Staphylococcus epidermidis - virulence factors and innate immune response

Contents

List of papers

Paper I

Hildegunn Norbakken Granslo, Claus Klingenberg, Elizabeth Gladys Aarag Fredheim, Arild Rønnestad, Tom Eirik Mollnes, Trond Flægstad. *The Arginine Catabolic Mobile Element is associated with low antibiotic resistance and low pathogenicity in Staphylococcus epidermidis from neonates.* Pediatric Research 2010; 68: 237-41

Paper II

Elizabeth G. Aarag Fredheim, **Hildegunn Norbakken Granslo**, Trond Flægstad, Yngve Figenschau, Holger Rohde, Irina Sadovskaya, Tom Eirik Mollnes, Claus Klingenberg. *Staphylococcus epidermidis Polysaccharide Intercellular Adhesin Activates Complement* FEMS Immunology and Microbiology 2011; 63: 269–280

Paper III

Hildegunn Norbakken Granslo, Claus Klingenberg, Elizabeth Aarag Fredheim, Ganesh Acharya, Tom Eirik Mollnes, Trond Flægstad. *Staphylococcus epidermidis biofilms induce lower complement activation in neonates compared to adults.* Submitted to Infection and Immunity October 27th 2011.

Appendix

Hildegunn Granslo, Karianne W. Gammelsrud, Elizabeth A. Fredheim, Trond Flægstad, Claus Klingenberg. *Coagulase-negative staphylococci- biofilm and antibiotic resistance.* Tidsskr Nor Laegeforen. 2008; 128: 2746-9. In Norwegian

Abbreviations

Aap	Accumulation associated protein
ACME	Arginine catabolic mobile element
AMP	Antimicrobial peptides
CARS	Compensatory anti-inflammatory response syndrome
CoNS	Coagulase-negative Staphylococci
CRP	C-reactive protein
DAMP	Danger-associated molecular pattern
DNA	Deoxyribonucleic acid
e.g.	Exempli gratia
GA	Gestational age
Ica	Intercellular adhesion
IS	Insertion Sequence
MBL	Mannose-binding lectin
MSCRAMMs	Microbial Surface Components Recognizing Adhesive Matrix Molecules
NEC	Necrotizing enterocolitis
Orf	Open reading frame
PAMP	Pathogen-associated molecular pattern
PIA	Polysaccharide intercellular adhesin
PRM	Pathogen recognition molecule
PRR	Pathogen recognition receptor
SIRS	Systemic inflammatory response syndrome
TCC	Terminal complement complex
TLR	Toll-like receptor

1. Introduction

1.1 Clinical relevance of the thesis

S. epidermidis rank first among the causative agent of nosocomial infections, and accounts for more than 50% of the late-onset sepsis episodes in neonates. *S. epidermidis* often cause infections in immune-compromised patients

Biofilm formation is the most important virulence factor of *S. epidermidis*. Its relevance has risen the past decades with the increased use of indwelling medical devices such as vascular and peritoneal catheters, prosthetic joints, heart valves and vascular grafts.

The frequency of *S. epidermidis* infections is increasing, mainly due to concurrent advances in medical practice with more people undergoing and surviving intensive care treatment, acquiring prosthesis, and the increased survival of patients with a compromised immune system, such as preterm neonates and HIV patients.

Although *S. epidermidis* infections only rarely develop into life-threatening diseases, they significantly increase morbidity in the affected groups. Their frequency and the fact that they are extremely difficult to treat, represent a serious burden for the public health system.

Therefore, increased knowledge of *S. epidermidis* virulence factors and their impact on the innate immune system is important to develop new methods to fight these infections.

1.2 Innate immune system

"Emergence of complex life was paralleled by immunologic demarcation against primitive organisms. Therefore worms, plants, and vertebrates share components of the innate immune system at the molecular level" (1)

The human immune system can be divided into two main parts, the innate ("the one we are born with") and the adaptive ("the one we acquire") (Figure 1). The hallmarks of the adaptive immune response are specificity, inducibility, discrimination of self vs. non-self and memory. The adaptive immune system will not be extensively covered in this thesis.

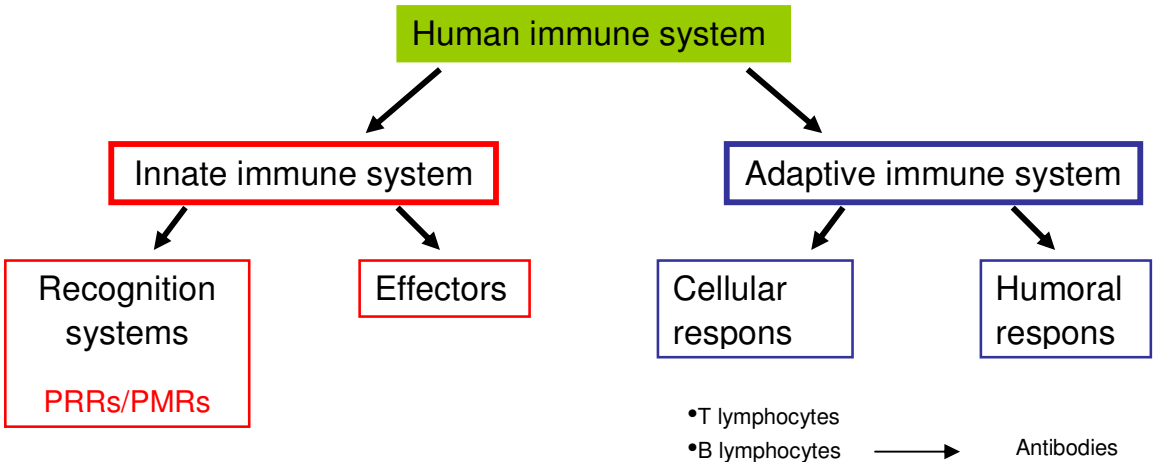


Figure 1: The human immune system, divided into the innate- and the adaptive immune system.

The innate immune system, also known as the "non-specific immune system", comprises cells and mechanisms that defend the host from danger. Components of the innate immune system recognize danger and responds with different effectors, but unlike the adaptive immune system, it does not confer long-lasting or protective immunity (2). The response of the innate immune system depends on recognition of i) pathogen-associated molecular patterns (PAMPs) signaling exogenous danger e.g. microbial antigens/surfaces **or** ii) damaged tissue in the host (damaged self), called alarmins (3). These structures are collectively called danger-associated molecular patterns (DAMPs) (4). The effectors of the innate immune system consist of components that are functional at all times (skin and mucus barrier, **antimicrobial peptides (AMPs**), normal bacterial flora, skin and mucosal pH) and the inducible components (**cells, complement and cytokines**). Typical for the inducible components of the innate system are the non-specific effectors and the rapid response (within minute to hours) after activation. In this thesis I will describe some of the main **recognition systems** and **effectors** (Figure 2) of the innate immune system, and particularly focus on their role in the innate immune system of neonates.

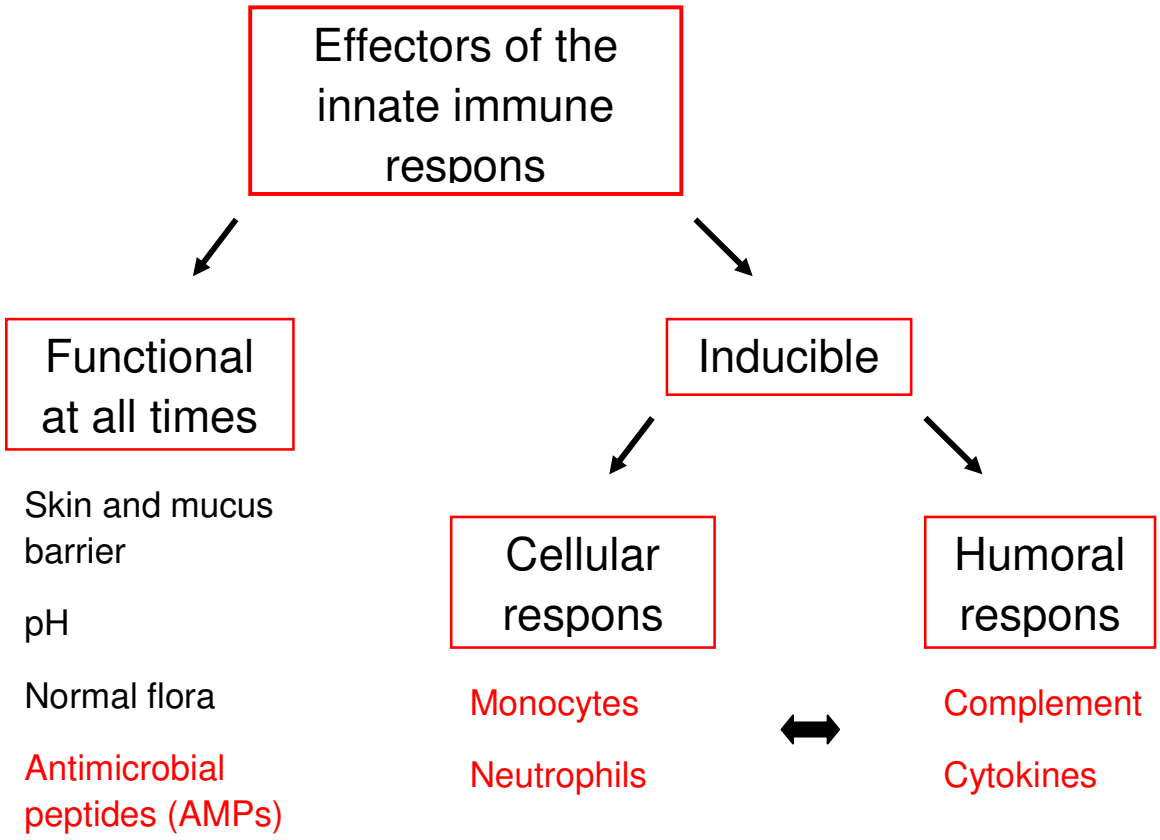


Figure 2: Effectors of the innate immune system. The effectors in red writing are specifically discussed in this thesis.

Specific aspects in neonates

"Birth probably constitutes the most important change of environment of our lifetime – the transition from a sterile intrauterine existence through the colonized birth canal into a largely peaceful coexistence with a myriad of microbes, both commensal and potentially invasive pathogens" (5).

For neonates to survive the transition from the sterile intrauterine life to the world "outside", their immune system needs to co-exist with the commensal bacteria, but still be able to recognize and fight the dangerous pathogens (5). Many components of the innate and adaptive immune system are not fully developed at birth. Skin and mucus membranes of neonates, especially those born preterm, are fragile (6, 7). The dramatic increase in number of bacteria on the skin and mucus membranes during the first days of life, make them more susceptible to invading bacteria by the dissemination of pathogens from the colonizing surface (8-10). Important for the initial development of both the innate and adaptive immune responses is the exposure to environmental antigens after birth (11). Repeated prenatal and postnatal exposure to environmental microbial products activating the innate immune system may accelerate the maturation process of the system (12).

Table 1: Timing of acquisition of a **mature** innate immune system

Function	Premature	Term	<1 year	1-2 years	2-5 years	5-10 years	10-20 years
Barriers							
Pathogen recognition							
Complement							
Cytokine production							
Phagocytosis							

Modified from (13)

The dark grey areas indicate the estimated age range at which near-adult-level function is attained. Light grey areas indicate that there are conflicting results in the literature (see cytokine section).

1.2.1 Recognition - Pathogen recognition receptors (PRRs)

Pathogen recognition receptors (PRRs) are recognition molecules bound to cell surfaces on both leukocytes and other cells (14, 15). However, as these important recognition molecules also can be found in soluble form they are sometimes coined pathogen recognition molecules (PRMs). PRRs recognize conserved microbial antigens such as lipoproteins and lipoteichoic acids, on the bacterial surface and alarmins from damaged self, collectively called DAMPs (16). Binding of DAMPs to PRRs activates downstream signaling cascades leading to activation of the different effectors of the innate immune system.

Toll-like receptors (TLR) were the first PRRs to be described by Hoffmann and co-workers in 1996 (17). TLRs belong to a large group of transmembrane receptor proteins expressed on the surface of different cells of the immune system or intracellular on endocytotic vesicles or organelles (18, 19). There are 10 known human types of TLR, recognizing different PAMPs, DNA and RNA in a variety of microbes (20-24). TLR activation leads to up-regulation of phagocytosis, maturation of leukocytes and cytokine release (16, 22). TLR-2, mainly distributed on the cell surface of neutrophils, monocytes and dendritic cells (22, 25, 26), plays a key role in the recognition of Gram-positive pathogens. TLR-2 alone or with co-factors, TLR-1, TLR-6 or CD14 (27), may detect Gram-positive cell wall components (e.g. peptidoglycan and lipteichoic acids), polysaccharide intercellular adhesin (PIA) and phenol-soluble modulins (PSMs) (28-33). TLR-4 and its co-factors, mainly recognize Gram-negative bacteria and lipopolysaccharides (LPS) (34, 35).

Additional recognition pathways of Gram-positive bacteria include β-integrins (36, 37), lectins (38), CD36 (39), and Nucleotide oligomerization domain proteins 1 and 2 (NOD1 and -2) (40,

41). There is cross-talk between the different groups of PRRs when recognizing bacteria, viruses and fungi (22, 42).

Specific aspects in neonates

Term-born neonates have a basal TLR–expression comparable to adults (43-45), while preterm neonates display reduced expression of some TLRs, such as TLR-4 (43, 46). Although the basal level of TLR-expression in term–born neonates is similar to adults, the downstream signaling cascades after the binding of agonists may be diminished, e.g. reduced production of multiple cytokines simultaneously (5, 45, 47-49).

TLR-4 and TLR-2 polymorphisms have been associated with a higher risk of preterm birth. These polymorphisms probably lead to increased rates of (subclinical) maternal and/or fetal infections triggering inflammatory processes that ultimately lead to preterm birth (50, 51).

1.2.2 Effector - Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) consist of 12-50 amino acids, and are secreted by epithelial cells, neutrophils, thrombocytes etc. (52). They are major players of the innate immune response in all living species (52)**.** Their immunological effects comprise initial lysis of bacteria, mast cell degranulation and histamine release, chemotaxis of neutrophils and T-lymphocytes, promotion of non-opsonic phagocytosis, inhibition of fibrinolysis by tissue plasminogen activator, and wound healing through fibroblast chemotaxis (52, 53). Most AMPs are solely “membrane active”, while a few also carry out enzymatic functions (54-57).

Most AMPs are cationic (58). Examples of major classes of cationic AMP in humans are; the defensins and cathelicidins produced and secreted by neutrophils and several other cells, and thrombocidins which are only released from platelets (52). The membrane effects of the cationic

AMPs are probably driven by electrostatic interactions with the negatively charged outer layer of the bacterial membrane (59-62).

Lactoferrin is a cationic AMP that has been widely studied. Lactoferrin is localized in the secondary granules of neutrophils as well as tear fluid, saliva and especially breast milk (63). It is found on the mucosal surface where it is a prominent component of the first line of host defense against infection (64, 65). Lactoferrin deprives microorganisms of an essential nutrient by binding iron (66), but it can probably also exert a directly microbicidal effect, presumably via membrane disruption or by regulation of different parts of the innate immune system (67-69). Bovine lactoferrins are effective against both Gram-positive and -negative organisms, including biofilms of *S. epidermidis* (70-73).

Anionic human AMPs are less common, but an example is the proteolytic product of dermicidin, DCD-1L, found in sweat (74).

AMPs and synthetic derivates of AMPs are currently under investigation for clinical application due to their antimicrobial effects (75-80).

Specific aspects in neonates

AMPs in neonates are detectable in early gestation, and levels generally show a positive correlation to GA (5). Still, there are generally reduced levels of AMPs in cord blood of both preterm and term born neonates (81).

The therapeutic use of oral bovine lactoferrins with or without adding probiotica (*Lactobacillus rhamnosus* GG) to prevent late-onset sepsis and the development of necrotizing enterocolitis (NEC) in preterm neonates have shown promising results (9, 82). The Cochrane collaboration

recently published two reviews on this topic and concluded that i) “Oral lactoferrin prophylaxis reduces the incidence of late-onset sepsis in infants weighing less than 1500g, but found no evidence of efficacy of oral lactoferrins in the prevention of NEC” (83), and ii) “Currently there is no evidence to recommend or refute the use of lactoferrin for the treatment of neonatal sepsis or NEC as an adjunct to antibiotic therapy” (84). Further studies regarding safety and efficacy of different preparations, dosage, long term safety, interactions with probiotics and human milk are needed before implementing oral lactoferrin as standard prophylactic care (9, 82, 84).

1.2.3 Effector - Complement

The complement system is a cascade system of more than 30 proteins in plasma and on cell surfaces (85). The system was called the complement system because it was first identified as a heat-labile component in serum “complementing” the heat-stabile antibodies killing bacteria (86). Many of the complement proteins circulate as pro-enzymes awaiting activation in order to further activate other proteins. There is a constant auto-activation of some of the factors, such as C3, but as long as this is balanced by the inhibiting factors, the full cascade will not “run”. A number of soluble and cell-bound regulatory proteins act to inhibit the complement system, keeping it under tight control (87) However, once the level of activated complement factors reaches a certain threshold (“the point of no return”), the full cascade is initiated. The main functions of the complement cascade are: i) defense against bacterial infections, ii) bridging innate and adaptive immunity, and iii) deposition of immune complexes and the products of inflammatory injury (85). There are three main initial complement pathways which usually distinguishes self from non-self targets (Figure 3).

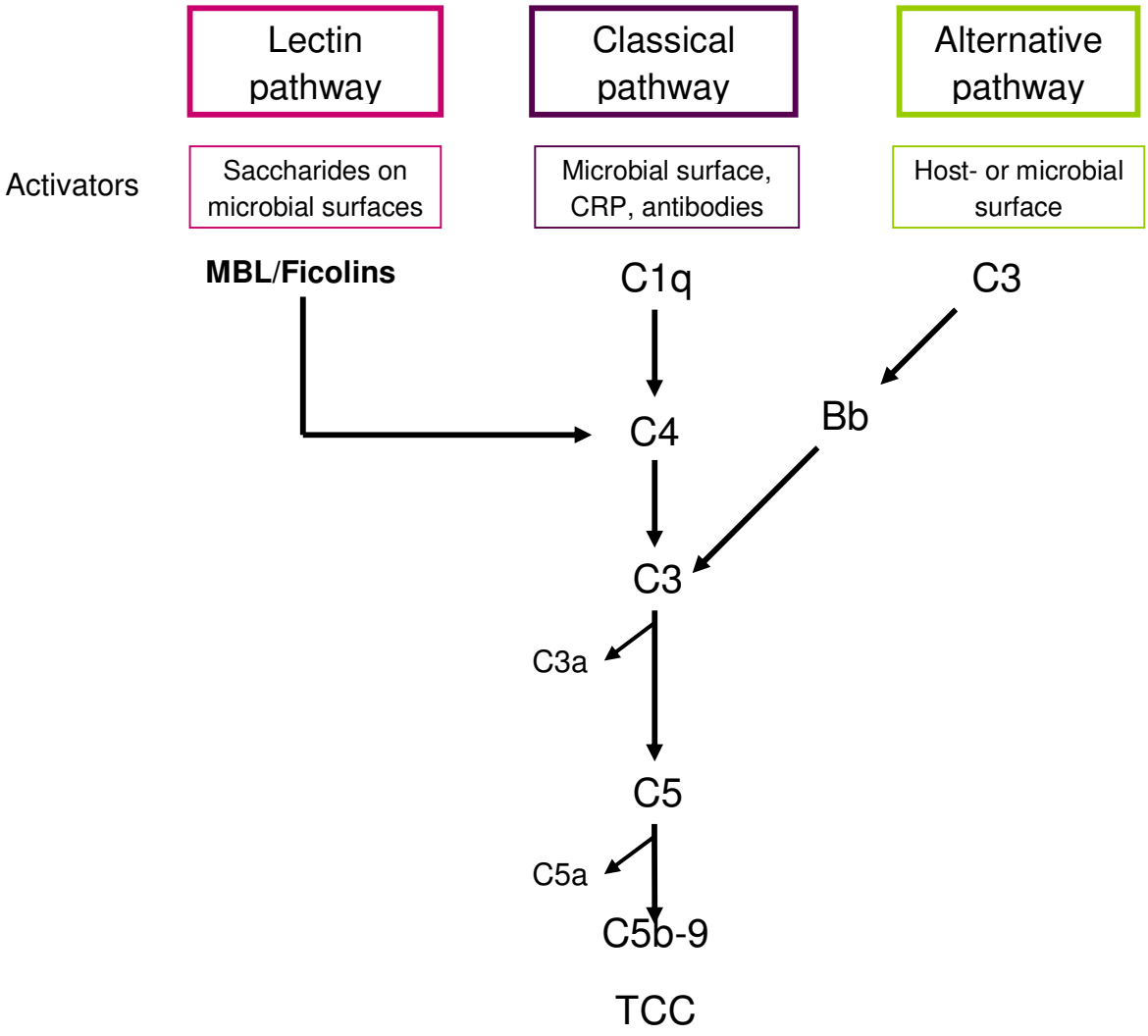


Figure 3: The complement cascade with its three initial pathways; the lectin pathway, the classical pathway and the alternative pathway. All three initial pathways converge in the final common pathway.

