Coagulation and Inflammation in Very Low Birth Weight Infants

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To my family

“Do your practice and all is coming”
Sri K Patthabi Jois
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This thesis includes unpublished results.
ABBREVIATIONS

APC activated protein C
ARDS Adult respiratory distress syndrome
AT antithrombin
BM Betamethasone
BPD bronchopulmonary dysplasia
CARS compensatory anti-inflammatory response syndrome
DIC disseminated intravascular coagulation
EPCR endothelial protein C receptor
F1+2 Prothrombin fragment 1+2
FIRS fetal inflammatory response syndrome
GA gestational age
GBS *Streptococcus G*
HLA-DR Human leukocyte antigen –DR
IVH intraventricular hemorrhage
LPS lipopolysaccharide
MHC major histocompatibility complex
NCPAP nasal continuous positive airway pressure
NEC necrotizing enterocolitis
PAI-1 Plasminogen activator inhibitor-1
PARs Protease activated receptors
PC protein C
PMN polymorphonuclear leukocytes
PPRs pattern recognizing receptors
PS Phosphatidylserine
RDS respiratory distress syndrome
ROC receiver operating characteristic
SIRS systemic inflammation response syndrome
SNAP-II score for neonatal acute physiology-II
SNAPPE-II SNAP-Perinatal Extension-II
TAF tracheal aspirate fluid
TAT thrombin-AT complex
TF tissue factor
TFPI tissue factor pathway inhibitor
TM thrombomodulin
TLR toll like receptor
VLBW infant very low birth weight infant
ABSTRACT

**Background:** A complex interaction prevails between coagulation and inflammation. The coagulation system is adapted to thrombotic challenges during and after birth, leading to specific features in the coagulation system of the newborn. In the very low birth weight (VLBW) infant, birth and intensive care present major immunological challenges, and the developmental immaturity of the immune system adds to the risks of intensive care. Inflammation and intra-alveolar fibrin formation characterize respiratory distress syndrome (RDS). Tissue factor (TF) is a link between inflammation and coagulation pathways. A protective response to a strong inflammatory stimulus downregulates the antigen-presenting molecules (Human leukocyte antigen (HLA)-DR) on monocytes. If severe, however, the response may lead to immunodepression or immunoparalysis, which in adults is associated with increased morbidity and death. Antenatal betamethasone treatment for mothers at risk for premature delivery effectively reduces neonatal morbidity and mortality, but exactly how this treatment in turn modulates the immune responses of VLBW infants remains unknown.

This thesis evaluates the bidirectional interaction between inflammation and coagulation and combines the findings in VLBW infants with clinical morbidity.

**Patients:** 56 VLBW infants, with a gestational age (GA) of < 32 weeks and a birth weight of < 1500 g. A control population comprised 25 healthy infants with a GA of > 34 weeks.

**Results:** We found that

1) VLBW infants showed low monocyte HLA-DR expression. On day 3, 45% of infants presented with immunodepression. This low HLA-DR expression associated with low gestational age, RDS and subsequent infections.

2) VLBW infants showed high plasma TF in circulation, but which did not lead to coagulation (thrombin formation).

3) In VLBW infants, tissue factor pathway inhibitor (TFPI) was the main anticoagulant in the first days; on day 3, however, the TF surge exceeded the TFPI capacity, leaving the VLBW infant with a pool of plasma TF.

4) In VLBW infants, antenatal betamethasone associated with immunodepression and suppressed both proinflammatory IL-6 and anti-inflammatory IL-10.

5) VLBW infants showed an inflammatory profile similar to adult sepsis or acute respiratory distress syndrome, with additional VLBW-specific features: a lower net anti-inflammatory cytokine effect without TF-induced activation of coagulation.

**Conclusions:** VLBW infants are postnatally in a state of immunodepression, which
associated with both RDS and low GA. This immunodepression is also associated with subsequent infections, and may represent a link between low GA and risk for infections. TF is expressed in the lungs of a VLBW infant and leaks into circulation. TFPI seems to be the main anticoagulant, but it could not control the TF surge on day 3. Plasma TF does not lead to thrombin formation, thereby making it available for inflammatory pathways (e.g. protease-activated receptors (PARs)). Antenatal betamethasone (BM) was associated in a time-dependent manner to immunodepression, those infants with BM <24 hours before birth showing the deepest nadir in HLA-DR expression. All these findings share similarities in adult inflammation and coagulation in clinical settings, yet still address the VLBW-specific features.
ABSTRACT IN FINNISH


Potilaat: 56 pienipainoista keskosta (alle 1500g), joiden gestaatioikä on alle 32 viikkoa. Kontrolleina 25 tervettä, yli viikolla 34 syntynyt vastasynty lasta.

Tulokset: Pienipainoisilla keskosilla oli matala monosyyttien HLA-DR-ekspressio, ja 3. päivänä 45%:lla keskosista todettiin immunodepressio (HLA-DR-ekspressio alle 60%). Tämä immunodepressio assosioituu gestaatioikään, RDS-tauksiin ja myöhempiin tehohoidon infektioihin. Keskosilla oli verenkierrossa korkea TF-pitoisuus, mutta hyytymisjärjestelmän aktivaatiota ei todettu (ei trombiinin muodostumista). TFPI (tissue factor pathway inibitor) oli keskosella merkittävä antiinflammatorinen sytokiinivainetta. Pienipainoisilla keskosilla syntymään edeltävä ahdistava kortikosteroidihoito assosioituu immunodepressioon sekä IL-6- ja IL-10-sytokiihien supressioon.

Yhteenvetona: Pienipainoisilla keskosilla on samankaltainen inflammatorinen profili kuin aikuisilla vaikeassa sepsisessa tai aikuisen RDS-taudissa, mutta keskosilla on matalampi anti-inflammatorinen sytokiini-vaikutus ja TF ei johda hyytymisjärjestelmän aktivoitumiseen. Syntyvän jälkeinen immunodepressio assosioituu matalaan gestaatioikään ja tehohoidon infektioihin; immunodepressio voi olla yksi merkittävä selitys tiedettynä matalaan gestaatioikään liittyvään infektioriskiin. RDS-taudissa TF-ekspression lisääntyminen keuhkoissa johtaa
systeemiseen TF:n vapautumiseen, mutta ei trombiinin syntyyn. TFPI pystyy kontrolloimaan ensi päivänä tätä TF-ylimääriä verenkierrossa, mutta ei enää päivänä
3. Koska TF ei kuitenkaan johda hyytymisjärjestelmän aktivoitumiseen, on
todennäköistä, että TF toimii inflammaation aktivoijana. Syntymää edeltävään
kortikosteroidi hoitoon liittyvän keskosilla immunodepressio, joka on voimakkain
keskosilla, joiden äidit ovat saaneet kortikosteroidi hoidon alle 24 tuntia ennen lapsen
syntymää. Kaikilla näillä löydöksillä on samankaltaisuksia aikuisen inflammaation
ja hyytymiseen liittyvien sairaustilojen kanssa, mutta pienipainoisilla keskosilla on
aivan omat erityispiirteensä.
INTRODUCTION

Preterm infants are born before 37 weeks of gestation, and very low birth weight infants (VLBW) are those with a birth weight under 1500g and usually a gestational age (GA) of less than 32 weeks. Preterm birth occurs in 5-13% of deliveries (Goldenberg et al. 2008, Muglia et al. 2010). In Helsinki, 150 VLBW infants are born annually. Their survival rates and the intact survival rates of preterm infants have improved, but their morbidities affect their childhood, even into adulthood (Tommiska et al. 2003, 2007, Mikkola et al. 2005, Hovi et al. 2007).

After birth, the lungs are exposed to a variety of antigens and often to ventilator-induced trauma and oxygen toxicity as well (Coalson et al. 1995). Respiratory distress syndrome (RDS) is the main morbidity in preterm infants during their first week of life (Speer 2011). Characteristic of RDS is inflammation and intra-alveolar fibrin formation (Jaarsma et al. 2004, Gitlin and Graig 1956). In adult RDS, tissue factor (TF) is the main mediator behind the interplay between inflammation and coagulation (Bastarache et al. 2007).

In acute injury, infection or trauma, the initial strong inflammatory reaction is attenuated to protect the body from overwhelming, harmful, systemic inflammation. This reaction known as the compensatory anti-inflammatory response syndrome (CARS) (Frazier and Hall 2008), is mediated in adults by anti-inflammatory cytokines, mainly IL-10 (Abe et al. 2008, Monneret et al. 2004). If prolonged or extensive, however, CARS can become harmful and increase the risk of infection and death (Livingston et al. 1988, Allen et al. 2002). A decrease in HLA-DR expression is characteristic of CARS (Döcke et al. 2005, Frazier and Hall 2008). Preterm infants show postnatal low HLA-DR expression in RDS and infection (Birle et al. 2003, Kanakoudi-Tsakalidou et al. 2001). Low HLA-DR associates with chorionamnionitis and sepsis and predicts mortality in sepsis (Azizia et al. 2012, Genel et al. 2010). However, the course of postnatal CARS and its possible risks have not been evaluated in preterm infants in intensive care.

In adults and in experimental studies, inflammation activates TF expression in the lungs and on monocytes, leading to the simultaneous activation of coagulation and inflammation, which increases one’s risk for organ injury (van Till et al. 2006, Bastarache et al. 2007, van der Poll 2008). In infants the activation of coagulation occurs postnatally (Hyytiäinen et al. 2003), in preterm infants, however, the role of TF in postnatal intensive care remains unknown.

Antenatal maternal glucocorticoids, mainly betamethasone (BM), are routinely used in case of risk for preterm delivery between weeks 23-34. The positive effects of antenatal BM are well documented (Roberts and Dalziel 2006): they reduce the risk
for RDS, intraventricular hemorrhage (IVH) and necrotizing enterocolitis (NEC) and improve survival rates. The effect of BM on the immune system in preterm infants has seen little study. In animal studies antenatal glucocorticoid treatment associates with changes in monocyte functions. Shortly before birth, the administration of glucocorticoid suppresses monocyte functions, both cytokine production and hydrogen peroxidase production, and a maturing immunomodulatory effect can be seen with increased monocyte response to stimuli (Kramer et al. 2004).

In VLBW infants, the developmental immaturity of the immune system (Hallwirth et al. 2004, Yerkovich et al. 2007) and immunological challenges after birth, together with the developmental immaturity of the coagulation and activated coagulation adds to the risks in intensive care. This thesis aims to explore the complex interactions between inflammation and coagulation in the VLBW infant and their associations with morbidity.
REVIEW OF THE LITERATURE

Inflammation is a reaction rubor, calor, tumor, dolor- to infection or tissue injury, that begins when innate immunity cells recognize foreign material or antigens. The major host defense systems, shared evolutionarily with multicellular organisms, include coagulation, phagocytosis, pattern-recognizing receptors, the production of reactive oxygen species, and complement activation (Iwanaga and Lee 2005).

Inflammation and coagulation are not separate entities but rather a cross-communicating system with several links at the cellular and molecular levels. In the interplay between inflammation and coagulation, tissue factor is the central mediator, resulting in the activation of coagulation and inflammation (Levi M et al. 2004, 2006).

**Inflammation**

*Innate immunity*

The immune system consists of innate and adaptive systems; of them the innate system is the first line response to foreign antigens. The innate immune system evolved early in evolution, is found in all multicellular organisms (Iwanaga and Lee 2005) and functions without prior contact with foreign material.

Innate immunity mechanisms include physical barriers such as skin and mucous membranes, antimicrobial peptides, a complement system, neutrophils, natural killer cells, monocytes, mast-cells and tissue macrophages (Medzhitow and Janeway 2000). Instead of antigen-specific receptors innate immunity receptors, known as pattern-recognizing receptors (PRRs), recognize conserved structures, such as lipopolysaccharide and peptidoglycan, which are common to microorganisms (Medzhitow and Janeway 2000). The recognition is specific to each genetically predetermined receptor, which can be divided into secreted pattern-recognizing receptors, such as mannan-binding lectin, responsible for activating complement when binding to microbes (Fraser et al. 1998), endocytic pattern-recognition receptors responsible for the uptake of microbes to be killed and processed to the HLA-DR system (Suzuki H et al. 1997), and signaling receptors, such as toll-like receptors (TLR), responsible for activating immune-response genes and resulting in cytokine production (Gay et al. 1991) (Figure 1).

Thus, the innate immunity system can eradicate foreign pathogens directly, or present the antigens to the adaptive immunity system in order to activate cell mediated and humoral T and B-cell responses.
Monocytes
Circulating monocytes are found in fetal blood at 18-20 weeks of gestation. A linear increase from 3 to 7% of blood count in monocytes begins at 30 weeks of gestation, and at birth term newborns have higher monocyte concentrations than adults (Clapp 2006). Christensen et al 2010, constructed monocyte reference ranges from a large cohort of infants from 22-42 weeks of gestational age, excluding infants with infection or necrotizing enterocolitis. The reference range for full-term infants is 0.3-3.3 (mean 1.4) 10E9/L, and for 28 weeks, 0.1-2.5 (mean 0.8) 10E9/L (Christensen et al. 2010).

Monocytes are important elements in innate immune responses by phagocytosis and by expressing HLA-DR molecules, thereby presenting antigens to cells of the adaptive immune system, and by releasing cytokines to mediate responses to other cells and to activate complement and coagulation cascades (Tonegawa 1988, Turina et al. 2006). Circulating monocytes migrate to tissues or to sites of inflammation within one day, becoming macrophages, Langerhans cells, Kupfer cells, microglial cells or osteoclasts (Christensen et al. 2010). Monocytes express upon activation tissue factor on their surface – forming a link between inflammation and coagulation (Østerud and Björklid 2006) (Figure 1).

Figure 1.
Figure 1. Monocyte activation. Activated monocytes express TF, phosphatidylserine (PS), CD11b and cytokines, all of which contribute to the activation of inflammation and coagulation. TF may shed to plasma either in free or microparticle-bound form. Protease-activated receptors (PARs) on monocytes are the receptors for coagulation factors that result in transmembrane signaling and the activation of monocytes, which leads to inflammation. TLR-4 is a pattern-recognition receptor, and the ligand is lipopolysaccharide (LPS) a cell membrane structure of Enterobacters. In the interplay between inflammation and coagulation, TF is a central mediator of activation for both coagulation and the innate immune system. Endothelium also plays an active, important role in regulating inflammation and coagulation. Physiological anticoagulants and modulating adhesion molecules are connected to the endothelium.

Major histocompatibility complex (MHC)
The discovery of similarities in the human and murine antigen systems in 1960 led to the classification of this antigen system as MHC, found in all vertebrates. All nucleated cells express MHC class I molecules, and these, together with B2-microglobulin, are responsible for immune reactions leading to the destruction of host cells by cytotoxic T-cells (reviewed in Turina et al. 2006). MHC class II molecules, expressed on antigen-presenting cells, serve as immunological recognition molecules and are responsible for the presentation of antigen to T-cells (Tonegawa 1998, Turina et al. 2006) (Figure 2).

In humans, MHC molecules are called human leukocyte antigen (HLA); MHC II molecules include HLA-DR, -DQ, and -DP. These HLA subgroups associate with different morbidities. HLA-DR, for example, serves as a marker of antigen presentation capacity (Livingston et al. 1988, Döcke et al. 2005).

