MIAC (43) and results of another study supports this idea (131). Large doses of intra-amniotic endotoxin do not cause fetal death or abortion (132, 133) but much smaller doses administered intramuscularly or into the peritoneal cavity do result in fetal death (133, 134). These findings suggest that intra-amniotic endotoxin does not tend to enter the fetal circulation rapidly. About one week after intra-amniotic endotoxin administration, fetal white blood cell counts increase (132, 135, 136), but only if the contents of the amniotic sac can gain access to the lungs (131). Concentrations of inflammatory cytokines are also increased in the umbilical arterial blood of sheep days after intra-amniotic endotoxin administration (135). Other routes of entry are possible as well, e.g. other mucosa in contact with AF or the placenta.

It has been proposed that onset of labor has a survival value and is initiated when the intrauterine environment is so hostile that it threatens the survival of the fetal-maternal pair. Elevated IL-6 in cord plasma from cordocentesis in patients with pPROM without contractions is associated with impeding the onset of spontaneous preterm delivery (126). This indicates that pro-inflammatory cytokines are involved in the initiation of labor since the elevation occurs before the clinical onset of labor (126). Another pro-inflammatory cytokine, IL-1β, did not have this relation to impeding delivery, which might be due to the time span of the IL-1β elevation and the sample size in the study (137).

Several observations support the notion that an inflammatory process already present in

umbilical cord blood at birth, may mediate short- and long-term complications of prematurity. Elevated umbilical cord IL-6 is associated with development of cerebral white matter lesions and congenital neonatal sepsis (138, 139). Elevated IL-18 in umbilical cord blood was found to be associated with PVL and CP (140).

Studies of SIRS in children and adults (129) may provide valuable information about FIRS. SIRS was initially viewed as a predominantly inflammatory phenomenon with increased levels of so-called pro-inflammatory cytokines in the blood and increased activation of endothelial cells and leukocytes. These pro-inflammatory mediators might be implicated in the multiple organ dysfunction and increased mortality in patients who develop SIRS (141-143). Today SIRS is regarded as having both an inflammatory and an anti-inflammatory component simultaneously. The anti-inflammatory phenomena in SIRS usually include both proteins (e.g. anti-inflammatory cytokines such as IL-10) and cells (monocyte deactivation) (144).The parallel (or, less likely, sequential) anti-inflammatory phenomena appear to explain the increased risk of infection seen in people with this disorder.

On the protein level, the anti-inflammation process often consists of increased plasma levels of cytokines that block the binding of pro-inflammatory cytokines to their cell surface receptors (interleukin-1 receptor antagonist, soluble TNF receptors) or induce the synthesis and release of so-called anti-inflammatory cytokines (IL-10, transforming growth factor-b) (144). On the cellular level, in blood, the anti-inflammation process is expressed as monocyte deactivation by disabling monocytes from processing antigen (characterized most commonly by reduced HLA-DR expression) and disabling polymorphonuclear leukocytes from up-regulating in response to inflammatory stimuli. People who have such defects are at increased risk of death due to overwhelming infection (144). There is one published study regarding the anti-inflammatory phenomena in FIRS (145). The soluble TNF-α receptors are increased in fetal plasma in FIRS (145).

Further observations on FIRS have been made lately. Funisitis and chorionic vasculitis seem to be the histological counterpart of this condition (146, 147). Neonates born to mothers with clinical chorioamnionitis had higher concentrations of umbilical plasma IL-6 than those born to women without clinical signs of infection. A fetal origin of the excess plasma IL-6 was suggested by the arteriovenous gradient observed in the group with chorioamnionitis (148). Also metalloproteinase 8 levels in AF have been related to funisitis, the histological hallmark of FIRS (149).

It has been a common misconception that fetuses and neonates are immunologically naive. However, neonatal human T-cells proliferate in response to an array of antigens, auto-antigens and allergens. Nevertheless, fetuses have a limited ability, both in quantitative and qualitative terms, to initiate immune responses, compared to adults and probably also to neonates (150). Some research findings suggest that preterm neonates are less able to initiate a vigorous inflammatory response than term counterparts (151-153). However, some findings support the view that very preterm neonates can initiate a more vigorous systemic inflammatory response than their term peers (152, 154-157). One study revealed that lower gestational age is associated with an increased ability to secrete proteins that can block progression of the inflammatory response (156). Other reports support the view that very preterm neonates are less able to initiate a vigorous systemic anti-inflammatory response than term neonates (158-160).

The concept of FIRS relates to the idea that plasma content is of fetal origin and mirrors the fetal response to certain stimuli. But data obtained from fetal plasma and umbilical cord blood must be interpreted with caution concerning to what extent the content is totally of fetal origin. Despite the fetal and maternal circulations being physically separated by villous trophoblast layers of the placenta, blood cells move from one circulation to the other. The proportion of neonates with maternal cells in their umbilical cord blood varies considerably (1-100% of neonates) (161-164). In a recent publication, unique maternal DNA

sequences were found in 43 of 57 (75%) cord plasma samples (165).

4.2.4 Sources of cytokines in the amniotic fluid

The suggested main possible sources of cytokines within the AF are decidua, fetal membranes, maternal circulation and possibly the fetus itself. The relative contribution of the maternal and fetal compartments to the overall inflammatory response is unknown (114). There are different cell types within the decidua that can act as sources of different cytokines, e.g. activated resident macrophages located throughout the reproductive tract, or macrophage-like decidual cells released in response to bacterial products (80, 111, 166). The idea of an extra-amniotic source of intra-amniotic cytokines, is contradicted by a study, the findings of which indicate that, AF does not reflect the cytokines produced by the decidua in case with intact and non-inflamed fetal membranes (80). The fetal membranes have been shown to be important producers of cytokines, which are probably released into the AF to some extent. Different layers in the membranes are involved in cytokine production in different ways (Table I). IL-6 and IL-8 are produced by both layers of the membrane (167-170). IL-18 is produced by the chorion membrane but not by the amnion (171). IL-1β is probably produced mostly in the chorion (168).

Maternal circulation can be another source but few reports have studied this issue. Researchers in one study proposed that IL-8 does not cross the placenta in either direction by simple diffusion. Likewise, no evidence of active transfer processes for IL-8 was found (172). This implies that activation and chemotactic effects of chorionic and amniotic IL-8 on neutrophil granulocytes appears to be limited to the fetal compartment (172).

The fetus itself could be a source of the cytokines in the AF. Many different cytokines and chemokines have been identified in umbilical blood from newborns, e.g. IL-6, IL-8 and IL-18. IL-6 has been identified in umbilical cord blood obtained by cordocentesis and in some of those cases with high intra-amniotic levels the blood levels are also increased (127).

4.2.5 Cytokines in amniotic fluid

The traditional view on the onset of parturition in the presence of infection has been that the bacteria or their products (endotoxins) directly stimulate prostaglandin biosynthesis. As knowledge has accumulated and understanding of the concept has changed, it has become obvious that there was an overlap between labor

Table I. The expression of mRNA (detected by in-situ hybridization) and cytokine peptides (detected by immunohistochemistry or by ELISA) in cultured amnio-chorion.

		Amnion membrane		Chorion membrane		Decidua	
		mRNA	protein	mRNA	protein	mRNA	protein
IL-1β	(168)	-	?	+	+		
IL-6	(168)	+	+	+	+		
IL-8	(167, 169)	+	+	+	+		+
IL-18	(171)	-	-	+	+	+	+
MCP-1	(167)		-		+		+
RANTES	(167)		-		+		+
IL-10	(167)		-		+		+

and non-laboring groups regarding the presence of endotoxins; a link was missing (173). After re-consideration the concept emerged that infection (but also other triggering agents) induced stimulation of immune cells to produce cytokines and the subsequent synthesis and release of prostaglandin E2 and prostaglandin F2α, which have been shown to induce cervical ripening, uterine contractions and labor at term (174-177). Cytokines also have been implicated in the physiological mechanisms of term labor and pathophysiological mechanisms of PTL (173). The association between several AF cytokines has been evaluated in relation to different preterm outcome variables (173). It has been proven that several cytokines in AF are increased in cases of MIAC e.g. TNF-α (176, 178), IL-1 (176, 179, 180), IL-6 (176, 179-189), IL-8 (190, 191), IL-18 (192), granulocyte colony stimulating factor (G-CSF) (191), macrophage inflammatory protein-1α (MIP-1α) (193) and growth related protein (GRO-α) (194, 195).

4.2.6 Intra-amniotic inflammation

The gold standard for the diagnosis of infection in clinical medicine is the isolation and identification of the microorganism from body fluid or tissue. However, microbial culture may take days, and results are often not available in time for important clinical decisions. This is in contrast to the diagnosis of inflammation that is more easily arrived at (e.g. blood cell count, cytokine determination). Therefore the prevalence and outcome of IAI has attracted interest. IAI is more common than MIAC and is associated with adverse maternal and neonatal outcome (122). The maternal and neonatal outcome of patients with only IAI does not differ from that of MIAC (122). IAI seems to be twice as common as MIAC (122, 185). These studies have been performed in patients with PTL. IAI in women with pPROM has not been studied to the same extent as in women in PTL, but there is one study that has provided indirect information regarding this issue (196). Without calling the condition IAI, the authors actually defined the condition similarly by drawing a receiver-operation-characteristic (ROC) curve for both cervical fluid and AF IL-6 in relation to MIAC and they found the same cut-off level (0.35 ng/mL) for both fluids. IAI in pPROM is also related to delivery within 7 days and significant neonatal morbidity (196). IAI seems to have different characteristics in women with pPROM (197). IAI without MIAC, may nonetheless be a bacterial reason for the inflammatory reaction. Studies using polymerase chain reaction (PCR) to detect bacteria in the AF have been able to demonstrate that the sensitivity for detecting MIAC is much higher with this technique than with standard procedures (198, 199). Another subgroup of IAI without MIAC is cases with extra-amniotic (between the amniotic and chorionic membranes) detectable bacteria. Previous studies have demonstrated that AF IL-6 may be elevated in these patients (185), but still IAI might nonetheless also have a non-bacterial cause.

4.3 Infectious and inflammatory mechanisms in cerebral palsy

Evidence suggests that 70-80 % of CP cases are due to prenatal factors and that birth asphyxia plays a relatively minor role (76, 200). The inflammatory/cytokine hypothesis, one of the important prenatal hypotheses, proposed initially by Leviton and Adinolfi, according to which a maternal infection may lead to elevated fetal blood and brain cytokine levels, which may result in central nervous damage and subsequent CP (201-203). Leviton also extended the hypothesis, suggesting that cytokines such as TNF-α, produced in response to MIAC, contributed to both PTB and periventricular white matter damage (203). Dammann and Leviton argued further for this hypothesis by finding support for four other sub-hypotheses: 1) Cytokines are present in all three relevant compartments (i.e. uterus, fetal circulation and brain), 2) cytokines are present in or may cross boundaries (placenta and blood brain barrier), 3) cytokines may contribute to the occurrence of intraventricular hemorrhage, 4) cytokines may be involved in the induction of white matter damage (204). An overview is presented in Fig.4.

Fig. 4

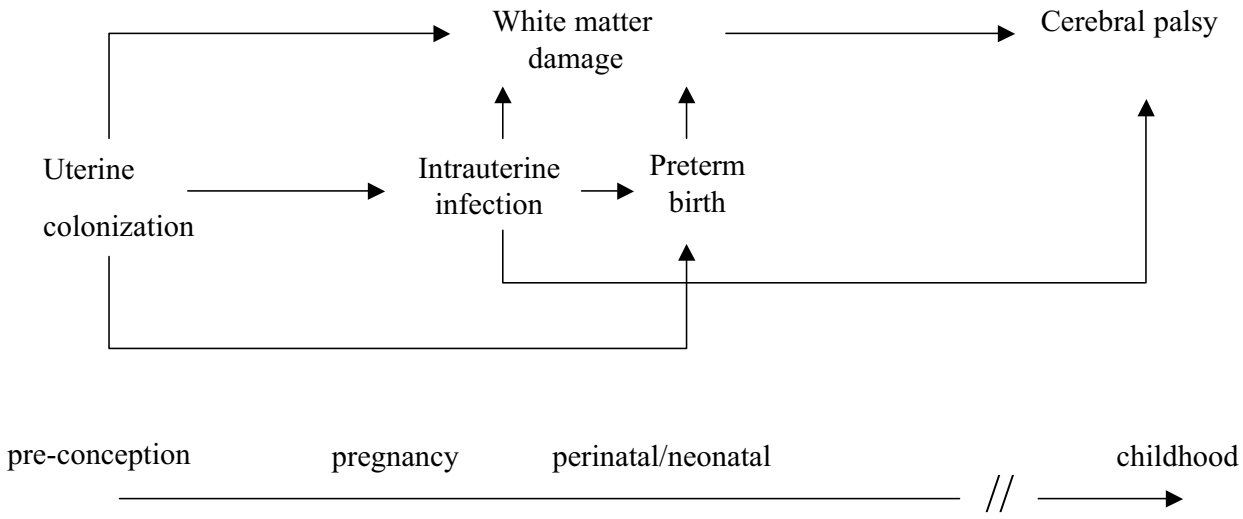

Fig. 4. Possible relationship between pre-conceptional uterine colonization, intra-uterine infection/inflammation, periventricular leukomalacia and cerebral palsy (Modified from Dammann et al Dev Med Child Neurol 1997;39:836-40).

An important study has shown that blood inflammatory cytokine levels were significantly higher in full term infants that developed CP than in controls (205).
In a preterm population, an association between pro-inflammatory cytokines such as IL-6 and IL-8 in AF and CP has been observed (206). IL-6 in AF is also related to intraventricular hemorrhage and elevated IL-6, IL-1β and TNF-α are associated with PVL (196, 207, 208). Intraventricular hemorrhage and PVL are associated with the development of CP.

OBJECTIVES

The main aim of this thesis was to investigate infectious and inflammatory mechanisms related to PTB and CP in a Scandinavian population with a low incidence of PTB.

Specific aims

1. To examine the relationship between the existence of intra-amniotic microorganisms and the levels of IL-6 and IL-8, and their relationship to PTB (delivery within 7 days and delivery at < 34 weeks of gestation, respectively) in a Swedish population of women in PTL (Paper I) and in women with pPROM (Paper II).

2. To investigate (Papers III and IV) how IL-18 and MCP-1 levels in cervical fluid and AF in PTL and in AF in pPROM are affected by microbial invasion of the AF, preterm delivery (< 34 weeks, ≤ 7 days) and IAI. We also compare the intra-amniotic IL-18 level in PTL with that in pPROM, as IL-18-induced apoptosis has been suggested to be important in the rupture of membranes. In addition, the levels of IL-18 and MCP-1 in cervical fluid and AF in women in PTL and in non-laboring women at term were compared.

3. To evaluate (Paper V) maternal, antenatal and intrapartal risk factors for spastic CP in preterm children, especially those factors related to feto-maternal infections. We also examine the risk factors for the subtypes of CP. The very preterm group (<32 weeks) and the moderately preterm group (32-36 weeks) were analyzed separately, as infection is considered to be a more common cause of very PTB.

MATERIAL AND METHODS

1.Patients

1.1 Cohort study of PTL patients (I, part of III and IV)

A total of 66 patients were recruited at the Department of Obstetrics and Gynecology at Sahlgrenska University Hospital in Göteborg, Sweden, between 1996 and 2001. The majority of the patients (n=59) were recruited at Sahlgrenska University Hospital/East (1997-2001) and some patients (n=7) were recruited at Sahlgrenska Hospital in the beginning of the study (1996-1997). Inclusion criteria were: women with a singleton pregnancy in PTL with altered cervical status (see below) and a gestational age of 22 - 34 weeks. PTL was defined as regular uterine contractions (at least 2 uterine contractions/10 minutes during ≥ 30 minutes) in combination with cervical alterations: 1) ≤ 2 cm length + ≥ 1 cm dilatation; 2) ≤ 2 cm length + cervical softening; 3) ≥ 1 cm dilatation + cervical softening; 4) cervical length <30 mm at endovaginal ultrasound. Exclusion criteria were: known uterine abnormalities, fetal malformations, significant vaginal bleeding, imminent delivery or fetal distress.
Gestational age was determined in all patients except three by routine ultrasound in the second trimester (16 to 19 weeks of gestation). Date of last menstrual period was used for gestational length determination when a routine ultrasound was not available. Tocolytic therapy (intravenous terbutaline and/or indomethacin, the latter, if the pregnancy was < 28 weeks of gestation) was administrered according to the department protocol.

1.2 Cohort study of pPROM patients (II, part of III and IV)

Fifty-eight women with singleton pregnancies and pPROM were recruited. The women presented at 2 delivery wards in Göteborg (Sahlgrenska Hospital (1996-1997; n=14) and Sahlgrenska University Hospital/East (1997-2001; n=44)). pPROM was defined as amniorrhexis (diagnosed by a speculum examination confirming pooling of AF in the vagina) before the onset of spontaneous labor. Digital examination was not performed. Women with contractions before rupture of membranes, known uterine abnormalities, fetal malformations, significant vaginal bleeding, imminent delivery or fetal distress were not included.

1.3 Cohort study of IL-18 and MCP-1 in PTL, pPROM and term patients (III and IV)

The PTL groups in these studies consisted of 87 patients in the IL-18 study and 75 patients in the MCP-1 study. These are partly the same patients as in paper I. The pPROM-group consists of the same patients as in paper II. Twenty-eight women at term (≥ 37 weeks) were also included in the IL-18 study and forty-five were included in the MCP-1 study. These women were scheduled for an elective CS with the following indications: psychosocial, breech presentation or two previous CS. None of the term patients had contractions or rupture of membranes prior to surgery.

1.4 Case-control study (V)

The population-based western Swedish CP-project was the case identification source (9, 209-213). When the case-control study started we used the two last four-year cohorts (birth years 1983-1986 and 1987-1990) (211, 212). Since then, another four-year cohort report has been published (9). The register is population-based in the sense that it includes all children born in Sweden and living in the study area on the date when the youngest child in the population is 4 years old. This date was December 31st, 1990 for the birth years 1983-

1986 and December 31st, 1994 for the birth years 1987-1990. The general methodology has been described in detail elsewhere (210, 213). All children with spastic CP were included (n=152), but not children with non-spastic CP (dyskinetic CP without spasticity and simple ataxia) (n=7). Children with an obvious postnatal (> 28 days after delivery) cause of CP were also excluded.

The controls were selected using the Swedish Medical Birth Register (MBR). The MBR is administrated by The National Board of Health and Welfare in Sweden. It contains prospectively collected medical record data from antenatal care, delivery and pediatric examination of neonates. The MBR is population-based, covers 98-99% of deliveries in Sweden and has been validated (214). The controls were matched for gestational age, gender, multiple gestation and delivery ward, all known risk factors for CP (9, 215-217). The births that met the criteria, occurring most closely before and after the case birth were chosen as controls.

2 Methods

2.1 Cohort studies (I, II, III, IV)

An ultrasound-guided transabdominal amniocentesis, at which 30-50 ml of AF was aspirated, was performed under antiseptic conditions within 12 hours after admission. In women at term, AF was retrieved during CS. A catheter was introduced into the amniotic cavity and 50 mL of AF was aspirated prior to opening the membranes. After retrieval, the AF was immediately placed in a refrigerator (+4°C) and was centrifuged within 5 hours of sampling for 10 minutes at 855 g in +4°C. The supernatant was stored at –80°C until analysis. Cervical fluid was obtained with a Cytobrush (Cytobrush Plus GT, Medscan Medical AB, Malmö, Sweden) from the external cervical os of PTL and term patients. The cervical mucus was weighed and kept in a refrigerator (+4°C) until processed within 5 hours. The Cytobrush with the cervical mucus was submerged in 1.0 mL NaCl, shaken for 30 minutes at +4°C, followed by centrifugation at 855 g at +4°C for 10 minutes and stored at –80°C until analysis.

2.1.1 Bacterial analyses

A sample of uncentrifuged AF was immediately transported to the microbiological laboratory for PCR analysis of *Ureaplasma urealyticum* and *Mycoplasma hominis* and for aerobic and anaerobic culture. Microbial invasion was defined as positive PCR and/or growth of any bacteria in the AF, except coagulase negative *Staphylococcus* (CoNS), which was considered to be a skin contamination. Bacterial isolates were characterized biochemically by using the API Rapid ID32STREP, Rapid ID32A, ID32STAPH, ID32E, API ZYM, API Coryne, API50CHL, ID32C according to the manufacturer's instructions (API bioMérieux). These systems identified anaerobes, coryneform bacteria, yeasts, streptococci, *Enterobacteriaceae* and other Gram-negative rods, the *Staphylococcus* and *Micrococcus* genera and the *Lactobacillus* genus and related organisms. PAGE analysis of whole-cell proteins was performed as described by Pot et al (218) . The GelCompar 4.1 software package (Applied Maths) was used for densitometric analysis, normalization and interpretation of protein patterns. Anaerobic bacteria were further analyzed by fatty acid analysis using the MIDI specifications (219). Some of the difficult bacterial isolates were identified by DNA sequencing. The 16S rRNA genes in the isolates were amplified by PCR and directly sequenced by using the *Big dye* terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 310, Applied Biosystems).

2.1.2 Cytokine analyses

IL-6, IL–8, IL-18 and MCP-1 in AF and cervical fluid were analyzed by enzyme-linked immunosorbent assay (ELISA). Commercially available paired antibodies and standards (from R&D Systems, Minneapolis, Minn, USA) were used in a sandwich ELISA setup. The samples were diluted 1:5, 1:20 and 1:100 and run in duplicates, except the MCP-1 samples which

were diluted 1:2, 1:3 and 1:6. The inter-assay variation was calculated at < 10% for IL-6 and IL-8 and at <25% for IL-18, based on analyses of several samples on three separate occasions. The coefficient of variation (29-76%) were higher for low values (≤ 700 pg/mL for IL-6 and IL-8 and ≤ 400 pg/mL for IL-18) The ELISA detection limit was 30 pg/mL for IL-6, IL-8 and IL-18, but the lower limit of detection in these studies were 150 pg/mL because the samples were run at a 1:5 dilution.

Comments:
Cytokines are considered to be stable at –80°C but a recent study showed that a partial degradation occurs even under these circumstances (220, 221). IL-6 seems to be most sensitive to higher temperatures (220, 221). No studies are available concerning the handling of AF before freezing but information on handling of blood samples indicates that AF should promptly be placed in a refrigerator (+4°C) and be centrifuged within 6 hours (222, 223). At any rate, the most important recommendation is that all samples be handled and frozen under identical conditions (222).

2.2 Case-control study (V)

This was designed as a case-control study, focusing on the infectious and inflammatory mechanisms that can be related to the development of spastic CP.

2.2.1 Variables studied

The primary variables in focus were those related to maternal infectious morbidity during pregnancy and delivery: clinical chorioamnionitis, histological chorioamnionitis, pyelonephritis, fever before/during/after delivery, postpartum endometritis, use of antibiotics during pregnancy. The secondary variables studied were complications related to pregnancy and delivery: second and third trimester bleeding, abruptio placentae, umbilical cord complications. Several descriptive variables were also collected. The reason to preterm delivery were classified: spontaneous, indicated – bleeding, indicated – hypertensive disease, indicated – pathological non-stress test, indicated – severe abdominal pain, indicated – suspected or clinical chorioamnionitis or other severe infections. A total of 154 variables categorized as maternal (40), antenatal (63), intrapartal or immediately postpartal (51) were investigated in this study. A database was constructed and tested in a pilot study by scrutinizing 48 medical records at Sahlgrenska University Hospital/Sahlgrenska. After analyzing the database function modifications were made and the records were re-scrutinized. One investigator, unaware of the pediatric outcome, examined all records.

3 Ethical considerations

Approval for the studies was obtained from the Ethics Committee at Göteborg University.

3.1 Cohort studies (I, II, III, IV)

In studies I-IV, the women gave informed consent before enrollment. The main ethical consideration in these studies was if the amniocentesis could in any way affect the pregnancy outcome. A review of the literature pertaining to this issue is presented in the Discussion section.

3.2 Case-control study (V)

A database was constructed using the collected data available only to the datacompiler. This database was protected by a user ID and a personalized password. The database was depersonalized before the basic analysis. The majority of the records were scrutinized at the archives; a minority (from small hospitals outside the region) were requested and sent by mail.

4 Statistics

4.1 Cohort studies (I, II, III, IV)

Continuous variables were analyzed with the Mann-Whitney U test and proportions were compared using Fisher's exact test. Spearman's rank correlations test was used for analysis of

correlation between continuous variables. We selected the best cut-off level for cytokines and chemokines, relating to different outcome variables of interest, from a receiver-operator characteristic (ROC) curve, in order to minimize the sum of type I and type II errors. Using this level to define the risk group, we compared time to delivery through a survival analysis. The risk ratio was calculated by using relative risk (RR) and 95% confidence interval (CI). A p-value < 0.05 was considered statistically significant, as was a CI not including 1.
Calculations were made using the computer programs StatView 5.01 (SAS Institute Inc, Cary North Carolina, USA) and InStat 2.01 (Graph Pad Software, San Diego California, USA).

Comments:
As these are prospective cohort studies RR is appropriate when calculating risk ratio.

4.2 Case-control study (IV)

Univariate logistic regression was used to estimate the odds ratio (OR) with a 95% CI for correlation between one factor and the outcome. Statistical significance was considered to exist if the 95 % CI did not include 1. Proportions were compared using Fisher's exact test. Wilcoxon's rank-sum test was used to test continuous variables for differences between two groups. A p-value < 0.05 was considered to be statistically significant.
Calculations were made using SAS (SAS Institute Inc, Cary North Carolina, USA) and InStat 2.01 (Graph Pad Software, San Diego, California, USA).

Comments:
In a prospective study, groups of subjects with different characteristics are followed up to explore whether an outcome of interest occurs and whether it is possible to calculate a risk ratio for that outcome. This study is a case-control study in which the subjects are selected according to outcome. This means that we cannot estimate the risk ratio by comparing the risk of the outcome in those with and without the characteristic. It is possible to obtain any risk ratio by varying the number of studied cases and controls. Therefore, in order to estimate a risk ratio in a case-control study, a comparison within the characteristic groups and an OR is calculated (224, 225).

RESULTS AND COMMENTS

1. Cohort studies (I, II, III, IV)

1.1 General description

1.1.1 PTL

Sixty-six patients underwent amniocentesis. In five women, no AF or an inadequate amount was obtained and no analyses could be performed. In one patient, the AF was only analyzed with PCR for *Ureaplasma urealyticum* and *Mycoplasma hominis*, leaving 60 patients with a sufficient amount of AF to allow the complete set of analyses. In the PTL group, 44.3% (27/61) delivered before 34 weeks and 63.9% (39/61) before 37 weeks of gestation (Fig. 5). Twelve patients were given antibiotics before or during delivery. Two patients were treated with antibiotics as prophylaxis, and 3 patients were treated because of bacteria in the AF. No patient was taking antibiotics at amniocentesis. Fourteen patients were given corticosteroids before amniocentesis, thirty-six patients after amniocentesis and eleven were not receiving any corticosteroids at all. In the PTL group, 18 % (11/61) of the patients were of non-Swedish origin according to their antenatal records: (Iran (n=3), Bolivia (n=2), Chile (n=1), China (n=1), Hungary (n=1), Lebanon (n=1), Somalia (n=1) and Turkey (n=1)). Labor did not begin spontaneously in five patients. Three of the inductions were at term (week 38, 40, 40) and two were preterm, the letter because of severe preeclampsia (week 32) and painful contractions (week 35), respectively. None of the inductions were undertaken within 7 days after amniocentesis.