The *classical pathway* can be initiated by three mechanisms; i) antibodies recognizing a microbial target with subsequent binding to the complement component C1q creating a complex (C1qrs), ii) binding of bacterial surface structures directly to the C1q component or iii) binding of C-reactive proteins (CRP) to bacterial surfaces and C1 (85, 88-90). Immunoglobulin M and G

(IgM and IgG) are the only groups of antibodies that are able to activate the classical pathway (91). Among the IgG subgroups, IgG1 and IgG3 activate complement most efficiently, while IgG2 is less efficient (92). Furthermore, immunoglobulins can influence the complement cascade in many steps, both as activators and inhibitors, indicating their important role in maintaining homeostasis in the inflammatory response (91).

The *lectin pathway* is initiated by recognition of specific patterns composed of saccharides on microbial surfaces. The recognition molecules of this pathway are the acute phase proteins, mannose-binding lectin (MBL) and ficolins (93, 94). From C4 the classical and lectin pathway share the same route.

The *alternative pathway* is activated by spontaneous cleavage of C3. However, the alternative pathway will only proceed when non-self surfaces (without inhibitory factors) or "own" surfaces lacking inhibiting factors (such as some tumor cells) are close to the cleaved components. Activators of the alternative pathway are lipids, carbohydrates and proteins (95-97). Another important function of the alternative pathway is amplification of the final complement response initiated by the other two pathways (98, 99). Recently, the alternative pathway has also been suggested to control and balance other parts of the innate immune system (100).

All three pathways converge at C3 and follow the same final pathway, from where the main inflammatory components are created. The end-product of the complement cascade is the terminal complement complex (TCC). TCC exists both in the fluid-phase and inserted into membranes where it is often called the membrane-attack complex (MAC) (101). The TCC is important in the defense against Gram negative bacteria, such as *meningococci* (102). In Gram

positive bacteria, however, the thick peptidoglycan cell wall is resistant to TCC-induced lysis (103).

There are several enzymes involved in the cascade, such as the C3 convertases created in the classical and lectin pathways, and the alternative C3 convertase created in the alternative pathway. Both these enzymes cleave C3 creating C3a (anaphylatoxins) and C3b (opsonin). After cleaving C3, the C3 convertase may bind to C3b and thus become a new convertase, the C5 convertase. The C5 convertase cleave C5 creating C5a (anaphylatoxins) and C5b (part of TCC) (85, 104).

The tree main inflammatory effects of complement are: i) recruitment and activation of inflammatory cells by the anaphylatoxins C3a and C5a, ii) microbial opsonization and phagocytosis by the opsonic effect of C3b interacting with complement receptor 3 (CR3) and iii) direct lysis of targeted pathogens by the C5b-9 terminal complement complex (TCC) (105).

C5a

C5a, an 11-kDa glycoprotein (106, 107), is one of the most potent pro-inflammatory peptides in the human immune system. C5a has a wide variety of functions, such as chemotaxis (108, 109), degranulation of inflammatory cells (108, 110, 111), enhancement of respiratory burst (112, 113), delayed neutrophil apoptosis (114), up-regulation of the expression of adhesion molecules (115, 116), induction of the pro-inflammatory cytokine secretion (117-121), vasodilatation and enhancement of vascular permeability (122-124) and smooth muscle contractions (122, 123). C5a exerts its effects mainly through the C5a receptor (C5aR), but can also act through the G-protein-uncoupled receptor of C5a, the C5L2 (negative modulator of C5aR) (125-127). C5a may also be initiated independently of the general complement cascade (128-131). Factors influencing C5a

levels in plasma are: i) generation of C5a independent the complement cascade, ii) degradation (availability and accumulation status of C5a degrading enzymes), and iii) number of C5a receptors (100, 132).

C3a

C3a is a less potent anaphylatoxin and mediates most of its effects after binding to C3aR, a receptor found on most hematopoietic cells (133). The main effects are chemotaxis (108, 109), granule release (109, 111), expression and shedding of adhesion molecules (116), increased oxidative burst in both neutrophils and eosinophils (134, 135) and modulator effects on the immune system by regulating the production of some cytokines (136, 137).

Still, even though the anaphylatoxins are important in the inflammatory response in order to remove "danger", an excessive generation of C3a and C5a may contribute to tissue damage in conditions such as sepsis (107, 138, 139), ischemia (140), arthritis (141, 142), and adult respiratory stress syndrome (143). The complement system has also a significant importance in the pathogenesis of membrano-proliferative glomerulonephritis, age-related macula degeneration and some autoimmune diseases (85, 144, 145). In all these above mentioned disorders, the complement system is performing its normal function, but the activation has occurred under inappropriate circumstances or under lack of tight regulation.

The C3 and C5 level in the final common pathway may be key-points for therapeutic modulation of the complement activation (146, 147). However, in the final common pathway only modulation of the complement cascade at the C5 level is in clinical use today. The C5 monoclonal antibody, eculizumab (Soliris®), is used for treatment of paroxysmal nocturnal

hemoglobinuria (PNH) and also for treatment of atypical variants of hemolytic uremic syndrome (HUS) (146-149). During the recent (summer 2011) outbreak of *Escherichia coli* O104:H4 in Germany, case-reports indicate a good therapeutic response of eculizumab in severe shiga-toxin-associated HUS (150).

Specific aspects in neonates

There is no transfer of complement factors across the placenta (151). The fetus begins synthesizing complement proteins at 6-14 weeks of gestation (152). However, the major development of the complement system in the fetus occurs late in the pregnancy, and complement factors does not reach adult levels until after birth (153). McGreal et al. recently published a paper summarizing the current knowledge on the complement system in term and preterm born neonates (154).

Compared to adults, the levels of complement factors in the *classical pathway* vary between ~50-80% in term infants and ~30-80% in preterm infants (153-157). The levels of some of the factors in the classical pathway, such as C1q and C4, are clearly correlated to the gestational age (153, 156, 158, 159). Davis et al. followed the classical complement factors during the first months of life and showed that C1q remained low the first 6 months, while C2 and C4 reached adult levels during the first months of life (160).

Compared to adults, the levels of complement factors in the *alternative pathway* vary between ~40-65% in term infants and ~50-60% in preterm infants (153-158, 161, 162). The only exception is factor D that seems to show higher titers in term infants than in adults (155, 163).

Levels of factor B (153, 164) and properdin (161) reach adult levels at around 6 months of age (factor B) or later (properdin) (160).

Functional studies of both the classical and alternative pathway show lower activity in both preterm and term infants compared to adults, ranging from ~30-80% for the classical pathway and ~40-70% for the alternative pathway (153, 155, 156, 161, 162).

MBL-genotypes associated with low MBL levels are the most common immune deficiency in the population, affecting up to 25 % Caucasians (165-168). Low-levels of MBL are associated with higher risk for neonatal sepsis and a longer duration of antibiotic treatment (169-173). In neonates without the low-level MBL genotypes, the MBL levels increases rapidly in the first week of life, reaching its highest level by one month of age (174). The ficolin level in neonates, especially L-ficolins, is lower in neonates compared to adults (169, 175, 176). Low L-ficolin levels are associated with low birth weight and increased risk of infections (169).

It is estimated that C3 levels in term infants are ~3/4 of the levels found in adults, and that C3 levels in preterm infants are even lower (154). By the age of 6 months the C3 levels reach adult levels (160).

The levels of many of the factors in the final common pathway are also lower in neonates compared to adults (155, 159, 160, 163). Especially factor C9 seems to be less than 20% of adult levels (159, 177, 178). In contrast, C7 levels are similar in neonates and adults (155). Neonates have also lower levels of the complement inhibiting factors compared to adults (155, 160, 163, 179, 180).

Complement activation is of often triggered by infections (102, 181-183). Meconium is also a strong activator of the complement cascade (184-186). Furthermore, the complement system is

activated in the cord blood of neonates born with significant acidosis (152, 187). Evidence indicates that complement activation is one of the pathologic mechanisms contributing to ischemia-reperfusion injury in the post-hypoxic-ischemic neonatal brain (188).

Immunoglobulins, especially IgG, probably participate in the down-regulation of complement attacks on host tissue, by controlling complement binding to target tissues or cells (189). Neonates in general and particularly preterm infants have lower levels of immunoglobulins (see also paragraph 1.2.6 Antibodies in neonates), leading to a reduced capacity to control complement activation (190, 191). Studies show that both the brain and lungs of neonates are vulnerable to damage caused by the complement cascade (192-194).

The lower complement factor concentration in neonates may theoretically contribute to decreased innate immunity of the newborn infant, through its role in chemotaxis, opsonization, and cross-talk with the adaptive immune system. In general, complement deficiencies early in life increase the risk of infections both in preterm and term neonates (159, 169-171, 178). However, occasionally lower complement levels may be a distinct advantage with fewer pathophysiological effects of an uncontrolled complement activation causing severe tissue damage (154, 195).

1.2.4 Effector - Leukocytes

Leukocytes are the “immune cells” of the body. There are several different groups of leukocytes with different functions, and all are produced in the bone marrow. Only monocytes/macrophages and neutrophils will be further reviewed in this thesis.

Monocytes continuously mature into macrophages, leave the circulation and migrate into tissues throughout the body, not only in association with inflammation. They are often the first cells to encounter a pathogen and belong to the group of cells called antigen presenting cells (APC).

Neutrophils are short lived cells, abundant in blood, and not present in normal tissue without an infection (196). They are the primary mediators of the innate cellular responses and important in the hosts defense especially against bacterial infections (25, 196, 197).

Both macrophages and neutrophils amplify cellular recruitment through the production of inflammatory mediators (cytokines), and they ingest and kill microorganisms by phagocytosis (25, 198).

Activated leukocytes express certain proteins on their surfaces, such as **CD11b/CD18.** CD11b/CD18 is also called the CR3 receptor. CD11b/CD18 function both as adhesion molecules and complement receptors binding C3b, and thus stimulates recognition and phagocytosis of the observed danger (199).

Phagocytosis is an active process where leukocytes "eat and kill" pathogens. Once a phagocyte recognizes a pathogen by a bound opsonin (such as C3b or antibodies) the pathogen is internalized and killed through lowering the pH in the phagocyte, release of enzymes degrading the pathogen or production of toxic molecules e.g. by the induction of oxidative burst (25). **Oxidative burst** is a process inside phagocytes where enzymes consume O_2 in the cell to produce toxic chemicals such as hydroxyl radicals ($OH^{-)}$, hydrogen peroxide and superoxide (O2-) (25, 104, 192, 200).

Accumulation of leukocytes to the site of the infection through chemotaxis is important to mediate an adequate immune response.

Specific aspects in neonates

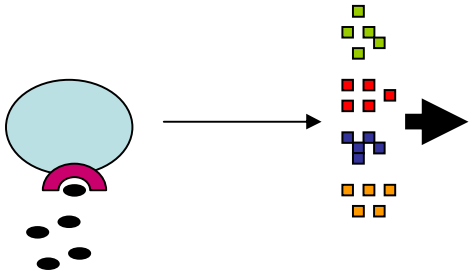
There is both a qualitative and quantitative impairment of the cellular response in neonates. A diminished precursor storage pool of both neutrophils and monocytes in the neonatal blood, and a reduced ability of the bone marrow of the newborn child to efficiently up-regulate the production of leukocytes during an inflammation cause a quantitative defect in the cellular innate immune response (201-204). There are also several qualitative impairments of neutrophils, such as deficiencies in the ability to accumulate leukocytes at the site of the infection (reduced chemotaxis, rolling, adhesion and transmigration) (204-208), reduced expression and function of surface molecules (such as CR2 and L-selectin) (209, 210), and reduced microbiocidal function (such as reduced up-regulation of oxidative burst) (206, 207, 211, 212). Neonatal neutrophils also respond differently to G-CSF and CM-CSF compared to adults. This may be of importance when considering treatment with CSF to improve the immune function of preterm infants (213). They also display different responses to other stimuli, such as hyporesponsivenes to LPS stimulation, maybe due to failure of TLR-4 up-regulation (214).

Macrophages do not have reduced phagocytic and intracellular killing capacity in term infants, but their capacity to further amplify the signal and activate the adaptive immune system seems to be diminished (215, 216).

1.2.5 Effector - Cytokines

Cytokines are small proteins synthesized and secreted from a variety of immune cells (e.g. monocytes, lymphocytes, neutrophils) and non-immune cells (e.g. endothelial cells) (217, 218). Cytokine production/secretion is most often mediated by binding of an agonist to a TLR (197) or other PRRs. This leads to transmission of signals through intracellular messenger systems,

activating transcription factors and inducing a change in gene expression (217, 218). Cytokines may act in an autocrine manner, affecting its own behavior, in a paracrine manner, affecting the adjacent cells, or in an endocrine manner, affecting the behavior of distant cells (25). Generally the functions of cytokines may be divided into one or more of the groups summarized in Figure 4.



Cytokine function	Example cytokines
I) Growth and differentiation factors	G-CSF, GM-CSF, IFNγ, FGF
II) Alarm signals (acute phase response)	TNF-α, IL-6, IL-1β
III) Chemotaxis	IL-8, MIP-1 α, IP-10
IV) Modulators of immune cells	IL-6, IFNγ, MIP-1 α
V) Tissue modeling	MIP-1 α
VI) Temperature regulation	IL-6, IL-1β, TNF-α
VII) Cell survival	IL-1β

(25, 219)

Figure 4: Pathogens bind to PRRs on the leukocyte surface, inducing secretion and new production of cytokines. The functions of cytokines can broadly be divided into 7 different groups. Each cytokine can have several different effects.

A vigorous production of pro-inflammatory cytokines in addition to the complement anaphylatoxins as a response to a microbes entering the blood stream, may lead to a systemic

inflammatory response syndrome (SIRS) (220). SIRS is a clinical entity with the following major symptoms in adults; High or low body temperature (<36 °C or >38 °C), heart rate > 90/min., respiratory rate >20/min (or PaCO2 < 4.3 kPa), and a high or low leukocyte count (<4x10^9/L, >12x10^9/L, or 10% bands) (220). Sepsis is a diagnosis used in patients with SIRS plus growth of bacteria from blood culture (221). The pro-inflammatory cytokines secreted during sepsis probably also induce secretion of anti-inflammatory cytokines attempting to limit inflammation (222-224). This anti-inflammatory cytokine secretion combined with expression of cytokine antagonists (such as TNF receptors, IL-1Ra) (223, 225, 226) are referred to as the compensatory anti-inflammatory response syndrome (CARS) (223, 227). A misbalance between the pro- and anti-inflammatory cytokine responses may either lead to an inflammatory catastrophe for the patient (too strong pro-inflammatory response) or failure to clear the infection (too strong anti-inflammatory response).

Specific aspects in neonates

Data in the literature are conflicting regarding the nature of the neonatal cytokine response to common pathogens and inflammatory conditions. Some authors describe the pro-inflammatory cytokine response in term infants (228-232) and preterm infants (232-234) as either equal or lower than that found in adults. A lower pro-inflammatory cytokine response is considered important to avoid alloimmune reactions between mother and fetus (235) and to make the transition from the sterile intrauterine life into the symbiosis with colonizing bacteria as smooth as possible (236, 237). However, a lower pro-inflammatory cytokine response may also render the neonate more susceptible to infections (201).

In contrast, other authors describe an increased pro-inflammatory cytokine secretion in response to infective stimuli has been described both in term and preterm infants (11, 238-242). The secretion of anti-inflammatory cytokines (e.g. IL-10 and TGF) is reduced in both term and preterm neonates (11, 224, 239, 243-245). These studies suggest that the pro-inflammatory cytokine production is adequate, but the compensatory anti-inflammatory response is diminished.

Several studies indicate an age-correlated maturation of cytokine production (246-249), representing a gradual development of the cytokine response, varying from cytokine to cytokine (Table 1).

Pro-inflammatory cytokines are important to eradicate an infection, but there is also growing evidence that this inflammatory response in neonates (especially in preterm neonates) plays a major role in the induction of several neonatal diseases of the brain, retina, lungs etc. (244, 250-254). The imbalance between the pro- (SIRS) and anti-inflammatory (CARS) cytokine response in preterm infants may to some extent explain the detrimental consequences of sepsis in preterm infants (13, 223, 224, 239, 250, 251).

Both the pro-and anti-inflammatory cytokine response in neonates (both term and preterm) vary depending on the pathogen involved (*E. coli,* Gr. B *streptococci, S. epidermidis*) (11, 238).

Variations in the findings of cytokine response in neonates, may also to some extent represent differences in experimental designs and assays used for cytokine detection (preparation of blood samples, types of stimulators, cells studied, duration of incubation of blood samples etc.). This must always be taken into account when interpreting the research finding.

1.2.6 Antibodies in neonates

Antibodies are produced by B-lymphocytes, cells in the adaptive immune system, as an adaptive response to an infection. The adaptive immune system is not generally covered in this thesis. However, as antibodies interact with the innate immune system, I will give a short description of their function in neonates. The fetus produce very little antibodies themselves due to immaturity in the adaptive immune system, poor signaling between the innate and the adaptive immune system and fetal life in a sterile environment (192). However, IgG antibodies (not IgM, IgA, IgE and IgD) are actively transported across the placenta in the last trimester of the pregnancy (192). Despite this active transfer, of antibodies, the total IgG level is lower in neonates (cord blood) than in adults (255). Furthermore, differences in transport kinetics between the IgG subclasses may cause quantitative differences in titers of IgG subclasses (191, 256), e.g. IgG1, IgG3 and IgG4 are fairly efficiently transported across placenta whereas transport of IgG2 is less efficient (256, 257). Titers of maternal IgG antibody decrease gradually during the first months after birth.

Preterm infants are often born before the active placental transfer of antibodies has occurred, and very low antibody titers in preterm neonates increase the risk for infections (258-260). Consequently, several clinical trials have investigated the effect of pooled intravenous immunoglobulin (IVIG) administration for prevention or treatment of neonatal sepsis (261-264). Many of these trials were small and of poor quality. It has therefore for a long time been difficult to make a clear recommendation whether immunoglobulins should be a part of sepsis treatment in neonates or not. In September 2011 the results of a large randomized controlled trial (INIS trial) including more than 3000 infants was published.(265) This study showed that therapy with IVIG had no effects on the outcome of suspected or proven sepsis (265).

1.2.7 Inflammation

When danger is observed by the recognition systems, the different effectors are activated and they collectively create inflammation. Inflammation is a coordinated process induced by microbial infection or tissue injury, initiated by the innate immune recognition system (266, 267). The inflammatory response activated by an infectious agent has traditionally been classified in 4 phases: i) recognition of infection ii) elimination of the microbe, iii) resolution of the inflammation, and vi) return to homeostasis (267, 268). The ideal inflammatory response is rapid and destructive, but also specific and self-limiting.

The first local inflammatory response starts within minutes after the microorganism has invaded the host or tissue damage has occurred (25). Macrophages (**leukocyte**) and the **complement cascade** respond quickly and inflammatory mediators such as **cytokines** and complement **anaphylatoxins** are created, followed by activation of other cellular and humoral parts of the innate immune system. There are five clinical signs of local inflammation; redness (rubor), warmth (calor), pain (dolor), swelling (tumor) and reduced function (function laesa) (Figure 5). These clinical signs reflect four changes in the local blood vessels during inflammation; i) increase in vascular diameter, causing increased local blood flow and a reduction in the velocity of blood flow (*warmth and redness*); ii) expression of adhesion molecules on the vessel endothelia attracting circulation leukocytes that migrate into the tissue (extravasation) to start clearing the microorganism; iii) increased vascular permeability with exit of fluid and proteins (complement factors etc.) to the inflamed tissue (*swelling and pain*); iv) activation of the coagulation and bradykinin system by changes in vessel wall, leading to blood clotting in micro vessels on the site of infections, limiting the spread of the pathogen via the blood (25). Both

swelling and pain reduces the function *(function laesa*) of the inflamed area. These vessel changes are initiated by different mediators of the innate immune system.

