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ANTISECRETORY FACTOR IN THE PERINATAL PERIOD

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Antisecretory factor in the perinatal period

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my Family, Science and Life

POPULAR SCIENCE SUMMARY OF THE THESIS

BACKGROUND

Preterm birth is when a baby is born too early, before 37 weeks of pregnancy have been completed. In the year 2020, preterm birth affected 6 of every 1000 infants born in Sweden. Globally, preterm birth is a major cause of disease and death in the early period after birth. Infants born preterm may be affected by complications in different organs of the body, as in the gut, lungs, and eyes which may cause both short- and long-term consequences leading to impaired infants' health, increased parental stress, and high costs for the society. The risk of complications increases with shorter length of pregnancy. The cause of preterm birth is not yet fully understood and effective prevention and treatments for both preterm birth and infant complications after birth are lacking. Human milk contains numerous components which are beneficial for infant health. Mothers own milk is the first choice of nutrition for all infants, and especially for preterm infants. However, maternal milk production may be affected by different factors, as inflammation or infection in the breast, and different factors related to giving birth too early. For preterm infants, pasteurized donor human milk, milk donated from other women to a milk bank, is given during hospital stay if the mothers own milk is not available or reaching the infant's needs. Furthermore, pasteurization in order to inactivate potential bacteria and viruses in human milk may also reduce or abolish beneficial components.

Inflammation has been suggested to be involved in the cause of preterm birth, maternal breast complications and complications in preterm infants. Inflammation is a part of the immune system protecting against infections, but too much or for too long, inflammation may cause tissue damage. The protein, antisecretory factor has been suggested to be involved in regulation of inflammation and may play a role in complications where inflammation is involved. Antisecretory factor has not previously been studied related to preterm birth and inflammation in humans.

AIMS

The overall aim of the thesis was to describe antisecretory factor in the period around birth and the first months after birth, related to preterm birth and inflammation.

METHODS AND RESULTS

Analyses were performed in placenta after term and preterm birth, in maternal plasma, in mothers' own milk and in donor human milk before and after pasteurization. Methods used to determine levels of antisecretory factor were immunohistochemistry, a method using antibodies to detect different proteins in different organs in the body, and enzyme-linked immunosorbent assay (ELISA), a method using antibodies to detect proteins in blood and other body fluids.

The thesis consists of four studies. The first study investigated antisecretory factor in placenta. The results showed lower levels of antisecretory factor and a higher degree of inflammation in placenta after preterm birth compared to after term birth. The second study investigated antisecretory factor in maternal plasma and breastmilk. The results showed that higher levels of antisecretory factor in maternal plasma was associated with higher levels in breastmilk. The third study investigated levels of antisecretory factor in mothers' own milk over time after term and preterm birth, and levels of antisecretory factor in donor human milk before and after pasteurization. The results showed higher levels of antisecretory factor in the early period of lactation (colostrum) compared to the later period (mature milk) after both term and preterm birth. Furthermore, antisecretory factor was preserved in donor human milk after pasteurization. The fourth study investigated levels of antisecretory factor in mothers' own milk after preterm birth related to infant outcome and inflammation. The results showed that higher levels of antisecretory factor in mother's own milk was associated with less inflammatory complications in preterm infants.

CONCLUSIONS

In conclusion, the findings in the thesis suggest that antisecretory factor may be involved in the regulation of inflammation during pregnancy and during the first months after birth. Since these findings are demonstrated for the first time and the studies were small, larger studies are needed to confirm these results. The findings of higher levels of antisecretory factor in mothers' own milk and less inflammatory complications in preterm infants support the hypothesis that antisecretory factor in human milk is a part of the protective components in human milk. The findings of preserved antisecretory factor in donor human milk after pasteurization may be a part of the explanation of the protective effect of donor human milk even though many other beneficial components might be destroyed and support the use of donor human milk in the neonatal intensive care unit when mothers own milk is not available or not reaching the infant's needs. Future research may involve both studies on the biological and immunological mechanism of antisecretory factor related to other important factors, as well as intervention studies with antisecretory factor as prevention or treatment.

POPULÄRVETENSKAPLIG SAMMANFATTNING

BAKGRUND

Att vara för tidigt född, prematur, är att födas före 37 hela veckors graviditet. År 2020 föddes 6 av 1000 barn för tidigt i Sverige. I ett globalt perspektiv är prematur förlossning en viktig orsak till sjukdom och död i den tidiga perioden efter födelsen. Barn som föds för tidigt kan påverkas av komplikationer i olika organ i kroppen, som i tarmen, lungorna och ögonen, vilket kan orsaka både kort- och långsiktiga konsekvenser och som kan leda till sämre hälsa hos barnet, ökad föräldrastress samt höga kostnader för samhället. Risken för komplikationer ökar med kortare graviditet. Orsaken till prematur förlossning är ännu inte helt klarlagd och effektiva förebyggande åtgärder och behandlingar för både prematur förlossning och komplikationer hos för tidigt födda barn efter födelsen saknas. Bröstmjölk innehåller många komponenter som har positiva effekter för barns hälsa. Mammans egen mjölk är det första valet av näring för alla spädbarn, och särskilt till för tidigt födda barn. Mammans produktion av bröstmjölk kan dock påverkas av olika faktorer, som inflammation eller infektion i bröstet, samt olika faktorer relaterade till att föda för tidigt. För barn som föds för tidigt ges under sjukhusvistelsen pastöriserad donatorbröstmjölk, mjölk som donerats från andra kvinnor till en mjölkbank, om mammans egen mjölk inte är tillgänglig eller når barnets behov. Vidare kan pastörisering för att inaktivera potentiella bakterier och virus i bröstmjölk också minska eller ta bort många fördelaktiga komponenter.

Inflammation har beskrivits vara en del i orsaken till prematur förlossning, bröstkomplikationer hos mamman samt komplikationer hos för tidigt födda barn. Inflammation är en del av immunsystemet som skyddar mot infektioner, men för mycket eller för länge kan inflammation orsaka vävnadsskada. Proteinet antisekretorisk faktor har beskrivits vara involverad i reglering av inflammation och kan spela en roll vid komplikationer där inflammation är involverad. Antisekretorisk faktor har inte tidigare studerats hos människor relaterat till prematur förlossning och inflammation.

SYFTE

Det övergripande syftet var att beskriva antisekretorisk faktor under perioden vid födelsen och de första månaderna efter födelsen relaterad till prematur förlossning och inflammation.

METOD OCH RESULTAT

Analyser har utförts av moderkaka efter prematur förlossning och efter förlossning i fullgången tid, i plasma och bröstmjölk från mammor samt i donerad bröstmjölk före och efter pastörisering. Metoder som använts för att bestämma nivåer av antisekretorisk faktor var immunhistokemi, en metod som använder antikroppar för att detektera olika proteiner i olika organ i kroppen, samt enzymkopplad immunabsoberande analys (ELISA), en metod som använder antikroppar för att detektera olika proteiner i blod och andra kroppsvätskor.

Avhandlingen består av fyra delstudier. Den första delstudien undersökte nivåer av antisekretorisk faktor och inflammatoriska markörer i moderkakan efter prematur förlossning eller förlossning i fullgången tid. Resultaten visade lägre nivåer av antisekretorisk faktor och högre grad av inflammation i moderkakan efter för tidig förlossning jämfört med förlossning i fullgången tid. Den andra studien undersökte nivåer av antisekretorisk faktor i mammans blod och bröstmjölk. Resultaten visade ett samband mellan högre nivåer av antisekretorisk faktor i mammans blod och högre nivåer i bröstmjölk. Den tredje studien undersökte nivåer av antisekretorisk faktor i mammans egen bröstmjölk över tid, samt nivåer av antisekretorisk faktor i donerad bröstmjölk före och efter pastörisering. Resultaten visade att nivåerna av antisekretorisk faktor var högre under den tidiga laktationsperioden (råmjölk) jämfört med den senare perioden (mogen mjölk) efter både prematur förlossning och förlossning i fullgången tid. Vidare bevarades antisekretorisk faktor i donerad bröstmjölk efter pastörisering. Den fjärde studien undersökte nivåer av antisekretorisk faktor i mammans egen bröstmjölk relaterat till inflammatoriska komplikationer hos barnet efter prematur förlossning. Resultaten visade att högre nivåer av antisekretorisk faktor i mammans egen mjölk var associerade med färre inflammatoriska komplikationer hos för tidigt födda barn.

SLUTSATS

Sammanfattningsvis tyder resultaten i avhandlingen på att antisekretorisk faktor kan vara en del i regleringen av inflammation under graviditeten och under de första månaderna efter födelsen. Eftersom dessa fynd demonstrerats för första gången och studierna var små, behövs större studier för att bekräfta resultaten. Resultaten av högre nivåer av antisekretorisk faktor i mammans egen mjölk och färre inflammatoriska komplikationer hos för tidigt födda barn stöder hypotesen att antisekretorisk faktor i bröstmjölk är en del av de skyddande komponenterna i bröstmjölk. Resultatet att antisekretorisk faktor är bevarad i donatorbröstmjölk efter pastörisering kan vara en del av förklaringen av donatorbröstmjölks skyddande effekt, även om många andra fördelaktiga komponenter kan bli förstörda, och stödjer användningen av donatorbröstmjölk på neonatalavdelning när mammas egen mjölk inte är tillgänglig eller inte når barnets behov. Framtida forskning kan omfatta både studier av hur biologiska och immunologiska mekanismer av antisekretorisk faktor är relaterade till andra faktorer av betydelse, samt interventionsstudier med antisekretorisk faktor som prevention eller behandling.

ABSTRACT

Preterm birth is the major cause of neonatal morbidity and mortality. Human milk, especially mothers' own milk, has many health benefits for infants, especially infants born preterm. The causes of preterm birth are not yet fully understood. Inflammation is suggested to be involved in the pathogenesis of preterm birth, in inflammatory complications in preterm infants as well as in complications during lactation. Antisecretory factor (AF) is a protein involved in regulation of secretory and inflammatory processes and has not previously been studied related to the perinatal period.

The aim of this thesis was to describe AF in the perinatal period related to preterm birth, human milk, inflammation, and infant outcome.

The included studies are sub-studies in three longitudinal cohort studies, with an exploratory approach related to antisecretory factor.

Methods used to determine levels of antisecretory factor were immunohistochemistry in paper I, enzyme-linked immunosorbent assay (ELISA) in paper II, and Sandwich ELISA in paper III and IV.

The results demonstrated lower levels of AF and a higher degree of inflammation in placenta after preterm birth (paper I), an association between higher levels of AF in maternal plasma and higher levels in mothers' own milk (paper II), higher levels of AF in colostrum versus mature milk in mothers own milk after term and preterm birth (paper III), preserved AF after Holder pasteurization (paper III), and that higher levels of AF in mothers own milk was associated with less adverse outcome and inflammation in preterm infants (paper IV).

In conclusion, this thesis is the most comprehensive description of antisecretory factor in the perinatal period to date. The results demonstrate a basic pattern of AF showing that AF levels in maternal plasma are reflected in maternal breastmilk, and that AF levels in breastmilk decreases with time after birth. Furthermore, Holder pasteurization of donor milk can be safely performed without concern that it may destroy AF. Additionally, the findings indicate that after preterm birth, lower levels of AF in both placenta and breastmilk are associated with more inflammation, and that higher levels of AF in mother's own breastmilk may be protective for the infant and reduce the risk for inflammatory complications, such as sepsis, in the neonatal period.

The significance of these novel findings implicates that AF may be involved in the complex pathophysiology related to inflammatory complications in the perinatal period and can serve as a base for further studies on mechanisms and interventions. Since effective prevention and treatments are lacking, AF may present as a possible modifiable factor to improve health in the perinatal period.

LIST OF SCIENTIFIC PAPERS

I. **Low levels of anti-secretory factor in placenta are associated with preterm birth and inflammation**
Gustafsson AM, Fransson E, Dubicke A, Hjelmstedt AK, Ekman-Ordeberg G, Silfverdal S-A, Lange S, Jennische E, Bohlin K.
Acta Obstet Gynecol Scand 2018; doi.org/10.1111/aogs.13282

II. **The Antisecretory Factor in Plasma and Breast Milk in Breastfeeding Mothers-A Prospective Cohort Study in Sweden**
Gustafsson A, Granström E, Stecksén-Blicks C, West CE, Silfverdal SA.
Nutrients. 2018 Sep 4;10(9):1227. doi: 10.3390/nu10091227.

