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"Do not go where the path may lead; go instead where there is no path and leave a trail."

Ralph Waldo Emerson

ABSTRACT

The aim of this project is to describe the pattern of innate immunity responses at birth with focus on the recruitment of leukocytes such as eosinophils and neutrophils and their content of cathelicidin antimicrobial peptide LL-37 associated to different degrees of fetal stress and delivery modes.

We know that delivery is associated to fetal stress including release of cortisol and cathecolamines. Stress has profound effects on the immune system, also by acting on the trafficking of leukocytes, a process in which adhesion and chemotaxis are primordial and critical events for the development of effective antimicrobial defences.

Birth and postnatal adaptation are associated with leukocyte recruitment and activation, an acute phase response and inflammatory skin reactions in the human newborn, all together indicating an enhanced innate immunity at that time-point in life.

We found that with a progressive increase in fetal stress, there are significant elevations in total white blood count, in particular neutrophils and monocytes, as well as an enhanced IL-8 and soluble E-Selectin level. Assisted delivery, associated with the highest degree of fetal stress in addition has an increased Interferon-γ level. The direct correlation between specific leukocytes and Interferon-γ respectively, with stress markers such as cortisol, β-endorphin and cathecolamines reflects the impact of stress on fetal immunity.

Also, after normal delivery cord plasma LL-37 levels were 3 times higher compared to C-section indicating fetal peptide release. Neutrophils from cord blood after normal delivery contain 10 times more cytoplasmatic cathelicidin peptide compared to corresponding cells after C-section where a strict granular localization is found, a possible sign of cell activation. A highly significant correlation was observed between maternal and cord plasma LL-37 levels, most probably reflects maternal cathelicidin placental transfer.

We also could show that cord eosinophils contain and to some degree release LL-37. Interesting, cord eosinophils contain 3 times more LL-37 compared to adults. Thus, it seems reasonable to deduce that these cells may exert important regulatory functions during the neonatal period, also including antimicrobial activity and immunomodulatory effects by peptide LL-37.

Our data analysis shows gene expression differences in eosinophils between the two delivery modes. We have identified the genes with most significant expression difference in C-section eosinophils compared to normal delivery eosinophils, here, being IFN-gamma receptor and STAT1. Our RT-PCR data confirms the upregulation of these genes in eosinophils from cord blood compared to normal delivery. This indicates a more enhanced immunity in eosinophils after normal delivery.

This study assembles a picture of newborn infant's ability to mount a powerful inflammatory immune response at birth and that the stress of normal labor and terrestrial adaptation induces profound structural and functional changes in fetal leukocytes.

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LIST OF PUBLICATIONS

I. **The stress of birth enhances in vitro spontaneous and IL-8-induced neutrophil chemotaxis in the human newborn.**

Yektaei-Karin E, Moshfegh A, Lundahl J, Berggren V, Hansson LO, Marchini G.

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II. **Maternal plasma level of antimicrobial peptide LL37 is a major determinant factor of neonatal plasma LL37 level.**

A Mandic Havelka*, E Yektaei-Karin*, K Hultenby, OE Sørensen, J Lundahl, V Berggren, G Marchini.

* Equal contribution

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III. **The cathelicidin antimicrobial peptide LL-37 is present in eosinophils isolated from human umbilical cord blood.**

Elham Yektaei-Karin, Ali Moshfegh, Kjell Hultenby, Eva Andersson, Chellakkan Selvanesan Blesson, Aleksandra Mandic Havelka[|], Joachim Lundahl, Giovanna Marchini

Submitted to Pediatrics Allergy and Immunology

IV. **Gene expression pattern of cord blood eosinophils in normal delivery compared to cesarean section.**

Elham Yektaie-Karin[1], Joachim Lundahl[2], Giovanna Marchini[1], Ali Moshfegh[3]

Manuscript

CONTENTS

LIST OF ABBREVIATIONS

25(OH)D	25-OHvitamine D
BSA	Bovine serum albumin
AMPs	Antimicrobial peptides and proteins
C-section	Cesarean section
ECP	Eosinophil Cationic Protein
EDN	Eosinophil Derived Neurotoxin
EPO	Eosinophil PerOxidase
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
hCAP18	human cationic antimicrobial protein of 18 kDa
HMGB1	High mobility box group protein 1
MBP	Major Basic Protein
IFN-γ	Interferon-gamma
IL	Interleukin
MMP9	Matrix metalloproteinase 9
MPO	Myeloperoxidase
PBS	Phosphate buffered saline
PI	Propidium iodide
qPCR	quantitative real-time Polymerase chain reaction
SEM	Standard error of mean
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS-T	Tris Buffered Saline-Tween
TLR	Toll Like Receptor
WBC	White blood cell count

1 BACKGROUND

1.1 INTRODUCTION

The birth process and postnatal adaptation are associated with leukocyte recruitment and activation (Steinborn, Sohn et al. 1999; Koenig, Stegner et al. 2005; Moshfegh, Lothian et al. 2005), an acute phase response (Marchini, Berggren et al. 2000) and inflammatory skin reactions in the human newborn (Marchini, Nelson et al. 2005), all together indicating an enhanced innate immunity at that time-point in life. Why should newborns be prone to inflammatory reactions? Our hypothesis is that the enhanced immunity may be an adaptive response designed to strengthen the vigilance and capacity of the immune system to respond to challenges at the major defence barriers of the body, skin and epithelial linings. In this way, the immune system of the newborn is alerted of pathogens and oncoming commensal microflora. It is also possible that leukocytes are primed at birth to execute other regulatory, immunologic effects, besides being antimicrobial, as indicated by a change in their transcriptional program upon transmigration into specific tissues (Theilgaard-Monch, Knudsen et al. 2004).
In our studies we have focused on the two different delivery modes; Normal delivery and elective C-section in order to investigate if different delivery modes can affect the immune response and leukocyte function- being neutrophils and eosinophils in this study- in the human newborn.

1.2 DELIVERY MODES

In the mankind history child birth has happened in different context, related to one's social class, economic state and culture.
With the development in modern societies in terms of technical and pharmaceutical methods, many dramatic changes have happened in the process of child birth. Pharmacological research made it possible to reduce the pain of delivery, and also to induce and speed up the labour. From biological point of view one of the most dramatic changes is the fact that nowadays an increasing number of infants are born via mothers' abdomen instead of her vagina (Waldenstrom 2007).
This biological change in the delivery form and its differences from the vaginal delivery has caused many debates in recent years and a lot of scientists are trying to define pros and cons of each method for the mothers and infants. This project also focuses on immunological alterations that happen in mothers and infants as an outcome of the delivery mode.

1.2.1 Normal Delivery

The definition of normal delivery according to the "Maternity Statistics Bulletin" is stated as: "a normal delivery is one without induction, without the use of instruments, not by cesarean section and without general, spinal or epidural anaesthetic before or during delivery. Excluded are any other procedures not relating to an unassisted delivery except repair of laceration." In this project normal delivery is used in the same sense.

1.2.2 Cesarean Section

Cesarean section on maternal request is defined as a cesarean delivery for a singleton pregnancy on maternal request at term in the absence of medical or obstetrical indications. Recently an increase in the rate of cesarean section has been observed in many countries. The appropriate rate of cesarean section suggested by WHO is 15% of deliveries (Waldenstrom 2007).
It has been shown that elective cesarean section induces a higher risk of neonatal respiratory morbidity (Hakansson and Kallen 2003).
Despite the data on the increase in neonatal death, individual discussions are needed for parents who consider elective C-section.

