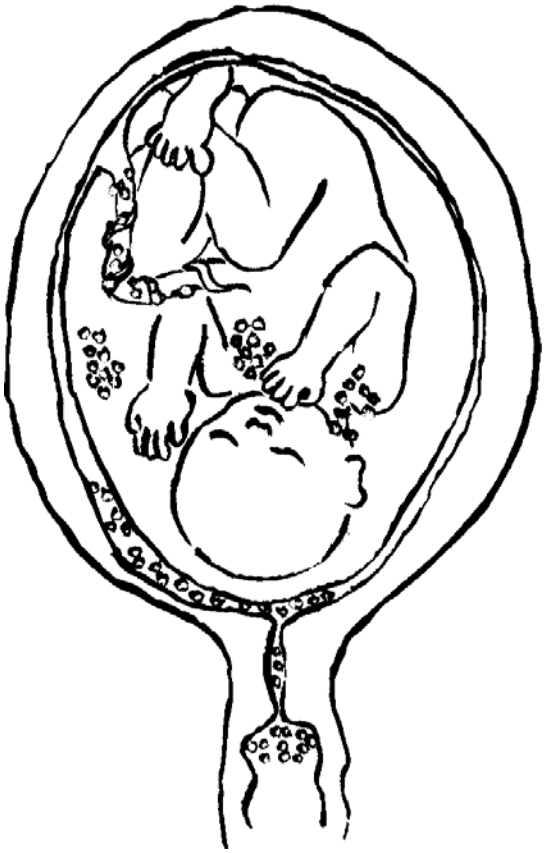
Inflammatory mediators in perinatal infections

Henrik Døllner



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Papers included in the thesis

I

Døllner H, Vatten L, Halgunset J, Rahimipoor S, Austgulen R. Histologic chorioamnionitis and umbilical serum levels of pro-inflammatory cytokines and cytokine inhibitors. Accepted for publication (Br. J. Obstet. Gynecol.).

II

Døllner H, Vatten L, Linnebo I, Zanussi GF, Lærdal Å, Austgulen R. Inflammatory mediators in umbilical plasma from neonates who develop early-onset sepsis. Biol. Neon. 2001; 80:41-47.

III

Døllner H, Arntzen KJ, Hæreid PE, Aag S, Austgulen R. Interleukin-6 concentrations in neonates evaluated for sepsis. J. Pediatr. 1998; 132:295-299.

IV

Austgulen R, Arntzen KJ, Hæreid PE, Aag S, Døllner H. Infections in neonates delivered at term are associated with increased serum levels of ICAM-1 and E-selectin. Acta Paediatr. 1997; 86:274-280.

V

Døllner H, Arntzen KJ, Hæreid PE, Aag S, Brubakk AM, Austgulen R. Increased serum concentrations of soluble tumor necrosis factor receptors p55 and p75 in early onset neonatal sepsis. Early Hum. Dev. 1998; 52:251-261.

VI

Døllner H, Vatten L, Austgulen R. Early diagnostic markers for neonatal sepsis: Comparing C-reactive protein, interleukin-6, soluble tumour necrosis factor receptors and soluble adhesion molecules. J. Clin. Epidemiol 2001; 54:1251-1257.

Abbreviations

AUC	Area Under the Curve
CRP	C-Reactive Protein
ELISA	Enzyme-Linked Immunosorbent Assay
GBS	β-hemolytic streptococci group B
ICAM-1	Intercellular Adhesion Molecule-1
IL-1β	Interleukin-1β
IL-1RA	Interleukin-1 Receptor Antagonist
IL-6	Interleukin-6
IL-8	Interleukin-8
I/T ratio	ratio between Immature and Total neutrophile counts
LPS	Lipopolysaccharide
NICU	Neonatal Intensive Care Unite
PROM	Premature Rupture of the Membranes
ROC	Receiver Operator Characteristic
SIRS	Systemic Inflammatory Response Syndrome
SWU	Sepsis Work-Up
TIS	Total Inflammatory Score
TNFα	Tumour Necrosis Factor-α
sTNFR	soluble Tumour Necrosis Factor Receptor
VCAM-1	Vascular Cell Adhesion Molecule-1
WBC	White Blood Cell

Background

Inflammation

Bacterial invasion is usually met by an inflammatory response with increased secretion of a number of mediators, such as cytokines and acute phase proteins, and accumulation of leukocytes in affected tissue [1].

Cytokines [1,2]

Cytokines are small hormone-like polypeptides or glycoproteins with a molecular weight less than 30 kD. They are synthesised and secreted from a variety of immune cells (e.g. monocytes, lymphocytes) and non-immune cells (e.g. endothelial cells), and after stimulation by microbes or other cytokines. They act at low concentrations by binding to specific cellular receptors. Binding transmits signals through intracellular messenger systems that activate transcription factors, and changes gene expression. Different intracellular signalling pathways are activated by different cytokine-receptor interactions. The receptors may also exist in soluble isoforms that have the ability to bind cytokines and modulate their activity. Most cytokines have multiple biologic effects, often overlapping the effects of others. The majority acts locally, but cytokines may also have systemic effects. Cytokines are important in host defence, but in high amounts cytokines may be detrimental to the host [2,3].

TNF and soluble TNF receptors

TNF-α belongs to a group of cytokines called the TNF superfamily [4]. It is a central inflammatory mediator in autoimmune disorders, cancer, and infection [5]. TNF exists as a transmembrane molecule, that can be cleaved, and circulates in a trimeric form [6]. Monocytes, tissue macrophages and many other cells produce TNF in response to a variety of stimuli, such as bacterial toxins (i.e. LPS), parasites, viruses, complement factor C5a, IL-1, IL-6, and TNF itself [6,7]. TNF binds to two receptors, p55 and p75, which are expressed on virtually all cells [8,9]. Both receptors can be shed from the cell membrane and exist as soluble isoforms, either constitutively [10], or in response to bacterial products (LPS), TNF or IL-6 [8,10]. It has been shown that different cell types

may release the receptors to different extent [11]. Soluble receptors interact with TNF and modulate its local and systemic effects [8]. TNF may increase transiently during the very early stage of the infection, whereas sTNFR likely remains elevated much longer [12,13]. In vivo, high levels of TNF and soluble receptors have been detected in adult sepsis [12,13], and high TNF-levels correlate with increased lethality from meningoccocaemia [3].

IL-1 and IL1-RA [1]

IL-1β and IL-1RA are synthesised by a wide range of cells, including monocytes and macrophages, in response to many of the same stimuli that activate TNF production. Together with TNF, IL-1β is a central mediator in inflammation and sepsis. The binding of IL-1β to its receptor can be blocked by high concentrations of IL-1RA, which is produced and released by nearly the same stimuli as IL-1β. Opposite to IL-1β, IL1-RA is produced constitutively.

IL-6 [1]

IL-6 is produced in at least five different forms with different molecular weights, by a variety of cells, e.g. B-lymfocytes, T-lymfocytes, and macrophages. IL-6 has important roles in immune regulation at the B and T cell level, and it stimulates proliferation and maturation at different levels in haematopoiesis. IL-6 also stimulates the acute phase reaction [14], and in vivo, high levels have been detected in sepsis, and other infectious and autoimmune diseases.

IL-8 [1]

IL-8 is a pro-inflammatory cytokine mainly with chemotactic actions, and belongs to a large group of cytokines called chemokines. IL-8 exists in several active forms, and it is synthesised by monocytes, macrophages, and endothelial cells, in response to endotoxins (LPS), TNF, and IL-1.