III. **Changes in Antisecretory Factor in Human Milk During the Postpartum and Length of Gestation**
Gustafsson A, Johansson E, Henckel E, Lange S, Bohlin K.
J Hum Lact. 2021 Jun 1:8903344211021306 doi: 10.1177/08903344211021306

IV. **Antisecretory factor in mothers own milk following preterm birth – association to neonatal inflammation and outcome**
Gustafsson A, Johansson E, Henckel E, Olin A, Rodriguez L, Brodin P, Lange S, Bohlin K
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

AF	Antisecretory factor
AA	Arachidonic acid
BMI	Body mass index
BPD	Bronchopulmonary dysplasia
C3	Complement factor 3
DHA	Docosahexaenoic acid
DHM	Donor human milk
ELBW	Extremely low birth weight
ELISA	Enzyme-linked immunosorbent assay
EOS	Early onset sepsis
EPT	Extremely preterm
FIRS	Fetal inflammatory response syndrome
FSMP	Food for special medical purposes
GBM	Glioblastoma
GBS	Group B Streptococcus
HLA	Human leukocyte antigen
HMO	Human milk oligosaccharide
HSE	Herpes simplex encephalitis
IBD	Inflammatory bowel disease
ICD-10	International statistical classification of diseases and health related problems
ICP	Intracranial pressure
IFP	Interstitial fluid pressure
IGF-1	Insulin-like growth factor 1
IL-1	Interleukin -1
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-12	Interleukin-12
IVH	Intraventricular hemorrhage

LOS	Late onset sepsis
LPT	Late preterm
mAb	Monoclonal antibody
MOM	Mothers own milk
MPT	Moderate preterm
NEC	Necrotizing enterocolitis
NICU	Neonatal intensive care unit
NO	Nitric oxide
NPX	Normalized Protein eXpression
PDA	Patent ductus arteriosus
PPROM	Preterm premature rupture of membranes
Psmd4	Proteasome 26S subunit nonATPase 4
PTB	Preterm birth
RCT	Randomized control trial
ROP	Retinopathy of prematurity
TB	Term birth
TBI	Traumatic brain injury
TGF-β	Transforming growth factor beta
TNF	Tumor necrosis factor
TRPV1	Transient potential vanilla receptor 1
VEGFA	Vascular-endothelial growth factor A
VLBW	Very low birth weight
VPT	Very preterm

1 INTRODUCTION

My interest in antisecretory factor began after listening to a fantastic and inspiring lecture on the immunobiology of human milk by Professor, Pediatrician, and Clinical Immunologist Lars Åke Hanson. I was fascinated of the immunobiology of human milk in general and of the antisectretory factor in particular.

Professor Hanson presented antisecretory factor, a protein involved in the regulation of inflammation and secretion, and the results from intervention studies on the treatment of children with diarrhea in Pakistan and the prevention of mastitis in breastfeeding mothers in Sweden.

As a midwife specialized in lactation, who had recently changed direction from working with mainly healthy mothers and infants in postnatal care to working with preterm or sick infants in neonatal care, the question was raised whether this could be of importance in this group as well. Inflammation has been described to contribute to several of the complications that are present during the period related to childbirth, and especially related to preterm birth.

After contact with Lars Åke Hanson, it turned out that he thought the question was of interest to study and introduced me to the research group working on the antisecretory factor in Gothenburg.

The antisecretory factor was first identified by Professor Stefan Lange and Ass. Professor Ivar Lönnroth in 1984. Further research on the mechanisms and biological effects have been performed by their research group since then.

Having had the opportunity to collaborate with research groups in different medical research areas as neonatology, pediatrics, obstetrics, immunology, and microbiology, as well as having been supervised by fantastic and supporting experts in these fields has been invaluable for completing this dissertation.

2 LITERATURE REVIEW

2.1 ANTISECRETORY FACTOR

Antisecretory factor (AF) is a 41kDa endogenous protein regulating secretory processes and inflammation(1, 2) that might be of importance in the perinatal period. AF have potent anti-inflammatory effects and regulates the transport of water and ions across cell membranes. It probably exerts its effects via nerves, but other mechanisms as receptors, binding proteins and transport channels in the cellular membrane may be involved(1, 3-7). AF is also known as Proteasome 26S subunit, nonATPase 4 (Psmd4) as well as AF-1, Rpn10 and S5a(8) (Human Protein Atlas available from http://www.proteinatlas.org), and is present mainly in the nucleosol and cytosol within eukaryotic cells. The multiprotein component proteasome 26S is involved in ATP-dependent degradation of ubiquitinated proteins with an important role in maintaining protein homeostasis by removing damaged or misfolded proteins which may affect cell functions. Psmd4 acts as a ubiquitin receptor subunit through ubiquitin-interacting motifs and selects ubiquitin-conjugates for destruction and has been described to be involved in 151 pathways(9) .AF is a subunit of the regulatory19S subunit of the 26S proteasome(1, 10) (Figure 1).

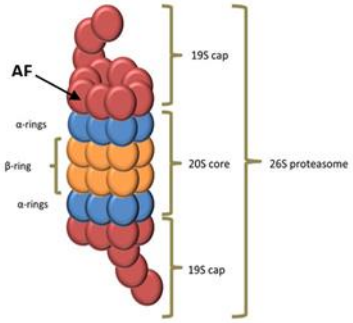


Figure 1. Composition of the proteasome. A schematic illustration of the 26S proteasome, where subunit AF on the regulatory 19S subunit is marked with an arrow. The figure is modified from Lub et al 2016(11). © 2016 Lub et al. Licence by Creative Commons Attribution (CC BY 3.0). https://creativecommons.org/licenses/by/3.0/

AF is present in most human tissues and body fluids, including the placenta(1, 12, 13) plasma(13) and breast milk(14, 15) with a suggested role in the immune system due to expression on macrophages, B-cells and dendritic cells, and in all secondary lymphoid organs(16). A high expression of AF is restricted to specific cell populations such as certain types of epithelia, neuron, endocrine cells and subgroups of leukocytes(1). Cells that store AF also have the capacity to synthesize AF(1). Normally present in an inactive form, AF becomes activated as part of the innate immune response, for example following exposure to bacterial toxins(1). Active AF converts complement factor 3 (C3) into its inactive form and thereby controlling inflammation(17). An early increased formation of complexes in cerebrospinal fluids in patients with herpes simplex encephalitis (HSE) suggest that the complex may be involved in host defense against HSE(18).

Since it is the activated AF which is of interest for the antisecretory and anti-inflammatory effects, method development has been needed due to the lack of ready to use commercial analysis kits. The method to determine activated AF has been developed in-house from an *in vivo* rat-loop model(19), but due to ethical, economical and temporal factors an *in vitro* model was developed. Monoclonal antibodies to detect the active site of AF was developed(13) for an in-house enzyme-linked immunosorbent assay (ELISA), which later was optimized to a sandwich ELISA(20). It has been shown that also complement factor C3c is increased in plasma after AF-induction(21). A complex is formed between the AF-including proteasome and complement factors, which is named "compleasome"(17). The sandwich ELISA disclose the binding of proteasomes to complement factor C3. During the formation of complex, the C3 is split into its inactive form C3c and also the hidden antisecretory peptide is exposed, which might explain the antisecretory and anti-inflammatory effect of AF complex formation(17).

2.1.1 Animal studies

In animals, the levels of active AF in breast milk is positively correlated to the levels in plasma with higher concentration in milk than in plasma, probably due to an active transport of active AF across the epithelial lining of the mammary gland(1). The AF activity in plasma of piglets has a cyclic variation, declines at weaning with the lowest levels on the third day after weaning(1) and there is a correlation with low levels of AF activity and the onset of diarrheal disease(22). AF has been demonstrated to be stress sensitive in rats as well as in chickens, with a rapid decrease of AF in plasma and in the pituitary gland(1, 23). Studies in animals have also demonstrated that levels of active AF in plasma and milk can be enhanced through an AF inducing diet with a protective effect in the offspring related to growth and health(24).

2.1.2 AF therapy

Active endogenous AF in plasma increases by exposure to enterotoxins and certain food constituents. An enhanced activation of endogenous AF synthesis improves the clinical outcome in diseases characterized by inflammation and secretory dysfunction in both humans and animals(1, 25-27). In farm animals, AF-inducing diets are used as an alternative to antibiotic growth promoters. When antibiotics in feeds were banned in Sweden in January 1986, it led to increasing health problems, mainly with diarrheal and inflammatory diseases. The development of feeds that activate endogenous AF synthesis successfully controlled these problems and have resulted in increased birth weight and growth of the offspring(24, 28). A more general use of this concept may generate an ecologic benefit combined with a reduced risk for the development of bacterial resistance to antibiotics. In Sweden, there are two products, SPC-flakes® and Salovum®, classified as food for special medical purposes (FSMP) by the EU (Commission Directive 1999/21/EC).

2.1.2.1 AF inducing diet

AF is mostly present in inactive form in healthy persons but can be stimulated to increased synthesis in both plasma and breast milk by oral intake of specially processed cereals, SPC-Flakes®(1, 13, 14).

An AF-inducing diet can decrease symptoms in diseases characterized by inflammation and secretory dysfunction, i.e., inflammatory bowel diseases (IBD), Meniere`s disease(29-32). An intervention study in breastfeeding mothers showed that induction of endogenous AF synthesis by means of specially processed cereals prevented mastitis(14).

SPC-flakes® are produced of oats subjected to malting, a hydrothermal process to achieve a specific content of amino acids and sugars. Extensive malting of wheat has been demonstrated to induce protection against intestinal secretion and diarrhea, with a 50% reduction caused by the substances guaiacol, ferulic acid and vanillic acid in malt(33). These substances also induced AF compleasome in blood, and induction of AF may involve the vanilloid receptor 1 (TRPV1) in the gut(33). Extensively malted cereals have been demonstrated to counteract diarrheal disease and IBD, and AF has been shown to be involved in this effect by induction of active AF in blood(34). In an intervention study investigating the effect of malting on cereal composition, the phenols cathecin, ferulic acid and sinapic acid were increased. Investigated in rats in the same study, intake of the malted cereals, as well as intake of the identified phenols gave an anti-inflammatory effect and also showed an induction of AF in rat blood, determined using sandwich ELISA. The results suggest a mechanism for the anti-inflammatory and antisecretory effect of malted cereals(34).

2.1.2.2 Preformed AF

Preformed active AF can be given directly through oral intake of an egg-yolk solution (Salovum®) with a verified high AF content and has been shown to significantly improve the condition of children with diarrhea(25, 26).

Eggs, mainly the egg yolk, are rich in AF possibly to provide protection to the chicken against gastrointestinal diseases until its own capacity to produce active AF is activated(28). Salovum® is produced as a spray dried egg yolk powder from hens being fed SPC-flakes(1, 28, 35). In a pilot study, nasogastric or rectal administration of Salovum® given to four patients with severe traumatic brain injury (TBI) demonstrated reduced intracranial pressure (ICP) after rectal administration, followed by increased AF levels in blood(36). In another pilot study, nasogastric administration of Salovum® given to five patients with TBI demonstrated that three of five patients had reduced ICP, five of five had favorable short-term outcome, four of five had favorable long-term outcome, and there were no toxicity observed(37). Furthermore, AF therapy with oral administration of Salovum® for patients with idiopathic normal pressure hydrocephalus and idiopathic intracranial hypertension did not have an effect on intracranial pressure (ICP), suggesting that brain swelling does not play a crucial role in these conditions(38).

2.1.2.3 AF-16

Antisecretory factor (AF)-16, includes the active site of the protein, and is located in the amino terminal part of the protein, derived from amino acids 36-51(39), however the active site of the human AF is suggested to reside in the short sequence between the amino acids 35-42(39). The AF-16 peptide has been used in experimental studies because of its stability and potency(40) (Figure 2).

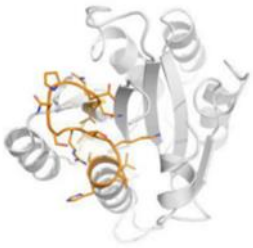


Figure 2. Computer produced model describing the structure of AF (amino acid 15-151). The biologically active peptide, AF-16 marked in yellow, Figure courtesy of Ass. Prof. Ewa Johansson and Prof. Stefan Lange, University of Gothenburg, Sweden.

In solid tumors, as the primary brain tumors glioblastoma (GBM), interstitial fluid pressure (IFP) presents a barrier to drug uptake. Induction of endogenous AF, or exogenous administration of the AF-peptide, reduced IFP and increased drug uptake in GBM xenografts. AF inhibited cell volume regulation of GBM cells *in vitro* and may represent a novel strategy to increase drug uptake and improve outcome in GBM(7). Furthermore, AF-16 has been demonstrated to improve injury related deficits in water and ion transport and decrease intracranial pressure after experimental cold lesion injury, encephalitis and traumatic brain injury in rats(41). Intranasal administration of AF-16 attenuated brain oedema and enhanced visuospatial learning and memory following traumatic brain injury in rats(41), and an early post injury treatment may offer a novel therapy for neuroprotection.