1.3 IMMUNOLOGY AT BIRTH

The process of birth induces a surge of stress hormones (Lagercrantz and Bistoletti 1977). Stress modulates immune system by regulation of leukocytes and by their distribution between different compartments of the body. This is a complex process and depends on the interactions between immune and endothelial cells (Muller 2002).
Neonates are protected in a microbiologically sterile, intra uterine environment from the outer world. At birth, there is a sudden exposure of skin and surface mucosal to the commensal flora. Host defence may have originated from skin glands initially involved in innate immunity (Marchini, Hultenby et al. 2007).
At birth, the newborn infant has receptors on mucosal surfaces and also leukocytes that express toll like receptors (TLR) such as monocytes/macrophages, neutrophils, eosinophils, mast cells, natural killer cells and dendritic cells (Hanson, Zaman et al. 2008).
To find the balance between eliminating the harmful pathogen and cooperating with the beneficial microorganisms is a crucial task for the newborn innate immunity at the moment of birth (Elias 2007).

1.4 GRANULOCYTES AT BIRTH

The culture of fetal blood at 12-19 weeks of gestation yields high levels of granulocytic/monocytic progenitor cells. Monocytes comprising 42-68%, neutrophils 27-41%, and eosinophils 5-30% (14). Despite this high number of granulocyte progenitors in the circulation at this time, granulocytes are not formed in large numbers in fetuses until after birth, neutrophils being actually the last population to appear in the blood during fetal life (Holt and Jones 2000).

Granulocytes are involved in the earliest responses to microbial and parasitic infections, and their bactericidal armoury includes the generation of reactive oxygen and nitrogen species and the release of a range of degradative enzymes and antimicrobial peptides. The appropriate initiation and resolution of their inflammatory responses is crucial to the clearance of infections and the prevention of nonspecific tissue damage leading to chronic inflammatory disease (Butcher and Lord 2004).

1.4.1 Neutrophils

Neutrophils comprise almost two third of leukocytes and they are the terminally differentiated cells with half-life of 7 hours.

Neutrophils are key players in the innate immune system and they take part in different steps of host-pathogen interaction. Neutrophils' recruitment to the immunological reaction sites is an important part of the inflammatory responses. They migrate fast to sites of infection, recognize and phagocyte microorganisms and kill them by the production of reactive oxygen species and antimicrobial peptides and other granule proteins. These components are delivered to the phagosomes and also to extracellular environment. Neutrophils produce chemokines and cytokines, which regulate their migration to the inflammation sites and also recruit inflammatory reactive leukocytes such as neutrophils themselves and T cells.

Although their migration is very important for the host defence, over activation of these cells in such conditions can cause severe tissue damage and exacerbated inflammation such as thrombosis and edema, by excessive release of cytokines and other cytotoxic mediators which can result in chronic inflammation and autoimmunity. However, in such conditions they are able to induce their own apoptosis to prevent further tissue damage. The apoptotic cells are engulfed by macrophages. Neutrophils are hallmarks of rheumatoid arthritis, vasculitis, inflammatory bowel diseases and chronic lung diseases and that's why their migration and immune reactions must be highly regulated.

Neutrophil recruitment requires orchestrated steps of adhesive and migratory events, which are mediated by three different groups of adhesion receptors, the selectins, integrins and adhesion receptors of the immunoglobulin superfamily.

Neutrophil recruitment can be categorized into several steps, namely mobilization from the bone marrow, rolling along and tight adhesion to endothelial cells, and transmigration (Choi, Santoso et al. 2009).

In the process of neutrophil migration from blood stream to the target tissue, neutrophils rolling and adherence to the vascular walls is generated by the force of blood circulation. As said previously, chemical factors are also importantly implicated in neutrophils recruitment to sites of infection. Most of them act as pro-adhesive factors. Examples of such mediators include histamine, platelet activating-factor, TNF-alpha, IL-8 and other chemokines. Understanding mechanisms of neutrophils recruitment is certainly a crucial area of research and can be attributed to the discovery and comprehension of the role for many receptors, ligands, secreted molecules and signaling pathways implicated in the complex process of host-pathogen interaction (Pinheiro da Silva and Soriano 2009).

1.4.1.1 Interleukin-8

During inflammation, neutrophils roll along the walls of vessel walls, CXC chemokines playing an important part in this process. Among various inflammatory mediators, CXC chemokines including IL-8 (CXCL8), are the most crucial chemokines for the recruitment of neutrophils to the sites of inflammation and infection (Kobayashi 2008).
For this purpose, tissue-derived CXC chemokines have to crosswise endothelial cells. It has shown that IL-8 (CXCL8) is presented to the adherent neutrophils on the endothelial cell membrane.
Expression of IL-8 is regulated by activator protein and nuclear factor-κB–mediated transcriptional activity. Also, expression of IL-8 has been shown to be regulated by a

number of different stimulating factors such as inflammatory signals like tumor necrosis factor α, chemical and environmental stresses as exposure to chemotherapy agents and hypoxia and steroid hormones; androgens and estrogens.
The biological effects of IL-8 are regulated by the binding of IL-8 to two cell-surface G protein–coupled receptors, named CXCR1 and CXCR2. These receptors show highly similar structures suggesting that these genes arose through gene duplication. However, CXCR1 and CXCR2 exhibit a markedly distinct ligand-binding in pharmacology (Waugh and Wilson 2008).

IL-8 (CXCL8) facilitates the oxidative burst induced by stimulants such as fMLP and P-selectin. IL-8 is also the first and the most studied chemokine shown to be produced by neutrophils. It is known that apart from producing IL-8 neutrophils are affected by this chemokine in many different ways such as being the primary targets for IL-8, responding to this mediator by chemotaxis, release of granule enzymes, respiratory burst activity, upregulation of adhesion molecules expression on the surface, and increased adherence to unstimulated endothelial cells and is reportedly an angiogenic factor. It has been known for a long time that neutrophils release IL-8 after exposure to lipopolysaccharide (LPS). Also, it is now well established that the other stimuli able to induce this response include, cytokines and growth factors such as tumour necrosis factor TNF-alpha, granulocyte macrophage- colony stimulating factor (GM-CSF), chemoattractants including N-formyl-methionyl-leucyl-phenylalanine (fMLP), various bacteria, fungi, protozoa, viruses and products derived from these microorganisms, all possess the capacity to trigger the secretion of considerable amounts of IL-8 by neutrophils. IL-8 is amongst the neutrophil- derived products which are synthesized in larger amounts compared to other chemokines or cytokines (Kobayashi 2008). IL-8 induced response seems to occur as soon as neutrophils come into contact with any sort of ligand or contact from foreign particles. The most obvious speculation explaining such a response is that a prompt release of IL-8 would represent a feedback mechanism to ensure greater numbers of PMN to inflammatory site.

1.4.2 Eosinophils

Eosinophils are a small population of leukocytes, constitute 1-3% of total white blood cells in human adults (Blanchard and Rothenberg 2009), and newborns (Kato 1935). Hematopoietic stem cells are the origin of eosinophils. Granulopoisis of these cells occurs at 5 weeks of gestation (Sohn, Kim et al. 1993) and their number increase during this period. At 20 weeks of gestation they can be seen in circulation (Holt and Jones 2000). Maturation of eosinophils is regulated by soluble mediators and transcription factors (Boyce, Friend et al. 1995).

Interleukin-5 is eosinophils' main cytokine and induces their maturation and release from bone marrow (Clutterbuck and Sanderson 1988). IL-5 also activates eosinophils and increases their survival rate (Dubucquoi, Desreumaux et al. 1994).
It has been shown previously that after intravenous injection of IL-5 to mice, they developed blood eosinophilia, and their bone marrow depleted from eosinophils. Also, a study on IL-5 deficient animals indicated a significant reduction in eosinophil levels both in blood and in allergic tissues (Mishra and Rothenberg 2003).