HLA-DR
HLA-DR is a transmembrane glycoprotein with a 36-kD α- and a 27-kD β- subunit (Lampson and Levy 1980). Monocytes, B-lymphocytes and dendritic cells all express HLA-DR (Turina et al. 2006). HLA-DR molecules are found on fetal mononuclear cells in the beginning of the second trimester (Azizia et al. 2012). Fetal and neonate monocytes express lower levels of HLA-DR than do adult monocytes (Azizia et al. 2012, Hallwirth et al. 2004, Kampalath et al. 1998).
Figure 2. HLA-DR molecules and antigen presentation (reproduced with permission from Turina et al. 2006). HLA-DR is an MHC class II protein which presents antigens to T helper-cells. The antigen is phagocytosed, degraded to fragments, and bound to an HLA-DR molecule. The antigen-HLA-DR complex is externalized to the cell surface to present this antigen to T helper-cells. These T-cells stimulate macrophages and cytotoxic T-cells, and activate B-cells to produce antigens, thereby linking the innate and adaptive immune systems.

Cytokines
Cytokines are small proteins that function as mediators and effectors in inflammatory processes. Cytokines also play an important role as mediators of normal cell signaling.

Cytokines are usually categorized as anti-inflammatory (IL-4, IL-10, IL-13) or proinflammatory (IL-1β, IL-6, IL-8, IL-12, IFN-γ, GM-CSFβ, TNFα), while some have functions unfit for this categorization (IL-2, IL-5, IL-7) (Ng et al. 2003, Gogos et al. 2000). IL-1, IL-6, IL-12, and TNF-α are released in tissue injury and from monocytes during inflammation, resulting in increasing TF expression and fibrin formation (Levi et al. 2004). This categorization to pro- and anti-inflammatory cytokines depends on biological processes, and many cytokines may have a different activity than that listed above (Dinarello 2000).

IL-6, IL-8 and IL-10 play important roles in the interplay between inflammation and coagulation, or in monocyte deactivation, where they receive more attention. TNF-α, though an important proinflammatory cytokine expressed by monocytes, is an unreliable marker for a clinical study due to its short half-life of 18.2 minutes (Oliver et al. 1993).
IL-6
Monocytes, lymphocytes and fibroblasts produce a 21-kDa glycoprotein known as IL-6. The main biological effects of IL-6 include the activation of T and B-lymphocytes, the modulation of hematopoiesis (Borden et al. 1994) and the activation of coagulation (Levi et al. 1997) (Figure 3). IL-6 is mainly responsible for the inflammatory activation of coagulation. In experimental studies, blocking IL-6 attenuates thrombin formation in endotoxemia (Levi et al. 1997). In addition to proinflammatory effects, IL-6 has anti-inflammatory effects, namely the suppression of IL-1β and TNF-α (Schindler et al. 1990, Aderka et al. 1989). These anti-inflammatory effects of IL-6 may play a role in sepsis (Xing et al. 1997) or lung injury (Ulich et al. 1991). The direction towards anti- or pro-inflammatory effects may depend on whether the IL-6 receptor is soluble (pro-inflammatory) or membrane bound (anti-inflammatory) resulting in different cell signaling and different target cells (Scheller et al. 2011). Plasma IL-6 is considered as a marker of activation of cytokine activation and a reflection of the inflammatory response and disease severity (Damas et al. 1992, Hack et al. 1989).

IL-8
Many cell types, including monocytes, polymorphonuclear leukocytes and endothelial cells, produce IL-8, a small protein in the chemokine (chemotactic cytokine) family of cytokines. The main biological effect of IL-8 is the activation, attraction and adhesion of neutrophils in inflammation sites (Strieter and Kunkel 1994, Blackwell and Christman 1996, Laudanna et al. 1996). High IL-8 concentrations correlate with mortality in sepsis and ARDS; IL-8 thus seems to be an important mediator in organ dysfunction following systemic inflammation (McClintock et al. 2008).

IL-10
IL-10, produced by monocytes, macrophages and lymphocytes, down-regulates MHC-II molecules on monocytes (Koppelmann et al. 1997), inhibits not only cytokine production from monocytes/macrophages and neutrophils, but also lymphocyte responses (Opal and DePalo 2000).
Figure 3. The procoagulant effects of cytokines. Cytokines TNF-α, IL-1, IL-6 and IL-8 activate both endothelial cells and monocytes. Increased monocyte TF and decreased monocyte and endothelial thrombomodulin (TM) increases available thrombin. Similarly, an increase in endothelial PAR-2 expression leads to FVIIa sensitization and increased thrombin formation. A decrease in endothelial protein C receptors (EPCR) and TM, however, results in a decrease in APC, thereby enhancing coagulation. Plasminogen activation inhibitor-1 (PAI-1) increases in cytokine activation, leading to decreased fibrinolysis.

Coagulation
Evolutionarily the blood coagulation system and innate immune system share a common ancestral cascade, and coagulation factors have evolved from complement system proteases (Krem and DiCera 2002). The history of coagulation cascade research began in the 19th century when Muller and Virchow discovered fibrinogen and fibrin and Schultze discovered thrombocytes. Toward the end of the 19th century, Arthus discovered the importance of calcium in coagulation. In 1905, Morawitz introduced the theory of tissue factor leading to thrombin formation (Shapiro 2003). Coagulation factors I-XII received their names in a consensus meeting in 1962, and factor XIII was added a year later. First came the idea of distinct intrinsic and extrinsic pathways, but over the years, the importance of tissue factor-initiated coagulation changed the view for the cascade; a common pathway is now considered important (Figure 4).

The amplification loops 1) TF-FVIIa->FIXa->Xa, 2) thrombin->FVa and FVIIIa, and 3) thrombin->FXa->FIXa and FXa (Figure 4), eventually lead to an abrupt increase in thrombin and fibrin formation (Levi et al. 2004). Several regulatory systems control coagulation (Figure 4) and coagulation factors play an important role in inflammatory processes (Figure 4).
TF

Tissue Factor (TF), formerly known as thromboplastin or coagulation factor III is a 47-kD transmembrane glycoprotein. It was discovered when tissue added to plasma led to activated coagulation cascade, hence the name tissue factor (Shapiro 2003); it was isolated in 1985. In 1989, Drake et al. introduced a concept of hemostatic envelope; TF is intact in the adventitia, but any rupture of the endothelial lining will expose TF to blood coagulation factors, leading to the formation of a repairing clot (Drake et al. 1989). The binding mechanism of the extracellular domain of TF binding to factor VII, was discovered 1996. The importance for animals and humans became evident that same year when Toomey et al. reported embryonic lethality in mice with knockout TF gene (Toomey et al. 1996). In 1999, Giesen et al. published the first reports of blood-borne circulating TF (Giesen et al. 1999), and this free TF activated the coagulation cascade.

TF was long considered only a tissue initiator of the coagulation cascade, leading to the activation of the (extrinsic) downstream coagulation cascade, the end product of
which is thrombin. Recent research depicts TF as the initiator of the coagulation, so division of the coagulation cascade into extrinsic and intrinsic parts is no longer relevant.

Providing tissue-specific hemostatic protection, TF is abundant in vascular organs, (Østerud and Björklid 2006) such as the brain, placenta and lungs, where both bronchoalveolar macrophages and alveolar epithelial cells express TF (Bastarache et al. 2007). In vessel walls, adventitial, but not endothelial cells (Østerud and Björklid 2006), express TF, thus creating a hemostatic envelope. Blood monocytes express TF (Østerud and Björklid 2006) with an ability to upregulate the expression 10- to 1000-fold upon cell activation (Levi et al 2006). Polymorphonuclear leukocytes (PMN) can acquire TF from microparticles, which are shed from activated monocytes and may fuse with the cell membranes of PMN (Egorina et al 2008).

In healthy adults, free circulating TF (plasma TF) is present in low concentrations in plasma (Giesen et al. 1999). TF reportedly also exists in an alternative spliced form, though its coagulation activity is uncertain (Bogdanov et al. 2003).

In systemic inflammation, lung injury or sepsis plasma TF increases (Gando et al. 2002 and 2003, Bastarache et al. 2007). The upregulation of monocyte TF expression is considered one mechanism for increasing the systemic availability of TF during inflammation (Giesen et al. 1999); another is lung-expressed TF leaking from the lungs into plasma (Bastarache et al. 2007).

TF mediates cytokine production and innate immunity responses through its cytoplasmic domain. In mice, a lack of this domain leads – in LPS challenge – to the transient enhancement of coagulation, inhibited inflammatory response and lower mortality (Sharma et al. 2004). TF in atherosclerotic plaques enhance thrombosis (Mackman 2009), and TF plays a role in tumor growth as well (Mackman 2009). As well alternatively spliced TF-integrin interaction seems to contribute to angiogenesis and monocyte-endothelial interactions (Srinivasan and Bogdanov 2012). TF is expressed already in the early stages of embryogenesis, and even in tissues where it cannot be observed in adults (Luther et al. 1996).

TF is the most important mediator in the interplay between coagulation and inflammation (Figure 5). TF-VII complex binds to protease-activated receptor-2 (PAR-2), thereby activating inflammation processes, such as the upregulation of HLA-DR expression (Coughling 2000, Cunningham et al. 1999, Veersteeg et al. 2001) (Figure 1). Blocking TF in sepsis prevents coagulation-induced inflammation, revealing its importance as an independent inflammatory mediator apart from thrombin (Miller et al. 2002, Welty-Wolf et al. 2001).

TFPI
Tissue factor pathway inhibitor (TFPI) is a serine protease inhibitor of the Kunitz
Hjort first showed the inhibitory activity of TFPI in 1957; the name TFPI was established as late as 1991 (Lwaleed and Bass 2006). TFPI is the only known inhibitor of TF and controls the coagulation effects of TF by binding together with Xa to TF-FVII-complexes, forming an inactive quaternary complex and thereby inhibiting thrombin formation (Broze 1995).

Endothelial cells synthesize TFPI, and most of the TFPI (50 to 80%) is bound to the endothelial cell surface. In addition to the endothelial bound form, TFPI circulates as lipoprotein-associated TFPI (80%) and as free TFPI. Only the free form of TFPI has anticoagulant activity. TFPI is also found in platelets, accounting for 5 to 10% of the total TFPI (Bridey et al. 1998). TFPI may also regulate the inflammatory TF signaling via PARs, because recombinant TFPI is known to inhibit TF-PAR signaling (Ahamed et al. 2005). Neutrophils activated by inflammation may cleave TFPI to a less active form, leaving TF available for coagulation and inflammation (Higuchi et al. 1992). The TFPI homozygous deletion gene phenotype is lethal in mice, thereby demonstrating its physiological importance (Huang et al. 1997).

**Thrombin**

TF initiates the coagulation cascade, which form the end-product thrombin. Thrombin serves many functions apart from fibrin formation, including inflammation, anticoagulation, anti-inflammation, cell protection, and feedback regulation of the coagulation cascade (Figures 4 and 5). The inflammatory effects of thrombin are mediated by PAR-1, -3 and -4. PARs, sensors of extracellular proteases, are found on mononuclear cells, endothelial cells, platelets and fibroblasts, and serve as their own ligands: an activated coagulation factor cleaves the extracellular end of the receptor, thus forming a neo-aminoterminus. This serves as the ligand for the same receptor, resulting in transmembrane signaling (Coughlin 2000). In endothelial cells, IL-6 and IL-8 secretion is enhanced, and in monocytes and macrophages, IL-8 production is stimulated (Drake et al. 1992). Thrombin is chemotactic for neutrophils and monocytes (Fujita et al. 2008), and induces the production and release of various adhesion molecules, growth factors and chemokines. PAR-1 activation by thrombin results in a proinflammatory and vascular permeability-enhancing response (Coughlin SR 2000, Feistrizer and Riewald 2005).

Thrombin serves as a negative feedback to its own production: when thrombin binds to thrombomodulin (TM), it can no longer convert to fibrinogen and loses its procoagulant activity (Fuentes-Prior et al. 2000, Conway 2012). This thrombin-TM complex acts as an anticoagulant enzyme converting protein C to activated PC (APC) (Esmon and Owen 1981) (Figure 5).

**Natural anticoagulants**

**Protein C**

Protein C (PC) is a liver-synthetized K-vitamin-dependent glycoprotein that circulates...
in zymogenic form in the blood with a half-life of eight hours. PC is activated by thrombin, and this activation is enhanced by the presence of TM or endothelial protein C receptors (EPCR) on the endothelial surface (Esmon and Owen 1981, Esmon 1989). APC proteolytically inactivates FVa and FVIIIa (Griffin et al. 2012) (Figure 2). If after activation APC is bound to EPCR, this complex then binds to PAR-1, thus mediating anti-inflammatory (Mosnier et al. 2007) and cell-protective effects (Riewald et al. 2003, Cheng et al. 2003). As a result of the cell-protective properties of APC-EPCR1 mediated by the activation of sphingosine-1-phosphate receptor, the endothelium retains a barrier against pro-apoptotic and proinflammatory factors (Feistrizer and Riewald 2005). On macrophages anti-inflammation and barrier protection is mediated by CD11b/CD18, not EPCR (Cao et al 2010). APC acts in vitro as an anti-inflammatory agent mainly by modulating monocyte activation during inflammation and by inhibiting neutrophil adhesion (Hancock et al 1995, Grey et al 1993, White et al 2000, Yuksel et al 2002) (Figure 6). In severe inflammation, the PC system malfunctions at many levels.

Antithrombin
Antithrombin (AT), a liver-synthesized 58-kDa glycoprotein with a half-life of three days, is the most important physiological inhibitor of thrombin (Quinsey et al. 2004). AT also inhibits other proteolytic coagulation factors such as FIxa, Fxa, and FIXa (Quinsey et al. 2004) (Figure 3). Thrombin binds to fibrin-clots protected from the anticoagulant effects of AT. As a cofactor, AT needs heparin. Endothelial heparin sulfate, the biological cofactor, localizes AT mainly to the endothelial lining of the vessels (Rau et al. 2007). AT also has anti-inflammatory effects on monocytes by reducing the expression of IL-6 and TF, and by reducing IL-8-induced chemotaxis (Dunzendorfer et al. 2001, Kaneider et al. 2002) (Figure 5).

Figure 5. The anti-inflammatory roles of natural anticoagulants. Thrombomodulin (TM) binds and inactivates thrombin, and acts as a cofactor for PC. Endothelial cell protein C receptor (EPCR) enhances the activation of PC by the thrombin-TM complex and, when bound to
APC, turns this natural anticoagulant into an anti-inflammatory mediator. AT antithrombin, TAFI thrombin activatable fibrinolysis inhibitor.

Special aspects of inflammation and coagulation in term and preterm infants
The special characteristics of newborn immune and coagulation systems are functionally adapted to best protect the term infant. In preterm infants, however, developing immune and coagulation systems show limitations compared with those of term infants and adults. At birth, the in utero environment without antigens changes dramatically to an antigen-rich environment. To avoid harmful in utero proinflammatory reactions, possibly leading to preterm delivery, the sterile environment will not activate adaptive immunity, and both proinflammatory IL-1B and TNF-alpha expression as well as T-helper activity are suppressed (Vitoratos et al. 2006). Adaptive immunity gradually matures postnatally, but for the newborn infant, innate immunity is the first-line immune defense (Krishnan et al. 2003).