AF has not previously been described related to the perinatal period in humans. My aim was to explore if it may be part of the mechanism of inflammatory processes around birth. The results may give a base to determine if AF may be a factor of interest to include in further studies and related to other factors involved in the mechanisms of preterm birth, inflammatory complications in preterm infants as well as mechanisms of protection via human milk.

2.2 INFLAMMATION IN THE PERINATAL PERIOD

2.2.1 The perinatal period

The definition of the perinatal period can vary between countries. In the definition used in the International Statistical Classification of Diseases and Health related Health Problems, ICD-10, the perinatal period starts at 22 completed weeks and ends at the seventh day after birth(42). Furthermore, other terms like the postpartum, postnatal- and neonatal period and

infancy are used to describe different length of periods after birth related to the infant and/or the mother. In this thesis I have chosen a wider definition of the perinatal period and include the period from 22 completed weeks of gestation to three months after birth.

2.2.2 The immune system- an overview

The immune system is complex, involving several mechanisms for sensing pathogens, cellular stress as well as tissue homeostasis and repair. Basically, the immune system can be divided into three levels of defense: physical and anatomical barriers, innate immunity and adaptive immunity(43), the two latter divided related to speed and specificity of the reaction(44).

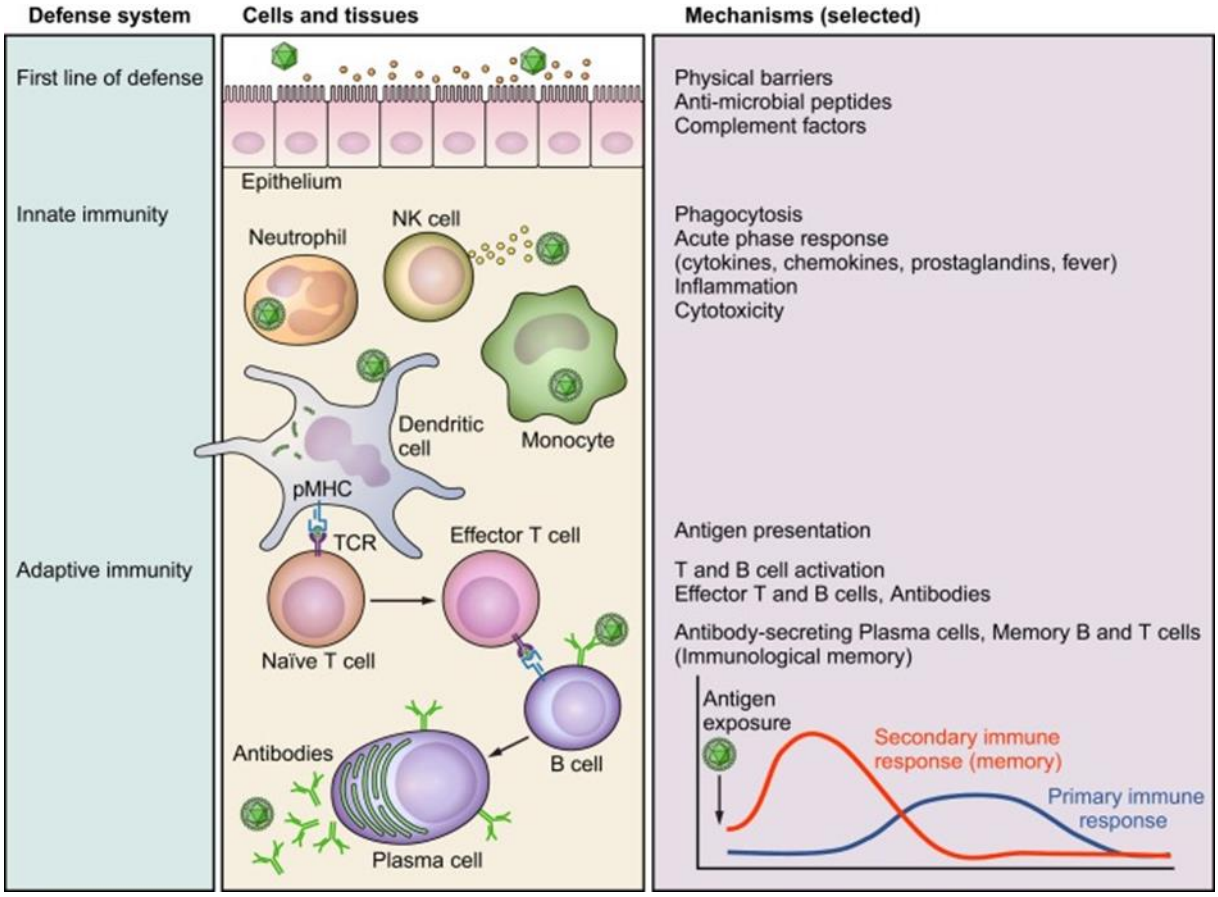


Figure 3. Major components of the immune system, a prototypical immune response to an infectious challenge, and immunological memory. The immune system is viewed as consisting of 3 levels: (1) first line of defense, consisting of anatomic and physiologic barriers; (2) innate immunity; and (3) adaptive immunity. © 2019 the American Physiological Society. Creative Commons Attribution CC-BY 4.0: © the American Physiological Society(45).

2.2.3 Inflammation- basic principles

Inflammation plays an essential role in the control of pathogens and is rapidly initiated by the innate immune system if pathogens breach the barriers of skin and mucosal surfaces. The innate immune system eliminates microbes by inducing an acute inflammatory response.

Inflammation is a tissue reaction that delivers mediators of host-defense (circulating cells and proteins) to sites subjected to tissue damage(46) or tissue infection. The process includes recruitment of cells and leakage of plasma proteins through blood vessels and activation of these in extravascular tissue(47). A local tissue accumulation of phagocytes, mainly neutrophils, in response to cytokines often follow. The cells bind to endothelial adhesion molecules which are induced by cytokines, as tumor necrosis factor (TNF) and interleukin-1 (IL-1), and migrate in response to chemo attractants (chemokines, complement fragments and bacterial peptides). Leukocytes are activated and destroy damaged cells or microbes. Cytokines are small proteins which are produced and secreted by many different cell types mediating communication between cells, immune response, and inflammation. Pro-inflammatory cytokines stimulate systemic inflammation, for example IL-1, interleukin-6 (IL-6) and TNF-α. Pro-inflammatory chemokines, as interleukin-8 (IL-8), are cytokines that induce chemotaxis and attract immune cells to inflammation sites(48).

Although inflammation has a protective function in controlling infections and promoting tissue repair, it can also cause tissue damage and disease(49). The regulation of inflammation is designed to prevent excessive tissue damage. Anti-inflammatory cytokines are molecules involved in regulation of inflammation and prevention of harmful effects of excessive or sustained inflammation. Examples of anti-inflammatory cytokines are IL-1 receptor antagonist, Interleukin-4 (IL-4), transforming growth factor beta (TGF-β) and interleukin 10 (IL-10)(48). The regulatory mechanisms include production of IL-10, which inhibits pro-inflammatory macrophage functions. The production of interleukin-1 receptor antagonist blocks the actions of IL-1. There is also other feedback mechanism where signals that induce pro inflammatory cytokines, also induce inhibitory cytokine expression(50, 51). Furthermore, a dysregulation between pro-inflammatory and anti-inflammatory cytokines has been described in patients with sepsis(48).

2.2.4 Immune tolerance in pregnancy

Immune tolerance is important for a successful pregnancy(52). During pregnancy, the immune system deviates from a Th1 to a Th2 phenotype with a stronger production of antibodies and less cellular immunity to protect against rejection(53). Allograft rejection initiated by cells and/or antibodies during pregnancy can present as chorioamnionitis or villitis of unknown etiology. Complement activation has been suggested to underlie antibody-mediated allograft rejection and human leukocyte antigen (HLA) resistance during the mid-trimester, which might elevate the risk factors for spontaneous PTB(54). As fetal tissues are semi allogenic, the fetus and the placenta may be at risk for complement- mediated immune attack with a potential risk for adverse pregnancy outcomes. In animal models, result shows that complement inhibition is essential for a successful pregnancy and that an uncontrolled complement activation may be a crucial effector in the pathogenesis of recurrent miscarriages, intrauterine growth restriction, preeclampsia, and preterm birth(55, 56). An association of inflammatory markers in amniotic fluid and chronic placental inflammatory

lesions has been demonstrated in women after unexplained fetal death, which may be due to a breakdown of maternal-fetal tolerance(57).

2.2.5 The placenta

The placenta is the fetal organ providing the interchange between the mother and the fetus and needs to provide its function even during its development(58).

The chorionic plate represents the fetal surface of the placenta and is covered by the amnion. The amnion is composed of a single layered epithelium and the amniotic mesenchyme, which is an avascular connective tissue weakly attached to the chorionic mesenchyme. The umbilical cord inserts into the chorionic plate, and the vessels in the chorionic mesenchyme is the continuous of the vessels of the umbilical cord. The two umbilical arteries branch in a centrifugal pattern into their final branches that supplies the villous trees and the chorionic vein give rise to a single umbilical vein(59).

The basal plate represents the maternal surface of the placenta and is a surface that is emerged from the separation of the placenta from the uterine wall during delivery. The basal plate includes fetal extravillous trophoblasts and maternal cells of the uterine decidua, as decidual stroma cells, NK cells, macrophages and other immune cells, and contains also a large amount of extracellular matrix, fibrinoid and blood clots. The basal plate can be divided into different lobes due to a system of grooves and clefts, and the lobes on the maternal surface correspond with the villous trees from the chorionic plate into the intervillous space. At the margin of the chorionic and basal plates merge and form the fetal membranes, which is composed of three layers; the amnion, the chorion, and the decidua capsularis(59, 60).

2.2.6 Inflammatory markers in placenta

Inadequate maternal and/or fetal vascularization and inflammation of placental tissue are pathophysiological aspects of extremely preterm deliveries(61). Studies have demonstrated elevated numbers of CD68-positive placental cells following miscarriage(62) and recurrent loss of pregnancy(63). Moreover, the incidence of acute chorioamnionitis falls gradually as the length of gestation increases(64). Both increased and decreased numbers of CD68-positive cells in placentas exhibiting chorioamnionitis have been reported(65, 66). Together, these results suggest that CD68-positive cells play a role in regulating placental infections and inflammation.

Preterm placentas display high levels of CD163(65) and a more pronounced expression of CD163 were also demonstrated in connection with higher-grade chorioamnionitis. During early pregnancy, decidual macrophages involved in immunomodulation and tissue remodeling express CD163(67, 68). However, CD163 has also been associated with production of the pro-inflammatory cytokine interleukin-12 (IL-12), suggesting that there is a plasticity and interaction between these molecules during complications of pregnancy(69). High levels of CD163 have been associated with inflammation and preterm birth(70). A change in the characteristics of macrophages from anti-inflammatory to pro-inflammatory has

been suggested to elevate the risk of preterm labor(71). *In vitro*, glucocorticosteroid up-regulates the expression of CD163 expression(72), which may be of importance related to glucocorticoids given antepartum for lung maturation in threatened preterm birth(73).

2.2.6.1 Chorioamnionitis, acute and chronic inflammation

The prevalence of chorioamnionitis is associated with gestational age at birth, and present in 94% of placentas after 21-24 weeks of gestation compared with in 3-5% of term placentas(74). Chorioamnionitis can be of acute or chronic origin. Acute inflammatory lesions consist of diffuse infiltrations of neutrophils in different parts of the placenta, and include acute chorioamnionitis, funisitis and chorionic vasculitis. Acute chorioamnionitis is associated with a maternal host response, while a fetal inflammatory response is associated with funisitis and chorionic vasculitis. The cause of acute chorioamnionitis has often been related to an intraamniotic infection, while new studies indicates that an intraamniotic inflammation can be induced by danger signals (chemokines) without the presence of microorganisms. Intraamniotic infection has been associated with chemokines (as interleukin-8) as a gradient that induce migration of neutrophils from the fetal or maternal circulation to the umbilical cord or the chorioamniotic membranes(74). Cellular stress or cell death can also release danger signals that induce the release of neutrophil chemokines(74). Chronic chorioamnionitis are associated with an infiltration of lymphocytes in the placenta. The cause may be an infection or be of an immune origin (maternal anti-fetal rejection)(54).

2.2.6.2 Fetal inflammatory response, FIRS

Fetal inflammatory response syndrome (FIRS) is characterized by an elevation of interleukin-6 in fetal plasma and is associated with funisitis, chorionic vasculitis and an onset of preterm labor and a higher rate of neonatal morbidity(74). FIRS has been associated with both fetal(75), and neonatal complications(76), enhanced perinatal morbidity and mortality(77) and been associated with an in increased risk for short- and long-term complications (inflammation in fetuses, neonatal sepsis, bronchopulmonary dysplasia, periventricular leukomalacia and cerebral palsy)(74). To detect occurrence of FIRS and signs of funisitis, a level of >11 pg/ml of IL-6 in cord blood has been suggested as a marker(74, 78).