Eotaxin acts as the main chemokine that involves in the cells' trafficking and it also activates eosinophils and induce the release of their granula proteins (Rothenberg 1999). Eotaxin is expressed in the thymus and also regulates the migration of eosinophils in the thymus (Matthews, Friend et al. 1998).
As shown in uterus, infiltration of eosinophils is regulated partly by eotaxin. In fact, in eotaxin deficient mouse eosinophils were seen in low numbers and a delay in uterus onset has been detected. These results suggest that eosinophils play an important role in maturation of uterus (Gouon-Evans and Pollard 2001).

Eosinophils are primary tissue cells and even under normal, non-inflammatory conditions they reside in proximity of mucous membranes colonized by microorganisms, such as the gastro-intestinal tract (Straumann and Simon 2004). Other eosinophil rich organs are lymph nodes, thymus, mammary glands, and uterus (Blanchard and Rothenberg 2009). In vitro studies show that eosinophils can inactivate bacteria by mounting respiratory bursts (Svensson and Wenneras 2005), release mitochondrial DNA (Yousefi, Gold et al. 2008) and mobilizing granule specific cytotoxic proteins, four of which, eosinophil cationic protein (ECP), major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil derived neurotoxin (EDN) have antibacterial effects (Blanchard and Rothenberg 2009). MBP, EPO and ECP, but not EDN, can also regulate and activate mast cells which results in histamine release (Piliponsky, Gleich et al. 2002).

Human eosinophils express Toll-like receptors (TLRs) for the recognition of conserved motifs in pathogens, including those for Gram-positive bacteria such as TLR2 and TLR5. Activation of these receptors leads to cytokine and ECP release and 50% increased eosinophils viability (Wong, Cheung et al. 2007). Eosinophils possess antigen-presenting capacity (Mawhorter, Kazura et al. 1994; Shi 2004) and can produce both Th1 and Th2 cytokines (Liu, Bates et al. 2007). They play a pivotal role in the clearance of parasites (Weller 1994). Taken together, these data indicate a role for eosinophils in innate immune response connected to microbes, besides being involved in allergy.
In neonates, eosinophils show abnormalities in their trafficking. Also the exudates from different neonatal compartments have increased number of eosinophils. Also, neonatal eosinophils are present in the inflammatory reactions such as bronchopulmonary dysplasia and also in infants with acute whizzing. There is a link between neonatal eosinophils and nonparasitic infections as well as a physiological role for them during the time of skin mucosal surface colonization at birth (Moshfegh, Lothian et al. 2005). In this project we hypothesize that the role of eosinophils in newborns are different from that in adults.

1.5 IMMUNOMODULATORS

1.5.1 Cathelicidin antimicrobial peptide LL-37

Endogenous antimicrobial peptides and proteins are the key player molecules of innate immunity (Sorensen, Borregaard et al. 2008). Cathelicidins are one of the main families of antimicrobial peptides in mammals. This family of AMPs has a conserved pro-region, cathelin, and a viable C-terminal domain. At the time of activation, the C-terminal domain is cleaved off which activates the antimicrobial peptide. Cathelicidins are stored in granules of leukocytes in their inactive form and are expressed in various epithelia in their active form (Zanetti 2004). hCAP18, the human cationic antimicrobial pro-peptide is the only human cathelicidin which cleaves and liberates its active form, LL-37. This active antimicrobial peptide was first identified in blood (Agerberth, Gunne et al. 1995). hCAP18 is mainly found in neutrophilic granules and they are cleaved extracellularly by serine protease (Sorensen, Follin et al. 2001). LL-37 strongly prevents the formation of bacterial biofilm in vitro (Overhage, Campisano et al. 2008) and inhibits growth of skin commensal staphylococci isolated from newborn infants (Nelson, Hultenby et al. 2009). Also it actively provides protection against a broad range of bacteria, virus and fungi and is active in the infection sites in human and mice (Chromek, Slamova et al. 2006).
In addition to the antimicrobial action, LL-37 also exhibits immunomodulatory effects on chemotaxis (Tjabringa, Ninaber et al. 2006), angiogenesis (Koczulla, von Degenfeld et al. 2003) and wound repair (Heilborn, Nilsson et al. 2003).
We previously showed that leukocytes, including eosinophils recruited in the skin of healthy newborns express cathelicidin antimicrobial peptide LL-37 (Marchini, Lindow et al. 2002).

1.5.2 Interferon-gamma

IFN-γ is secreted by activated immune cells. Schroder and colleagues have shown that, mice lacking either IFN-γ or its receptor have increased susceptibility to bacterial and viral pathogens (Schroder, Hertzog et al. 2004). Other functions of IFNs are leukocyte homing, cellular adhesion, immunoglobulin class switching, Th cell polarity, antigen presentation, cell cycle arrest and apoptosis (Boehm, Klamp et al. 1997). IFN-γ signaling is initiated by the interaction between IFN-γ and cell surface receptors IFN-γR1 and IFN-γR2, resulting in oligomerization of the receptor. After tyrosine phosphorylation of Janus Kinase 1 (JAK1), JAK2, and signal transducers and activators of transcription (STAT1), they migrate into the nucleus and mediate transcription of cytokine-specific genes.
Previously, it has been shown that human peripheral eosinophils express the functional IFN-γ receptor (Ishihara, Ochiai et al. 1997). IFN-γ regulates eosinophils functions (Valerius, Repp et al. 1990), (Fujisawa, Abu-Ghazaleh et al. 1990) differentiation (Ochiai, Iwamoto et al. 1995) and apoptosis (Ochiai, Kagami et al. 1997). IFN-γ acts as primary mode of signaling in the Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathways (Greenlund, Farrar et al. 1994). Also it has been shown that tyrosine phosphorylation of STAT1 is directly regulated by tyrosine phosphorylation of IFN-γR (Ochiai, Ishihara et al. 1999). The increased expression of IFN-γR and its direct effects on the activation of STAT1 signaling cascade after normal delivery prolong cord blood eosinophils survival and cytokine production.
IFN-γ also affects adhesion and transmigration of polymorphonuclear leukocytes which is mediated via Receptor for advanced glycation endproducts (RAGE) and CD11b/CD18 (Zen, Chen et al. 2007). This data is in line with our previous data that

shows the stress of birth in normal deliveries activates the immune function of the leukocytes such as transmigration capacity and expression of CD11b in neonatal neutrophils (Yektaei-Karin, Moshfegh et al. 2007).

2 AIMS

The overall aim of this study was to investigate the inflammatory immune response in newborns in relation to different delivery modes and different degree of fetal stress. This study has focused on neutrophils and eosinophils and the changes in their immune reactions in normal delivery and elective cesarean section.

The specific aims of each paper were:

Paper I: To analyze spontaneous and IL-8-induced chemotaxis as well as CD11b expression of neutrophils obtained after different delivery modes, measure circulating IL-8, IFN-γ, and soluble E-Selectin levels as well as the major leukocyte populations.

Paper II: To analyze if the circulating LL-37 level measured in the umbilical cord blood, was related to the stress of delivery and neutrophil activation or to maternal peptide levels. Also if the peptide's level is related to vitamin D status of both mothers and infants. Finally, we wanted to describe the subcellular location of the peptide in neutrophils purified from cord blood.

Paper III: To further investigate the link between eosinophils and their main immunomediators, IL-5 and eotaxin, and LL-37, to show that neonatal eosinophils contain and release hCAP18/LL-37 and the peptide's subcellular distribution in eosinophils from cord blood.

Paper IV: To focus on umbilical cord blood eosinophils and the changes that their gene expression profile goes through after different delivery modes.

3 MATERIALS AND METHODS

Materials and methods that have been used in paper I-IV have been briefly described in this section. A more detailed description is available in material and method section of each paper.
The ethics committee of Karolinska University Hospital approved the study and parents gave their informed consent. All singleton pregnancies were uncomplicated and at term gestation (37 weeks or more). No mother obtained antibiotic treatment in connection to delivery. All infants were healthy at birth, had adequate birth weight and an uncomplicated neonatal period.