1.1.2 pPROM

Fifty-eight patients with pPROM, before uterine contractions started, underwent amniocentesis. In five patients, no AF or an inadequate amount was obtained and no analyses could be performed. In another six patients, the AF was only analyzed for bacteria with PCR and culture, leaving 47 patients with a sufficient amount of AF to allow a complete set of analyses. In the pPROM-group, 70.2%

Fig.5

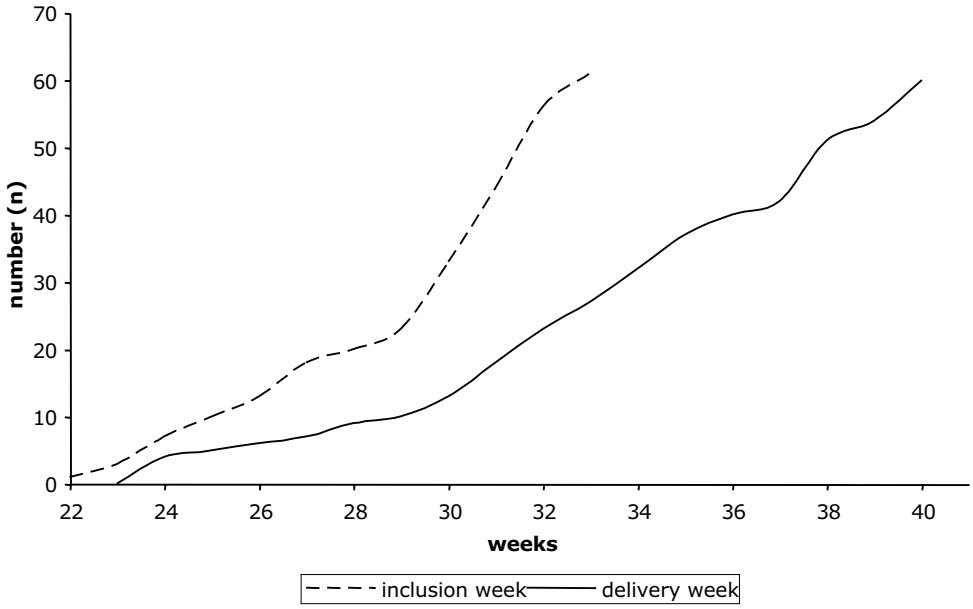

Fig. 5. Gestational age at inclusion and birth in the preterm labor group.

(33/47) delivered before 34 weeks and 89.4% (42/47) before 37 weeks of gestation (Fig. 6). Contractions began prior to amniocentesis in seven cases of pPROM. Thirteen patients were given antibiotics before or during delivery, two of which because of bacteria in the AF. No patient was taking antibiotics at amniocentesis. All but two patients were treated with corticosteroids, eleven before amniocentesis. In the pPROM-group, 23 % (11/47) were of non-Swedish origin according to their antenatal records: (Iraq (n=2), Turkey (n=2), Bosnia-Herzegovina (n=1), El Salvador (n=1), Iran (n=1), Poland (n=1), Romania (n=1), Somalia (n=1) and Yugoslavia (n=1)). Delivery did not begin spontaneously in twelve patients. Labor was induced in eight patients (week 33, 33, 33, 34, 34, 34, 38, 38) and 4 had CS before onset of delivery (week 29, 32, 34, 40). Indication for the preterm inductions were prolonged pPROM, with fever as additional indication in one case, vaginal bleeding in another and in three cases that the gestational age exceeded week 34. The indications for the three preterm CS were fever (week 29), suspected abruptio placentae (week 32) and transverse lie (week 34). Three of the inductions and none of the CS occurred within 7 days after amniocentesis. The indications for the three inductions were: passed 34 weeks (week 34, 34) and vaginal bleeding (week 33). There were three inductions before 34 weeks, the indications for which were low platelets (week 33), fever (week 33) and vaginal bleeding (week 33), respectively .

1.2 Microbial invasion of the amniotic cavity

MIAC is examined in papers I and II and the relevant bacteria are presented in Table 2 in both papers. The prevalence of MIAC was 16% in women in PTL and 25% in women with pPROM. We found bacteria that have been identified in AF before, except for one case of *Sneathia sanguinegens*. CoNS was considered to be contamination.

Comments:
We have identified 36 amniocentesis sampling studies, all non-Scandinavian, in which the prevalence of MIAC in women in PTL was reported (108, 110, 121, 127, 176, 181, 182, 184, 186, 199, 226-250). It is difficult to compare these studies, because of differences in study design, inclusion criteria, bacterial identification technique and frequency of

Fig.6

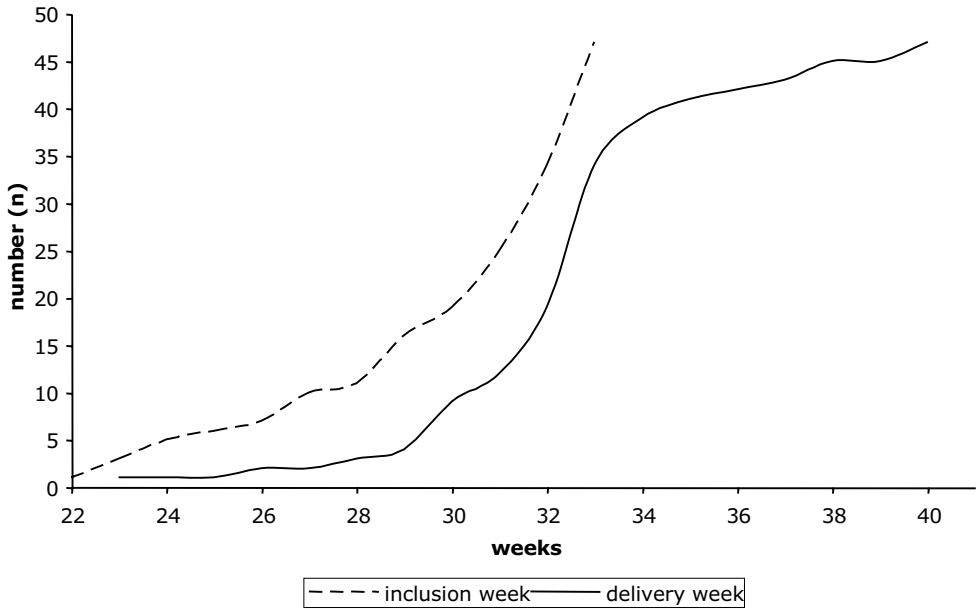

Fig. 6. Gestational age at inclusion and birth of the women in the preterm pre-labor rupture of membrane

Fig. 7

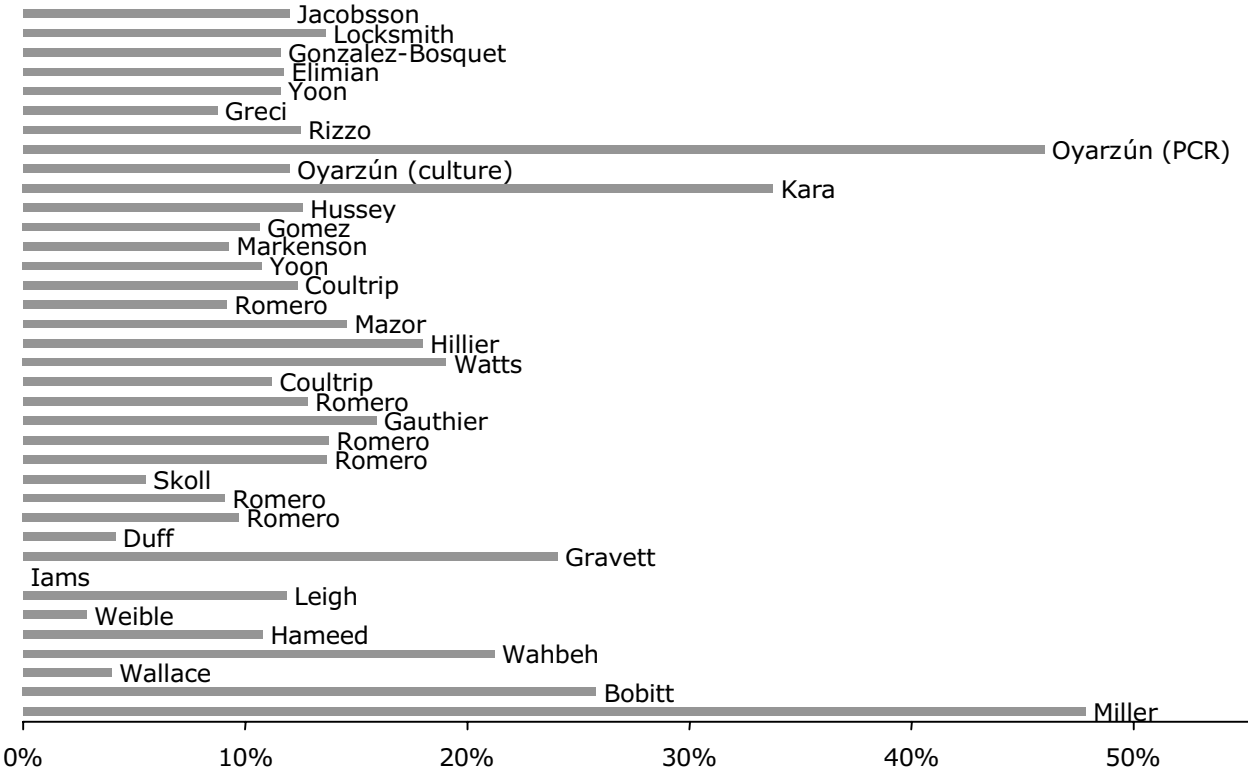

Fig. 7. The prevalence of microbial invasion of the amniotic cavity in women in preterm labor. Modified from Goncalves LF, Chaiworapongsa T, Romero R. Intrauterine infection and prematurity. Ment Retard Dev Disabil Res Rev 2002;8(1):3-13.

amniocentesis in eligible patients. However, according to these studies, 13% (415/3108) of successful amniocenteses were culture or PCR positive (Fig. 7).

With a slightly different spelling one study have previously reported a case of *Actinomyces odontolyticus* in AF (228). CoNS has both been reported as a contamination, as we have done, and as an infection causing microorganism in the literature (122, 184, 199, 226, 227, 232, 239, 240).

The prevalence of MIAC was 25 % in the pPROM study. We have identified 18 studies (127, 130, 198, 229, 248, 251-262) that report the prevalence of MIAC in women in pPROM (all non-Scandinavian, two European (England, Italy)). According to these studies, culture or PCR from 34 % (509/1512) of successful amniocentesis have had a positive bacterial culture (Fig. 8). At any rate, with the limitations mentioned above, our results are comparable to those of others. The use of PCR to identify the Ureaplasmas might have yielded a comparatively higher level of MIAC. The use of culture for detection of the Ureaplasmas might give false negative results. Among the 43 patients with a positive PCR for *Ureaplasma urealyticum*, AF culture was negative in 42% (18/43) (198). CoNS has both been reported as a contamination, as we have done, and as an infection causing microorganism in the literature (196, 198, 254).

In international studies, more than one microorganism is isolated from AF in fifty percent of patients with MIAC (107). In these two studies, we found only two patients (2/23) with two types of bacteria in the AF. The fact that *Ureaplasma urealyticum* and certain anaerobes predominate in our patients with pPROM must affect the choice of antibiotic if amniocentesis is not performed in the clinical situation (is most often the case in Sweden).

1.3 IL-6

ELISA for IL-6 in AF was performed in 61 patients in the PTL group and in 47 patients in the pPROM group. IL-6 was detectable in the

Fig. 8

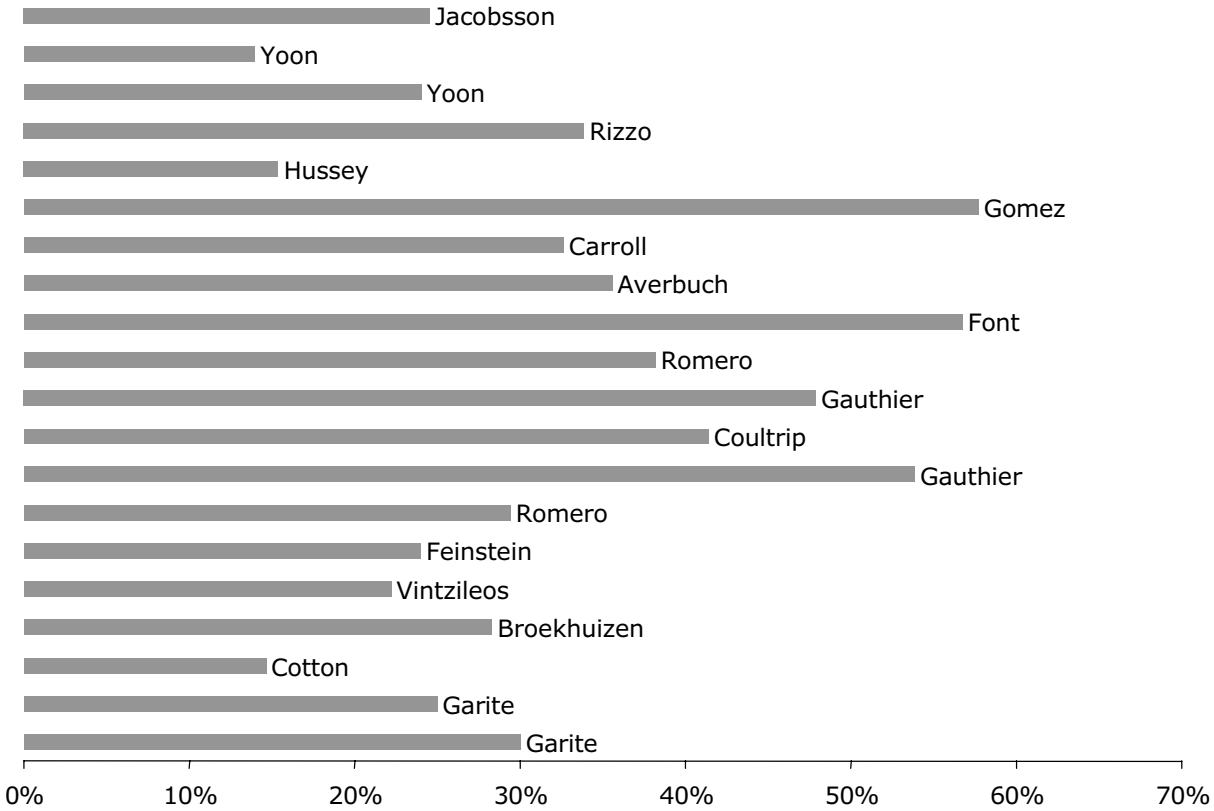

Fig. 8. The prevalence of microbial invasion of the amniotic cavity in women with preterm pre-labor rupture of membranes. Modified from Goncalves LF, Chaiworapongsa T, Romero R. Intrauterine infection and prematurity. Ment Retard Dev Disabil Res Rev 2002;8(1):3-13.

amniotic samples in 80% (49/61) of the women in PTL and in 79% (37/47) of the women with pPROM.

In the PTL group, AF IL-6 was related to MIAC, delivery within 7 days and delivery < 34 weeks. In the pPROM group, AF IL-6 was related to MIAC and delivery within 7 days but not to delivery < 34 weeks. Three patients in the pPROM group underwent induction of labor within 7 days from amniocentesis, and the cases might therefore be misclassified as spontaneous delivery within 7 days. Exclusion of these patients did not alter results. There were also 3 inductions and 2 CS before the onset of contractions in the pPROM group, so they could be misclassified as spontaneous delivery < 34 weeks. Exclusion of these patients did not alter the results either.

Comments:

IL-6 in AF of patients in PTL or with pPROM has well-documented relations to MIAC (176, 179-189), histological chorioamnionitis (176, 182, 183, 189, 263-266), PTB (176, 182-184, 186) and significant neonatal morbidity and mortality (183, 266). Our results concur with those in the literature. This indicates that IL-6 follows the same pattern in a Scandinavian population with its low incidence of PTB as in populations with higher incidences of PTB. We found that nearly 80% of women in PTL leading to PTB had elevated IL-6 levels (≥1.5 ng/mL), compared to 11% of those that did not deliver preterm. Our data support the concept that intra-amniotic IL-6 is one of the best predictors of PTB (186).

1.4 IL-8

ELISA for IL-8 in AF was performed in 61 patients in the PTL group and in 47 patients in the pPROM group. IL-8 was detectable in the amniotic samples in 64% (39/61) of the women in PTL and in 66% (31/47) of women with pPROM.

In the PTL group, AF IL-8 was related to MIAC, delivery within 7 days and delivery < 34 weeks. In the pPROM group, AF IL-8 was

related to MIAC and delivery < 34 weeks but not to delivery within 7 days. Three patients in the pPROM group underwent an induction of labor within 7 days and these cases might be misclassified as spontaneous delivery within 7 days. If these patients are excluded, a significant relationship was also found between IL-8 and delivery within 7 days. The cut-off level for IL-8 in defining IAI was not altered by these exclusions. There were also 3 inductions and 2 CS before the onset of contractions in the pPROM group, which might be misclassified as spontaneous delivery < 34 weeks. Exclusion of these patients did not alter results.

Comments:
IL-8 in AF in patients in PTL or with pPROM is related to MIAC (190, 191, 195), PTB (267), histological chorioamnionitis (267) and bronchopulmonary dysplasia (268). We found that the alpha chemokine IL-8, which induces neutrophil attractant and activating responses, was highly expressed in the AF both in the PTL and pPROM cases. Our results concur with those in the literature. Previous studies have shown that pPROM is associated with a cytokine response including expression of IL-1, IL-6, IL-8 and TNF-α (123).

1.5 Intra-amniotic inflammation

IAI seems to be twice as common as MIAC in women in PTL (122, 185). In our study, the frequency of IAI in women in PTL actually approached three times the MIAC rate, which might be partly due to the fact that we used a combination of IL-6 and IL-8 levels to define the condition. An inflammatory response, defined in our study as IL-6 ≥ 1.5ng/mL and/or IL-8 ≥ 1.3 ng/mL, was found in 45.9 % (28/61) of the patients. Other researchers have found the best cut-off level for IL-6 in AF to be 2.6 ng/mL (122) or 2.0 ng/mL (109). Our cut-off levels for IL-6 in defining IAI in both the PTL and the pPROM groups, is in accordance with other studies, even if the definitions differ (122, 196). Other investigators have made their calculations in relation to different outcome variables (microbes present in the AF (122) and IL-6 elevation over the 70th percentile (109), respectively).
Only half of the patients with an inflammatory response, as defined above, in the PTL group and only 37 % (10/27) of the patients with pPROM, defined as IL-6 ≥ 0.80 ng/mL or IL-8 ≥ 0.42 ng/mL, had detectable microorganism levels in the AF despite PCR being used for detection of *Ureaplasma urealyticum* and *Mycoplasma hominis* (198). This concurs with previous reports (122), and probably indicates that some microorganisms cannot be detected with the techniques used or that PTB can result from non-infectious inflammation.

1.6 IL-18

The PTL group in this study consisted of 87 patients. ELISA for IL-18 was performed in cervical fluid (n=87) and in AF (n=59). In the patients with accessible AF, IL-6 and IL-8 levels were available from the previous studies. The pPROM group included 47 patients with available IL-18, IL-6 and IL-8 samples from AF. IL-18 was detectable in the cervix in 60% (52/87) of women with PTL and in 11% (3/28) of women at term. IL-18 was detectable in the amniotic samples in 73% (43/59) of the women in PTL and in 70% (33/47) of the women with pPROM. IL-18 was detectable in AF in 11% (3/28) of the term cases.
There were higher levels of IL-18 in cervical fluid of women in PTL, compared with non-laboring women at term. The patients with IAI were excluded in order to facilitate assessment of the influence of gestational age on the IL-18. The levels of IL-18 in cervical fluid in women in PTL were still higher than in non-laboring women at term (PTL: IL-18 0.41 ng/mL; term: median 0.15 ng/mL; $p<0.001$). No correlation was seen between gestational age at sampling and IL-18 levels in cervical fluid.
In AF there were also higher levels of IL-18 of women in PTL, compared with non-laboring women at term. The patients with IAI were excluded in order to facilitate assessment of the influence of gestational age on the IL-18 level in AF. The IL-18 levels in the AF of women in PTL were still higher than compared with non-

laboring women at term (median IL-18 0.38 ng/mL; median IL-18 0.15 ng/mL; p<0.001). No correlation was seen between gestational age at sampling and IL-18 levels in AF.
IL-18 levels were studied in AF in women in PTL; MIAC, delivered within 7 days after amniocentesis or before 34 completed weeks as well as IAI had significantly increased levels in this group. In women with pPROM, these associations were not significant. No difference was found between IL-18 levels in the PTL and pPROM groups.

Comments:
IL-18 in cervical fluid and AF is of great interest when it comes to the outcome of PTB, especially since IL-18 has been proven to affect long-term neurological outcome of preterm children (140). No study concerning IL-18 in cervical fluid is available, but two previous studies have presented data regarding IL-18 in AF (171, 192). Both Pacora et al and Menon et al were able to detect IL-18 in all their samples. We detected IL-18 in 71.8 % of our samples. We found IL-18 levels similar to those reported by Menon et al and 10-100 times higher than those found by Pacora et al (Table 2). Menon's and our study were performed with antibody from the same provider (R&D Systems), but Pacora used an antibody from another provider. The IL-18 antibody from R&D System is directed towards an epitope on the active part of IL-18.
IL-18 levels in both cervical fluid and AF seems to decrease with increasing gestational age. This relationship is consistent if patients with IAI are excluded. This is new information as is the fact that IL-18 in cervical fluid is related neither to MIAC, PTB nor IAI. This result contradicts results obtained by Pacora et al who found a significant increase with gestational age.
MIAC was associated with increased AF IL-18 in PTL but not in pPROM cases. Bacteria previously identified in AF, e.g. *Corynebacteria, Pseudomonas aeruginosa, Haemophilus influenzae* and *Chlamydia tracomatis*, have been shown to provoke an IL-18 response in vivo in other compartments of the human body (90). It is also important to note that the amniotic concentrations of IL-18 in patients with pPROM and MIAC were not higher than in cases of pPROM with sterile AF. This does not apply to IL-6 and IL-8, levels of which were higher in patients with microbial invasion both in the PTL and pPROM groups. These data suggest that IL-18 plays different roles in PTL and pPROM. A similar dichotomy between PTL and pPROM has previously been observed when it comes to TNF-α and IL-1α (180, 192).

Table II. IL-18 levels detected in amniotic fluid in the three published studies on this subject . Data are given in ng/mL.

	Pacora et al (192)	**Menon et al (171)**	**Jacobsson et al (III)**
PTL	~ 0.016 (0.004-0.28)	0.92 (0.1-2.7)	0.59 (0.15 – 6.7)
pPROM	~ 0.014 (0.004-0.34)	1.86 (0.3 – 15.2)	0.35 (0.15 – 3.4)
term	~ 0.019 (0.006-0.14)	0.16 (0.1-1.2)	0.15 (0.15 – 0.79)

(median and range)

Women in PTL who gave birth within 7 days (or ≤34 weeks) had higher levels of IL-18 in the AF than those who delivered after 7 days (or ≥34 weeks). This relationship between IL-18 and interval to delivery was not found by Pacora et al (192), a discrepancy that might be due to differing gestational age (<34 weeks vs. <37 weeks, respectively) (12), since inflammation may be more important at low gestational ages (122). In women with pPROM, the opposite relationship was found, i.e. high IL-18 levels were associated with longer interval to delivery; this should, however, be interpreted with caution since few patients (8/47) had high levels ≥ 1.0 ng/mL.

1.8 MCP-1

There were higher levels of MCP-1 in cervical fluid and AF in women in PTL compared with non-laboring women at term.

There were higher levels of MCP-1 in the cervical mucus in women in PTL who gave birth within 7 days than in those with a longer sample-delivery interval. Women in PTL who gave birth within 7 days after amniocentesis had significantly higher levels of MCP-1 in AF than those who gave birth after 7 days. In the pPROM group, the levels of MCP-1 in patients that delivered in ≤ 7 days did not differ from those with an interval > 7 days. Three patients were allocated to the spontaneously delivered group despite labor being induced. However, the results were not affected by exclusion of these cases. We performed a log rank survival analysis of the best MCP-1 cut-off level in AF (≥ 2.0 ng/mL) for delivery within seven days of women in pPROM, with censor of patients with induction of labor or CS before onset of delivery (n=12). A significant difference between the two groups regarding long interval to delivery was found, in contrast to short interval to delivery (Fig. 3 in article IV).

In AF there were higher levels of MCP-1 in PTL women who gave birth before 34 weeks of gestation than in those who delivered at ≥ 34 weeks of gestation). In women with pPROM, there was no association between AF MCP-1 and delivery < 34 weeks. There were 3 inductions and 2 CS before the onset of labor; these cases might be misclassified as spontaneous delivery < 34 weeks. Exclusion of these patients did not affect the results.

MCP-1 levels were higher in cervical mucus in women in PTL with MIAC than in those with sterile AF. In AF, the levels of MCP-1 were not significantly higher in women in PTL with MIAC than in those with sterile AF. In women with pPROM, the levels of MCP-1 in AF were higher in women with than in those without MIAC.

There were also higher levels of MCP-1 in the cervical mucus in women in PTL who had IAI. MCP-1 in AF correlated to general IAI both in women in PTL and in women with pPROM.

MCP-1 in cervical fluid predicted PTB and MIAC with a high negative but low positive predictive value. The AF levels of MCP-1 also predicted MIAC and IAI in patients with pPROM.

A correlation was seen between MCP-1 in cervical fluid and AF ($r=0.35$ and $p=0.02$). There was a high degree of correlation between MCP-1, IL-8 and IL-6 in AF in both the PTL (IL-8 $r=0.74$ and $p<0.001$; IL-6 $r=0.70$, $p<0.001$) and the pPROM groups (IL-8 $r=0.64$, $p<0.001$; IL-6 $r=0.41$, $p=0.005$).

Comments:

MCP-1 is the prototypic β-chemokine. It recruits and activates monocytes and macrophages. The macrophages in the decidua and membranes are presumed to play a critical role in the preterm labor syndrome. It is thus most interesting that MCP-1 seems to be associated with MIAC and IAI both at the cervical and amniotic level.

Cervical ripening has been described as an inflammatory process (269). The infiltration of white blood cells (both neutrophils and macrophages) into the cervix is known to occur in women at term (270). Neutrophils are responsible for most of the connective tissue changes that take place during cervical ripening, but macrophages are also involved, although their specific role is still unclear (271). There is a tenfold increase in macrophages in cervical tissue from early to late pregnancy (270) and another increase in number during the final cervical ripening at birth (272).