2.3 PRETERM BIRTH

2.3.1 Definitions and risk factors

Globally, more than one of ten births are preterm and preterm birth (PTB) is the most common cause of neonatal death(79). The rate of PTB is increasing(80). Preterm birth is defined by the WHO as all birth before 37 completed weeks of gestation, and can be divided further related to gestational weeks; late preterm 34-37 weeks of gestation (LPT), moderately preterm 32-34 weeks of gestation (MPT), very preterm 28-32 weeks of gestation (VPT) and extremely preterm <28 weeks of gestation (EPT)(80). The etiology of PTB is multifactorial and preventive treatment often ineffective. In addition, PTB elevates the risk for morbidity during the perinatal period and can lead to lifelong disability with high societal costs(81).

PTB can be spontaneous or initiated for medical reasons(82). Risk factors for spontaneous PTB vary regarding to gestational length(82) and social and environmental factors. Maternal factors associated with increased risk are an advanced maternal age, shorter inter-pregnancy interval and low maternal body mass index (BMI)(79, 83). Approximately 70% of all preterm births is proceeded by preterm labor(79, 84). Furthermore, it is still unknown what triggers half of the cases spontaneous PTB and a better understanding of the underlying pathophysiology would be of considerable value(61, 80).

Inflammation may play a role in the pathological process of spontaneous preterm labor(85) and can be a result of the activation of innate immunity(86, 87), microorganisms(88) or by endogenous signals from cellular stress or necrosis(89). The adaptive immune system has also been demonstrated to be involved in the pathology of inflammation in preterm labor(90).

Preterm premature rupture of membranes (PPROM) occurs in one to two percent of pregnancies and is associated with elevated maternal and neonatal morbidity(91). Moreover, cervical ripening is an inflammatory reaction that is required for a vaginal PTB to occur(92). No signs of infection are normally associated with cervical ripening, but there is an influx of inflammatory cells including dendritic cells(93). The controlled inflammation associated with normal pregnancy may be more severe and/or uncontrolled in the case of PTB(71).

2.3.2 Immune system development in newborn infants

A new approach to study the immune system, systems immunology, focuses on the interplay between different components of the immune system measured at the same timepoint instead of focusing on individual components(94). The immune system is highly variable between individuals but quite stable over time within individuals. The variation between individuals is due to both heritable and non-heritable factors, with the interplay between symbiotic and pathogenic microbes and other non-heritable factors explaining most of the variation(95).

Newborn infants, especially infants born preterm are susceptible to infections(96). An important period for the development of the immune system seems to be the first 100 days of life(97, 98). Preterm and term infants differ at birth but converges to a similar trajectory during the first 3 month of life(98) and this process seems to be driven by interaction with microbes and may be negatively affected by dysbiosis.

Preterm birth is associated with a strong inflammatory response, and the difference related to term are probably related to multifactorial causes like maturation, perinatal conditions associated with PTB and responses to environmental exposure(98). PTB is associated with high expression of T-cells and IL-8 in cord blood, which is not explained by immaturity alone(98) and dramatic changes in protein profiles have been described during the first weeks in life(99).

Furthermore, a newborn infant have received, via the placenta, maternal IgG antibodies(100). In a recent study, preterm infants were demonstrated to receive a comparable repertoire of

maternal IgG antibodies as term infants, but in lower concentration and with a shorter half-life(101).

2.3.3 Inflammatory complications in preterm infants

Inflammation may contribute to complications of prematurity, such as bronchopulmonary dysplasia(102, 103), necrotizing enterocolitis(104) and retinopathy of prematurity(105). The etiology of these adverse outcome are multifactorial and inflammatory processes is a major contributor(106). The gut of the newborn infant is susceptible to inflammation and inflammatory conditions that may affect the growth of the infant and cause complications especially in infants born preterm(79, 80). Maternal infection and inflammation are risk factors for preterm birth and may lead to exposure to inflammatory stimuli for premature infants prior to birth(79, 88). Additional inflammatory stimuli often occur as a result by the care and treatment needed in the Intensive Care Unit, necessary for survival(102, 106). Procedures like resuscitation after birth, where endotracheal intubation and/or umbilical intravascular catheter or other invasive procedures are needed but represent risk factors for bacterial infections(107). Furthermore, in preterm infants, antibiotics given to prevent or treat infections may be associated with intestinal dysbiosis and an increased risk for complications of prematurity(108).

2.3.3.1 Sepsis

Neonatal sepsis is defined as a systemic condition associated with hemodynamic changes and other clinical manifestations leading to substantial morbidity and mortality. Globally, neonatal sepsis is the third leading cause of neonatal mortality and contributes to 13% of all neonatal mortality(109). When assessing the global burden of disease, more than half of worldwide sepsis in 2017 occurred in children under 5 years of age(110). Of those, many are neonates, approximately 2200 of 100000 with a mortality rate of 11-19%(111). The origin can be bacterial, viral, or fungal(107). There are a lack consensus how to determine neonatal sepsis, suspected or confirmed, and both isolation of a pathogen in blood as well as inflammatory cytokines has been described(107). Furthermore, the symptoms of neonatal sepsis may be subtle in early stages of disease, with only signs of hypothermia and enteral intolerance present (112).

Neonatal sepsis, defined as occurring during the first 28 days of life(113), can be classified as early onset sepsis (EOS, onset< 72 hours after birth) or late onset sepsis (LOS, onset >72 hours after birth). EOS is often associated with a mother-infant transmission during pregnancy or birth, while LOS is associated with the interaction to the hospital- or the community environment(107).The incidence of EOS , reported in a Cochrane review, is 0.9-3.5 per 1000 live births(114), and for LOS 3-6 per 1000 infants, more prevalent in preterm infants(109). In a study of VPT, infants with LOS have been described to have elevated levels of both pro- and anti-inflammatory cytokines with a shift to anti-inflammation suggested to be associated with a hypo-responsiveness(115). Different regimes of antibiotics are used for treatment of sepsis(114). However, even though there has been development of neonatal care

and an availability of antibiotics, sepsis remains a serious condition and may cause long term consequences(113). Other strategies for prevention have been studied using different supplementation of proteins, peptides or probiotics, components naturally found in maternal milk, and shown to be of interest(116).

2.3.3.2 Neonatal enterocolitis (NEC)

NEC is a gastrointestinal disease associated with high mortality in preterm infants. In a meta-analysis of 27 cohort studies, the global incidence of NEC was 7 % in preterm infants(117) with an estimated mortality of 20-30%(118). Risk factors are multifactorial and NEC has been associated with for example: low gestational age, low birth weight, chorioamnionitis, mechanical ventilation(119) as well as microbial dysbiosis, formula feeding and excessive inflammation(120). NEC is characterized by inflammation in the intestinal wall and may include different diseases leading up to severe intestinal injury, perforation, or necrosis(120). Increased inflammatory markers as IL-6, IL-8, procalcitonin and CRP have been demonstrated, as well as thrombocytopenia and neutropenia as a result of inflammatory processes(104). Treatments for NEC can be medical (bowel rest, antibiotics) or surgical(118). Human milk feeding is associated with lower risk for NEC compared to formula feeding(121, 122), where the presence of human milk oligosaccharides (HMOs) may be a part of the preventive effect(123). HMOs may act as prebiotics for a beneficial microbiota in the infant gut(124) and the composition of HMOs varies between individual mothers and may be influenced of preterm birth(125). Probiotics as prevention for NEC has been studied extensively, however with a variation of bacterial strains given, as well as age at initiation and time of duration of treatment(126). Summarized in a meta-analysis, probiotics was demonstrated to have a strong treatment effect to reduce the incidence of NEC(126). However, there are still questions to be addressed related to quality of preparations, safety, optimal dose, age and time for initiation and duration, and long-term outcomes(126).

2.3.3.3 Patent ductus arteriosus (PDA)

Delayed closure of the ductus arteriosus is a common cardiovascular disturbance in preterm infants, with a prevalence around 33% in VLBW infants and 65% in ELBW infants(127). Inflammation has been suggested to play a role in PDA and in a retrospective study of infants ≤30 weeks of gestation, an association between PDA and higher levels of CRP was demonstrated(128). Prostaglandins play a role in PDA and cyclo-oxygenase inhibitors are used as treatment but may have adverse effects(129). Management of PDA can be conservative including for example fluid restriction, diuretics, and minimal oxygen supplementation. Pharmaceutical management with ibuprofen or paracetamol is also used as well as surgical ligation and transcatheter closure (127). PDA has been shown to be related to several other complications of prematurity, as sepsis, NEC, bronchopulmonary dysplasia (BPD), and intraventricular hemorrhage (IVH)(130). Enteral feeding is a challenge in infants with a hemodynamically significant PDA related to the risk of gastrointestinal complications, and therefore enteral feeding may be withheld(131). When enteral feeding, the benefits of

human milk feeding, and especially MOM feeding has been demonstrated due to its positive effect on maturation of the gastrointestinal system(132).

2.3.3.4 Bronchopulmonary dysplasia (BPD)

BPD is a respiratory condition of aberrant alveolar and vascular development resulting in impaired gas exchange(133). The most common definition of BPD is need for oxygen at 36 weeks postmenstrual age(133). The condition affects 32-59% of VPT infants(134) and may lead to later chronic respiratory impairment(135). The cause is complex and may be influenced by both prenatal and postnatal factors, and the mechanisms leading to BPD are not yet fully understood(135). Contributing factors are associated with oxygen toxicity, ventilator induced lung injury, impaired lung vascular development and inflammation(136). Early inflammation has been suggested to play role in BPD, and treatment with steroids may reduce the incidence of BPD but may also have adverse effects(137). In a meta-analysis, human milk feeding has been shown to be a protective factor and reduce the incidence of BPD(138).

2.3.3.5 Intraventricular hemorrhage (IVH)

IVH is the most important adverse neurologic event in preterm infants. IVH, defined as blood leakage into the ventricular space, occur in approximately 20% of preterm and very low birth weight (VLBW) infants. More than 50% of cases occur within the first 24 hours after birth, and 90 % within the first week(139). Vascular fragility and fluctuations in cerebral blood flow contributes to the risk of IVH(139). Furthermore, exposure to intrauterine inflammation has been associated with an increased risk of IVH(140). In preterm infants, increased levels of IL-6 and changes in the coagulation system have suggested inflammation to be related to IVH(141). A grading system of severity from I-IV is used and determined by ultrasound(139). Antenatal steroid treatment is used for prevention and different pharmacological treatments and shunts may be needed(139). In a retrospective study, exclusive human milk feeding was associated with reduced incidence of IVH in preterm infants(142).

2.3.3.6 Retinopathy of prematurity (ROP)

ROP is a vaso-proliferative disease which affects the retina of preterm infants and is the main cause of childhood blindness in the world(143). The cause of the disease is multifactorial and risk factors are for example: low gestational age, treatment with oxygen, maternal hypertension, maternal diabetes, maternal age, PPROM and chorioamnionits(143). However, postnatal inflammatory factors as supplemental oxygen, sepsis, or NEC, have been suggested to have a stronger association to ROP than prenatal factors(144). ROP has been associated with comorbidities as pulmonary complications, anemia, thrombocytopenia, PDA, IVH, NEC and sepsis. Furthermore, impaired infant growth has been associated with ROP and infant nutrition, both parenteral and enteral, seems to influence the disease. Human milk feeding has a protective effect for ROP(143). Different preventive treatments have been suggested related to growth factors insulin-like growth factor-1 (IGF-1) and vascular-endothelial growth factor

A (VEGFA)(145) and fatty acids arachidonic acid (AA) and docosahexaenoic acid (DHA)(146).

2.4 HUMAN MILK AND BREASTFEEDING

Human milk is the optimal nutrition for all infants(147), and in particular for preterm infants(148, 149). Recent studies have demonstrated a higher consumption of MOM to be associated with an enhanced cardiac performance in preterm infants at 1 year of age(150) and intake of even a relatively small amount of colostrum to be associated with lower blood-pressure at 3 years of age demonstrated in late preterm and term infants(151). Furthermore, oropharyngeal administrated colostrum given to preterm infants < 32 weeks of gestation has in a RCT been demonstrated to be associated with lower incidence of NEC, severe IVH and LOS(152). Sub-optimal breastmilk feeding may lead to negative health effects for the individual infant and mother as well as to high societal costs(153, 154). However, the onset of lactation may be compromised in mothers who gives birth preterm(155), which may affect both the composition(156, 157) and the amount of milk(158), leading to a lack of mothers own breastmilk for the most vulnerable preterm infants.