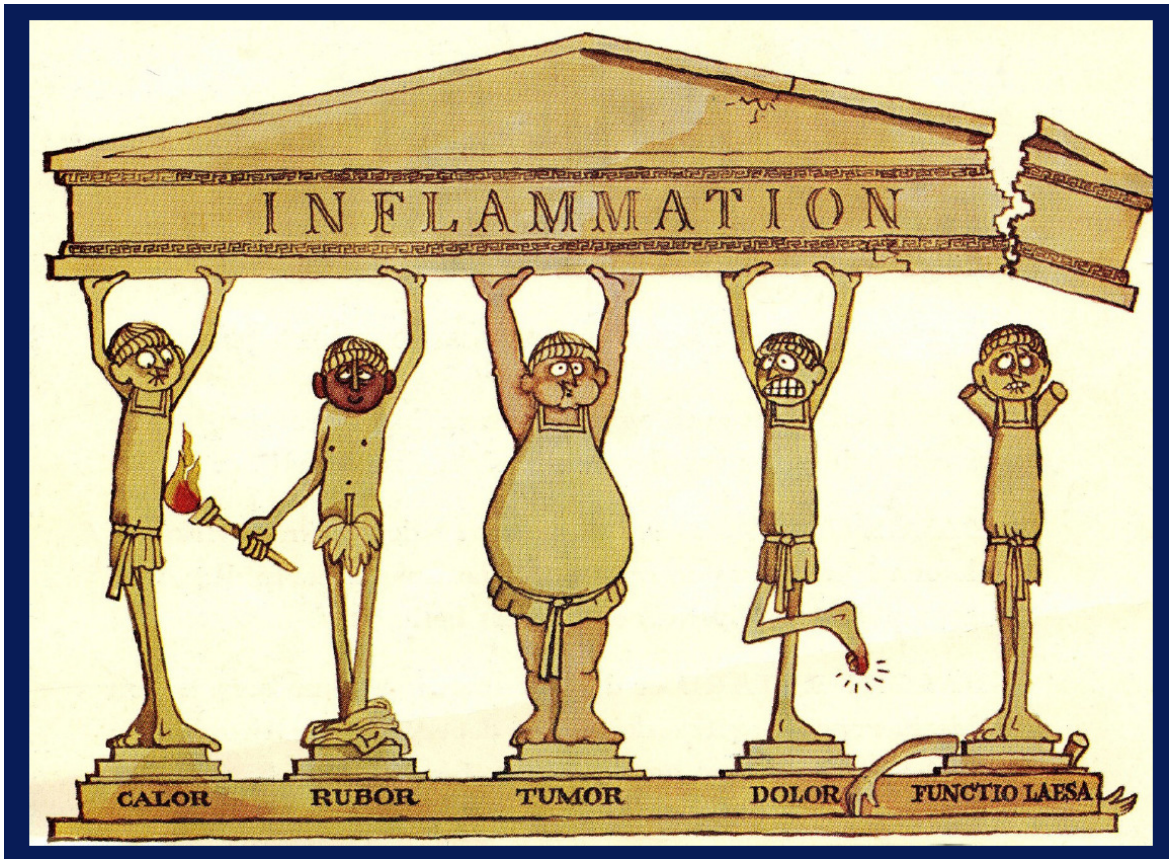


Figure 5: The five clinical signs of inflammation

If the microorganism invades the bloodstream or the local inflammatory response fail to clear the infection before it spreads to other parts of the body, SIRS may be initiated. Here the same mechanisms causing the local responses are activated, but with a more systemic release/effect of the different factors including major new production of leukocytes in the bone marrow and production of acute phase proteins (such as C-reactive protein (CRP)(269), mannose-binding lectin (MBL) (174) and soluble CD14 in the liver (43). This massive inflammatory response

affecting the whole body is a double-edged sword; crucial to clear the infection, but if not tightly regulated the effects may be catastrophic for the patient.

1.3 Staphylococcus epidermidis

1.3.1 General characteristics of *Staphylococcus epidermidis*

Staphylococci are Gram-positive cocci, which often stick together in grape-like clusters. They belong to the family *Micrococcacea.* There are 45 species and 24 subspecies of the genus *Staphylococcus* (www.bacterio.cict.fr/s/staphylococcus.html). With a few exceptions, all species are catalase-positive, and they are all facultative anaerobe. The genus can be separated into two groups based on the ability to produce coagulase, an enzyme that causes clotting of blood plasma: The coagulase-positive *staphylococci* (*Staphylococcus aureus* and a few others) and the coagulase-negative staphylococci (CoNS) (270)

Among the CoNS, *S. epidermidis* are the most frequently isolated species from human epithelia, predominantly colonizing the axilla, head and nares as part of the normal commensal skin flora (271). Their ability to produce adhesion factors and withstand high salt concentrations is important to colonize human tissues.

1.3.2 *S. epidermidis* - clinical significance

Ubiquitous colonization of *S. epidermidis* on the human skin and mucus membranes gives them the opportunity to cause infections under special circumstances. However, in general *S. epidermidis* are low virulent bacteria with few virulence factors (272, 273). *S. epidermidis* has emerged as an important opportunistic human pathogen, reflecting the increased use of indwelling medical devices and an increasing number of patients with impaired immune systems,

e.g. patients receiving immune-suppressive therapy, preterm infants, AIDS patients, and drug abusers (272, 274, 275). *S. epidermidis* is now considered one the most frequent causes of nosocomial infections (275) (The Nosocomial Infections Surveillance System (NNIS); http://www.cdc.gov/ncidod/hip/NNIS/2004NNISreport.pdf).

In immune competent humans, *S. epidermidis* mainly become pathogenic i) when associated with indwelling medical devices (biofilm), such as arteriovenous shunts, contact lenses, urinary and central venous catheters, orthopedic devices, and peritoneal dialysis catheters (272, 276-280) and ii) in rare cases, when associated with native valve endocarditic (281, 282).

S. epidermidis infections are seldom lethal, but they significantly contribute to morbidity and health care costs (275, 283).

There are also risks of both under- and over-reporting of *S. epidermidis* infections, because of difficulties in finding a common standard to determine clinical relevance of a strain, since clinically relevant *S. epidermidis* also are common contaminants of clinical samples.

1.3.3 *S. epidermidis* – antibiotic resistance

Hospital-acquired *S. epidermidis* often display resistance against many antimicrobials in use today, such as methicillin and aminoglycosides (284-289). About 70-90% of all CoNS, both in Norway and in the rest of the world are resistant to methicillin (284, 290, 291). The methicillin resistance in staphylococci is mediated by the *mecA*-gene encoding a penicillin-binding protein (PBP2a) with reduced affinity for all betalactam antibiotics (292-294). The *mecA* gene is integrated in the *SCCmec* element (292, 295-297). Glycopeptide resistance in *S. epidermidis* is still relatively rare (285, 298), and Vancomycin is the drug of choice for methicillin-resistant *S. epidermidis* (285). An increasing prevalence of antibiotic resistance in *S. epidermidis* is partly

due to the increasing use of broad-spectrum antibiotics, which encourage selection of multiresistant strains (299).

1.3.4 *S. epidermidis* infections in neonates

S. epidermidis may cause a wide spectrum of infections in neonates. Isolation of *S. epidermidis* has been associated with wound abscesses, pneumonia, urinary tract infections, necrotizing enterocollitis (NEC), endocarditis, omphalitis and meningitis (300, 301). However, clearly the most important and prevalent *S. epidermidis* infection in neonates is sepsis with or without the association to indwelling catheters.

Neonatal sepsis is an important cause of morbidity in neonatal intensive care units (280, 302-304). Over the past twenty years there has been a substantial shift in pathogen patterns for late-onset sepsis in neonates (305), where the nosocomial pathogens have become more important. CoNS are now the most prevalent pathogen causing late-onset sepsis, accounting for more than 50% of the episodes (280, 302, 304, 305). These infections are associated with low birth weight, low gestational age, need of mechanical ventilation, parenteral nutrition (PN) and a history of intravascular catheterization (280, 304, 306). Infections with *S. epidermidis* are preceded by skin colonization, and usually occur after the second or third week of life (280, 307), a time where a large number of *S. epidermidis* colonizes the skin. A large proportion of systemic infections due to *S. epidermidis* in the neonatal period are associated with indwelling catheters or other devices that causes a break in the skin. Many neonatal infections are caused by bacteria that colonize the patients own skin (10), indicating that invasive *S. epidermidis* infections often are derived from the skin and that indwelling vascular lines may be a major source of infection (308). Late onset-sepsis caused by *S. epidermidis* is seldom fatal, but they cause significant morbidity with longer in-hospital time, and a significant increase in total hospital costs (280, 304, 309).

The diagnosis of *S. epidermidis* late onset-sepsis in neonates is difficult. The clinical signs of infection in neonates, and especially in premature neonates, are subtle and non-specific, and the laboratory tests including the "gold standard" blood culture are not always reliable (310) (311). Distinguishing between true *S. epidermidis* bacteriemia and blood culture contamination is also difficult (312, 313). To minimize the amount of blood drawn and puncture of the skin of the neonates, usual practice in many neonatal intensive care units (NICUs) are to obtain only a single blood culture. The most used definition for *S. epidermidis* sepsis in neonates is: One positive blood culture and the addition of clinical signs of sepsis such as apnea, tachypnea, need for increased respiratory support, bradycardia, hypotonia, feeding intolerance, abdominal distention or in the early phase only "the baby is just not right" (314, 315), **and** either i) at least 5 days of appropriate antibacterial therapy (316) or ii) elevated CRP values (280, 317). There is unfortunately no uniform agreement how to define late-onset sepsis depending on time of onset after delivery. Definitions range from occurring at least 48 hours after delivery (318), 72 hours after delivery (319, 320) to more than 1 week after delivery (321, 322).

S. epidermidis infections may have a significant impact on the innate immune response of the neonate. The preterm neonates are especially vulnerable because of an immature functioning immune system. *S. epidermidis* infections induce significant secretion of both pro-and anti-inflammatory cytokines (11, 238), but the secretion of pro-inflammatory cytokines seems to be gestational age dependent (234). It has been reported that glucose and especially intravenous lipids may modulate host defense and increase the risk of infections in neonates (323-325). The use of total parenteral nutrition (TPN) may also reduce the function of neutrophils (317, 326). Recently it was shown that the pro-inflammatory cytokine response to *S. epidermidis in vitro* was affected by both lipids and glucose. However, further studies are needed to investigate whether

these findings are applicable to clinical settings and to evaluate the role of cytokine monitoring in infants receiving long-term parenteral nutrition (327). *S. epidermidis* and *S. epidermidis* biofilms also activate leukocytes, but their ability to up-regulate oxidative burst, induce opsonophagocytosis and bacterial killing is impaired in infants compared to adults. This is probably due to the immaturity of their immune system, with a significant hypogammaglobulinemia and reduced complement activity both in the classical and alternative pathway (164, 259, 328, 329). Also, the inflammatory response in neonates, assessed by CRP, is compromised when challenged with *S. epidermidis* biofilm producing strains (291). Deficiency of complement factor C3 and IgG have been related to greater risk for CoNS associated infections in neonates (258).

In conclusion, defects in the neonatal immune response, may partly explain why this otherwise low virulent pathogen, causes such serious infections among these patients.

1.3.5 Virulence factors –general

Virulence has been defined in several different ways, such as: "Harmfulness, and describes the ability of a pathogen to reduce host fitness" (330), or "The ability of a microorganism to establish an infection and cause disease in a host" (331, 332). Factors important for the pathogens virulence generally contributes to either i) immune evasion, ii) immune stimulation, iii) colonization, or iv) factors that cause damage to the host (331, 332) (Table 2). In general, *S. epidermidis* has few virulence factors which directly cause damage to the host, compared to its more virulent relative, *S aureus. S. epidermidis* therefore have to rely on factors modulating the immune system of the host in order to maintain a persistent infection. It has been suggested that *S. epidermidis* actually could have an evolutionary advantage of this low aggressiveness (330). In fact, many of the factors important for sustaining the commensal life of *S. epidermidis* are

beneficial as virulence factors during an infection. I will in this thesis mainly focus on two *S. epidermidis* virulence factors that we have studied more closely; biofilm formation and the Arginine Catabolic Mobile Element (ACME).

Table 2 is a summary of the main strategies *S. epidermidis* uses to modulate the immune system of the host. Some of these immune modulating strategies also participate in skin and mucus membrane colonization.

Table 2: Immune modulating strategies of *S. epidermidis*

Name (gene)	**Function**
Immune evasion strategies	
Biofilm	Colonization, immune evasion and antibiotic resistance
Attachment	
AtlE (*atle)*	Autolysin, initial attachment; abiotic surface and host proteins
Aae (*aae)*	Autolysin, initial attachment; abiotic surface and host proteins
SSP1/2	Surface associated protein: initial attachment to abiotic surfaces
Teichoic and lipoteichoic acid	Initial attachment to abiotic surfaces and host proteins
Bhp (*sesD)*	Initial attachment and intercellular adhesion
SdrG *(fbe/sdrG)*	*S. epidermidis* surface protein (Ses-protein). Binds fibrinogen. Inhibit phagocytosis
SdrF *(sdrF)*	Ses-protein. Bind collagen
SdrH *(sdrH)*	Putative binding function
SesI	Unknown ligand
SesC	Fibrinogen binding, biofilm accumulation?
Accumulation	
Polysaccharide Intercellular adhesion (PIA) *(ica)*	Intercellular adhesion and accumulation, cell adherence. Immune evasion.
Accumulation associated Protein (Aap) *(aap)*	Biofilm accumulation, immune evasion
Extracellular matrix-bindig protein	Initial attachment to fibronectin. Biofilm

(*embp)*	accumulation. Immune evasion
Poly-γ-glutamic Acid (PGA) *(capA/B/C/D)*	Resistance to AMPs and neutrophil phagocytosis. Increased survival during high salt concentrations
VraF/G *(vraF/G)*	AMP resistance
Aps system *(aps)*	AMP sensing system, AMP resistance
Metalloprotease *(sepA*)	Exoenzyme. Lipase maturation, AMP degradation and resistance

Immune stimulatory strategies

PIA	Cytokine secretion
Peptidoglycan	Component of cell wall, induce cytokine secretion
Lipopetides	Component of cell wall, induce cytokine secretion
Phenol-soluble Modulins (*hld, psm α/β/γ/ε/δ)*	Chemotaxis, decreased apoptosis of leukocytes, cytokine secretion, degranulation, oxidative burst. Colonization. Cell lysis. Biofilm maturation

Based on references: (29, 183, 333-353).

1.3.6 Biofilm

Biofilms are microorganisms encased in an extracellular matrix consisting of components produced by the microorganism and derived from the environment they grow in. They can be formed by both bacteria and fungi (277). It has been estimated that 99% of all bacteria live in biofilms (354). In nature, biofilms are primarily multispecies communities, where the different species engage in favorable metabolic interactions. For many organisms biofilms actually seems to be their preferred mode of growth. Biofilms are found in the everyday life; in the drain, on shower curtains, in the oil industry and deposited on our teeth. In clinical medicine, biofilms are usually monospecies, and they are considered an evil in that they complicate a range of infections. Many bacteria are associated with biofilm-associated infections, and most are hospital acquired (277, 278, 355-359).

Living in a biofilm gives the bacteria the advantage of a better adaptation to environmental factors and increased resistance to hostile conditions (272, 277). Additionally, increased levels of horizontal gene transfer in biofilm can be important for the survival of a species, giving it new tools to adapt to environmental changes and driving evolution forward (360, 361). There is a significant metabolic shift from the planktonic to the biofilm mode of growth (352), towards anaerobic or micro aerobic metabolism, decreased transcription and translation, and induction of a dormant state of life for the bacteria (362). Most likely these changes are a result of the low concentration of oxygen in biofilms and the restricted availability of nutrients (352).

Biofilm formation in *S. epidermidis*

Biofilm formation is the most important virulence factor of *S. epidermidis*. The adaptation to environmental factors and the metabolic shift contributes to *S. epidermidis* success in colonization of host tissue and medical devices, and protects the bacteria against the hosts immune system (363) and attempts of antibiotic treatments (364, 365). It is now generally accepted that *S. epidermidis* infections are dependent on the species ability to adhere to artificial surfaces and to assemble biofilm consortia (366, 367).

Biofilm formation is commonly described as two-step process with i) initial attachment to surfaces with ii) a subsequent aggregation and maturation into multicellular structures. A final detachment phase after steady-state has been acquired then follows. The detachment phase involves the detachment of single cells or cell cluster by various mechanisms and is believed to be crucial for the dissemination of the bacteria. The process of biofilm formation and detachment will be reviewed in more detailed below and in Figure 6.

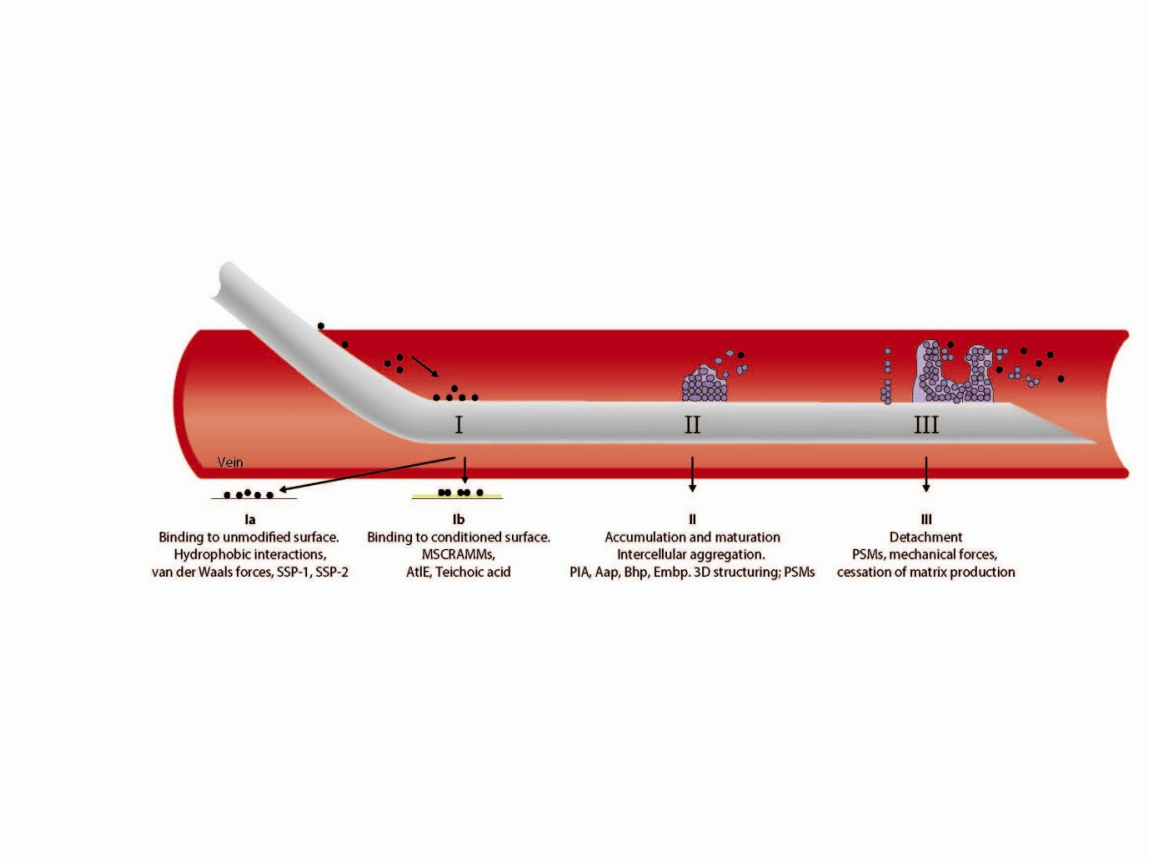


Figure 6: Formation of *S. epidermidis* biofilm; an overview of the steps in biofilm formation and the main factors involved, displayed on an intravascular catheter. Phase I: attachment of the bacteria to an unmodified surface (Ia) or a modified surface with a conditioning film (Ib). Phase II: accumulation of biofilm and cell-cell interaction, followed by the detachment phase (III) of cells and cell clusters from the biofilm.

Attachment:

The initial attachment of *S. epidermidis* to the foreign body (e.g. medical implanted device) may occur either directly to an unmodified synthetic surface or to host proteins that already have coated the surface (modified surface).

The initial attachment to an unmodified surface involves hydrophobic interactions, van-der Waals forces and electrostatic interaction (368), mediated through physico-chemical properties of the bacteria and the abiotic surface, (272). The abiotic surfaces implicated in *S. epidermidis* biofilm associated infections are hydrophobic surfaces of plastic, often used in catheters or other indwelling devices (272). *S. epidermidis* surface (SES) proteins, that are involved in the attachment to unmodified surfaces are SSP1 and 2 (369), AAE (334), AtlE (333), and teichoic (TA) and lipoteichoic acids (LTA) (370). The latter four can also take part in attachment to modified surfaces (333, 334).

Accumulation and maturation:

This phase is characterized by i) intercellular aggregation between cells accomplished by a variety of molecules such as polysaccharides and adhesive proteins, and ii) biofilm structuring forces leading to the typical 3-dimensional appearance of a mature biofilm. The biofilm matrix may consist of exopolysaccharides (e.g. PIA), DNA, proteins and accessory macromolecules (such as teichoic acids) aiding intercellular aggregation.