Chemokines have been proposed to be involved in cervical ripening via their chemoattractant and activating effects on neutrophils and monocytes (273). Although functional redundancy exists with other chemokines in vitro, MCP-1 is exclusively responsible for mononuclear cell infiltration in several inflammatory animal models in vivo (104). It is thus possible that MCP-1 is also involved in the cervical ripening process via activation and recruitment of monocytes and macrophages.
Monocytes and macrophages play a critical role in the immune response, both by being potent producers of pro-inflammatory cytokines, matrix metalloproteinases and prostaglandins and by processing and presenting antigens to T-cells for recognition. In that sense, they regulate both the innate and the specific immune systems. It has been proposed that the β-chemokines are likely to induce chemotaxis of monocytes and macrophages into the amniotic cavity and activate them (274). β-chemokines may be involved in the host defense against MIAC and in IAI. It was not surprising that there was a significant association between MCP-1 and MIAC in women with pPROM, which is in accordance with preliminary data reported by Esplin et al. (275). MCP-1 is thus the third β-chemokine (MIP-1α, RANTES) that has been shown to increase in response to MIAC (193, 274).
The poor correlation between the levels of MCP-1 in cervical fluid and AF in our study may indicate different production sites. MCP-1 is known to be produced by term placenta, decidua and chorion and, to a lesser extent, by the amniotic epithelium (167). It has been shown that cell cultures of cervical fibroblasts can produce MCP-1 (276) and immunohistologically stained cervical biopsies from pregnant women are positive for MCP-1 (277). MCP-1 is probably produced locally by cervical tissue. However, the decidua and membranes can be an alternative source of MCP-1 as the inflammatory process leads to disruption of the chorio-decidual interface, leading to its possible release into the cervical fluid (278). Kent et al found that AF does not reflect the cytokines produced by the decidua in cases with intact and non-inflamed fetal membranes (279). Thus, and since the amniotic epithelium appears to be a minor producer of MCP-1, the fetus itself may be a source of MCP-1. There are no reports of MCP-1 levels

Table III. Distribution of 148 preterm spastic CP cases according to type and gestational age

Type of CP	**< 32 w**		**≥ 32 w**		**Total**	
	n	**%**	**n**	**%**	**n**	**%**
Spastic diplegia	60	66%	28	49%	88	59.5%
Spastic hemiplegia	11	12%	20	35%	31	21%
Ataxic-spastic diplegia	11	12%	4	7%	15	10%
Spastic tetraplegia	9	10%	5	9%	14	9.5%
Total	91		57		148	

w = weeks of gestation

Table IV. Clinical chorioamnionitis and CP.
Modified from Wu Y W. Systematic review of chorioamnionitis and cerebral palsy. Ment Retard Dev Disabil Res Rev 2002;8(1):25-9 and Wu YW, Colford JM. Chorioamnionitis as a risk factor for cerebral palsy: A meta-analysis. JAMA 2000;284(11):1417-24.

	Study design	Risk ratio
Allan et al	cohort	2.6 (1.2-5.9)
Gray et al	cohort	1.2 (0.1-10.2)
Kim et al	cohort	2.3 (0.5-10.7)
Neson and Ellenberg	cohort	3.4 (1.6-7.4)
Yoon et al	cohort	2.5 (0.3-26.0)
Cooke	case-control	3.3 (1.2-8.9)
Grether et al	case-control	0.95 (0.5-2.0)
Grether and Nelson	case-control	1.2 (0.8-1.8)
Jacobsson et al	case-control	1.8 (0.9-3.6)
O'Shea et al	case-control	2.5 (0.9-6.9)
Wilson-Costello et al	case-control	2.1 (0.7-6.1)
Summary		1.9 (1.4-2.5)

in umbilical cord blood but MCP-1 is present in dried neonatal blood spots obtained from 3-day-old neonates (205) and the respiratory tract of the preterm baby is a known producer of MCP-1 (280). Further studies are required to determine the source of MCP-1 in cervical fluid and AF.

MCP-1 is also part of the IAI in both PTL and pPROM. However, in contrast to the findings in the PTL group, we found no correlation between MCP-1 in AF and delivery within 7 days or before 34 weeks of gestation in patients with pPROM. Similar results were recently pertaining to IL-18 (281), supporting the idea that IAI has different characteristics and outcome in pPROM and PTL, respectively (282).

The levels of MCP-1 in cervical fluid and AF were higher in women in PTL than in non-laboring women at term. MCP-1 in AF probably reflects the feto-placental inflammatory response, whereas cervical MCP-1 reflects both inflammation related to cervical ripening and the generalized feto-placental inflammatory response.

2. Case-control study (V)

All medical records including all documents required for the study were available. Two cases and their controls were excluded because the correct gestational age was ≥ 259 days. Two cases were excluded together with their controls due to unequivocal perinatal cytomegalovirus infection. One hundred and forty-eight cases and 296 controls were included in the final analysis. The distribution of sub-types of CP and gestational age is described in Table III. The MBR was used to match the controls and cases. In certain cases, in which the MBR did not contain correct information about the

Table V. Histological chorioamnionitis and CP.
Modified from Wu YW. Systematic review of chorioamnionitis and cerebral palsy. Ment Retard Dev Disabil Res Rev 2002;8(1):25-9 and Wu YW, Colford JM. Chorioamnionitis as a risk factor for cerebral palsy: A meta-analysis. JAMA 2000;284(11):1417-24..

	Study design	Risk ratio
Yoon et al	cohort	7.6 (0.9-66.6)
Grether and Nelson	case-control	1.2 (0.7-2.0)
Jacobsson et al	case-control	3.9 (1.2-14.0)
O'Shea et al	case-control	1.3 (0.3-5.1)
Redline et al	case-control	1.2 (0.5-2.8)
Summary		1.5 (1.5-2.5)

matching variables, the next patient with correct matching variables was chosen.
The matching was complete for gestational age, gender, single and multiple pregnancy. Matching for delivery ward was complete in only 84.5%, as controls could not always be recruited from small units. In these cases, controls were recruited from another unit of similar level and size.

2.1 Infection related variables

Clinical chorioamnionitis/pyelonephritis (OR 2.02 (1.02-3.99)), histological chorioamnionitis (OR 3.61 (1.16-12.1)) and long duration of pPROM were associated with an increased risk of CP, whereas treatment with anti-inflammatory corticosteroids was associated with a significantly lower risk (OR 0.42 (0.20-0.90)). Fever before onset of delivery was of borderline significance (OR 2.30 (0.99-5.17)). Fever during delivery and postpartum endometritis were not associated with CP (Table 3 in paper V).
In the sub-group analysis, infectious indicators such as administered antibiotics during pregnancy (OR 2.39 (1.12—5.09)) and fever before the onset of delivery (OR 3.10 (1.14-8.44)), but not histological chorioamnionitis (OR 2.80 (0.76-10.3) were associated with diplegic CP. Fever before onset of delivery was also an antecedent of CP in the very preterm (<32 weeks) (Table 4 in paper V).

Comments:
Clinical chorioamnionitis and pyelonephritis were grouped together since severe infections outside the genital tract have also been shown to be associated with perinatal brain damage (283). There were no cases of appendicitis or any other abdominal cavity infection in the study, but there were two cases of severe pyelonephritis; both those children developed CP. Appendicitis and pyelonephritis are rare conditions in pregnancy and calculation, when testing the infectious/inflammation hypothesis requires that they be grouped together in compiled diagnosis, even in a large case-control study as ours. Clinical chorioamnionitis may expose the fetus to infectious material more directly than pyelonephritis. The incidence of clinical chorioamnionitis in the case group was 10.8 % (16/148) and 6.4 % (19/296) in the control group (OR 1.77 (0.88-3.55)). The OR in this study, whether pyelonephritis cases are excluded or not, is close to the OR that Wu et al found in their meta-analysis (OR 1.9 (1.5-2.5)) (284, 285) (Table IV).

The histological chorioamnionitis factor might be burdened with selection bias since only 73 of 444 placentas were analyzed, but the proportion of analyzed placentas was the same in both groups (18.9% cases, 15.2% controls; p=0.69). Since so few placentas were analyzed in the different sub-groups there might be power problems in the analysis. According to two recent meta-analyses, eight studies (206, 207, 284, 286-291) examine the relationship between histological chorioamnionitis and CP (284, 285). These studies were found to be somewhat heterogeneous in design; it was only in our study and one other that a significant association was found (Table V). In another population-based control study of the etiology of CP from the USA, they examined factors that influence whether placentas were submitted for pathologic examination (292). Within birth weight groups the main determinant of placental submission was surgical delivery. Maternal and infant conditions had little influence on the submission rate. Whether this is applicable to Swedish conditions is unknown.

There might also be a selection bias towards less infectious cases, when it comes to corticosteroids since corticosteroids were not a part of the clinical routine during the study period due to concerns about increasing the risk of severe infection. If all patients at less than 34 weeks of gestation would have been considered eligible for corticosteroid treatment, then only 13.9 % (47/345) were actually treated.

2.2 Other variables

Hypertensive disease (OR 0.54 (0.28-0.99)), cervical insufficiency (OR 0.85 (0.70-0.98) and iatrogenic reason to delivery (OR 0.54 (0.28-0.99)) were all associated with a lower occurrence of CP, whereas no difference was found between cases and controls with regard to spontaneous onset of labor (pPROM and PTL) (Table 4 in paper V). Decreased viability at birth (low Apgar scores at 1, 5 and 10 min) was associated with higher occurrence of CP. The OR was higher the later after birth the child had a low Apgar score (Table 5 in paper V).

No case of hypothyroidism and only one case of thyrotoxicosis (control) were diagnosed in the study.

Risk factors for CP were also analyzed separately for very and moderately preterm infants, as well as for spastic diplegic and hemiplegic forms of CP (Table 6, paper IV). Abruptio placentae and low Apgar scores were associated with a higher risk of CP, especially in the hemiplegic and moderately preterm (32-36 weeks) group.

Comments:

Hypertensive disease and iatrogenic reason to delivery are partly found in the same group of patients. Several studies have indicated that preeclampsia and/or hypertensive disease might be related to a decreased risk of developing CP (293-295). This has raised concerns about preeclampsia as a potential confounder, raising the estimated degree of association between chorioamnionitis and CP (284).

We confirmed the fact that Apgar scores are related to CP (66), especially after 5 and 10 minutes. In the subgroup analysis it is possible to observe that a Apgar score < 7 yielded a higher OR for CP in the moderately preterm group and in the hemiplegic group.

In the case of cervical insufficiency, it is of course difficult to ascertain, in a retrospective study, whether the diagnostic criteria were met.

GENERAL DISCUSSION

1. Preterm birth

It is currently acknowledged that genital tract infection can occur in a subset of pregnant women and persist in a chronic and subclinical state, eventually resulting in spontaneous PTB (115). This type of infection may also have additional negative effects on the neonate, including adverse neurological outcomes such as CP (204). The majority of these children belong to a small proportion of children born after spontaneous onset of labor before 32 weeks of gestation (<1%). Sweden has a much lower incidence of PTB than the USA (1, 3). Also, the incidences of perinatal infections in Sweden and the USA/North America differ: clinical chorioamnionitis in patients with pPROM: 0.8% vs. 6.7 % (296, 297); endometritis in patients with pPROM; 0.3 % vs. 8.3 % (297, 298); neonatal infection in cases of pPROM: 1.9% vs. 2.4 % (296, 299). This difference had led us to speculate on infection related spontaneous PTB as an explanation for the differences in the incidence of PTB.

An appropriate preventive strategy directed at this group of high-risk pregnancies might entail a major opportunity to reduce the prevalence of CP and the perinatal mortality rate. Several preventive strategies have been attempted without any substantial success (1). One main issue in understanding these issues and suggesting effective preventive strategies is the lack of basic knowledge regarding the protection of the fetus from the bacterial hazards surrounding it – proximity to the bacteria in the vagina and rectum – and how the fetal immune system actually deals with invading bacteria. Recent discoveries in immunology suggest that the powerful innate immune system is an important contributor to the epithelial defense (300-302).

The prevalence of MIAC in our studies approximate the median in studies based on amniocentesis (Fig. 7 and 8). The use of PCR is probably not the explanation for this data in the PTL group since there were only two patients with positive PCR for *Ureaplasma urealyticum* (2/10), but it might have led to a false high level of MIAC in the pPROM group in which *Ureaplasma urealyticum* (n=9) is the most frequent bacterium. But on the other hand, only 37 % (10/27) of the patients with IAI had detectable bacteria in AF, even less than in the PTL group. However, as mentioned in Results, it is difficult to compare the data on MIAC from different studies, since they differ to such an extent. Bearing this limitation in mind a Scandinavian population apparently has the same prevalence of MIAC as a USA population, although just half of the PTB incidence. This is somewhat surprising as Sweden also has low incidence of perinatal infections. Several lines of evidence, reviewed in a number of articles, indicate that MIAC is involved in the pathogenesis of PTL, leading to PTB (29, 106, 107, 111, 112). In three studies (study I, II, III) we have designated CoNS a skin contamination but we had from study IV, and in the future, considered CoNS to be MIAC if accompanied by IAI (PTL, n=1; pPROM n=1). This means that one of the four CoNS found in the PTL study were regarded as MIAC in the MCP-1 study (IV). At present, MIAC is not the optimal marker for the intra-amniotic situation, as it requires an invasive amniocentesis and because of the low sensitivity of the technique used. Sensitivity can be improved by the use of broad-spectrum bacterial rDNA PCR (16S rDNA) (199), but test results are not available for days in current clinical practice.

IL-6 and IL-8 have turned out to be the most AF valuable markers of MIAC and PTB, no other pro-inflammatory cytokines have been studied with such consistent results. IL-6 and IL-8 levels are also increased in term normal labor but to a lesser extent (303).

There is a need for clinical studies of patients in PTL and with pPROM, stratified according to presence/absence of IAI. The identification of patients with the most substantial risk of adverse outcome would have important clinical implications. It is possible that delivery is

preferable to tocolysis and/or antibiotics for patients with IAI (or infection). On the other hand, if individual fetal genetic constitution is not disposed toward initiation of inflammatory response, the risk of intra-uterine fetal death or bad neonatal outcome may be even higher.
There is a need for randomized controlled studies in order to clarify whether a fetus should be kept in an inflamed/infected uterine environment by the use of tocolytic and antibiotic treatment. All future studies concerning tocolytic and antibiotic treatment of patients with pPROM or PTL should provide information on IAI, as it is probably one of the most important confounders of different outcome variables of interest. Tocolytic and antibiotic studies should provide information (primary outcome variables) on short-term and long-term infant morbidity outcome rather than interval to delivery. A study, results of which indicate that a certain treatment prolongs pregnancy one week, is of little importance without information on the intra-amniotic environment during this week, as well as the short-term and long-term outcomes for the children in both treatment and non-treatment groups. Information on the intra-amniotic situation might be obtainable with a cervical test, although the optimal cervical/vaginal test is yet not available.

2. CP

The diversity of the CP diagnosis and etiology is one of the major challenges in the study of its etiology. Since CP is not defined according to etiology or pathology, it is not surprising that it is etiologically and pathological diverse. There are several known antenatal causes of CP: developmental, vascular, infective, genetic, metabolic and toxic (304). Thus, it seems that a variety of clinical pictures can arise from a single cerebral pathology and that many different clinical cerebral pathologies can result in similar clinical entities (10). Adverse antenatal events may either cause brain damage themselves or make the infant more vulnerable to the normal asphyxiating events during delivery (10). Experimental animal studies indicate that bacterial endotoxin sensitizes the immature brain to the effect of an asphyxiating event (305). There are indications in the results of clinical studies, that maternal infections may increase the effect of asphyxia (306).
For a long time, AF infection was thought to primarily represent a maternal condition, while it may instead actually primarily be of fetal origin (307). However, there are no possibilities to study the AF in large retrospective case-control studies, necessitating the interpretation of fetal infections based on maternal symptoms and signs. Several studies focus on maternal infections and CP; as described above, maternal infection moderately increases the RR of CP. Our study agrees with the meta-analysis presented by Wu et al (285). Only one major study of term infants (>2500g) has been presented and the results indicate that maternal infections (excluding TORCH infections) at delivery are a major risk factor (OR 9.3) for development of unexplained spastic CP (308). Hypothetically, if maternal infection was a sufficient cause of CP, then the estimated etiologic proportion attributable to this diagnosis of total spastic CP in term children would be 12 % (95% CI, 4-20%). In other subgroups of CP, this percentage would be even higher (unexplained spastic quadriplegia) according to the same study (308).
There is no international consensus on the definition of clinical chorioamnionitis; a variety of definitions have been applied in the past. Fever, maternal tachycardia, fetal tachycardia, uterine tenderness, malodorous AF and maternal leukocytosis in various combinations are the most commonly used. The cut-off temperature was 37.8˚C in study I-IV because that is the most common definition in studies of AF infection/inflammation based on Gibbs et al. (309, 310). We believed that it was important to be able to compare our results to previously published data. In the case-control study of preterm children with CP, a slightly higher cut-off temperature, 38.0˚C, was chosen in order to allow comparison with most other studies in that particular area (285, 308).
Many studies define chorioamnionitis or intrauterine infection as described above. These signs, chosen for their relationship with maternal sepsis, are not sensitive indicators for

infections with non-purulent agents common to the genital tract, such as *Mycoplasma* and *Ureaplasma*. Moreover, these signs are not specific to intrauterine infection, because some of them can result from pharyngitis, urinary tract infection, dehydration or prolonged epidural anesthesia. This terminology requires improvement but today there is no widely accepted alternative (311).

Fever before the onset of delivery and antibiotics during pregnancy might indicate a longer-lasting and more intense infectious and/or inflammatory intrauterine environment (47). Fever during delivery might be confounded by increased temperature due to physical exertion during delivery, epidural analgesia and thus fetal exposure to a hostile intrauterine environment might be shorter and less intense in cases of intrauterine infection. Another infectious variable of interest is the use of antibiotics during pregnancy or delivery. There are indications that this variable can serve as not only an indicator of ongoing or previous infection, but also of prolonged intrauterine exposure to bacteria, bacterial products and cytokines (42).

3. Amniocentesis in clinical practice and safety

Amniocentesis in the third trimester is quite common in an international context but more rarely used in the Nordic countries. Indications for this procedure are assessment of fetal lung maturity before delivery, sampling AF for karyotype analysis in cases of severe intrauterine growth retardation, isoimmunization and evaluation of intrauterine conditions in patients with PTL and pPROM. Complications to third trimester amniocentesis have been discussed; e.g. need of urgent delivery, rupture of membranes, shorter latency to delivery, fetal injury, feto-maternal bleeding and contamination of the amniotic cavity by skin bacteria. Most studies on the safety of third-trimester amniocentesis have been performed before the era of ultrasound-guided punctures and complications were than indeed common (252, 312-314). When the first ultrasound guided amniocenteses were performed, an AF pool was located by ultrasound examination and then a blind puncture was performed.

Yeast et al studied 91 patients with pPROM using ultrasound for identification of an AF pool but performing punctures without ultrasound guidance. They did not find any shortening of the latency period but they used patients with oligohydramnios (insufficient AF for amniocentesis) as a control group which might have influenced the results (315). There were no fetal or cord complications in this study but there was one maternal abdominal wall hematoma (315).

In another study, a protocol entailing continuous CTG monitoring of the fetal heart rate for at least one hour after amniocentesis, resulted in detection of six cases of urgent fetal jeopardy, making appropriate interventions possible. The amniocenteses were performed without ultrasound guidance in this study (313).

Piiroinen et al performed 501 diagnostic amniocenteses in order to assess pulmonary maturity or to enable spectrophotometric analysis of the AF in cases of isoimmunization. In 92 patients, the procedure was repeated 2-5 times. An ultrasound examination preceded amniocentesis which was performed without a real-time examination. In nine cases (1.8%), the membranes ruptured within 24 hours of the puncture although it is unclear if these particular women were subjected to more than one puncture (314).

Only three studies, published during the last 5 years, have studied modern ultrasound-guided amniocentesis (316-318). Ultrasound-guided amniocentesis with real-time observation of needle passage has a high success rate (98-99%) and is considered to be a safe procedure with a complication rate of less than 1 % (316-318). Stark et al studied 913 amniocenteses, indications for which were assessment of the fetal lung maturity, with a focus on complications necessitating urgent delivery. Six cases required urgent delivery (five emergency CS and one vaginal induced delivery that ended in CS). Complications included fetal heart rate abnormalities (n=3), placental bleeding (n=1), abruptio placentae (n=1) and uterine rupture (n=1). Four of the six complications were

associated with two punctures, bloody fluid was withdrawn in two of six procedures and two were unsuccessful. The uterine rupture occurred in a patient with a former classic cesarean incision (316). Spontaneous labor did not contribute to any complication requiring immediate delivery or that led to the decision to deliver before the results of the maturity studies were available (316). In this study, 3% had transient amniotic leakage after the procedure (5 minutes to 8 hours). Haeusler et al also found amniocentesis (192 procedures), performed under continuous ultrasound guidance, to be a safe procedure (318). They also studied the acceptance of third trimester amniocentesis and found that the expectations of procedure–related pain were generally higher than the experienced pain, the degree of which was low (318). Gordon et al found a high success rate (99.2%) and a low risk of complications (0.7%) in their recently published study. The complications were rupture of membranes (n=1), PTL (n=1), feto-maternal bleeding (n=1) and placental abruption (n=1) and no subject required an emergency CS (317). Transplacental needle passage had occurred in the case of placental abruption and was unclear in the other cases (317). In this study, 50 patients required more than one puncture (9%) and eight patients (1.4%) required more than two. Bloody fluid was obtained in 55 of the procedures (10%) (317).

Most studies are performed with patients undergoing amniocentesis for reasons other than PTL or pPROM. A review of the role of amniocentesis in the diagnosis of subclinical infections has recently been published (319), according to which amniocentesis plays a potential role in the management of PTL and pPROM, and its results may offer guidance for administration of appropriate antibiotics to mother and neonate. Amniocentesis may also provide information on IAI, as a basis for decisions on withdrawal of tocolytics, timing of corticosteroid administration and delivery. The authors argue that there is enough evidence at present (in comparison to other obstetric routines in clinical practice) to use amniocentesis in the context of threatening PTB, but they also say that there is a critical need for a well-designed randomized trial of amniocentesis in the handling of pPROM and PTL (319).

According to our protocol, we checked the fetal heart rate with ultrasound immediately after the invasive procedure and CTG was performed for 30-60 minutes after the procedure. We tried to avoid more than one puncture. No maternal complications were registered in our study. No fetal complications of amniocentesis were registered in the PTL group, but an urgent CS was performed in the pPROM group due to fetal bradycardia. This occurred in the beginning of the study period; the patient had oligohydramnios and the neonatal outcome was good.

Although a third trimester amniocentesis is considered to be a relatively safe and well accepted procedure in a modern context (316-318), the procedure should be performed under ideal conditions and the procedure should be restricted to one puncture (316). A third trimester amniocentesis should be performed in a hospital-based environment with access to operation facilities and a strict surveillance scheme should be applied after the puncture (316-318). If puncture occurs under ultrasound surveillance and passing the needle though the placenta is avoided, the success rate is increased (316, 317) and it may be a safer procedure (316, 317). Since only three studies have been performed under modern conditions with continuous ultrasound surveillance, the power is too low to detect if the skill of the operator is significant (317).

Amniocentesis has been used internationally for several decades to handle patients with threatening PTB. Nevertheless, no clinical study has shown that amniocentesis and analysis of AF lead to improvement of the neonatal outcome. However, amniocentesis may serve as an important research tool, e.g. to identify different sub-groups of PTL that may have different etiologies. Division of patients with threatening PTB into different subgroups is urgently required in treatment studies of tocolysis and antibiotics (122).

CONCLUSIONS

Preterm birth is related to microbial invasion of the amniotic cavity and measurable inflammation in the AF. Although the Scandinavian countries have a low incidence of preterm delivery, the prevalence of microbial invasion of the amniotic cavity is comparable to that in populations with higher incidence of preterm birth.

IL-6 and IL-8 are related to microbial invasion of the amniotic cavity and preterm delivery (delivery within 7 days or < 34 weeks) in patients with preterm labor also in a Scandinavian population. The prevalence of intra-amniotic inflammation found in a population with a low incidence of preterm labor seems to be comparable to that found in other populations. Intra-amniotic inflammation was three times more common than microbial invasion of the amniotic cavity in women in preterm labor.

IL-6 and IL-8 are also related to microbial invasion of the amniotic cavity in patients with preterm prelabor rupture of membranes.

IL-18 in cervical fluid is not related to microbial invasion of the amniotic cavity, intra-amniotic inflammation or preterm delivery.

IL-18 in AF in patients in PTL is related to microbial invasion of the amniotic cavity, intra-amniotic inflammation and preterm delivery. IL-18 seems to be part of the intra-amniotic inflammation process in women in preterm labor. These associations were not found in the pPROM group.

MCP-1 in cervical fluid is related to microbial invasion of the amniotic cavity, intra-amniotic inflammation and preterm delivery.

MCP-1 in AF in patients in PTL is related to microbial invasion of the amniotic cavity, intra-amniotic inflammation and preterm delivery. MCP-1 was associated to microbial invasion of the amniotic cavity and intra-amniotic inflammation in the pPROM group, but not to preterm delivery. MCP-1 appears to be part of the intra-amniotic inflammation both in women in preterm labor and in those with preterm prelabor rupture of membranes.

Antenatal infections marginally increased the risk of CP concurring with data from populations with higher incidences of perinatal infections and preterm birth. Low Apgar score and abruptio placentae were associated with CP, especially in moderately preterm infants with hemiplegic CP.

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Errata paper I:

page 122, par 10, line 22 should be : AF, IL-6 (≥ 1.5 ng/mL) and IL-8 (≥ 1.3 ng/mL) in .....
page 122, par 11, line 2-3 should be : ≥ 1.5 ng/mL, ≥ 1.3 ng/mL
page 122, par 11, line 6 should be: ≥ 1.3 ng/mL
page 123, Table I, line 2 should be ≤ 7 days

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ORIGINAL ARTICLE

Microbial invasion and cytokine response in amniotic fluid in a Swedish population of women in preterm labor

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Background. Previous studies indicate an association between intra-amniotic microbial invasion and/or inflammation and spontaneous preterm birth, but there is a limited amount of data available from Europe. The aim of this study was to investigate the occurrence of intra-amniotic microorganisms and cytokines (interleukin-6 and interleukin-8) in a Swedish population of women in preterm labor and their correlation with preterm birth.
Methods. Amniotic fluid was retrieved transabdominally from 61 patients in preterm labor before 34 weeks of gestation. Polymerase chain reaction analyses for *Ureaplasma urealyticum* and *Mycoplasma hominis* and culture for aerobic and anaerobic bacteria were performed. Interleukin-6 and interleukin-8 were analyzed with enzyme-linked immunosorbent assay.
Results. Microorganisms in amniotic fluid were detected in 10 patients (16%). Patients with detected bacteria in the amniotic fluid had significantly higher levels of interleukin-6 and interleukin-8. There was also an association between interleukin-6/-8, the amniocentesis-delivery interval ($\leq$ 7 days) and preterm birth ($<$34 weeks). An amniotic fluid concentration of interleukin-6 $\geq$ 1.5 ng/mL or interleukin-8 $\geq$ 1.3 ng/mL was associated with an increased risk of delivery within 7 days (interleukin-6: relative risk 7.3; 95% confidence interval: 2.8–19; sensitivity 83%, specificity 87%; interleukin-8: relative risk 14, 95% confidence interval: 3.6–55, sensitivity 91%, specificity 87%).
Conclusions. The occurrence of intra-amniotic microbial invasion and inflammation in this population of Swedish women in preterm labor was similar to data reported from populations with a higher incidence of preterm delivery. Amniotic interleukin-6 and interleukin-8 correlated with the presence of microorganisms and with preterm birth.