3.1 STUDY MATERIAL

Blood was collected from the umbilical cord after clamping according to hospital routines. Maternal blood was obtained by peripheral venipuncture before C-section or within 15 min after normal vaginal delivery. Umbilical cord arterial blood was obtained for determination of pH.

3.2 METHODS

3.2.1 Purification

Granulocytes were isolated using Ficoll density gradient (Amersham, Buckinghamshire, United Kingdom).
Erythrocytes were specifically hemolysed by adding NH4Cl-EDTA buffer [154 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA (pH 7.2)]. Eosinophils were isolated by Eosinophil isolation kit (Milteny Biotech, Bergisch Gladbach, Germany) after negative selection from MACS LS columns (Milteny Biotech). Neutrophils were isolated by anti CD16 micro beads (Milteny Biotech) after positive selection from MACS LS columns (Milteny Biotech).

3.2.2 Transmigration assay

Blood samples after normal delivery (n=6), C-section (n=3) and from adults (n =3) were analyzed. The transmigration assay was performed using 24-well microchemotaxis chamber with a polyethylene terephthalate treated membrane (BD FalconTM, San Jose, CA, USA) which has a high pore density membrane (2.0×10^6 pores/cm^2, 3.0 μm in diameter) for maximum permeability. Insert filters were coated with collagen IV. Briefly, for IL-8-induced neutrophil transmigration, Ficoll-separated (Amersham, Buckinghamshire, United Kingdom) granulocytes were resuspended in RPMI alone and added to the upper chamber. Buffer supplemented with IL-8 (100 ng/ml; BD) was added to the lower chamber. Cells that transmigrated into the lower chamber (transmigrated cells) were, together with the original cell suspension (total cells), subjected to the further analysis. A ratio between transmigrated cells and total cells was calculated for each experimental setting and expressed as percentage of transmigrated cells (transmigration index), as previously reported (25). The transmigration assay was run for 180 min, and the transmigration index was calculated at stated time-points.

3.2.3 Flowcytometric studies

3.2.3.1 Purity check

Purity of eosinophils and neutrophils was assessed by flowcytometry (Epics XL, Coulter, Krefeld, Germany) after incubating the cell pellets with FITC conjugated CD16 (BD Biosciences, CA, USA) antibody. Neutrophil population shows positive staining for CD16 and eosinophil population shows negative staining for CD16. The purity of both cell type populations were > 95%.

3.2.3.2 Apoptosis

Cultivated eosinophils with RPMI + 10% FCS were collected at the indicated time points as well as cells from an overnight culture. Cells were washed with 2 ml PBS, and resuspended in 100 µl binding buffer (Immunotech, Marseille, France). Then 1 µl of Annexin V-FITC solution and 5 µl dissolved propidium iodide (Immunotech, Marseille, France) were added and the cells were incubated for 10 min in dark at 4°C. 400 µl of binding buffer was added to the cells, and annexin V and propidium iodide (PI) staining was detected by flowcytometry (Epics XL, Coulter, Krefeld, Germany).

3.2.3.3 Surface marker expression (CD11b)

Leukocytes were incubated with phycoerythrin-conjugated mAb directed against CD11b (DAKO A/S, Glostrup, Denmark) at a final concentration of 50 µg/ml for 30 min on ice and thereafter were washed once in 3 ml of 4ºC PBS-EDTA (300 g, 6 min). The final preparations of mixed leukocytes were analyzed and counted in an EPICS XL flowcytometer (Coulter, Krefeld, Germany).

3.2.3.4 Intracellular expression (LL-37)

Blood samples of 150 µl were collected from the umbilical cord after normal deliveries (n=9) and from adults (n=6) in citrate tubes.
Erythrocytes were hemolysed by addition of NH4Cl-EDTA buffer [154 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA (pH 7.2)]. After washing with PBS, reminding cells were fixed with 200 µl of 4% paraformaldehyde (10 minutes at room temperature). Pellet was then washed with PBS and cells were permeabilized by incubation with 0.74% OG (n-octyl β-D-glukopyranoside) for 6 minutes at room temperature. After washing with PBS cells were incubated with 10 µg/ml of monoclonal antibody to human LL-37/CAP18 (Hycult Biotechnology, PB Uden, The Netherlands) for 30 minutes on ice. Cells were washed and incubated with FITC-conjugated anti mouse IgG1 for 30 minutes on ice. The pellet was then washed, resuspended in 500 µl of PBS and analyzed by flowcytometry (Epics XL, Coulter, Krefeld, Germany). Eosinophils and neutrophils were gated based on their position on the forward and side scatter.

3.2.4 Immunoblotting

Cord blood eosinophils and immunoprecipitated supernatants from activated eosinophils, denaturised in sample buffer and boiled for 5 minutes and loaded on a 12 % Bis-Tris Gel (Invitrogen Life Technologies, CA, USA). Unstimulated cord blood

neutrophils' lysates and rLL-37 peptide were run in parallel and served as controls. The electrophoresis was run for 45 minutes in 200 constant voltages according to NuPAGE Technical Guide (Invitrogen Life Technologies, CA, USA). The proteins were transferred to Hybond ECL nitrocellulose membrane (Amersham, Buckinghamshire, UK) in an Xcell II Blot Module (Invitrogen, CA, USA) for 35 min in 30 constant voltages. Non-specific sites were blocked in 5% non-fat dry milk (Bio-Rad, CA, USA) in tris-buffer saline with 0.05 % Tween 20 (TBS-T), incubated with 2 µg/ml mouse monoclonal antibody to human LL-37/CAP18 (Hycult Biotechnology, PB Uden, The Netherlands) at 4°C over night and after repeated washing steps, a horseradish peroxidase conjugated secondary antibody (Pierce Biotechnology, IL, USA) was added to the membrane and incubated in room temperature for one hour, repeated washing steps were done as above. The protein was visualized with ECL advanced detection kit (Amersham Life Science, UK) and was laid on autoradiograph film, Hyperfilm ECL (Amersham Life Science, Buckinghamshire, UK) for 1 min.

3.2.5 Immunofluorescence

Eosinophils were spun on slides and were air dried for 20 minutes. Cells were fixed in 4% phosphate buffered formaldehyde (4 min, RT), methanol (1 min, -20^{o}C) and acetone (4 min, -20^{o}C). The slides were then stored at -20^{o}C in storage buffer (PBS containing 50% glycerol, 250mM sucrose and 7mM $MgCl_2$) until further processing. The slides were dipped in deionized water for a few seconds to remove the excess storage buffer and were incubated in PBS twice for 5 min each. They were then incubated in PBS containing 10mM glycine for 3 minutes. Permeabilization was performed by incubating the cells in PBS containing 0.1% Triton X 100 for 5 minutes. Blocking was done for 30 minutes with 0.2% BSA in TBS (50mM Tris-HCl, 150mM NaCl and 1mM $CaCl_2$, pH 7.4). The primary antibodies, mouse monoclonal to human LL-37/CAP18 (Hycult Biotechnology, PB Uden, The Netherlands) dilution 1:50 and EG2 (A gift from Pharmacia Diagnostic, Uppsala, Sweden) dilution 1:200, were incubated overnight at 4^{o}C. For negative controls, respective non-immune serums with same concentration to that of the primary antibodies were used. The slides were washed thrice with PBS for 5 min each and were incubated with fluorescent conjugated secondary antibodies (1:200, Anti mouse FITC and anti rabbit Cy3, Jackson ImmunoResearch, PA, USA) for 60 min at RT. The slides were washed with PBS thrice and then briefly with deionized water. The slides were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, CA, USA). A Nikon E800 microscope was used to capture the images and the fluorescent images were merged using DP Manager software.