The composition of human milk varies over the lactation period, starting with colostrum, produced in low quantity and rich in immunologic components(159). The timing of secretory activation varies, but often starts within a few days postpartum and determines the onset of the transitional milk. Preterm birth is a factor that can delay the onset of secretory activation in women(160). Transitional milk is often described as the period from five days to two weeks postpartum and share some of the composition of colostrum but is characterized with an increased milk production. After two weeks milk is often considered mostly mature and after four to six weeks fully mature. The composition of mature milk is relative stable with smaller variation over time, in contrast to the dramatic changes during the first month postpartum(161).

2.4.1 Human milk and bioactive components

Human milk provides a multifactorial anti-inflammatory defense, including secretory IgA antibodies directed particularly against the microbial flora of the mother and her environment(162). Human milk contains a variety of biologically active components(163, 164), involved in the development of the infant immune system and intestinal microbiota(165, 166). Lactoferrin, a major milk protein, reduces inflammatory responses and the non-absorbed human milk oligosaccharides (HMOs) block attachment of microbes to the infant's mucosa, preventing infectious diseases(167). Transfer of numerous cytokines and growth factors via milk may also activate the infant's immune system. Interactions between microbes and infant immune system early in life may influence the risk of inflammatory diseases, and a lack of bifidobacteria and depletion of genes required for HMO utilization has been shown to be associated with systemic inflammation and immune dysregulation(168). Breastfed infants given a supplement product with Bifidobacterium infantis, expressing HMO utilization genes, was associated with a change in polarization of T-cells from Th2 towards

Th1 phenotype which may influence mucosal immunity toward intestinal tolerance of microbes(168).

Furthermore, recent studies have detected a human milk microbiome(169), which is suggested to be influenced by factors like mode of delivery, duration of breastfeeding as well as place of living. Different components in human milk may help to explain why breast milk can reduce infant mortality, protect against infections, necrotizing enterocolitis, and other immunological diseases(167).

A relation between the health status of both the mother and the infant and leucocyte count in breast milk has previously been described(170, 171), with low baseline levels of leukocytes and with increasing leukocyte levels, a proxy for immune response, if mother or infant had an infection. When the mother and infant are healthy, the origin of maternal cells in human milk are mainly from the mammary epithelium. However, during the first days postpartum (colostrum) and during periods of infection in either the mother or the infant, the human milk cells are dominated by immune cells from the maternal circulation(170, 172, 173). Breast milk of a mother who is breastfeeding a sick infant has also been suggested to have enhanced levels of lactoferrin, expressed by endothelial cells and activated neutrophils(173).

2.4.2 Donor human milk (DHM)

If mother's own milk is not available, donated pasteurized human milk (DHM) is often given to preterm infants instead of preterm formula. The clinical routines for pasteurisation of DHM varies between and within countries(174). Pasteurisation is performed to inactivate potential viral and bacterial agents in the milk(175). Pasteurisation affects many of the bioactive components in human milk and abolish or reduce their activity(176). Holder pasteurization is a common method used, heating DHM to 62.5 ℃ for 30 minutes(175). Even though pasteurisation affect bioactive components, an exclusive human milk diet have been associated with less NEC in preterm infants compared with preterm formula(177), but also with less growth(178). To improve growth, different strategies as standard volume with added supplementation of bovine or human supplement of protein, fat and or carbohydrates, or high volume without supplementation are used(179). However, in a RCT comparing DHM versus preterm formula related to adverse outcome of LOS and/or NEC, the authors did not find any differences between groups(180, 181). In contrast, a diet of mother's own milk was associated with less infection and shorter hospital stay(180). During the first critical period after birth there is a lack of fit between MOM an DHM, and strategies to support MOM during this period in the NICU is important(182). Recent studies suggest that strategies to personalize DHM by inoculating with small amount of fresh or frozen MOM when available, may add beneficial bacteria with positive effects for the infant(183).

2.4.3 Inflammatory complications in lactation

Lactation mastitis is an inflammatory condition, with severe and painful symptoms that can lead to discontinuation of lactation/breastfeeding. Mastitis is often treated with antibiotics in high doses, with possible side effects in both mother and infant(184, 185). As previously

described, human milk provides a broad anti-inflammatory defense for the infant(161) but sometimes lactation is threatened by inflammatory complications like mastitis that can lead to cessation of breastfeeding(186). Recent studies have suggested that inflammation in subclinical mastitis may cause low milk supply and hence increase the risk of impaired growth of the infant as well as the cessation of breastfeeding(187). A study by Li et al(188) demonstrated higher levels of calcium related to subclinical mastitis and a positive correlation between calcium and the pro-inflammatory cytokine IL-6. Calcium is known to be involved in the recruitment of neutrophils to sites of inflammation and changes in intracellular calcium levels play an important role in neutrophil activation and function(189). Different strategies have been suggested for prevention of mastitis, as anti-secretory factor cereals (SPC-flakes®)(14) and probiotics(190), with positive results, but the studies are small and further studies are needed. There is an urgent need to develop strategies to prevent lactation mastitis and the evidence from intervention studies are low(191).

3 RESEARCH AIMS

3.1 GENERAL AIM

The overall aim of this thesis was to increase knowledge regarding the antisecretory factor in the perinatal period, focusing on describing AF in placenta, maternal plasma and human milk related to term and preterm birth and inflammation.

3.2 SPECIFIC AIMS

- To test the hypothesis that AF may play a role in immune reactivity and homeostasis during pregnancy by examining the level of AF in placental tissue in relation to the degree of inflammation and length of gestation.

- To evaluate if there is a correlation between AF levels in maternal plasma and breastmilk in a cohort of breastfeeding mothers in Sweden.

- To investigate AF-compleasome levels over time in MOM related to term and preterm birth

- To investigate AF-compleasome levels in donor human milk before and after pasteurization.

- To investigate possible associations between AF-compleasome levels in MOM and infant outcome and inflammation following preterm birth.

4 MATERIALS AND METHODS

4.1 STUDY DESIGN AND SETTING

The data in this thesis was collected in three different cohort studies. All studies were prospective, descriptive, and exploratory related to AF. All cohorts had a longitudinal design, however only one of the cohorts (paper III and IV) had longitudinal samples collected and analyzed for AF. The cohort studies were all performed in Sweden, with recruitment of participants in delivery, postnatal care, neonatal intensive care unit (NICU), or child health care. The three cohorts include different samples and focuses on different parts of the perinatal period. An overview of the included studies is presented in Table 1.

4.1.1 Paper I

Paper I is based on 61 women after VPT (n=11), MPT (n=20) and TB (n=30) included in a sub-study of a prospective cohort study on preterm birth at Karolinska University hospital in Stockholm. Inclusion criteria was women ≥ 18 years of age with spontaneous onset of delivery. Exclusion criteria were multiple pregnancy, use of tobacco, presence of preeclampsia, diabetes, or other systemic disease.

Obstetrical data was collected prospectively from medical records. At birth, a placenta biopsy (1x1 cm) through full thickness was collected.

4.1.2 Paper II

Paper II is based on 95 mother-infant pairs recruited at four weeks postpartum at a Well Baby Clinic in Umeå between April 2011 and September 2012. The study was a sub-study of a cohort study investigating oral Candida in infants. Mother-infant pairs in which the mother had both breastmilk and plasma samples collected were included in this study.

At entry of the study four weeks postpartum, maternal plasma and breastmilk samples were collected. Information on living conditions, maternal dietary habits, smoking, delivery mode, infant birth weight and length, and use of antibiotics in mother and infant were collected using a questionnaire. Information on maternal age and BMI were collected from medical records. At 12 months postpartum, mothers were asked on history of breast complications

4.1.3 Paper III and IV

Paper III and IV are sub- studies in a cohort study, the TELLUS study (TELomers, LUngdisease and oxidative Stress in preterm infants), a prospective, longitudinal cohort study of cellular aging in preterm infants less than 30 gestational weeks and full-term controls. Exclusion criteria was infants born with severe malformations. The TELLUS study included 181 infants during the study period, April 2014 to April 2019.

Table 1. Overview of the four included studies in the thesis				
	Paper I	**Paper II**	**Paper III**	**Paper IV**
Participants	61 women (11 VPT, 20 MPT and 30 TB)	95 women and their infants	I) 87 women (41 TB, 46 PTB <30 weeks of gestation) II) 20 DHM samples	38 women and their 49 preterm infants < 30 weeks of gestation
Data collection	Medical records	Questionnaire	Prospective and Medical records	Prospective and Medical records
Timepoints	Delivery	Postnatal week 4, plasma and MOM collected. Questionnaires at 4 weeks and 12 months	Postnatal week 1, 4 (preterm group only) and 12	Postnatal week 1, 4 and 12
Samples	Placenta biopsies	MOM, Maternal plasma	MOM, DHM	MOM, Infant plasma
AF analyses	Immuno-histochemistry (*placenta*)	ELISA (*maternal plasma, MOM*)	Sandwich-ELISA (*MOM, DHM*)	Sandwich-ELISA (*MOM*)
Other analyses	IL-6 (*cord blood*)	Lactoferrin (*MOM*)		Plasma proteins, ProSeek, Olink (*infant plasma*)
Statistics	Descriptive statistics, Spearman rank correlation, Chi-square test, ANOVA, Kruskall-Wallis test	Descriptive statistics, Spearman rank correlation, Pearson correlation, Chi-square test, Independent t-test, Mann-Whitney U-test, Wilcoxon signed rank test, Kolmogorov Smirnov test, Fishers exact test	Descriptive statistics, Pearson correlation, Chi-square test, Independent t-test, Paired t-test, GLS random effects model	Descriptive statistics, Spearman rank correlation, Mann-Whitney U-test, Kruskall-Wallis test, General linear model, repeated measures

Abbreviations: VPT = Very preterm, MPT = Moderate preterm, TB =Term birth, MOM = Mothers own milk, DHM = Donor human milk

Paper III is based on 87 mothers of term (n=41) and preterm (n=46) infants, and 20 anonymized donor milk samples from the Milk bank in Stockholm. Information on maternal and infant data were collected from medical records. Samples of MOM was collected at approximately week 1, 4 and 12 postpartum. Anonymized samples of DHM were collected in the Milk bank in Stockholm.

Paper IV is based on 38 mothers and their 49 infants (9 twins and 1 triplets) born less than 30 weeks of gestation. Inclusion criteria for the sub-study was AF-compleasomes analysed in mothers' own milk (MOM) at the first timepoint, week 1. Longitudinal samples of AF-compleasomes in MOM was analysed in MOM from 15 of these mothers and their 19 infants (4 twins). Samples of MOM were collected at approximately week 1, 4 and 12 postpartum. Maternal and infant data was collected from medical records. Nutritional data was collected for age at start of enteral nutrition and age at full enteral nutrition. At all timepoints for sampling, percentage MOM of enteral nutrition and percentage enteral nutrition of total nutrition was recorded. Neonatal morbidities were followed until term corrected age, and included sepsis, BPD, ROP, NEC, PDA and IVH.

4.2 EXPERIMENTAL METHODS

This section presents a brief description and overview of the methods used for analysis in the included papers, a more detailed description is presented in each paper.

4.2.1 Immunohistochemistry

In paper I, immunohistochemistry was used to determine AF levels in placenta biopsies. For immunohistochemical analysis, two different antibodies were used for AF visualization, the monoclonal mAb43 to detect the active form of AF, and the polyclonal P8 to detect the total amount of AF i.e., the active as well as the inactive form. The polyclonal P8 antibodies bind primarily to a variety of amino acid sequences in the P8 peptide and may cross-react with other unrelated peptides as well, whereas the anti – AF mAb43 antibodies recognize the three-dimensional structure of the AF protein reducing the risk for cross reactivity(13). Tissue sections were incubated with one of the two different antibodies against AF described above, i.e., mAb43, a mouse monoclonal IgM antibody, or P8, a polyclonal affinity purified rabbit antiserum.

4.2.2 ELISA

In paper II a single ELISA was used to determine AF levels in maternal plasma and MOM. The ELISA method for analyzing AF was developed by Johansson et al, first as a non-commercial, in-house, version of a single ELISA which has later been further developed as an in-house sandwich ELISA(13, 17). A monoclonal antibody of IgM isotype (mAb43) was used for detecting the active form of the protein(13). The concentrations of AF were expressed in arbitrary units against the reference peptide AF1–105, a peptide at the N-terminal part of AF, including the active part of the full-length protein.

4.2.3 Sandwich ELISA

In paper III and IV a sandwich ELISA was used to determine AF levels in MOM, and in donor human milk in paper III. The sandwich ELISA has been developed to improve and optimize the analysis (Figure 4). The analysis in the sandwich ELISA required 10 times less sample and is time effective since no primary purification of sample is needed and have a higher sensitivity than the previous single ELISA(17).