LPS challenge models indicate that the function of innate immunity cells, monocytes and antigen-presenting cells in infants, is normal in the production of certain cytokines (IL-6, IL-8 IL-10, IL-23) (Angelone et al. 2006, Vanden Eijnden et al. 2006, Chelvarajan et al. 2004, Schultz et al. 2002). HLA-DR expression, however, is lower in infants than in adults, leading to the impaired activity of antigen-presenting cells (Kanakoudi-Tsakalidou et al. 2001).

Fetal cytokine activation occurs in maternal inflammatory conditions and can continue after birth as a systemic inflammation reaction (Lyon et al. 2010). Birth-stress activates a short-lived proinflammatory cytokine response in term infants, but may continue longer and contribute to morbidity in preterm infants (Lyon et al. 2010). Newborn infants exhibit inhibitory activity in plasma against Toll-like receptors (TLR), leading to a 10- to 1000-fold reduction in TNF-alpha production. This protects the fetus from inflammation and the risk for preterm delivery, but also poses a risk for postnatal infections in preterm and newborn infants (Levy et al 2004). In healthy newborns, complement proteins, a part of innate immunity, reach 10-70% of adult levels, possibly limiting the eventual capacity to reduce and clear bacteria from blood (Firth et al 2005).

In newborn infants, and especially in preterm infants, the physical aspects of innate immunity (e.g., the skin, gut and mucosal linings) are fragile and more easily invaded by bacteria (Larson and Dinulos 2005). On the other hand, the vernix caseosa, rich in innate immunity molecules, serve as the infant’s antimicrobial defense in the transition to the outer world (Tollin et al. 2005). Elevated postnatal levels of IL-6 induce an acute phase response, activating anti-infective proteins and peptides on both mucosal and epithelial linings and in the blood, thus protecting the newborn infant against infection during microbial colonization (Angleone et al. 2006).
In preterm infants, colonization of the skin with normal bacterial flora does not follow the normal postnatal course in a sterile environment or in selected bacterial flora settings in intensive care, where the use of antibiotics also disturbs colonization (Larson and Dinulos 2005). Intensive care, with its intravenous lines and central catheters passing through the skin and with intubation tubes and tracheal suction causing micro-trauma to the respiratory tract, also adds to the risks of this immaturity.

The developmental immaturity of the immune system increases the infant’s risk for postnatal infections. Preterm infants are at 5- to 10-fold greater risk for infections than are term infants (Clapp 2006). In addition, near-term infants with GA < 37 weeks might also be at increased risk for infections (Sadeghi 2007).

In the newborn, coagulation is effective, optimal, and adapted to birth and postnatal life, but exhaustion in longer coagulation needs can occur. The levels of coagulation factor depend on gestational age, and increase towards term (Andrew et al. 1987, 1988, Kuhle et al. 2003, Monagle et al. 2006). Birth activates the coagulation system (Surace et al. 1985, Yuen et al. 1989, Kulkarni et al. 2013). In healthy infants, this activation is short-lived, and clinical complications are rare; in sick infants, however, elevated thrombin markers are present as a marker of ongoing coagulation (Hyytiäinen et al. 2003, Schmidt et al. 1993).

Concentrations of specific k-vitamin-dependent coagulation factors, as well as concentrations of protein C (zymogen), are lower in infants than in adults (Monagle et al. 2006). The balance of coagulant factors and anticoagulants in newborns differs from that in adults, thus ensuring efficient hemostasis despite lower levels of coagulation factors than in adults (Hyytiäinen et al. 2003). In cord blood, levels of both TFPI and AT are physiologically low, and thrombin formation is effective (Cvirn et al. 2003). During longer coagulational needs, exhaustion of both the coagulation and anticoagulation systems is possible, but they are quite efficient for short-lived needs.

TF procoagulant activity in term infants is low in cord blood (Cvirn et al. 2003), even though studies have reported TF antigen levels in cord blood to be two-fold higher than adult levels (Uszynski et al. 2011). The postnatal regulation of TF is unknown, but as a K-vitamin-independent coagulation factor, the regulation and expression of TF may be more similar to those of adults than are the K-vitamin-dependent factors. Both TFPI activity and total TFPI in cord blood are low in term infants compared those in adults (Cvirn et al. 2003) Healthy newborns have 50% of adult TFPI levels (Lwaleed and Bass 2006), but the postnatal regulation of TFPI is unknown (Monagle et al 2006). In vivo thrombin formation in newborns begins earlier than in adults, but the total amount of thrombin generated is smaller (Hyytiäinen et al. 2003, Cvirn et al. 2003).
At 15% of adult levels, fetal levels of PC zymogen are low. In healthy newborns, the levels are at 30-40% of adult levels (Monagle et al. 2006). APC levels in cord blood, however, are similar to or higher than those in adults (Petäjä et al. 1998). This suggests that in newborns, keeping the K-vitamin-dependent zymogen form at low levels is favorable in cases of K-vitamin deficiency, as is maintaining appropriate anticoagulation by enhanced activation of the zymogen form. On the other hand, this leads to exhaustion of the system if the need for anticoagulant exceeds zymogen levels (Petäjä et al. 1998, Petäjä and Manco-Johnson 2003). At 20% of adult levels, fetal levels of AT are low. In healthy preterm infants, the levels are around 40% of adult levels (Andrew et al. 1988), and in healthy newborns, 60-70% of adult levels (Monagle et al 2006). Increased heparane sulphates lining the endothelium, however, may upregulate the functional activity of AT (Nitschmann et al. 1998).

Systemic inflammation response syndrome and fetal inflammatory response syndrome

In 1983, Nelson introduced the concept of systemic inflammation response syndrome (SIRS), to describe acute inflammatory reactions after hypotensive shock. In 1992, the American College of Chest Physicians/ the Society of Critical Care Medicine defined SIRS as the systemic inflammatory response to a variety of clinical conditions. However, if infection is the cause of SIRS it should be called sepsis (Bone et al 1992). In SIRS, a proinflammatory cytokine burst predominates (Bone 1996a). Fetal inflammatory response syndrome (FIRS) is a similar condition in the fetus characterized by systemic inflammation and elevated fetal plasma IL-6 (Gotsch et al. 2007).

Compensatory anti-inflammatory response syndrome

Compensatory anti-inflammatory response syndrome (CARS), first noted 1997 by Bone, to give a name on the processes limiting inflammation in severe illness (Bone 1996b). In acute illness or trauma the inflammatory burst may lead to CARS (Frazier and Hall 2008, Adib-Conquy and Cavallon 2009), the immune system is downregulated to protect the body from overwhelming systemic inflammation. Anti-inflammatory cytokines may mediate this downregulation (Monneret et al. 2004, Abe et al. 2009).

Indicative of CARS is the downregulation of HLA-DR molecules on monocytes (Döcke et al. 2005) and monocyte hyporesponsiveness (Majetschak et al. 1999). The mechanism of this process is not fully understood, however. In experimental, LPS-challenge models, possible intracellular mechanisms for HLA-DR downregulation are the impairment of TLR signaling or the inhibition of proinflammatory transcription factor NF-κB (Frazier and Hall 2008), or the inhibition of exocytosis and inhibition of the recycling of existing HLA-DR molecules by IL-10 (Fumeaux and Pugin 2002).
Persistent low HLA-DR expression is a sign of immunoparalysis. In adults, immunoparalysis is associated with increased risk for infection and death (Volk et al. 1996, Mentula et al. 2004, Livingston et al. 1988) (Figure 6). The definition most often used for immunoparalysis is HLA-DR expression < 30% on circulating monocytes, quantified by flow cytometry. Monocyte HLA-DR expression between 30 and 60% indicate moderate to severe immunodepression (Döcke et al. 2005). LPS-challenge in vitro on monocytes may reveal hyporesponsiveness (Allen et al. 2006), indicating immunoparalysis on higher HLA-DR expression than in these definitions (Azizia et al. 2012). IFN-gamma may reverse anti-inflammatory response to strong inflammation (Kox et al. 1997).

Figure 6. Hershman et al. 1990 showed in trauma patients the course of protective CARS with all patients surviving (circles), immunodepression with sepsis as complication in intensive care (rectangles) and immunoparalysis with mortality (triangles). Reproduced with permission from: Hershman et al. 1990.

Coagulation and inflammation in clinical disease in adults

Acute lung-injury ARDS
In adult RDS, the activation of coagulation occurs simultaneously with inflammation. The systemic activation of coagulation may lead to disseminated intravascular coagulation (DIC) with a risk for multi-organ failure (Levi 2007). Vascular permeability is enhanced leading to pulmonary edema. Mononuclear cells and polymorphonuclear leukocytes invade the lungs and increase cytokine production, IL-8 is an important cytokine associating with disease severity (McClintock et al. 2008, Lin et al. 2010).
Free TF expressed in bronchoalveolar macrophages, leaks into plasma (Bastarache et al. 2007). In lung injury, however, TFPI production is insufficient to inactivate TF (Bastarache et al. 2008), yielding an imbalance in the form of increased intra-alveolar fibrin formation and inflammation. On the other hand, experimental studies show that blocking TF upregulation by inhibiting proinflammatory cytokines attenuates lung injury (Welty-Wolfe et al. 2001, Miller et al. 2002).

Sepsis
In sepsis, the localized immune response to pathogens becomes widespread leading to endothelial injury, tissue damage, and – if severe to septic shock (Astiz and Rackow 1998). Systemic inflammation often precedes widespread infection, and as a second phase, anti-inflammatory CARS will reduce the strong and possibly harmful inflammatory reaction, if prolonged or extensive CARS becomes harmful. In adult patients with septic shock, survivors show a recovery of HLA-DR expression on days 3-4. An HLA-DR expression < 30% on days 3-4 independently associates with mortality (Monneret et al 2006). Those sepsis patients who can react with proinflammatory cytokines despite CARS have better prognoses than do those with no proinflammatory response (Pachot A et al. 2006). Among adults with sepsis and children with multi-organ failure non-survivors showed an anti-inflammatory profile with elevated IL-10 concentrations or IL-10 transcription factors (Lekkou et al. 2004, Hall et al. 2007).

The coagulation system becomes active simultaneously with the immune response, leading in severe cases to disseminated intravascular coagulopathy with the consumption of coagulation factors and micro-thrombus formation in the vasculature (Levi 2007). Supplementation of APC in sepsis reduces mortality (Bernard et al. 2001), but the risk for bleedings as well as controversial results in large studies have discouraged its use (Wiedermann and Kaneider 2005). Typical of a septic shock is a reduction in AT concentrations (Eisele and Lamy 1998). In endotoxemia- challenging animal models AT diminishes the capillary leak by reducing the interaction of inflammatory cells with the vessel wall (Neviere et al. 2001). However, AT has shown no benefit in human sepsis studies (Warren et al. 2001). In experimental studies in baboons, the use of recombinant TFPI infusion showed promising results by attenuating TF-induced activation in coagulation and lung injury (Creasey A et al. 1993).

Clinical sepsis studies show that TFPI blocks coagulation, but does not prevent inflammatory effects and tissue damage (De Jonge et al. 2000). In adult sepsis and ARDS, an imbalance between plasma TF and TFPI associates with poor prognosis (Bastarache et al. 2008, Gando et al. 2002, 2003).
Coagulation and inflammation in clinical disease in preterm infants

Maternal morbidity

Preeclampsia is a maternal hypertensive disease occurring in 12-22% of pregnancies. Preeclampsia is one cause of prematurity. In preeclampsia maternal inflammation may prime and activate fetal inflammation, leading to stronger postnatal systemic inflammatory reaction (Turunen et al. 2011). The effects of preeclampsia on postnatal morbidity may also derive partly from placental dysfunction, leading to small size for gestational age (Campbell et al. 2012).

Chorioamnionitis, an infection of the placental membranes and amniotic fluid, is one cause of prematurity and an important factor in postnatal morbidity, and especially in neurodevelopmental impairment (Adams-Chapman and Stoll 2005). Chorioamnionitis caused by *E coli* or *Streptococcus G* (GBS), however, induces lung maturation by a TLR-mediated increase in IL-6 production: consequently, direct proinflammatory stimuli in the lungs, (Nogueira-Silva et al. 2006) and initial RDS may therefore be less severe. On the other hand, *Ureaplasma urealyticum* as a causative microbe for chorioamnionitis induces TNF-α production, which can lead to both preterm labor and abnormal lung development manifesting as BPD (Waites et al. 2005). Histological chorioamnionitis, often without clinical signs, associate with preterm delivery (Goldenberg et al. 2000).

In fetal sheep, endotoxin-induced chorioamnionitis leads to low production of active oxygen species and IL-6 in endotoxin-challenged monocytes, as well as low monocyte HLA-DR expression, all findings consistent with immunoparalysis (Kramer et al. 2005). This association between chorioamnionitis and immunoparalysis, defined as persistently low HLA-DR expression and low LPS-stimulated TNF-α concentration, also occurs in extremely preterm infants (Azizia et al. 2012).

The gold standard for the diagnosis of chorioamnionitis is histological examination of the placenta. Clinical chorioamnionitis correlates poorly with histological chorioamnionitis, thus leading to underestimation of the percentage of chorioamnionitis involved in preterm birth (Kallapur and Jobe 2006).

RDS

RDS is a multifactorial condition in the immature lung with surfactant deficiency (Speer 2011). In RDS, both the activation of inflammation (Nupponen et al. 2002, Jaarsma et al. 2004) and coagulation (Gitlin and Craig 1956) play significant roles. Mechanical ventilation causes leucocytes and monocytes to become trapped in the lungs (Merrit et al. 1981 a and b), as well as cell activation with concomitant cytokine production (Bohrer 2010). The activation of inflammation in RDS associates with low HLA-DR (Kanakoudi-Tsakalidou et al. 2001).
Activation of coagulation and the pulmonary inhibition of fibrinolysis with high plasminogen activator inhibitor-1 (PAI-1) results in fibrin deposition (Cederqvist et al. 2006); hence previous name hyalin membrane disease (Avery and Mead 1959).

The clinical course of RDS has been differed since the era of antenatal corticosteroids and postnatal surfactant therapy, increasing survival rates dramatically (Engle et al. 2008, Saigal et al. 2008). However, RDS remains the main cause of morbidity among preterm infants during their first postnatal week.

Low-grade chorioamnionitis seems to be protective against RDS (Been et al 2009), whereas severe chorioamnionitis with a possible secondary postnatal hit in the form of problems with initial stabilization, mechanical ventilation, infection or oxygen toxicity, may lead to severe or relapsing RDS with a strong inflammatory response, structural lung abnormalities and poor surfactant response (Paananen et al. 2009; Björklund et al. 1997, Kramer et al. 2002).

BPD
Bronchopulmonary dysplasia (BPD), lung injury in preterm infants, was described 1967 (Northway et al. 1967). In the preterm weeks (23-32), lung development is at the saccular stage. Alveolarization begins after week 36 and continues up to two years of life (Langston et al. 1984). The mechanisms behind BPD (old BPD) involved injury following oxygen administration and mechanical ventilation, resulting in inhibition of the lung alveolar and vascular development (Coalson et al. 1995).