3.2.6 Immunoelectron microscopy

In order to locate hCAP18/LL-37 within cord blood eosinophils, immunogold electron microscopy was performed on leukocytes obtained from umbilical cord blood after normal delivery (n=1) and cesarean section (n=2), stained with LL-37 antibody. A pellet of leukocytes was fixed in 3% paraformaldehyde and 0.1% glutaraldehyde in 0.1M phosphate buffer. The pellet was then infiltrated with 10 % gelatin and fixed in the same fixative. Small pieces were then cut from the pellet and all specimens were then infiltrated into 2.3M of sucrose and frozen in liquid nitrogen. Sectioning was

performed at −95°C, and grids were placed directly on drops of 0.15M NaCl containing 20mM glycine followed by incubation in 2% BSA and 2% gelatin in 0.1M phosphate buffer to block non-specific binding. Sections were then incubated with the rabbit polyclonal anti human LL-37 (Innovagen, Lund, Sweden) diluted 1:200 in 0.1M of phosphate buffer containing 0.1% BSA and 0.1% gelatin overnight in a humidified chamber at room temperature. The sections were thoroughly washed in the same buffer and bound antibodies were detected with protein A, coated with 10 nm gold (Amersham Biosciences, Piscataway, NJ, USA) at a final dilution of 1:100. Sections were rinsed in buffer and fixed in 2% glutaraldehyde, contrasted with 0.1% uranyl acetate, and embedded in 2% methylcellulose.

3.2.7 PCR

First-strand cDNA was synthesized for each RNA sample, using SuperScript II reverse transcriptase (Invitrogen, CA, USA) 200U/µl. The cDNA was then used as the target template for amplification in qPCR. Primer and probe for LL-37 (Gene hCG18336 Celera Annotation) as well as the house keeping gene GAPDH were commercially purchased (Applied Biosystems, Stockholm, Sweden). Probes were labelled at the 5'end with the reporter dye molecule FAM (6-carboxy-fluorescein) and at the 3'end with quencher molecule TAMRA (6-carboxytetramethylrhodamine). Each cDNA sample was used as a template for LL-37 genes and GAPDH were simultaneously tested. RT-PCR of cDNA specimens was conducted in a total volume of 25 µl with 2x TaqMan Master Mix (Applied Biosystems, Stockholm, Sweden), primers at 300 nM and probes at 200 nM.
The relative quantitation of gene expression was performed by using the arithmetic equation $2^{-\Delta\Delta CT}$ according to the Applied Biosystems manual (1997). The amount of RNA was normalized to the endogenous reference gene (GAPDH) at each stage in order to distinguish for differences in the total amount of nucleic acid added to a reaction mixture. The values are expressed relative to a reference sample. The calculation of ΔΔCT was determined by the subtraction of target CT and the corresponding CT for the internal control (=ΔCT). The ΔCT for each sample was subtracted from ΔCT reference sample (=ΔΔCT). The relative quantitative expression or fold difference of sample compared to reference sample is acquired by the equation $2^{-\Delta\Delta CT}$

3.2.8 Microarray studies

Samples were processed according to the Affymetrix GeneChip Expression Analysis Manual (Affymetrix Inc., Santa Clara, UK). Briefly, total RNA from purified eosinophils was extracted with the Qiagen RNeasy kit (Düsseldorf, Germany) according to the manufacturer's instructions. The integrity of the extracted RNA was confirmed by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). GeneChip arrays were scanned using fluorometric scanners (Affymetrix scanner). The scanned images were inspected and analyzed using established quality control criteria.

3.2.8.1 Data analysis

Comparative analysis between expression profiles for Affymetrix experiments was carried out using GeneSpring™ software version GX (Silicon Genetics, CA, USA).

The “Cross gene error model for deviation from 1.0” was active. Gene expression data were normalized in two ways: “per chip normalization” and “per gene normalization” For “per chip normalization” all expression data on a chip are normalized to the 50th percentile of all values on that chip. For “per gene normalization” the data for a given gene are normalized to the median expression level of that gene across all samples. The data sets were then assigned to two groups for vaginally delivered and elective cesarean section. The expression profiles from experiments were compared using t-test to identify genes that were differentially expressed between the groups. For sample clustering, standard correlation was applied to measure the similarity of the expression pattern between different samples. Lists were compiled of all genes for which expression was differentially either induced or suppressed >1.5-fold between groups. The gene lists were categorized according to their biological functions as described in GeneSpring, Ingenuity and GeneCards.

3.2.9 Statistical analysis

Paper I: Because the values of most of the studied metabolic endocrine variables followed a non-Gaussian distribution, these data are given as median (range) and we used the Kruskal–Wallis rank test, followed by the Mann–Whitney U-test to evaluate possible differences between groups. Simple regression analysis was performed to evaluate the relationship between variables by group. Data on cell migration kinetics and CD11b expression are given as medial and interquartile range. We used analysis of variance followed by Mann–Whitney U-test to establish differences between groups. Interactions within groups were calculated with the Tukey test.
PaperII: Clinical and anthropometric data are given as mean±SD, biomarkers are given as median (range). Difference between groups were analysed using Mann-Whitney U-test and Wilcoxon rank-test for paired groups. Correlations between variables were obtained with Spearman’s test.
Paper III, IV: Student t-test was used for analysis of the statistical differences between groups, and a p-value of < 0.05 was considered significant.

4 RESULTS

4.1 BIOMARKERS AND WBC

Paper I

We found that the stress of birth and the mode of delivery have direct impact on white blood count and immuno regulatory factors in umbilical cord blood. The results are summarized in Table I.

Table I. Biomarkers, total WBC and major leukocyte populations in cord blood of newborns born at term gestation after C-section, normal and assisted delivery. We compared C-section with normal delivery, and normal delivery with assisted delivery (Mann-Whitney U-test). Values are median (range). nd=not determined. The number of infants for each variable analyzed was 17-47 for C-Section, 18-73 for normal delivery and 9-26 for assisted delivery.

	C-section (n=47)	**normal** delivery (n=73)	**surgical/assisted** delivery (n=26)
a pH	7.31 (7.23-7.40)	7.29 (7.17-7.39) *p<0.01*	7.19 (7.02-7.34) *p<0.001*
Lactate (mmol/L)	1.7 (1.3-3.9)	4.4 (1.8-7.0) *p<0.001*	8.1 (4.6-11.1) *p<0.001*
IL-8 ng/L	2.2 (0.7-4.1)	2.8 (0.7-20.7) *p<0.05*	3.8 (1.7-34) *p<0.01*
INF-gamma (pg/mL)	0	0 (0-17.7)	0.6 (0-16.7) *p<0.0001*
E-Selectin (µg/L)	43.1 (25-85.3)	52.6 (29.2-92.2) *p<0.05*	70.7 (27.8-127.7) *p<0.01*
Cortisol (nmol/L)	155 (95-312)	477 (241-964) *p<0.0001*	538 (340-901)
Prolactin (µg/L)	177 (96-413)	187 (89-648)	247 (106-848) *p<0.05*
ß-endorphin (pmol/L)	87 (5-152)	125 (33-314)	124 (41-566)
Noradrenalin (pmol/mL)	nd	26.5 (4.2-449.8)	48.5 (29.2-166.7) *p<0.05*