Keywords: interleukin-6, interleukin-8, microbial invasion, preterm birth, preterm labor

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Preterm birth (PTB) is still a major unresolved problem in modern obstetrics. It is related to increased perinatal mortality and morbidity, including cerebral palsy and suboptimal school achievement (1–4). The incidence of PTB has not decreased over the last decades, despite new pharmacological therapies and prevention programs (5). In Sweden, the incidence of PTB has been approximately 5% since the beginning of the 1970s (2). In the United States and many other countries, the incidence of PTB is significantly higher (12%) and has increased slightly

Abbreviations:
AF: amniotic fluid; ELISA: enzyme-linked immunosorbent assay; IL: interleukin; PCR: polymerase chain reaction; PTB: preterm birth; PTL: preterm labor; ROC: receiver-operator characteristic.

from the 1980s to the mid-1990s (5). One possible explanation for the difference in PTB incidence could be a lower number of patients with infection-related PTB, as the rate of infection in the genito-urinary tract (e.g. urinary tract infections, bacterial vaginosis, clinical chorioamnionitis) and of neonatal sepsis appear to be comparatively low in Sweden (6–8). Several recent studies have shown that microbial invasion of the amniotic fluid (AF) is one important risk factor for PTB (9,10). Previous studies have indicated a wide range in the prevalence of microbial invasion of the AF (3–48%) among women with preterm labor (PTL) (10,11). This variability in prevalence is probably because of differences in the defintions of PTL, including gestational age groups, microbiological techniques and studied populations (11). It has also been shown in several studies that proinflammatory cytokines such as interleukin (IL)-6 and chemokines such as IL-8 are elevated in AF preceding PTB (12–14). There are, however, no studies from northern Europe based on transabdominal AF retrieval and focusing on the relationship between microbial invasion of the amniotic cavity, intra-amniotic inflammation and PTL. Such basic information is required in order to develop strategies for the prevention and treatment of PTL and its consequences in this region. In addition, it may be beneficial to compare mechanisms of PTL in regions with high and low incidences of PTB/perinatal infections.

The aim of this study was to examine the relationship between the intra-amniotic occurrence of microorganisms and the levels of IL-6 and IL-8, and their relationship with PTB (delivery within 7 days and delivery at <34 weeks of gestation, respectively) in a Swedish population of women in PTL.

Methods

The study population consisted of 66 women in PTL, with singleton pregnancies, who presented at two delivery wards in Göteborg (Sahlgrenska Hospital, 1996–97, $n=7$; Sahlgrenska University Hospital/East, 1997–2001, $n=59$) at a gestational age of less than 34 weeks. Preterm labor was defined as regular uterine contractions (at least two uterine contractions/10 min during 30 min) in combination with cervical changes: (one, ≤ 2 cm in length and ≥ 1 cm in dilatation; two, ≤ 2 cm in length and cervical softening; three, ≥ 1 cm in dilatation and cervical softening; or four, cervical length <30 mm at endovaginal ultrasound). Women with preterm prelabor rupture of membranes, known uterine abnormalities, fetal malformations, significant vaginal bleeding, imminent delivery or fetal distress were not included.

Gestational age was determined in 64 pregnancies by routine ultrasound in the second trimester (16th to 19th weeks of gestation) and in two patients by the date of their last menstrual period. Tocolytic therapy (intravenous terbutaline and/or indomethacin, the latter if the pregnancy was <28 weeks of gestation) was used according to department protocol.

An ultrasound-guided transabdominal amniocentesis was performed under antiseptic conditions within 12 h after admittance. A 0.8-mm diameter needle was used and 30–50 mL amniotic fluid (AF) was aspirated. After sampling, the AF was immediately placed in a refrigerator ($+4\,^{\circ}$C) and processed within 5 h. It was centrifuged at 855 g (3000 r.p.m.) in $+4\,^{\circ}$C for 10 min and stored at $-80\,^{\circ}$C until the time of analysis.

A sample of uncentrifuged AF was immediately transported to the microbiological laboratory for polymerase chain reaction (PCR) analysis of *Ureaplasma urealyticum* and *Mycoplasma hominis* and for aerobic and anaerobic culture. Microbial invasion was defined as positive PCR and/or growth of any bacteria in the AF, except for coagulase negative *Staphylococcus*, which was considered to be a skin contamination. Bacterial isolates were characterized biochemically by using the API Rapid ID32STREP, Rapid ID32A, ID32STAPH, ID32E, API ZYM, API Coryne, API50CHL, ID32C according to the manufacturer's instructions (API bioMérieux Marcy-Etoile, France). These identification systems identified anaerobes, coryneform bacterium, yeasts, streptococci, *Enterobacteriaceae* and other Gram-negative rods, the genera *Staphylococcus, Micrococcus* and the genus *Lactobacillus,* and related organisms. PAGE analysis of whole-cell proteins was performed as described by Pot et al. (15). For densitometric analysis, normalization and interpretation of protein patterns, the GelCompar 4.1 software package (Applied Maths, Gent, Belgium) was used. Anaerobic bacteria were further analyzed by fatty acid analysis using the MIDI specifications (16). Some of the difficult bacterial isolates were identified by DNA sequencing. The 16S rRNA genes of the isolates were amplified by PCR and directly sequenced using the *Big dye* terminator cycle sequencing kit (Applied Biosystems, Foster City, USA) and an automatic DNA sequencer (model 310, Applied Biosystems).

IL-6 and IL-8 in the AF were analyzed with enzyme-linked immunosorbent assay (ELISA) (paired antibodies from R & D Systems, Minneapolis,

MN). The AF samples were diluted 1 : 5, 1 : 20 and 1 : 100 and run in duplicates. The interassay variation was calculated at <10%, based on analysis of several samples on three separate occasions. Low values (≤700 pg/mL) showed a higher coefficient of variation (29–76%). The detection limit of the ELISA test was 30 pg/mL for both IL-6 and IL-8, but because the samples were run at a 1 : 5 dilution, the lower limit of detection was 150 pg/mL for both IL-6 and IL-8.

Clinical chorioamnionitis was defined as fever at ≥37.8 °C if occurring on two occasions of at least 4 h apart and if at least two or more of the following criteria were present: uterine tenderness, malodorous vaginal discharge, fetal tachycardia (>160 beats/minute), maternal tachycardia (>100 beats/minute) and maternal leukocytosis (>15000 cells/mm^3 (17)).

Two investigators (BJ, RMH) scrutinized the medical records and entered the maternal and perinatal data into a database.

Ethical approval for the study was obtained from the local Ethics Committee in Göteborg. The patients gave their informed consent before enrolment in the study.

Calculations were made using the computer programs StatView 5.01 (SAS Institute Inc, Cary, NC) or InStat 2.01 (Graph Pad Software, San Diego, CA). Continuous variables were analyzed with the Mann–Whitney *U*-test and proportions with Fisher's exact test. A *P*-value <0.05 was considered statistically significant, as was a confidence interval not including 1.

Sensitivity, specificity, positive and negative predictive values were calculated for different concentrations of IL-6 and IL-8 in relation to delivery within 7 days. A receiver-operator characteristic (ROC) curve was used to identify the best cut-off levels for IL-6 and IL-8. A survival analysis of the amniocentesis-delivery interval according to best cut-off levels for AF IL-6 and IL-8 was carried out.

Results

Characteristics of the study population are shown in Table I. Women who gave birth preterm (<34 weeks or within 7 days) did not differ from those who delivered later, regarding age, parity, number of previous gestations, number of previous PTD, smoking habits or antenatal treatment with tocolytics, antibiotics or corticosteroids. Women who delivered within 7 days had a significantly higher Bishop's score at inclusion than those who gave birth after 7 days. Sixty-six patients underwent amniocentesis. In five patients, no or an inadequate amount of AF was obtained and no analyses could be performed. In one patient, the AF was only analyzed with PCR for *U. urealyticum* and *M. hominis*, leaving 60 patients with a sufficient amount of AF to allow the complete set of analyses. Delivery before 34 weeks of gestation occurred in 44% (27/61) of the patients and delivery within 7 days occurred in 38% (23/61). Patients were included at a median of 30 weeks + 6 days (range: 22 weeks + 6 days – 33 weeks + 5 days).

Microbial invasion of the AF was identified in 16% (10/61) (Table II). Different bacterial species were found in all patients except *U. urealyticum*, which was found in two patients. In addition, there was growth of coagulase negative *Staphylococcus* in the AF in four cases. No patient had more than one species isolated in the AF. Nine of 10 patients with microbial invasion of the AF delivered before 34 weeks of gestation and one patient (*Streptococcus mitis)* delivered at term. All patients with coagulase negative *Staphylococcus* delivered after 34 weeks (three between 34 and 37 weeks and one at term).

Patients with microbial invasion of the AF had significantly higher levels of IL-6 and IL-8 than those without microbial invasion (median IL-6: 33.20 ng/mL vs. 0.72 ng/mL, p = 0.01; median IL-8: 26.39 ng/mL vs. 0.37 ng/mL, p = 0.002) (Fig. 1). The bacterial species and corresponding levels of IL-6 and IL-8 are presented in Table II.

Patients who delivered within 7 days had significantly higher levels of IL-6 and IL-8 than those who delivered after 7 days (Table I and Fig. 2). Patients who delivered before 34 weeks of gestation had significantly higher levels of IL-6 and IL-8 than those who delivered at ≥34 weeks (median IL-6 6.21 ng/mL vs. 0.29 ng/mL, $p < 0.001$; median IL-8 4.87 ng/mL vs. 0.15 ng/mL; $p < 0.001$) (Fig. 3). A ROC curve analysis of delivery at ≤7 days for various cut-off levels of IL-6 and IL-8 is shown in Fig. 4A,B. An IL-6 level of ≥1.5 ng/mL and an IL-8 level of ≥1.3 ng/mL were found to be the best cut-off points. The diagnostic indices for microbial invasion of the AF, IL-6 (1.5 ng/mL) and IL-8 (1.3 ng/mL) in cases with delivery at ≤7 days are presented in Table III. Survival analyses of the amniocentesis-delivery interval according to the AF IL-6 and IL-8 cut-off levels are presented in Fig. 5.

An inflammatory response, defined as an IL-6 level of 1.5 ng/mL was found in 39% (24/61) and an IL-8 level of 1.3 ng/mL was found in 43% (26/61) of the cases. An inflammatory response, defined as IL-6 ≥ 1.5 ng/mL and/or IL-8 = 1.3 ng/mL was found in 46% (28/61) of the patients. Of patients who delivered at <34 weeks,

Table I. Clinical background variables in women delivering within or after 7 days

Variable	Women delivering at ≥7 days ($n=23$)	Women delivering >7 days ($n=38$)	*p*-value
Maternal age (year) (median, range)	30(19–37)	29(19–38)	0.28
Nulliparous (*n*)	16	22	0.58
Number of previous gestations (median, range)	1(0–5)	1(0–9)	0.54
Previous preterm delivery (*n*)	4	7	1.00
Smoking in first trimester (*n*)	1	6	0.23
Gestational age at amniocentesis (days) (median, range)	214(167–236)	218.5(160–235)	0.30
Bishops score at inclusion (median, range)	7(3–10)	4(1–9)	<0.001
Use of corticosteroids before delivery (*n*)	22	28	0.07
Use of antibiotics before delivery (*n*)	8	8	0.37
Use of tocolysis before delivery (*n*)	22	33	0.64
Microbial invasion of the amniotic fluid (*n*)	8	2	0.0042
Interleukin-6, ng/mL (median)	7.15	0.32	<0.001
Interleukin-8, ng/mL (median)	5.57	0.15	<0.001

78% (21/27) had an inflammatory response with an IL-6 level ≥1.5 ng/mL and 85% (23/27) had an IL-8 level ≥1.3 ng/mL.

Two bacterial species have not previously been identified in AF: *Sneathia sanguinegens* and *Actinomyces odontolyticus*. A patient with *Sneathia sanguinegens* delivered at 24 weeks and 5 days. She had fever during delivery but did not fulfil the diagnostic criteria of chorioamnionitis. A patient with *Actinomyces odontolyticus* had no signs of any clinical infection and delivered at 24 weeks and 5 days.

None of the 10 patients with microbial invasion in the AF developed clinical chorioamnionitis. One other patient did, however, develop clinical chorioamnionitis, and had a negative culture/PCR but showed a clear inflammatory response (IL-6 23.10 pg/mL, IL-8 8.89 pg/mL); she delivered at 25 weeks of gestation (15 days after the amniocentesis).

Table II. Microbes isolated from amniotic fluid and the corresponding levels of interleukin-6 and interleukin-8

Organism	Interleukin-6 (ng/mL)	Interleukin-8 (ng/mL)
Ureaplasma urealyticum	35.43	17.54
Ureaplasma urealyticum	30.80	35.25
Fusobacterium species	2720	184.8
Corynebacterium	0.84	0.33
Eubacterium species	>50.0	866.3
Actinomyces odontolyticus	4166	822.3
Snethia sanguinegens	15080	149.1
Listeria monocytogenes	1.00	2.95
Streptococcus mitis	<0.15	<0.15
Difteriodic rods	1.62	2.14
Coagulase negative *Staphylococcus*	5.69	1.49
Coagulase negative *Staphylococcus*	0.71	0.38
Coagulase negative *Staphylococcus*	<0.15	<0.15
Coagulase negative *Staphylococcus*	<0.15	<0.15

Discussion

The main findings in this study were that in a Scandinavian population presenting with PTL

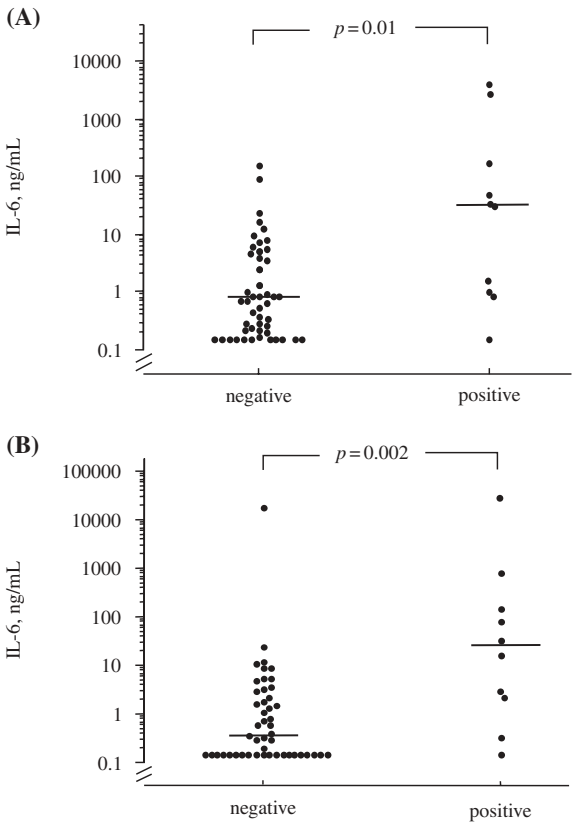

Fig. 1. (A) Interleukin (IL)-6 and (B) interleukin-8 levels in microbe-positive and microbe-negative amniotic fluid tested by polymerase chain reaction analyses or culture. Horizontal bars indicate medians. Values on the *Y*-axis are given on a logarithmic scale (ng/mL) and statistical significance was evaluated with the Mann–Whitney *U*-test.

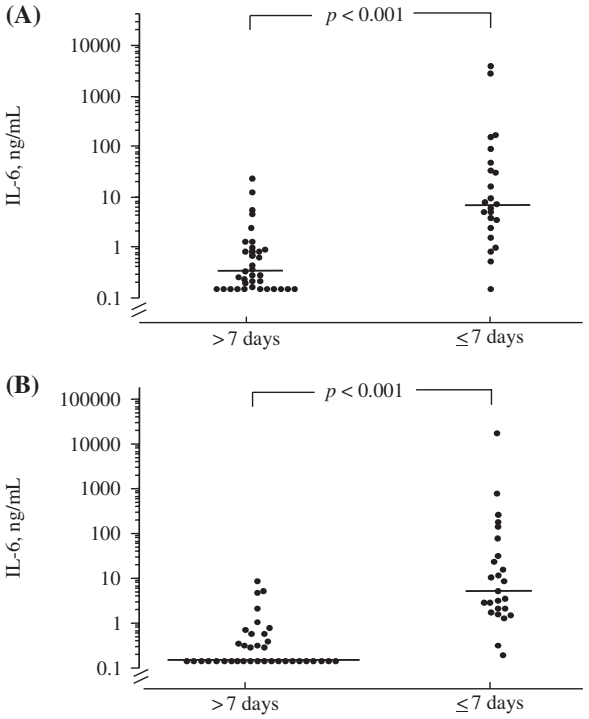

Fig. 2. (A) Interleukin (IL)-6 and (B) interleukin-8 levels in amnion related to amniocentesis-delivery interval. Horizontal bars indicate medians. Values on the *Y*-axis are given on a logarithmic scale. Statistical significance (delivery at ≤7 days vs. >7 days) was evaluated with the Mann–Whitney *U*-test.

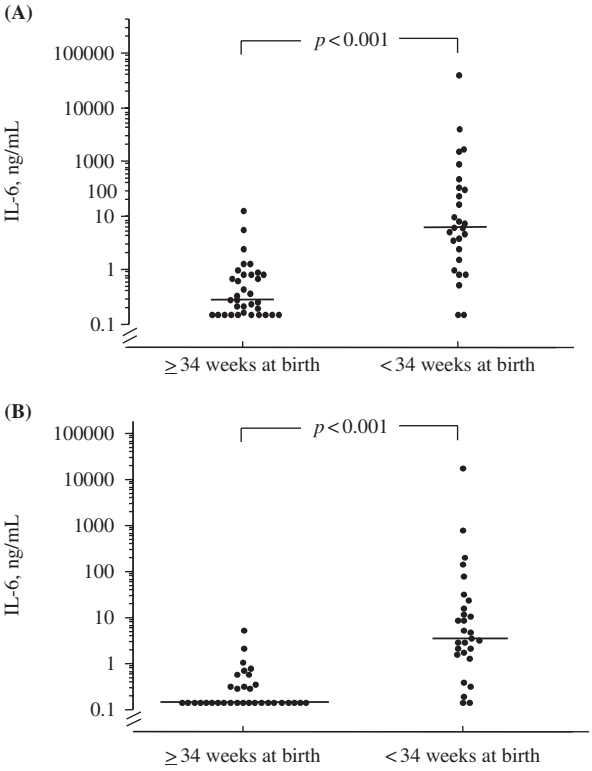

Fig. 3. (A) Interleukin (IL)-6 and (B) interleukin-8 levels in amnion related to birth at <34 weeks of gestation. Horizontal bars indicate medians. Values on the *Y*-axis are given on a logarithmic scale (ng/mL) and statistical significance (delivery at <34 weeks vs. ≥34 weeks of gestation) was evaluated with the Mann–Whitney *U*-test.

16% had detectable levels of microorganisms in the AF and approximately 49% had intra-amniotic inflammation. Intra-amniotic inflammation is a good predictor of PTB (<34 weeks) and delivery within 7 days, and is a better marker of PTB than intra-amniotic infection. These findings concur with those of earlier reports (18).

The rate of microbial invasion of the AF in this study (16%) in women in PTL is within the wide range (3–48%; median: 13%) of earlier reports (9,11,19,20). This is somewhat surprising because the incidence of PTB is only 5–6% and the rate of urogenital infections and neonatal sepsis is usually reported to be low in Sweden (6,7). Comparisons are difficult, however, because of differences in the definition of PTL, e.g. contractions and cervical change, gestational age at inclusion and microbiological technique (13,21–25). In this study, 90% of the patients with positive cultures or with PCR-detectable microorganisms in the AF delivered before 34 weeks of gestation. Most of them delivered within a few days, which is also highly consistent with previous reports (9,13, 21,26) demonstrating that 70–100% of patients with microbial invasion deliver shortly after presenting. In adding the four patients with coagulase negative *Staphylococcus*, only one patient with intra-amniotic growth of *S. mitis*

Table III. Diagnostic indices for amniotic fluid interleukin-6, interleukin-8 and presence of microbes as predictors of delivery at 7 days

	IL-6 ≥ 1.5 ng/mL	IL-8 ≥ 1.3 ng/mL	Microbes detected in amnion
Sensitivity	83%	91%	35%
Specificity	87%	87%	95%
Positive predictive value	79%	81%	80%
Negative predictive value	89%	94%	70%
Relative risk and 95% confidence interval	7.3 (2.8–19)	14 (3.6–55)	2.7 (1.6–4.6)

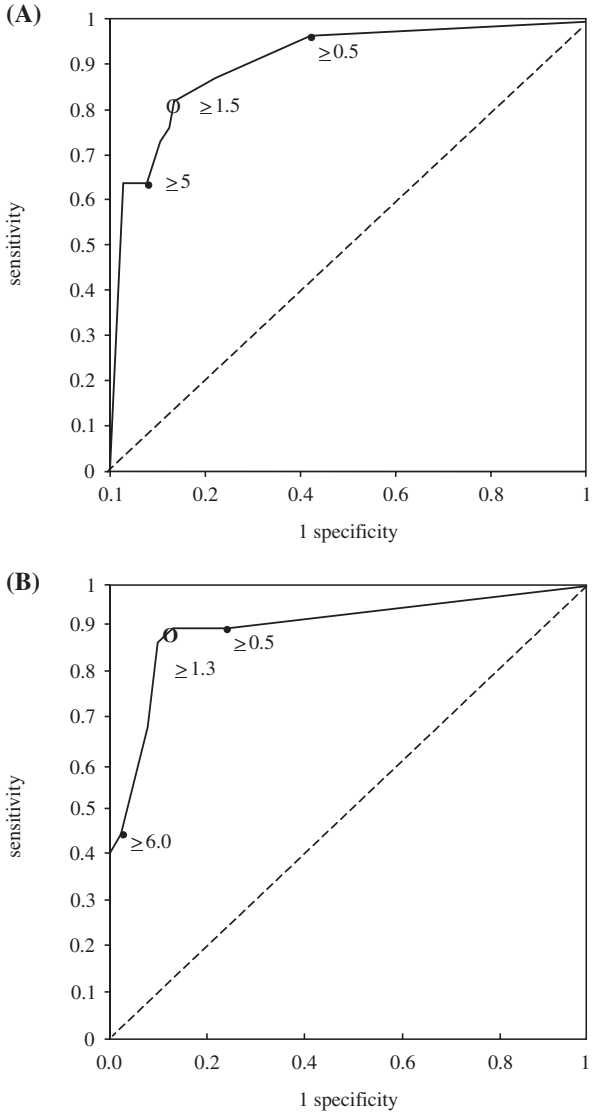

Fig. 4. Receiver-operator characteristic curve analysis of amniotic fluid A) Interleukin (IL)-6 and (B) interleukin-8 concentrations (ng/mL) as a predictor of preterm birth within 7 days. Numbers next to solid or open dots represent some of the cut-off values of the amniotic fluid concentrations (ng/mL) used in the analysis. The chosen cut-off value was (A) 1.5 ng/mL for IL-6 and (B) 1.3 ng/mL for IL-8.

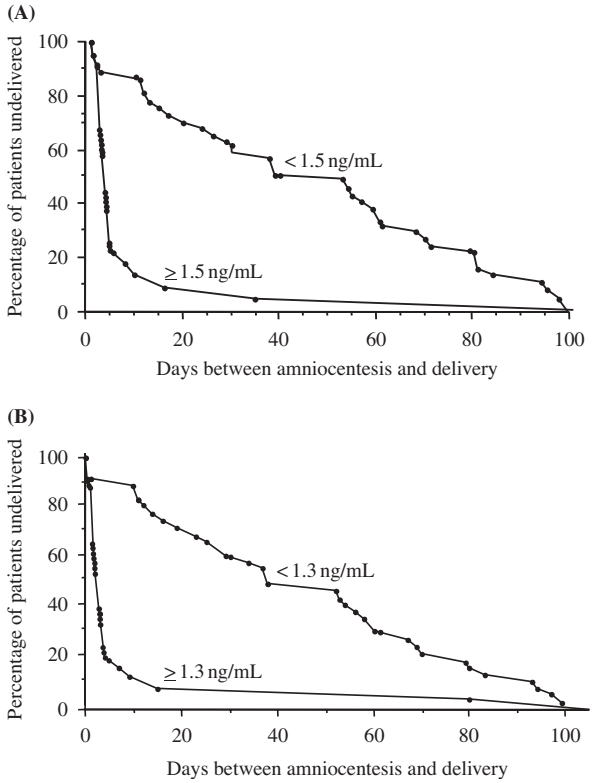

Fig. 5. Survival analysis of the elapsed time between amniocentesis and delivery, according to levels of (A) interleukin-6 (cut-off level ≥ 1.5 ng/mL) and (B) interleukin-8 (cut-off level ≥ 1.3 ng/mL) in amniotic fluid.

delivered after 34 weeks. This patient had normal levels of IL-6 and IL-8.

We have considered coagulase negative *Staphylococcus* to be a skin contamination because it is a well-known skin contaminant in blood cultures. Even if amniocentesis is performed under sterile conditions, bacteria from the maternal skin can contaminate AF culture. However, one patient had an inflammatory response, indicating that coagulase negative *Staphylococcus* could be related to an intra-amniotic inflammation. We think that coagulase negative *Staphylococcus* might, depending on the circumstances, be both a skin contamination and an ascending bacterium from the vagina, initiating an inflammatory response.

Intra-amniotic growth of *Sneathia sanguinegens* and *Actinomyces odontolyticus* has not been shown previously in patients with PTL. Both patients exhibited a robust inflammatory response with high levels of IL-6 and IL-8 in the AF. *Actinomyces odontolyticus* is a natural habitant of the oral cavity and an occasional pathogen in humans (27). This is interesting in light of the fact that periodontitis seems to correlate with PTB (28). *Sneathia sanguinegens* has recently been described as a gram-negative anaerobic rod; it has been isolated from human blood and now also from AF (29).

In international studies, more than one microorganism has been isolated from the AF in 50% of patients with microbial invasion (11). No patient had more than one species in this study.

Previous studies have shown that PTL is associated with a cytokine response, including expression of IL-6, IL-8, granulocyte colony-stimulating factor and tumor necrosis factor-alpha (10). Preterm birth has been associated with increased levels of IL-6 in the cervico-vaginal

fluid (30,31), AF (26) and fetal blood (32). We found that nearly 80% of women in PTL leading to PTB had an elevated IL-6 level ($\geq$1.5 ng/mL) compared with 11% of those who did not deliver preterm. Our data agree with the fact that intraamniotic IL-6 is one of the best predictors of PTB (26). We also found that the alpha-chemokine IL-8, which induces neutrophil attracting and activating responses, was highly expressed in the AF in PTL and was also an excellent predictor of PTB (18). Other researchers have found the best cut-off level for IL-6 in amniotic fluid to be 2.6 ng/mL (33) or 2.0 ng/mL (34). Even if these calculations have been made in relation to different outcome variables [microbes present in the amniotic fluid (33) and IL-6 elevation over the 70th percentile (34), respectively], they are comparable to our cut-off level. Our calculations regarding these outcome variables yield similar values [microbes present in the amniotic fluid (2.5 ng/mL) and IL-6 elevation over the 70th percentile (4.8 ng/mL)].

Interestingly, only half of the patients with an inflammatory response (indicated by IL-6 $\geq$ 1.5 ng/mL or IL-8 $\geq$ 1.3 ng/mL) had detectable levels of microorganisms in the AF, although PCR was used for *U. urealyticum and M. hominis* (35). This is in agreement with previous reports (33), and most likely indicates that some microorganisms cannot be detected with the techniques presently employed or that PTB could result from noninfectious inflammation.

In a recent study, Hebisch et al. showed that both IL-6 and IL-8 increase in AF with increased cervical dilatation as part of the normal delivery process (36). Yoon et al. also found that patients in PTL with high IL-6 levels had a more advanced cervical dilatation at inclusion (33). This might also be the case regarding PTB in this study, as the patients delivering within 7 days and before 34 weeks of gestation had a higher Bishop's score at inclusion. It is feasible that the process involved in uterine contractions contributed to the elevations of amniotic cytokines in our study. It is important to make the point, however, that most of these women were not in actual labor at the time of amniocentesis as they did not deliver until a few days later.