Polysaccharide Intercellular Adhesin (PIA) - biofilm

PIA is the best described matrix component of *S. epidermidis* biofilms. PIA is a linear unbranched homopolymer of β-1,6-linked N-acetylglucosamine residues, of which 15-20% are deacetylated (335, 344). Deacetylation of PIA is important for both biofilm formation and immune evasion (342). PIA is encoded by the *ica* operon consisting of *icaA* and *icaD* (expressing N-acetylglucosamine transferases) producing chains of the N-acetylglucosamines (GlcNAc) monomers*, icaC* (a putative PIA exporter) causes elongation of the GlcNAc monomers, and *icaB (*PIA deacetylace) which encodes a surface enzyme that deacetylate the monomers after export

(335, 342, 371). Proteins such as Aap and Bhp have domains with putatively PIA-binding properties and might contribute to create a strong biofilm (372).

PIA-independent biofilm formation:

During the past years it has been recognized that PIA is not essential for *S. epidermidis* biofilm formation. A number of biofilm producing strains lacking the *ica* operon have been identified (373-376).These *ica*-negative strains have also been isolated from biofilm-associated infections (375). Adhesive proteins most likely substitute PIA in these biofilm. Especially two proteins have been described as important in the formation of proteinaceous biofilms.

Accumulation-associated protein (Aap) is a cell surface associated protein bound covalently to the cell surface. It consists of two domains (A and B), where the B domain requires proteolytic activation (345) and Zn ions for Aap to give its biofilm promoting effect (377). Domains in this biofilm may interact with GlcNAc and form protein-polysaccharide biofilm networks (345, 372).

*Extracellular matrix-binding protein (*Embp*)* is proteinaceous intercellular adhesin, non-covalently attached to the cell surface. Embp binds fibronectin and is essential for biofilm formation in some strains (347).

PIA and non-PIA biofilms seems to have a different architecture. The PIA-biofilms have regions of e a flat architecture with other areas consisting of prominent large cell aggregates and cavities, leading to an overall irregular biofilm surface structure. The non-PIA biofilms are usually thinner and display an even, smooth surface (378). The PIA independent biofilms are considered "weaker" than the PIA-dependent biofilms (375); that means they have often a lower amount of extracellular matrix material.

A significant number of clinical *S. epidermidis* isolates carry the *ica* operon, *aap,* and *embp* (375, 379-381)*,* indicating that under *in vivo* conditions *S. epidermidis* biofilms are probably formed by parallel expression of all these biofilm forming genes. There are numerous reports describing *S. epidermidis* wild type (wt) strains that produce biofilm in a PIA independent manner (345, 346, 374, 376, 382). In a study with isolates from prosthetic joint infections 27% of the biofilm forming strains formed PIA-independent biofilms, and in most cases biofilm formation appeared to be mediated by Aap (375). However, Embp may also mediate biofilm formation in *ica* and *aap* negative strains (347).

Detachment:

The detachment phase involves the detachment of single cells or cell clusters by various mechanisms, causing spread of the infections to new sites and secures the survival of the bacteria. Our understanding of the detachment process is limited, but several mechanisms for *S. epidermidis* biofilm detachment have been suggested: Enzymatic degradation of the biofilm matrix and disruption of non-covalent interactions by detergent-like molecules, such as phenol-soluble modulins (PSMβ) (383-386). Mechanical forces, like fluid share, nutrient starvation (387) and the cessation of matrix production may also contribute to the detachment.

Immune modulation by biofilms

The ability of biofilms to protect the bacteria against and modulate the host innate immune system is important in *S. epidermidis* pathogenesis. PIA protects *S. epidermidis* from effective phagocytosis by reducing opsonization of C3b and IgG binding on the bacterial surface (183, 326, 343, 388). PIA also acts as a mechanical barrier to block the effects of both cationic and anionic AMPs, probably by electrostatic repulsion of the cationic peptides, while the mechanism

of anionic AMP protection is still unclear (343). A significant activation of the complement cascade mediated by PIA biofilm has also been noted (183). *S. epidermidis* induce cytokine production by human mononuclear cells (such as monocytes) *in vitro* (234, 389, 390). Both, PIA-biofilms and non-PIA- biofilms protects *S. epidermidis* from phagocytosis, probably due to lack of contact between the bacteria and PRRs on the leukocytes, and the induction of a poor NF-κB mediated macrophage inflammatory response (347, 378). It was recently demonstrated that LPS-induced NF-κB activation of macrophages after 2 hours of contact with biofilm forming *S. epidermidis* was low. Interaction of macrophages and *S. epidermidis* biofilms seems to render the macrophages hyporesponsive to strong pro-inflammatory compounds such as LPS (378). So, in addition to the lack of contact between the macrophages and the bacteria, also events modifying the macrophage function seem to take part in the host failure to eradicate *S. epidermidis* during infections.

1.3.6 The Arginine Catabolic Mobile Element (ACME)

ACME is a genomic island that contains one or both of two characteristic gene clusters (*arc*- and/or *opp3*- operon) that are homologs of virulence determinants in other bacterial species (391). The ACME-*arc*-operon is a characteristic cluster of six genes that encode several enzymes in the arginine deiminase catabolic pathway, converting L-arginine into carbondioxide, carbamoyl ornithine, ammonia and ATP (391). The exact function of the *arc*-operon in staphylococci is not fully understood. However, several mechanisms have been suggested. First, the ACME-encoded arginine deiminase pathway generates ammonia, which would allow for staphylococci to maintain pH homeostasis on the acidic human skin and mucosal surfaces (391). Second, the production of ATP under anaerobic conditions may be important for energy production in wound environments low in oxygen or in biofilms where oxygen levels can be low

(391). Finally, arginine deiminase, the main enzyme coded for by the operon, is important in the inhibition of human peripheral blood mononuclear cell proliferation in *Streptococcus pyogenes* (392). The *opp3*- operon encodes an ABC transporter system. Similar *opp*-operons in other bacterial species have a wide array of functions, such as pheromone transport, chemotaxis and expression of virulence determinants (391, 393). ACME –opp3 belong to the same family as *opp1* and *opp2,* two natural chromosomal operons encoding ABC transporters involved in nutrient up-take from the bacterial environment (394).

ACME integrates into *orfX* in both the USA300 clone and ATCC12228 strain, and is flanked by *SCCmec*- specific repeat sequences (391). The *SCCmec* elements nearby and their cassette chromosome recombinases *(ccrA/ccrB)* is believed to be important when moving this island from one strain to another (391, 395-397)

ACME was first described in the community associated methicillin-resistant *S. aureus* (Ca-MRSA) USA300 strain and the biofilm-negative *S. epidermidis* strain ATCC12228 (391). In *S. aureus* ACME was initially considered as a new and putative important virulence factor. This was due to its presence in the pathogenic and widespread *S. aureus* strain, USA300 (398-401), and its correlation to methicillin resistance through it co-localization in to the *SCCmec* element (391, 396), and because an isogenic ACME-negative mutant showed significantly reduced fitness in a rabbit infection model (402). However, and in contrast to Diep`s study from 2008, a recent study did not show that the presence of ACME was associated with increased virulence in a rat model of necrotizing pneumonia (403) Thus, currently ACMEs role as a virulence factor in *S. aureus* is unclear.

ACME is divided into three allotypes (391, 396, 397, 402, 404). ACME-I was first found in the *S. aureus* strain USA300, and it contains the *arc*-operon and the *opp3*-operone. There are several subtypes of ACME-I, where ACME-I.01 is found in the USA300 type while ACME-I.02 is the most common subtype in *S. epidermidis* (397). There are only 11 nucleotides in difference between these two subtypes, indicating recent common origin (397). ACME-II was first found in the *S. epidermidis* strain ATCC12228, and consists of only the *arc*-operon. ACME-III has only the *opp3*-operone and lacks the *arc*-operon. All these three allotypes have been detected in *S. epidermidis* (397, 404).

ACME is widespread in staphylococcal strains colonizing human skin and mucus membranes. The USA300 strain colonize sites such as axilla, inguinal, perineum and rectum (405), which usually are uncommon sites for *S. aureus* colonization, but more common for CoNS colonization. A horizontal transfer of ACME from *S. epidermidis* to *S. aureus* was probably important in the evolution of the virulent USA300 strain (391, 397).

The prevalence of ACME in CoNS is high (~20 - 70%) in both hospital and community settings (391, 397, 404, 406). ACME has been found in different CoNS species, such as *S. epidermidis, S. capitis, S. haemolyticus* (391, 397, 406).

Current perception is that ACME is mainly an advantage for strains colonizing skin and mucus membranes, rather than accounting for enhanced capacity of infection (397, 403)

2. Aims of the thesis

The overall aim for this thesis was to study interactions between *S. epidermidis* virulence factors (ACME and biofilm) and the innate immune system in both adults and neonates

Paper I

ACME is proposed as a new virulence factor in *S. aureus*. However, at the time when the experiments for Paper I were performed little was known about ACMEs role in *S. epidermidis*. We therefore aimed to evaluate the prevalence of ACME allotypes in a large collection of *S. epidermidis* blood culture isolates from neonates. Furthermore, we assessed antibiotic resistance and compared different components of the inflammatory response in ACME-positive and ACME-negative isolates

Paper II

S. epidermidis is a frequent cause of biofilm associated and often low-grade infections. We aimed to study how two *S. epidermidis* biofilms, with different extracellular matrix, modulated innate immune response, with a particular focus on the complement system.

Paper III

S. epidermidis accounts for more than 50% of the late-onset sepsis episodes in neonates. Data indicate that the complement system is important in the host defense against *S. epidermidis* biofilm associated infections in adults. In this study we aimed to compare the complement and cytokine response between neonates (cord blood) and adults in an experimental *S. epidemidis* biofilm sepsis model.

3. Material and methods

3.1. Materials

3.1.1 Bacterial collection

Paper I: *S. epidermidis*

A collection of 128 *S. epidermidis* blood culture isolates from neonates admitted to the neonatal intensive care unit (NICU) at Rikshospitalet University Hospital (Oslo, Norway) was used. The strains were collected during the period from January 1989 through April 2000. Blood culture isolates that were accompanied by clinical signs of sepsis in a neonate older than 72 hours of age, and with a C-reactive protein (CRP) level >10 mg/L were considered "true" invasive isolates. All other isolates were considered contaminants. Based on this definition, 64 isolates were defined as invasive and 64 isolates as contaminants. In addition to the clinical signs of sepsis, information on birth weight (BW), gestational age (GA) and maximum C-reactive protein (CRP) value of the patients were collected.

Paper II-III: *S. epidermidis*

A clinical *S. epidermidis* (SE1457) isolate and its isogenic *ica* – negative mutant (SE1457-M10) were used in these papers. The original isolate, *S. epidermidis* 1457 (SE1457), was collected from an infected central venous catheter at the University Hospital Hamburg-Eppendorf, Germany (407). The isogenic mutant was constructed by insertion of Tn917, carrying erythromycin resistance, into the *icaA* gene abolishing PIA synthesis (408).

3.1.2 Study group and blood donors

Paper I, II

Blood from healthy adults where used in these experiments; two adult controls were used in paper I and six adult donors were included in paper II.

Paper III

Pregnant women were recruited from a study investigating maternal, placental and fetal hemodynamic in low risk pregnancies. A convenience sample of cord blood from 20 term born neonates was initially included in this study. The umbilical cord was clamped immediately after birth and blood was obtained from the umbilical cord. Five samples where later discarded due to signs of perinatal infection, small sample volume or a delay of > 30 min from delivery until the experiment was started. Thus, the final study group constituted cord blood from 15 infants (9 girls) with a median (range) birth weight of 3678 (2898 – 4360) g, median Apgar scores (range) where 9 (6-9) after 1 minute and 10 (7-10) after 5 minutes. Median umbilical cord pH (range) was 7.27 (7.14-7.40). Media Base Excess (BE) (range) was -2.5 (-7.2 – 7.2). Blood from a total of six healthy adults (4 women) were used as controls in this study.

3.1.3 Ethical considerations

Paper I and II

The regional committee for medical research ethics approved the collection of blood for the immune response studies. Informed written consent was obtained from each blood donor before collection of blood.

Paper III

The regional committee for medical research ethics approved collection of blood from the umbilical cord of the neonate, and obtaining clinical information of the neonate and the mother from the hospital-records after birth. Informed written consent was obtained from each mother during the second trimester of the pregnancy.

3.2. Methods

3.2.1 Species identification

Paper I:

The species of each strain was identified by the use of several well-known phenotypic tests: Catalase test, Coagulase test, Gram-stain, and ID32Staph (bioMèrieux, Marcy l'Etoile, France). A selection of the strains had their species verified by sequencing the housekeeping gene 16sRNA (409). Heterogeneity in the housekeeping gene was identified by cycle sequencing of both strands with a Big Dye Terminator (Applied Biosystems, Warrington, UK), and run on an ABI Prism 377 sequence analyzer.

3.2.2 Biofilm analysis

Paper I:

Semi-quantitative determination of biofilm production for all isolates was performed in a microtiter plate assay as previously described (291, 410). Briefly, each strain (in Tryptic-Soy-Broth (TSB) with 3% NaCl) was inoculated in eight parallel wells in polystyrene micro titer plates (Nunclon, Roskilde, Denmark). All strains were tested independently on three separate occasions. We determined the optical density (OD) of the crystal violet-stained adherent biofilm with a spectrophotometer at 570nm. The highest and lowest OD_{570} value of each parallel was

excluded from the analyses, and the remaining 18 values were averaged. *S. epidermidis* ATCC 35984 (RP62A) was the positive and *S. epidermidis* ATCC 122228 was negative control. Isolates with $OD_{570} > 0.12$ were defined as biofilm producers.

3.2.3 Antimicrobial susceptibility testing

Paper I:

We determined the minimal inhibitory concentration (MIC) of oxacillin, gentamicin, fusidic acid, clindamycin and vancomycin with Etest (AB Biodisk, Solna, Sweden) and ciprofloxacin by disk diffusion test. Antibiotic susceptibility was interpreted according to the Clinical and Laboratory Standards Institute guidelines (411).

3.2.4 Detection of resistance and virulence genes

Paper I:

Detection of central resistance- and virulence genes was done by polymerase chain reaction (PCR). Bacterial DNA was extracted by the boiling method (409). We performed PCRs using previously reported primers for i) the methicillin resistance gene (*mecA*) (412), *ii)* SCCmec type (413) iii) one central aminoglycoside resistance gene (*aac(6')-Ie-aph(2")-Ia*) (288), iv) a marker of the *ica*-operon encoding biofilm accumulation (*icaD*) (312) and v) ACME-*arcA* and ACME-*opp3* (391, 397).

3.2.5 Phylogenetic analysis

Paper I:

Pulsed Field Gel Electrophoresis (PFGE) was used to determine the relatedness between the bacterial isolates as described previously (409). PFGE patterns were analyzed by GelCompar II version 2.5 (Applied Maths, Belgium). Isolates with ≥ 95 % similarity were considered to be

indistinguishable strains and isolates with ≥ 80 % similarity were considered to be related strains (291, 414).

3.2.6 *Ex vivo* whole blood sepsis model

Paper I-III

An *ex vivo* whole blood sepsis model was used to study the inflammatory response of different parts of the innate immune system and the interaction between them (104). We used lepirudin as anticoagulation.

Collecting blood samples

Blood from *healthy adults* was collected into sterile polypropylene tubes (4.5 ml Nunc cryotubes; Nagle Nunc International) containing 50 µg/ml Lepirudin (Refludan®, Hoechst). The blood samples were obtained immediately before each experiment and kept at 37°C throughout the experiment **(Paper I, II, and III)**.

Neonatal cord blood was collected in sterile polypropylene tubes (4.5 ml Nunc cryotubes; Nagle Nunc International) containing 50 µg/ml lepirudin (Refludan®, Hoechst) after puncture of the umbilical cord. The cord blood samples were obtained within the first minutes after birth, and no more than 30 minutes before starting the experiment **(Paper III).**

Preparation of inoculum

Bacteria in planktonic growth:

Bacterial solutions of all the strains studied in the planktonic growth phase were prepared to a concentration of 2 McFarland. To verify the bacterial count, the inoculum was diluted and spread

on blood-agar plates and CFU was determined by counting retrospective of the experiments. **(Paper I and II).** The final bacterial concentration in blood was ~10^8 CFU/ml.

Bacteria in biofilm growth:

Overnight cultures of the bacterial strains were diluted 1:100 in Tryptic soy broth with 1% glucose (TSB 1% glucose). 1.8 ml of this bacterial solution was added to polyvinyl chloride (PVC) tubes (length 30cm, internal diameter 3mm, Mediplast, Malmø, Sweden), and the segments closed end-to-end to form small loops. The loops were incubated for 24 hours at 37°C while slowly rotating. They were then emptied and carefully washed once with sterile phosphate-buffered saline (PBS) **(Paper II and III).**

Preparation of highly-purified PIA

PIA was prepared from a biofilm extract of a PIA-producing clinical strain, *S. epidermidis* CIP 109562, as described previously (29, 415). Briefly, the crude extract was treated with DNase I (Sigma) for 2 h at 37 °C in 1mM Tris-HCl, in presence of 1 mM $MgCl_2$, followed by treatment with proteinase K (Sigma, 100 µg ml^{-1} for 2 h at 37 °C), and H_2O_2 (1%, 37°C, 4 h). This treatment leads to degradation of DNA, proteins and inactivation of highly active lipopeptides and lipoproteins (29, 416). The extract was then subjected to gel-filtration chromatography on a Sephacryl S-300 column. This fractionation affords complete separation of PIA from teichoic acids and lipoteichoid acids, as well as other low molecular weight (LMW) impurities. The purity of such preparations has previously been investigated and verified by ^{1}H-Nuclear Magnetic Resonance, monosaccharide analysis and gas liquid chromatography (417).

A proportion of the PIA preparation was treated with sodium periodate ($NaIO_4$, 50 mM, 20 °C 18 h), dialysed and lyophilized to give $NaIO_4$-treated PIA. $NaIO_4$ aids in degrading PIA, rendering

the substance inert (29). This $NaIO_4$-PIA was included as a control. O-polysaccharide from *Proteus mirabilis* (kindly provided by Dr. Vinogradov, National Research Council, Ottawa, Canada), which is a high molecular weight zwitterionic polysaccharide (418) similar to PIA, was used as a negative control to ensure that the observed response to purified PIA was not a general response to this type of polysaccharides **(Paper II)**.

PIA purification was performed in Dr. Sadovskaya`s laboratory in Boulogne-sur-mer, France.

Complement activation

For the studies in planktonic growth, 1.5 ml of blood and 300 µl of the bacterial solution (final concentration of~ 10^8C FU/ml) were added to PVC tubing loops without biofilm (**Paper I and II).** For studies in biofilm growth, 1.8 ml **(Paper II)** or 900 µl **(Paper III)** of blood was added to PVC tubing loops with pre-made biofilms. The loops were incubated at 37 °C slowly rotating for 30 minutes. At the end of the incubation EDTA was added to a final concentration of 10mM, before plasma was separated and stored at -70 °C before further analysis.

Central complement activation products were quantified by ELISA techniques (Table 4).

Table 4: Complement activation products

Activation products	**Pathway**	**Paper I**	**Paper II**	**Paper III**	**References**
C1rs-C1inh *	Classical	X	X	X	(419)
C4d	Classical/lectin	X	X		Micro Vue C4d EIA Kit. (Cat. No A008, Quidel Corp., San Diego, USA)
C4bc	Classical/lectin			X	(420)
Bb	Alternative	X	X		Micro Vue Bb Plus EIA Kit (Cat. No A027, Quidel Corp., San Diego, USA)
C3bBbP	Alternative			X	(104)
C3a	Common**	X	X		Micro Vue C3a EIA Kit (Cat. No A015, Quidel Corp., San Diego, USA)
C5a	Common**	X	X		OptEIA^{TM} Human C5a ELISA Kit II (Cat. No 557965, BD Biosciences, San Jose, USA)
C3bc	Common			X	(421)
TCC***	Final-common	X	X	X	(422, 423)

* C1rs-C1inhibitor complex, ** Anaphylatoxins, *** Terminal Complement Complex

Solid phase TCC (C5b-9) analyses. TCC deposition on the biofilm in the PVC tubing was performed as described earlier (424). All incubations were done for 30 min at room temperature in slowly rotating loops unless otherwise noted. After incubation, blood was drained from the tubing and treated for analysis of soluble complement components. The tubing was washed 3-5

times with PBS/0.1% Tween (also used for antibody dilutions), before further incubation with aE11 ascites (anti-TCC) diluted 1/2000. Subsequently the tubing was washed before adding anti-mouse IgG horse radish peroxidase (Cat. no NA9310, GE Healthcare, UK) diluted 1/1000 and incubated before being washed again. Substrate solution (0.15 M sodium-acetate-buffer, pH 4.0, with 0.18 g L^{-1} 2,2'-azino-di-3 ethylbenzothiasoline sulfoniacide and $2.4x10^{-3}$% H_2O_2) was added before incubation for 10-30 min. The samples were collected in test tubes, and 100 µl aliquots were transferred to microtiter plates (Nunclon, Roskilde, Denmark) for determination of OD at 405/490 nm in a VERSAmax microplate reader (Molecular devices Corp., Sunnyvale Ca, USA). Blank control and a control incubated without monoclonal aE11 antibodies were included in all experiments **(Paper II).**

Cytokine secretion

Bacteria (either in planktonic growth or biofilm growth) and blood was added to PVC tubing loops as described for complement activation (**Paper I and II).** The loops were incubated rotating slowly at 37°C for three hours (102, 104) **(Paper I and II)**.