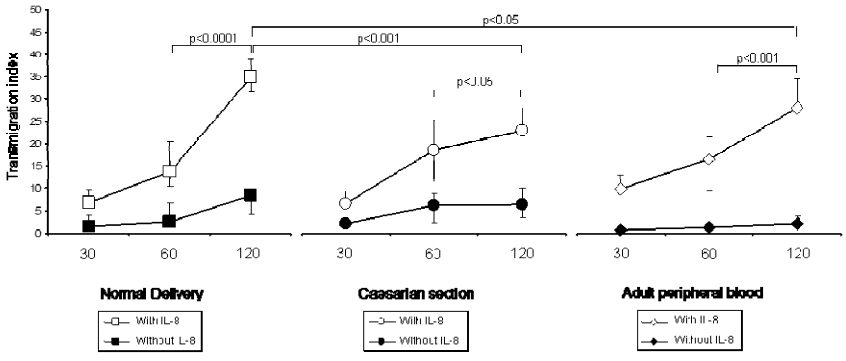
Adrenaline (pmol/mL)	nd	3.2 (0.5-38.7)	7.6 (0.8-29.9) *p<0.05*
Dopamine (pmol/mL)	nd	1.4 (1-1.1)	1.8 (3.3-2.5) *p<0.05*
Total WBC (10^9/L)	11.5 (5.8-17.9)	14.7(8.2-32.2) *p<0.0001*	20.4 (14.1-32.3) *p<0.0001*
Neutrophils	5.7 (1.9-11.3)	8.3 (3.1-19.1) *p<0.0001*	11.7 (3.7-21.7) *p<0.001*
Basophils	0.1 (0-0.6)	0.1 (0-1.3)	0.1 (0-0.8)
Eosinophils	0.4 (0-0.1)	0.4 (0-1.3)	0.5 (0.3-2.5)
Lymphocytes	4.5 (4.0-7.4)	4.4 (2.2-11.3)	7.2 (4.7-11.8) *p<0.0001*
Monocytes	0.8 (0.4-2)	1.1 (0.1-3.9) *p<0.001*	2.6 (0.4-2.6) *p<0.05*

4.2 NEUTROPHILS' FUNCTIONALITY IN DIFFERENT DELIVERY MODES

Paper I

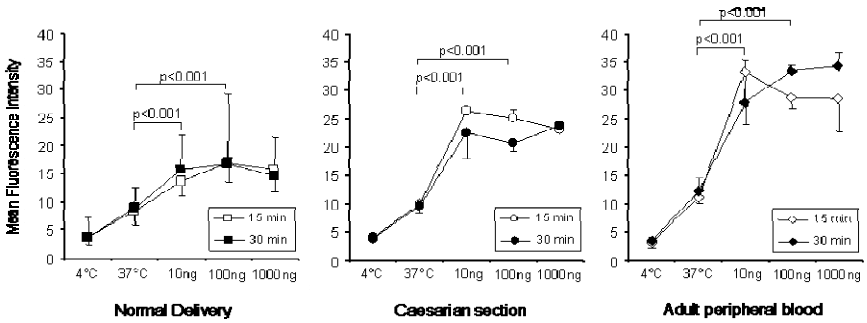
We found that spontaneous and IL-8 induced transmigration ability of neutrophils from newborns after normal delivery was significantly higher compared to that of neutrophils from cesarean section or from adults after 120 minutes (Figure 1).

Figure1. The effect of interleukin (IL)-8 on in vitro transmigration of neutrophils obtained from cord blood after normal delivery (n= 6) and Cesarean-section (C-section; n=3) and from adults (n=3). Results expressed as transmigration index are presented as median and interquartile range.



Neutrophils that were incubated with 10 ng IL-8 showed an elevated expression in CD11b compared to the ones incubated with RPMI, both after 15 and 30 minutes. After incubation with 10 ng IL-8 for 15 min and100 ng for 30 min, expression of CD11b in adults' neutrophils was higher compared to the corresponding cells from normal delivery and C-section (Figure 2).

Figure 2. Expression of CD11b on neutrophils obtained from cord blood after normal delivery (n= 8) and Cesarean-section. (n=5) and from adults (n=4). The neutrophils are treated at +4ºC with RPMI alone, and at +37ºC with interleukin (IL)-8 at three different concentrations: 10, 100, and 1000 ng/ml for 15 and 30 min. Results, expressed as MFI units, are presented as median and interquartile range.



Paper II

Cord plasma LL37 levels were higher ($p<0.03$) after normal delivery compared to C-section. Maternal plasma LL37 levels were not significantly affected by delivery mode. There is a positive and highly significant correlation between maternal and cord plasma LL37 levels**,** regardless of delivery mode. No relationship was found between LL37 and 25(OH)D levels.

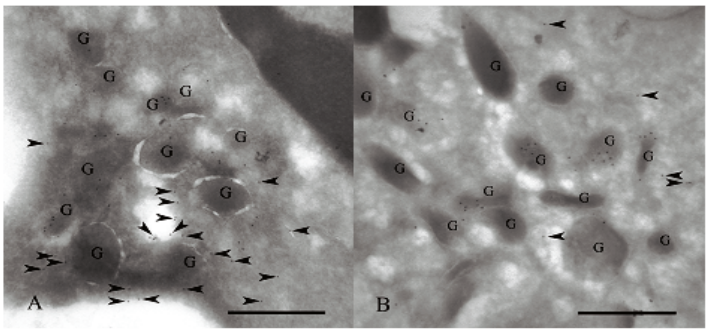
There was no significant correlation between and WBC, MMP9, MPO or stress markers, neither in mothers nor infants (Table II).

Tabel II. Circulating levels of biomarkers in infants (cord blood) and mothers. Note that plasma LL37 levels were measured in a total number of 115 infants, while in a subpopulation of these; 27 normal deliveries and 23 C-sections, also maternal plasma LL37 levels as well as additional biomarkers were analysed in paired samples. ND=normal delivery.

LL37 (ng/ml)					
ND	1.11	(0-38.45)	n=68	1.74	(0.15-17.54) n=27
C-Section	0.38	(0-13.5)	n=47	0.82	(0.11-21.38) n=23
25(OH)D nmol/L					
ND	40.2	(8.7-98.5)	n=27	40.2	(8.7-98.5) n=27
C-Section	58.1	(13.3-99.8)	n=23		
				42.7	(10.5-89.4) n=23
WBC (10^9/L)					
ND	15.6	(10.1-24.8)	n=27	15.7	(6.5-28.5) n=27
C-Section	10.8	(7.6-21.8)	n=21	8.3	(5.8-15.2) n=23
Neutrophils(10^9/L)					
ND	8.8	(3.7-14.0)	n=27	13.9	(6.0-23.1) n=27
C-Section	5.1	(2.4-11.4)	n=21	5.4	(3.6-10.6) n=23
MMP9 (ng/ml)					
ND	37.7	(19.4-89.4)	n=27	35.9	(7.9-116.9) n=27
C-Section	35.0	(21.1-133.7)	n=23	8.9	(3.3-128.8) n=23
MPO (ng/ml)					
ND	121.5	(46.5-325.5)	n=25	140.0	(35-1272.7) n=25
C-Section	86.5	(21.1-980.0)	n=15	51.0	(13.7-561.9) n=15
Arterial pH					
ND	7.27	(7.14-7.38)	n=27		
C-Section	7.30	(7.27-7.37)	n=23		
Glucose (mmol/L)					
ND	5.3	(3.5-7.7)	n=27	6.3	(4.8-9.5) n=27
C-Section	2.9	(2.1-4.6)	n=23	4.4	(3.8-5.1) n=23
Lactate (mmol/L)					
ND	4.5	(1.8-8.8)	n=27	4.0	(2.4-8.0) n=27
C-Section	2.2	(1.1-4.0)	n=23	1.4	(0.9-2.7) n=23

Neutrophils from cord blood after normal delivery contained 10-times more cytoplasmatic cathelicidin peptide compared to corresponding cells after C-section where a strict granular localization was found (Figure 3).

Figure 3. Immunoelectron micrographs showing the distribution of LL37 in neutrophils purified from cord blood (A) after normal delivery (B) and C-section. Note the increased level of LL37 in the cytoplasm (arrowhead) in normal delivery (A) compared to the labelling mostly restricted to granules in C-sectioned. G = granules, bar = 0.5 µm.