Amniocentesis has been used for decades to handle patients with threatening PTB. Analysis of the lecithin/sphingomyelin-ratio in AF provides information about the lung maturity of the fetus and is used worldwide but rarely in the Nordic countries. Our amniocentesis success rate was 91%. No complications to the procedure occurred in this study, but several have been described in the literature, such as fetal bradycardia requiring immediate delivery, as well as risk of bacterial contamination of the AF (37). Theoretically, detection of bacteria in the AF/or intraamniotic inflammation may offer guidance for administration of appropriate antibiotics to mother and neonate, withdrawal of tocolytics and timing of corticosteroid administration and delivery. Nevertheless, no clinical studies have shown that amniocentesis and analysis of AF lead to improved neonatal outcome. However, amniocentesis may serve as an important research tool, e.g. to identify different subgroups of PTL that may have different etiologies. Infection/inflammation-related PTB can be separated from PTB resulting from cervical insufficiency, stress, bleeding or disturbances in hydroxyprostaglandin dehydrogenase metabolism (38). It is also of great importance to examine whether cervical IL-6 and IL-8 are good indicators of the presence of microbes in the amniotic fluid, intraamniotic inflammation and neonatal outcome in a Scandinavian population, as a cervical sample is less invasive than amniocentesis (30,34).

In conclusion, a significant proportion of women in PTL, in a Scandinavian population, had microbial invasion (16%) and/or a cytokine inflammatory response (46%) in the AF. High levels of IL-6 and IL-8 were excellent predictors of PTB (delivery at $\leq$7 days, delivery $<$34 weeks) irrespective of the presence of microbes in the AF. These data may have clinical implications and are of importance in understanding the mechanisms involved in PTB. It will serve as background information for future studies on strategies to predict and reduce PTB, perinatal mortality and long-term morbidity in the Scandinavian context.

Acknowledgments

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II

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Microbial invasion and cytokine response in amniotic fluid in a Swedish population of women with preterm prelabor rupture of membranes

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Background. Previous studies have shown an association between intra-amniotic microbial invasion and/or inflammation and spontaneous preterm birth. The aim of this study was to investigate the occurrence of intra-amniotic microorganisms and cytokines (interleukin-6 and interleukin-8) in a Swedish population, with low incidence of preterm birth, of women with preterm prelabor rupture of membranes and their correlation to preterm birth.
Methods. Amniotic fluid was retrieved transabdominally from 58 patients with preterm prelabor rupture of membranes before 34 weeks of gestation. Polymerase chain reaction analyses for *Ureaplasma urealyticum*
and *Mycoplasma hominis* and culture for aerobic and anaerobic bacteria were performed. Interleukin-6 and interleukin-8 were analyzed with enzyme-linked immunosorbent assay.
Results. Microorganisms in amniotic fluid were detected in 13 patients (25 %). Patients with bacteria detected in the amniotic fluid had significantly higher levels of interleukin-6 and interleukin-8. An amniotic fluid concentration of interleukin-6 ≥ 0.80 ng/mL (relative risk 1.93, 95 % confidence interval: 1.13-3.29, sensitivity 63 %, specificity 75 %) was associated with an increased risk of delivery within 7 days. There was also an association between interleukin-8 and preterm birth (< 34 weeks).
Conclusions. Intra-amniotic microbial invasion and inflammation in this population of Swedish women with preterm prelabor rupture of membranes were similar to data reported from populations with a higher incidence of preterm delivery. Amniotic interleukin-6 correlated to the presence of microorganisms and delivery within 7 days and interleukin-8 to delivery before 34 weeks.

Background

Preterm birth (PTB) is the leading cause of perinatal mortality, perinatal morbidity, childhood neurological morbidity and is associated with suboptimal school achievements (1-5). Despite advances in obstetric care during the last decades the incidence of PTB has not decreased (6). In Sweden, the incidence of PTB has been 5-6 % since the beginning of the seventies, but in the United States and many other countries, the incidence of PTB is much higher (12 %) (2, 6). The cause of PTB seems to be multifactorial but several studies indicate that microbial invasion of the amniotic fluid (AF) is an important risk factor for PTB (7). One possible explanation for the difference in PTB incidence in the Scandinavian countries and USA could be a lower number of patients with infection-related PTB, since the rate of

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infections in the reproductive tract and of neonatal sepsis appears to be comparatively low in Sweden (8-10).There are two forms of spontaneous PTB: preterm labor (PTL) and preterm prelabor rupture of membranes (pPROM). In a previous study, we have not been able to confirm the hypothesis of less infection-related PTB in a Swedish population of patients in preterm labor (11).

Microbial invasion of the AF occurs in approximately one third of patients with pPROM (7, 12). Previous studies have indicated a wide range in the prevalence (15 - 57 %) and is probably due to differences in population characteristics, gestational age and microbiological techniques (7, 12). It has also been shown that proinflammatory cytokines like interleukin (IL)-6 and chemokines like IL-8 are elevated in AF preceding PTB (13, 14). There is, however, only one study available from northern Europe concerning the relationship between microbial invasion of the amniotic cavity in patients with pPROM (15) and no study regarding intra-amniotic inflammation and PTB using transabdominal technique for retrieval of AF. Such basic information is important in order to develop strategies for prevention and treatment of pPROM and its consequences in this region.

The aim of this study was to examine the relationship between intra-amniotic occurrence of microorganisms and the levels of IL-6 and IL-8 and their relationship to PTB (delivery within 7 days and delivery at < 34 weeks of gestation) in a Swedish population of women with pPROM.

Methods

The study population was recruited prospectively and consisted of 58 women with singleton pregnancies with pPROM, who presented at 2 delivery wards in Göteborg (Sahlgrenska Hospital (1996-1997; n=14) and Sahlgrenska University Hospital/East (1997-2001; n=44)) at a gestational age of less than 34 weeks. pPROM was diagnosed by a speculum examination confirming pooling of AF in the vagina. Digital examination was not performed. Women with contractions before rupture of membranes, known uterine abnormalities, fetal malformations, significant vaginal bleeding, imminent delivery or fetal distress were not included.

Gestational age was determined in all 58 pregnancies by routine ultrasound in the second trimester (16^{th} to 20^{th} weeks of gestation). Tocolytic therapy (intravenous terbutaline and/or indomethacin, the latter if the pregnancy was < 28 weeks of gestation) was used according department protocol.

An ultrasound-guided transabdominal amniocentesis was performed under antiseptic conditions within 12 hours after admittance and 30 – 50 mL AF was aspirated (needle diameter: 0.8 mm). After sampling, AF was immediately placed in a refrigerator (+4°C) and processed within 5 hours. It was centrifuged at 855 g in +4°C for 10 minutes and stored at –80°C until time of analysis.

A sample of uncentrifuged AF was immediately transported to the microbiological laboratory for polymerase chain reaction (PCR) analysis of *Ureaplasma urealyticum* and *Mycoplasma hominis* and for aerobic and anaerobic culture. Microbial invasion was defined as positive PCR and/or growth of any bacteria in the AF, except for coagulase negative *Staphylococcus*, which was considered to be a skin contamination.

Bacterial isolates were characterized biochemically by using the API Rapid ID32STREP, Rapid ID32A, ID32STAPH, ID32E, API ZYM, API Coryne, API50CHL, ID32C according to the manufacturer's instructions (API bioMérieux). These identification systems identified anaerobes, coryneform bacterium, yeasts, streptococci, *Enterobacteriaceae* and other Gram-negative rods, the genera *Staphylococcus*, *Micrococcus* and the genus *Lactobacillus* and related organisms. PAGE analysis of whole-cell proteins was performed as described by Pot et al (16). For densitometric analysis, normalization and interpretation of protein patterns, the GelCompar 4.1 software package (Applied Maths) was used. Anaerobic bacteria were further analyzed by fatty acid analysis using the MIDI specifications (17). Some of the difficult bacterial isolates were identified

Table I. Clinical background variables in women delivering within or after 7 days.

	Women delivering ≤ 7 days (n=27)	Women delivering >7 days (n=20)	p-value
Maternal age (year) (median, SD, range)	32 (6.04) (20-43)	27 (5.74) (19-42)	0.01
Nulliparous (n)	11	12	1.00
Previous preterm delivery (n)	0	2	0.18
Smoking at the beginning of pregnancy (n)	8	5	1.00
Bleeding during pregnancy (n)	3	4	0.44
Gestational age at amniocentesis (days) (median, SD, range)	229 (17.91) (158-236)	204 (22.74) (162-234)	< 0.001
Use of corticosteroids before delivery (n)	26	19	1.00
Use of antibiotics before delivery (n)	8	5	1.00
Use of tocolysis before delivery (n)	23	9	0.005
Microbial invasion of the amniotic fluid (n)	10	2	0.047

by DNA sequencing. The 16S rRNA genes of the isolates were amplified by PCR and directly sequenced by using the *Big dye* terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 310, Applied Biosystems).

IL-6 and IL–8 in AF were analyzed with enzyme-linked immunosorbent assay (ELISA) (paired antibodies from R&D Systems, Minneapolis, Minn, USA). AF samples were diluted 1:5, 1:20 and 1:100 and run in duplicates. An arithmetic mean was calculated. The interassay variation was calculated at < 10 %, based on analysis of several samples on three separate occasions. Low values (≤ 700 pg/mL) showed a higher coefficient of variation (29-76 %). The detection limit of the ELISA test was 30 pg/mL for both IL-6 and IL-8, but because the samples were run at a 1:5 dilution, the lower limit of detection was 150 pg/mL.

Before amniocentesis, cervico-vaginal fluid was sampled with a plain cotton-viscose swab. No bacteriostatic lubricant was used. The swabs were placed in Stuart's transport medium for aerobic and anaerobic standardized culture. If the laboratory reported an ordinary flora it was considered to be negative.

Clinical chorioamnionitis was defined as fever ≥ 37.8˚C on two occasions at least 4 hours apart and ≥ 2 of the following criteria were present: uterine tenderness, malodorous vaginal discharge, fetal tachycardia (> 160 beats/minute), maternal tachycardia (>100 beats/minute) and maternal leukocytosis (> 15000 cells/mm^3) (18).

Two investigators (BJ, RMH) scrutinized the medical records and entered maternal and perinatal data into a database.

Ethical approval for the study was obtained from the local ethic committee in Göteborg. The patients gave informed consent before enrollment in the study.

Calculations were made using the computer programs StatView 5.01 (SAS Institute Inc, Cary North Carolina, USA) or InStat 2.01 (Graph Pad Software, San Diego, California, USA). Continuous variables were analyzed with the Mann-Whitney U test and proportions with Fisher's exact test. A p-value < 0.05 was considered statistically significant, as was a 95 % confidence interval not including 1.00. A logistic regression analysis was performed for differences in gestational age at inclusion and maternal age between patients who gave birth at ≤ 7 days vs. > 7 days.

Table II.

Microbes isolated from amniotic fluid and the corresponding levels of interleukin-6 and interleukin-8.

Organism	Interleukin-6 (ng/mL)	Interleukin-8 (ng/mL)
Ureaplasma urealyticum	53.6	21.4
Ureaplasma urealyticum	0.52	0.49
Ureaplasma urealyticum	3.50	3.24
Ureaplasma urealyticum	1.79	3.30
Ureaplasma urealyticum	41.1	22.0
Ureaplasma urealyticum	>50.0	6.01
Ureaplasma urealyticum	33.8	13.1
Ureaplasma urealyticum	-	-
Ureaplasma urealyticum+ Mycoplasma hominis	1.67	2.26
Haemophilus influenzae	1.40	0.43
Streptococcus agalactiae	833	45.0
Bacteroides fragilis + Bifidobacterium adolecentis	0.21	<0.15
Anaerob gram-negative rods	1736	420
Coagulase negative staphylococcus	4.15	0.65

Sensitivity, specificity, positive and negative predictive values were calculated for different concentrations of IL-6 and IL-8 in relation to delivery within 7 days. A receiver-operator characteristic (ROC) curve was used to identify the best cut-off levels for IL-6 and IL-8 for delivery within 7 days. The same cut-off levels were used to define intra-amniotic inflammation and to perform survival analysis of the amniocentesis-delivery interval.

Results

Clinical background information of the study population is presented in Table I. Women who gave birth within 7 days where not different from those who delivered > 7 days except for gestational age at inclusion and maternal age. Fifty-eight patients with pPROM, before uterine contractions started, underwent amniocentesis. In five patients, no or inadequate amount of AF was obtained and no analyses could be performed. In another six patients, the AF was only analyzed for bacteria with PCR and culture, leaving 47 patients with sufficient amount of AF to allow a complete set of analyses.

Patients were included at a median of 31 weeks + 4 days (range: 22weeks+4 days – 33 weeks+5 days). Delivery occurred before 34 weeks of gestation in 33/47 (70 %) patients and within 7 days in 27/47 (57 %) patients.

Microbial invasion of the AF was identified in 25 % (13/53) (Table II). *Ureaplasma urealyticum* was found in nine patients. In addition, one patient had growth of coagulase negative *Staphylococcus* in the AF. Two patients had 2 species of bacteria isolated from the AF. All patients with microbial invasion of the AF delivered prior to 34 weeks of gestation. The patient with coagulase negative *Staphylococcus* delivered at 33 weeks of gestation.

The strain with anaerobic Gram-negative rods died during identification at the laboratory, so no further information of the strain is available. The strain was associated with a very intense

Fig. 1

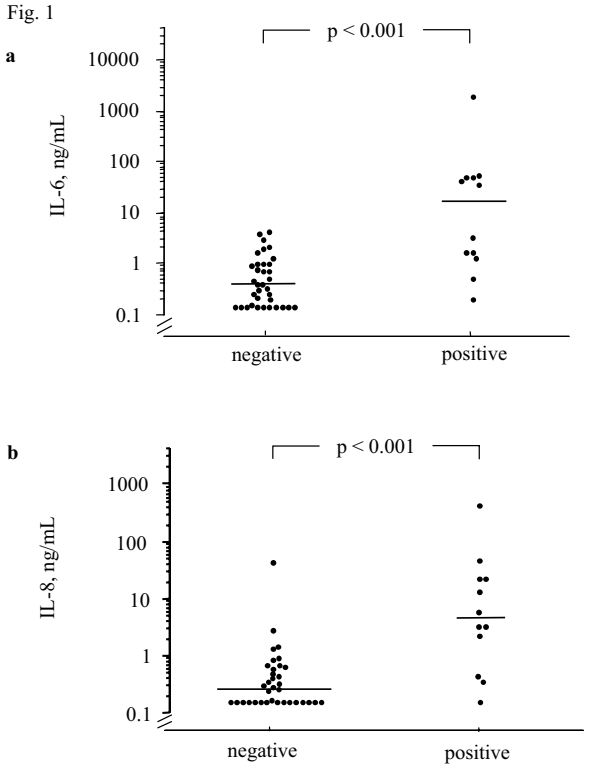

Fig. 1. Interleukin (IL)-6 (a) and interleukin-8 (b) levels in microbe-positive and microbe-negative amniotic fluid tested by PCR and culture. Horizontal bars indicate medians. Values on the Y-axis are given on a logarithmic scale (ng/mL) and statistical significance was evaluated with the Mann-Whitney U test.

Fig. 2

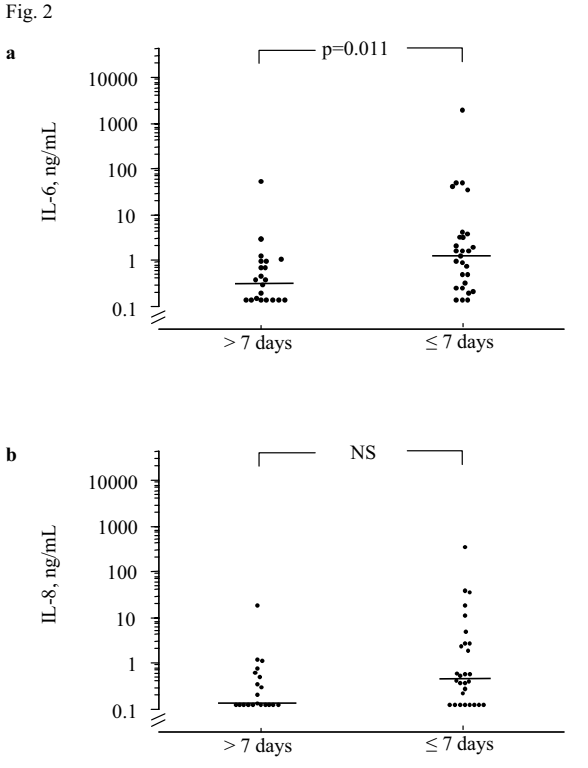

Fig. 2. Interleukin (IL)-6 (a) and interleukin-8 (b) levels in amniotic fluid related to amniocentesis-delivery interval. Horizontal bars indicate medians. Values on the Y-axis are given on a logarithmic scale (ng/mL). Statistical significance (delivery at ≤ 7 days vs. >7 days) was evaluated with the Mann-Whitney U test.

inflammatory response in the AF with high levels of IL-6 and IL-8.

None of the thirteen patients with microbial invasion in AF developed clinical chorioamnionitis. The patient with Haemophilus influenzae was suspected to suffer from clinical chorioamnionitis but did not fulfill all the criteria. Clinical chorioamnionitis developed in one patient with a negative culture/PCR and no inflammatory response (IL-6 <0.15 ng/mL, IL-8 <0.15 ng/mL) and she delivered 7 hours after amniocentesis at 33 weeks of gestation by cesarean section due to pathological CTG and clinical chorioamnionitis.

Patients with microbial invasion of the AF had significantly higher levels of IL-6 and IL-8 than those without microbial invasion (median IL-6 18.6 ng/mL versus 0.42 ng/mL; p<0.001) (median IL-8 4.66 ng/mL versus 0.25 ng/mL; p<0.001) (Fig. 1). The bacterial species and corresponding levels of IL-6 and IL-8 are presented in Table II.

Patients who delivered within 7 days had significantly higher levels of IL-6 than those who delivered after 7 days (median IL-6 1.40 ng/mL versus 0.37 ng/mL; p=0.011) but the IL-8 levels were not significantly higher (median IL-8 0.43 ng/mL versus 0.22 ng/mL; p=0.089) (Fig. 2). A logistic regression analysis was performed with correction for gestational age at inclusion and maternal age without any significant changes in the results (IL-6 p=0.048, IL-8 NS). However, three patients had an induction of labor within 7 days and might therefore be misclassified as spontaneous delivery within 7 day. There were no changes of the results if these patients were excluded, except for the correlation between IL-8 and time of delivery that became slightly stronger (median IL-8 0.54 ng/mL in delivery ≤ 7 days vs. 0.22 ng/mL in delivery > 7 days; p=0.04). Patients who delivered before 34 weeks of gestation had significantly higher levels of IL-8 than those who delivered ≥ 34 weeks (median

Fig. 3

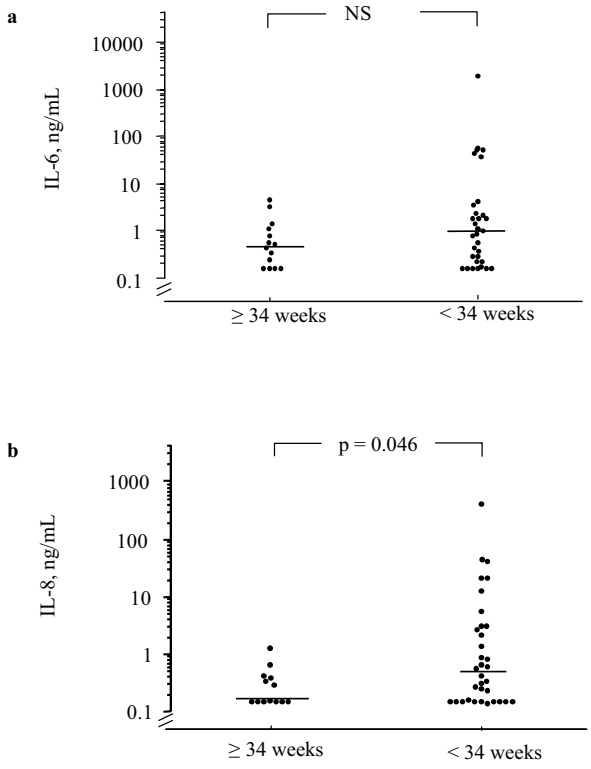

Fig. 3. Interleukin (IL)-6 (a) and interleukin-8 (b) levels in amniotic fluid related to birth at <34 weeks of gestation. Horizontal bars indicate medians. Values on the Y-axis are given on a logarithmic scale (ng/mL) and statistical significance (delivery at ≤ 34 weeks vs. > 34 weeks of gestation) was evaluated with the Mann-Whitney U test.

Fig. 4

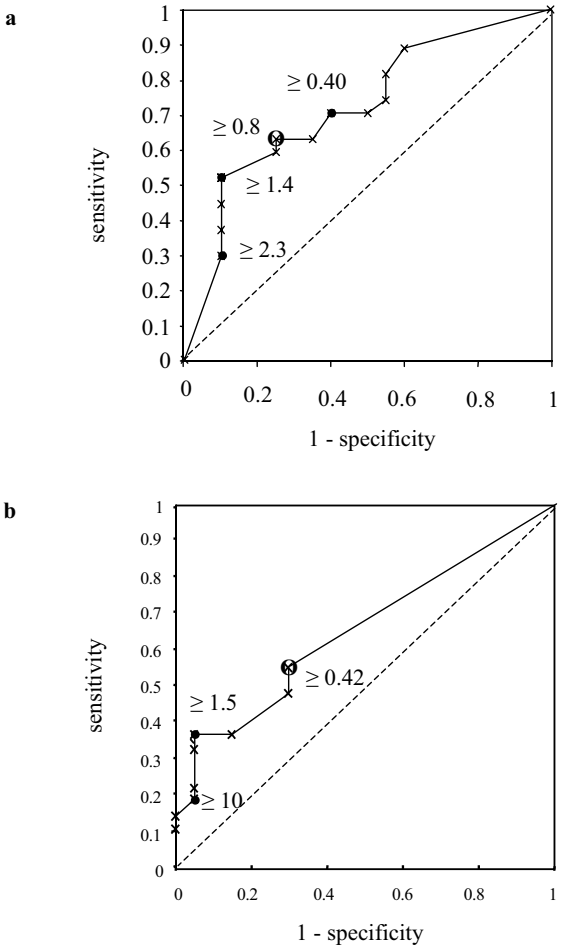

Fig. 4. Receiver-operator characteristic curve analysis of amniotic fluid interleukin-6 (a) and interleukin-8 (b) concentrations (ng/mL) as a predictor of preterm birth within 7 days. Numbers next to solid or open dots represent some of the cut-off values of amniotic fluid

IL-6 1.0 ng/mL versus 0.46 ng/mL; p=0.18) (median IL-8 0.51 versus 0. 16 ng/mL; p=0.046) (Fig. 3). There were 3 inductions and 2 cesarean sections performed before onset of contractions that were included in the group "spontaneous delivery < 34 weeks". However, the results were not affected by exclusion of these patients.

A ROC curve analysis of delivery at ≤ 7 days for various cut-off levels of IL-6 and IL-8 is shown in Fig. 4 a and b. An IL-6 level of 0.80 ng/mL and IL-8 level of 0.42 ng/mL were found to be the best cut-off points. The diagnostic indices for microbial invasion of the AF, IL-6 (0.80 ng/mL) and IL-8 (0.42 ng/mL) in cases with delivery at ≤ 7 days are presented in Table III. Survival analyses of amniocentesis-delivery interval according to AF IL-6 (0.80 ng/mL) and IL-8 (0.42 ng/mL) are presented in Fig 5.

An inflammatory response, defined as an IL-6 level ≥ 0.80 ng/mL was found in 47 % (22/47) and an IL-8 level ≥ 0.42 ng/mL was found in 44 % (21/47) of the cases. An inflammatory response, defined as IL-6 ≥ 0.80 ng/mL and/or IL-8 ≥ 0.42 ng/mL was found in 57 % (27/47) of the patients.

A positive endocervical culture was found in 19 patients. In 4 patients the result of the endocervical culture were missing. Bacteria found in the endocervical culture were *Streptococcus agalactiae* (n=12), *Candida albicans* (n=6), *Gardnerella vaginalis* (n=2), *Staphylococcus aureus* (n=1), *alpha-Streptococcus* (n=1), coagulase negative *Staphylococcus* (n=1) and one patient had a disturbed vaginal flora (gram positive aerobe and anaerobe mixed flora). One patient with a positive culture in AF *Streptococcus agalactiae* had *Streptococcus agalactiae* and *Staphylococcus aureus* in the endocervical

Table III.
Diagnostic indices for amniotic fluid interleukin-6, interleukin-8 and presence of microbes in the amniotic fluid as predictors of delivery at ≤ 7 days.

	IL-6 ≥0.80 ng/mL (n=47)	IL-8 ≥0.42 ng/mL (n=47)	IAI (n=47)	Microbes detected in amniotic fluid (n=53)
Sensitivity	63 %	56 %	70 %	33 %
Specificity	75 %	70 %	60 %	90 %
Positive predictive value	77 %	71 %	70 %	84 %
Negative predictive value	60 %	54 %	60 %	45 %
Relative risk and 95% confidence interval	1.93 (1.14– 3.29)	1.55 (0.94-2.54)	1.76 (0.98-3.17)	1.71 (1.12-2.62)

IL – interleukin
IAI – intra-amniotic inflammation

Fig. 5

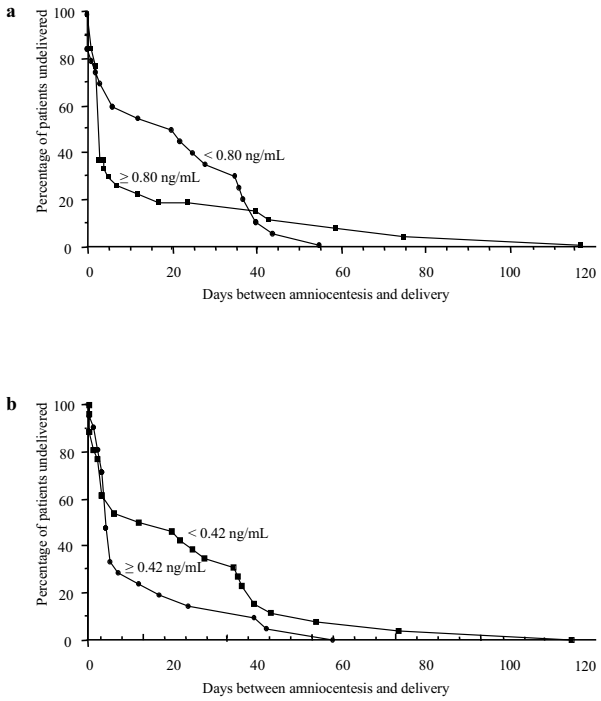

Fig. 5. Survival analysis of elapsed time between amniocentesis and delivery, according to levels of interleukin-6 (a; cut-off level ≥ 0.80 ng/mL) and interleukin-8 (b; cut-off level ≥ 0.42 ng/mL) in amniotic fluid.

fluid. Eleven other patients had *Streptococcus agalactiae* in endocervix without detectable levels of the bacteria in AF.