Or 900µl of umbilical cord blood/adult control was added to loops with premade biofilm, incubated for 30 minutes, and EDTA to a final concentration of 10mM was added in the end of the incubation. After incubation plasma was separated and stored at -70°C until analysis.

The secretion of the pro- and anti-inflammatory cytokines and chemokines (TNF-α, IL-1β, MIP-1α, MIP-1β, IL-6, IL-8, IL-17, FGF, G-CSF, G-MCSF, IP-10, MCP-1, VEGF, IL-1ra and IFN-γ) was measured by the use of a Bioplex cytokine assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA).

Leukocyte activation

The activation of leukocytes was studied by two different markers: oxidative burst and the expression of CD11b **(Paper I and II)**.

Oxidative burst. For analysis of response to planktonic bacteria, 0.6 ml of the blood-bacteria suspension was incubated for 10 min at 37°C in polystyrene tubes (4.5 ml Nunc cryotubes; Nagle Nunc International) **(Paper I and II)**. For analysis of response to bacteria embedded in biofilms, blood and biofilms were incubated as described for complement activation, but with an incubation time of 10 min **(Paper II)**. Immediately after the 10 minutes incubation a lysis-buffer containing paraformaldehyd (PFA) was added to the samples, causing lysis of erythrocytes and fixation of leukocytes. Cells were analyzed on a FACS Aria flow cytometer (Becton Dickenson, San Jose, CA, USA). Granulocytes and monocytes were identified and gated for in a forward-scatter (FSC)/side-scatter (SSC) plot by their typical patterns. Median fluorescence intensity (MFI) of the total population and percentage bursted cells was calculated for each cell type.

CD11b expression. For analysis of response to planktonic bacteria, 0.6 ml of the blood-bacteria suspension was incubated for 10 min at 37°C in polystyrene tubes (4.5 ml Nunc cryotubes; Nagle Nunc International) **(Paper I and II)**. For analysis of response to bacteria embedded in biofilms, blood and biofilms were incubated as described for complement activation, but with an incubation time of 10 min **(Paper II)**. Thereafter, the blood-bacteria suspensions were fixed with 0.5% PFA in PBS for 5 min, and further stained with IgG2a anti-CD11b APC (Becton Dickenson, San Jose, CA, USA), IgG1 anti-CD45 PerCP (Becton Dickinson, San Jose, CA, USA), and IgG1 anti-CD64 PE (Dako, Glostrup, Denmark) for 15 min. Red blood cells were lysed using a PFA-based lysisbuffer (0.04% PFA pH 7.4, 0.15 M NH_4Cl, 0.01 M $NaHCO_3$). Cells

were then analyzed on a FACS Calibur flow cytometer (Becton Dickenson, San Jose, CA, USA). Threshold was set for CD45 to exclude debris. Then monocytes and granulocytes were separately gated in a SSC-CD64 plot. CD11b expression was measured as MFI.

3.2.6 Titration of anti-PIA IgG antibodies

ELISA technique was used to define the anti-PIA IgG titers in non-stimulated blood from the umbilical cord of neonates and adult controls, as previously described (415). Briefly, Microlon 600 plates (Greiner-Bio) were coated with 100µl highly-purified PIA at 1 µg ml^{-1} in 40mM sodium phosphate buffer overnight in room temperature and then washed. The plates were blocked with 5% skim milk in Tris-buffered saline (TBS). Plasma was added at a concentration of 1:1600 (diluted in TBS-0.05% tween 20) and incubated for 1 hour, washed with TBS-0,05% tween 20, and then incubated for 1 hour with Horseradish peroxidase-conjugated rabbit anti-human IgG (Sigma-Aldrich, St. Louis, USA) diluted 1:2000 in TBS-0.05% tween 20. Color was developed with 100 µl of substrate solution (RnD systems, Minneapolis,USA) for 15 minutes in the dark, and then 50 µl H_2SO_4 was added to stop the reaction. OD was measured at 450 nm. Background readings corresponding to the control wells incubated with blocking solution not containing sera were subtracted automatically. Anti-PIA IgG depleted plasma was used as a cut-off marker for non-specific IgG binding. Each experiment was conducted twice in triplicates. Antibody titers were expressed as units of OD **(Paper III)**.

3.3 Statistics

Paper I:

- Mann-Whitney-U was used to compares differences between continuous variables without normal distribution
- χ^2 test or Fisher´s were used to compare differences for dichotomous variables
- Linear multivariable regression was used to identify bacterial and clinical variables potentially influencing CRP. To reduce skewness of residuals CRP was log transformed.

Paper II:

- Wilcoxon signed-rank test was used to compare differences in innate immune response when stimulating with a PIA and a non-PIA biofilm. The same six blood donors were used for both groups.

Paper III:

- Wilcoxon signed-rank test was used to compare immune response to the PIA positive and the PIA negative strains within each group (neonate or adult).
- Mann-Whitney-U was used to compare complement response, cytokine secretion and anti-PIA IgG titers between neonates and adults.

4. Summary of the main results

Paper I

- We found a high prevalence (32%) of ACME in *S. epidermidis* strains from blood culture of neonates. Most of these were found in the group classified as contaminants.

- ACME positive strains were most commonly isolated from the blood culture of the most mature neonates (highest gestational age, GA)

- We found lower levels of antibiotic resistance (methicillin and gentamicin) among ACME positive compared to ACME negative strains, and no association between the *SCCmec* type IV element and ACME.

- There were lower levels of biofilm formation among ACME positive compared to ACME negative strains.

- We found a high clonal diversity among the ACME positive strains

Paper II

- We found that SE1457 (PIA biofilm) produced a metaperiodate sensitive polysaccharide biofilm (PIA), while M10 (non-PIA biofilm) produced a biofilm mainly composed of DNA and proteins.
- Both strains (SE1457 and M10) induced a powerful activation of the complement cascade, cytokine secretion and leukocyte activation compared to non-stimulated control.
- SE1457 induced a higher complement activation compared to M10.
- Highly purified PIA was a strong activator of complement activation.
- *S. epidermidis* biofilms activated the complement cascade through the classical pathway, with a probable engagement of the alternative amplification loop.
- M10 induced higher expression of CD11b on the surface of leukocytes and a higher secretion of pro-inflammatory cytokines compared to SE1457.

Paper III:

- Both *S. epidermidis* biofilms induced strong complement activation through the classical/lectin pathway in cord blood, with further activation through the alternative pathway amplification loop.

- The PIA-positive biofilm induced stronger complement activation than the PIA-negative biofilm.

- Both *S. epidermidis* biofilms induced much stronger complement activation in adult compared to cord blood.

- We found significantly lower titers of anti-PIA IgG antibodies in cord blood compared to adult blood.

- An immature complement response in neonates may contribute to their increased susceptibility to *S. epidermidis* infections.

- *S. epidermidis* biofilms induced a significantly higher cytokine secretion in the neonatal compared to the adult blood, but with no differences in the cytokine secretion induced by the two different biofilms.

5. General discussion

5.1. Arginine Catabolic Mobile Element (ACME) - a new virulence factor?

ACME was first reported by Diep et al. in 2006 as a "new" virulence factor in *S. aureus* (391). The question was then raised whether ACME also is an important virulence factor in *S. epidermidis*?

In line with others (391, 397, 404, 406), we found in our study on blood culture isolates from neonates a high prevalence of ACME in *S. epidermidis*. The highest prevalence of ACME positive strains was observed in the group of bacteria that were considered as contaminants **(Paper I)** and in the blood cultures of the most mature babies (highest GA). The lowest prevalence of ACME was seen among "invasive" isolates (**Paper I**).

The contaminant strains are most likely commensal strains from the skin of the patient or health care workers. It has been suggested that L-arginine catabolism contributes to optimize staphylococcal life on the acidic human skin through generation of ammonia (396, 396). This may explain the high prevalence of ACME in typically skin commensals, such as *S. epidermidis* and *S. haemolyticus* (396, 406). Our results indicate that ACME in *S. epidermidis* may be considered as an indicator of a benign, skin flora isolate.

Antibiotic resistance, especially methicillin resistance, has been associated with *S. epidermidis* isolates from hospital settings, the skin of patients after antibiotic therapy (425), health-care workers in intensive care units (426) and on surfaces in the hospital environment (427). Many studies on ACME are performed on selected cohorts of methicillin resistant staphylococci only (e.g. MRSE, MRSA, MRSH) (403, 404, 406, 428). However, a few studies have included both

methicillin resistant and –sensitive isolates, showing that ACME in *S. aureus* seems to be associated with methicillin resistance (396).In *S. epidermidis*, no such association has been identified (397). Indeed, we found a higher prevalence of antimicrobial resistance among the ACME negative strains.

We also investigated whether there was any association between the ability to form biofilms and the prevalence of ACME. We found that biofilm formation is more common in the ACME-negative isolates (**Paper I**). Biofilm formation and carriage of the *ica* operon is often found in endemic *S. epidermidis* clones and is prevalent in hospital strains (414, 429). The ACME-positive strains in paper I was associated with high clonal diversity as also described for S. *haemolyticus* (406) and were rarely present in the local endemic *S. epidermidis* clones (414). This is in contrast to a study by Miragaia et al. who found that the vast majority of ACME positive isolates belonged to one large clonal complex in a collection of geographically diverse *S. epidermidis* isolates (397). However, studies on *S. epidermidis* indicate that ACME is not associated with the typical endemic/hospital clones often causing disease.

We did not find any difference in the inflammatory response (CRP) between patients with ACME-positive and -negative invasive isolates, even after adjusting for possible confounding factors, such as biofilm formation, methicillin resistance and GA. We also studied the innate inflammatory response in a whole blood model using ACME positive and -negative isolates, matched for some possible confounding factors (invasiveness, methicillin and gentamicin resistance, biofilm formation and the presence of *ica)*. A limitation of our approach was the lack of an isogenic ACME negative mutant that could have limited confounding factors (402, 403). However, we found no significant differences in the inflammatory response evoked by ACME positive vs. ACME negative *S. epidermidis* isolates.

To conclude, and answer the question in the beginning of this section: ACME does not seem to be a "new" important virulence factor in *S. epidermidis*, but rather contributing to bacterial growth, survival, transmission and colonization in the host (391, 396, 397, 403).

5.2 The *Ex vivo* whole blood sepsis model – advantages and limitations

The *ex vivo* whole blood sepsis model used in **Papers I-III** enable us to study the host inflammatory response including detailed analysis of complement and leukocyte activation and cytokine secretion in blood from different patient groups/donors and after different stimuli. Crucial for this model is the use of lepirudin as anticoagulation. Lepirudin is a specific thrombin inhibitor, not affecting other biological systems such as the complement cascade (104, 430).

In vivo the different parts of the immune system, both innate and adaptive, interact and communicate with each other. Compared to many *in vitro* models using isolated cell lines, serum and plasma, we capture more of the complex interplay between the different parts of the innate immune system, and thus get a more correct, but complex picture of the inflammatory response

However, there are some limitations and concerns regarding this model and our experiments:

Time-limitations

Being an *ex vivo* model the experiments are run under physiological conditions. However, in this *ex vivo* model we can only maintain human physiological conditions for around four hours. Therefore, the choice of incubation-times for cellular activators, complement and cytokines was based on these time-limits. Circulating leukocytes are ready to rapidly express CD11b and display oxidative burst. In this whole-blood model oxidative burst declines if samples are incubated longer than 15 minutes (104) and early analysis is thus essential. It is also essential to analyse activation of free circulating complement factors early in the course. The cytokines

increase in serum somewhat later and only those responding within 4 hours were possible to include in our studies. To avoid non-specific activation of inflammatory response and to "keep" the physiological time-limits, the experiments had to be started as quick as possible within collection of the blood samples, and no later than 30 minutes. This was a challenge in **paper III**, since few births are "time scheduled". Four of the included neonates were excluded because of a time-delay from blood sampling to the start of the experiment. Therefore, such studies are time consuming and require available personnel 24 hours a day during the inclusion period.

Cooperation with other organs

Studying whole blood *ex vivo* we lack information about the cooperation between blood and other tissues in the body, such as endothelia, which is known to be an important part of the inflammatory network in sepsis (431)

High planktonic bacterial load

The concentration of planktonic *S. epidermidis* was high in the whole blood model (**Paper I and II**). However, this concentration is similar to lethal and sub lethal doses of staphylococci used in primate models of sepsis (432, 433). During sepsis, bacteria often demonstrate massive growth within the first 12–24 h after entering the blood stream (434). The natural bactericidal activity of blood subsequently reduces the viability of organisms that can be recovered from blood cultures (435). Quantitative neonatal blood cultures often reveal CoNS concentration around 10^3 CFU/mL. However, sepsis studies assessing total bacterial DNA load have shown that the difference between dead and live bacteria may be three to four orders of magnitude, and that both dead and live bacteria induce the innate immune system (434, 436). Still, even though none of these studies

were done on staphylococci, we assessed the inflammatory response only up to three hours and, thus, argue that the bacterial concentration used in our experiments still was adequate.

Purity of PIA

When using PIA preparations there are always concerns of purity, especially in the context of recent findings that some contaminating compounds, such as glycopeptides, are active in nanogram quantities (416). For **paper II** we therefore improved the previously published PIA purification protocol (29) to achieve an extensive PIA purification. We also used a PIA concentration similar to the one used in previous studies (29, 437). Furthermore, we used proper control substances to exclude that observed effects were due to non-specific binding to polysaccharides instead of specific binding to PIA.

All these considerations must be taken into account when designing studies using this method and when interpreting the results. Still, the model reveals essential knowledge on the role of leukocytes, cytokines and complements the first few hours after *S. epidermidis* "infections".

5.3 *S. epidermidis* biofilms and the inflammatory response

Biofilm formation has an important role in the evasion of the host immune defense. It protects *S. epidermidis* from neutrophil phagocytosis, AMPs and deposition of antibodies and complement, and induces a significantly lower CRP response in neonatal blood compared to non-biofilm producing strains (183, 275, 291, 343, 438-440).

In **paper II and III** we used the strains SE1457 and the *ica* knock-out mutant SE1457-M10 to study the inflammatory response to *S. epidermidis* biofilms in adult and neonatal blood (cord blood). The same strains have been used in previous studies (183, 343), but in these papers the

knock-out mutant was treated as a non-biofilm producing strain. In **paper II** we found that SE1457-M10 incubated in a glucose-rich media formed a DNA-rich and proteinaceous biofilm, although with less biofilm mass compared to SE1457 (343), this in line with a previous study (374).

Both the PIA and non-PIA biofilm producing strains induced a significant activation of the complement cascade in both adults and neonates (**Paper II and III**). The most powerful activation was induced by the PIA biofilm producing strain, although the differences did not reach significance in neonatal blood. In **paper II**, we confirmed the complement activating properties of PIA by using highly-purified PIA. Both the PIA biofilm and purified PIA significantly increased the formation of the C1rs-C1inhibitor complex and C4d/C4dc. This strongly suggests activation through the classical pathway, and can indicate activation of the lectin pathway. Exactly how PIA activates the classical pathway is still unknown. We speculate that there either is activation by direct binding of molecules on the bacterial surface or factors in the biofilm to the first classical component C1q **or** binding through anti-PIA antibodies. Sadovskaya et al. demonstrated relatively high anti-PIA IgG titers in humans (415), as also demonstrated by us in **paper III.**

In addition to activation of the classical pathway, and possible activation of lectin pathway, we observed an increase in Bb/C3bBbP indicating that the alternative amplification loop is engaged in the activation (**Paper II and III).** Throughout literature, one proposed main function of the alternative pathway is to amplify the final complement response to reach higher levels of complement activation than the classical/lectin pathway can on their own (98, 99, 441).

The increased complement activation after exposure to *S. epidermidis* biofilms as shown in **Paper II and III** and by others (183) could indicate an important role of complement cascade when fighting *S. epidermidis* biofilm infections.

In contrast to the complement activation induced by the PIA biofilm producing strain (**Paper II and III),** the PIA biofilm induced a lower activation of leukocytes, shown by lower expression of CD11b and less up-regulation of oxidative burst **(Paper II).** This was in accordance with previous studies (183, 343, 388). The exact reasons for this lower leukocyte activation induced by a PIA biofilm compared to a non-PIA biofilm are not known. An explanation could be that the PIA-biofilm hides PAMPs located on the bacterial surface, and thus reduce deposition of antibodies and/or complement important for leukocyte recognition and activation (182, 442). The recent study by Schommer et al. has also shed further light on the mechanisms behind our observations (378): An already activated leukocyte (e.g. by LPS as in the Schommer study) may be inactivated by unknown substances/mechanisms in the biofilm.

In accordance with the low leukocyte activation induced by PIA biofilms, we also found a lower cytokine secretion after stimulation with a PIA biofilm **(paper II).** We have previously reported a reduced inflammatory response (CRP) in biofilm positive versus biofilm negative CoNS (291). This may be explained by our current finding (**Paper** II) that PIA biofilm induces lower levels of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) compared with a non-PIA biofilm. The explanation for a lower cytokine secretion after PIA biofilm challenge may be the same mechanisms as those causing lower leukocyte activation. Indeed, although we found differences between the PIA and non-PIA biofilm, both biofilms induced significantly stronger cytokine secretion than the non-stimulated control (**Paper II**). A previous study has demonstrated that PIA induces cytokine secretion in isolated human astrocytes (29).

5.4 The neonatal versus the adult innate immune response

Biofilm-associated *S. epidermidis* infections are common in modern neonatal intensive care (280, 304), and the knowledge about the pathogenesis of the infections is crucial to develop new methods to treat these infections. We (**paper II)** and others (183) have found data that indicates an important role of the complement cascade in fighting these infections. Thus, we believe new knowledge about the neonatal complement activation is important. **Paper III** is to my knowledge the first report on neonatal complement activation upon stimulation with *S. epidermidis* biofilm.

We found that both the PIA biofilm and the non-PIA biofilm induced a higher activation of the complement cascade in blood from adults compared to cord blood (**Paper III**). From previous studies we know that the complement system in neonates is immature (152, 153, 155-160, 163), which may explain our findings.

There is a classical initial activation of the complement cascade after stimulation with *S. epidermidis* biofilms, and also engagement of the alternative amplification loop (**Paper I and II**). The classical pathway can be activated through mainly three different mechanisms, where activation through antibodies is one of them. In our experimental model, any antibody dependent complement activation relies on pre-formed antibodies.

In general, the total IgG level is lower in neonates (cord blood) compared adults (255). Preterm neonates have considerably lower titers of anti-CoNS IgG antibodies compared to adults (328). Additionally, PIA antibodies mainly belong to the IgG2 subclass (415, 443), which are modest complement activators. **Paper III** showed that there was significantly less anti-PIA IgG in cord blood compared to adult blood. A contribution to the difference in classical pathway activation

between adults and neonates may thus be a combination of lower PIA antibody titers in cord blood and the inefficient complement activating potency of IgG2.

We observed that both the PIA and non-PIA biofilm induced significantly higher activation of the alternative pathway in adults compared to neonates, which may be caused by low levels of alternative pathway factors (155, 161). A lower activity of the amplification loop in neonates may thus explain the general differences observed between adult and neonate complement activation (**Paper III**), decreasing the amount of complement activation products in the final common pathway. Lower activation of the alternative pathway may also partly explain the many adverse effects of neonates during sepsis, since they then lack the newly suggested regulatory role of the alternative pathway that probably limits damages caused by the complement cascade (100).

In contrast to the lower complement activation in neonates, we found an increased activation of pro-inflammatory cytokines in cord blood compared to adult blood (**Paper III**). Many studies agree that there is an immaturity of the cytokine response in neonates compared to adults. Whether this immaturity is due to reduced production of pro-inflammatory cytokines in neonates as some authors suggest (228-230, 232) or an imbalance in pro- and anti-inflammatory response as other authors suggest (11, 238-242) is still up for discussion. However, due to limitations in our model regarding incubation times, available volumes of neonatal blood and that we wanted to capture the complement activation as well as cytokine secretion, we could only incubate the neonatal blood for 30 minutes. Within this time only the very early pro-inflammatory cytokines have been activated. We can therefore not comment on the balance between pro- and anti-inflammatory cytokines. From our study we therefore can conclude that the very early cytokine secretion from cells in the cord blood of neonates is comparable to the adult response (**Paper III**). Important further studies would be to study cord blood incubated with *S. epidermidis*

biofilms with a longer incubation time, to include more cytokines, both pro- and anti-inflammatory.