Advances in neonatal care with antenatal steroids, surfactant use and better ventilation techniques, have improved the survival rates of immature infants. This has led to a new type of BPD with an initially mild course of lung injury/ RDS, but with increasing ventilatory support and oxygen needs during the weeks in intensive care (Charafeddine et al. 1999). The definition of BPD is the need for oxygen treatment within 28 days of age with a second evaluation of the need for oxygen in week 36 in order to grade the BPD as mild-severe (Bancalari and Claure 2006).

Chorioamnionitis or postnatal infections (i.e. exposition for inflammation) increase the risk for BPD. Proinflammatory cytokines (IL-8), in both the lungs and tracheal lavation fluid, associate with BPD (Munshi et al. 1997). Low polymorphonuclear leukocyte count soon after birth also associates with BPD severity (Palta et al. 2008) - addressing the importance of the cells becoming trapped in lungs as one mechanism involved in BPD formation. The activation of coagulation and fibrin formation may play role in the pathogenesis of BPD or reflect the severity of initial lung injury, since PAI-1 is higher in infants with developing BPD (Cederqvist et al. 2006).
Infection

Postnatal infections can be classified as early and late infections. Early infections occur within 72 hours of birth, and late infections from 72 hours up to several months after birth in intensive care (Stoll et al. 2002). The incidence of late infections is 20%, and late infections increase risk for prolonged hospitalization and death (Stolle et al. 2002). GBS and *E coli* are among the early pathogens transmitted from the mother. In late infections *Staphylococcus epidermidis* predominates in the infections (Isaacs et al. 1996). Other causative agents include gram-negative bacteria and fungi.

The concomitant measurement of CRP and IL-6 provides the most accurate early diagnosis of sepsis; in the future, however, CD 64 may be added to these measurements (Beniz 2010, Buck et al. 1994, Ng et al. 2006). HLA-DR is more as a prognostic marker of infections predicting either susceptibility to infections or outcome (Beniz 2010), than a diagnostic tool.

HLA-DR is lower in infants with signs of infection than in those without infection (Birle et al. 2003). Developing sepsis associates with immunoparalysis in cord monocytes, with low HLA-DR and low stimulated TNF-α concentration (Azizia et al. 2012). In neonatal sepsis HLA-DR at diagnosis predicts mortality with a cut off value of monocyte HLA-DR expression < 30% (i.e. immunoparalysis) (Genel et al. 2010). In neonatal sepsis IL-1, TNF-α and IL-6 levels rise (Pickler et al. 2010).

Intraventricular hemorrhage (IVH)

IVH is a multifactorial complication with long-term neurodevelopmental consequences (Papile et al. 1978, Merciera et al. 2010). The incidence of IVH varies between different neonatal intensive care units, with 20-25% in preterm infants born before week 28, to 26-37% in infants with a birth weight under 1000g (McCrea and Ment 2008, Tommiska et al. 2007). IVH associates with postnatal elevated IL-6 concentrations (Poralla et al. 2012), and neurological insult (IVH and periventricular leukomalacia) shows concentrations of IL-1, IL-6 and IL-8 (Pickler et al. 2010). Coagulation disturbances associate with IVH; Low prothrombin activity and APC resistance due to Factor V Leiden mutation increase the risk for IVH, especially in infants born before 30 weeks of gestation (Salonvaara et al. 2004, Petäjä et al. 2001). A recent study showed that coagulation factors II, VII, X and AT are lower in infants with IVH, and that low FVII and low hematocrit are independent risk factors for IVH (Poralla et al. 2012).

Although evaluating the risk for IVH with routine laboratory testing in intensive care is impossible, however, a decrease in hemoglobin may nevertheless be if the hemorrhage is extensive. IVH is diagnosed with routine ultrasound scans performed on days 1,3 and 7, and which are later checked a couple of times during intensive care.
**Antenatal corticosteroids**

The use of antenatal glucocorticoids is based on the findings in animal studies of accelerated lung development and surfactant production (DeLemos et al. 1970, Motoyama et al. 1970). Liggins 1972 did the first trials on preterm infants with RDS. Large studies have shown the effect of antenatal glucocorticoids in preventing RDS, IVH and NEC as well as in reducing mortality (Roberts and Dalziel 2006).

The use of antenatal glucocorticoids constitutes routine care and is recommended in imminent preterm labor during weeks 23-34. The recommended glucocorticoid, betamethasone (BM), is administered twice in doses of 12 mg 12-14 hours apart. Although they reduce the risk for RDS, multiple courses associate with an elevated risk for cerebral palsy, and are therefore seldom recommended (Wapner et al. 2007). The optimal timing of antenatal BM is one to seven days before birth, and an additional dose can be considered if delivery has not occurred in seven days (Miracle et al. 2006). In a clinical trial (Peltoniemi et al. 2007) an additional dose administered < 24 hours before birth has raised concerns, because of findings of increased risk for RDS and a decreased intact survival rate (without RDS or IVH).

**Glucocorticoids and inflammation**

Lungs are a glucocorticoid target tissue due to their specific glucocorticoid receptors (Ballard and Ballard 1974). In the lungs, the favorable effects of glucocorticoids include accelerated maturation, enhanced antioxidant enzyme production, and lung fluid absorption (Grier and Halliday 2004). The suppressive effects of glucocorticoids on innate immunity and cytokine production (Schacke et al. 2002) may be one mechanism for the favorable effects of glucocorticoids. In animal studies a dose of betamethasone administered immediately before birth decreases monocyte function, in both hydrogen peroxidase production and cytokine expression (Kramer et al. 2004).

Data on the effects of glucocorticoids on immunodepression are available only from studies on adult. Both high endogenous cortisol and administered methylprednisolone associate with immunodepression (Volk et al. 2001, Le Tulzo et al. 2004). In adult patients who underwent bypass surgery, methylprednisolone treatment associates with immunodepression (Volk et al. 2006). Further, in adult sepsis, high endogenous cortisol associates with low HLA-DR expression (Le Tulzo et al. 2004). One mechanism might be a decrease in a HLA-DR transactivator or a non-DNA-binding class II transactivator A. Dexamethasone *in vitro* causes downregulation of a transcription factor for HLA-DR (Le Tulzo et al. 2004).
Timing of antenatal BM and its effects on glucocorticoid concentrations

Mothers who received BM show brief suppression in endogenous cortisol concentrations (Ballard et al. 1975). Similarly, in preterm infants a suppression of endogenous cortisol occurs after BM, but a stress response is evident after birth (Nykänen et al. 2007). In cord blood, BM is undetectable > 60 hours after the first maternal BM dose; and cord blood cortisol suppression reaches its nadir within 48 hours but subsequently recovers to pretreatment levels in six days (Ballard et al. 1975, 1980). The BM receptor occupancy may be longer than a week, which explains some BM effects occurring after the wash-out time (Kramer et al. 2004). However, a longer functional suppression of the hypothalamus-pituitary-adrenal axis (HPA) for even 4 to 6 weeks is possible (Davis et al. 2006). The effect of antenatal BM on circulating glucocorticoid activity in cord blood, measured by recombinant cell assay, depends on the time between the last dose of BM and birth. If BM is administered > 72 hours before birth, circulating glucocorticoid bioactivity depends on cord cortisol (Kajantie et al. 2004).
AIMS OF THE STUDY

This study aimed to investigate the complex interaction between inflammation and coagulation in VLBW infants in intensive care, and to understand the role of coagulation and the regulation of inflammation in common neonatal morbidities.

The specific aims were:

1) To define the course of HLA-DR expression and immunodepression in VLBW infants in intensive care, and to study the clinical associations of changes in HLA-DR expression

2) To define the course of plasma TF and its inhibitor TFPI during the first postnatal week, and to relate these findings to the known perinatal activation of coagulation and to the inflammatory changes described in the other parts of this study

3) To relate the above-mentioned postnatal changes with cytokines

4) To separately study the potential effects of maternal BM on the above-mentioned inflammatory change
PATIENTS AND METHODS

Patients and controls
The study was conducted at the Neonatal Intensive Care Unit at the Children's Hospital, University of Helsinki with the approval of the hospital ethics committee. All parents provided their written informed consent.

Altogether 56 consecutive VLBW infants, were enrolled between June 2007 and November 2008. All patients with a gestational age (GA) of < 32 weeks and a birth weight of < 1500 g were eligible for the study. A control population for cord plasma samples and postnatal flow cytometry samples on days 1 and 3 comprised 25 healthy infants with a GA of > 34 weeks. Samples from six healthy adults (age 29-45) served to validate the sample-handling procedures. In study III additional control populations consisted of stored blood samples of 16 infants with hyperbilirubinemia prior to exchange transfusion and of 14 children with pneumonia. In study IV, infants of mothers with antenatal BM more than seven days before delivery served as a comparison group.

All mothers to VLBW infants received 1 or 2 doses, 12 mg each, of antenatal BM treatment at 12- to 24-hour intervals. Criteria for preeclampsia (n = 16) were elevated blood pressure and proteinuria (ACOG Committee on Obstetric Practice). The attending obstetrician made the diagnosis of clinical chorioamnionitis (n = 15). Preterm rupture of membranes > 24 h before birth was found in 11 infants.

Of the VLBW infants, 16 were delivered vaginally. The male:female ratio was 37:19. The infants’ gestational age ranged from 23.7 to 31.9 weeks (mean 28 weeks) and birth weight from 605 to 1500 g (mean 1034 g). At birth, 41 VLBW infants were intubated and received prophylactic surfactant (Curosurf®, 100 mg/kg) within 15 min of birth.

Postnatal morbidity
Of the VLBW infants 35 were diagnosed with RDS, which served as a model of acute lung injury in preterm infants, bearing in mind the somewhat inexact diagnosis of RDS. The RDS diagnosis, made by an attending clinician unaware of the study allocation, was defined as a need for respiratory support at the age of 24 hours and a typical radiological picture (Edwards et al. 1985). Of the RDS infants only three received nasal continuous positive airway pressure (CPAP), the remaining 32 required ventilatory care. Of the NoRDS infants, only three required no respiratory support at 24 hours of age; 18 NoRDS infants required nasal CPAP.

Of the 56 infants, 12 received no surfactant, 18 received one dose, 12 two doses and 14 three or more.
BPD was diagnosed in 16 infants (15 RDS infants and 1 NoRDS infant). The definition of BPD was a need for supplemental oxygen at the age of 36 gestational weeks (Bancalari and Claure 2006). Four infants died before the gestational age of 36 weeks.

All VLBW infants routinely received benzylpenicillin 100 000 IU/kg and netilmicin 2.5mg/kg twice daily for two to five days. During the first postnatal week, benzylpenicillin was changed to vancomycin in eight infants. The indications for vancomycin were: clinical suspicion of sepsis, high CRP, perforated bowel, pathological bowel distension (not due to infection). The blood cultures of two infants tested positive for *Staphylococcus epidermidis*, and in two infants the indication was surgical closure of patent ductus arteriosus (no infection).

Infection was diagnosed in 31 infants (55%), 17 of which had positive blood cultures and the remaining, a clinical infection (Figure 7). On the day of birth, the blood culture was positive (*E coli* and *Bifidobacter*) in two infants. As a standard regimen in suspected infection, blood cultures were obtained and antibiotics administered. Infections appeared late (after 72 hours) in all but two infants, and in all but four infants after seven days.

Figure 7. Late infections occurring after the age of 72 hours. Four infections (one clinical and three blood-culture positive) occurred before one weeks of age. Reproduced with permission from Palojarvi et al. Pediatr Res 2013.

Intraventricular hemorrhage (IVH) (Papile et al. 1978) was diagnosed in routine ultrasound scans on days 1, 3, or 7 in 19 (34%) infants. Bilateral grade III/IV IVH was diagnosed in seven infants, and one infant had unilateral grade II/III IVH. The remaining 11 infants had either uni- or bilateral grade I-II IVH.

Of the infants, 29 needed closure of the patent ductus arteriosus. Of them, 23 received indomethacin, 4 received ibuprofen, and 2 had a primary surgical closure. In seven infants, the patent ductus arteriosus was closed surgically after the pharmacological treatment failed.
Six infants died. The causes of death (and time) included lung hypoplasia (at 1 day), severe intraventricular hemorrhage (at 4 days), necrotizing enterocolitis (at 11 days), persistent infection (two infants: one at 18 days and the other at six weeks), and severe BPD (at the age of three months).

Markers and indexes for morbidity
As an index of neonatal illness severity, we used the Score for Neonatal Acute Physiology–II (SNAP-II score) and SNAPPE-II (SNAP-II with perinatal extension). The SNAP-II score includes mean arterial pressure, urine output, PaO2/FiO2, seizures, blood pH and temperature calculated for the first 12 hours, perinatal extension at birth weight, small for gestational age, and Apgar score at 5 min (Richardson et al. 2001).
In studies I and II, we used lowest PaO2/FiO2 during the first 12 hours as an independent marker for early respiratory morbidity.

Methods
Sample collection
Blood samples
Cord blood was drawn at birth from the umbilical vein with a pyrogen-free syringe. Postnatal samples were drawn through an arterial cannula concurrently with clinical samples on days 1 (under 24 hours), 3 (72 ± 12 hours), and 7 (5-7 days). In infants with a birth weight from 751 to 1000 g, research blood samples (1.8 mL) were drawn simultaneously with a red-cell transfusion given on clinical indication. If birth weight was 500 to 750 g, samples were drawn only for flow cytometry (0.4 mL). In control infants, blood samples for flow cytometry were obtained concurrently with samples from the clinically required heel stick, and cytokines were determined only in samples of cord blood.

Once drawn, blood samples were immediately divided into two or three aliquots. To study monocyte surface markers with flow cytometry, a 0.4-mL aliquot was added to a pyrogen-free tube containing citrate phosphate dextrose (ACD, Baxter health care LTD, Norfolk, UK), was immediately cooled to 0°C, and kept until analysis in an ice-water bath to minimize cell activation. To determine the HLA-DR cell surface on monocytes, samples were stained within 6 hours, kept on ice, and analyzed within 24 hours (Mentula et al. 2003).

Blood for plasma samples was added to tubes of 3.8% sodium citrate (BD; Becton Dickinson, Franklin Lakes, NJ). Plasma was then separated by centrifugation at 2500 xg for 15 min at +4°C without delay, immediately frozen, and stored at -80°C.
Tracheal aspirates
Tracheal aspirates were obtained on day 1 from 17 intubated RDS infants concurrently with routine tracheal suctions. A total of 1 mL of saline was inserted into an intubation tube in aliquots and subsequently suctioned. Samples were stored at -80°C.

Flow cytometric assays
Flow cytometry was performed in the HUSLAB, Department of Clinical Chemistry and Hematology, Helsinki University Hospital.