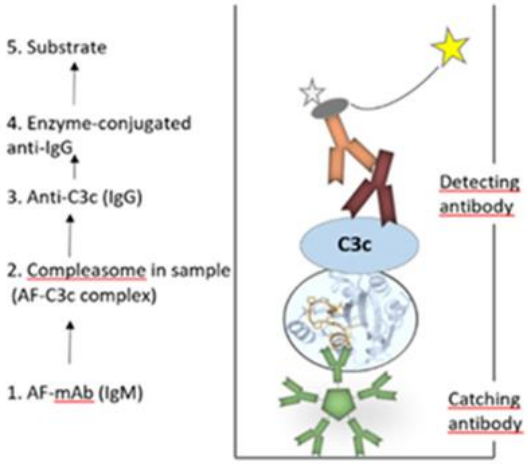


Figure 4. Illustration of the principle and steps in sandwich ELISA for determination of AF-compleasome in sample. Kindly provided by Ass. Prof. Ewa Johansson, University of Gothenburg, Sweden.

In the sandwich ELISA, in-house produced monoclonal antibodies against AF (mAb43) were used as catching antibodies and polyclonal antibodies against C3c were used as detecting antibodies. The catching antibodies, mAb43 or phosphate-buffered saline (PBS) as control was coated on Maxisorb microtiter plates. After incubation with the sample, the detecting antibody against C3c was applied. The Absorbance was read in a photometer at 405nm after development by a secondary antibody coupled to alkaline phosphate and the difference between antibody coated samples and controls were calculated and demonstrated as netAbs405nm.

A pre-treatment with ethyl-acetate to remove disturbing fat, was performed before using the sandwich ELISA for analysis of human milk(192).

4.2.4 Plasma proteins

In paper IV, we used an inflammation panel (ProSeek, Olink AB Uppsala) consisting of 92 proteins to determine markers of inflammation in infant plasma. The method has previously been described(98, 193). In short, proteins in the sample bind to paired oligo-nucleotide antibodies with overlapping sequences. When the paired oligo-nucleotide antibodies are bound to the target protein and close to each other, they can be measured using Real-time PCR. Normalized Protein eXpression (NPX) are used to demonstrate the results from the analyses. NPX is an arbitrary unit on the log2 scale and high levels on the NPX represent high protein concentration(193, 194).

4.3 STATISTICAL ANALYSIS

The included studies were all observational cohort studies with an explorative approach related to AF. Descriptive statistics was analyzed using Chi square test for dichotomous variables. To determine correlations, we mainly used the non-parametric Spearman rank correlation when the sample size was small, not normally distributed or with a variable on the ordinal scale. Both non-parametric as well as parametric (Pearson product-moment) correlation was used after log2 transformation and where the sample size was larger. Log2 transformation is sometimes used to make the variables more normally distributed to increase the power and reliability of parametric tests in small samples. Effects on log2 transformed scale can be interpreted as proportional, so results remain interpretable despite the transformation. Spearman rank correlation test is less sensitive to outliers in the data(195). To interpret the size of correlation, a value of 0.5 (-0.5) may be considered moderate to high/strong, values between 0.3 (-0.3) and 0.5 (-0.5) to be low/weak, and 0 to 0.3 (-0.3) to negligible(195). Importantly, correlations are used to assess strength and direction of the linear relationship between variables and not to determine causal relationship, and cannot be used to determine if the variables are dependent or independent(195).

To determine differences between two or more groups and for longitudinal samples, we used both non-parametric (Mann-Whitney U test, Kruskall Wallis test, Wilcoxon test) and parametric tests (Independent Students t-test, ANOVA, paired samples t-test) as described for correlations above. When assumptions hold, parametric tests can have more statistical power than non-parametric tests, thus we used both when applicable. Furthermore, different tests to determine normal distribution, as the Kolmogorov-Smirnoff test and the Shapiro Wilks W test can be used. However, both tests are influenced by sample size and outliers. In addition, histograms can be used to visually assess if the data is normally distributed. A p-value < 0.5 was considered statistically significant in all studies.

In paper I, data with normal distribution were presented as means and standard deviations, and other data as median and ranges. Differences between groups related to maternal and infant characteristics was determined using Chi square test, ANOVA and Kruskall Wallis test and placental levels of AF and inflammatory markers using Mann Whitney U-test or Kruskall Wallis test. Correlations between length of gestation related to placental AF, vascularization and inflammatory markers were determined using Spearmans rank correlation.

In paper II we used both the original data as well as log2 transformed data, and data were analyzed with both parametric (Pearson correlation, independent sample t-test) and non-parametric tests (Spearmans rank correlation, Mann-Whitney U-test and Wilcoxon signed rank test). Chi square test was used for dichotomous variables and Fischer exact test was used when there was a small number of observations. Kolmogorov-Smirnov test was used to test for normality.

In paper III, AF-compleasome in MOM was analyzed related to demographics using independent t-test and Pearson correlation. AF-compleasome was further analyzed related to

term and preterm birth, timepoint of sampling and in longitudinal samples using Pearson correlation, independent sample t-test, two-sample t-test with unequal variance and paired t-test. General Least Squares (GLS) random effects model was used to determine within group differences between timepoints. A random effects model was used to take into account some but not all participants had samples measured at all timepoints. Kernel densities was used to compare distributions between groups at the different timepoints in longitudinal samples.

In paper IV we used non-parametric tests due to the small sample size. We used Spearmans rank correlation to determine associations of AF-compleasome between timepoints of sampling and related to nutritional parameters. For analyses of AF-compleasome related to infant outcomes, we used Mann Whitney U-test, Kruskall Wallis test and General linear model, repeated measures.

4.4 ETHICAL CONSIDERATIONS

4.4.1 Informed consent

All included studies have been ethically reviewed and approved by the Regional Ethical Review Board (Etikprövningsnämnden, EPN). When recruiting participants, informed written consent has been obtained for participation in the study and information was given that participation was voluntary and could be terminated at any time. Oral and written study information was provided with the opportunity to ask questions and time to think about possible participation in the study. Information was given about the right to receive registry extracts once a year on the data and any samples collected. Information was given about the right to have trial material destroyed and that incorrect information is corrected or deleted from registers as well.

4.4.2 Blood sampling

Blood sampling can be painful and was therefore coordinated as far as possible with sampling for clinical purposes. When taking blood samples from newborn and preterm infants, the greatest possible efforts is done to coordinate with clinical blood sampling to avoid extra pain. Pain relief according to clinical guidelines, is given as required. A lot of work has preceded the studies to reach as small sampling amounts of blood as possible. The amounts of blood taken were small and were judged not to affect the participants.

4.4.3 Breastmilk analysis

The analysis of the mother's own breast milk may implicate that the infant receives less breast milk. Only a very small amount of breast milk was needed for analysis as a minimum if only small amounts were available. Furthermore, breastmilk production is stimulated by more frequent stimulation/expression. In paper III and IV, donated breast milk was given to preterm infants during the first period of time, if the mother's own breast milk was not available or reaching the infant’s needs. Analysis of donated breast milk could have a

disadvantage by less access of donated breast milk to infants who may need it. However, the analysis required only a few milliliters and therefore the risk of lack of donated breastmilk was negligible.

4.4.4 Placenta and umbilical cord samples

It can be difficult to give study information and obtain informed consent during active labor due to pain and stress in the mother. In some studies, informed consent is obtained during pregnancy which gives the advantage of more time for reflection. Unfortunately, it can be difficult to ask for participation during pregnancy if a study is undertaken in one specific hospital site and pregnant women may not be able to choose hospital site in advance for their delivery.

In paper I, we investigated AF in placenta biopsies already collected material, with an ethical approval by the EPN.

For paper III and IV, the ethical approval contains the possibility to take samples from umbilical cord and placenta after childbirth without asking for consent at the time. These samples were taken care of and stored in the freezer. If parents did not want to participate in the study, these samples were discarded. An ethical approval to request parents who do not consent to participate in the entire study, for consent to save these already collected samples together with information on childbirth / pregnancy was later approved by the EPN. These samples do not affect the child, are not painful and use no blood volume from the infant. These are already existing samples that are otherwise discarded, and thus utilized, were then available from a participant where blood sampling is minimized. One disadvantage could be that parents who have not consented to participate in the research study, may experience it stressful to be asked for consent to analyze the samples already collected. Furthermore, they may experience discomfort that information about the delivery is saved, however all information was coded and followed the same rules as for a given consent for the entire study. Our experience in conversation with parents who do not consent to the study was that they mainly reject the continued blood sampling but were not concerned about samples already taken at childbirth.

4.4.5 General risk/benefit

By collaborating with other research groups and thus utilizing already collected material and coordinating new collection, the effort it entails in collecting and implementing the study, for both participants and researchers, can provide the greatest possible exchange and results. Interprofessional, as well as interdisciplinary collaboration may strengthen research and add different perspectives to the subject.

There is a great need to increase knowledge of the pathophysiological mechanisms of inflammatory complications during the perinatal period (such as PTB, NEC, BPD, ROP, mastitis) in order to prevent and treat them. Increased knowledge of basic levels of AF could

lead to intervention studies, aiming at prevention and treatment of inflammatory complications during the perinatal period.

4.4.6 Confidentiality

Information about participants was collected in a register, i.e., day of birth, treatments, and results from surveys. Names and social security numbers were not included on any study documents or in the electronic database. Instead, a study code was used and the only persons with access to the code key were the investigators of the study. The studies followed the current legislation under the EU Data Protection Ordinance (GDPR). Personal data controller for the registry is the Data Protection Officer at Karolinska University Hospital. The information was be stored in a database at Karolinska Institutet and samples were saved in accordance with the Biobanks Act in the Biobank at Karolinska University Hospital (according to PUL, 1998: 204). Participants have the right to find out in writing which personal data are registered, correct any incorrect information, and the right to revoke the consent and then delete the identifiable personal data that has been registered.

5 RESULTS

A summary of the main results is presented here. Detailed results are found in the individual papers included in the thesis.

5.1 ANTISECRETORY FACTOR IN MATERNAL PLASMA AND HUMAN MILK

5.1.1 Associations between maternal plasma and breastmilk

In paper II, a positive correlation (Spearmans rho 0.403, $p < 0.001$) was demonstrated at 4 weeks postpartum between maternal plasma and breastmilk in the cohort of breastfeeding, with higher levels in breastmilk associated with higher levels in plasma.

Furthermore, higher AF levels in breastmilk were correlated with higher lactoferrin levels in breastmilk (Spearmans rho 0.341, $p < 0.01$), but there was no correlation between lactoferrin levels in breastmilk related to maternal AF levels in plasma (Spearmans rho 0.193 $p = 0.06$).

5.1.2 Changes in mothers' own milk over time

In paper III, AF-compleasome in mothers' own milk (MOM) were present in all samples (n=128) and were higher week 1 compared to week 12 postpartum ($p < 0.001$). In longitudinal samples of individual mothers (n=24) a higher AF-compleasome level was demonstrated week 1 vs week 12 in both the term and preterm group ($p < 0.01$ and $p < 0.01$, respectively).

Furthermore, some of the mothers in the preterm group (n=15) contributed with samples of MOM at 4 weeks postpartum demonstrating lower levels compared to week 1 ($p < 0.001$). Even if only analyzed in the preterm group, the result suggest that AF levels change early.

5.1.3 Effect of Holder pasteurization on donor human milk

In anonymized donor human milk (DHM) (n=20) AF-compleasome levels were higher after Holder pasteurization than before ($p < 0.001$) (paper III).

5.2 ANTISECRETORY FACTOR RELATED TO TERM AND PRETERM BIRTH

5.2.1 In placenta

In paper I, AF was present in the outer syncytiotrophoblast layer of all placenta biopsies included.

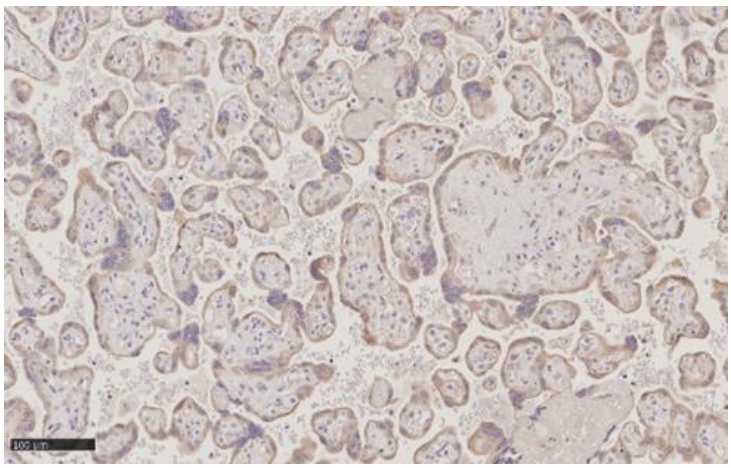


Figure 5. Picture demonstrating AF in brown color in the outer syncytiotrophoblast layer of the placenta stained with monoclonal antibody mAb43. Kindly provided by Professor Eva Jennische, University of Gothenburg, Sweden.