Soluble adhesion molecules [1,15]

Leukocyte adhesion to endothelium, and subsequent migration into the tissue is mediated through interactions between molecules on leukocytes and vascular endothelium. There are three classes of adhesion molecules: selectins, integrins, and various

members of the immunoglobulin gene superfamily. L-selectin is expressed constitutively on leukocytes, and E-selectin and P-selectin are expressed on activated endothelial cells in response to TNF and IL-1. The integrins, composed of an α and a β chain, are expressed on leukocytes. Neutrophils constitutively express those with a β2 chain (CD18), but expression and avidity for endothelial ligands may increase in response to chemotactic factors such as IL-8. Integrins bind endothelial ligands of the immunoglobulin-like family, including intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1). These molecules are also markedly expressed after exposure to LPS, TNF and IL-1. Adhesion molecules (L-selectin, E-selectin, ICAM-1, VCAM-1) may be shed from cell surfaces, and exist as soluble isoforms [15]. In adults, high serum concentrations of soluble adhesion molecules have been detected in sepsis and various immunologic diseases. It has been suggested that soluble isoforms may bind to ligand bearing cells and modulate leukocyte adhesion [15]. It is also possible that high concentrations simply may reflect leukocyte adhesion and activation [15].

Acute phase proteins

Acute phase proteins are mainly produced in the liver, and they serve important functions during inflammation, and in restoring homeostasis after inflammation [16]. Several factors regulate the production of acute phase proteins among which the most important are cytokines (IL-6, IL-1, TNF), growth factors (e.g. insulin, hepatocyte growth factor), and corticosteroids [17].

CRP was originally named for its ability to bind the C-polysaccharide of *Streptococcus pneumoniae.* CRP may have several functions; i.e. it binds cell-wall components, and opsonises bacteria, parasites and immune complexes. CRP may also activate the classical complement pathway [18]. In clinical medicine, CRP is widely used to diagnose inflammatory diseases and infections, and to monitor the efficacy of treatment.

The inflammatory response

Local effects [1]

The inflammatory response is characterised by accumulation and activation of leukocytes in affected tissues. The process is initiated at the site of invasion when tissue

macrophages produce TNF and IL-1 in response to bacterial exposure or trauma. These cytokines in turn activate a complex cytokine cascade with generation of other pro-inflammatory cytokines (e.g. IL-6), and chemokines (e.g. IL-8). IL-1 and IL-6 stimulates hematopoiesis and release of neutrophils from the bone marrow, acting synergistically with colony stimulating factors (G-CSF, GM-CSF). The accumulation of leukocytes results from a series of events: (1) increased expression of adhesion molecules on endothelial cells and leukocytes; (2) establishment of leukocyte-endothelial cell adhesion; (3) leukocyte migration through the endothelium; and (4) leukocyte migration along a chemotactic gradient. At the site of inflammation, leukocytes are activated (5) and invading pathogens are phagocytosed and killed by release of a variety of microbicidal products. Cytokines are involved at each step of this process (Table 1). As inflammation progresses, TNF and IL-1 may also increase local production of vasodilators (e.g. nitric oxide, platelet activating factor, prostaglandins), and vasoconstrictors such as endothelins. In addition, TNF and IL-1 may stimulate the extrinsic coagulation pathway and inhibit fibrinolysis leading to local clot formation (Table 1).

Systemic effects [1]

A number of physiologic, behavioural, biochemical, and nutritional changes involving many organ systems may accompany inflammation [16]. Pro-inflammatory cytokines may induce fever, anorexia, somnolence, and increased secretion of corticosteroids and acute phase proteins [16]. The local and systemic inflammatory responses are maintained until regulatory mechanisms are activated and cytokine synthesis and biologic activities are attenuated [1]. Anti-inflammatory mediators are synthesised, including IL-4, IL-10, IL-13 and Transforming Growth Factor-β, and naturally occurring inhibitors such as soluble TNF receptors and IL-1RA may be secreted in large amounts [1]. In most cases, the inflammatory response is successfully resolved. However, vigorous production of pro-inflammatory cytokines can lead to increasing systemic cytokine concentrations, and the development of a systemic inflammatory response syndrome (SIRS) [19]. This syndrome is characterised by increased vascular permeability, and may be complicated by hypotension, hypoxia, and intravascular thrombosis and haemorrhage. SIRS may occur in association with severe infections (sepsis), in trauma, severe burns, and haemorrhagic shock [19].

Table 1 Summary of local and systemic effects of pro-inflammatory cytokines during inflammation.*

	TNF	IL-1	IL-6	IL-8
Local effects				
Expression of adhesion molecules	+	+	–	+
Leukocyte chemotaxis	–	–	–	++
Activation of leukocytes	+	±	±	++
Release of endogenous mediators (PG, PAF, NO)	+	+	±	±
Procoagulant activity	+	+	–	–
Synthesis of TNF, IL-1, IL-6, chemokines	+	+	–	–
Systemic effects				
Fever	++	++	+	–
Acute phase reaction	+	+	++	–
B- and T-cell activation	+	+	++	–
Systemic inflammatory response syndrome	++	+	–	–

PG, prostaglandin; PAF; platelet activation factor; NO, nitric oxide.

*adapted from Kilpatrick [1].

Intrauterine infection

Intrauterine infection may present with fever, uterine tenderness, maternal or fetal tachycardia, or foul smelling vaginal discharge (clinical chorioamnionitis) [20]. Clinically, intrauterine infection occurs in 1-2% of all deliveries [20]. In a large retrospective study of nearly 9600 deliveries, Seo et al. found that a clinical chorio-amnionitis was present in 1.7% of term, and 5.7% of preterm deliveries [21]. Intrauterine infection may also cause preterm PROM, and preterm labor (subclinical chorioamnionitis) [22-24]. Intrauterine infection occurs when vaginal bacteria ascend through the cervix, or more seldom, when blood-borne bacteria settle in utero [25]. Most commonly, when infection appears after membrane rupture [21], high-virulent organisms such as β-hemolytic streptococci group B (GBS), and *E Coli* easily invade the amniotic cavity [20]. Bacteria may also cross intact membranes. Such cases most often occur early in pregnancy, and with relatively low virulent bacteria (e.g. *Ureaplasma urealyticum, Mycoplasma hominis*, peptostreptococci, bacteroides species) [23,26]. There are indications that vaginal bacteria initially invade decidua and the chorio-decidual space in the lower part of the uterine cavity, then spread into the chorion and amnion, and possibly cross into the amniotic cavity [23]. Ultimately, the umbilical cord, and the foetus itself may be invaded (Figure 1) [23,24].

Histologic findings

It has long been assumed that maternal leukocytes constitute the majority of inflammatory cells that infiltrate intrauterine tissues in response to bacterial invasion [25]. Indeed, in 1997 McNamara et al. confirmed that approximately 90% of leukocytes are maternal, using flourescent in situ hybridization [27]. When the bacterial focus is in the amniotic cavity, maternal leukocytes are believed to migrate through blood vessels in decidua toward the cavity, giving rise to deciduitis and chorioamnionitis [25]. In the placenta, maternal leukocytes may accumulate in the subchorionic fibrin of the intervillous space, before infiltrating the chorionic plate, and migrating toward the amniotic cavity [25]. It has also been assumed that maternal cells constitute the majority in amniotic fluid. However, in a recent small case series with amniotic fluid infection almost all leukocytes were fetal [28]. Usually, the fetal immune system has been assumed to be involved late during an intrauterine infection, and activated fetal leukocytes are believed to migrate through chorionic and umbilical blood vessels

toward the amnion cavity, giving rise to chorionic and umbilical vasculitis, and funisitis [25,29].