HLA-DR expression
The method for determining monocyte HLA-DR expression was modified from that of Mentula et al. 2003 to fit preterm infants. Monocytes in 25-µL aliquots of whole blood were double-labeled with CD 14 FITC antibodies (BD) and HLA-DR R-phycocerythrin (PE) conjugated antibodies (BD). Mouse IgG-gamma1 PE served as a control (BD). After labeling, contaminating erythrocytes were lysed by adding 2 mL of ice-cold FACS lysing solution (BD). After a 3 min of incubation on ice, the leukocytes were centrifuged for 5 minutes at 4˚C at 400 x g. A second incubation with 2 mL of FACS lysing solution was carried out for 5 minutes at room temperature. After centrifugation, the cells were resuspended in 1% formalin at 0˚C. A BD FACSort™ flow cytometer and CellQuest analysis software (BD) served to acquire and analyze the data. Monocytes were identified by CD14 positivity and on the basis of their light-scattering properties.

In each sample, 2000 monocytes were recorded. HLA-DR expression is reported as (HLA-DR %). The fluorescence of HLA-DR antibodies on monocytes compared with the fluorescence of control mouse IgG antibodies on monocytes enabled the calculation of the percentage of HLA-DR-positive monocytes (HLA-DR%). Monocyte HLA-DR expression < 60% means that 60% of the infants’ monocytes show fluorescence of HLA-DR antibodies, greater than their background fluorescence.

In immunodepression monocyte HLA-DR expression is between 30 and 60% and in immunoparalysis below 30%. In our studies, we defined immunodepression as low monocyte HLA-DR, more accurately called as immunodepression characterized by a low number of HLA-DR-expressing monocytes.
Figure 8. Flow cytometry graphics. Original flow cytometry graphics from a VLBW infant on day 1. Monocytes were identified by their scatter properties and CD14 positivity. In graph A all cells are included and neutrophils are gated. Graph B shows 2000 monocytes gated and graph C CD14 positivity. Graph D shows HLA-DR histogram. The marker on D is set on 5% positivity of the control Mouse IgG-PE (not shown).

**TF expression**

The method for determining monocyte TF expression was modified from that of Amirkoshravi et al. 1996. Monocytes in 25-µL aliquots of whole blood were double-labeled with saturating concentrations of the CD 14 R-phyco-erythrin antibodies (BD) and TF FITC-conjugated mab against human tissue factor 4508CJ from American Diagnostica Inc. (ADI, Stamford, CT). Mouse IgG-gamma1 FITC served as a control (BD). Determination was completed as in the HLA-DR determination method. TF expression is reported as a percent of TF-positive monocytes.
Cytokines

Cytokines were analyzed with the Luminex® technique. The test kit MILLIPLEX MAP High Sensitivity Human Cytokine Panel - Premixed 13 Plex (Millipore, Billerica, MA) measured 13 different cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IFN-γ, GM-CSFβ, and TNF-α).

Assays from plasma

**TF, TFPI, F1+2 and TAT**

TF was analyzed with the IMUBIND Tissue Factor Elisa Kit (ADI). Results are reported as pg/mL. Free and total TFPI were analyzed with the Asserachrom® free and total TFPI ELISA (Diagnostica Stago, Asnieres-Sur-Seine, France). Results are reported as ng/mL. Prothrombin fragment 1+2 (F1+2) was analyzed with the Entzynogst F1+2 Elisa test (Dade Behring/ Siemens Healthcare Diagnostics, Deerfield, IL). Results are reported as pmol/L. Thrombin-antithrombin complexes TAT were analyzed with Entzynogst TAT Elisa test (Dade Behring/ Siemens Healthcare Diagnostics, Deerfield, IL). Results are reported as µg/ml. Each sample was determined in duplicate.

ELISA measurements were read with the Thermolabsystems Multiskan and analyzed with Ascent software.

**TF activity**

In a subset of samples (six intratracheal aspirates from intubated RDS infants, six VLBW infants during their first week of life, three hyperbilirubinemic infants, four infants with pneumonia, five cord blood samples from healthy control infants, and four adults), the procoagulant activity of TF was analysed with ACTICHROME TF assay (ADI). In this assay, TF reacts with factor VII, and the complex converts factor X to Xa. The activity of factor Xa was then measured by its ability to cleave SPECTROZYME® FXa. Measurements were read with the Thermo Labsystems Multiskan and analyzed with Ascent software.

**Glucocorticoids**

Cortisol and BM were analyzed in plasma with liquid chromatography tandem-mass spectrometry (LC-MS/MS). We converted the nmol/L values to ng/mL; for BM 1 nmol/L = 0.392 ng/mL and cortisol 1 nmol/L = 0.362 ng/mL. We calculated the BM to cortisol equivalent (BM/ cortisol = 33.3:1) and the total glucocorticoid index (cortisol + BM).

Assays from the tracheal aspirate samples

We analyzed TF in TAF with the IMUBIND Tissue Factor Elisa Kit (ADI) and TFPI total and free in TAF with the Asserachrom® free and total TFPI ELISA kit (Diagnostica Stago, Asnieres-Sur-Seine, France). TFPI kit Results are reported as
ng/mL. Each sample was determined in duplicate.

Routine plasma laboratory measurements
Blood samples taken on clinical basis were analyzed at the HUSLAB laboratory at the Children’s Hospital. These samples include TT, D-dim, AT, PC, infant arterial blood gases, blood count, and cord blood gases.

Data Analysis
We analyzed the data with SPSS 18.0, 19.0 and 20.0 software (SPSS Inc. Chicago, IL). Patient data are presented as mean ± SD and results as medians and ranges. Non-normally distributed data required Mann-Whitney, Kruskal-Wallis, and Spearman correlation tests. Linear regression models allowed the statistical evaluation of correlations, and we performed normalizing transformation (HLA-DR: reflect square root).

p values of < 0.05 were considered statistically significant. In Studies I and II, we performed power analyses for patients with and without RDS. With sample sizes of 20 NoRDS infants and 35 RDS infants a difference of 0.80 SD was detectable at a power of 0.80. In Study III we performed power analysis for results concerning patients with antenatal betamethasone administered < 7 d and > 7d before birth. With sample sizes of 32 and 24 infants a difference of 0.77 SD was detectable at a power of 0.80. In study IV we performed power analysis for VLBW infants and control infants. With sample sizes of 50 VLBW infants and 25 control infants, a difference of 0.70 SD was detectable at a power of 0.80.
RESULTS

Patients
The RDS infants had lower GA (27.4 vs. 28.9 weeks, p = 0.012) and birth weight (985 g vs. 1121 g, p = 0.035) than did the NoRDS infants. Infants with subsequent infection had lower GA than did infants without infections (27 vs. 29 weeks, p = 0.001).

Inflammation
Monocytes
The median monocyte count in VLBW infants, both in cord and on day 1, was comparable to the reference range for preterm infants (Table 1). The monocyte percentage in cord was 6.5 (2-18), and on day 1 7.5 (2-26).

HLA-DR
In the VLBW infants, median monocyte HLA-DR in the cord blood was 87%, with a decrease after birth to a nadir of 57% on day 3 (Table 1). HLA-DR expression showed no correlation with cord or day 1 monocyte counts or percentages. Male infants had lower HLA-DR expression on all days (all p < 0.009) than did female infants.

Cytokines
VLBW infants showed a postnatal increase in proinflammatory IL-6 and IL-8. IL-4, IL-7 and IFN-γ decreased from birth to day 1 in all infants (Table 2). High postnatal proinflammatory cytokines IL-6 and IL-8 correlated with low HLA-DR.
Table 1. Results of measured parameters in VLBW and control infants. Reference values for infants and adults are shown if known. Volk et al. 2006, showed in adult perioperative patients monocyte counts comparable to those of our VLBW infants.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>cord (mean)</th>
<th>day 1 (mean)</th>
<th>day 3 (mean)</th>
<th>day 7 (mean)</th>
<th>reference preterm (mean)</th>
<th>reference term (mean)</th>
<th>reference adult (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monocyte 10E9/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.1-2.5 (mean 0.8)</td>
<td>0.3-3.3 (mean 1.4)</td>
<td>0.2-0.8</td>
</tr>
<tr>
<td>Leukocyte 10E9/L</td>
<td>5.3 (1.5-21)</td>
<td>6.2 (1.2-25.9)</td>
<td>6.2 (1.2-25.9)</td>
<td>6.2 (1.2-25.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-DR VLBW %</td>
<td>87 (60.4-98.6)</td>
<td>69.8 (36.4-91.9)</td>
<td>56.7 (14.4-93.2)</td>
<td>81.2 (22.1-97.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-DR control %</td>
<td>92.1 (84.1-98.8)</td>
<td>86.5 (71-92.9)</td>
<td>87.7 (67.8-95.7)</td>
<td>81.2 (22.1-97.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma TF VLBW pg/mL</td>
<td>16.4 (0-192)</td>
<td>220.6 (0-2462)</td>
<td>407 (20-1810)</td>
<td>156.8 (7.6-430)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma TF control pg/mL</td>
<td>36</td>
<td>18 (0-47)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocyte TF VLBW %</td>
<td>1.2 (0-3.3)</td>
<td>0.8 (0-4.6)</td>
<td>1.3 (0-4.7)</td>
<td>1 (0-3.9)</td>
<td>6.9 (0-17) (Hodge)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocyte TF control %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.6 (0-9.9) (Hodge)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TF in TAF (12 RDS infants) pg/mL</td>
<td>4888 (2167-24000)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TFPIf VLBW ng/mL</td>
<td>6.8 (4.2-58.3)</td>
<td>12.9 (6-34.8)</td>
<td>11.9 (7.1-26.6)</td>
<td>9.8 (6.1-31.9)</td>
<td>unavailable</td>
<td>10.0 ± 4.8</td>
<td></td>
</tr>
<tr>
<td>TFPIt VLBW ng/mL</td>
<td>34.9 (19-1-70.7)</td>
<td>49.6 (27.7-76.9)</td>
<td>43.1 (24.2-77.6)</td>
<td>38.3 (27.2-109.1)</td>
<td>21(13-33)% (Kuhle)</td>
<td>81.2 ± 30.4</td>
<td>73% (Kuhle)</td>
</tr>
<tr>
<td>TFPIf control ng/mL</td>
<td>5.2 (3.4-7.9)</td>
<td></td>
<td></td>
<td></td>
<td>unavailable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TFPIt control ng/mL</td>
<td>26.1 (13.7-37.9)</td>
<td></td>
<td></td>
<td></td>
<td>38 (23-56)% (Kuhle)</td>
<td>73% (Kuhle)</td>
<td></td>
</tr>
<tr>
<td>F1+2 VLBW pmol/L</td>
<td>1385.8 (140-2316)</td>
<td>392.3 (141-1254)</td>
<td>234.3 (112-692)</td>
<td>226.9 (124-474)</td>
<td>1150 (690-2290) / 20-1200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1+2 control pmol/L</td>
<td>460</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAT VLBW µg/ml</td>
<td>108.7 (4.7-137)</td>
<td>6.3 (1.2-24)</td>
<td>3.3 (1-54)</td>
<td>3.1(0.8-29)</td>
<td>1.5 (1.0-4.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-dimer VLBW mg/L</td>
<td>1.7 (0.3-7.6)</td>
<td>1.2 (0.3-15.8)</td>
<td>1.3 (0.5-13.3)</td>
<td></td>
<td>day 1 0.41-2.47, (Monagle)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Cytokines (pg/mL) in VLBW infants (cord – day 7) and in control infants (cord). Shown as medians and ranges.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Cord plasma</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
<th>TT VLBW %</th>
<th>reference preterm</th>
<th>reference term</th>
<th>reference adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>0.9, 0-5.5</td>
<td>0.3, 0-15.7</td>
<td>0.2, 0-3.8</td>
<td>0.3, 0-4.7</td>
<td>27.5 (15-58)</td>
<td>43.5 (25-72)</td>
<td>49 (26-78)</td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>3.1, 0.5-18.1</td>
<td>2.5, 0-13.6</td>
<td>11.8, 0.5-10.5</td>
<td>2.2, 0.4-8.3</td>
<td>22 (9-38)</td>
<td>22.5 (11-42)</td>
<td>27 (14-56)</td>
<td>36 (24-44) (Monagle)</td>
</tr>
<tr>
<td>IL-4</td>
<td>31.1, 0-295.9</td>
<td>21.3, 0-225</td>
<td>19.1, 0-55.8</td>
<td>25.4, 0-205</td>
<td>36.5 (18-58)</td>
<td>47 (22-64)</td>
<td>53.5 (18-83)</td>
<td>76 (58-90) (Monagle)</td>
</tr>
<tr>
<td>IL-5</td>
<td>1.3, 0.3-14.8</td>
<td>1.7, 0-12-70</td>
<td>2.9, 0.4-34.9</td>
<td>2.3, 0.1-15.9</td>
<td>36 (15-58)</td>
<td>25 (11-42)</td>
<td>27 (14-56)</td>
<td>36 (24-44) (Monagle)</td>
</tr>
<tr>
<td>IL-6</td>
<td>17.2, 1-9-627</td>
<td>46.9, 0-2132</td>
<td>19.3, 4-3-198</td>
<td>17.8, 5.3-173</td>
<td>43.5 (25-72)</td>
<td>49 (26-78)</td>
<td>49 (26-78)</td>
<td>76 (58-90) (Monagle)</td>
</tr>
<tr>
<td>IL-7</td>
<td>9.7, 1-4-59.3</td>
<td>7.4, 1-3-23.4</td>
<td>8.1, 0.6-29.7</td>
<td>9.4, 1.5-36</td>
<td>43.5 (25-72)</td>
<td>49 (26-78)</td>
<td>49 (26-78)</td>
<td>76 (58-90) (Monagle)</td>
</tr>
<tr>
<td>IL-8</td>
<td>27, 7.6-252.6</td>
<td>46.3, 5.5-582</td>
<td>33.7, 2-193</td>
<td>29.2, 7.9-73.4</td>
<td>36 (15-58)</td>
<td>25 (11-42)</td>
<td>27 (14-56)</td>
<td>36 (24-44) (Monagle)</td>
</tr>
<tr>
<td>IL-10</td>
<td>53.3, 9.8-500</td>
<td>66.4, 19.6-605</td>
<td>38.6, 14.4-274</td>
<td>38.6, 11.6-1970</td>
<td>43.5 (25-72)</td>
<td>49 (26-78)</td>
<td>49 (26-78)</td>
<td>76 (58-90) (Monagle)</td>
</tr>
<tr>
<td>IL-12</td>
<td>2.2, 0-21.2</td>
<td>2, 0.2-13.3</td>
<td>1.8, 0-10.9</td>
<td>2.8, 0.2-107</td>
<td>22.5 (11-42)</td>
<td>27 (14-56)</td>
<td>27 (14-56)</td>
<td>36 (24-44) (Monagle)</td>
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<tr>
<td>IL-13</td>
<td>11.3, 0-217</td>
<td>5.4, 0-178</td>
<td>6.1, 0-36.9</td>
<td>8.8, 0-213</td>
<td>22.5 (11-42)</td>
<td>27 (14-56)</td>
<td>27 (14-56)</td>
<td>36 (24-44) (Monagle)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>7.7, 0.2-48.6</td>
<td>4.3, 0-31.</td>
<td>3.6, 0-22.5</td>
<td>5.7, 0-53.4</td>
<td>22.5 (11-42)</td>
<td>27 (14-56)</td>
<td>27 (14-56)</td>
<td>36 (24-44) (Monagle)</td>
</tr>
<tr>
<td>GM-CSFβ</td>
<td>2.7, 0.2-14.7</td>
<td>3.4, 0.3-19.6</td>
<td>3.7, 0.6-17.3</td>
<td>4.1, 0.6-11.76</td>
<td>22.5 (11-42)</td>
<td>27 (14-56)</td>
<td>27 (14-56)</td>
<td>36 (24-44) (Monagle)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>24.2, 13.1-223</td>
<td>23.8, 11.4-112</td>
<td>24.9, 10.9-49</td>
<td>22.2, 11-121</td>
<td>22.5 (11-42)</td>
<td>27 (14-56)</td>
<td>27 (14-56)</td>
<td>36 (24-44) (Monagle)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control</th>
<th>Cord plasma</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>1, 0.3-15</td>
<td>2.5, 0.9-22.1</td>
<td>33.5, 4.7-473</td>
<td>1.3, 0.4-9.5</td>
</tr>
<tr>
<td>IL-2</td>
<td>2.5, 0.9-22.1</td>
<td>33.5, 4.7-473</td>
<td>1.3, 0.4-9.5</td>
<td>8.5, 1.9-114</td>
</tr>
<tr>
<td>IL-4</td>
<td>8.5, 1.9-114</td>
<td>10, 0.9-93</td>
<td>5.9, 1.9-29.5</td>
<td>11.2, 0-330</td>
</tr>
<tr>
<td>IL-5</td>
<td>10, 0.9-93</td>
<td>5.9, 1.9-29.5</td>
<td>11.2, 0-330</td>
<td>11.2, 0-330</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5.8, 0.3-59</td>
<td>5.8, 0.3-59</td>
<td>5.8, 0.3-59</td>
<td>5.8, 0.3-59</td>
</tr>
<tr>
<td>GM-CSFβ</td>
<td>2.5, 0.3-15.5</td>
<td>2.5, 0.3-15.5</td>
<td>2.5, 0.3-15.5</td>
<td>2.5, 0.3-15.5</td>
</tr>
<tr>
<td>TNF-α</td>
<td>27.1, 8.1-47.2</td>
<td>27.1, 8.1-47.2</td>
<td>27.1, 8.1-47.2</td>
<td>27.1, 8.1-47.2</td>
</tr>
</tbody>
</table>