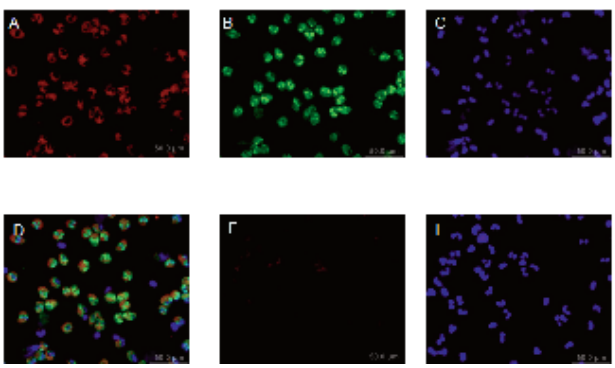


4.3 EOSINOPHILS' FUNCTIONALITY IN DIFFERENT DELIVERY MODES

Paper III

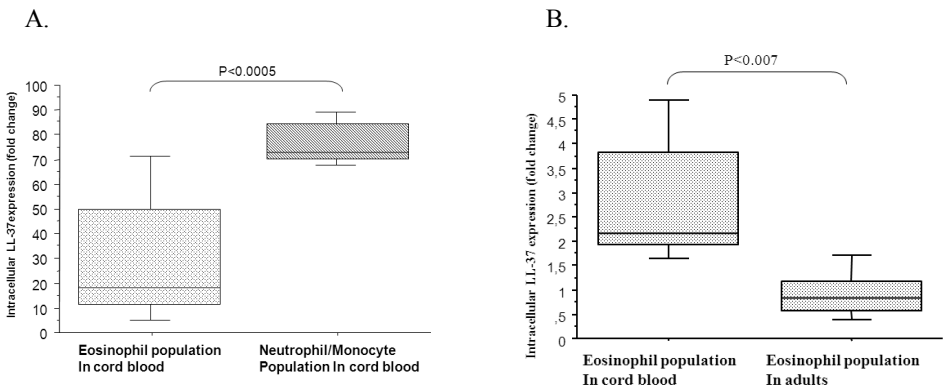
The study showed that circulating eosinophils in umbilical cord blood contain cathelicidin antibacterial peptide LL-37 (Figure 4.).

Figure 4. Co-localization of LL-37 and EG-2 in purified cord blood eosinophils. (A) Representative image stained for LL37 (red), (B) EG2 (green), (C) and counterstained with DAPI for nuclear staining (Blue). (D) The overlaid image showing the co-localization of LL-37 and EG2. Panels E and F are the images of negative control where the primary antibodies were replaced by non-immune IgG (E) and DAPI stained nuclei of eosinophils of the same field (F). Magnification = 400X, The scale bars represents 50 microns.



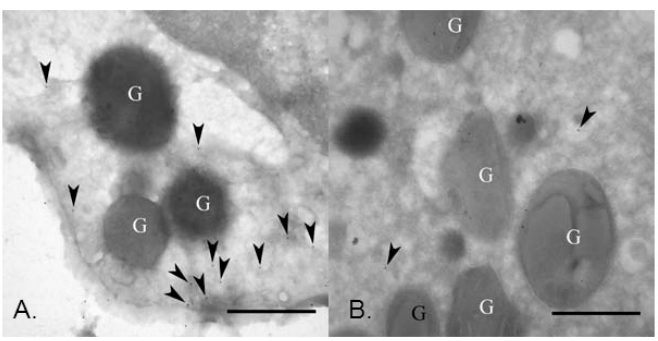
Results from flowcytometry and western blot optical density showed that LL-37 content of cord blood eosinophils was 3-5 fold less than the peptide content in neutrophils (Figure 5A).
Eosinophils obtained from newborns possessed significantly higher peptide content compared to eosinophils from adults (Figure 5B).

Figure 5. Expression of intracellular hCAP18/LL37 in (A)neutrophil/monocyte and eosinophil population, isolated from cord blood after normal delivery (B) and comparison of intracellular expression of cord eosinophils with adult eosinophils.



Immunoelectron microscopy figures showed that in cord blood eosinophils LL-37 is mainly located in the cytoplasmatic granules. A 15-fold increase was seen in cytoplasmatic compartment after normal delivery compared to eosinophils after scheduled cesarean section, which indicates peptide mobilization in response to delivery stress (Figure 6).

Figure 6. Immunoelectron microscopy of eosinophil isolated from cord blood showing the distribution of LL-37 in the cytoplasm of cells obtained after (A) normal delivery and, (B) C-section. Note the increased level of hCAP18/LL-37 in the cytoplasm (arrowheads) of cells obtained after normal delivery compared to C-section.
G = granules, bar = 0.5 µm.

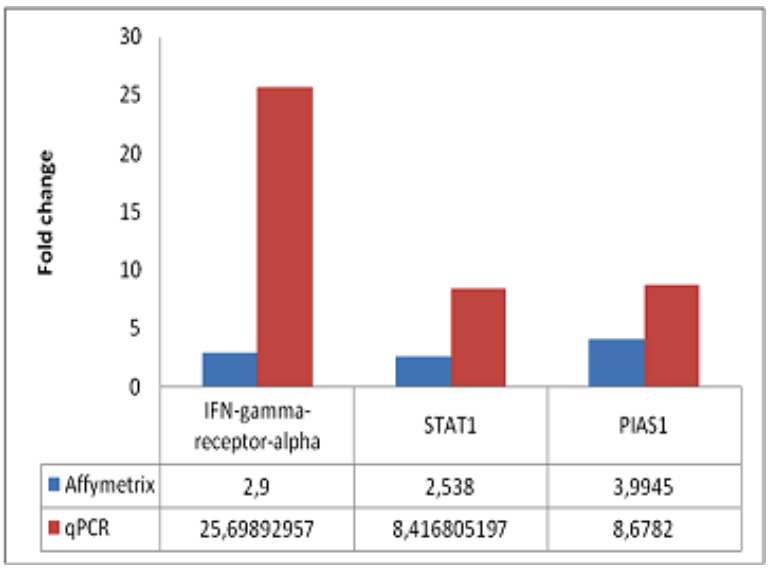


Release of LL-37 from eosinophils was observed in 2 of 5 samples after incubation of eosinophils with IL-5 and eotaxin as their main immunomodulatory factors. Expression of the cathelicidin gene in eosinophils after the above stimulation remained unchanged, as analysed by qPCR.

Paper IV

Our preliminary data analysis shows gene expression differences in eosinophils between the two delivery modes. We have identified the biological pathways that contain the genes with the most fold change difference in C-section eosinophils compared to normal delivery eosinophils.
We observed higher expression of genes critical for interferon signaling such as IFN-γ alpha receptor 2.9 fold and STAT1 protein 2.5 fold upregulated in cord blood eosinophils compared to C-section. The data has also been confirmed by qPCR (Figure 7).

Figure 7. Upregulation of IFN gamma receptor alpha, STAT1 and PIAS1 assessed by microarray analysis and Real Time PCR.



5 DISCUSSION

The main finding of our research is that the stress of birth can affect leukocytes and their immunoregulatory factor at the moment of birth. In the context of immunology we have looked at these changes from different angles.

We showed that neutrophil count in umbilical cord blood increases after normal delivery compared to C-section and this increase was even more after assisted surgical delivery. Also our data provides evidence that IL-8 released at low doses during labor may be a physiologic mechanism of neutrophil priming leading to enhanced chemotaxis. The increased IL-8 level observed after birth may reflect a spill over from activated vascular endothelial cells and/or circulating immune cells and implicates neutrophil activation and inflammation.
E-selectin, that is only expressed by endothelial cells (Koenig, Stegner et al. 2005), is increased after normal delivery in cord blood. This shows that fetal vascular endothelial cells are activated during labor. The redistribution and activation of immune cells under the stress of labor may be an adaptive response designed to enhance the vigilance and capacity of the immune system reaction to challenges at the major defence barriers of the body, skin, and mucosal linings. In this way, the immune system is alerted of the oncoming commensal microflora and potential pathogens. The interaction between microbes and host at the epithelial linings is an active process where the early, innate immune response plays a pivotal role for survival but also constitutes a factor for the maturation of the immune system of the newborn (Hakansson and Kallen 2003).