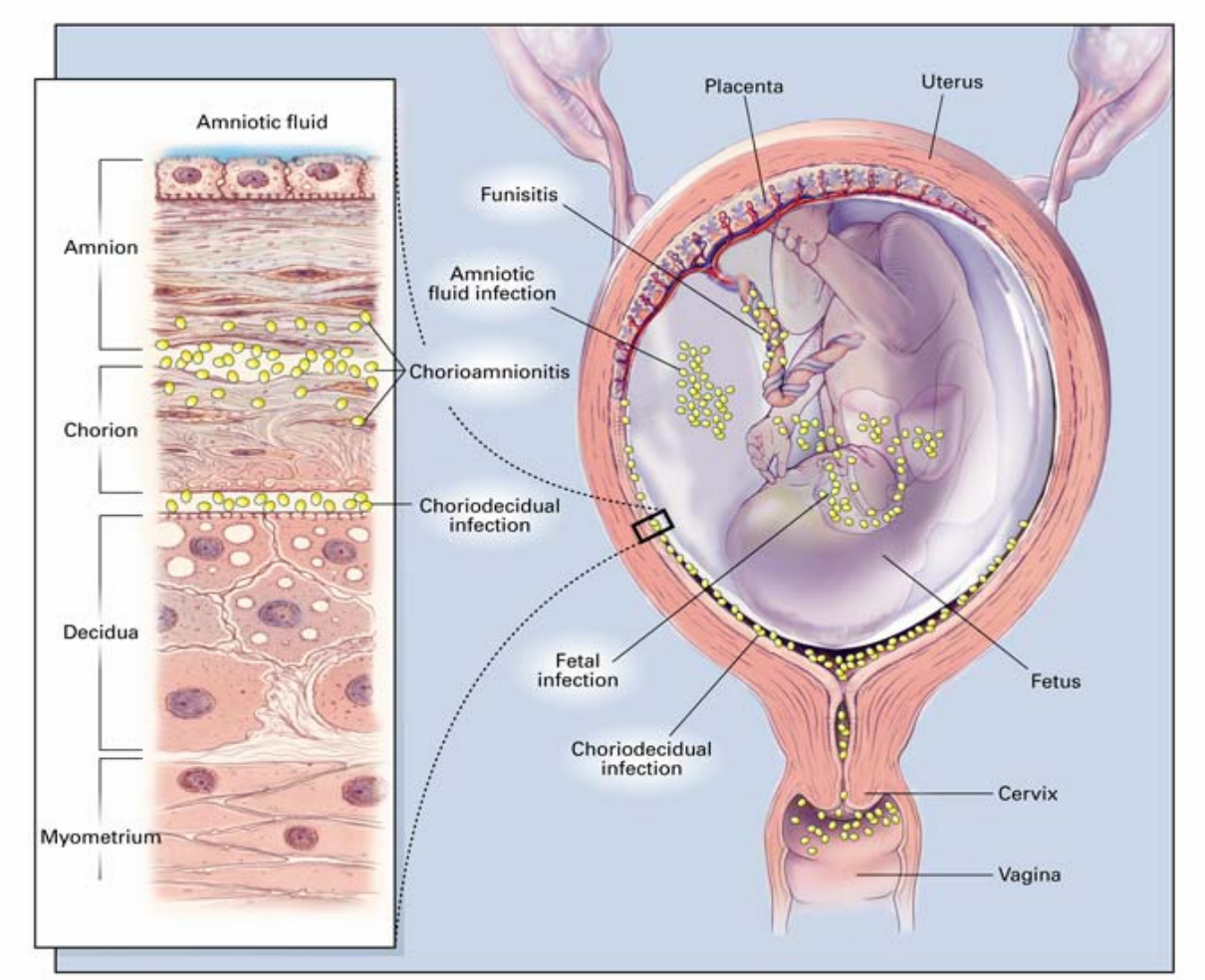

Figure 1 Possible sites of infection within the uterus (Goldenberg [23]).

Various histopathologic criteria have been used to define an intrauterine infection. Blanc defined three grades of chorioamnionitis based on the degree of leukocyte infiltration in the chorionic plate [25]. Salafia used a more detailed classification with four degrees of infiltration in the chorionic plate, membranes, and umbilical cord [30]. Regardless of the classification used, high-grade infiltration in placenta and the membranes is associated with the presence of bacteria in amniotic fluid [31], and in the membranes [32]. It has also been shown that histologic chorioamnionitis occurs in nearly all cases with clinically evident infection [21,33,34]. On the other hand, leukocyte infiltration may be present in non-infected deliveries [20,33,35]. In a one-year

cohort of 2774 deliveries, Guzick et al. found that chorioamnionitis including all three grades in the Blanc classification occurred 5-10 times more frequently than clinically evident infection [33]. In normal deliveries, Salafia reported that high-grade infiltration is seldom, but a low-grade infiltration in decidua and in the subchorionic fibrin of the chorionic plate appeared in nearly half [30]. Hence, it seems that histologic chorioamnionitis with high-grade leukocyte infiltration is a sensitive indicator of clinically and microbiologically evident infection, whereas a low-grade infiltration often may be present in normal pregnancies.

Intrauterine infection and cytokines

Human gestational tissues may secrete cytokines. Inflammatory cytokine mRNA have been detected in placenta and membranes from infected and non-infected deliveries [36]. It has been shown that cytokines can be secreted both from local cells in decidua, chorion, and amnion [37-41], and from infiltrating macrophages [37,42]. During infection the majority may be produced from infiltrating inflammatory cells [42]. Cytokine levels may increase to some extent in amniotic fluid toward term in a normal pregnancy [35], but in intrauterine infection several studies have detected very high levels of pro-inflammatory cytokines (TNF, IL-1, IL-6, IL-8, G-CSF) [23,42,43], and natural cytokine inhibitors (p55, p75 and IL1-RA) [44,45]. Cytokine levels (TNF, IL-6, G-CSF) and CRP may also be elevated in maternal serum [23,46-49].

Until recently, only a few investigators had studied fetal/neonatal cytokine levels and intrauterine infection (Table 2). Most used histological criteria, and found elevated umbilical cord blood IL-6 [37,50-52]. IL-1, IL-8 and IL-1RA may also be elevated [37,44,50,53]. In three recent studies, it has been shown that funisitis is associated with elevated IL-6 [52,54,55], and neonatal sepsis [52,54].

Table 2 Summary of studies reporting umbilical cytokine levels in intrauterine infection.