45
Coagulation

TF
In VLBW infants, plasma TF was low in cord plasma, with a median value of 16 pg/mL, and peaked on day 3 (Table 1, Figure 9). In adults and in control infants, TF concentrations in cord plasma were similar to those in VLBW infants (Table 1). Monocyte TF expression in VLBW infants, control infants and adults ranged from 0 to 5%, with a median below 2% (Table 1). At no sampling point did plasma TF show any significant correlation with monocyte TF.
Figure 9. The coagulation panel: Plasma TF (A), F1+2 (B) and TAT (C) TFPIf (D), TFPIt (E). Plasma TF increased from cord and peaked on day 3. F1+2 and TAT decreased from cord to postnatal days. TFPIf and TFPIt peaked on day 1. TF and F1+2 or TF and TAT did not correlate positively at any time point. Lines represent medians.

On day 1, plasma and TAF TF-pairs were concomitantly available for statistical analysis in 14 infants, 12 of whom had RDS. TF concentrations between plasma and TAF correlated significantly in 12 RDS infants (R = 0.692, p = 0.013). TF concentration in TAF was 10-fold higher than in plasma (Table 1), and taking into account the dilution due to sampling, estimates indicated a 100-fold higher TF concentration in the lungs than in plasma.

TF activity
In a subset of samples TF activated FX; this activity correlated with the level of plasma TF (R = 0.544, p = 0.016).

Plasma TFPI
TFPI showed a postnatal pattern similar to plasma TF, but the peak was on day 1 (Table 1, Figure 9). Both TFPIt and TFPIf correlated with plasma TF postnatally in the VLBW infants (for TFPIf as the active form: day 1 R = 0.595, p < 0.001; day 3 R = 0.582, p < 0.001; day 7 R = 0.433, p = 0.005).

In tracheal aspirates (n = 15), free TFPI was low (3.5ng/mL), but total TFPI was unmeasurable. Our healthy adults (internal control group for test validation) showed values similar to the reference values (total 54.5 ng/mL and free 6.9 ng/mL).
F1+2, TAT (Thrombin markers)
In VLBW infants the concentration of F1+2 in cord plasma was high (median 1385 pmol/L, adult reference values 20-1200 pmol/L), but decreased to approximately 17% of the cord plasma level during the first week despite the high plasma TF concentration (Table 1, Figure 9).

TAT showed a similar profile with a decrease to 3% postnatally. In control infants, F1+2 in cord plasma was significantly lower than in preterm infants (median 460 vs. 1385 pmol/L, p = 0.001). Plasma TF and F1+2 showed no correlation in cord blood or on days 1 or 7. On day 3, however, a negative correlation emerged (R = -0.480, p = 0.005). TAT showed no correlations with TF, but F1+2 and TAT correlated in cord blood and on day 3.

D-dimer
In VLBW infants D-dimer remained unchanged during the first postnatal week, with a median level below 2 mg/L (Table 1). D-dimer correlated with F1+2 on days 1 and 3 (R = 0.431, p = 0.02 and R = 0.415, p = 0.035).

TT
If TT was below 20, the VLBW infant received fresh frozen plasma as clinical practice, and therefore no TT was used in the data analyses.

PC and AT
PC levels were consistently low (22-27%), and AT levels on day 1 were 36.5% (Table 1). Some infants received AT concentrate after the day 1 measurement, so no day 3 and 7 levels were used in the data analysis (unpublished data).
CARS and immunodepression

VLBW infants showed postnatally low monocyte HLA-DR, with a nadir on day 3, but high levels of proinflammatory cytokines IL-6 and IL-8. Of the VLBW infants, 14 on day 1, 22 on day 3, and 5 on day 7 met the criteria for immunodepression (HLA-DR expression on 30-60% of monocytes) (Figure 10). Three infants on day 3 and one on day 7 met the criteria for immunoparalysis (HLA-DR expression on < 30% of monocytes). CARS was evident in the control infants, but with a shorter duration and less severity, and the nadir occurred on day 1 (Figure 10).

Postnatal immunodepression correlated with GA and RDS. Of the cytokines, IL-6 on day 1 was an independent predictor for low HLA-DR expression.

Figure 10. Monocyte HLA-DR expression in VLBW and control infants. HLA-DR expression was lower in VLBW infants (Mann-Whitney t-test). Lines represent medians.
In receiver operating characteristic (ROC) analysis (Figure 11), low HLA-DR expression on day 3, with a cut off value of 60% (the definition for immunodepression), gave AUC 0.761 (CI 0.614-0.909) for infections (appearing after 72 h of age), AUC 0.778 (CI 0.634-0.923) for BPD and AUC 0.701 (CI 0.525-0.877) for IVH (coinciding or appearing after day 3). The HLA-DR nadir was predictive and specific for late infection (sensitivity 75%, specificity 68%) and BPD (sensitivity 86%, specificity 65%), but non-specific for IVH (sensitivity 69%, specificity 51%).

![Figure 11. The ROC –analysis for infections, BPD and IVH.](image)

**HLA-DR expression, cytokines and TF in VLBW infants**

Maternal morbidity: preeclampsia and chorioamnionitis

A comparison of HLA-DR expression in preterm infants divided into three groups – 1) infants born to mothers with chorioamnionitis (n = 13), 2) infants born to mothers with preeclampsia (n = 16), and 3) infants born to mothers with neither condition (n = 24, no mother had either condition) – showed significant differences at birth and on day 7 (Kruskall-Wallis). Of these three groups, the lowest HLA-DR in cord was in infants born to mothers with chorioamnionitis. On day 3, although not significantly, infants born to mothers with neither chorioamnionitis nor preeclampsia showed the deepest (though non-significant) HLA-DR nadir, with incomplete recovery to day 7 (Figures 12 A and B) (unpublished data).
Figure 12 A. Monocyte HLA-DR expression in infants born to mothers with chorioamnionitis (Chorion) and infants born to healthy mothers (No). In cord HLA-DR expression was lower in infants born to mothers with chorioamnionitis (Mann-Whitney t-test). Lines represent medians.

Figure 12 B. Monocyte HLA-DR expression in infants born to mothers with preeclampsia and in infants born to healthy mothers. On day 7 HLA-DR expression was higher in infants born to mothers with preeclampsia (Mann-Whitney t-test). Lines represent medians.

Infants born to mothers with chorioamnionitis had a significantly higher concentration of TF in cord plasma and a lower concentration of TF in postnatal plasma samples than did infants born to mothers without chorioamnionitis (Figure 13). In infants born to mothers with preeclampsia, TF did not differ from that of infants born to mothers without it.
With regard to cytokines, the following associations with chorioamnionitis emerge:
day 3 IL6, \( p = 0.018 \) (higher without chorioamnionitis); in cord IL10, \( p = 0.006 \) (higher with chorioamnionitis); day 1 IL5, \( p = 0.028 \) (higher without chorioamnionitis); in cord IL-7, \( p = 0.04 \) (higher with chorioamnionitis); day 3 IL12, \( p = 0.026 \) (higher with chorioamnionitis); in cord TNF-\( \alpha \), \( p = 0.002 \) (higher with chorioamnionitis); others non-significant (unpublished data).

**Respiratory morbidity**

In RDS infants, HLA-DR expression was higher in cord blood, but significantly lower at the nadir on day 3 than that in NoRDS infants (Figure 14 A). RDS infants had higher plasma TF concentrations postnatally than did NoRDS infants (Figure 14 B). However, concentrations of TF in cord plasma from RDS and NoRDS infants showed no differences.

Using the lowest PaO2/FiO2 for the first 12 hours as an independent marker for early respiratory morbidity confirmed these findings. PaO2/FiO2 correlated with HLA-DR % on day 3 (\( R = 0.295, p = 0.047, n = 51 \)), and with plasma TF concentrations postnatally (day 1 \( R = -0.345, p = 0.034, n = 38 \); day 3 \( R = -0.402, p = 0.022, n = 32 \); day 7 \( R = -0.506, p = 0.001, n = 42 \)).
In VLBW infants, the subsequent development of BPD associated with low HLA-DR on day 3 (45% vs. 67%, p = 0.003) (Table 3) and a higher plasma TF on day 1 than in infants who did not develop BPD (598 pg/mL, n = 11 vs. 199 pg/mL, n = 27, p = 0.013).

Figure 14 A. Monocyte HLA-DR expression in RDS and NoRDS infants. In cord RDS infants had higher and postnatally lower HLA-DR expression with a nadir on day 3, compared with NoRDS infants with nadir on day 1 (Mann-Whitney t-test). Lines represent medians.

Figure 14 B. Plasma TF in RDS and NoRDS infants. RDS infants had postnatally higher plasma TF compared with infants without RDS (Mann-Whitney t-test). Lines represent medians.
Infection
Comparisons of infants with blood culture positive infection, clinical infection, and no infection revealed that HLA-DR on days 3 and 7 differed significantly between these groups: (Kruskal-Wallis) 50% vs. 44% vs. 74%, p = 0.001 and 75% vs. 70% vs. 85%, p = 0.032. Plasma TF on day 1 was highest in infants with blood culture positivity (529 vs. 352 vs. 111 pg/ml (Kruskall-Wallis), p = 0.029).

IVH
HLA-DR % on days 3 and 7 in infants with IVH (n = 19) differed significantly from that of infants without IVH (day 3, 45% vs. 67%, p= 0.028 and day 7, 68% vs. 85%, p = 0.005). On day 7, infants with IVH had significantly higher TF (289 pg/ml vs. 112 pg/ml, p = 0.030).

Mortality
On days 3 and 7, those infants who died had lower HLA-DR than did the survivors (Figure 15). Of the four infants who died before the postnatal age of three weeks, three had the lowest (below 35%) HLA-DR during the first week (14.4%, 22%, 34.9%) (Figure 15).

Figure 15. Monocyte HLA-DR expression and mortality. 6 of 56 infants died. On days 3 and 7 infants who died had lower monocyte HLA-DR expression compared with infants who lived (Mann-Whitney t-test).
Table 3. HLA-DR results from our study and relevant other studies (grey shading). HLA-DR expression in our study is divided into different morbidities. The relevant studies by Azizia et al. and Genel et al. in neonates are also shown. Allen et al. showed postoperative immunodepression in children from 2 days to 16 years, which related to later sepsis/SIRS. * HLA-DR expression values are extrapolated from the figures. # Genel et al. measured HLA-DR at the onset of symptoms (the non-infected group proved to have a non-infective cause) and in controls simultaneously with bilirubin or other blood sampling.

<table>
<thead>
<tr>
<th>HLA-DR %</th>
<th>cord</th>
<th>day 1</th>
<th>day 3</th>
<th>day 7</th>
<th>Onset of symptoms #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=25)</td>
<td>92.1</td>
<td>86.5</td>
<td>87.8</td>
<td>81.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(84.1-98.8)</td>
<td>(71.0-92.9)</td>
<td>(67.8-95.7)</td>
<td>(22.1-97.4)</td>
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</tr>
<tr>
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<td>56.7</td>
<td>81.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(60.4-98.6)</td>
<td>(36.4-91.9)</td>
<td>(14.4-93.2)</td>
<td>(22.1-95.4)</td>
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</tr>
<tr>
<td>RDS (n=35)</td>
<td>90.5</td>
<td>67.5</td>
<td>49.6</td>
<td>81.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(60.4-98.6)</td>
<td>(36.4-91.9)</td>
<td>(14.4-93.2)</td>
<td>(22.1-95.4)</td>
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</tr>
<tr>
<td>No RDS (n=21)</td>
<td>76.1</td>
<td>69.8</td>
<td>75.3</td>
<td>81.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(66.5-86.4)</td>
<td>(48.2-90.8)</td>
<td>(48.0-84.7)</td>
<td>(62.6-97.4)</td>
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</tr>
<tr>
<td>Preeclampsia (n=16)</td>
<td>93.6</td>
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<td>62.1</td>
<td>86.4</td>
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<tr>
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<td>(38.5-91.9)</td>
<td>(31.9-93.2)</td>
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<td>Chorioamnionitis (n=15)</td>
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<td>64.9</td>
<td>61.8</td>
<td>80.3</td>
<td></td>
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<tr>
<td></td>
<td>(60.4-90.5)</td>
<td>(36.4-88.8)</td>
<td>(14.4-84.7)</td>
<td>(22.1-97.4)</td>
<td></td>
</tr>
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<td>Infection, clinical (n=12)</td>
<td>82.4</td>
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<td>44.3</td>
<td>68.7</td>
<td></td>
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<tr>
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<td>(15.4-93.2)</td>
<td>(34.9-95.4)</td>
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<tr>
<td>Infection, blood culture positive (n=17)</td>
<td>86.1</td>
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<td>50.1</td>
<td>74.5</td>
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<tr>
<td></td>
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<td>(41.0-88.6)</td>
<td>(14.4-76.4)</td>
<td>(22.1-90.5)</td>
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<tr>
<td>Preterm + IUGR (n=13)(Azizia *)</td>
<td>73 (40-95)</td>
<td>Day 2 34</td>
<td>Day 2 34</td>
<td>Day 2 34</td>
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<tr>
<td></td>
<td>(28-55)</td>
<td>(28-55)</td>
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<tr>
<td>Preterm + PTL (n=19)(Azizia *)</td>
<td>66 (10-87)</td>
<td>Day 2 29</td>
<td>Day 2 29</td>
<td>Day 2 29</td>
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<tr>
<td></td>
<td>(10-90)</td>
<td>(10-90)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preterm + PPROM (n=33) (Azizia *)</td>
<td>52 (15-85)</td>
<td>Day 2 35</td>
<td>Day 2 35</td>
<td>Day 2 35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(10-95)</td>
<td>(10-95)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Term (Azizia *)</td>
<td>92 (27-100)</td>
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<td></td>
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<tr>
<td>Neonate +infected (Genel) (n=40)</td>
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<td></td>
<td></td>
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<td>37.1 (25.2-57.9)</td>
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<tr>
<td>Neonate non-infected (Genel) (n=24)</td>
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<td></td>
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<td>62.2 (48.6-81.8)</td>
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<tr>
<td>Neonate healthy (Genel)(n=25)</td>
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<td></td>
<td></td>
<td>53.7 (43.9-71.0)</td>
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<tr>
<td>Sepsis survivor (32) vs non-survivor (8) (Genel)</td>
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<td></td>
<td></td>
<td></td>
<td>45.2 vs 16.6</td>
</tr>
<tr>
<td>Child:cardiopulmonary bypass (Allen *) (n= 82)</td>
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Table 4. Plasma TF results from our study and relevant other studies (grey shading). Plasma TF in our study is divided into different morbidities. The table shows the relevant studies (Långström, Bastarache, Gando).