5.5 Crosstalk in the innate immune system

The innate immune system is a complex network of many factors acting in synchrony, opposing and balancing each other. The *ex vivo* whole blood sepsis model enables us to study some of these interactions.

In **paper II** we saw a higher activation of the complement cascade induced by the PIA biofilm, while the non-PIA biofilm induced the highest leukocyte activation and cytokine secretion. As stated earlier, an explanation for our findings could be that the PIA biofilm inhibit deposition of opsonins (C3b and antibodies), and thus activation of leukocytes and further cytokine secretion (183, 343, 388, 440). Another explanation could be the interplay between factors of the complement cascade and the leukocytes. Excessive C5a production reduces the effects of neutrophils (107, 444-447), acting via the C5a-receptor (C5aR) (448). Neutrophils play a major role in the defense against invading bacteria (449). The massive inflammatory response induced by the complement cascade causing adverse effects in the patient combined with reduced bacterial clearance due to neutropenia, can therefore be very crucial for the patient.

Both **paper II** and **III** showed contradicting cytokine-complement results, with a "delicate balance" between complement activation and cytokine secretion. In **paper II** we saw that the PIA biofilm induced a higher complement activation compared to the non-PIA biofilm, while the cytokine response was opposite. In **paper III** we found lower complement activation in the neonates, but a higher cytokine secretion compared to adults.

Most publications on the crosstalk in the innate immune system use Gram-negative bacteria or LPS as the stimulating agent (104, 449-451). We can therefore not draw general conclusions that the same mechanisms are present in our system with Gram positive bacteria and for biofilms. However, based on these studies, we can hypothesize on the mechanisms in the crosstalk between these two systems.

The interplay between complement activation and cytokine secretion is complex, with some cytokines being complement dependent, whereas others are complement independent (104, 452, 453). High levels of C5a may also both up- and down-regulate the transcription of different cytokines (118-120, 450, 454). Cytokines may, however, indirectly contribute to complement activation e.g. by increasing the expression of anaphylatoxin receptors (120). There is a complex interplay between the different parts of the innate immune system and the interaction with the microbe. Understanding the underlying mechanisms and the crosstalk between the different parts leading to impaired or inadequate responses, may be a crucial step for the development of new therapeutic targets for treatment of *S. epidermidis* sepsis.

6. Main conclusions

The Arginine Catabolic mobile element (ACME) was proposed as a "new" virulence factor in *S. aureus*. We showed that ACME in *S. epidermidis* was associated with low antimicrobial resistance, negatively associated with biofilm formation, most prevalent in blood cultures of the most mature babies and the cultures categorized as contaminants, indicating its role as a factor most prominent in low-virulent, commensal skin flora.

In a *S. epidermidis* sepsis model we found that *S. epidermidis* biofilms are strong inducers of the inflammatory response. Especially PIA induces a strong activation of the complement cascade, primarily through the classical pathway with engagement of the alternative amplification loop. This indicates a putative important role of the complement cascade when fighting PIA biofilm infections.

The neonatal inflammatory response to *S. epidermidis* biofilms is complex, with a cytokine response comparable to the adult response, while the complement activation was significantly reduced compared to adults. Our results may partly explain the increased susceptibility neonates have to *S. epidermidis* biofilm infections.

There is a complex interplay between the different parts of the inflammatory response when challenged with *S. epidermidis* biofilms. Further knowledge on the basic mechanisms could be important in the development of new treatment strategies.

7. Future aspects

S.epidermidis biofilm infections are common, especially in the hospital setting and in preterm infants. They cause a high morbidity, high public costs and are difficult to eradicate. In line with previous studies we have shown that, *S. epidermidis* can evade some parts of the host immune system and cause imbalances in other parts. Therefore, future research should focus on developing new treatment strategies to modulate the immune system to maximize its effects when fighting these infections. To reach this goal we need more studies looking at:

Inflammatory response in preterm neonates

Preterm neonates have the highest risk of acquiring *S.epidermidis* biofilm infections in the NICU, due to their general immaturity, and increased need of intensive care. Studying the inflammatory response, especially the complement activation, in this group, would probably bring us one step closer to understanding the pathogenesis of these infections, and maybe add to the knowledge on new treatment strategies for this vulnerable group of patients.

Increased knowledge on the initiating mechanisms of the innate immune response and the interaction between the different parts of the system

By the use of PRR inhibitors (such as anti-TLR2 antibodies), inhibitors of the different pathways of the complement cascade (defect serum), and general complement inhibitors (147), we can acquire new information on the initial activation mechanisms of the inflammatory response and how they interact with each other. Such studies can increase our knowledge on which factors are important in the infection response and how they may balance the system.

Animal models

It is a long way from the lab-benches to the real clinical life. Studying the inflammatory response, its initial mechanisms of activation, and interactions after challenging with *S. epidermidis* biofilms *in vivo* is important to more closely understand "nature" and in the long run maybe be able to use information from *ex vivo* and *in vitro* studies in clinical settings.

Reference List

(1) Kenzel S, Henneke P. The innate immune system and its relevance to neonatal sepsis. Curr Opin Infect Dis 2006 Jun;19(3):264-70.

(2) Alberts B, Johson A, Lewis J, Raff M, Robers K, Walters P. Molecular Biology of the Cell. 4th ed. New York and London: Garland Science; 2002.

(3) Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol 2007 Jan 1;81(1):1-5.

(4) Köhl J. The role of complement in danger sensing and transmission. Immunologic Research 2006 Feb 1;34(2):157-76.

(5) Strunk T, Currie A, Richmond P, Simmer K, Burgner D. Innate immunity in human newborn infants: prematurity means more than immaturity. J Matern Fetal Neonatal Med 2011 Jan;24(1):25-31.

(6) Evans NJ, Rutter N. Development of the epidermis in the newborn. Biol Neonate 1986;49(2):74-80.

(7) Kalia YN, Nonato LB, Lund CH, Guy RH. Development of skin barrier function in premature infants. J Invest Dermatol 1998 Aug;111(2):320-6.

(8) Saiman L. Strategies for prevention of nosocomial sepsis in the neonatal intensive care unit. Curr Opin Pediatr 2006 Apr;18(2):101-6.

(9) Manzoni P, Mostert M, Stronati M. Lactoferrin for prevention of neonatal infections. Curr Opin Infect Dis 2011 Jun;24(3):177-82.

(10) Liljedahl M, Bodin L, Schollin J. Coagulase-negative staphylococcal sepsis as a predictor of bronchopulmonary dysplasia. Acta Paediatr 2004 Feb;93(2):211-5.

(11) Tatad AMF, Nesin M, Peoples J, Cheung S, Lin H, Sison C, et al. Cytokine Expression in Response to Bacterial Antigens in Preterm and Term Infant Cord Blood Monocytes. Neonatology 2008;94(1):8-15.

(12) Ng N, Lam D, Paulus P, Batzer G, Horner AA. House dust extracts have both TH2 adjuvant and tolerogenic activities. J Allergy Clin Immunol 2006 May;117(5):1074-81.

(13) Wynn J, Cornell TT, Wong HR, Shanley TP, Wheeler DS. The host response to sepsis and developmental impact. Pediatrics 2010 May;125(5):1031-41.

(14) Hallman M, Ramet M, Ezekowitz RA. Toll-like receptors as sensors of pathogens. Pediatr Res 2001 Sep;50(3):315-21.

(15) Zarember KA, Godowski PJ. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. J Immunol 2002 Jan 15;168(2):554-61.

(16) Heine H, Ulmer AJ. Recognition of bacterial products by toll-like receptors. Chem Immunol Allergy 2005;86:99-119.

(17) Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 1996 Sep 20;86(6):973-83.

(18) Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S. Host Innate Immune Receptors and Beyond: Making Sense of Microbial Infections. Cell Host & Microbe 2008 Jun 12;3(6):352-63.

(19) Tosi MF. Innate immune responses to infection. Journal of Allergy and Clinical Immunology 2005 Aug;116(2):241-9.

(20) Hoffmann JA. The immune response of Drosophila. Nature 2003 Nov 6;426(6962):33-8.

(21) Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006 Feb 24;124(4):783-801.

(22) Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity 2011 May 27;34(5):637-50.

(23) Beutler BA. TLRs and innate immunity. Blood 2009 Feb 12;113(7):1399-407.

(24) Medzhitov R. Recognition of microorganisms and activation of the immune response. Nature 2007 Oct 18;449(7164):819-26.

(25) Janeway, Travers, Walport, Shlomchik. Immunobiology the immune system in health and disease. 6th edition ed. 2005.

(26) Kawai T, Akira S. The roles of TLRs, RLRs and NLRs in pathogen recognition. Int Immunol 2009 Apr;21(4):317-37.

(27) Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc Natl Acad Sci U S A 2000 Dec 5;97(25):13766-71.

(28) Hashimoto M, Tawaratsumida K, Kariya H, Kiyohara A, Suda Y, Krikae F, et al. Not lipoteichoic acid but lipoproteins appear to be the dominant immunobiologically active compounds in *Staphylococcus* aureus. J Immunol 2006 Sep 1;177(5):3162-9.

(29) Stevens N, Sadovskaya I, Jabbouri S, Sattar T, O`Gara JP, Humphreys H, et al. *Staphyococcus epidermidis* polysaccharide intercellular adhesin induces IL-8 expression

in human astrocytes via a mechanism involving TLR2. Cellular Microbiology 2009;11(3):421-32.

(30) Hajjar AM, O'Mahony DS, Ozinsky A, Underhill DM, Aderem A, Klebanoff SJ, et al. Cutting Edge: Functional Interactions Between Toll-Like Receptor (TLR) 2 and TLR1 or TLR6 in Response to Phenol-Soluble Modulin. J Immunol 2001 Jan 1;166(1):15-9.

(31) Takeuchi O, Kaufmann A, Grote K, Kawai T, Hoshino K, Morr M, et al. Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway. J Immunol 2000 Jan 15;164(2):554-7.

(32) Brightbill HD, Libraty DH, Krutzik SR, Yang RB, Belisle JT, Bleharski JR, et al. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. Science 1999 Jul 30;285(5428):732-6.

(33) Aliprantis AO, Yang RB, Mark MR, Suggett S, Devaux B, Radolf JD, et al. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science 1999 Jul 30;285(5428):736-9.

(34) Gangloff M, Gay NJ. MD-2: the Toll "gatekeeper" in endotoxin signalling. Trends in Biochemical Sciences 2004 Jun 1;29(6):294-300.

(35) Poltorak A, He X, Smirnova I, Liu MY, Van HC, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998 Dec 11;282(5396):2085-8.

(36) Cuzzola M, Mancuso G, Beninati C, Biondo C, Genovese F, Tomasello F, et al. Beta 2 integrins are involved in cytokine responses to whole Gram-positive bacteria. J Immunol 2000 Jun 1;164(11):5871-6.

(37) Levy O, Jean-Jacques RM, Cywes C, Sisson RB, Zarember KA, Godowski PJ, et al. Critical role of the complement system in group B *streptococcus*-induced tumor necrosis factor alpha release. Infect Immun 2003 Nov;71(11):6344-53.

(38) Albanyan EA, Edwards MS. Lectin site interaction with capsular polysaccharide mediates nonimmune phagocytosis of type III group B streptococci. Infect Immun 2000 Oct;68(10):5794-802.

(39) Stuart LM, Deng J, Silver JM, Takahashi K, Tseng AA, Hennessy EJ, et al. Response to *Staphylococcus aureus* requires CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain. J Cell Biol 2005 Aug 1;170(3):477-85.

(40) Kapetanovic R, Nahori MA, Balloy V, Fitting C, Philpott DJ, Cavaillon JM, et al. Contribution of phagocytosis and intracellular sensing for cytokine production by *Staphylococcus aureus*-activated macrophages. Infect Immun 2007 Feb;75(2):830-7.

(41) Shaw MH, Reimer T, Kim YG, Nunez G. NOD-like receptors (NLRs): bona fide intracellular microbial sensors. Curr Opin Immunol 2008 Aug;20(4):377-82.

(42) Kleinnijenhuis J, Oosting M, Joosten LA, Netea MG, Van CR. Innate immune recognition of *Mycobacterium tuberculosis*. Clin Dev Immunol 2011;2011:405310.

(43) Levy O, Zarember KA, Roy RM, Cywes C, Godowski PJ, Wessels MR. Selective Impairment of TLR-Mediated Innate Immunity in Human Newborns: Neonatal Blood Plasma Reduces Monocyte TNF-{alpha} Induction by Bacterial Lipopeptides, Lipopolysaccharide, and Imiquimod, but Preserves the Response to R-848. J Immunol 2004 Oct 1;173(7):4627-34.

(44) Viemann D, Dubbel G, Schleifenbaum S, Harms E, Sorg C, Roth J. Expression of toll-like receptors in neonatal sepsis. Pediatr Res 2005 Oct;58(4):654-9.

(45) Yan SR, Qing G, Byers DM, Stadnyk AW, Al-Hertani W, Bortolussi R. Role of MyD88 in diminished tumor necrosis factor alpha production by newborn mononuclear cells in response to lipopolysaccharide. Infect Immun 2004 Mar;72(3):1223-9.

(46) Forster-Waldl E, Sadeghi K, Tamandl D, Gerhold B, Hallwirth U, Rohrmeister K, et al. Monocyte toll-like receptor 4 expression and LPS-induced cytokine production increase during gestational aging. Pediatr Res 2005 Jul;58(1):121-4.

(47) Kollmann TR, Crabtree J, Rein-Weston A, Blimkie D, Thommai F, Wang XY, et al. Neonatal innate TLR-mediated responses are distinct from those of adults. J Immunol 2009 Dec 1;183(11):7150-60.

(48) Levy O, Coughlin M, Cronstein BN, Roy RM, Desai A, Wessels MR. The adenosine system selectively inhibits TLR-mediated TNF-alpha production in the human newborn. J Immunol 2006 Aug 1;177(3):1956-66.

(49) Sadeghi K, Berger A, Langgartner M, Prusa AR, Hayde M, Herkner K, et al. Immaturity of infection control in preterm and term newborns is associated with impaired toll-like receptor signaling. J Infect Dis 2007 Jan 15;195(2):296-302.

(50) Lorenz E, Hallman M, Marttila R, Haataja R, Schwartz DA. Association between the Asp299Gly polymorphisms in the Toll-like receptor 4 and premature births in the Finnish population. Pediatr Res 2002 Sep;52(3):373-6.

(51) Krediet TG, Wiertsema SP, Vossers MJ, Hoeks SB, Fleer A, Ruven HJ, et al. Toll-like receptor 2 polymorphism is associated with preterm birth. Pediatr Res 2007 Oct;62(4):474-6.

(52) Hancock RE, Diamond G. The role of cationic antimicrobial peptides in innate host defences. Trends Microbiol 2000 Sep;8(9):402-10.

(53) Otto M. Bacterial sensing of antimicrobial peptides. Contrib Microbiol 2009;16:136-49.

(54) Suzuki YA, Lonnerdal B. Characterization of mammalian receptors for lactoferrin. Biochem Cell Biol 2002;80(1):75-80.

(55) Harwig SS, Tan L, Qu XD, Cho Y, Eisenhauer PB, Lehrer RI. Bactericidal properties of murine intestinal phospholipase A2. J Clin Invest 1995 Feb;95(2):603-10.

(56) Arnold RR, Cole MF, McGhee JR. A bactericidal effect for human lactoferrin. Science 1977 Jul 15;197(4300):263-5.

(57) Adamik B, Zimecki M, Wlaszczyk A, Berezowicz P, Kubler A. Lactoferrin effects on the in vitro immune response in critically ill patients. Arch Immunol Ther Exp (Warsz) 1998;46(3):169-76.

(58) Peschel A, Sahl HG. The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nat Rev Microbiol 2006 Jul;4(7):529-36.

(59) Levy O. Antimicrobial proteins and peptides: anti-infective molecules of mammalian leukocytes. J Leukoc Biol 2004 Nov;76(5):909-25.

(60) Robert EW H. Cationic peptides: effectors in innate immunity and novel antimicrobials. The Lancet Infectious Diseases 2001 Oct;1(3):156-64.

(61) Wiese A, Gutsmann T, Seydel U. Towards antibacterial strategies: studies on the mechanisms of interaction between antibacterial peptides and model membranes. J Endotoxin Res 2003;9(2):67-84.

(62) Hristova K, Selsted ME, White SH. Critical role of lipid composition in membrane permeabilization by rabbit neutrophil defensins. J Biol Chem 1997 Sep 26;272(39):24224-33.

(63) Levy O. Antimicrobial proteins and peptides of blood: templates for novel antimicrobial agents. Blood 2000 Oct 15;96(8):2664-72.

(64) Ward PP, Paz E, Conneely OM. Multifunctional roles of lactoferrin: a critical overview. Cell Mol Life Sci 2005 Nov;62(22):2540-8.

(65) Legrand D, Pierce A, Elass E, Carpentier M, Mariller C, Mazurier J. Lactoferrin structure and functions. Adv Exp Med Biol 2008;606:163-94.

(66) Jurado RL. Iron, infections, and anemia of inflammation. Clin Infect Dis 1997 Oct;25(4):888-95.

(67) Chapple DS, Mason DJ, Joannou CL, Odell EW, Gant V, Evans RW. Structure-function relationship of antibacterial synthetic peptides homologous to a helical surface region on human lactoferrin against *Escherichia coli* serotype O111. Infect Immun 1998 Jun;66(6):2434-40.

(68) Gahr M, Speer CP, Damerau B, Sawatzki G. Influence of lactoferrin on the function of human polymorphonuclear leukocytes and monocytes. J Leukoc Biol 1991 May;49(5):427-33.

(69) Lima MF, Kierszenbaum F. Lactoferrin effects on phagocytic cell function. I. Increased uptake and killing of an intracellular parasite by murine macrophages and human monocytes. J Immunol 1985 Jun;134(6):4176-83.

(70) Ochoa TJ, Cleary TG. Effect of lactoferrin on enteric pathogens. Biochimie 2009 Jan;91(1):30-4.

(71) Valenti P, Antonini G. Lactoferrin: an important host defence against microbial and viral attack. Cell Mol Life Sci 2005 Nov;62(22):2576-87.

(72) Drago-Serrano ME, Rivera-Aguilar V, Resendiz-Albor AA, Campos-Rodriguez R. Lactoferrin increases both resistance to *Salmonella typhimurium* infection and the production of antibodies in mice. Immunol Lett 2010 Nov 30;134(1):35-46.

(73) Venkatesh MP, Pham D, Kong L, Weisman LE. Prophylaxis with lactoferrin, a novel antimicrobial agent, in a neonatal rat model of coinfection. Adv Ther 2007 Sep;24(5):941-54.

(74) Schittek B, Hipfel R, Sauer B, Bauer J, Kalbacher H, Stevanovic S, et al. Dermcidin: a novel human antibiotic peptide secreted by sweat glands. Nat Immunol 2001 Dec;2(12):1133-7.

(75) Samuelsen O, Haukland HH, Jenssen H, Kramer M, Sandvik K, Ulvatne H, et al. Induced resistance to the antimicrobial peptide lactoferricin B in *Staphylococcus aureus*. FEBS Lett 2005 Jun 20;579(16):3421-6.

(76) Samuelsen O, Haukland HH, Kahl BC, von EC, Proctor RA, Ulvatne H, et al. *Staphylococcus aureus* small colony variants are resistant to the antimicrobial peptide lactoferricin B. J Antimicrob Chemother 2005 Dec;56(6):1126-9.

(77) Haug BE, Stensen W, Kalaaji M, Rekdal O, Svendsen JS. Synthetic antimicrobial peptidomimetics with therapeutic potential. J Med Chem 2008 Jul 24;51(14):4306-14.

(78) Flemming K, Klingenberg C, Cavanagh JP, Sletteng M, Stensen W, Svendsen JS, et al. High in vitro antimicrobial activity of synthetic antimicrobial peptidomimetics against staphylococcal biofilms. J Antimicrob Chemother 2009 Jan 1;63(1):136-45.

(79) Gordon YJ, Romanowski EG, McDermott AM. A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. Curr Eye Res 2005 Jul;30(7):505-15.

(80) Andres E, Dimarcq JL. Cationic antimicrobial peptides: update of clinical development. J Intern Med 2004 Apr;255(4):519-20.

(81) Strunk T, Doherty D, Richmond P, Simmer K, Charles A, Levy O, et al. Reduced levels of antimicrobial proteins and peptides in human cord blood plasma. Arch Dis Child Fetal Neonatal Ed 2009 May;94(3):F230-F231.