Author year[reference]	Population	Neonatal outcome	Evidence of intrauterine infection	Cytokine levels in umbilical cord blood
Shimoya 1992[53]	72 preterm deliveries	-	Histologic CAH grade 1–3 (n=38)	↑ IL-8 in CAH grade 1-3
Romero 1994[44]	102 preterm and 102 term deliveries	-	Positive amniotic fluid culture: preterm (n=45), term (n=31)	↑ IL1-RA in preterm, but not in term infection
Stallmach 1995[37]	64 preterm and term cesarian sections	-	Histologic CAH (n=18)	↑ IL-6, ↑ IL-8, ↑ G-CSF, → TNF, → IL-1
Singh 1996[56]	32 term deliveries	Healthy (n=32)	Clinical CAH (n=6)	↑ IL-6
Salafia 1997[50]	32 preterm deliveries	-	Amnionitis (n=16) Umbilical vasculitis (n=17)	↑ IL-6 in amnionitis grade 3-4 ↑ IL-1, ↑ IL-6
Miyano 1998[51]	215 preterm and term deliveries	-	Histologic acute CAH (n=37) and subacute CAH (n=30)	↑ IL-6 ↑ IL-6
Kashlan 2000[52]	43 preterm deliveries	Sepsis (n=21)	Histologic CAH grade 1-3 (n=20) Funisitis (n=11)	↑ IL-6 in CAH grade 2-3. ↑ IL-6. Funisitis associated with neonatal sepsis
Yoon 2000[54]	315 preterm deliveries	Sepsis (n=11)	Funisitis (n=78)	↑ IL-6. Funisitis associated with neonatal sepsis
Naccasha 2001[55]	94 preterm deliveries	-	Funisitis (n=15)	↑ IL-6

CAH = Chorioamnionitis; ↑ higher than controls; → not higher than controls.

Neonatal sepsis

Sepsis in neonates is a life threatening condition with an incidence of 1-8 per 1000 newborn infants (culture proven infection) [57]. The occurrence is inversely related to gestational age; Seo et al. found that among 9600 neonates the incidence increased from 0.6% in neonates at term to 16.6% among those with a gestational age less than 28 weeks [21]. Despite the advances in life support therapy, and development of broad-spectrum antibiotic treatment, the mortality is still considerable, in particular among preterms [58].

Sepsis, defined as a systemic response to infection, usually implies isolation of a known pathogenic micro-organism in a normally sterile tissue fluid (blood, cerebrospinal fluid) [57]. However, this diagnostic criterion may have a low sensitivity when it is applied to neonates [59,60]. Squire et al., and Pierce et al. compared blood culture results with post-mortem findings in neonates who died from sepsis, and found that only about 70-80% of cases with reliable post-mortem findings of a septic infection had a positive blood culture before they died [61,62]. Bacterial isolation may be hindered by antenatal antibiotic therapy [63], small blood volumes [64], or because standard microbial techniques may be unable to detect possible pathogens [65]. Thus, most recent studies have defined neonatal sepsis as an ill neonate with either a positive culture (blood/CSF), or an ill neonate with sterile cultures, but a significant inflammatory response.

Early-onset sepsis appears less than 4-7 days after delivery, often after premature rupture of the membranes, or after intrauterine infection [21,57]. In the study of Seo et al., early-onset sepsis (culture proven or clinical) appeared after clinical chorio-amnionitis in 34.7% of preterms with a gestational age less than 28 weeks, among 22.2% with gestational age 31-33 weeks, and among 9.1% of term neonates [21]. Compared to non-infected deliveries, clinical chorioamnionitis increased the risk of early-onset neonatal sepsis 8 to 10 times [21]. Infants with late-onset sepsis (≥ 4-7 days of age) are less likely to have a history of obstetric complications, and may be infected mainly with nosocomially acquired organisms [57].

Pathogenesis

The foetus is protected from bacterial exposure by the membranes and placenta [59]. It has also been shown that the amniotic fluid has inhibitory properties against bacterial

growth [66,67]. However, foetal bacteremia may occur in preterm labor [68], and term neonates may have bacteremia or present symptoms at birth [69], suggesting that bacterial colonisation may take place before birth. Some bacteria (e.g. *Listeria monocytogenes*) cause transplacental infections via the mother's bloodstream. More commonly, however, bacterial exposure takes place in the amniotic cavity, or during the passage through the birth canal (Figure 1) [57,59]. Pyati et al. studied neonatal GBS sepsis among 30000 newborn infants [69]. Nearly all with sepsis and a birth weight less than 2000 gram presented with symptoms less than one hour after birth, whereas more than two-thirds of those with a higher birth weight developed symptoms later than four hours [69]. These findings suggest that preterm neonates may be exposed to GBS in utero, whereas term neonates often may be exposed during the passage through the birth canal. Foetal colonisation is likely to take place by aspiration of contaminated amniotic fluid [61,62], or by bacteria penetrating through injured skin or natural body openings [59]. In most cases this colonisation proceeds without causing disease [59]. The mechanism by which bacterial colonisation converts to invasive disease is not fully understood, but it is likely to reflect bacterial virulence, maternal immunological factors, and the competence of the neonatal immune system [59].

The neonatal host response

Many components of the innate and adaptive immune system may not be fully developed at birth, and neonates may be susceptible to infection because they have an immature host response [70]. Secretory IgA may be undetectable in mucosal secretions at birth and may not appear until several weeks, making neonates susceptible to colonisation [71]. The most critical is, however, limited capacity to produce and deliver adequate numbers of well functioning phagocytes (neutrophils and monocytes) at the site of bacterial invasion [70,72]. In animal studies, it has been shown that fetal and neonatal bone marrow contain expanded stem cell populations that proliferate at nearly maximal rates already during basal conditions [73-75]. Nevertheless, the neutrophile storage pool that serves as a reservoir of functionally mature neutrophils, is considerably smaller in neonates than in adults [76]. Recently, it has also been shown that human preterm liver and bone marrow contain a considerably smaller neutrophile storage pool than liver and bone marrow from term neonates and adults [77]. The frequent finding of neutropenia in association with neonatal sepsis is likely to reflect the

limited neutrophile stores, and diminished capacity to accelerate proliferation in response to bacterial invasion [78]. There are also indications that foetal bone marrow, liver, and blood cells secrete less G-CSF and express less G-CSF mRNA than cells of adults [77]. Neonatal neutrophils may function less efficiently than adult cells [70]. Their adherence to endothelial surfaces may be looser [70], probably because they express less L-selectin [79], and have an impaired up-regulation of integrins (Mac-1, LFA-1) in response to inflammatory cytokines and chemotactic agents [70,80]. Moreover, neonatal neutrophils and monocytes migrate slower into affected tissues [70]. Once at the site of infection, they may bind, ingest, and kill bacteria as efficiently as adult cells under optimal in vitro conditions [70]. However, under suboptimal conditions, such as a limiting opsonin concentration (complement, immunoglobulin), or a high bacteria/polymorphonuclear leukocyte ratio, neonatal neutrophils likely ingest and kill bacteria less efficiently [70].

Neonatal sepsis and cytokines

Inflammatory cytokines stimulate leukocyte migration and activation (Table 1), and one may speculate whether immature cytokine secretion could impair neonatal resistance to bacteria. In vitro, it has been shown that cord blood monocytes from term neonates produce equal amounts, or somewhat less TNF [81-87], IL-6 [85,88,89], and IL-8 [81,90-92] than adult monocytes (Table 3). However, it seems that monocytes from preterm neonates produce less TNF [82], IL-6 [88,93,94], and IL-8 [91] than term neonatal monocytes (Table 3). Several studies have shown that monocytes from adults, and preterm and term neonates produce equal amounts of IL-1 (Table 3) [82,84,85,95,96]. It has also been shown that cord blood monocytes express receptors for TNF (p55, p75), IL-1 and IL-6 [97].