Discussion

The result of this study demonstrates that in this Swedish population presenting with pPROM (median gestation: 31 weeks), 25 % had detectable levels of microorganisms in the AF and approximately 57 % had amniotic inflammation (indicated by IL-6 ≥ 0.80 ng/mL and IL-8 ≥ 0.42 ng/mL). IL-6, IL-8 and intra-amniotic inflammation were better predictors for imminent PTB (Table III) than intra-amniotic infection, which agrees with earlier reports (19).

The prevalence of microbial invasion of the AF found presently (25 %) in women with pPROM, using PCR for *Mycoplasma hominis* and *Ureaplasma urealyticum* combined with aerobic and anaerobic cultures, is within the wide range (15-57 %; median: 34 %) of earlier reports (12, 15). Although, all studies seem to underestimate the occurrence of microbial invasion in women with pPROM since microbial invasion is related to oligohydramnios and therefore less accessible for amniocentesis (20). Comparisons between different reports are difficult, however, because of differences in the definition of pPROM with respect to detection method of AF in the vagina,

gestational age of included patients and microbiological technique used (19, 21-29). Our data show that the occurrence of culture-positive patients in pPROM was not different from previous studies performed in other countries (if expressed as percentage of patients sampled). This is somewhat surprising as the incidence of birth <37 weeks of gestation is only 5-6% (and birth <34 weeks 1.5 %) (2) and the rate of bacterial vaginosis, postpartum endometritis and neonatal sepsis is reported to be comparatively low in Sweden (8-10). Furthermore, all the patients with positive cultures or with PCR-detectable microorganisms in the AF delivered prior to 34 weeks of gestation.

Coagulase negative *Staphylococcus* is a well-known skin contaminant in blood cultures. Even if amniocentesis is performed under sterile conditions, bacteria from the maternal skin can contaminate the amniotic culture. It's likely that coagulase negative *Staphylococcus*, depending on the circumstances, might be a skin contamination in certain cases and an ascending bacterium from vagina in other cases. There was an inflammatory response in the patient with coagulase negative *Staphylococcus*, which might indicate that coagulase negative *Staphylococcus* could be related to the intra-amniotic inflammation in this case and therefore, should not be regarded as a skin contamination.

In international studies, fifty percent of patients with microbial invasion have more than one microorganism isolated from the AF (12). In this study we found only two patients (2/13) with two types of bacteria in the AF. The fact that *Ureaplasma urealyticum* and certain anaerobes predominate in our patients with pPROM must be taken into consideration when the type of antibiotic is chosen in cases when amniocentesis is not performed (which is most often the case in Sweden). A combination of e.g. erythromycin and clindamycin is preferable to cefuroxime that is often used today in the Scandinavian countries.

Previous studies have shown that pPROM is associated with a cytokine response, including expression of IL-1, IL-6, IL-8 and tumor necrosis factor-alpha (30). PTB has been associated with increased levels of IL-6 in AF (19) and fetal blood (31). We presently found that nearly 53 % of the women with pPROM leading to PTB (< 34 weeks of gestation) had an elevated IL-6 level (≥ 0.80 ng/mL) compared to 31 % of those that delivered ≥ 34 weeks. Our data agree with the fact that intra-amniotic IL-6 is one of the best predictors of preterm delivery (13) even if the sensitivity and specificity are not as high as in PTL patients (11). It has previously been shown that IL-6 is not as good predictor of microbial invasion of the AF in pPROM as in PTL patients, and microbial invasion of the AF is related to a short amniocentesis-delivery interval (19). We also found that the alpha chemokine IL-8, that induces neutrophil attractant and activating responses, was highly expressed in the AF with pPROM but did not have as high sensitivity and specificity as in the PTL group (11). This difference in prediction of PTB between the PTL and pPROM groups is not surprising, since different mechanisms appear to be important in pPROM as compared to PTL (32).

It is interesting to note that only 37 % (10/27) of the patients with an inflammatory response (indicated by IL-6 ≥ 0.80 ng/mL or IL-8 ≥ 0.42 ng/mL) had detectable levels of microorganisms in the AF in spite of that PCR was used for *Ureaplasma urealyticum* and *Mycoplasma hominis* (33). This is in agreement with previous reports (34), and most likely indicate that some microorganisms cannot be detected with the techniques presently employed or that PTB could result from non-infectious inflammation.

In summary, a significant percentage of women with pPROM (< 34 weeks), even in a Swedish population with low prevalence of genital tract infections, have microbial invasion (25 %) and/or a cytokine inflammatory response (57 %) in the AF.

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III

Interleukin-18 in cervical mucus and amniotic fluid: relationship to microbial invasion of the amniotic cavity, intra-amniotic inflammation and preterm delivery

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Objective. To evaluate the relationship between interleukin (IL)-18 in cervical mucus and amniotic fluid and microbial invasion of amniotic fluid, preterm delivery and intra-amniotic inflammation in women in preterm labour (PTL), with preterm prelabour rupture of the membranes (pPROM) and at term.
Design. A prospective follow-up study
Setting. Sahlgrenska University Hospital, Göteborg, Sweden.
Sample. Women with singleton pregnancies (<34 weeks) presenting with PTL (n=87) or pPROM (n=47) and women, not in labour, at term (n=28).
Methods. Amniotic fluid was retrieved transabdominally. Cervical mucus was taken from the uterine cervix of women in PTL and at term. IL-18 was analyzed with enzyme-linked immunosorbent assay.
Main outcome measures: IL-18 in relation to microbial invasion of the amniotic fluid, delivery within 7 days or <34 weeks of gestation and intra-amniotic inflammation.
Results. The levels of IL-18 in the cervical and amniotic fluid were higher in women with PTL than in those not in labour at term. In the PTL group, significant associations were found between elevated IL-18 in amniotic fluid and microbial invasion of the amniotic fluid, as well as between delivery within 7 days or <34 weeks of gestation and intra-amniotic inflammation. Delivery was delayed longer in the pPROM sub-group with IL-18 ≥1.0 ng/mL than in that with IL-18 <1.0 ng/mL.
Conclusions. In the PTL group, high IL-18 in amniotic fluid (but not in the cervix) was associated with microbial invasion of the amniotic fluid, intra-amniotic inflammation and prompt delivery. On the other hand, elevated IL-18 in the pPROM group correlated with a longer interval to delivery.

Introduction

Interleukin (IL)-18 is a recently identified cytokine (1). It is considered to have pleiotrophic qualities that regulate both the innate and acquired immune responses and it can stimulate both a T helper 1 and a T helper 2 response depending on the local cytokine environment (2, 3). IL-18 is synthesized as a pro-form and is activated through cleavage by caspase-1 (IL-1β converting enzyme) (4). IL-18 is important in the host defence against severe infections via induction of other cytokines and effector cells and molecules (2). It is mainly synthesized by macrophages, monocytes and keratinocytes, but can also be produced by epithelial cells (4-8). IL-18 enhances the inflammatory process by stimulating the production of interferon γ (INF-γ), tumor necrosis factor (TNF)-α and IL-1β (2). IL-12 can act synergistically with IL-18 to provoke a T helper 1 response (4, 9). The IL-

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18-receptor in the cell membrane activates the same intracellular signal pathway as the IL-1-receptor, through activation of nuclear factor κ§ (2, 10). Several human epithelial cell lines express pro-IL-18 and *Chlamydia trachomatis* infection has been shown to cause epithelial cells to produce mature IL-18 through caspase-1 activation (5). IL-18 can also activate apoptosis by enhancing Fas ligand and Fas expression (11).

IL-18 is present in the amniotic fluid (AF) as well as in maternal and foetal plasma (12-14). The levels of IL-18 appear to increase with advancing gestational age (12). Menon et al detected IL-18 mRNA in chorion and in the interface between decidua and chorion, whereas the amniotic epithelium was devoid of mRNA and protein for IL-18. Higher levels of IL-18 were found in women with preterm prelabour rupture of the membranes (pPROM), no microbial invasion of the AF or contractions, in comparison with women with preterm labour (PTL) (13). On the other hand, Pacora et al found that the concentration of IL-18 in the AF increases with microbial invasion of the AF in PTL and in women in labour at term (12). The elevated level of IL-18 in pPROM was suggested (13) to initiate apoptosis in the foetal membranes through the Fas-Fas-ligand pathway but this has not been confirmed. Thus, the role of IL-18 in PTL and pPROM remains uncertain, and no data are available on the levels of IL-18 in cervical mucus.

Considering its critical role in host defence, IL-18 may participate in providing a cervical/decidual barrier against microbial invasion of the AF and might, conceivably, serve as a cervical marker for intra-amniotic infection. Therefore, our primary aim was to investigate the relationship between IL-18 in cervical mucus and AF in PTL and in AF in pPROM and microbial invasion of the AF, preterm delivery (< 34 weeks, ≤ 7 days) and intra-amniotic inflammation. Secondly, the intra-amniotic IL-18 level in PTL was compared with that in pPROM, as IL-18-induced apoptosis has been suggested to be important in the rupture of membranes (13). Third, cervical mucus and AF levels in women in PTL and in non-labouring women at term were compared.

Methods

The study population consisted of women with singleton pregnancies in PTL (n=87) or with pPROM (n=47) who presented at 2 delivery wards in Göteborg (Sahlgrenska Hospital (1996-1997) and Sahlgrenska University Hospital/East (1997-2001) at a gestational age less than 34 weeks of gestation. PTL was defined as regular uterine contractions (at least 2 uterine contractions/10 minutes during ≥ 30 minutes) in combination with cervical changes at admittance to the clinic: ((1) ≤ 2 cm length + ≥ 1 cm dilatation or (2) ≤ 2 cm length + cervical softening or (3) ≥ 1 cm dilatation + cervical softening or (4) cervical length < 30 mm at endovaginal ultrasound. pPROM was defined as amniorrhexis (visible AF in the vagina) before the onset of spontaneous labour. Contractions began prior to amniocentesis in seven women with pPROM.

Women with known uterine abnormalities, foetal malformations, significant vaginal bleeding, imminent delivery or foetal distress were not included. Twenty-eight women at term (≥ 37 weeks) were included. These women were scheduled for an elective caesarean section (CS) with the following indications: psychosocial, breech presentation or two previous CS. None of the women at term had contractions or rupture of membranes prior to surgery.

Gestational age was determined in all women except three by routine ultrasound in the second trimester (16^{th} to 19^{th} weeks of gestation). Tocolytic therapy (intravenous terbutaline and/or indomethacin if the pregnancy was < 28 weeks of gestation) was administered according to the local protocol at the department.

Cervical mucus was obtained with a Cytobrush (Cytobrush Plus GT, Medscan Medical AB, Malmö, Sweden) from the external os of cervix in all women in PTL (n=87) and in all women at term (n=28). The cervical mucus was weighed and kept in a refrigerator (+4°C) until processed within 5 hours. The Cytobrush with the cervical mucus was submerged in 1.0 mL NaCl, shaken for 30 minutes at +4°C, followed by centrifugation at 855 g at +4°C for 10 minutes and storage at –80°C until analysis.

Ultrasound-guided transabdominal amniocentesis, aspirating 30 – 50 mL of AF, was performed in 59 women in PTL and in 47 women with pPROM under antiseptic conditions within 12 hours after admittance. In women at term, AF was retrieved during CS. A catheter was introduced into the amniotic cavity and 50 mL of AF was aspirated prior to opening the membranes. After retrieval, the AF was immediately placed in a refrigerator (+4°C) and was centrifuged within 5 hours of sampling at 855 g in +4°C for 10 minutes. The supernatant was stored at –80°C until analysis. A sample of uncentrifuged AF was immediately transported to the microbiological laboratory for polymerase chain reaction (PCR) analysis of *Ureaplasma urealyticum* and *Mycoplasma hominis* and for aerobic and anaerobic culture. Microbial invasion was defined as positive PCR and/or growth of any bacteria in the AF except *Coagulase negative Staphylococcus* that was considered to be skin contamination

IL-18 in AF was analysed with enzyme-linked immunosorbent assay (ELISA) (paired antibodies from R&D Systems, Minneapolis, Minn, USA). AF samples were diluted 1:5, 1:20 and 1:100 and run in duplicates. The inter-assay variation was calculated to < 25%, based on analysis of several samples on three separate occasions. Low values (≤ 400 pg/mL) showed the highest variation coefficient. The detection limit of the ELISA test was 30 pg/mL, but because the samples were run at 1:5 dilution, the actual lower limit of detection was 150 pg/mL.

We have analysed IL-6 and IL-8 in AF in the same population in previous studies (15, 16) and calculated the diagnostic indices for delivery within 7 days. A receiver-operator characteristic curve was used to identify the best cut-off levels for IL-6 and IL-8. Intra-amniotic inflammation was defined as IL-6 ≥ 1.5 ng/mL and/or IL-8 ≥ 1.3 ng/mL for women in PTL and as IL-6 ≥ 0.80 ng/mL and/or IL-8 ≥ 0.42 ng/mL for women with pPROM.

Two investigators (BJ, RMH) scrutinised the medical records and entered maternal and perinatal data into a database.

Ethical approval for the study was obtained from the local ethics committee in Göteborg. The women gave informed consent before enrolment in the study.

Calculations were made using the computer programs StatView 5.01 (SAS Institute Inc, Cary North Carolina, USA) and InStat 2.01 (Graph Pad Software, San Diego California, USA). Continuous variables were analysed with the Mann-Whitney U test and proportions with Fisher's exact test. A p-value < 0.05 was considered statistically significant, as was a confidence interval not including 1.00.

Results

Demographics, presence of bacteria in the AF, and pregnancy outcome are presented in Table I. IL-18 was detectable in the cervix in 60% (52/87) of women with PTL and in 11% (3/28) of the women at term. IL-18 was detectable in the amniotic samples in 73% (43/59) of the women in PTL and in 70% (33/47) of women with pPROM. In the term group, IL-18 was detected in AF in 11% (3/28) of the cases. Microorganisms isolated from the amniotic cavity were Ureaplasma urealyticum (n=8), *Mycoplasma hominis (n=1), Fusobacterium species* (n=1), *Corynebacterium* (no intra-amniotic inflammation) (n=1), *Eubacterium species* (n=1), *Actinomyces odontolyticus* (n=1), *Snethia sanguinegens* (n=1), *Listeria monocytogenes* (n=1), *Streptococcus mitis* (no intra-amniotic inflammation) (n=1), *Haemophilus influenzae* (n=1), *Streptococcus* agalactiae (n=1), *Bacteroides fragilis* (no intra-amniotic inflammation) (n=1), *Bifidobacterium adolecentis* (no intra-amniotic inflammation) (n=1), *Difteriodic rods* (n=1), *Anaerob gram-negative rods* (n=1) and Coagulase negative *Staphylococcus* (no intra-amniotic inflammation, n=3) (n=5) (15, 16). None of these women developed clinical chorioamnionitis.

<u>IL-18 in Cervical mucus</u>

There were higher levels of IL-18 in the cervical mucus in women in PTL compared with non-labouring women at term (median 0.51 ng/mL versus <0.15 ng/mL; p<0.001) (Fig. 1a). In women with PTL, no significant associations were found between cervical IL-

Table 1. Demographics and pregnancy outcome in women undergoing cesarean section at term, women in preterm labour (PTL) and with preterm prelabour rupture of the membranes (pPROM).

	Term	**PTL (CF)**	**p-value term/ PTL (CF)**	**PTL (AF)**	**PPROM**	**p-value (PTL(AF)/ pPROM)**
Number of patients (n)	28	87		59	47	
Maternal age (years)	34 (22-41)	29 (19-43)	0.002	29 (19-38)	30 (19-43)	0.49
Nulliparous (n)	11 (39%)	51 (59%)	0.09	36 (62%)	26 (55%)	0.55
Gestational age at sampling (days)	271 (263-288)	216 (160-237)	0.0003	216 (160-236)	221 (158-236)	0.07
Gestational age at delivery (days)	271 (263-288)	252 (168-299)	0.0003	242 (168-288)	232 (162-281)	0.04
Patients giving birth at <34 weeks (n)	NA		NA	25 (43%)	34 (72%)	0.003
Birth weight (gram)	3505 (2575-4340)	2785 (705-4675)	<0.0001	2550 (705-4675)	2170 (470-3940)	0.07
Microbial invasion of the amniotic fluid (n)	1 (3.6%)	NA	NA	9 (15.5%)	11 (23.4%)	0.32

Data are presented as median and range except for nulliparity, births < 34 weeks and microbial invasion of the amniotic fluid for which the number (n) and percentage (%) of the women in preterm and term population are given, respectively.
AF – amniotic fluid
CF – cervical fluid
NA – not applicable

18 and microbial invasion in the AF, time of delivery (≤ 7 days, < 34 weeks) or intra-correlation was found between IL-18 in cervical mucus and AF (r=0.30 and p=0.028).

Amniotic fluid IL-18

There were higher levels of IL-18 in the AF of women in PTL compared with non-labouring women at term (median 0.59 ng/mL versus < 0.15 ng/mL; p=0.004) (Fig. 1b).
Women in PTL with microbial invasion of the AF had significantly higher levels of IL-18 in the AF than those without microbial invasion. In women with pPROM, this association was not significant. In the term group, IL-18 was not detectable in the AF from the woman with positive PCR for neither *Ureaplasma urealyticum* nor the woman with coagulase negative *Staphylococcus*.
Women in PTL who delivered within 7 days after amniocentesis had significantly higher levels of IL-18 than those who delivered after 7 days (median 1.01 ng/mL versus 0.57 ng/mL, p=0.046) (Fig. 2b). In the pPROM group, the levels of IL-18 in women that delivered ≤ 7 days did not differ from those with an interval > 7days (median 0.24 ng/mL versus median 0.38 ng/mL; p=0.19). However, women with pPROM and low IL-18 in AF had shorter interval to delivery than those with high IL-18 levels (< 1.0 ng/mL: median interval 4 days; ≥ 1.0 ng/mL: median latency 36 days; p=0.03).

Table 2. Interleukin (IL)- 18 in cervical fluid in women in preterm labour related to different outcome variables

	IL-18 (ng/mL)	p-value
MIAC (n=7) / no MIAC (n=47)*	1.72 / 0.42	0.34
Delivery ≤ 7 days (n=26) / > 7 days (n=61)	1.26 / 0.41	0.16
Delivery < 34 weeks (n=37) / ≥ 34 weeks (n=50)	0.92 / 0.40	0.26
IAI (n=25) / No IAI (n=29)*	1.33/0.41	0.51

MIAC – microbial invasion of the amniotic cavity
IAI - Intra-amniotic inflammation
* cervical sample missing in 5 women

Women in PTL that delivered before 34 completed weeks had significantly higher levels of IL-18 than those who gave birth ≥ 34 weeks of gestation (median 1.0 ng/mL versus 0.47 ng/mL; p=0.02) (Fig. 2c). In women with pPROM, there was no association between AF IL-18 and delivery < 34 weeks (median 0.42 ng/mL versus 0.23 ng/mL; p=0.22).
Intra-amniotic IL-18 correlated to general intra-amniotic inflammation in women in PTL (median 0.81 ng/mL versus 0.39 ng/mL; p=0.02) (Fig. 2d), but no such relationship was found in the pPROM group (median 0.46 ng/mL versus 0.28 ng/mL; p=0.13).
No difference was found between IL-18 levels in the PTL and pPROM groups (median 0.59 ng/mL versus 0.35 ng/mL; p=0.068). Women with pPROM in the absence of both contractions and microbial invasion of the AF (n=31) did not have higher concentrations of IL-18 than those women with pPROM that had contractions and/or microbial AF invasion (n=16) (median 0.39 ng/mL versus 0.35 ng/mL; p=0.75).

Fig. 1

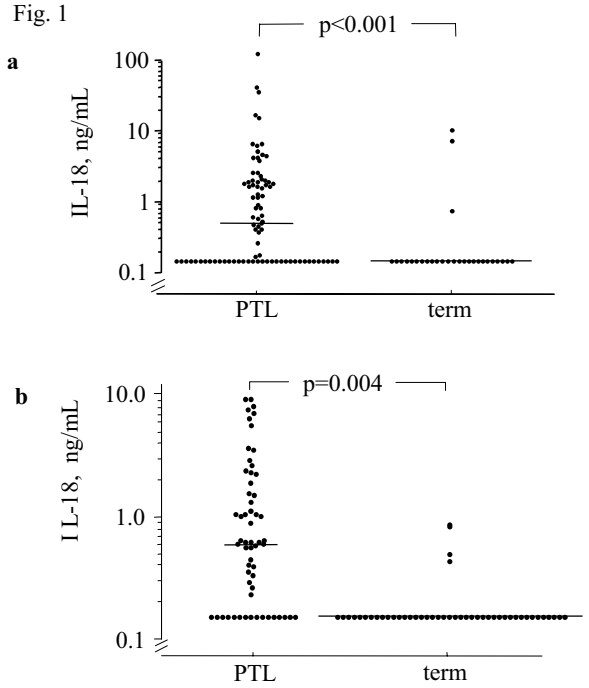

Fig. 1 Interleukin-18 in cervical fluid from women in PTL and at term (a) and in amniotic fluid from women in PTL and at term (b). Horizontal bars indicate medians.

Discussion and Conclusion

This is the first study to document IL-18 in cervical mucus and its relationship to PTL. The major finding of this study is that high levels of AF IL-18 correlated to intra-amniotic inflammation and prompt delivery in women presenting with PTL, whereas IL-18 levels ≥ 1.0 ng/mL were associated with a longer interval to delivery in the pPROM group. In agreement with a previously published study (12), we found that IL-18 in AF is associated with microbial invasion of the AF and preterm delivery in PTL women (12).
The level of IL-18 in cervical mucus and AF was higher in women in PTL than in non-labouring women at term. A poor correlation was observed between the levels of IL-18 in cervical mucus and AF. Pacora et al have shown that the levels of IL-18 in AF increases with

Fig. 2

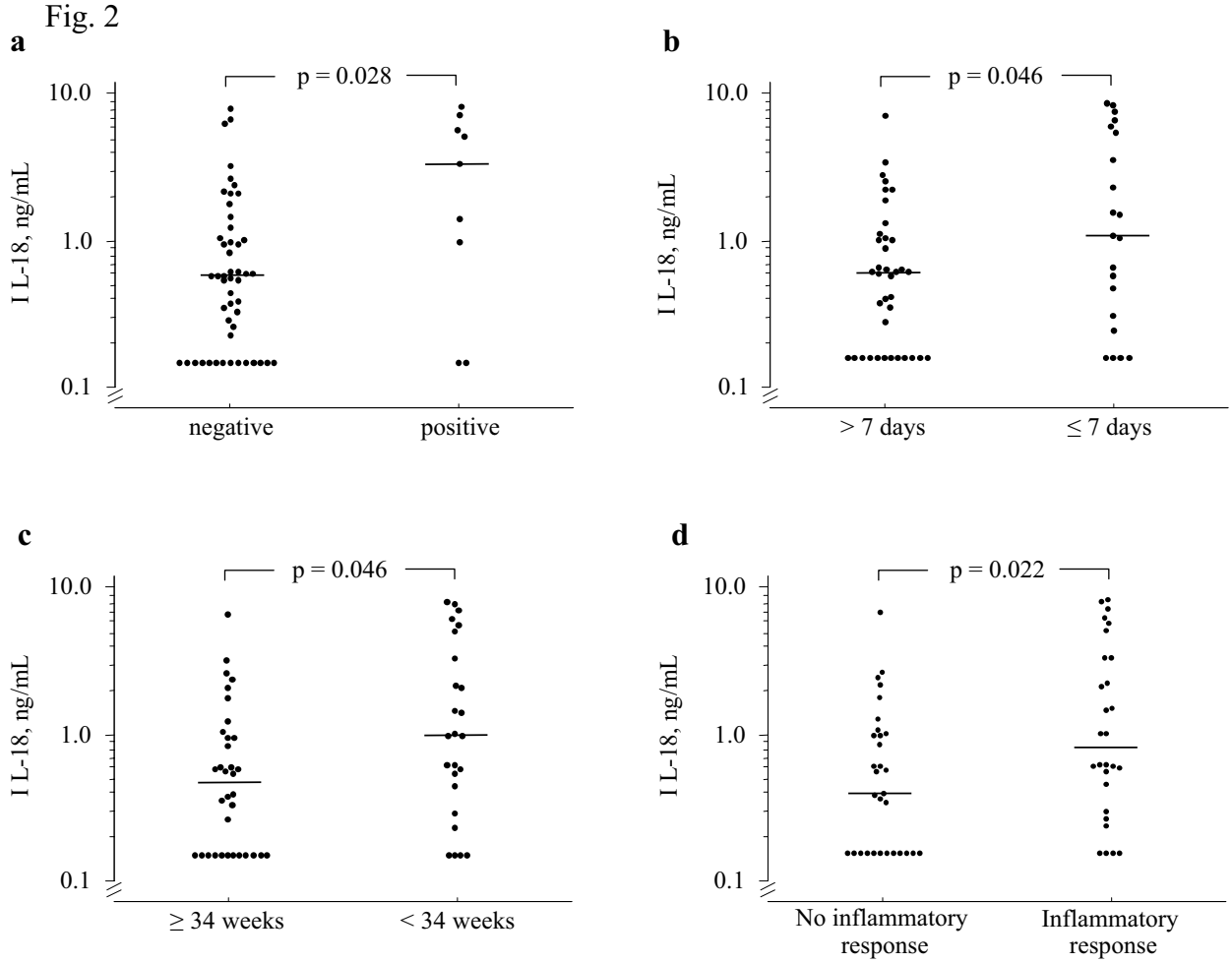

Fig. 2. Interleukin-18 in amniotic fluid from women in PTL. The levels are related to microbial invasion in the amniotic fluid, as defined by a positive culture or PCR (a), to interval between amniocentesis and delivery (> 7 vs. ≤ 7 days) (b), to gestational age at delivery (>34 vs. <34 weeks of gestation) (c) and presence of an inflammatory response (defined as increased levels of IL-6 (≥ 1.5 ng/mL) and/or IL-8 (≥ 1.3 ng/mL)) (d). Horizontal bars indicate medians.

gestational age (12). Our interpretation of the differences between women in PTL and non-labouring at term is that the higher levels of IL-18 are related to the general inflammatory response in PTL; this is reflected both in the cervical mucus and AF.

There was no association between cervical IL-18 and microbial invasion of the AF, intra-amniotic inflammation and delivery within 7 days or < 34 weeks, suggesting that this cytokine is unlikely to be of value as a cervical marker of preterm birth or infection in women in PTL.

IL-18 plays a well-documented role in the development of inflammatory disease, e.g. rheumatoid arthritis and inflammatory bowel disease (3, 6). In this study, it is shown that intra-amniotic IL-18 correlate to the general intra-amniotic inflammation in women in PTL. The IL-18 level was also higher in women that delivered shortly after admission, indicating that IL-18 is part of the inflammatory response in PTL. This is interesting as IL-18 could also be involved in the foetal inflammatory response syndrome, which might have pathophysiological consequences, as this cytokine could exert adverse effects in the foetus. A recently published study shows that preterm infants who later developed periventricular leukomalacia or cerebral palsy had elevated concentrations of IL-18 in cord blood (17). Experimental studies also show that IL-18 mediates liver cell injury in response to bacteria (18) and, like IL-1β, induces injury in the CNS (19).

IL-18 plays a role in the defence against both extra- and intracellular bacterial infection (20). Initially, IL-18 was called INF-γ–inducing factor and INF-γ is known to play a central role in the immune response against infectious agents (4, 9). This is also supported by data from several animal studies (21-23). IL-18 has the capacity to stimulate cytotoxic natural killer cells and stimulates T-cells to produce INF-γ and granulocyte/macrophage colony-stimulating factor (8). Bacteria found in the AF in women in PTL, e.g. *Corynebacteria, Pseudomonas aeruginosa, Haemophilus influenzae* and *Chlamydia tracomatis*, have been shown to provoke a IL-18 response in vivo in other compartments of the human body (20). It is therefore interesting that we find, in agreement with Pacora (12), higher IL-18 levels in cases of PTL with positive cultures. It is a tentative hypothesis that IL-18 is part of the immune response to bacteria within the amniotic cavity of women in PTL.