(82) Manzoni P, Rinaldi M, Cattani S, Pugni L, Romeo MG, Messner H, et al. Bovine lactoferrin supplementation for prevention of late-onset sepsis in very low-birth-weight neonates: a randomized trial. JAMA 2009 Oct 7;302(13):1421-8.

(83) Pammi M, Abrams SA. Oral lactoferrin for the prevention of sepsis and necrotizing enterocolitis in preterm infants. Cochrane Database Syst Rev 2011;(10):CD007137.

(84) Pammi M, Abrams SA. Oral lactoferrin for the treatment of sepsis and necrotizing enterocolitis in neonates. Cochrane Database Syst Rev 2011;(10):CD007138.

(85) Walport MJ. Complement First of two parts. New England Journal of Medicine 2001 Apr 5;344(14):1058-66.

(86) Herter. Cytotoxins and cytotoxic immunity - The Herter lectures. Boston Medical and Surgical Journal 1904;150:448-58.

(87) Mollnes TE, Song WC, Lambris JD. Complement in inflammatory tissue damage and disease. Trends Immunol 2002 Feb;23(2):61-4.

(88) Serruto D, Rappuoli R, Scarselli M, Gros P, van Strijp JAG. Molecular mechanisms of complement evasion: learning from staphylococci and meningococci. Nat Rev Micro 2010 Jun;8(6):393-9.

(89) Biro A, Rovo Z, Papp D, Cervenak L, Varga L, Fust G, et al. Studies on the interactions between C-reactive protein and complement proteins. Immunology 2007 May;121(1):40-50.

(90) Paidassi H, Tacnet-Delorme P, Garlatti V, Darnault C, Ghebrehiwet B, Gaboriaud C, et al. C1q binds phosphatidylserine and likely acts as a multiligand-bridging molecule in apoptotic cell recognition. J Immunol 2008 Feb 15;180(4):2329-38.

(91) Milan B. Ambivalent effect of immunoglobulins on the complement system: Activation versus inhibition. Molecular Immunology 2008 Oct;45(16):4073-9.

(92) Bindon CI, Hale G, Bruggemann M, Waldmann H. Human monoclonal IgG isotypes differ in complement activating function at the level of C4 as well as C1q. J Exp Med 1988 Jul 1;168(1):127-42.

(93) Runza VL, Schwaeble W, Mannel DN. Ficolins: novel pattern recognition molecules of the innate immune response. Immunobiology 2008;213(3-4):297-306.

(94) Roos A, Bouwman LH, Munoz J, Zuiverloon T, Faber-Krol MC, Fallaux-van den Houten FC, et al. Functional characterization of the lectin pathway of complement in human serum. Mol Immunol 2003 Jan;39(11):655-68.

(95) Bexborn F, Andersson PO, Chen H, Nilsson B, Ekdahl KN. The tick-over theory revisited: formation and regulation of the soluble alternative complement C3 convertase (C3(H2O)Bb). Mol Immunol 2008 Apr;45(8):2370-9.

(96) Spitzer D, Mitchell LM, Atkinson JP, Hourcade DE. Properdin can initiate complement activation by binding specific target surfaces and providing a platform for de novo convertase assembly. J Immunol 2007 Aug 15;179(4):2600-8.

(97) Pillemer L, Blum L., Lepow IH, Ross OA, Todd EW, Wardlaw AC. The properdin system and immunity. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena. Science 1954 Aug 20;120(3112):279-85.

(98) Harboe M, Ulvund G, Vien L, Fung M, Mollnes TE. The quantitative role of alternative pathway amplification in classical pathway induced terminal complement activation. Clin Exp Immunol 2004 Dec;138(3):439-46.

(99) Harboe M, Garred P, Karlstrom E, Lindstad JK, Stahl GL, Mollnes TE. The down-stream effects of mannan-induced lectin complement pathway activation depend quantitatively on alternative pathway amplification. Mol Immunol 2009 Dec;47(2-3):373-80.

(100) Dahlke K, Wrann CD, Sommerfeld O, Sossedorf M, Recknagel P, Sachse S, et al. Distinct different contributions of the alternative and classical complement activation pathway for the innate host response during sepsis. J Immunol 2011 Mar 1;186(5):3066-75.

(101) Cole DS, Morgan BP. Beyond lysis: how complement influences cell fate. Clin Sci (Lond) 2003 May;104(5):455-66.

(102) Sprong T, Moller AS, Bjerre A, Wedege E, Kierulf P, van der Meer JWM, et al. Complement activation and complement-dependent inflammation by *Neisseria meningitidis* are independent of lipopolysaccharide. Infect Immun 2004 Jun 1;72(6):3344-9.

(103) Bestebroer J, Aerts PC, Rooijakkers SH, Pandey MK, Kohl J, van Strijp JA, et al. Functional basis for complement evasion by staphylococcal superantigen-like 7. Cell Microbiol 2010 Oct;12(10):1506-16.

(104) Mollnes TE, Brekke OL, Fung M, Fure H, Christiansen D, Bergseth G, et al. Essential role of the C5a receptor in *E coli*-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation. Blood 2002 Aug 13;100(5):1869-77.

(105) Hajishengallis G, Lambris JD. Crosstalk pathways between Toll-like receptors and the complement system. Trends in Immunology 2010 Apr;31(4):154-63.

(106) Fernandez HN, Hugli TE. Primary structural analysis of the polypeptide portion of human C5a anaphylatoxin. Polypeptide sequence determination and assignment of the oligosaccharide attachment site in C5a. Journal of Biological Chemistry 1978 Oct 10;253(19):6955-64.

(107) Ward PA. The dark side of C5a in sepsis. Nat Rev Immunol 2004 Feb;4(2):133-42.

(108) Hartmann K, Henz BM, Kruger-Krasagakes S, Kohl J, Burger R, Guhl S, et al. C3a and C5a stimulate chemotaxis of human mast cells. Blood 1997 Apr 15;89(8):2863-70.

(109) Daffern PJ, Pfeifer PH, Ember JA, Hugli TE. C3a is a chemotaxin for human eosinophils but not for neutrophils. I. C3a stimulation of neutrophils is secondary to eosinophil activation. J Exp Med 1995 Jun 1;181(6):2119-27.

(110) Haeger M, Unander M, Norder-Hansson B, Tylman M, Bengtsson A. Complement, neutrophil, and macrophage activation in women with severe preeclampsia and the syndrome of hemolysis, elevated liver enzymes, and low platelet count. Obstet Gynecol 1992 Jan;79(1):19-26.

(111) Takafuji S, Tadokoro K, Ito K, Dahinden CA. Degranulation from human eosinophils stimulated with C3a and C5a. Int Arch Allergy Immunol 1994;104 Suppl 1(1):27-9.

(112) Ehrengruber MU, Geiser T, Deranleau DA. Activation of human neutrophils by C3a and C5A. Comparison of the effects on shape changes, chemotaxis, secretion, and respiratory burst. FEBS Lett 1994 Jun 13;346(2-3):181-4.

(113) Goldstein IM, Roos D, Kaplan HB, Weissmann G. Complement and immunoglobulins stimulate superoxide production by human leukocytes independently of phagocytosis. J Clin Invest 1975 Nov;56(5):1155-63.

(114) Perianayagam MC, Balakrishnan VS, King AJ, Pereira BJ, Jaber BL. C5a delays apoptosis of human neutrophils by a phosphatidylinositol 3-kinase-signaling pathway. Kidney Int 2002 Feb;61(2):456-63.

(115) Foreman KE, Vaporciyan AA, Bonish BK, Jones ML, Johnson KJ, Glovsky MM, et al. C5a-induced expression of P-selectin in endothelial cells. J Clin Invest 1994 Sep;94(3):1147-55.

(116) Jagels MA, Daffern PJ, Hugli TE. C3a and C5a enhance granulocyte adhesion to endothelial and epithelial cell monolayers: epithelial and endothelial priming is required for C3a-induced eosinophil adhesion. Immunopharmacology 2000 Mar;46(3):209-22.

(117) Ember JA, Sanderson SD, Hugli TE, Morgan EL. Induction of interleukin-8 synthesis from monocytes by human C5a anaphylatoxin. Am J Pathol 1994 Feb;144(2):393-403.

(118) Okusawa S, Dinarello CA, Yancey KB, Endres S, Lawley TJ, Frank MM, et al. C5a induction of human interleukin 1. Synergistic effect with endotoxin or interferon-gamma. J Immunol 1987 Oct 15;139(8):2635-40.

(119) Okusawa S, Yancey KB, van der Meer JW, Endres S, Lonnemann G, Hefter K, et al. C5a stimulates secretion of tumor necrosis factor from human mononuclear cells in vitro. Comparison with secretion of interleukin 1 beta and interleukin 1 alpha. J Exp Med 1988 Jul 1;168(1):443-8.

(120) Riedemann NC, Neff TA, Guo RF, Bernacki KD, Laudes IJ, Sarma JV, et al. Protective Effects of IL-6 Blockade in Sepsis Are Linked to Reduced C5a Receptor Expression. J Immunol 2003 Jan 1;170(1):503-7.

(121) Schindler R, Gelfand JA, Dinarello CA. Recombinant C5a stimulates transcription rather than translation of interleukin-1 (IL-1) and tumor necrosis factor: translational signal provided by lipopolysaccharide or IL-1 itself. Blood 1990 Oct 15;76(8):1631-8.

(122) Cochrane CG, Muller-Eberhard HJ. The derivation of two distinct anaphylatoxin activities from the third and fifth components of human complement. J Exp Med 1968 Feb 1;127(2):371-86.

(123) Gorski JP, Hugli TE, Muller-Eberhard HJ. C4a: the third anaphylatoxin of the human complement system. Proc Natl Acad Sci U S A 1979 Oct;76(10):5299-302.

(124) Schumacher WA, Fantone JC, Kunkel SE, Webb RC, Lucchesi BR. The anaphylatoxins C3a and C5a are vasodilators in the canine coronary vasculature in vitro and in vivo. Agents Actions 1991 Nov;34(3-4):345-9.

(125) Rittirsch D, Flierl MA, Nadeau BA, Day DE, Huber-Lang M, Mackay CR, et al. Functional roles for C5a receptors in sepsis. Nat Med 2008 May;14(5):551-7.

(126) Chen NJ, Mirtsos C, Suh D, Lu YC, Lin WJ, McKerlie C, et al. C5L2 is critical for the biological activities of the anaphylatoxins C5a and C3a. Nature 2007 Mar 8;446(7132):203-7.

(127) Bamberg CE, Mackay CR, Lee H, Zahra D, Jackson J, Lim YS, et al. The C5a Receptor (C5aR) C5L2 Is a Modulator of C5aR-mediated Signal Transduction. Journal of Biological Chemistry 2010 Mar 5;285(10):7633-44.

(128) Vogt W. Complement activation by myeloperoxidase products released from stimulated human polymorphonuclear leukocytes. Immunobiology 1996 Aug;195(3):334-46.

(129) Huber-Lang M, Sarma JV, Zetoune FS, Rittirsch D, Neff TA, McGuire SR, et al. Generation of C5a in the absence of C3: a new complement activation pathway. Nat Med 2006 Jun;12(6):682-7.

(130) Vogt W. Cleavage of the fifth component of complement and generation of a functionally active C5b6-like complex by human leukocyte elastase. Immunobiology 2000 Jan;201(3-4):470-7.

(131) Wetsel RA, Kolb WP. Expression of C5a-like biological activities by the fifth component of human complement (C5) upon limited digestion with noncomplement enzymes without release of polypeptide fragments. J Exp Med 1983 Jun 1;157(6):2029-48.

(132) Ward PA. The harmful role of C5a on innate immunity in sepsis. J Innate Immun 2010;2(5):439-45.

(133) Puri TS, Quigg RJ. The Many Effects of Complement C3- and C5-Binding Proteins in Renal Injury. Seminars in Nephrology 2007 May;27(3):321-37.

(134) Elsner J, Oppermann M, Czech W, Dobos G, Schopf E, Norgauer J, et al. C3a activates reactive oxygen radical species production and intracellular calcium transients in human eosinophils. Eur J Immunol 1994 Mar;24(3):518-22.

(135) Elsner J, Oppermann M, Czech W, Kapp A. C3a activates the respiratory burst in human polymorphonuclear neutrophilic leukocytes via pertussis toxin-sensitive G-proteins. Blood 1994 Jun 1;83(11):3324-31.

(136) Takabayashi T, Vannier E, Clark BD, Margolis NH, Dinarello CA, Burke JF, et al. A new biologic role for C3a and C3a desArg: regulation of TNF-alpha and IL-1 beta synthesis. J Immunol 1996 May 1;156(9):3455-60.

(137) Takabayashi T, Vannier E, Burke JF, Tompkins RG, Gelfand JA, Clark BD. Both C3a and C3a(desArg) regulate interleukin-6 synthesis in human peripheral blood mononuclear cells. J Infect Dis 1998 Jun;177(6):1622-8.

(138) Raby AC, Holst B, Davies J, Colmont C, Laumonnier Y, Coles B, et al. TLR activation enhances C5a-induced pro-inflammatory responses by negatively modulating the second C5a receptor, C5L2. Eur J Immunol 2011;41(9):2741-52.

(139) Guo RF, Riedemann NC, Ward PA. Role of C5a-C5aR interaction in sepsis. Shock 2004 Jan;21(1):1-7.

(140) Woodruff TM, Arumugam TV, Shiels IA, Reid RC, Fairlie DP, Taylor SM. Protective effects of a potent C5a receptor antagonist on experimental acute limb ischemia-reperfusion in rats. J Surg Res 2004 Jan;116(1):81-90.

(141) Grant EP, Picarella D, Burwell T, Delaney T, Croci A, Avitahl N, et al. Essential role for the C5a receptor in regulating the effector phase of synovial infiltration and joint destruction in experimental arthritis. J Exp Med 2002 Dec 2;196(11):1461-71.

(142) Banda NK, Thurman JM, Kraus D, Wood A, Carroll MC, Arend WP, et al. Alternative Complement pathway activation is essential for inflammation and joint destruction in the passive transfer model of collagen-induced arthritis. J Immunol 2006 Aug 1;177(3):1904-12.

(143) Stevens JH, O'Hanley P, Shapiro JM, Mihm FG, Satoh PS, Collins JA, et al. Effects of anti-C5a antibodies on the adult respiratory distress syndrome in septic primates. J Clin Invest 1986 Jun;77(6):1812-6.

(144) Pickering MC, Cook HT, Warren J, Bygrave AE, Moss J, Walport MJ, et al. Uncontrolled C3 activation causes membranoproliferative glomerulonephritis in mice deficient in complement factor H. Nat Genet 2002 Aug;31(4):424-8.

(145) Thurman JM, Holers VM. The central role of the alternative complement pathway in human disease. J Immunol 2006 Feb 1;176(3):1305-10.

(146) Ricklin D, Lambris JD. Compstatin: a complement inhibitor on its way to clinical application. Adv Exp Med Biol 2008;632:273-92.

(147) Emlen W, Li W, Kirschfink M. Therapeutic complement inhibition: new developments. Semin Thromb Hemost 2010 Sep;36(6):660-8.

(148) Tschumi S, Gugger M, Bucher BS, Riedl M, Simonetti GD. Eculizumab in atypical hemolytic uremic syndrome: long-term clinical course and histological findings. Pediatr Nephrol 2011 Nov;26(11):2085-8.

(149) Kelly RJ, Hill A, Arnold LM, Brooksbank GL, Richards SJ, Cullen M, et al. Long-term treatment with eculizumab in paroxysmal nocturnal hemoglobinuria: sustained efficacy and improved survival. Blood 2011 Jun 23;117(25):6786-92.

(150) Lapeyraque AL, Malina M, Fremeaux-Bacchi V, Boppel T, Kirschfink M, Oualha M, et al. Eculizumab in severe Shiga-toxin-associated HUS. New England Journal of Medicine 2011 May 25;364(26):2561-3.

(151) Berger M. Complement deficiency and neutrophil dysfunction as risk factors for bacterial infection in newborns and the role of granulocyte transfusion in therapy. Rev Infect Dis 1990 May;12 Suppl 4:S401-S409.

(152) Petrova A, Mehta R. Dysfunction of innate immunity and associated pathology in neonates. Indian J Pediatr 2007 Feb;74(2):185-91.

(153) Wolach B, Dolfin T, Regev R, Gilboa S, Schlesinger M. The development of the complement system after 28 weeks' gestation. Acta Paediatr 1997 May;86(5):523-7.

(154) McGreal EP, Hearne K, Spiller OB. Off to a slow start: Under-development of the complement system in term newborns is more substantial following premature birth. Immunobiology. In press 2011 .

(155) Sonntag J, Brandenburg U, Polzehl D, Strauss E, Vogel M, Dudenhausen JW, et al. Complement system in healthy term newborns: Reference values in umbilical cord blood. Pediatric and Developmental Pathology 1998 Mar 21;1(2):131-5.

(156) Drew JH, Arroyave CM. The complement system of the newborn infant. Biol Neonate 1980;37(3-4):209-17.

(157) Wagner MH, Sonntag J, Strauss E, Obladen M. Complement and contact activation related to surfactant response in respiratory distress syndrome. Pediatr Res 1999 Jan;45(1):14-8.

(158) Miyano A, Miyamichi T, Nakayama M, Kitajima H, Shimizu A. Effect of chorioamnionitis on the levels of serum proteins in the cord blood of premature infants. Arch Pathol Lab Med 1996 Mar;120(3):245-8.

(159) Hogasen AK, Overlie I, Hansen TW, Abrahamsen TG, Finne PH, Hogasen K. The analysis of the complement activation product SC5 b-9 is applicable in neonates in spite of their profound C9 deficiency. J Perinat Med 2000;28(1):39-48.

(160) Davis CA, Vallota EH, Forristal J. Serum complement levels in infancy: age related changes. Pediatr Res 1979 Sep;13(9):1043-6.

(161) Adamkin D, Stitzel A, Urmson J, Farnett ML, Post E, Spitzer R. Activity of the alternative pathway of complement in the newborn infant. J Pediatr 1978 Oct;93(4):604-8.

(162) Strunk RC, Fenton LJ, Gaines JA. Alternative pathway of complement activation in full term and premature infants. Pediatr Res 1979 May;13(5 Pt 1):641-3.

(163) Johnson U, Truedsson L, Gustavii B. Complement components in 100 newborns and their mothers determined by electroimmunoassay. Acta Pathol Microbiol Immunol Scand C 1983 Apr;91(2):147-50.

(164) Notarangelo LD, Chirico G, Chiara A, Colombo A, Rondini G, Plebani A, et al. Activity of classical and alternative pathways of complement in preterm and small for gestational age infants. Pediatr Res 1984 Mar;18(3):281-5.

(165) Super M, Gillies SD, Foley S, Sastry K, Schweinle JE, Silverman VJ, et al. Distinct and overlapping functions of allelic forms of human mannose binding protein. Nat Genet 1992 Sep;2(1):50-5.

(166) Skattum L, van Deuren M, van der Poll T, Truedsson L. Complement deficiency states and associated infections. Molecular Immunology 2011 Aug;48(14):1643-55.

(167) Garred P, Larsen F, Seyfarth J, Fujita R, Madsen HO. Mannose-binding lectin and its genetic variants. Genes Immun 2006 Jan 5;7(2):85-94.

(168) Madsen HO, Garred P, Thiel S, Kurtzhals JA, Lamm LU, Ryder LP, et al. Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. J Immunol 1995 Sep 15;155(6):3013-20.

(169) Swierzko AS, Atkinson AP, Cedzynski M, Macdonald SL, Szala A, Domzalska-Popadiuk I, et al. Two factors of the lectin pathway of complement, l-ficolin and mannan-binding lectin, and their associations with prematurity, low birthweight and infections in a large cohort of polish neonates. Mol Immunol 2009 Feb;46(4):551-8.

(170) Dzwonek AB, Neth OW, Thiebaut R, Gulczynska E, Chilton M, Hellwig T, et al. The role of mannose-binding lectin in susceptibility to infection in preterm neonates. Pediatr Res 2008 Jun;63(6):680-5.

(171) Frakking FN, Brouwer N, van Eijkelenburg NK, Merkus MP, Kuijpers TW, Offringa M, et al. Low mannose-binding lectin (MBL) levels in neonates with pneumonia and sepsis. Clin Exp Immunol 2007 Nov;150(2):255-62.

(172) De Benedetti F, Auriti C, D'Urbano LE, Ronchetti MP, Rava L, Tozzi A, et al. Low serum levels of mannose binding lectin are a risk factor for neonatal sepsis. Pediatr Res 2007 Mar;61(3):325-8.

(173) Ozdemir O, Dinleyici EC, Tekin N, Colak O, Aksit MA. Low-mannose-binding lectin levels in susceptibility to neonatal sepsis in preterm neonates with fetal inflammatory response syndrome. J Matern Fetal Neonatal Med 2010 Sep;23(9):1009-13.