At the time we planned the present investigations, only a few and small in vivo studies had been reported on the relation between neonatal sepsis and cytokine levels. Recently, several papers have been published. Table 4 summarises the main findings. It seems that cord blood and peripheral blood levels of TNF, IL-6, and IL-8 are elevated in early-onset sepsis (Table 4). There are also some indications that IL-1, p55, p75, and ICAM-1 may be high (Table 4). None of the studies have investigated preterm neonates separately. Based on experimental and clinical data, it seems that neonates have capacity to produce inflammatory cytokines in response to bacterial exposure. However,

some cytokines may be produced less efficiently compared to adults, in particular among preterm neonates.

Table 3 In vitro cytokine production by cord blood monocytes.**

TNF	Adult ≥ term > preterm	[81]*[82-84,86,87]
IL-1	Adult = term = preterm	[82,84,85,95,96]
IL-6	Adult ≥ term > preterm	[85,88,89,93,94]
IL-8	Adult ≥ term > preterm	[81,90-92]

*reference.

**adapted from Kilpatrick [1].

Table 4 Summary of studies reporting cytokine levels in neonatal sepsis.

	Early-onset sepsis		Late-onset sepsis
	Cord blood	Peripheral blood	
TNF	↑ [98]*	↑ [98-103]; →[52,104]	↑ [105,106]
IL-1	↑ [98]	↑ [100,107]; → [99,103]; ↓ [108]	
IL-6	↑ [52,98,109]	↑ [98-100,103,110-112]	↑ [105,113,114]
IL-8	↑ [98,109]	↑ [98,115,116]	↑ [113]
p55/p75	↑ [112]	↑ [112]	
IL1-RA		↑ [107]	↑ [114]
E-selectin			↑ [105]
ICAM-1	↑ [98,109]	↑ [117]	↑ [113]

*reference.

↑ higher than controls; → not higher than controls; ↓ lower than controls.

Neonatal sepsis: some clinical aspects

Infected neonates should be recognised as quickly as possible, in order to initiate antibiotic therapy. However, quite often the clinical manifestations are similar to those of other neonatal conditions, and in the early stages of infection may be subtle [57,59]. Numerous studies have evaluated whether a diagnostic test could assist in early identification. Total white blood cell count (WBC) and differential have a low sensitivity and specificity [57]. Some studies have shown that neutropenia, or a total WBC less than 5000 per μl has moderate to high negative predictive values [60,118]. The ratio between immature and total neutrophil cell counts (I/T-ratio) is a quite sensitive, but not very specific diagnostic method [57,60]. In clinical medicine it is a disadvantage that the ratio must be determined by microscopy, and the inter-observer variability is substantial [60,119]. CRP is rather specific for bacterial infection in neonates, but CRP has a low sensitivity during the first 12-24 hours of infection [120]. Although the relative CRP increase may be similar in infected preterm and term neonates, it is furthermore a disadvantage that CRP may never rise to detectable levels in infected preterms [119]. Hence, in the absence of sensitive and clinically applicable diagnostic tests, it has been recommended to initiate treatment with broad-spectrum antibiotics in every neonate that is suspected to suffer from infection [57,59]. This liberal antibiotic policy may have contributed to lower mortality [57], but it has also led to a disturbing overuse of antibiotics [121].

Objectives

Bacterial invasion of intrauterine tissues is met by an inflammatory response with leukocyte infiltration in placenta, membranes, and umbilical cord, and high levels of inflammatory cytokines in amniotic fluid. The infection increases the risk of fetal/neonatal sepsis.

1. The first objective was to examine whether leukocyte infiltration in placenta, membranes or umbilical cord is reflected by elevated levels of pro-inflammatory cytokines (TNFα, IL-1β, IL-6, IL-8), cytokine inhibitors (p55, p75, IL1-RA), and CRP in the fetal/neonatal circulation (umbilical cord blood).

Early-onset sepsis in neonates may be caused by bacterial exposure in utero or at delivery. Clinical manifestations may be present at birth or develop during the first days of life. Although the neonatal host response to bacterial exposure may not be fully developed, previous studies have suggested that neonatal blood cells may have capacity to secrete cytokines in vitro.

2. The second objective was to examine whether early-onset neonatal sepsis is associated with a) a prenatal immune response with elevated levels of pro-inflammatory cytokines, cytokine inhibitors, and CRP in umbilical cord blood, and b) elevated levels of IL-6, p55, p75, soluble adhesion molecules (E-selectin, ICAM-1, and VCAM-1), and CRP in peripheral serum when clinical symptoms develops.

Neonatal sepsis is associated with high morbidity and mortality. Infected neonates should be identified as early as possible, but clinical examination and existing laboratory tests do not provide accurate diagnostic tools.

3. The third objective of the study was to evaluate the accuracy of the various mediators (TNFα, IL-1β, IL-6, IL-8, p55, p75, IL1-RA, E-selectin, ICAM-1, CRP) in diagnosing early-onset neonatal sepsis.

Materials and methods

The work is based on three study populations. Population I (paper I) consists of 221 women who delivered at the University Hospital of Trondheim, Norway, in 1998. This population was included prospectively in order to establish a sample with varying degree of placenta tissue inflammation, and included 139 deliveries complicated by premature rupture of the membranes, clinical signs of intrauterine infection or preterm labour, and 82 normal deliveries.

Population II (paper II) was recruited among 7073 neonates born at the Rogaland Central Hospital, Stavanger, Norway, from May 1994 to the end of December 1995. In all, 335 neonates were admitted to the NICU suspected to suffer from infection. We used a case-control design and included 52 neonates with infection, and 99 healthy controls. In addition, we included a group of sick controls, i.e. neonates who were suspected to suffer from infection but in whom infection was not confirmed (n=33).

Population III (paper III-VI) was recruited among 2881 neonates born at the University Hospital of Trondheim, Norway from February to the end of December 1993. We included prospectively 241 neonates who were admitted to the NICU due to a suspected infection (n=166), or other neonatal diseases.

Classification

All clinical information was abstracted from medical records. In the classification, we used a combination of clinical information, culture results, and evidence of an inflammatory response. Neonates were classified retrospectively, blinded to cytokine levels and I/T-ratio, but not to CRP and total WBC. Signs and symptoms associable with neonatal sepsis were divided into six categories: 1) pallor or icterus; 2) lethargy, apnoea, bradycardia, irritability or seizures; 3) tachypnea, retractions or respiratory distress; 4) poor peripheral perfusion, tachycardia or hypotension; 5) abdominal distension or vomitus and 6) fever or temperature instability. *Neonatal sepsis* (called probable sepsis in paper III) was defined as a combination of at least one clinical sign or symptom from each of at least three categories of clinical signs and symptoms, and a

positive blood culture. The criteria used to classify *clinical sepsis* and *pneumonia* differed somewhat in the papers, due to practical possibilities and the demands of various reviewers. Besides negative cultures, neonates with *clinical sepsis* had at least one clinical sign or symptom from each of at least three categories of clinical signs and symptoms. Neonates with *pneumonia* had sterile blood culture, respiratory signs or symptoms, and radiographic evidence of lung infection. In addition, both diagnoses demanded some evidence of an inflammatory response. In paper I, we used a maximum CRP >30 mg/l as a criterion. In paper III and IV we used an abnormal white blood cell count (elevated I/T-ratio >0.20 or white blood cell count $<5.0 \times 10^3/mm^3$ or $>25.0 \times 10^3/mm^3$), *and* a maximum CRP value >10 mg/l. In paper V and VI abnormal white blood cell counts were used alone. Probable/possible infection was diagnosed when some but not all criteria for being infected were fulfilled.