Neutrophils are known to be the main containers of cathelicidine antimicrobial peptide LL-37 in humans (Sorensen, Arnljots et al. 1997). In paper II we showed that there is a correlation between maternal and fetal LL-37 level that can indicate transplacental passage of this peptide during late pregnancy and delivery. LL-37 on cord blood increased by three fold after normal delivery compared to C-section in relation to the stress of birth. In cord neutrophils the amount of cytoplasmic LL-37 increased by 10 fold after normal delivery which yet again shows the effect of stress at birth on leukocyte activation. It seems that LL-37 has an important role in protection against infection. Patients with renal diseases have low levels of LL-37 and they are more prone to infections (Chromek, Slamova et al. 2006). LL-37 also inhibits the growth of *Staphylococcus epidermidis*, which is the main cause of sepsis in infants (Nelson, Hultenby et al. 2009). These facts indicate the importance of LL-37 in the protection of newborn infant against infection at birth and since the levels of LL-37 in infants are regulated by the levels of this peptide in mothers, it is of importance to induce a higher production of this peptide in mothers, normal delivery being an example. In case of preterm babies this aspect is very critical.
The increase of LL-37 in cytoplasmic compartment of cord neutrophils after normal delivery was shown by immunoelectron microscopy pictures. That indicates the mobilization of this peptide due to the stress of birth. This goes in line with the increase of IL-8 after normal delivery and the fact that IL-8 affects neutrophils' functions in a wide spectrum. The mobilization of the peptide does not seem to be a result of cord

neutrophils degranulation, since the level of MMP9, neutrophils granule factor did not increase at birth.
Previously, it has been shown that the regulation of hCAP18, the gene responsible for LL-37 production, is regulated by vitamin D (Yim, Dhawan et al. 2007). We have shown that vitamin D levels correlate in mothers and infant but we did not find the same correlation between the levels of LL-37 and vitamin D in mothers or in infants. This shows that LL-37 production during pregnancy and delivery are not regulated by vitamin D. In fact recently a placenta specific methylation has been found during pregnancy and delivery at the mother-infant interface.

All the above results show that a moderate stress at birth, as induced by normal delivery enhances immunological functions of neonatal neutrophils, such as the increase in their number, migration capabilities and antimicrobial impacts.

We have also studied the immunological characteristics of eosinophils at birth. This leukocyte subtype plays an important role in the immunological reactions in neonates. They are present in the lesions of *Erythema toxicum* rash in newborn infants.
We found that cord blood eosinophils contain cathelicidine antimicrobial peptide both in its proform hCAP18 and its active form LL-37. Immunofluorescence images showed a co-localization of LL-37 with intracellular marker of eosinophils, EG2, which agrees with the previous finding (Marchini, Lindow et al. 2002). Also eosinophils had 3-4 folds less LL-37 compared to the main containers of this peptide, neutrophils, as shown by flowcytometry and optical density analysis of western blot results.
Immunoelectron microscopy images showed that LL-37 in cytoplasmic compartments of eosinophils increase up to 15 fold in connection to normal delivery compared to C-section. The figures did not show any signs of degranulation in eosinophils. Also it seems unlikely that this results from apoptosis of eosinophils, since the cells are immediately fixated after purification and the annexin V/PI staining in these cells did not show signs of apoptosis until 120 minutes.
Therefore, it seems that an active mechanism takes part in the peptide transport after normal delivery. The results also show that eosinophils follow the same trend as neutrophils when being under the stress of normal delivery. The mechanism of this intracellular transport system is still not defined.
We stimulated eosinophils with their known immunomodulators IL-5 and eotaxin to induce the secretion LL-37, but only in 2 of 5 samples we saw secretion of this peptide. Also the stimulated cells were used in a qPCR experiment and the results did not show an increase in the expression level of hCAP18/LL-37 gene. This might be due to maximal cell activation in the process of birth.
In a comparative study between neonatal eosinophils and adult eosinophils, neonatal eosinophils showed a higher content of intracellular LL-37 compared to adults. This finding is an evidence, showing the leukocyte activation at birth. Neonatal eosinophils show enhanced transmigration in response to stimulation with IL-5 and eostaxin compared to adults. This agrees with our results on the enhanced transmigration index in neutrophils due to the stress of birth compared to adults.

To investigate how the stress of birth affects eosinophils we analyzed gene expression of cord blood eosinophils isolated after normal delivery and compared them to cord eosinophils isolated after C-section. We identified genes in pro-inflammatory pathways

that have higher expression in eosinophils after normal delivery compared to C-section. After normal delivery eosinophils showed a more pronounced expression of STAT1 gene via IFN-γ receptor pathway.
As it has been shown previously, IFN-γ and its receptors are playing an important role in protection against viral and bacterial infections; lack of these factors cause susceptibility to pathogens.
Also IFN-γ is known to affect adhesion and transmigration of polymorphonuclear leukocytes via receptor for advanced glycation endoproducts and CD11b/CD18 (Zen, Chen et al. 2007). This is in line with our previous results that showed enhanced IL-8 induced migration and CD11b expression in neutrophils after normal delivery.
IFN-γ primes the signaling pathways of Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) (Greenlund, Farrar et al. 1994) and tyrosine phophorylation of STAT1 directly regulates tyrosine phosphorylation of IFN-γ. The increase of IFN-γR and subsequently the activation of STAT1 signaling pathway after normal delivery can prolong eosinophils' survival and also cytokine production in these cells.
Although these results are not fully investigated in a physiological context, but we hypothesize that eosinophils affected by stress of birth show a more enhanced proinflammatory characteristics that result in a higher transmigration rate of leukocytes and increased cytokine production.

6 FUTURE PERSPECTIVES

All the above evidence indicates a more activated immune reaction in eosinophils and neutrophils after normal delivery. However, these conclusions are based on results at the moment of birth and it's not yet known that how these results will be altered in later stages of neonatal period and later during childhood and adulthood.
Specifically eosinophils seem to go through significant alterations in adulthood compared to infancy. Considering the presence of this leukocyte subtypes in atopic reaction our future studies will focus on eosinophils and the changes that they go through after neonatal period.
Our areas of interest in this context are:

Levels of LL-37 in eosinophils changes with age: As mentioned in paper III, LL-37 levels in eosinophils decreases in adulthood compared to neonatal period. It will be of importance to further investigate this result and find out when and how this decrease starts in eosinophils' LL-37 level. We will collect samples from infants of 6 months old, from children at 4-5 years old and later from different age groups of adults.

Alterations of eosinophils' gene expression and the factors that induce that after birth: As well as measuring LL-37 levels the same samples can be used to study epigenetic changes of eosinophils and neutrophils after different time points from the moment of birth.
In particular it is of interest to study the expression of atopy related genes in eosinophils after each delivery mode and follow the infants as explained above to find out about the epigenetic changes that occur in those genes in normal delivery and C-section.

7 MAJOR CONCLUSION

Delivery by C-section for humanitarian reasons continues to increase, nationally and internationally, although both maternal and infant mortality and morbidity are enhanced compared to normal delivery. Atopic disease in children is also reported to be increased after C-section delivery. Our findings indicate that both mothers and newborns may benefit from the stress connected to normal delivery, also from an immediate, immunological point of view. Our main findings are that the stress of normal labor induces profound structural and functional changes in fetal leukocytes, besides a simple increase in total cell number, which may give the newborn certain advantages in the protection against infection, particularly at the time of commensal colonization, but also by priming the immune system towards a Th1 orientation.

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