Active AF, reflected by the number of mAb43 positive staining cells with high intensity, was higher in term placentas versus preterm placentas. Thus, a shorter gestation was associated with a lower level of the active form of AF (Spearmans rho = 0.286, $p = 0.025$). The total number of AF-P8 positive staining cells reflecting both active and inactive forms of AF demonstrated an inverse relation to length of gestation (Speamans rho = -0.249, $p = 0.053$) and higher levels in VPT versus TB ($p = 0.046$).

Vascularization reflected by the number of blood vessels per villus was not associated with length of gestation, but the diameter of blood vessels was smaller in placentas in the VPT group versus the TB ($p < 0.001$) and the MPT ($p = 0.001$) groups. A positive correlation was also demonstrated between infant birth weight and diameter of blood vessels in the placenta (Spearmans rho 0.328, $p = 0.010$). Active AF reflected by mAb43 positive staining cells was positively associated with the diameter of blood vessels (Spearmans rho = 0.368, $p = 0.004$).

5.2.2 In mothers' own milk

There was no difference in AF-compleasome levels in MOM at week 1 between the term and preterm group ($p = 0.82$). At 12 weeks postpartum, AF-compleasome in MOM of mothers in the preterm group were higher with a wider distribution compared with mothers in the term group ($p < 0.05$). Furthermore, there were two mothers in the preterm group with similar or slightly higher AF-compleasome levels week 12 versus week 1 (paper III).

5.3 ANTISECRETORY FACTOR RELATED TO INFLAMMATION

5.3.1 Inflammatory markers in placenta

In paper I, a higher degree of inflammation in the placental tissue, reflected as higher numbers of CD68 positive cells, was associated with a shorter length of gestation (Spearmans rho = -0.576, $p < 0.001$). Furthermore, the numbers of CD163 positive staining cells were

also associated with a shorter length of gestation (Spearmans rho = -0.504, $p < 0.001$). There were higher numbers in both the MPT ($p < 0.01$) and the VPT ($p < 0.001$) groups related to the TB group. Higher levels of mAb43 positive staining cells, representing active AF, were associated with lower levels of both CD68 positive staining cells (Spearmans rho = -0.310, $p = 0.015$) and CD163 positive staining cells (Spearmans rho = -0.282, $p = 0.028$).

For a subgroup of participants (n=12), IL-6 in cord blood was determined. There was a difference between groups with a higher IL-6 level in cord blood in the VPT group ($p < 0.01$) than in the MPT and TB groups. There was no difference in mAb43 levels, CD68 levels or CD163 levels related to IL-6 in cord blood of higher or lower than 11 pg/ml, irrespective of length of gestation.

5.3.2 AF in mothers' own milk and inflammatory proteins in infant plasma

In paper IV, AF levels in MOM week 1 related to inflammatory proteins in infant plasma week 4 (n=36), had a moderate to strong inverse correlation for IL-8 and CCL25, and a weaker inverse correlation to IL6 and VEGFA.

Furthermore, AF levels in MOM week 4 had a moderate to strong inverse correlation to VEGFA and several other proteins in infant plasma week 4, however only measured in 13 infants. Moreover, infants who developed sepsis had higher levels of IL-8, IL-6, ENRAGE and CCL25 in plasma at week 4 compared to infants without sepsis. Similarly, infants who developed BPD had higher levels of IL-8 compared with infants without BPD.

5.4 ANTISECRETORY FACTOR RELATED TO INFLAMMATORY COMPLICATIONS

5.4.1 AF in mothers' own milk and outcome for preterm infants

In paper IV, lower levels of AF in MOM week 1 was demonstrated in mothers of infants who later developed an adverse outcome, only statistically significant for sepsis.

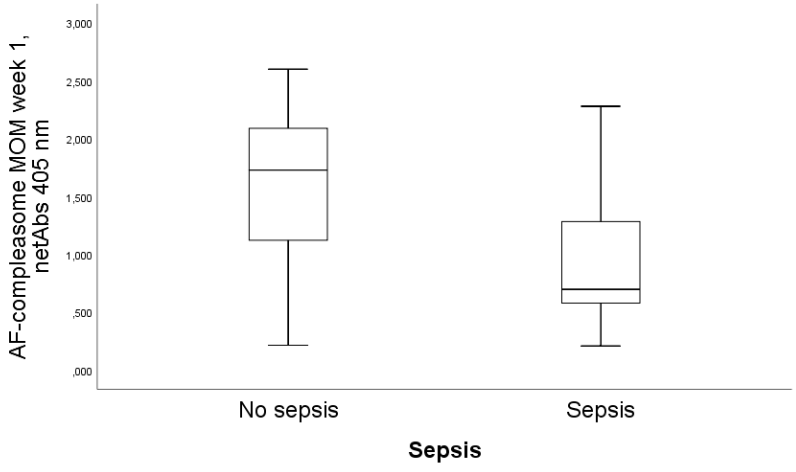
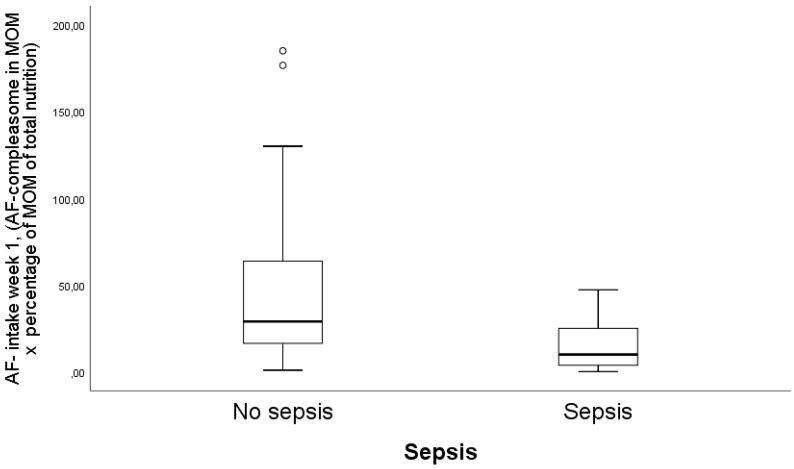


Figure 6. AF-compleasome in MOM week 1 related to infant sepsis ($p = 0.005$).

In longitudinal samples (n=15), the result demonstrated lower levels of AF in MOM from mothers of infants who developed sepsis or ROP versus AF levels in MOM of mothers of infants without these adverse outcomes.

Several infants in the cohort had more than one adverse outcome. AF levels in MOM week 1 was inversely correlated to the number of adverse outcomes in infants (Spearmans rho -0.361, $p = 0.011$, Kruskall Wallis test $p = 0.002$). Visualized, increasing numbers of adverse outcomes were related to subsequently lower levels of AF-compleasome in MOM in longitudinal samples, however not reaching statistical significance. Moreover, a higher number of adverse outcomes was associated with a lower gestational age and later attainment of full enteral nutrition

Since both enteral nutrition and MOM intake may differ between infants, AF intake was calculated at the different timepoints (AF-compleasome level in MOM x proportion of MOM of total nutrition) to try to adjust for differences in intake. AF-compleasome intake week 1 was lower in infants who later developed sepsis or BPD, and there was no difference between these groups related to MOM intake.



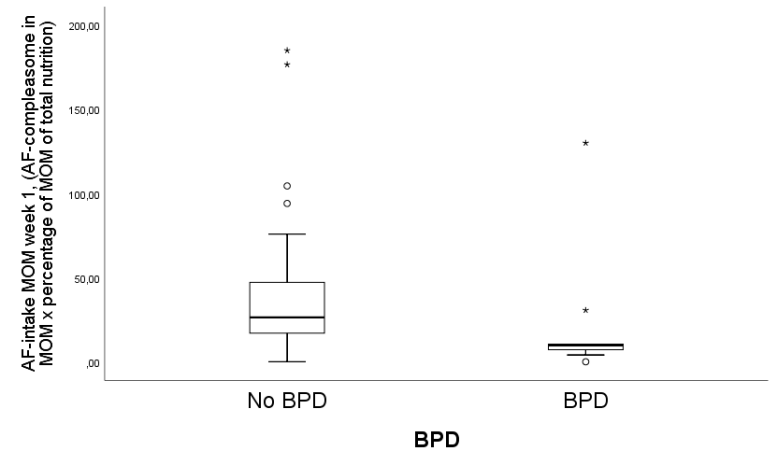


Figure 7. AF intake in MOM week 1 related to infant sepsis and bronchopulmonary dysplasia ($p = 0.011$ and $p = 0.027$).

5.4.2 AF in maternal plasma and breastmilk and breast complications

In paper II, breast infection was reported by only three mothers at 12 months postpartum. At 4 weeks postpartum, these mothers had lower AF levels in plasma (independent t-test $p < 0.05$), but not in breastmilk (independent t-test $p > 0.05$) compared to mothers without breast infection.

5.5 ANTISECRETORY FACTOR RELATED TO MATERNAL AND INFANT CHARACTERISTICS

Apart from gestational age, the levels of AF did not seem to be significantly affected by any perinatal characteristics. The inclusion criteria differed between the studies in the thesis. Overall, there were no association between AF levels related to mode of delivery or infant sex (paper I-IV). Furthermore, there were no differences between AF levels and maternal age or parity (paper II-IV).

In paper IV, we found a week correlation between AF levels in maternal plasma related to BMI (paper II), not found related to breastmilk (paper II-IV). In paper III, mothers in the term group who were multiparas had higher AF levels in MOM week 1 than mothers who were primiparas, but there was no difference in the preterm group or at any other timepoint.

Furthermore, in the preterm cohort, there was no difference in AF levels in MOM related to maternal Group B Streptococcus (GBS) in urine, rupture of membranes >18 hours, pre- or intrapartal antibiotics or preeclampsia (Paper IV).

6 DISCUSSION

In this thesis, AF levels in placenta, maternal plasma, MOM and DHM have been described.

The findings of lower AF levels in placenta, higher level of inflammatory marker and less developed vascular architecture after shorter length of gestation indicate that AF may be involved in regulating inflammation in the placenta during pregnancy. However, the possibility that AF levels normally increases over the time of gestation cannot be excluded. Since it is difficult to analyze levels of AF in placenta during pregnancy, it may be of interest to investigate AF levels in maternal plasma during pregnancy and relate to AF level in placenta at birth. Although, as preterm birth is multifactorial, the role of AF may be difficult to determine in our study (paper I) only women with spontaneous onset of labor were included, and women with preeclampsia and systematic inflammatory diseases excluded to minimize risk for confounding by these factors. Moreover, further studies are needed to determine if AF in placenta have a role in vascular development or if the finding of less developed vascular architecture only is associated with length of gestation. Vascular disorders have been described to have an impact on preterm labor in women with intact membranes or preterm PROM(196). Doppler technology has been suggested as a non-invasive technique to assess the vascularity of placenta during pregnancy and to determine intrauterine growth restriction (IUGR) and preeclampsia (PE)(197). A study combining AF levels in maternal plasma with the use of doppler technology to determine vascularity in the placenta during pregnancy may give information on the role of AF related to vascularization.

Immune tolerance is important for a successful pregnancy(52). Results in paper I demonstrate lower AF levels in placenta to be associated with higher levels of CD68-positive cells and CD163-positive cells in placenta after preterm birth suggesting a higher degree of inflammation. This is in line with the study of Berezhna et al(198) demonstrating higher levels of CD68 positive cells and CD163 positive cells in placentas after preterm birth. They suggest that the increased CD68 activation may be associated with disorders in the vascular and stromal component of the villus.

High AF levels in maternal plasma was demonstrated to be weak-moderate associated to high levels in MOM in a cross-sectional sample collected 4 weeks postpartum in breastfeeding mothers of term infants. This association suggest a possible systemic co-regulation of AF levels between plasma and MOM, however a local regulation in each compartment may also be possible as previously suggested for AF in other tissue compartments(1). Only three mothers in the study reported having had a breast infection, and all three mothers had AF levels below the 25th percentile. The small sample makes it difficult to draw firm conclusions of the result. High AF levels in MOM has previously been described to be associated with less mastitis in a RCT were mothers were given SPC-flakes® compared to placebo flakes(14). A disadvantage of the study was that information on breast complications was collected at 12 months postpartum, several months after the collection of plasma and MOM samples. Additionally, we did not know whether the breast complication developed before or

after the sample was taken. However, many breast complications occur during the first 6 weeks postpartum (199, 200). Since breast complications as mastitis and infection are associated with an early cessation of breastfeeding(201), and subclinical mastitis associated with lower milk supply(187), detection of modifiable factors for breast health during lactation are of importance.