Samples

Umbilical cord blood was collected from all deliveries in population I and II using tubes without additives (population I), and heparin treated tubes (population II). We are not aware that cytokine measurements should differ between serum and plasma. Samples were kept on refrigerator until next morning, centrifuged, and frozen at -80° C until assayed. In population III, a peripheral blood sample was collected by heel-stick from all neonates on admission or the next morning (S1), and after three to four days (S2). Using tubes without additives, samples were centrifuged earlier than two hours after collection, and the serum stored at –20° C until assayed.

Histologic examination

We used histologic chorioamnionitis as an indicator of intrauterine infection. In population I, the placenta, including foetal membranes and umbilical cord was collected immediately after delivery and immersed in 4% formaldehyde. Sections from eight blocks of placenta tissues (two from the periphery and two from the central placenta, one membrane roll, and three transverse sections of the umbilical cord) were prepared routinely, and examined by light microscopy. The degree of polymorphonuclear leukocyte infiltration was evaluated separately in the chorionic plate, in the membranes (amnion and chorion-decidua), and in the umbilical cord, according to criteria given by

Salafia et al. with some modifications [30]. One medical doctor and three bioengineer students who were blinded to mediator levels and clinical information made the examinations. The inter-observer agreement was good, as evaluated by a mean weighted kappa score = 0.74 (95% CI: 0.64-0.83) in 20 examinations.

Measurements

IL-6 bioassay

IL-6 was determined by a bioassay (paper III and VI) and by ELISA (paper I and II). The bioassay measures activity, whereas the ELISA measures concentration, and the results cannot be compared directly. The bioassay utilises that growth in a mouse hybridoma cell line (B 13.29, clone B9) is IL-6 dependent [122]. Heat-inactivated (56^{o} C, 30 min) serum samples were added in duplicate, and cell viability was measured in a colorimetric assay with a tetrazolium salt after 72 hours of incubation (Sigma Chemical Co., St.Louis, MO) [123]. The IL-6 concentration was calculated by comparing growth in samples with growth induced by a recombinant IL-6 standard (kindly provided by L. Arden, University of Amsterdam, NL). The specificity of detected activity was tested by a monoclonal antibody (mAb) against IL-6 (Genzyme, Cambridge, MA). Inter- and intra-assay variability was below 10%.

ELISA

Double sandwich enzyme linked immunoassays were used to measure the concentrations of IL-1β, IL-6, IL-8, IL-1RA (Quantikine, R&D Systems Europe Ltd., Abingdon, UK), TNFα (TNFα EASIA, Medgenix Diagnostics SA, Nivelles, Belgia), E-selectin, ICAM-1 and VCAM-1 (Parameter, British Bio-technology, Abingdon, UK). The analyses were performed according to the manufacture instructions. The TNF assay uses several monoclonal antibodies, and detects TNF bound to soluble TNF receptors, as well as free TNF. Soluble TNF receptors (p55 and p75) were analysed using ELISA assays as described by Liabakk et al. [124]. CRP levels were analysed by turbidimetric assays (Cobas Mira, Roche Diagnostic Systems, and Hitachi 917, Hitachi, Japan). Single samples were analysed in appropriate serial dilutions. In paper V, the inter-assay variability of p75 was < 12.5%, in all other analyses the inter- and intra-assay variability

was below 10%. All measurements were made without knowledge of clinical and histologic data.

Statistics

Descriptive statistics included mean and standard deviation for symmetrically distributed data, and median, range, and interquartile range for skewed distributions. Differences across several groups were tested by use of ANOVA, or Kruskall-Wallis test (skewed distributions), and differences between two groups were compared by Student's test, and Mann-Whitney *U*-test (skewed distributions). Differences between categorical parameters were tested by the χ^2 –test. Correlation between postnatal age and mediator levels was expressed by Spearman's correlation coefficient *r*. Multivariate logistic regression analysis was used to evaluate possible confounding effects of several factors on the relation between leukocyte infiltration and mediator levels (paper I). A two-sided *p*-value less than 0.05 was considered statistically significant. All analyses were performed using the Statistical Package of Social Sciences (SPSS), version 8.0, 9.0, or 10.0 (SPSS, Inc., Chicago, IL).

Diagnostic accuracy

Diagnostic accuracy was evaluated by comparing infected neonates with non-infected sick controls, i.e. neonates who initially were suspected to suffer from infection, but in whom infection was not verified [125]. In paper VI, two separate analyses were performed by including possibly infected neonates as either cases or controls.

Fitted ROC curves with AUC were calculated by use of the ROCKIT software (Metz CE, Department of Radiology, Chicago Medical Centre, Chicago, IL, USA). A ROC curve describes the relation between true positive rate (sensitivity) and false positive rate (1 – specificity) for a range of threshold levels of a continuos parameter, and the AUC illustrates overall accuracy [126]. Sensitivity, specificity, and positive and negative predictive values were used to describe test performance at one mediator level. The positive likelihood ratio (true positive rate/false positive rate) and the negative likelihood ratio (false negative rate/true negative rate) were used to illustrate the weight that a given positive or negative test result could be assigned in predicting or excluding infection, respectively (paper VI) [127]. We used a stepwise multivariate logistic regression to analyse which of six inflammatory mediators independently predicted

infection, and thus might be used in combination with benefit (paper VI) [128]. In the logistic regression analyses, 24 infected and 18 probably infected neonates were available. We compared several models by entering various combinations of two to five variables. Mediators with a skewed distribution were logarithmic transformed in order to obtain nearby linearity.

Main results

Histologic chorioamnionitis and inflammatory mediators

Paper I

Severe chorioamnionitis with high-grade leukocyte infiltration was associated with elevated umbilical serum levels of TNFα, IL-1β, IL-6, IL-8, p55, p75, IL-1RA and CRP. Mediator levels in mild chorioamnionitis were not higher than in non-inflamed placentas.

Severe chorioamnionitis with subsequent neonatal disease had higher levels of all mediators except IL-1β and CRP, than severe chorioamnionitis without neonatal disease. However, severe chorioamnionitis was also accompanied by a more intense and widely distributed leukocyte infiltration, when neonatal disease developed. Umbilical vasculitis mainly appeared in severe chorioamnionitis, and had higher levels of all mediators except p75 and CRP than other cases with chorioamnionitis. Umbilical vasculitis frequently was followed by neonatal disease/neonatal sepsis.

Severe chorioamnionitis that was followed by a normal neonatal progress had higher IL-1β, IL-6, IL-8, and IL-1RA than non-inflamed placentas.

Early onset neonatal sepsis and inflammatory mediators

Paper II

Neonates with early-onset sepsis had higher umbilical serum levels of TNFα, IL–1β, IL-6, IL-8, p55, p75 and IL-1RA than healthy controls. Among preterm neonates, those with infection had higher levels of IL–1β, IL-6, IL-8, p55 and p75 than non-infected sick controls, whereas infected term neonates did not have higher mediator levels than term sick controls. Levels of all mediators, but not CRP, were very high among six preterm infected neonates who presented symptoms already at delivery.