Women in PTL who gave birth delivery within 7 days (or ≤34 weeks) had higher levels of IL-18 in the AF than those who delivered after 7 days (or >34 weeks). Such a relationship between IL-18 and interval to delivery was not found by Pacora et al (12). This discrepancy could be due to the difference in gestational age (<34 weeks vs. <37 weeks, respectively) in our study compared and Pacora's et al study (12), since inflammation might be more important at low gestational ages (24). In women with pPROM, the opposite relationship was found, i.e. high IL-18 was associated with longer interval to delivery. It is also important to note that the amniotic concentrations of IL-18 in women with pPROM and microbial invasion of the AF were not higher than in cases of pPROM with sterile AF. The response is different for IL-6 and IL-8, levels of which were higher in women with microbial invasion both in the PTL and pPROM groups (15, 16). These data suggest that IL-18 plays different roles in PTL and pPROM. A similar dichotomy between PTL and pPROM has previously been observed regarding TNF-α and IL-1α (12, 25). The mechanisms involved are unclear, but IL-18, TNF-α and IL-1α all possess cytotoxic properties, in contrast to IL-6 and IL-8 that seem to be part of the general activation of the inflammatory response (26).

Neither our study, nor Pacora's (12), could confirm the findings by Menon et al (13) of higher levels of IL-18 in pPROM. On the contrary, our data indicated a tendency towards lower values in the pPROM group.

In conclusion, IL-18 is involved in intra-amniotic inflammation related to PTL and host defence. IL-18 in cervical mucus cannot serve as a marker of microbial invasion of the AF or preterm delivery. Our data supports, the idea that IL-18 play a different role in pPROM than in PTL.

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IV

Monocyte chemotactic protein-1 in cervical and amniotic fluid: relationship to microbial invasion of the amniotic cavity, intra-amniotic inflammation and preterm delivery

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Objective: To evaluate the role of monocyte chemotactic protein-1 (MCP-1) in cervical and amniotic fluid in women in preterm labor (PTL) and with preterm prelabor rupture of membranes (pPROM).
***Study design*:** Women with singleton pregnancies (≤ 34 weeks) in PTL (n=75), pPROM (n=47) and term women (n=45) undergoing elective cesarean section were included. Cervical and amniotic fluid were sampled.
Results: MCP-1 in cervical and amniotic fluid was higher in women in PTL than at term. Cervical MCP-1 in women in PTL was associated with microbial invasion of the amniotic cavity, intra-amniotic inflammation, delivery within 7 days and ≤ 34 weeks. Amniotic MCP-1 correlated to microbial invasion of the amniotic cavity in women with pPROM, intra-amniotic inflammation in PTL and pPROM, delivery within 7 days and delivery ≤ 34 weeks in women in PTL.
Conclusions: MCP-1 in cervical and amniotic fluid is elevated in PTL and pPROM, and correlates to intra-amniotic infection/inflammation.

Introduction

Some normal functions of the female reproductive tract such as parturition and cervical ripening depend on inflammatory processes (1). In addition, inflammation appears to be critical in certain pathological processes, e.g. in the preterm labor syndrome (2). Modern understanding of reproductive functioning indicates the existence of local paracrine-autocrine regulatory mechanisms (1). Inflammatory processes are regulated by a coordinated expression of pro- and anti-inflammatory cytokines and chemokines. Chemokines (short for chemoattractant cytokines) play a crucial role in this process by regulating the leukocyte traffic. A great deal of research has identified the chemoattractants and activators responsible for neutrophil and lymphocyte traffic but less is known about the molecules regulating monocyte migration. Certain chemokines, such as monocyte chemotactic protein (MCP)-1, have been demonstrated to recruit monocytes into foci of active inflammation (3). MCP-1 is considered to be a prototypic b-chemokine. It has been proven that MCP-1 is involved in several aspects of uterine function and in local regulation of endometrial processes such as menstruation and implantation (1).
The preterm labor syndrome has been characterized as an inflammatory-like condition (2). Several cytokines with proinflammatory properties, such as interleukin (IL)-6, IL-1b, IL-18 and tumor necrosis factor-a, are known to be involved in this process (2). Information on chemokines in pregnancy has focused on IL-8, macrophage inflammatory

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protein (MIP)-1a, GRO-a and RANTES (4-7). RANTES and MIP-1a are other b-chemokines that have been studied in relation to preterm birth and proven to be increased in patients with microbial invasion of the amniotic cavity (MIAC) (4, 6).

Considering its critical role in the traffic of important cells in inflammatory processes and the host defense, MCP-1 might participate in providing a cervical/decidual defense line against MIAC and might conceivably serve as a cervical marker for MIAC and intra-amniotic inflammation. The clinical signs of intra-amniotic infection are often non-existent, and it is only by the invasive procedure of amniocentesis that intra-amniotic conditions can be revealed. A good cervical marker of MIAC, intra-amniotic inflammation and preterm delivery would be clinically important as cervical sampling is less invasive than amniocentesis. Preliminary reports indicate that amniotic MCP-1 may play a role in term parturition (8) as well as in preterm labor (PTL) and preterm prelabor rupture of membranes (pPROM)(9) but no information has been published on cervical MCP-1 concentrations.

The primary aim was to compare the levels of MCP-1 in cervical and amniotic fluid (AF) in women in PTL and in non-laboring women at term. Secondly, the relationship between MCP-1 in AF and/or cervical fluid and MIAC, intra-amniotic inflammation and preterm delivery was investigated in patients with PTL and pPROM.

Material and Methods

A prospective cohort study was performed. The study population consisted of women with singleton pregnancies in PTL (n=75) or with pPROM (n=47) who presented at 2 delivery wards in Göteborg (Sahlgrenska Hospital (1996-1997) and Sahlgrenska University Hospital/East (1997-2001)) at a gestational age less than 34 weeks of gestation. PTL was defined as regular uterine contractions (at least 2 uterine contractions/10 minutes for ≥ 30 minutes) in combination with cervical changes: ((1) ≤ 2 cm length + ≥ 1 cm dilatation or (2) ≤ 2 cm length + cervical softening or (3) ≥ 1 cm dilatation + cervical softening or (4) cervical length <30 mm at endovaginal ultrasound). pPROM was defined as amniorrhexis (visible AF in the vagina) before the onset of spontaneous labor. Contractions began prior to amniocentesis in seven cases of pPROM. Women with known uterine abnormalities, fetal malformations, significant vaginal bleeding, imminent delivery or fetal distress were not included. Forty-five women at term (≥ 37 weeks) were included. These women were scheduled for an elective cesarean section (CS) with the following indications: psychosocial, breech presentation or two previous CS. None of the term patients had contractions or rupture of membranes prior to surgery.

Gestational age was determined in all patients except three by routine ultrasound in the second trimester (16^{th} to 19^{th} weeks of gestation). Date of last menstruation was used for gestational length determination when a routine ultrasound was not available. Tocolytic therapy (intravenous terbutaline and/or indomethacin, the latter if the pregnancy was < 28 weeks of gestation) was administered according to department protocol.

Cervical fluid was obtained with a Cytobrush (Cytobrush Plus GT, Medscan Medical AB, Malmö, Sweden) from the external cervical os in all patients in PTL (n=75) and in all term patients (n=45). The cervical mucus was weighed and kept in a refrigerator (+4°C) until processed, which occurred within 5 hours. The Cytobrush with the cervical mucus was submerged in 1.0 mL NaCl, shaken for 30 minutes at +4°C, followed by centrifugation at 855 g at +4°C for 10 minutes and storage at –80°C until analysis.

Ultrasound-guided transabdominal amnio-centesis, aspirating 30-50 ml of AF, was performed in 61 women in PTL and in 47 women with pPROM under antiseptic conditions within 12 hours after admission. In women at term, AF was retrieved during CS. A catheter was introduced into the amniotic cavity and 50 mL of AF was aspirated prior to opening the membranes. After retrieval, the AF was immediately placed in a refrigerator (+4°C) and centrifuged, within 5 h of sampling, at 855 g in

Table I. Demographics and pregnancy outcome in women undergoing to cesarean section at term, women in preterm labor (PTL) and with preterm prelabor rupture of membranes (pPROM).

	Term	PTL (CF)	p-value term vs. PTL (CF)	PTL (AF)	pPROM	p-value (PTL(AF) vs. pPROM)
Number of patients (n)	45	75		61	47	
Maternal age (years)	33 (22-41)	29 (19-43)	<0.001	29 (19-38)	30 (19-43)	0.42
Nulliparous (n)	19 (42%)	45 (60%)	0.09	38 (62%)	26 (55%)	0.55
Gestational age at sampling (days)	270 (263-288)	216 (160-237)	<0.001	216 (160-236)	222 (158-236)	0.08
Gestational age at delivery (days)	270 (263-288)	254 (168-299)	<0.001	242 (168-288)	232 (162-281)	0.06
Birth weight (gram)	3500 (2575-4340)	2930 (705-4675)	<0.001	2425 (705-4675)	2175 (470-3940)	0.11
Patients giving birth at <34 weeks (n)	NA	22 (29%)	NA	27 (44%)	34 (72%)	0.005
Term delivery (≥ 37 weeks)	45 (100%)	28 (37%)	<0.001	22 (36%)	5 (11%)	0.003
Delivery ≤ 7 days	NA	17	NA	23 (38%)	27 (57%)	0.05
Microbial invasion of the amniotic fluid (n)	1 (2%)	8/45 (18%)	0.03	11 (18%)	13 (27%)	0.25
Intra-amniotic inflammation	0 (0%)	19/45 (42%)	<0.001	28 (46%)	27 (57%)	0.25

Data are presented as median and range except for nulliparity, births < 34 weeks, term delivery (≥ 37 weeks), delivery ≤ 7 days, microbial invasion of the amniotic fluid and intra-amniotic inflammation for which the number (n) and percentage (%) of the women in the preterm and term population, respectively, are given.
AF – amniotic fluid
CF – cervical fluid
NA – not applicable

+4°C for 10 minutes. The supernatant was stored at –80°C until analysis.

A sample of uncentrifuged AF was immediately transported to the microbiological laboratory for polymerase chain reaction (PCR) analysis of *Ureaplasma urealyticum* and *Mycoplasma hominis* and for aerobic and anaerobic culture. MIAC was defined as positive PCR and/or growth of any bacteria in the AF except for coagulase-negative *Staphylococcus*, which was considered to be a skin contamination. However, coagulase-negative *Staphylococcus* in AF from patients with an intra-amniotic inflammation (higher levels of IL-6 and IL-8), was considered to be MIAC (10, 11).

MCP-1 in cervical fluid and AF was analyzed with enzyme-linked immunosorbent assay (ELISA) (paired antibodies from R&D Systems, Minneapolis, Minn, USA). The samples were diluted 1:2, 1:3 and 1:6 in different analysis sets and run in duplicates.

We have analyzed IL-6 and IL-8 in AF in the same population in previous studies (10, 11) and calculated the diagnostic indices for delivery within 7 days. A receiver-operator characteristic curve (ROC) was used to define intra-amniotic inflammation. Intra-amniotic inflammation was defined as IL-6 ≥ 1.5 ng/mL and/or IL-8 ≥ 1.3 ng/mL for women in PTL and as IL-6 ≥ 0.80 ng/mL and/or IL-8 ≥ 0.42 ng/mL for women with pPROM.

Sensitivity, specificity and positive and negative predictive values were calculated for different concentrations of MCP-1 in cervical fluid and AF in relation to delivery within 7 days after amniocentesis, MIAC and intra-amniotic inflammation. ROC curves were used to identify the best cutoff levels of MCP-1 in cervical fluid and AF for the different outcome variables of interest.

Two investigators (BJ, RMH) scrutinized the medical records and entered maternal and perinatal data into a database. The study was approved by the local ethics committee in Göteborg. The women gave informed consent before enrollment in the study.

Calculations were made using the computer programs StatView 5.01 (SAS Institute Inc, Cary, North Carolina, USA) and InStat 2.01 (Graph Pad Software, San Diego, California, USA). Continuous variables were analyzed with the Mann-Whitney U test and proportions with Fisher's exact test. Spearman's rank correlations test was used for analysis of correlation between continuous variables. A p-value < 0.05 or a confidence interval not including 1 was considered to be statistically significant.

Results

Demographics, presence of bacteria in the AF, and pregnancy outcome are presented in Table I. Analysis of MCP-1 in the cervical sample was successful in the 75 women in PTL and in all term patients. A MCP-1 analysis of the AF was available in all 61 PTL patients, in all 47 patients with pPROM and in 44 of the 45 term patients. In 45 of the PTL and in 44 of the term women, MCP-1 analyses were available both in cervical and AF. MCP-1 was detectable in cervical fluid in 95% (71/75) of the women in PTL and in 87% (39/45) of the term women. MCP-1 was detectable in all AF samples. Microorganisms isolated from the amniotic cavity and the corresponding inflammatory response have been presented in two previous papers (10, 11).

Fig. 1

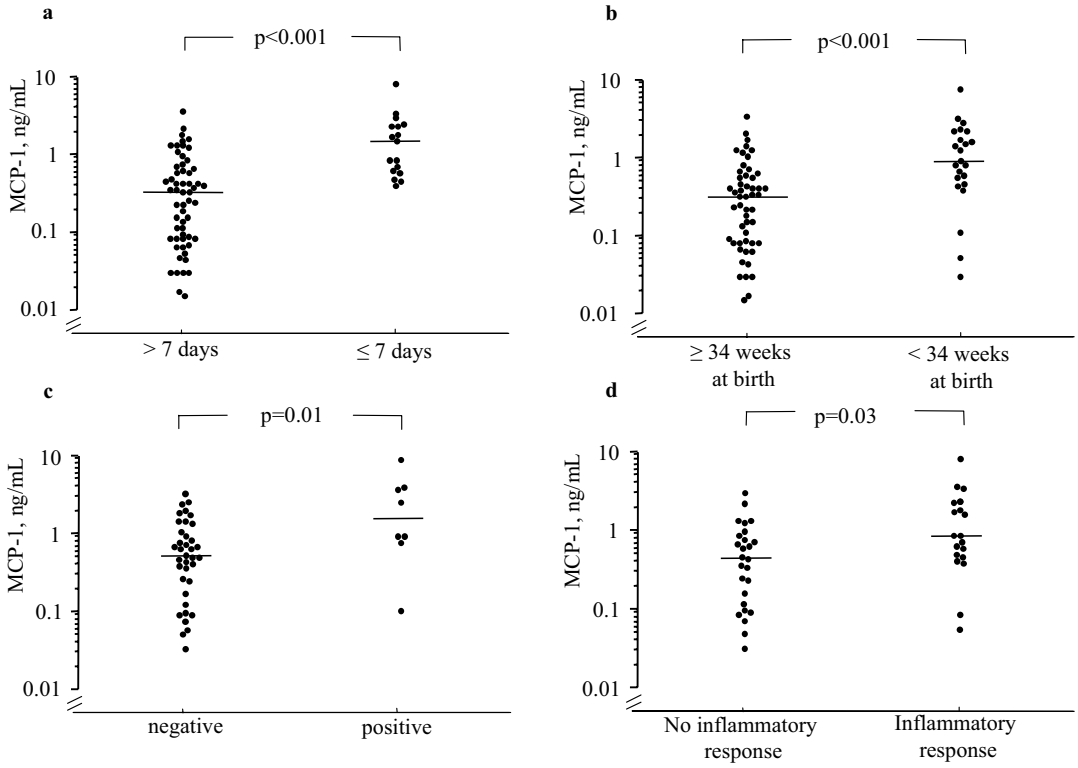

Fig. 1. Monocyte chemotactic protein (MCP)-1 in cervical fluid from women in preterm labor. The levels are related to interval between sampleing and delivery (> 7 vs. ≤ 7 days) (a); to gestational age at delivery (≥34 vs. <34 weeks of gestation) (b); to microbial invasion in the amniotic cavity, defined by a positive or negative culture or PCR (c); and to inflammatory response in the amniotic fluid, defined as increased levels of IL-6 (≥ 1.5 ng/mL) and/or IL-8 (≥ 1.3 ng/mL) (d). Horizontal bars indicate medians.

Cervical fluid MCP-1

There were higher levels of MCP-1 in the cervical fluid in women in PTL compared with non-laboring women at term (median 0.42 ng/mL, range 0.016-8.5 ng/mL versus median 0.22 ng/mL, range 0.010-8.5 ng/mL; p=0.016).

There were higher levels of MCP-1 in the cervical mucus in women in PTL who gave birth within 7 days than those with a longer sample-delivery interval (median 1.5 ng/mL, range 0.41-8.5 ng/mL versus median 0.34 ng/mL, range 0.016-3.7 ng/mL; p<0.001) (Fig. 1a) and higher levels of MCP-1 in PTL women who gave birth before 34 weeks of gestation than in those ≥ 34 weeks of gestation (median 0.99 ng/mL, range 0.031-8.5 ng/mL versus 0.34 ng/mL, range 0.016-3.7 ng/mL; p<0.001) (Fig. 1b).

MCP-1 levels were higher in the cervical mucus in women in PTL with than in those without MIAC (median 1.63 ng/mL, range 0.099-8.5 ng/mL versus median 0.50 ng/mL, range 0.031-3.1 ng/mL; p=0.01) (Fig. 1c). There were also higher levels of MCP-1 in the cervical mucus in women in PTL who had intra-amniotic inflammation (median 0.85, range 0.055-8.5 ng/mL versus median 0.37, range 0.031-2.2 ng/mL; p=0.02) (Fig. 1d).

Amniotic fluid MCP-1

There were higher levels of MCP-1 in the AF in women in PTL compared with non-laboring women at term (median 0.66 ng/mL, range 0.19-12 ng/mL versus median 0.32 ng/mL, range 0.13-1.3 ng/mL; p<0.001).

Women in PTL with MIAC did not have significantly higher levels of MCP-1 in the AF than women without MIAC (median 2.0 ng/mL, range 0.21-12 ng/mL versus median 0.61 ng/mL, range 0.19-4.0 ng/mL; p=0.063) (Fig. 2a). In women with pPROM, the levels of MCP-1 were higher in women with than in those without MIAC (median 2.2 ng/mL, range 0.37-4.0 ng/mL versus median 0.74 ng/mL, range 0.13-3.9 ng/mL; p=0.0017) (Fig. 2b).

Women in PTL who gave birth within 7 days after amniocentesis had significantly higher levels of MCP-1 than those who gave birth after

Fig. 2

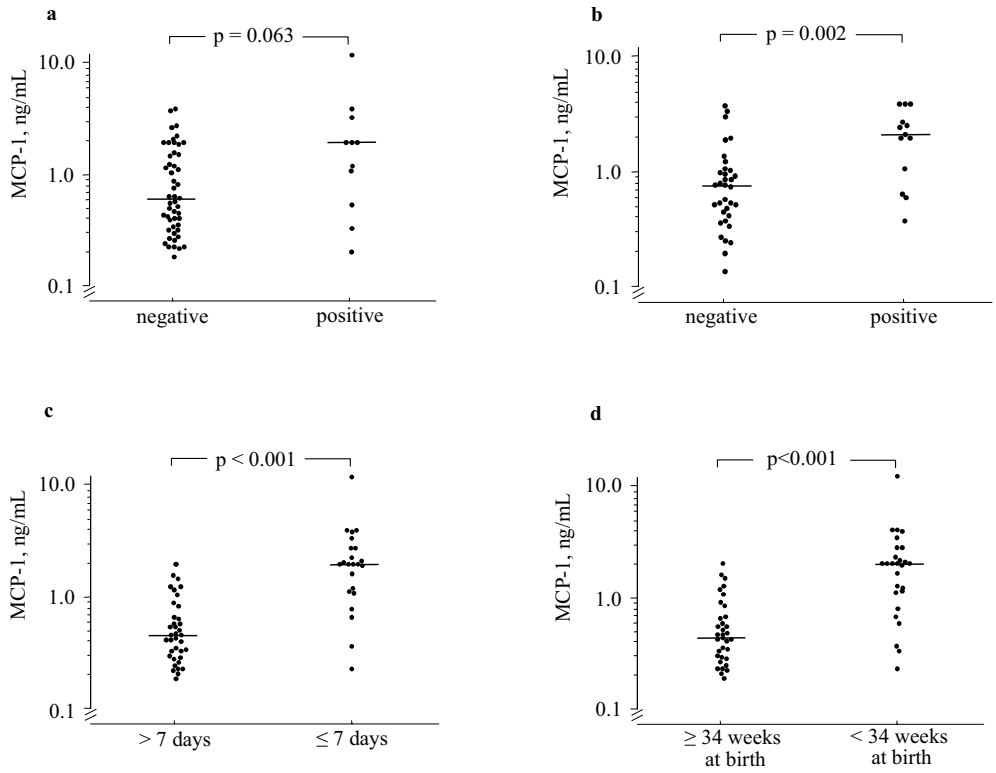

Fig. 2. Monocyte chemotactic protein (MCP)-1 in amniotic fluid from women in preterm labor (PTL) (a, c, d) or with preterm prelabor rupture of membranes (pPROM) (b). The levels are related to microbial invasion in the amniotic cavity, defined by a positive or negative culture or PCR, in PTL (a); to microbial invasion in the amniotic fluid in pPROM (b); to time between amniocentesis and delivery (> 7 vs. ≤ 7 days) (c); and to gestational age at delivery (≥34 vs. <34 weeks of gestation) (d). Horizontal bars indicate medians.

7 days (median 2.0 ng/mL, range 0.23-12 ng/mL versus median 0.46 ng/mL, range 0.19-2.0 ng/mL; p< 0.001) (Fig. 2c). In the pPROM group, the levels of MCP-1 in patients that delivered within 7 days did not differ from those with an interval > 7 days (median 0.73 ng/mL, range 0.13-4.0 ng/mL versus median 0.90 ng/mL, range 0.24-4.0 ng/mL; p=0.65). Three patients were allocated to the spontaneously delivered group despite labor being induced. However, the results were not affected by exclusion of these cases. We have performed a log rank survival analysis regarding the best MCP-1 cut-off level in AF (≥ 2.0 ng/mL) for delivery within seven days of women in pPROM, with censor of patients with induction of labor or cesarean section before delivery had started (n=12). A significant difference between the two groups regarding long interval to delivery (p=0.01) was found, in contrast to short interval to delivery (Fig. 3).

Women in PTL who delivered before 34 weeks had significantly higher levels of MCP-1 than those who gave birth ≥ 34 weeks of gestation (median 2.0 ng/mL, range 0.23-12 ng/mL versus median 0.44 ng/mL, range 0.19-2.0 ng/mL; p<0.001) (Fig. 2d). In women with pPROM, there was no association between AF MCP-1 and delivery < 34 weeks (median 0.94 ng/mL, range 0.13-4.0 ng/mL versus median 0.77 ng/mL, range 0.24-1.9 ng/mL; p=0.21). There were 3 inductions and 2 cesarean sections before the onset of contractions; these cases could be misclassified as spontaneous delivery < 34 weeks. If these patients were excluded, no differences were seen in results.

MCP-1 in AF correlated to general intra-amniotic inflammation both in women in PTL (median MCP-1 2.0 ng/mL, range 0.23-12 ng/mL versus median 0.41 ng/mL, range 0.19-1.6 ng/mL; p<0.001) and in women with pPROM (median MCP-1 1.0 ng/mL, range 0.26-4.0 ng/mL versus median 0.52 ng/mL, range 0.13-1.9 ng/mL; p=0.006).

MCP-1 in cervical fluid predicted preterm birth and MIAC with a high negative but low positive predictive value (Table II, III). The AF levels of MCP-1 also predicted MIAC and intra-amniotic inflammation in patients with pPROM (Table IV).

A correlation was seen between MCP-1 in cervical fluid and AF (r=0.35 and p=0.02). Data concerning IL-8 and IL-6 in these patients has been published previously (13,14) and was used for comparison in this study. There was a good correlation between MCP-1, IL-8 and IL-6 in AF in both the PTL (IL-8 r=0.74 and p<0.001; IL-6 r=0.70, p<0.001) and the pPROM groups (IL-8 r=0.64, p<0.001; IL-6 r=0.41, p=0.005).

Fig. 3

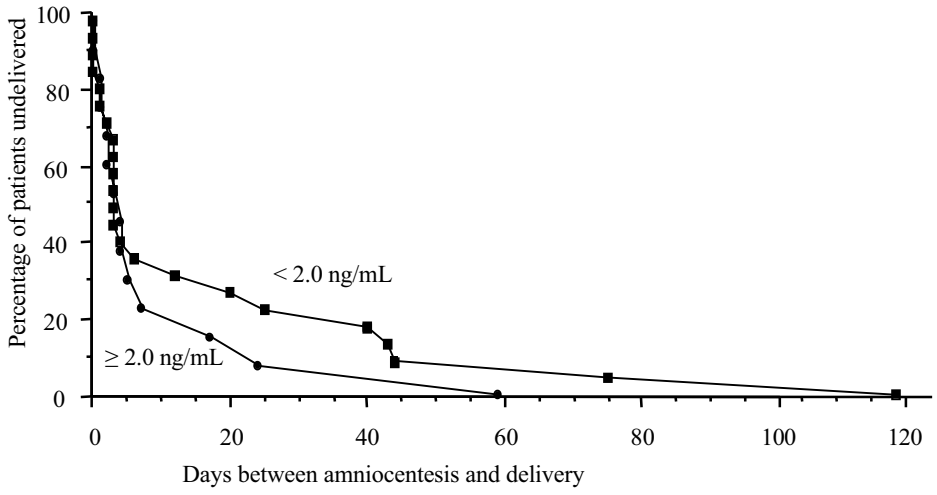

Fig. 3. Survival analysis of elapsed time between amniocentesis and delivery in women with pPROM, according to the best cut-off level of monocyte chemotactic protein (MCP)-1 (≥ 2.0 ng/mL) for delivery within 7 days.

Table II. Diagnostic indices for cervical and amniotic fluid monocyte chemotactic protein-1 (MCP-1) as predictor of delivery at ≤ 7 days in women in preterm labor.

	Cervical fluid	Amniotic fluid
	MCP-1 ≥0.60 ng/mL	MCP-1 ≥1.0 ng/mL
Sensitivity	76 %	83 %
Specificity	72 %	79 %
Positive predictive value	45 %	70 %
Negative predictive value	91 %	88 %
Relative risk and 95% confidence interval	5.2 (1.9– 14)	6.0 (2.3-16)

Table III. Diagnostic indices for cervical and amniotic fluid monocyte chemotactic protein-1 (MCP-1) as predictor of microbial invasion of the amniotic cavity and inflammatory response in the amniotic fluid women in preterm labor.