In the results of the included studies in the thesis, AF did not appear to be associated to other maternal and infant characteristics, except gestational length. However, since composition of other factors as HMOs(202), fatty acids(203) and microbiota(157) in human milk has been described to be associated with different characteristics and related to both fixed and modifiable factors(202), further investigations of possible factors influencing AF levels may be of interest. Furthermore, maternal diet has been suggested to be a modifiable factor influencing the immune system, and in a recent RCT on probiotics given during the second half of the pregnancy resulted in modulation of circulating active and resting Treg cells (204). Another recent study of maternal probiotic intake during pregnancy and breastfeeding demonstrated conflicting results with an increased risk for breast complications but associated to longer duration of breastfeeding. The authors highlight a risk of socio-economic confounding and call for larger studies on the subject(205). Since induction of AF is possible through diet, and several studies have been performed in both humans and animals in other contexts, intervention studies may be of interest.

Furthermore, AF levels was demonstrated to be high in colostrum and decrease over the first 12 weeks postpartum. This finding is in line with high levels in colostrum and decreasing levels of other bioactive factors over the first weeks postpartum(162). Colostrum is known to be rich in immune-active components(206) and are suggested to play a role educating the immune system and in programming for later health(207). In longitudinal samples, AF levels in MOM was demonstrated to be higher and with a wider distribution after preterm birth. The composition of human milk microbiota has been described to be associated with maternal factors (BMI, parity, and mode of delivery), breastfeeding practices and other components in milk(157). Furthermore, the variation in composition of human milk fatty acids have been described to be related to dietary, genetical, sociodemographic and environmental factors, but not to maternal age, parity, mode of delivery or infant sex (203). The composition of other factors in human milk like HMO: s also varies between mothers and are associated with genetic secretor status, environmental factors and feeding practices(202). Further studies are needed to determine factors associated with AF levels in maternal milk as well as possible associations to other components in human milk.

Since donor human milk (DHM) is often used for preterm infants when MOM is not available or not reaching infant needs, we examined the effect of Holder pasteurization on DHM. Interestingly, higher AF levels in DHM was demonstrated after pasteurization than before. Complement factors in milk has been described to be inactivated when heated to 56°C(208). Furthermore, AF is activated in complex with complement factor C3, which is converted to the inactive form C3c(20) and heating during Holder pasteurization may thereby

lead to both higher AF levels and inactivation of complement. This finding suggests a sustained anti-inflammatory effect of DHM even though many other bioactive factors are reduced or abolished during pasteurization(209) and may contribute to the protective effect of DHM on the development of NEC(178).

Thereafter, investigation of potential associations between AF-levels in MOM related to infant outcome after preterm birth was performed. The results of higher AF- levels in MOM week 1 and in longitudinal samples to be associated with less sepsis in infants may suggest AF to be one of the protecting components in human milk. Furthermore, the findings of higher AF levels, AF intake or in longitudinal samples associated with less BPD and ROP as well as a smaller number of adverse outcomes may further support the potential protective effect. In experimental pilot studies in pigs, the peptide AF-16 has been suggested to inhibit sepsis-induced liver edema(210), and improve edema resolution in experimental acute respiratory distress syndrome (ARDS)(211). In animals, a biological effect of induced AF levels in milk has been described with improved weight gain, less diarrheal diseases, and increased survival in the offspring(1).

Related to proteins in the inflammatory panel, higher AF levels in MOM week 1 was associated to lower levels of IL-8 in infant plasma week 4, and a weaker correlation to IL-6 and ENRAGE. Furthermore, infants who developed sepsis or BPD had higher levels of IL-8 in plasma week 4. Elevated levels of IL-6 and IL-8 have previously been described related to sepsis(212-214). A sustained inflammatory condition can continue for weeks in preterm infants and has been associated with increased levels of IL-6, IL-8 and TNFα(106).The chemokine IL-8 is a mediator involved in acute inflammation in recruitment and degranulation of neutrophils(215). IL-6 is induced related to tissue damage or inflammation until homeostasis is restored, and dysregulation may be involved in immune-mediated diseases(216). ENRAGE also known as S100A12, has been suggested as a biomarker for sepsis in neonates, with higher levels related to sepsis(217). In line with this, we found higher levels of IL-8, IL6 and ENRAGE in plasma from infants who developed sepsis. Additionally, higher levels of these proteins were correlated to lower levels of AF levels in MOM in the early perinatal period.

Furthermore, higher levels of AF levels in MOM week 1 was associated to lower levels of CCL25, a chemokine involved in chemotaxis and inflammation, in infant plasma week 4. Additionally, infants who developed sepsis had higher levels of CCL25 week 4 than infants without. CCL25 is expressed in thymus and the small intestine and suggested to be of importance for mucosal immunity(218).

Higher AF levels in MOM week 1 had a weak correlation to lower levels of VEGFA in infant plasma week 4. A stronger inverse correlation was shown between AF levels in MOM week 4 and VEGFA in infant plasma week 4, however only measured in 13 infants. High levels of VEGFA have been associated with the development of both BPD(219) and ROP(220) however, in this cohort we did not find any differences between levels of VEGFA in infant plasma week 4 related to having the condition or not.

The findings of the association between higher AF levels in MOM and less adverse outcome in preterm infants was supported by our findings of higher levels of IL-8 in infants with these outcomes. Additionally, an association was demonstrated between higher levels of AF in MOM and lower levels of IL-8 in preterm infants. Interestingly, higher levels of IL-8 have been described in MOM of mothers with subclinical mastitis(221). Unfortunately, analysis of IL8 was not performed in MOM in this study, which would have been interesting related to AF levels in MOM as well as IL-8 levels in infant plasma. However, the studies included in this thesis were explorative and can only describe association, not causality and further studies are needed to address to address that question.

To identify modifying factors to decrease the burden of sepsis as well as other adverse outcome in preterm infants is important to increase health and quality of life for infants and families, as well as reduce societal costs. Since the pathophysiology of preterm birth as well as of adverse outcomes in preterm infants are not yet fully understood, studies addressing the underlaying mechanisms are needed to be able to develop interventions.

Furthermore, mechanistical as well as intervention studies of AF biology and AF therapy are ongoing and have been performed in other areas of health and disease. In intervention studies in humans, AF therapy to increase AF levels as a complement to other treatments has been suggested to have an effect on childhood diarrhea(25), inflammatory bowel diseases (IBD)(29), Meniere disease(32), and reducing intracranial pressure (ICP) after severe traumatic brain injury (TBI)(36, 37). In a xenograft, AF-16 given to increase AF levels have been suggested to decrease interstitial fluid pressure in glioblastoma and tumor growth (GBM) and increase drug uptake (7). In a rat model, treatment with AF-16 decreased interstitial fluid pressure(222) and increased vascular access in rat mammary tumors was demonstrated(223) which may further suggest AF as a candidate to increase drug uptake.

An increased AF plasma level has not been associated with any severe medical side effects(29, 37). However, in a small study on AF inducing diet or administration of exogenous AF in patients with short bowel syndrome (SBS) did not improve symptoms and appeared to aggravate fluid loss and induce side effects like abdominal pain, increased stoma effluent and decreased diuresis(224). Intervention studies to investigate both potential beneficial effects as well as adverse effects are important to perform before starting to use treatments clinically.

Methodological considerations

Having the opportunity to perform sub-studies within larger studies enabled these exploratory studies of AF in the perinatal period. The collaboration between and within research groups and an open mind in how to use already collected material and data within the scope of the research and ethics frame, are important in respect to all participants contributing to the studies. It may be difficult to design, get funding and to recruit participants in studies without having done exploratory/pilot studies as a base. However, there are some difficulties in performing sub-studies in larger cohorts' studies. First, in studies where the material and data

are already collected the design are set, which set the boundaries for what can be analyzed. Second, in prospective studies with a broad approach in collection of material and data, limitations for what is collected needs to be set to be able to handle both the practical collection of samples as well as entering data into a database.

Performing clinical studies in the perinatal period can be challenging since it is difficult to know when birth will occur. To be flexible when recruiting as well as to be sensitive for participants needs and facilitate in timing for making appointments and collecting samples are crucial in longitudinal studies. Having a team collaborating in these aspects are an important factor in both recruiting participants and maintain participation throughout the study period.

Collection, handling and storing of samples may differ between studies and protocols with standardized operation procedures are important within the study to be able to make comparisons. There are many factors that may affect the results of the analyses which needs to be considered when comparing between different studies. In human milk studies, a standardized approach is needed to better determine human milk composition and health outcomes(225). Furthermore, there is also a need to study human milk as a biological system and not only the contribution of individual components in milk composition(226). A multidisciplinary panel of researcher have identified several prioritized research areas related to breastfeeding and the origin of health(227).

However, due to the small sample sizes and explorative design, larger studies designed to answer these questions are needed. Additionally, the method for determine AF in plasma has been under development during the period of the thesis. This method development has been important, for example a reduction of the amount of human milk or plasma needed for analyses which allow analyses also in vulnerable populations as after preterm birth. Further development is under way to develop a standard enable comparison between batches to allow larger studies.

7 CONCLUSIONS

Altogether the findings in the thesis suggest that AF may be involved in the complex pathology of preterm birth, and that AF in human milk may be a part of the bioactive components involved in protecting preterm infants against inflammation and infection. The results of the thesis contribute to the most comprehensive description of antisecretory factor in the perinatal period to date.

In conclusion:

- AF follows a basic pattern in the postnatal period where AF levels in maternal plasma are reflected in breastmilk and the levels of AF in breastmilk decrease with time after birth.
- In MOM, the highest levels of AF were found in colostrum, strengthening the importance of an early start of breastmilk feeding.
- Higher AF levels in MOM in the first week after birth were associated with less inflammatory complications, specifically sepsis, in the infant. This finding highlights the importance of supporting mothers to an early start of breastmilk stimulation to provide colostrum for the infant.
- The findings of less adverse outcome in preterm infants related to higher AF levels in longitudinal samples of MOM, further strengthen the need of lactation support for mothers of preterm infants to be able to initiate and maintain a milk supply, due to a more complicated start of lactation.
- The finding of an association between higher AF levels in MOM and less inflammatory complications and infection, in preterm infants may be a base for intervention studies with AF aiming to increase levels in MOM to prevent inflammatory processes in the infant.
- Holder pasteurization of donor milk can be safely performed without concern that it may destroy AF, and preserved AF may be a part of the explanation for the beneficial effect of DHM related to inflammatory complications and supports the use in the NICU when mothers own milk is not available or reaching the infant’s needs.
- The finding that lower levels of AF in both placenta and breastmilk are associated to more inflammation supports our hypothesis that AF has a role in the regulation of inflammation and warrant further investigation of mechanisms involved.

8 POINTS OF PERSPECTIVE

Globally, PTB is the leading cause of neonatal morbidity and mortality. The pathophysiology is multifactorial and not yet fully understood. There is a need for predictive biomarkers and preventive treatments for PTB itself and for inflammatory complications in preterm infants. Human milk is rich in bioactive factors and have preventive effects on adverse outcome in preterm infants. However, lactation may be compromised by inflammatory conditions like mastitis or breast infections, leading to decreased milk supply and cessation of milk production.

The focus for this thesis has been to, for the first time, perform basic studies on AF in the perinatal period to investigate if AF may play a role in inflammatory complications related to term and preterm birth, human milk, and lactation, as well as infant outcome. The studies have been exploratory and aimed to be a basis for further studies, with focus to explore the mechanisms of AF in the perinatal period related to other factors involved in the pathogenesis of inflammatory complications. Since these complications are multifactorial and AF has been described to be involved in several pathways, it would be of interest to include AF in analysis on a system level related to maternal and infant microbiome, other bioactive factors, and macronutrients in human milk, within the perinatal period.

The significance of these novel findings implicates that AF may be involved in the complex pathophysiology related to inflammatory complications in the perinatal period and may be a base for further studies on mechanisms and interventions. Since effective prevention and treatments are lacking, AF may present as a possible modifiable factor to reduce inflammation and improve health in the perinatal period. Furthermore, since there are AF inducing therapies as well as active AF available, intervention studies may be of interest.

Further method development for AF analyses may facilitate comparison between larger sample sizes, and work is ongoing to develop standards and to be able to determine concentrations of AF-compleasome. The continued work on method development is also aiming to make the analyses assessable in the clinical setting not only for research.

Clinical research can be complex and difficult, but also very interesting and rewarding. Collaboration in a research team including clinicians with different specialties, laboratory technicians, statisticians and researchers improves clinical research in practice and broadens the scientific ideas and discussions.

There is still a lot to learn about the mechanisms of AF in the perinatal period and these novel findings implicate further research to be of interest.

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