Paper III, IV and V

Preterm neonates with early-onset sepsis had higher peripheral serum levels of IL-6, CRP, p55, and p75, than non-infected sick controls. Infected term neonates in addition had higher E-selectin, and ICAM-1. There were no differences in levels of VCAM-1 between infected neonates and sick controls.

Among infected neonates, levels of IL-6, CRP, and p55 decreased, whereas levels of p75, E-selectin, ICAM-1, and VCAM-1 did not change from admission and until three to four days after admission. Among non-infected neonates, no differences in IL-6, CRP, and p75 were detected during the same time period, whereas E-selectin and ICAM-1 increased, and p55 and VCAM-1 decreased.

Diagnostic accuracy of inflammatory mediators

Paper II

Eleven preterm neonates had early-onset sepsis, and fourteen preterms were classified as non-infected sick controls. Receiver operator characteristic plots showed that umbilical IL–1β, IL-6 and IL-8 identified infected preterm neonates with a quite high accuracy (area under the ROC plots: 0.82-0.87).

Paper VI

Among 166 consecutive neonates admitted with a suspected early-onset infection, twenty-four had an infection, eighteen had a possible infection, and 124 were classified as non-infected but sick controls. Receiver operator characteristic plots showed that CRP was the single best diagnostic test. Multivariate logistic regression modelling showed that IL-6, in addition to CRP, independently predicted sepsis. With infected and possibly infected neonates as the reference standard, a combined test of CRP ≥10 mg/l and/or IL-6 ≥20 pg/ml had a sensitivity of 85%, specificity of 62%; and a negative likelihood ratio of 0.24. Using infected neonates as the reference standard alone, and including possibly infected neonates as controls, sensitivity increased to 96%, whereas specificity decreased to 58%; a negative test result (CRP <10 mg/l *and* IL-6 <20 pg/ml) ruled out sepsis with high certainty (likelihood ratio = 0.07).

Discussion

Histologic chorioamnionitis with high-grade leukocyte infiltration in placenta tissues was associated with elevated levels of pro-inflammatory cytokines (TNFα, IL-1β, IL-6, IL-8), natural cytokine inhibitors (p55, p75, IL1-RA), and CRP in umbilical serum. This finding is in accordance with the results from previous studies that reported elevated levels of IL-1β, IL-6, IL-8, and IL1-RA [37,44,50,51]. We have not documented a bacterial aetiology to the histologic findings, but a high-grade leukocyte infiltration is likely to indicate intrauterine infection with a high accuracy [31,32,34]. With a bacterial focus in the amniotic cavity, maternal leukocytes initially may infiltrate decidua and accumulate in the subchorionic fibrin of the intervillous space. We found that elevated IL-6 and IL-8 reflected high-grade infiltration in these locations. More advanced cases with infiltration in chorion, amnion, and umbilical cord were reflected by high levels of all pro-inflammatory cytokines, and cytokine inhibitors. Thus, a certain correlation may exist between the severity of tissue inflammation and the cytokine response. In the placenta study (paper I), neonatal sepsis preferentially developed after cases with severe chorioamnionitis/umbilical vasculitis, and with high mediator levels. The population based data in paper II confirmed that neonates with early-onset sepsis have higher mediator levels than healthy neonates at delivery. Recently, three other studies have described an association between funisitis, early-onset neonatal sepsis, and high umbilical IL-6 [52,54,55]. Our results demonstrate that a much more comprehensive cytokine response accompanies umbilical vasculitis/funisitis and neonatal sepsis.

It is difficult to assess if high umbilical mediator levels were only caused by a marked placenta inflammation, or if a fetal immune activation was also present. Usually, funisitis/umbilical vasculitis may reflect a fetal inflammatory response [25,29]. The frequent finding of this manifestation in cases followed by neonatal sepsis suggest that high cytokine levels could be a result of a fetal immune response. Berner et al. found that early-onset neonatal sepsis had high levels of IL-8 in cord blood, and cells expressing mRNA for IL-8 at a high level [129], which may support this interpretation. On the other hand, we found that less marked chorioamnionitis had high umbilical levels of IL-1β, IL-6, IL-8 and IL1-RA in healthy neonates, i.e. in a situation where a fetal/neonatal contribution was unlikely. Singh et al. have reported a similar

observation. In a small study they found that clinical chorioamnionitis was associated with elevated IL-6 in umbilical serum, even in the absence of neonatal disease [56], and that cord blood cells were an unlikely source of IL-6 when neonates were clinically unaffected, because the cells did not express mRNA for IL-6. It is unknown whether cytokines can cross from inflamed placental tissues into the fetal circulation, but in general an increased vascular permeability is a central phenomenon in inflammation. Thus, there are indications that umbilical cytokine levels could be affected by a fetal immune response, although inflamed placenta tissues could also contribute.

We found that infected preterm neonates with symptoms at birth had particularly high mediator levels (paper II). Further, we observed a certain relation between severe chorioamnionitis and prematurity, in accordance with the results from several previous studies (paper I). Thus, it may be suggested that preterm neonates frequently were exposed to bacteria in utero in relation to an intrauterine infection. In contrast, a majority of term neonates developed symptoms after delivery (paper II), as previously reported [69]. Term infected neonates only had moderately elevated mediator levels in umbilical serum not higher than sick controls (paper II). However, on admission to the NICU they had higher IL-6, CRP (paper III), ICAM-1, E-selectin (paper IV), p55 and p75 (paper V) than sick controls. These findings should be interpreted carefully, because mediators were measured in two different populations, and IL-6 was measured by different assays. However, it may be suggested that term neonates could be exposed later than preterms, possibly during the passage through the birth canal. The fact that term infected neonates only had moderately elevated umbilical IL-6, but high IL-6 later may also support that term neonates have the capacity to secrete IL-6. We detected p55 and p75 in all umbilical and peripheral blood samples from healthy and ill neonates, and elevated levels were detected in both preterm and term infected neonates. Recently, other investigators have detected p55 and p75 in fetal blood [130]. We also found that p55 and p75 were elevated in severe chorioamnionitis, with a possible effect on fetal/neonatal serum levels. Nevertheless, these results suggest that shedding of soluble TNF receptors may be established in preterm and term neonates. Umbilical CRP was undetectable in nearly all neonates with infection. On admission to the NICU, CRP was elevated in the majority, and increased to maximum levels one to two days later (data not shown). These observations may suggest that increased secretion in the neonate was likely to cause high neonatal CRP levels.

Antibiotics should be used on wide indications in neonates, but it would be preferable if infection could be ruled out by use of a diagnostic test. We found that umbilical IL-1β, IL-6, and IL-8 were quite accurate in diagnosing early-onset sepsis in preterm neonates. Our results also suggest that measuring IL-6 and CRP in peripheral serum may be useful when clinical symptoms develop. If the history and clinical findings do not indicate high risk of infection, low levels of both markers may allow the physician to consider withholding antibiotics. On the other hand, high levels of one or both increased the probability of infection six-fold, and would strongly support the initiation of antibiotic treatment. Due to limited sample size, we have not validated these findings, but other investigators have also shown that CRP in combination with IL-6 or IL-8 is very sensitive [110,115]. Before clinical prediction rules can be developed, our findings should be confirmed and complemented in a larger study.