<table>
<thead>
<tr>
<th>TF (pg/ml)</th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>cord</td>
<td>day 1</td>
<td>day 3</td>
<td>day 7</td>
<td>Tracheal aspirate</td>
<td>reference /healthy control</td>
<td></td>
</tr>
<tr>
<td>VLBW (n=56)</td>
<td>16(0-192)</td>
<td>221 (0-2462)</td>
<td>407 (20-1810)</td>
<td>157 (8-430)</td>
<td>control cord 36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RDS (n=35)</td>
<td>9 (0-72)</td>
<td>552 (0-2462)</td>
<td>626 (26-1810)</td>
<td>224 (12-430)</td>
<td>4888 (2167-24000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NoRDS (n=21)</td>
<td>19 (0-192)</td>
<td>111 (30-341)</td>
<td>138 (20-596)</td>
<td>61 (8-285)</td>
<td>5243 (1152-32200)</td>
<td>(n=4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13 (0-54)</td>
<td>352 (198-2462)</td>
<td>734 (118-1810)</td>
<td>162 (9-420)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preeclampsia (n=16)</td>
<td>60 (0-192)</td>
<td>104 (0-893)</td>
<td>142 (20-438)</td>
<td>71 (8-160)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Chorioamnionitis (n=15)</td>
<td>46 (0-72)</td>
<td>352 (10-1610)</td>
<td>551 (98-654)</td>
<td>160 (30-426)</td>
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<tr>
<td>Infection, clinical (n=12)</td>
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<td>529 (55-2462)</td>
<td>505 (124-1810)</td>
<td>161 (8-414)</td>
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<tr>
<td>Infection, blood culture positive (n=17)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHILD</td>
<td>pneumonia (n=28) (Långström 2012)</td>
<td>360 (41-3996)</td>
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<tr>
<td>ADULT</td>
<td>ARDS (n=54) (Bastarache)</td>
<td>336 (0-1300)</td>
<td>37113 (0-150000)</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>ARDS (n=) (Gando 2003)</td>
<td>Day 2</td>
<td>0-750</td>
<td>118 ± 12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trauma + ARDS (n=76) (Gando 1999)</td>
<td>Day 0</td>
<td>0-750</td>
<td>165 ± 12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sepsis + ARDS (n=37)(Gando 1999)</td>
<td>Day 2</td>
<td>0-800</td>
<td></td>
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</tr>
</tbody>
</table>
**Antenatal betamethasone**

Glucocorticoid panel

We analyzed the glucocorticoid data against three distinct time categories for antenatal BM administration: 24 h to 7 d as ‘optimal timing’, > 7 d as ‘too early’, and < 24 h as ‘too late’. Antenatal BM administration < 24 h showed the most pronounced effects on those infants’ glucocorticoid panel. In this group, the BM concentration and total glucocorticoid index were high, and the endogenous cortisol concentration was low in the cord (Figure 16). However, this effect was of short duration; by day 1, total glucocorticoid indexes were similar for all three time categories (Figure 16, F). Moreover, the relative increases in endogenous cortisol cord concentrations up to day 1 were similar for the three time categories: 5.5-fold at < 24 h, 8.5-fold at 24 h to 7 d, and 7.0-fold at > 7 d (Figures 16 A and B), (unpublished data).

Antenatal BM and immunodepression

Antenatal BM showed no correlation with TF at any time point. The timing of antenatal BM correlated with HLA-DR expression, with infants with BM shortly before birth showing the lowest HLA-DR expression (cord: R = 0.419, p = 0.033, n= 26; day 1: R = 0.354, p = 0.011, n = 51). On day 7, infants with BM < 24 h before birth showed prolonged immunodepression, with significantly lower HLA-DR expression compared to infants with HLA-DR > 7 d before birth (66% vs. 85%, p = 0.047).
Figure 16. Infants’ glucocorticoids relative to timing of maternal betamethasone (BM) administration: 24 h to 7 days representing the optimal timing. In 9 infants exposed to BM
within 24 hours before birth, cord endogenous cortisol was lower (A) \((p = 0.031)\), and BM (C) \((p = 0.009)\) and total glucocorticoid index (E) \((p = 0.018)\) were higher, than in 24 infants exposed to BM \(> 7\) d before birth. In Kruskall-Wallis test, BM concentrations both in cord (C) \((p = 0.002)\) and on day 1 (D) \((p < 0.001)\), and total glucocorticoid index in cord (E) \((p = 0.021)\) differed significantly between the three groups.

### Antenatal BM and cytokines

In infants with BM \(> 7\) d before birth, concentrations of IL-6 and IL-10 increased from birth to day 1 (Figure 17 A), but in infants with BM \(< 7\) d before birth, IL-6 remained unaltered and IL-10 decreased. IL-5 increased in infants with BM \(< 7\) d before birth, while the remaining cytokines remained more or less stable (Figure 17 B).

![Figure 17 A. Cytokines IL-6, IL-8 and IL-10 in infants with BM > 7 days (left black bars) and < 7 days (right grey bars) before birth. In infants with BM > 7 days before birth IL-6 and IL-10 increased from cord to day one, while in infants with BM < 7 days before birth IL-6 was stable and IL-10 decreased. The IL-6 response in infants with BM > 7 days before birth may represent physiological stress without BM suppression or augmented response due to maturating effect of BM on monocyte responses.](image-url)
Figure 17 B. The cytokine panel except IL-6, IL-8 and IL-10. No significant changes occurred when comparing infants with BM < 7 days (grey bars) and infants with BM > 7 days (black bars) before birth except IL-5 (number 4) with a postnatal increase in infants with BM < 7 days before birth.
DISCUSSION

In this thesis, we evaluated the postnatal immunological state of VLBW infants, along with findings of the interplay between coagulation and inflammation.

CARS and immunodepression
Postnatal, transient CARS occurred in both VLBW infants and near-term controls. One could speculate that the rapid decline in HLA-DR after birth could be due to monocytes with high HLA-DR-expression gathering on the site of new antigens (e.g. the lungs), and a new pool of monocytes released from bone marrow expressing lower HLA-DR.

On day 3, 45% of VLBW infants showed immunodepression, and on day 7, 11% of the infants expressed HLA-DR at less than 60%. Pediatric postoperative patients recovered completely from CARS within a week, with HLA-DR expression returning to preoperative levels (> 95%) (Allen et al. 2002) (Table 3). In adult trauma patients, HLA-DR expression of 75% was considered a sufficient recovery from CARS and showed no association with increased morbidity. Patients with physiological CARS or short-duration immunodepression recovered by day 7; in immunodepression and sepsis, patients recovered only after day 25-30 (Figure 7). In pediatric patients with multi-organ failure, HLA-DR < 30% for three or more days was associated with increased risk for infection and death.

Non-survivors showed lower ex vivo TNF-α production than did survivors (Hall et al. 2000). In our study, a comparison of TNF–α levels in survivors and non-survivors proved impossible to obtain, due to missing cytokine samples among the non-survivors.

By day 7, 60% of our VLBW infants recovered (HLA-DR expression > 75%) indicating prolonged immunodepression in 40% of infants at a postnatal age of one week. This prolonged immunodepression associated with morbidity (SNAP-II and SNAPP-II). HLA-DR expression recovered (HLA-DR expression > 75%) by day 3 in all control infants but one.

VLBW infants are immature and suffer from multi-trauma, so similarities to CARS and immunodepression postnatally and to adult trauma and multi-organ failure patients are logical.

The findings of immunoparalysis as defined by the hyporesponsiveness of monocytes (with HLA-DR expression not yet meeting the criteria for immunodepression or – paralysis) (Azizia et al. 2012), could indicate that our results have underestimated the proportion of infants with actual immunoparalysis. However, our serial measurements
showing the postnatal nadir on day 3 can be considered reliable, and are in line with the results of several published works on children and adults (Allen et al. 2002, Gouel-Chéron et al. 2012, Hershman et al. 1990, Livingston et al. 1988, Mentula et al. 2004, Volk et al. 1996 and 2001). Defining CARS and immunodepression by only one measurement – HLA-DR expression – is a weakness of our study. An elegant alternative could have been the LPS stimulation of monocyte-produced cytokines (Azizia et al. 2012), but the minute volumes and pre-planned exhaustive number of measured parameters resulted in this study design.

RDS, the main morbidity during the first week, and gestational age both independently associated with the HLA-DR nadir on day 3. In adults CARS, and immunodepression occur in a variety of clinical conditions such as trauma, sepsis, ARDS and post-surgery (Azizia et al. 2012).

In adults and children, normal monocyte HLA-DR expression (< 95%) (Allen et al. 2002, Volk et al. 2001) is higher than in cord blood monocytes (Birle et al. 2003).

**Innate immunity**

HLA-DR is responsible for antigen presentation, linking innate immunity to adaptive immunity. In newborn infants immune response relies on innate immunity, and adaptive immunity matures during the first months (Krishnan et al. 2003). Therefore the presentation of impaired HLA-DR antigen to adaptive immunity cells may be more tolerated or more protective in VLBW infants than in adults. The transient decline of HLA-DR expression encountered in healthy near-term and term infants after birth may therefore be considered partly physiological.

**CARS and cytokines**

Our VLBW infants showed a proinflammatory cytokine profile, in contrast to adults who showed an anti-inflammatory cytokine profile during immunodepression. In LPS challenge cord blood monocytes from VLBW infants show hyporesponsiveness in TNF-α production, but not in IL-6 production (Azizia et al. 2012).

The ability to express proinflammatory cytokines despite immunodepression could maintain innate immune functions in preterm infants. In adults, the ability to produce proinflammatory cytokines despite immunodepression increases survival in sepsis (Pachot et al. 2006). In the activation of inflammation that occurs after birth, immunodepression may, if moderate or transient, protect from potentially overwhelming postnatal inflammation, thereby offering protection from tissue injury.

We showed high proinflammatory cytokines IL-6 and IL-8 postnatally, both of which correlated with respiratory morbidity, thus suggesting that prematurity with respiratory distress is associated with a high inflammatory burden. Cytokines IL-6 and IL-8 both correlated with low HLA-DR and high TF.
**TF in VLBW infants**

We showed that VLBW infants have high levels of plasma TF. Only Långström et al. (2012) have shown plasma TF levels similar to those of VLBW infants; adult levels are lower (Table 4). In the thrombogram studies (Hyytiäinen et al. 2003), neonates show lower thrombin formation than do adults, and prothrombin is lower in neonates than in adults (Andrew et al. 1987, 1988, Monagle et al. 2006).

In our study, the F1+2 peak occurred in cord blood, with postnatal F1+2 values comparable to adult reference values. TFPI levels failed to track TF beyond day 3. Interestingly, F1+2 in VLBW infants decreased to 17% during the first week. Simultaneously, TAT decreased to 3%, which suggests that more thrombin formed than thrombin-AT complexes, this is indicative of available thrombin for inflammatory or other pathways.

We cannot exclude the contribution of plasma TF to local coagulation in, for example, vascular beds or sites of vascular injury. However, a stable postnatal d-dimer concentration does not support enhanced fibrin formation. In conclusion, VLBW infants showed a significant TF burden that does not contribute to thrombin formation or actual coagulation.

Monocyte TF expression was surprisingly constant and low, showing no correlation with plasma TF – except for monocytes as a main source of plasma TF. Tracheal aspirates, however, revealed a 10- to 100-fold higher TF concentration in tracheal fluid than in plasma. Since no mechanism is known for how TF could accumulate against a concentration gradient towards the lungs, a major part of plasma TF evidently originated in the lungs, where both bronchoalveolar macrophages and alveolar epithelial cells express TF. This is in accordance with the results of other studies on adults (Bastarache et al. 2007).

Our study showed that TFPIf was low and that TFPIt was undetectable in tracheal aspirates; in adult studies TFPI in the lungs was high, but majority of it was inactive by truncation (Bastarache et al. 2008).

**TF and thrombin formation**

In VLBW infants, TF in cord blood was low, and F1+2 and TAT were high, possibly because in cord blood, disruption of the placenta and cell-bound TF, rather than plasma TF, triggered coagulation. In control infants, however, F1+2 was lower than in VLBW infants. A mechanism for why VLBW infants show higher thrombin markers in cord blood could be a stronger procoagulant effect of placental disruption following preterm birth; placental disruption could be considered preterm or more traumatic in preterm infants than in term infants.
The finding that circulating plasma TF does not translate to thrombin formation raises several questions. Does circulating plasma TF have procoagulant properties? We showed in vitro, with the actichrome assay, that plasma TF was coagulationally active and had the potential to contribute to systemic thrombin formation in circulation, but did not do so. The following logical question is whether plasma TF could be inactive in the blood, but active under the experimental conditions in vitro. The mechanism could logically be some inactivating factor, other than TFPI, in plasma or an inactivating factor in the TF molecule itself. The handling procedures for blood and plasma were kept at a minimum in order to exclude the in vitro activation of plasma proteins.

TF is present in plasma in encrypted, inactive form on monocytes, and in active form circulating freely or microparticle bound. The decryption or activation of TF occurs when phosphatidylserine-rich phospholipid surfaces on microparticles or platelets are available (Bach 2006). Could this TF activation occur in sample handling or after removing platelets by centrifugation and result in a change in activity measured by in vitro testing? This question remains to be answered further.