	Cervical fluid	Cervical fluid	Amniotic fluid	Amniotic fluid
	MCP-1 ≥0.80 ng/mL (microbial invasion of the amniotic cavity)	MCP-1 ≥0.50 ng/mL (inflammatory response)	MCP-1 ≥1.0 ng/mL (microbial invasion of the amniotic cavity)	MCP-1 ≥0.70 ng/mL (inflammatory response)
Sensitivity	75 %	74 %	73 %	89 %
Specificity	70 %	54 %	62 %	85 %
Positive predictive value	35 %	54 %	30 %	83 %
Negative predictive value	93 %	74 %	91 %	90 %
Relative risk and 95% confidence interval	4.9 (1.1– 22)	2.0 (0.89– 4.7)	3.4 (0.98-12)	8.6 (2.9-26)

Comments

This is the first study that documents MCP-1 in cervical fluid and its relationship to MIAC, intra-amniotic inflammation and preterm delivery. The cervical level of MCP-1 was a predictor of delivery within 7 days but with a low positive predictive value (44 %). MCP-1 in AF was also a predictor for delivery within 7 days. MCP-1 in AF is a marker of intra-amniotic inflammation and correlating well with IL-6 and IL-8 in AF. Cervical MCP-1 was also a predictor of MIAC but with a low positive predictive value (35%). However, cervical MCP-1 may not be the ultimate non-invasive tool to screen for MIAC, intra-amniotic inflammation and preterm delivery.

Cervical ripening has been described as an inflammatory process (12). The infiltration of white blood cells (both neutrophils and macrophages) into the cervix is known to occur in women at term (13). Neutrophils are responsible for most of the connective tissue changes that take place during cervical ripening, but macrophages are also involved, although their specific role is still unclear (14). There is a tenfold increase of the number of macrophages in cervical tissue from early to

Table IV. Diagnostic indices for amniotic fluid monocyte chemotactic protein-1 (MCP-1) as predictor of microbial invasion of the amniotic cavity and inflammatory response in the amniotic fluid in women with preterm prelabor rupture of membranes.

	Amniotic fluid	Amniotic fluid
	MCP-1 ≥1.0 ng/mL (microbial invasion of the amniotic cavity)	MCP-1 ≥0.90 ng/mL (inflammatory response)
Sensitivity	77 %	82 %
Specificity	74 %	73 %
Positive predictive value	53 %	63 %
Negative predictive value	89 %	88 %
Relative risk and 95% confidence interval	4.9 (1.5-16)	5.3 (1.7-16)

late pregnancy (13) and they also increase in number in relation to the final cervical ripening at birth (15). Chemokines have been proposed to be involved in cervical ripening via their chemoattractant and activating effects on neutrophils and monocytes (1). Although functional redundancy exists with other chemokines in vitro, MCP-1 alone is responsible for mononuclear cell infiltration in several inflammatory animal models in vivo (16). It is thus possible that MCP-1 is also involved in the process of cervical ripening, since it is a key chemokine in the activation and recruitment of monocytes and macrophages.

Further studies are required to determine the source of MCP-1 in cervical fluid and AF. The poor correlation between the levels of MCP-1 in cervical fluid and AF in our study may indicate different production sites. MCP-1 is known to be produced by term placenta, decidua and chorion and, to a lesser extent, by the amniotic epithelium (17). It has been shown that cell cultures of cervical fibroblasts can produce MCP-1 (18) and immunohistologically stained cervical biopsies from pregnant women are positive for MCP-1 (19). MCP-1 is most probably produced locally by cervical tissue. However, the decidua and membranes can be an alternative source of MCP-1 as the inflammatory process leads to disruption of the chorio-decidual interface, leading to its possible release into the cervical fluid (20). Kent et al found that AF does not reflect the cytokines produced by the decidua in case of intact and non-inflamed fetal membranes (21). As AF does not reflect the cytokines/chemokines produced by the decidua and the amniotic epithelium appears to be a minor producer of MCP-1, this suggests that the fetus itself may be a source of MCP-1. There are no reports of MCP-1 levels in umbilical cord blood but MCP-1 is present in dried neonatal blood spots obtained from 3 day old neonates (22) and the respiratory tract of the preterm baby is a known producer of MCP-1 (23).

The level of MCP-1 in cervical fluid and AF was higher in women in PTL than in non-laboring women at term. MCP-1 in AF probably reflects the feto-placental inflammatory response, whereas cervical MCP-1 reflects both inflammation related to cervical ripening and the generalized feto-placental inflammatory response.

Monocytes and macrophages play a critical role in the immune response, both by being potent producers of proinflammatory cytokines, matrix metalloproteinases and prostaglandins

and by processing and presenting antigens to T-cells for recognition. In that sense, they regulate both the innate and the specific immune systems. It has been proposed that the b-chemokines are likely to induce chemotaxis of monocytes and macrophages into the amniotic cavity and activate them (4). b-chemokines may be involved in the host defense against MIAC and in intra-amniotic inflammation. It was not surprising that there was a significant association between MCP-1 and MIAC in women with pPROM, which is in accordance with preliminary data reported by Esplin et al. (9). MCP-1 is thus the third b-chemokine (MIP-1-a, RANTES) that has been shown to increase in response to MIAC (4, 6). It is also interesting to note that MCP-1 is part of the intra-amniotic inflammation in both PTL and pPROM. However, in contrast to the findings in the PTL group, we found no correlation between MCP-1 in AF and delivery within 7 days or before 34 weeks of gestation in patients with pPROM. A similar result was recently obtained for IL-18 (24), supporting the idea that intra-amniotic inflammation has different characteristics and outcome in pPROM and PTL, respectively (25).

The major finding of this study is that cervical MCP-1 correlated to MIAC, intra-amniotic inflammation and preterm delivery in women in PTL. In addition, we found that AF MCP-1 is also associated with MIAC in women with pPROM, with intra-amniotic inflammation in women in PTL or with pPROM, and preterm birth in women in PTL.

Acknowledgments

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V

Errata paper V

Abstract Page 946, line 11 should be: .. entire group, clinical chorioamnionitis ....
Table 2 should be: median
Table 3 should be: median
Table 3 line12 should be: 12/146 (8)

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Cerebral palsy in preterm infants: a population-based case–control study of antenatal and intrapartal risk factors

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Jacobsson B, Hagberg G, Hagberg B, Ladfors L, Niklasson A, Hagberg H. Cerebral palsy in preterm infants: a population-based case–control study of antenatal and intrapartal risk factors. Acta Pædiatr 2002; 91: 946–951. Stockholm. ISSN 0803-5253

Previous studies have indicated that foetomaternal infection increases the risk of spastic cerebral palsy (CP) in term infants, whereas this association appears to be less evident in preterm infants. The aim of this study was to analyse infection-related risk factors for spastic CP in preterm infants. A population-based series of preterm infants with spastic CP, 91 very preterm (<32 wk) and 57 moderately preterm (32–36 wk), born in 1983–90, were included and matched with a control group ($n = 296$). In total, 154 maternal, antenatal and intrapartal variables were retrieved from obstetric records. In the entire group, histological chorioamnionitis/pyelonephritis, long interval between rupture of membranes and birth, admission–delivery interval <4 h and Apgar scores of <7 at 1 min just significantly increased the risk of CP, and Apgar scores of <7 at 5 and 10 min were strongly associated with an increased risk. Abruptio placentae, Apgar scores <7 at 1 min and pathological non-stress test (reason for delivery) were significant risk factors of CP only in the moderately preterm and hemiplegic groups, whereas fever before delivery was a significant risk factor in the very preterm and spastic diplegic groups. Antibiotics during pregnancy was associated with CP only in the spastic diplegic CP group.

Conclusion: Antenatal infections marginally increased the risk of CP. Low Apgar score and abruptio placentae were associated with CP, especially in moderately preterm infants with hemiplegic CP.

Key words: *Antenatal risk factors, case–control study, cerebral palsy, pregnancy complications, preterm infants*

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Preterm birth is the most important risk factor for cerebral palsy (CP). The risk of CP is inversely proportional to gestational age and the relative risk is 60 times higher at <28 wk of gestation than at term (1, 2). Only 6.1% of infants were born preterm (<37 wk of gestation) during the period 1983–1990 in western Sweden, but they accounted for 41.5% of that region's CP cases (1, 2). The number of preterm infants with CP has increased since 1970, mainly related to the parallel decrease in perinatal mortality (1, 2). Although perinatal and neonatal risk factors for CP appear to dominate in the preterm group, further investigation into antenatal and intrapartal risk factors is interesting, as they can act as antecedents to the brain damage resulting in CP. In previous analyses of antenatal risk factors for CP in preterm infants no single risk factor has been consistent across all or even most studies (3–16). Recent studies suggest that foetoplacental uterine infection/inflammation is important in the initiation of preterm labour and for the development of central nervous system injury and CP (16, 17). The western Swedish population is characterized by a low frequency of perinatal infections (18, 19) and a low rate of preterm birth. Testing the hypothesis that infection is a risk factor for CP in this population as part of the ongoing CP project (1, 2, 20), applying the uniform and internationally accepted definition of this condition (21), may thus be interesting from a pathophysiological standpoint.

The aim of this study was to evaluate maternal, antenatal and intrapartal risk factors for CP, especially those related to foetomaternal infections (1, 2). A case–control study was designed, based on the preterm cases of spastic CP from the birth cohorts 1983–1990 in the western Swedish CP project. The very preterm group (<32 wk) and the moderately preterm group (32–36 wk) were analysed separately, as infection is considered to be a more common cause of very preterm birth (22). Furthermore, the moderately preterm group

has usually not been investigated in previous studies (4–16), despite its comprising 44% of preterm CP and 18% of all CP (1, 2).

Material and methods

Selection of subjects

This study is part of the CP project in western Sweden; it is population-based and its geographical area consists of the western healthcare region of Sweden, with a total population of 1.7 million inhabitants. The total number of livebirths was 168 627 during the period 1983–1990. Of the births, 1063 occurred before 32 wk and 8829 occurred at 32–36 wk of gestation, which corresponds to a preterm birth rate (<37 wk) of 6.1%.

CP was defined as a group of non-progressive, but often changing, motor impairment syndromes, secondary to lesions or abnormalities of the brain arising in the early stages of development (21). The internationally accepted Swedish classification was used in this context (21). Owing to increasing interest in antecedents of preterm CP, especially the importance of inflammatory processes in the development of diplegic CP types (12), the study was restricted to the spastic type of CP in children born preterm (152 of 159 cases of CP in these cohorts).

Preterm children with spastic CP were included if they were born in Sweden and lived in the study region on 31 December 1990 (for the years 1983–1986) and 31 December 1994 (for the years 1987–1990) and lacked any obvious postnatal cause of CP. All children were at least 4 y old at the time of diagnosis (1, 2)

Using the Swedish National Birth Register each case was matched with two controls. The closest births occurring before and after the case birth were chosen. The controls were matched for gestational age, gender, multiple gestation and delivery ward. In most cases (97.1%), calculation of gestational age was based on ultrasound scans performed between gestational weeks 16 and 19. In cases that had not been dated by ultrasound, gestational age was estimated from the date of last menstrual period and clinical assessment of the child at birth.

The total number of spastic preterm CP cases was 152. Four cases with their respective controls were excluded: two cases had a proven perinatal cytomegalovirus infection and two cases had ultrasound-corrected gestational age exceeding 37 wk. Subsequently, analysis was performed on 148 CP cases and 296 matched controls. Matching was complete for gestational age, gender and multiple gestation. Matching with regard to delivery ward was complete in only 84.5%, as controls could not always be recruited from small units. In these cases, controls were recruited from another unit of similar level and size.

Subgroup analysis was performed with regard to the two major types of CP, spastic diplegia and hemiplegia, and the two gestational age groups, very preterm (<32 wk) and moderately preterm (32–36 wk).

Approval was obtained from the Ethics Committee in Göteborg.

Data collection

One investigator (BJ), unaware of the paediatric outcome, examined all 456 records, recording a total of 154 variables including maternal (40), antenatal (63), intrapartal and immediately postpartal (51) data. Clinical chorioamnionitis was defined as fever (≥38°C recorded on two occasions ≥4 h apart) and/or uterine tenderness and/or foetal tachycardia in the absence of other focus of infection. Fever before onset of delivery was defined as fever (≥38°C) prior to the occurrence of regular contractions and cervical dilation. Postpartum endometritis was defined as fever (≥38°C recorded on two occasions ≥4 h apart) and uterine tenderness or foul-smelling cervicovaginal discharge. Histological chorioamnionitis was defined as increased infiltration of polymorphonuclear white blood cells in the chorioamniotic membranes. Histopathological examination was performed in 73 of the 444 placentas (18.9% in cases, 15.2% in controls). Clinical chorioamnionitis and pyelonephritis were grouped together, as previous studies implied that severe infection in close relation to the genital tract may be associated with brain injury (23). Hypertensive disease was used as a compound diagnostic term including pre-eclampsia (≥140/90 and ≥0.3 g protein in urine), gestational hypertension (≥140/90 after 20 wk of gestation) and essential hypertension (≥140/90 before the pregnancy or <20 wk of gestation). Cervical insufficiency was defined as opening of the cervix without uterine contractions. The term "bad obstetric history" was used when one of following criteria was fulfilled: more than three subsequent spontaneous abortions, one spontaneous abortion after 20 wk of gestation, intrauterine foetal death or an earlier case of perinatal death. Maternal disease was defined as the presence of any of the following diseases at the onset of the pregnancy: diabetes, hypertension, severe psychiatric disease, asthma, active neoplasia, epilepsy and glomerulonephritis. Antenatal cardiotocographic (CTG) tracings were classified according to FIGO standards (24).

Standardization of birthweight according to gestational age and gender was performed using a recent study of ultrasonically estimated foetal weights to avoid underestimating the degree of size deviation in preterm infants (25). Growth restriction was defined as below −24% weight deviation compared with the mean birthweight for a given gestational age and gender (25), corresponding to below −2 standard deviations (SD) from the mean; the latter is a more common definition in paediatric contexts (25).

Table 1. Distribution of 148 preterm spastic cerebral palsy (CP) cases by type and gestational age group.

Type of CP	<32 wk[a]	32–36 wk[a]	Total
Spastic diplegia	60 (66)	28 (49)	88 (59.5)
Spastic hemiplegia	11 (12)	20 (35)	31 (21)
Ataxic–spastic diplegia	11 (12)	4 (7)	15 (10)
Spastic tetraplegia	9 (10)	5 (9)	14 (9.5)
Total	91	57	148

Data are *n* (%).
[a] Weeks of gestation.

Statistical methods

Univariate logistic regression was used to estimate the odds ratio (OR) with a 95% confidence interval (CI) for correlation between one factor and the outcome. Statistical significance was considered to exist if the 95% CI did not include 1.0.

Calculations were made using SAS (SAS Institute, Cary, NC, USA) and InStat 2.01 (Graph Pad Software, San Diego, CA, USA). Proportions were compared using Fisher's exact test. Wilcoxon's rank-sum test was used to test continuous variables for differences between two groups. A p-value <0.05 was considered to be statistically significant.

Results

The distribution of spastic CP types according to gestational age is shown in Table 1. Most antenatal/intrapartal factors were unrelated to CP, and the results are given for the more frequently reported factors and for variables with significant or borderline significant association to outcome. Maternal characteristics were comparable in CP and controls (Table 2). The birthweight, standardized for gestational age and gender, did not differ significantly between cases and controls (12% cases and 14% controls were below −2 SD). Infectious factors [clinical chorioamnionitis/pyelonephritis, histological chorioamnionitis and long duration of preterm prelabour rupture of membranes (pPROM)] were associated with an increased risk of CP, whereas

Table 2. Selected maternal factors in cerebral palsy (CP) cases and controls.

	CP cases (n = 148)	Controls (n = 296)	OR (95% CI) or p-value
Maternal age[a]	27 (24–33)	28 (24–33)	$p = 0.46$
Nulliparous	66 (45)	159 (54)	0.83 (0.67–1.02)
Infertility >1 y	13 (9)	32 (11)	0.78 (0.38–1.50)
Maternal disease	10 (7)	19 (6)	1.05 (0.50–2.21)
Bad obstetric history	17 (11)	19 (6)	1.89 (0.94–3.76)
Previous legal abortion	37 (25)	51 (17)	1.60 (0.99–2.58)

Data are a mean (interquartile range), or *n* (%).
OR: odds ratio; 95% CI: 95% confidence interval.

Table 3. Selected factors, related to infection and inflammation, in cerebral palsy (CP) cases and controls.

	CP cases (n = 148)	Control (n = 296)	OR (95% CI) for CP or p-value
Clinical chorioamnionitis or pyelonephritis	18 (12)	19 (6)	2.02 (1.02–3.99)
Clinical chorioamnionitis	16 (11)	19 (6)	1.77 (0.88–3.55)
Histological chorioamnionitis	10/28 (36)	6/45 (13)	3.61 (1.16–12.1)
Fever before onset of delivery	12/146 (26)	11/289 (4)	2.30 (0.99–5.17)
Fever during delivery	13/147 (9)	25/295 (8)	1.05 (0.52–2.12)
pPROM (h to delivery)[a]	63 h (28–252)	37 h (12–90)	$p = 0.01$
Postpartum endometritis	9 (6)	18 (12)	1.00 (0.44–2.28)
Antenatal corticosteroids	9 (6)	39 (13)	0.42 (0.20–0.90)

Data are a mean (interquartile range), or *n* (%).
OR: odds ratio; 95% CI: 95% confidence interval; pPROM: preterm prelabour rupture of membrane.

treatment with anti-inflammatory corticosteroids was associated with a significantly lower risk (Table 3). There was a significant association between CP and an admission–delivery interval <4 h (Table 4). Hypertensive disease, cervical insufficiency and iatrogenic reasons for delivery were all associated with a lower occurrence of CP, whereas no difference was found between cases and controls with regard to spontaneous onset of labour [pPROM and preterm labour (PTL)] (Table 4). Decreased viability at birth (low Apgar scores at 1, 5 and 10 min) occurred more frequently in CP cases than in controls (Table 5).

Risk factors for CP were also analysed separately for very and moderately preterm infants, as well as for spastic diplegic and hemiplegic forms of CP (Table 6). Abruptio placentae and low Apgar scores were asso-

Table 4. Selected factors related to preterm birth and route of delivery in cerebral palsy (CP) cases and controls.

	CP cases (n = 148)	Controls (n = 296)	OR (95% CI)
pPROM	42 (28)	104 (35)	0.73 (0.48–1.09)
PTL	60 (41)	119 (40)	1.01 (0.68–1.52)
pPROM or PTL	102 (69)	223 (75)	0.91 (0.81–1.04)
Iatrogenic reason for delivery	14 (9)	48 (16)	0.54 (0.28–0.99)
Cervical insufficiency	2 (1)	16 (5)	0.85 (0.70–0.98)
Hypertensive disease	14 (9)	48 (16)	0.54 (0.28–0.99)
Pre-eclampsia	13 (9)	32 (11)	0.79 (0.40–1.56)
Caesarean section	99 (67)	169 (57)	1.11 (1.00–1.23)
<4 h from admission to delivery	34 (23)	42 (14)	1.80 (1.09–2.98)

Data are *n* (%).
OR: odds ratio; 95% CI: 95% confidence interval; pPROM: preterm premature rupture of membrane; PTL: preterm labour.

Table 5. Selected factors related to viability at birth and urgent delivery in cerebral palsy (CP) cases and controls.

	CP cases ($n = 148$)	Controls ($n = 296$)	OR (95% CI)
Apgar <7 at 1 min	66/146	96/289	1.36 (1.06–1.74)
Apgar <7 at 5 min	38/145	30/288	2.52 (1.64–3.85)
Apgar <7 at 10 min	18/140	6/279	5.98 (2.72–13.2)
Maternal bleeding, reason for delivery	29/148	37/296	1.71 (1.00–2.90)

OR: odds ratio; 95% CI: 95% confidence interval.

ciated with a higher risk of CP, especially in the hemiplegic and moderately preterm (32–36 wk) group. Indicators of infection, such as antibiotics during pregnancy, were associated with diplegic CP. Fever before onset of delivery was an antecedent of CP in the very preterm (<32 wk) and the diplegic groups (Table 6).

Discussion

In studies of CP, there is an unavoidable delay of 4–5 y from birth before a reliable diagnosis can be established in all cases. In addition, a large number of cases is required if subgroups of CP types and different gestational age groups are to be analysed. The strengths of the present study are that virtually all cases of spastic CP in a geographically defined area (birth 1983–1990) were included, the size of the study and the fact that all children with CP were at least 4 y old at diagnosis. The problems encountered were some missing information in the records and, in both cases and controls, the limited number of histopathological examinations of the placentas (data available in only 73 of the 444 records).

The more frequently observed risk factors for CP in previous studies are clinical chorioamnionitis (3–5, 7, 11, 26), pPROM of long duration (3, 5, 8, 27), multiple pregnancy (4, 6), maternal antibiotics during pregnancy (11, 13), antepartum fever (11), rapid vaginal delivery lasting <4 h (6, 13), CTG abnormalities/low Apgar scores at birth (5, 13), vaginal bleeding (6, 13) and vaginal preterm delivery subsequent to PTL or pPROM (12). In contrast, delivery without labour (5, 13) and pre-eclampsia have been found to be associated with a reduced risk of CP (5, 13). The variable results obtained may relate to differences in study design, outcome (all CP or subgroups of CP), weight/gestational age groups included (most studies covered the <1500 g group), occurrence of risk factors in the population and data retrieval methods (records or birth registers).

In this study, there was an increased occurrence of CP in infants whose histories reported clinical chorioamnionitis/pyelonephritis or histological chorioamnionitis, and in cases with a delay between pPROM and delivery. Fever before onset of delivery was a significant antecedent of CP in the spastic diplegic and the very preterm (<32 wk) groups, and the use of antibiotics during pregnancy was correlated with diplegic CP. This is in agreement with previous reports demonstrating that infectious risk factors are, to some extent, associated with CP in preterm infants (3–5, 7, 8, 10, 11, 13). The present data support the general concept of foeto-placental infection as a possible pathogenic factor of both preterm birth and brain injury (16, 17), even in a population with a low occurrence of perinatal infections (18, 19). However, the association between CP and infectious factors was not particularly strong (Table 3) and these results differ from those of previous studies, which demonstrated a markedly increased risk of brain lesions (28) or a moderately increased risk of CP (12) in the PTL/pPROM group, compared with groups born preterm for other reasons. This may reflect the fact that infection was associated with spontaneous delivery (and CP) in only a minority of cases in the preterm population (see Table 3). It is important to point out that the associations found between infection-related factors and CP are not proof of direct causality; instead, infection/inflammation may play an intermediary role

Table 6. Risk of cerebral palsy (CP) for selected variables, given separately for the two major subgroups of CP (spastic diplegia and hemiplegia) and for the very preterm (<32 wk) and moderately preterm (32–36 wk) gestational groups. In the subgroup analyses, the cases are compared to their own matched controls.

	All patients	Spastic diplegia	Hemiplegia	<32 wk	32–36 wk
Abruptio placentae	1.64 (0.90–3.02)	1.05 (0.50–2.22)	7.20 (1.63–31.9)	1.31 (0.65–2.63)	4.31 (1.14–16.3)
Pre-eclampsia	0.79 (0.40–1.56)	0.94 (0.41–2.18)	0.17 (0.03–1.16)	1.32 (0.55–3.19)	0.40 (0.13–1.20)
Apgar <7 at 1 min	1.36 (1.06–1.74)	1.30 (0.77–2.21)	3.42 (1.40–8.36)	1.02 (0.61–1.70)	5.48 (2.61–11.5)
Apgar <7 at 5 min	2.52 (1.64–3.85)	2.44 (1.18–5.06)	4.58 (1.46–14.3)	2.30 (1.25–4.26)	7.90 (2.79–22.4)
Apgar <7 at 10 min	5.98 (2.72–13.2)	3.72 (1.06–13.1)	30.9 (1.7–568)	6.12 (2.34–16.0)	10.4 (1.74–62.0)
Antibiotics during pregnancy	1.18 (0.64–2.20)	2.39 (1.12–5.09)	0.47 (0.10–2.29)	1.35 (0.68–2.68)	0.65 (0.13–2.30)
Fever before onset of delivery	2.30 (0.99–5.17)	3.10 (1.14–8.44)	0.66 (0.07–6.56)	2.60 (1.06–6.36)	1.00 (0.09–11.3)
Pathological non-stress test (CTG), reason for delivery	2.89 (0.95–8.84)	2.04 (0.4–10.0)	4.20 (0.43–40.9)	1.01 (0.09–11.3)	4.3 (1.14–16.3)

Data are odds ratio (95% confidence interval).
CTG: cardiotocography.

between CP and some other, yet unknown, factor (29). To investigate this further, more detailed information regarding the presence of microbes and inflammatory mediators in the membranes, amniotic fluid and blood of the newborn is required.

The use of antenatal corticosteroids (betamethasone) was associated with a decreased risk of CP, which is in agreement with a recent report indicating that betamethasone (but not dexamethasone) reduces periventricular leukomalacia in preterm infants (30), possibly through its beneficial effects on respiration during the neonatal period, anti-inflammatory actions or neuroprotective properties. Alternatively, there may have been a selection bias among cases receiving treatment towards those delivered for iatrogenic reasons and with a longer latency between admission and delivery, and this group may therefore have been at lower risk even without corticosteroids. This issue is of great importance and must be addressed in prospective randomized controlled trials.

Traditionally, preterm birth has been subdivided into a very preterm (<32 wk) and a moderately preterm (32–36 wk) group. Periventricular leukomalacia, known to be strongly correlated with spastic diplegic CP in preterm children, predominantly occurs in the 24th to 32–34th wk of gestation, whereas cortical/subcortical insults (which correlate with moderate–severe neonatal encephalopathy) occur after that time (20). Hence, the observed rate of CP in low-birthweight infants with low Apgar at 10–20 min is relatively low, whereas the CP rate in term babies with low Apgar at 10–20 min is very high, probably reflecting that the immaturity of the nervous system of preterm infants confers cerebral resistance to asphyxia, whereas the brain of term infants is more vulnerable (31). Accordingly, from an aetiological point of view, the moderately preterm group is an intermediate group between the very preterm and term groups, which is, to some extent, illustrated in this study. In the very preterm group, fever before delivery and low Apgar scores at 5 and 10 min were significant risk factors for CP, as in the spastic diplegic group that dominated the very preterm group. In the moderately preterm group, abruptio placentae, low Apgar scores at 1, 5 and 10 min and pathological non-stress test (reason for delivery) were significant risk factors, as in the hemiplegic group (Table 4).

These results indicate that foetal distress and precipitous delivery constitute a risk for CP, not a consistent finding in previous studies, which may be explained by the exclusion of moderately preterm cases in most studies (4–14). However, the present data are in agreement with Cooke, who found an association between CTG abnormalities and preterm CP (4). Although the data may indicate that intrapartal distress is contributory in the aetiology of spastic CP in preterm infants, drawing a firm conclusion is complicated by the fact that infants born with low Apgar scores at birth (or delivered after abruptio) are probably more likely to have suffered from other adverse events antenatally and/or neonatally as well. Since adjustment for all of these possible confounders was not made, the results must be interpreted with caution.

The risk of CP was lower in infants born to mothers with hypertensive disease (but not significantly for pre-eclampsia as a separate group). Pre-eclampsia appears to be associated with a reduced risk, irrespective of whether $MgSO_4$ was used or not (5, 6, 13). It has been suggested that pre-eclampsia does not decrease the risk in itself but rather represents a group delivered preterm in the absence of adverse infectious/inflammatory factors, as opposed to the PTL–pPROM group (5, 12). As mentioned previously (Table 5), no higher risk of CP in the PTL/pPROM group was found compared with the pre-eclamptic group, suggesting that other mechanisms may be in play.

In summary, among the antenatal variables studied, infectious factors were significantly, but weakly, associated with CP. Low Apgar scores and abruptio placentae correlated with hemiplegic CP in moderately preterm infants, whereas a similar relationship was less evident in the very preterm diplegic cases. Postpartal and neonatal variables were not studied, but are known to be associated with brain damage resulting in CP in preterm births (1, 26).

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