There are a number of limitations of the studies in the thesis. In papers I, and III-VI, patients were not selected randomly, and we only included a fraction of eligible individuals. Thus, the possibility of selection bias may limit the interpretation of the results. Paper II was population based and it may be safer to generalise the results from this study. In paper III-VI, antibiotic therapy was initiated before blood sampling in some of the neonates. It may be a source of error since antibiotics are likely to reduce cytokine secretion. However, other factors may also have affected cytokine measurements, because the treatment of the patients was not standardised. For instance, antibiotic therapy was likely to be initiated at various time during the infection in individual neonates, because time from bacterial exposure until clinical manifestations may have varied. Non-standardised clinical management may also lead to work-up bias [131]. Work-up bias means that physicians unconsciously may increase their efforts to diagnose certain individuals, for instance if a standardised protocol is missing. It may systematically increase the differences between cases and controls, leading to differential misclassification with erroneously high accuracy for negative prediction [131]. The fact that the physicians were not blinded to CRP may have increased the risk of this particular bias further. In the present studies, all neonates suspected to suffer from infection were treated with antibiotics. Some truly infected neonates therefore might have been classified as controls, because they benefited from treatment, and experienced an uncomplicated clinical course. This possible bias may have caused differential misclassification with affection of the control groups, reducing the accuracy

for positive prediction. The classification also implied various problems. In order to study a clinically credible spectrum of infected neonates, we included those with negative cultures if clinical evidence was strong, and a significant inflammatory response was present. However, inflammatory cytokines influence leukocytes and acute phase proteins, and the markers we used in the classification (I/T-ratio, WBC counts, peak CRP) may be related at some level to the mediators we examined. In a diagnostic study, this could give rise to some sort of incorporation bias [131]. Using culture results alone might have overcome this problem, but it would probably have caused a spectrum bias [131]. In paper II-VI, scientists who were not blinded to CRP classified neonates. Awareness of the test result may affect a classifier systematically [131], and possibly such a diagnostic review bias may have caused some misclassification. However, it is difficult to evaluate whether it would be principally differential, or non-differential. It was also a problem that some neonates could not be clearly classified, because they did not fulfil the criteria for being infected. In paper VI, we included these probably infected neonates as cases or as controls, and examined the influence on test accuracy.

Conclusion

Chorioamnionitis with high-grade leukocyte infiltration was reflected by elevated levels of pro-inflammatory cytokines (TNFα, IL-1β, IL-6, IL-8), natural cytokine inhibitors (p55, p75, IL1-RA), and CRP in umbilical serum. Chorioamnionitis with extensive leukocyte infiltration in placenta and the membranes had very high mediator levels, and often was followed by neonatal disease. Umbilical vasculitis appeared in the most severe cases, with high mediator levels, and frequently was followed by neonatal disease.

Neonates with early-onset sepsis had higher umbilical serum levels of all pro-inflammatory cytokines, and cytokine inhibitors than healthy controls. Infected preterms who were symptomatic at birth had particularly high mediator levels, whereas infected term neonates did not have higher levels than non-infected sick controls. On admission to the NICU, preterms with infection had higher peripheral serum levels of IL-6, p55, p75, and CRP than sick controls. Term infected neonates in addition had higher E-selectin, and ICAM.

Umbilical cord blood levels of IL-1β, IL-6, and IL-8 diagnosed early-onset sepsis in preterm neonates with a quite high accuracy. When clinical manifestations developed, peripheral serum levels of IL-6 and CRP independently predicted infection. A combined test of IL-6 and CRP was sensitive, and low levels of both mediators ruled out infection with a high likelihood.

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Errata

Thesis

1) Page 27, line 7: "In paper I, we used a maximum CRP >30 mg/l as a criterion. In paper III and IV we used an abnormal white blood cell count (elevated I/T-ratio >0.20 or white blood cell count <5.0 x 10^3/mm^3 or >25.0 x 10^3/mm^3), *and* a maximum CRP value >10 mg/l. In paper V and VI abnormal white blood cell counts were used alone." Replaced by: "In paper II, we used a maximum CRP ≥30 mg/l as a criterion. In paper III and IV, we used I/T-ratio ≥0.20, *and* a maximum CRP ≥10 mg/l. In paper V and VI abnormal white blood cell counts (I/T-ratio >0.20 or total white blood cell count <5.0 x 10^3/mm^3 or >25.0 x 10^3/mm^3) were used alone."

2) Page 31, in "Paper II", line 1: "Neonates with early-onset sepsis had higher umbilical serum levels of TNFα, IL–1β, IL-6, IL-8, p55, p75 and IL-1RA than healthy controls." Replaced by "Neonates with early-onset sepsis had higher umbilical plasma levels of TNFα, IL–1β, IL-6, IL-8, p55, p75 and IL-1RA than healthy controls."

3) Page 34, second paragraph, line 7: "Term infected neonates only had moderately elevated mediator levels in umbilical serum not higher than sick controls (paper II)." Replaced by "Term infected neonates only had moderately elevated mediator levels in umbilical plasma not higher than sick controls (paper II)."

4) Page 37, second paragraph, line 1: "Neonates with early-onset sepsis had higher umbilical serum levels of all pro-inflammatory cytokines, and cytokine inhibitors than healthy controls." Replaced by "Neonates with early-onset sepsis had higher umbilical plasma levels of all pro-inflammatory cytokines, and cytokine inhibitors than healthy controls."

Paper IV

5) Page 274, in the Abstract, line 17: "The use of ICAM-1 concentration (cut-off level: 250 μg l^{-1}) as a diagnostic test for infection in term neonates yielded a sensitivity of 80% and a specificity of 61%, whereas a sensitivity of 70% and a specificity of 61% were found when E-selection concentration (cut-off level: 150 μg l^{-1}), was used." Replaced by: "The use of ICAM-1 concentration (cut-off level: 250 μg l^{-1}) as a diagnostic test for infection in term neonates yielded a sensitivity of 80% and a specificity of 61%, whereas a sensitivity of 79% and a specificity of 64% were found when E-selection concentration (cut-off level: 150 μg l^{-1}), was used."

6) Page 276, in *"Statistical analysis"*, line 2: "Differences between groups were assessed by one-way analysis of variance (Kruskall-Wallis and Mann-Whitney *U* test)." Replaced by:

"Differences between groups were assessed by one-way analysis of variance (Kruskall-Wallis test), and Mann-Whitney *U*-test."

7) Page 278, in the second paragraph of *Adhesion molecules in term infants with infection*, line 7: "These cut-off levels yielded a sensitivity of 80% and a specificity of 61% for ICAM-1, and a sensitivity of 70%, and a specificity of 79% for E-selectin in detection of infections in neonates delivered at term." Replaced by: "These cut-off levels yielded a sensitivity of 80% and a specificity of 61% for ICAM-1, and a sensitivity of 79%, and a specificity of 64% for E-selectin in detection of infections in neonates delivered at term."

8) Page 278, in the Discussion, line 13: "Both ICAM-1 (cut-off level: 250 μg l^{-1}) and E-selectin (cut-off level: 150 μg l^{-1}) concentrations demonstrated a high sensitivity (80% and 79%, respectively) in the detection of infections in term neonates, whereas the corresponding specificity was moderate (both 61%)." Replaced by: "Both ICAM-1 (cut-off level: 250 μg l^{-1}) and E-selectin (cut-off level: 150 μg l^{-1}) concentrations demonstrated a high sensitivity (80% and 79%, respectively) in the detection of infections in term neonates, whereas the corresponding specificity was moderate (61% and 64%, respectively)."

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