Microparticles are cell membrane structure bearing, cell-membrane particles shed from activated cells. They provide an excellent activation for coagulation by bringing together phospholipids, especially inner-coat PS, and TF (Bogdanov et al. 2009, Levi 2004). We do not know whether our VLBW infants have microparticles in plasma, but on the results of other studies (Uszynski et al. 2011), suggest that they very well may do so. If TF was microparticle bound it should potentiate its activity in coagulation. Bogdanov et al (2009) showed that in patients with coronary artery disease, 25%, and in normal controls 42%, of TF activity is on microparticles. (Bogdanov et al. 2009) The actichrome TF activity assay features some problems in measuring TF activity, mainly because microparticles may interfere the results (Bogdanov et al. 2009). Based on this actichrome assay, stating whether the soluble free TF in plasma was active in our study is impossible. In the plasma of our VLBW infants, however, microparticles and microparticle TF are not excluded. When we question whether the ELISA-measured (free or microparticle bound) plasma TF is capable to form thrombin, this actichrome assay should logically prove useful in our study setting.

In adults increased in plasma TF associates with disseminated intravascular coagulation (Gando 2001,) and in children with pneumonia, high TF correlates with TAT (Långström et al. 2012). Why are VLBW infants different? TFPI could partly account for this inactivation of TF, but other possible mechanisms remain poorly understood. In adult acute pancreatitis, a known inducer of strong inflammation, mortality associates with high TFPIf (30-200 ng/mL) and impaired thrombin formation studied with thrombogram. F1+2 levels were similar in all patients, although TF was not measured (Lindström et al. 2011). Our VLBW infants showed
TFPIf levels below 45 ng/mL during the first week, and based on previous studies, thrombograms of infants in vitro should not indicate low thrombin formation (Hyytiäinen et al. 2003). These findings together suggest that in VLBW infants, TFPI is not solely responsible for the inactivation of TF.

If plasma TF is coagulationally active in vivo, are our thrombin markers able to detect thrombin formation? F1+2 and TAT form a good laboratory base to test our hypothesis of whether plasma TF does or does not contribute to thrombin formation. F1+2 is a highly sensitive thrombin marker, since it is formed in 1:1 stoichiometric relation to thrombin, with F1+2 released into the blood stream. While prothrombin circulates at µM concentrations, F1+2 is detected at pM levels. The biochemical specificity of the assay to true F1+2 is generally considered excellent. Both TAT and F1+2 are sensitive enough to detect the rapid escalation of in vivo thrombin generation (Raivio et al. 2006). On the other hand, the half-lives of TAT (15 min) and F1+2 (90 min) (Bauer 1993) are short enough to allow detection of reductions in thrombin generation. In our VLBW infants, F1+2 and TAT were high in cord blood, and decreased thereafter.

This finding of plasma TF not turning into thrombin is a new, relevant addition to our knowledge of developmental hemostasis, and the data about TFPI in our VLBW infants add logic and significance to this knowledge.

The complex, partly unknown biology of TF and the lack of direct measurements for TF proinflammatory properties make the interpretation of our results challenging. No direct indicator of the inflammatory effects of TF is available. Our study showed an excess of plasma TF not turning into thrombin and this TF exceeding TFPI levels, as well as a correlation between TF and proinflammatory cytokines. A basic understanding of TF as an activating factor of monocyte cytokine production and our findings together could suggest a proinflammatory role for TF in VLBW infants. TFPI, in turn, regulates TF activity following the downstream activation of factor X to FXa; TFPI is therefore no inhibitor of TF inflammatory properties, but may regulate the profiles of inflammatory or procoagulant TF.

**Chorioamnionitis**

In infants born to mothers with chorioamnionitis, HLA-DR expression in cord blood was lower and TF higher than in infants born to mothers without chorioamnionitis. Postnatally, however, infants born to healthy mothers showed the deepest nadir in HLA-DR expression. In cord blood IL-7, IL-10 and TNF-α were higher, and on day 3, IL-6 was lower and IL-12 higher, in infants born to mothers with chorioamnionitis than in infants born to mothers without it. This is in good accordance with the findings of Paananen et al. (2013); in chorioamnionitis inflammatory cytokines decline from birth to day 1, possibly due to the inflammation-activated hypothalamus-pituitary axis and the postnatal suppression of cytokine production, when maternal
infection no longer triggers cytokine response.

Chorioamnionitis may lead to LPS hyporesponsiveness and the inability to produce cytokines. Postnatal inhibition of cytokines after chorioamnionitis seems to be important in avoiding further inflammatory burden and susceptibility to BPD (Paananen et al. 2013). This same immunomodulatory or anti-inflammatory effect was evident in our VLBW infants born to mothers with chorioamnionitis with lower IL-6 on day 3, as well as postnatally lower TF and less deep HLA-DR nadir and. Prenatal inflammation followed by postnatal infection is considered a risk factor for BPD (Paananen et al. 2013), and postnatal infection show lower HLA-DR expression than do infants without infection (Azizia et al. 2012).

**Postnatal infection**

Infection in intensive care is a well-known risk factor that increases morbidity and mortality. In children and adults, infection or susceptibility to infection is linked to immunodepression (Allen et al. 2002, Hall et al. 2007, Livingstone et al. 1988). Infants with chorioamnionitis and infection show lower cord HLA-DR expression than do infants without infection (Azizia et al. 2012); in our infants, HLA-DR expression in cord blood showed no differences between infants who developed infection and those who did not.

When evaluating HLA-DR in infants with signs of sepsis, Genel et al. (2009), found lower HLA-DR at the time of diagnosis in infants with infection than in infants without infection. HLA-DR below 30% was predictive for mortality. In our VLBW infants, infants with infection also showed postnatally low HLA-DR expression on days 3 and 7. An HLA-DR nadir on day 3 could predict later infection with a sensitivity of 75% and a specificity of 68%.

In adult trauma patients, both the slope of HLA-DR expression from days 1-2 to days 3-4, and IL-6 together predicts the development of sepsis (Gouel-Chéron et al. 2012). In pediatric cardiac bypass patients, postoperatively low HLA-DR expression was predictive for sepsis and a long stay in intensive care (Allen et al. 2002). In our VLBW infants, plasma TF on day 1 was the highest in infants developing a blood-culture positive infection after 72 hours than infants with a clinical infection and no infection.

**Respiratory morbidity**

Low HLA-DR expression and high plasma TF both associated with respiratory morbidity (RDS, BPD, Pao2/FIO2). Infants without RDS showed postnatally a similar HLA-DR expression profile to that of the controls, with recovery after day 1. Infants without RDS had lower cord HLA-DR and higher HLA-DR expression on postnatal days than did infants with RDS. This might attributable to of chorionamnionitis in these infants without RDS: inflammatory burden in utero followed by postnatal
maturation or immunomodulation.

**Mortality**
The heterogeneity in causes of death and low mortality rate made the evaluation of the effect of immunodepression on mortality unfeasible. On day 7, however, all surviving infants had HLA-DR expression over 40%, whereas two of the non-survivors had HLA-DR below 25%.

**Antenatal BM and postnatal glucocorticoid profile**
Antenatal BM not only led to enhancement of total glucocorticoid index, but also caused a brief suppression of endogenous cortisol production. However, irrespective of the timing of BM, the infants’ adrenals retained their ability for at least a short-term response to stress factors. These results are in good accordance with the literature (Nykänen et al. 2007, Kajantie et al. 2004, Ballard P et al. 1975). However, a longer functional suppression of the hypothalamus-pituitary-adrenal axis (HPA) of even four to six weeks is possible (Davis et al. 2006).

The infants retained their short-term stress response even with the administration of BM immediately before birth. The five- to eight-fold increase in the endogenous cortisol concentration from birth to day 1 in all infants suggests that this stress response in VLBW infants depends more on postnatal factors than on birth (vaginal or caesarean section) alone. However, if the BM was administered very shortly before birth, the endogenous cortisol values were lower, even if the relative increase in cortisol remained unchanged.

**Antenatal BM and immunodepression**
BM may directly modulate HLA-DR expression, since dexamethasone in vitro decreases a transcription factor for HLA-DR expression (Le Tulzo et al. 2004). In our VLBW infants close to birth, the administration of BM resulted in lower HLA-DR expression and prolonged immunodepression with incomplete HLA-DR recovery by day 7 than in infants with BM administered > 7 days before birth.

**Antenatal BM and cytokines**
Infants with BM > 7 d before birth saw an increase in both IL-6 and IL-10, which may represent a physiological or augmented (Kramer et al. 2006) response to postnatal stress. Our finding of the suppression of IL-6 and IL-10 concentrations in VLBW infants with BM < 7 d before birth may represent a pattern unique to VLBW infants.

The net anti-inflammatory effect on VLBW infants may be less than that on adults, thereby potentially offering protection (Pachot A et al. 2006) against low monocyte HLA-DR-induced immunological and monocyte dysfunctions.
The cytokine profile associating with immunodepression in VLBW infants was proinflammatory, with high levels IL-6 and IL-8. Antenatal BM changed this profile in an anti-inflammatory direction with not only low IL-6, but also low anti-inflammatory IL-10. In adults, immunodepression associates with high IL-10, resulting in an anti-inflammatory cytokine profile.

**Methodological aspects**

The ethical requirements for different sample volumes for various birth weight categories and the additional requirement of concurrent blood transfusion in the smallest infants resulted in protocol-associated, but individually varying missing values. In VLBW infants, cord blood coagulated unexpectedly rapidly after sampling despite sufficient anticoagulants and efficient sampling procedures, leading to loss in the samples.

One weakness of our study is the lack of postnatal plasma samples from control infants. The blood sample volume (1.8ml) and the need for venipuncture to obtain these samples made doing so unethical as healthy term infants do not need a needle in their veins.

Döcke et al. (2005) have described the consequences of pre-analytical storage: four hours on ice before staining lead to a 4% increase in HLA-DR expression. Stained samples are stable for at least 24 hours when stored at 4°C in the dark. Mentula et al. (2003) considered six hours on ice before staining safe. Because we reported low HLA-DR values, the handling procedures (consistent throughout the study) should underestimate rather than overestimate HLA-DR expression. Amirkoshravi et al. (1996) reported stable TF four hours after sampling, in our experiments, however, six hours on ice was considered safe.

Analyzing 2000 monocytes for each flow cytometric analysis, randomly offering a selection wide enough to provide reliable results, provides a representative picture of circulating monocytes. Nevertheless, one might speculate about the differences in adult and neonate monocyte counts and their effect of that on HLA-DR expression. Although the adult reference values are lower than in preterm infants, Volk et al showed perioperative monocyte counts similar to our neonate monocyte counts (Volk et al. 2001)- rendering the HLA-DR results comparable.

We used an American diagnostica TF ELISA assay. This assay might show higher TF values than with other assays (Parhami-Seren B et al. 2005). However, this assay has served in the most important studies on adults (Bastarache et al. 2007, Gando et al. 2002, 2003), making their results comparable with ours.

Red-cell transfusions

VLBW infants received red-cell transfusions on clinical indications; only 18 infants
received no red-cell transfusions. Before sampling on day 1, 6 infants (11%); before sampling on day 3, 22 infants (39%); and before sampling on day 7, 17 infants (30%) received red-cell transfusions. Before sampling on day 3, 6 of the 22 infants; and before sampling on day 7, 1 of 17 infants received two red-cell transfusions.

Red-cell transfusions may activate mononuclear cells and endothelium, Keir et al. (2013) showed in infants with <28 weeks of gestation and two to eight weeks of postnatal age that red-cell transfusion raises IL-1, IL-8 and TNF-α levels two to four hours after transfusion. In our study of patients with birth weight 751 to 1000 g, we drew study samples when red-cell transfusion, administered on clinical indication, began, thereby minimizing the possible effects of transfusion.

Red-cell transfusions correlated with morbidity: receiving a red-cell transfusion before sampling on day 3 correlated with SNAP-II (R = 0.412, p = 0.002) and SNAPPE- II (R = 0.386, p = 0.003). Similarly indicative of morbidity, high TF on day 1 correlated with receiving red-cell transfusions before day 3, and high TF on day 3 correlated with red-cell transfusions received before day 7. The nadir in HLA-DR expression on day 3 was lower in infants who received a red-cell transfusion on day 3; HLA-DR was also lower on days 1 and 3 in those infants who received a red-cell transfusion before day 7. These associations with morbidity show that, logically, the sickest and smallest VLBW infants received red-cell transfusions more than did other infants. The initial analyses failed to take into account the timing of red-cell transfusions.

Fresh-frozen plasma
During patient enrollment almost all (48 infants, 86%) VLBW infants received fresh-frozen plasma on a clinical basis, if not as an initial volume expander in the stabilization of the infant, then later in intensive care if the TT% was below 25%. Hyytiäinen et al. (2003), showed that in VLBW infants with high F1+2 (≥ 2350 pmol/L), fresh-frozen plasma has transient lowering effect on the F1+2 concentration, whereas in infants with a lower F1+2 concentration (< 2350 pmol/l) this lowering effect was unseen. In the high F1+2 group the lowering effect was absent after two hours. Our infants could all be classified in the lower F1+2 group (the highest F1+2 in cord was 2316 pmol/L, and on day 1, 1254 pmol/L). We therefore estimated a low or no transient effect of fresh-frozen plasma on F1+2.

AT
As part of our study design, we routinely analyzed the AT, D-Dimer, PC and TT of the VLBW infants. Measuring the AT of these infants more frequently and revealing in low or physiologically low AT percentages led to the enhanced use of AT concentrate (Atenativ) during the study period. We therefore excluded the AT values of days 3 and 7 from the data analysis.
Dividing infants into RDS and NoRDS groups in our study enabled us to compare TF and immunodepression with the severity of respiratory morbidity in the two groups. Of course, the limitation of this is the diffuse diagnosis of RDS, but the diagnosis in our infants was clinical and made by the attending clinician, thus reflecting the clinical status of the infant. PaO2/FiO2 correlated with TF and HLA-DR similarly to RDS/NoRDS in statistical manners.

Diagnosis of chorioamnionitis
In our study, the diagnosis of chorioamnionitis was clinical (maternal fever, CRP). Nowadays, however, the more accurate diagnosis is possible with a quick verification test for amnion fluid glucose, because bacterial infection lowers glucose levels in the amniotic fluid.
CONCLUSIONS

Developmental immaturity of the immune system is evident in VLBW infants as low HLA-DR expression on monocytes. Lower GA correlated with lower HLA-DR expression. Immunodepression associated with late infections, and may reflect a link between gestational age and risk for infections. Immunodepression in VLBW infants associated with RDS and GA.

On day 3, 45% of infants showed immunodepression and on day 7, 40% of infants had incomplete recovery, with HLA-DR expression below 75%. This prolonged immunodepression associated with morbidity, this adding to the risks in intensive care.

We showed that in VLBW infants TF was high in the lungs and possibly leaked to circulation, under the partial control of TFPI, but without contributing to thrombin formation. This leads to the speculation that TF in VLBW infants takes an inflammatory direction over coagulation. Plasma TF associated with proinflammatory cytokines and morbidity. The postnatal increase in plasma TF, concomitantly high proinflammatory cytokines, and immunodepression may offer new insights into the pathophysiology of prematurity.

Antenatal BM associated in a time-dependent manner with immunodepression, such that infants with BM administered closest to birth showed the lowest HLA-DR expression. Antenatal BM associated with low IL-6 and IL-10, whereas immunodepression alone associated with proinflammatory cytokines, high IL-6 and IL-8. This more anti-inflammatory cytokine profile after antenatal BM administration may strengthen the adverse effects of prolonged immunodepression.
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