(174) Aittoniemi J, Miettinen A, Laippala P, Isolauri E, Viikari J, Ruuska T, et al. Age-dependent variation in the serum concentration of mannan-binding protein. Acta Paediatr 1996 Aug;85(8):906-9.

(175) Kilpatrick DC, Fujita T, Matsushita M. P35, an opsonic lectin of the ficolin family, in human blood from neonates, normal adults, and recurrent miscarriage patients. Immunol Lett 1999 Apr 1;67(2):109-12.

(176) Sallenbach S, Thiel S, Aebi C, Otth M, Bigler S, Jensenius JC, et al. Serum concentrations of lectin-pathway components in healthy neonates, children and adults: mannan-binding lectin (MBL), M-, L-, and H-ficolin, and MBL-associated serine protease-2 (MASP-2). Pediatr Allergy Immunol 2011 Jun;22(4):424-30.

(177) Adinolfi M, Dobson NC, Bradwell AR. Synthesis of two components of human complement, beta 1H and C3bINA, during fetal life. Acta Paediatr Scand 1981 Sep;70(5):705-10.

(178) Lassiter HA, Watson SW, Seifring ML, Tanner JE. Complement factor 9 deficiency in serum of human neonates. J Infect Dis 1992 Jul;166(1):53-7.

(179) Malm J, Bennhagen R, Holmberg L, Dahlback B. Plasma concentrations of C4b-binding protein and vitamin K-dependent protein S in term and preterm infants: low levels of protein S-C4b-binding protein complexes. Br J Haematol 1988 Apr;68(4):445-9.

(180) de Paula PF, Barbosa JE, Junior PR, Ferriani VP, Latorre MR, Nudelman V, et al. Ontogeny of complement regulatory proteins - concentrations of factor h, factor I, c4b-binding protein, properdin and vitronectin in healthy children of different ages and in adults. Scand J Immunol 2003 Nov;58(5):572-7.

(181) Hellerud BC, Stenvik J, Espevik T, Lambris JD, Mollnes TE, Brandtzaeg P. Stages of meningococcal sepsis simulated in vitro, with emphasis on complement and Toll-like receptor activation. Infect Immun 2008 Sep 1;76(9):4183-9.

(182) Clark LA, Easmon CSF. Opsonic requirements of *staphylococcus epidermidis*. J Med Microbiol 1986 Aug 1;22(1):1-7.

(183) Kristian SA, Birkenstock TA, Sauder U, Mack D, Götz F, Landmann R. Biofilm formation induces C3a release and protects *staphylococcus epidermidis* from IgG and complement deposition and from neutrophil-dependent killing. Journal of Infectious Diseases 2008 Apr 1;197(7):1028-35.

(184) Salvesen B, Fung M, Saugstad OD, Mollnes TE. Role of Complement and CD14 in Meconium-Induced Cytokine Formation. Pediatrics 2008 Mar 1;121(3):e496-e505.

(185) Salvesen B, Nielsen EW, Harboe M, Saugstad OD, Mollnes TE. Mechanisms of complement activation and effects of C1-inhibitor on the meconium-induced inflammatory reaction in human cord blood. Mol Immunol 2009 Feb;46(4):688-94.

(186) Mollnes TE, Castellheim A, Lindenskov PH, Salvesen B, Saugstad OD. The role of complement in meconium aspiration syndrome. J Perinatol 2008 Dec;28 Suppl 3:S116-S119.

(187) Hecke F, Hoehn T, Strauss E, Obladen M, Sonntag J. In-vitro activation of complement system by lactic acidosis in newborn and adults. Mediators Inflamm 2001 Feb;10(1):27-31.

(188) Lassiter HA. The role of complement in neonatal hypoxic-ischemic cerebral injury. Clin Perinatol 2004 Mar;31(1):117-27.

(189) Frank MM, Miletic VD, Jiang H. Immunoglobulin in the control of complement action. Immunol Res 2000;22(2-3):137-46.

(190) Miletic VD, Frank MM. Complement-immunoglobulin interactions. Curr Opin Immunol 1995 Feb;7(1):41-7.

(191) Landor M. Maternal-fetal transfer of immunoglobulins. Ann Allergy Asthma Immunol 1995 Apr;74(4):279-83.

(192) Schelonka RL, Infante AJ. Neonatal immunology. Seminars in Perinatology 1998 Feb;22(1):2-14.

(193) Davies J, Turner M, Klein N. The role of the collectin system in pulmonary defence. Paediatr Respir Rev 2001 Mar;2(1):70-5.

(194) Watford WT, Wright JR, Hester CG, Jiang H, Frank MM. Surfactant protein A regulates complement activation. J Immunol 2001 Dec 1;167(11):6593-600.

(195) Schultz SJ, Aly H, Hasanen BM, Khashaba MT, Lear SC, Bendon RW, et al. Complement component 9 activation, consumption, and neuronal deposition in the post-hypoxic-ischemic central nervous system of human newborn infants. Neurosci Lett 2005 Apr 11;378(1):1-6.

(196) Faurschou M, Borregaard N. Neutrophil granules and secretory vesicles in inflammation. Microbes Infect 2003 Nov;5(14):1317-27.

(197) Wynn JL, Levy O. Role of innate host defenses in susceptibility to early-onset neonatal sepsis. 37 ed. 2010. p. 307-37.

(198) Nathan CF. Secretory products of macrophages. J Clin Invest 1987 Feb;79(2):319-26.

(199) Ehlers MR. CR3: a general purpose adhesion-recognition receptor essential for innate immunity. Microbes Infect 2000 Mar;2(3):289-94.

(200) Björkqvist M, Jurstrand M, Bodin L, Fredlund H, Schollin J. Defective neutrophil oxidative burst in preterm newborns on exposure to Coagulase-negative staphylococci. Pediatric Research 2004;55(6).

(201) Levy O. Innate immunity of the newborn: basic mechanisms and clinical correlates. Nat Rev Immunol 2007 May;7(5):379-90.

(202) Gessler P, Luders R, Konig S, Haas N, Lasch P, Kachel W. Neonatal neutropenia in low birthweight premature infants. Am J Perinatol 1995 Jan;12(1):34-8.

(203) Koenig JM, Christensen RD. Incidence, neutrophil kinetics, and natural history of neonatal neutropenia associated with maternal hypertension. N Engl J Med 1989 Aug 31;321(9):557-62.

(204) Carr R. Neutrophil production and function in newborn infants. Br J Haematol 2000 Jul;110(1):18-28.

(205) Henneke P, Berner R. Interaction of neonatal phagocytes with group B *streptococcus*: recognition and response. Infect Immun 2006 Jun;74(6):3085-95.

(206) Hallwirth U, Pomberger G, Zaknun D, Szepfalusi Z, Horcher E, Pollak A, et al. Monocyte phagocytosis as a reliable parameter for predicting early-onset sepsis in very low birthweight infants. Early Hum Dev 2002 Apr;67(1-2):1-9.

(207) Klein RB, Fischer TJ, Gard SE, Biberstein M, Rich KC, Stiehm ER. Decreased mononuclear and polymorphonuclear chemotaxis in human newborns, infants, and young children. Pediatrics 1977 Oct;60(4):467-72.

(208) Eisenfeld L, Krause PJ, Herson V, Savidakis J, Bannon P, Maderazo E, et al. Longitudinal study of neutrophil adherence and motility. The Journal of Pediatrics 1990 Dec;117(6):926-9.

(209) Reddy RK, Xia Y, Hanikyrova M, Ross GD. A mixed population of immature and mature leucocytes in umbilical cord blood results in a reduced expression and function of CR3 (CD11b/CD18). Clin Exp Immunol 1998 Dec;114(3):462-7.

(210) Rebuck N, Gibson A, Finn A. Neutrophil adhesion molecules in term and premature infants: normal or enhanced leucocyte integrins but defective L-selectin expression and shedding. Clin Exp Immunol 1995 Jul;101(1):183-9.

(211) Kallman J, Schollin J, Schalen C, Erlandsson A, Kihlstrom E. Impaired phagocytosis and opsonisation towards group B streptococci in preterm neonates. Arch Dis Child Fetal Neonatal Ed 1998 Jan;78(1):F46-F50.

(212) Peden DB, VanDyke K, Ardekani A, Mullett MD, Myerberg DZ, VanDyke C. Diminished chemiluminescent responses of polymorphonuclear leukocytes in severely and moderately preterm neonates. J Pediatr 1987 Dec;111(6 Pt 1):904-6.

(213) Molloy EJ, O'Neill AJ, Grantham JJ, Sheridan-Pereira M, Fitzpatrick JM, Webb DW, et al. Granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor have differential effects on neonatal and adult neutrophil survival and function. Pediatr Res 2005 Jun;57(6):806-12.

(214) Molloy EJ, O'Neill AJ, Doyle BT, Grantham JJ, Taylor CT, Sheridan-Pereira M, et al. Effects of heat shock and hypoxia on neonatal neutrophil lipopolysaccharide responses: altered apoptosis, Toll-like receptor-4 and CD11b expression compared with adults. Biol Neonate 2006;90(1):34-9.

(215) Hallwirth U, Pomberger G, Pollak A, Roth E, Spittler A. Monocyte switch in neonates: high phagocytic capacity and low HLA-DR expression in VLBWI are inverted during gestational aging. Pediatr Allergy Immunol 2004 Dec;15(6):513-6.

(216) Jones CA, Holloway JA, Warner JO. Phenotype of fetal monocytes and B lymphocytes during the third trimester of pregnancy. J Reprod Immunol 2002 Jul;56(1-2):45-60.

(217) Vilcek J, Le J. Immunology of cytokines: An introduction. In: The cytokine handbook. Editor: Thomson A. London: Academic Press Limited; 1994. p. 1-19.

(218) Kilpatrick L, Harris MC. Cytokines and the inflammatory response. In: Fetal and Neonatal Phsyiology. Editors: Polin RA, Fox WW.Philadelphia: W.B. Saunders Company; 1998. p. 1967-79.

(219) Fitzgerald KA, O'Neill L, Gearing AJH., Callard RE. The cytokine Facts book. 2nd edition. San Diego. Academic Press; 2001.

(220) American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. Crit Care Med 1992 Jun;20(6):864-74.

(221) Goldstein B, Giroir B, Randolph A. International pediatric sepsis consensus conference: definitions for sepsis and organ dysfunction in pediatrics. Pediatr Crit Care Med 2005 Jan;6(1):2-8.

(222) Bone RC. Sir Isaac Newton, sepsis, SIRS, and CARS. Critical Care Medicine 1996;24(7).

(223) Ward NS, Casserly B, Ayala A. The compensatory anti-inflammatory response syndrome (CARS) in critically ill patients. Clin Chest Med 2008 Dec;29(4):617-25, viii.

(224) Schultz C, Temming P, Bucsky P, Gopel W, Strunk T, Härtel C. Immature anti-inflammatory response in neonates. Clin Exp Immunol 2004 Jan;135(1):130-6.

(225) Opal SM, DePalo VA. Anti-inflammatory cytokines. Chest 2000 Apr;117(4):1162-72.

(226) Adib-Conquy M, Cavaillon JM. Compensatory anti-inflammatory response syndrome. Thromb Haemost 2009 Jan;101(1):36-47.

(227) Bone RC, Grodzin CJ, Balk RA. Sepsis: a new hypothesis for pathogenesis of the disease process. Chest 1997 Jul;112(1):235-43.

(228) Pillay V, Savage N, Laburn H. Circulating cytokine concentrations and cytokine production by monocytes from newborn babies and adults. Pflugers Arch 1994 Oct;428(3-4):197-201.

(229) Weatherstone KB, Rich EA. Tumor necrosis factor/cachectin and interleukin-1 secretion by cord blood monocytes from premature and term neonates. Pediatr Res 1989 Apr;25(4):342-6.

(230) Schibler KR, Liechty KW, White WL, Rothstein G, Christensen RD. Defective production of interleukin-6 by monocytes: a possible mechanism underlying several host defense deficiencies of neonates. Pediatr Res 1992 Jan;31(1):18-21.

(231) Chelvarajan RL, Collins SM, Doubinskaia IE, Goes S, Van WJ, Flanagan D, et al. Defective macrophage function in neonates and its impact on unresponsiveness of neonates to polysaccharide antigens. J Leukoc Biol 2004 Jun;75(6):982-94.

(232) Faust K, Demmert M, Bendiks M, Gopel W, Herting E, Härtel C. Intrapartum colonization with Streptococcus pneumoniae, early-onset sepsis and deficient specific neonatal immune responses. Arch Gynecol Obstet 2011 Jul 30.

(233) Strunk T, Temming P, Gembruch U, Reiss I, Bucsky P, Schultz C. Differential maturation of the innate immune response in human fetuses. Pediatr Res 2004 Aug;56(2):219-26.

(234) Härtel C, Osthues I, Rupp J, Haase B, Röder K, Göpel W, et al. Characterisation of the host inflammatory response to *Staphylococcus epidermidis* in neonatal whole blood. Archives of Disease in Childhood - Fetal and Neonatal Edition 2008 Mar 1;93(2):F140-F145.

(235) Makhseed M, Raghupathy R, Azizieh F, Omu A, Al-Shamali E, Ashkanani L. Th1 and Th2 cytokine profiles in recurrent aborters with successful pregnancy and with subsequent abortions. Hum Reprod 2001 Oct;16(10):2219-26.

(236) Marchini G, Nelson A, Edner J, Lonne-Rahm S, Stavreus-Evers A, Hultenby K. Erythema toxicum neonatorum is an innate immune response to commensal microbes penetrated into the skin of the newborn infant. Pediatr Res 2005 Sep;58(3):613-6.

(237) Karlsson H, Hessle C, Rudin A. Innate immune responses of human neonatal cells to bacteria from the normal gastrointestinal flora. Infect Immun 2002 Dec;70(12):6688-96.

(238) Mohamed MA, Cunningham-Rundles S, Dean CR, Hammad TA, Nesin M. Levels of pro-inflammatory cytokines produced from cord blood in-vitro are pathogen dependent and increased in comparison to adult controls. Cytokine 2007 Sep;39(3):171-7.

(239) Schultz C, Rott C, Temming P, Schlenke P, Moller JC, Bucsky P. Enhanced interleukin-6 and interleukin-8 synthesis in term and preterm infants. Pediatr Res 2002 Mar;51(3):317-22.

(240) Hebra A, Strange P, Egbert JM, Ali M, Mullinax A, Buchanan E. Intracellular cytokine production by fetal and adult monocytes. J Pediatr Surg 2001 Sep;36(9):1321-6.

(241) Dembinski J, Behrendt D, Reinsberg J, Bartmann P. Endotoxin-stimulated production of IL-6 and IL-8 is increased in short-term cultures of whole blood from healthy term neonates. Cytokine 2002 Apr 21;18(2):116-9.

(242) Schultz C, Zimmer J, Härtel C, Rupp J, Temming P, Strunk T. Attenuation of monocyte proinflammatory cytokine responses to Neisseria meningitidis in children by erythropoietin. Clinical & Experimental Immunology 2008;154(2):187-91.

(243) Ng PC, Li K, Wong RP, Chui K, Wong E, Li G, et al. Proinflammatory and anti-inflammatory cytokine responses in preterm infants with systemic infections. Arch Dis Child Fetal Neonatal Ed 2003 May;88(3):F209-F213.

(244) Jones CA, Cayabyab RG, Kwong KY, Stotts C, Wong B, Hamdan H, et al. Undetectable interleukin (IL)-10 and persistent IL-8 expression early in hyaline membrane disease: a possible developmental basis for the predisposition to chronic lung inflammation in preterm newborns. Pediatr Res 1996 Jun;39(6):966-75.

(245) Chheda S, Palkowetz KH, Garofalo R, Rassin DK, Goldman AS. Decreased interleukin-10 production by neonatal monocytes and T cells: relationship to decreased production and expression of tumor necrosis factor-alpha and its receptors. Pediatr Res 1996 Sep;40(3):475-83.

(246) Härtel C, Adam N, Strunk T, Temming P, Muller-Steinhardt M, Schultz C. Cytokine responses correlate differentially with age in infancy and early childhood. Clin Exp Immunol 2005 Dec;142(3):446-53.

(247) Gasparoni A, Ciardelli L, Avanzini A, Castellazzi AM, Carini R, Rondini G, et al. Age-related changes in intracellular TH1/TH2 cytokine production, immunoproliferative T lymphocyte response and natural killer cell activity in newborns, children and adults. Biol Neonate 2003;84(4):297-303.

(248) Chipeta J, Komada Y, Zhang XL, Deguchi T, Sugiyama K, Azuma E, et al. CD4+ and CD8+ cell cytokine profiles in neonates, older children, and adults: increasing T helper

type 1 and T cytotoxic type 1 cell populations with age. Cell Immunol 1998 Feb 1;183(2):149-56.

(249) Smart JM, Kemp AS. Ontogeny of T-helper 1 and T-helper 2 cytokine production in childhood. Pediatr Allergy Immunol 2001 Aug;12(4):181-7.

(250) Leviton A, Kuban K, O`Shea TM, Paneth N, Fichorova R, Allred EN, et al. The Relationship between Early Concentrations of 25 Blood Proteins and Cerebral White Matter Injury in Preterm Newborns: The ELGAN Study. The Journal of Pediatrics 2011 Jun;158(6):897-903.

(251) Leviton A, Kuban KCK, Allred EN, Fichorova RN, O'Shea TM, Paneth N. Early postnatal blood concentrations of inflammation-related proteins and microcephaly two years later in infants born before the 28th post-menstrual week. Early Human Development 2011 May;87(5):325-30.

(252) Lee J, Dammann O. Perinatal infection, inflammation, and retinopathy of prematurity. Seminars in Fetal and Neonatal Medicine 2011 Sept.

(253) Bagchi A, Viscardi RM, Taciak V, Ensor JE, McCrea KA, Hasday JD. Increased activity of interleukin-6 but not tumor necrosis factor-alpha in lung lavage of premature infants is associated with the development of bronchopulmonary dysplasia. Pediatr Res 1994 Aug;36(2):244-52.

(254) Yoon BH, Romero R, Yang SH, Jun JK, Kim IO, Choi JH, et al. Interleukin-6 concentrations in umbilical cord plasma are elevated in neonates with white matter lesions associated with periventricular leukomalacia. Am J Obstet Gynecol 1996 May;174(5):1433-40.

(255) Ghoshal AK, Soldin SJ. Evaluation of the Dade Behring Dimension RxL: integrated chemistry system-pediatric reference ranges. Clinica Chimica Acta 2003 May;331(1-2):135-46.

(256) van den Berg JP, Westerbeek EA, van der Klis FR, Berbers GA, van Elburg RM. Transplacental transport of IgG antibodies to preterm infants: a review of the literature. Early Hum Dev 2011 Feb;87(2):67-72.

(257) Wang AC, Faulk WP, Stuckey MA, Fudenberg HH. Chemical differences of adult, fetal and hypogammaglobulinemic IgG immunoglobulins. Immunochemistry 1970 Aug;7(8):703-8.

(258) Lassiter HA, Tanner JE, Cost KM, Steger S, Vogel RL. Diminished IgG, but not complement C3 or C4 or factor B, precedes nosocomial bacterial sepsis in very low birth weight neonates. Pediatr Infect Dis J 1991 Sep;10(9):663-8.

(259) Baker CJ, Rench MA, Noya FJ, Garcia-Prats JA. Role of intravenous immunoglobulin in prevention of late-onset infection in low-birth-weight neonates. The Neonatal IVIG Study Group. Rev Infect Dis 1990 May;12 Suppl 4:S463-S468.

(260) Bussel JB. Intravenous gammaglobulin in the prophylaxis of late sepsis in very-low-birth-weight infants: preliminary results of a randomized, double-blind, placebo-controlled trial. Rev Infect Dis 1990 May;12 Suppl 4:S457-S461.

(261) Ohlsson A, Lacy JB. Intravenous immunoglobulin for suspected or subsequently proven infection in neonates. Cochrane Database Syst Rev 2004;(1):CD001239.

(262) Ohlsson A, Lacy JB. Intravenous immunoglobulin for preventing infection in preterm and/or low-birth-weight infants. Cochrane Database Syst Rev 2004;(1):CD000361.

(263) Alejandria MM, Lansang MA, Dans LF, Mantaring JB. Intravenous immunoglobulin for treating sepsis and septic shock. Cochrane Database Syst Rev 2002;(1):CD001090.

(264) Laupland KB, Kirkpatrick AW, Delaney A. Polyclonal intravenous immunoglobulin for the treatment of severe sepsis and septic shock in critically ill adults: a systematic review and meta-analysis. Crit Care Med 2007 Dec;35(12):2686-92.

(265) Treatment of Neonatal Sepsis with Intravenous Immune Globulin. New England Journal of Medicine 2011 Sep 28;365(13):1201-11.

(266) Nathan C. Points of control in inflammation. Nature 2002 Dec 19;420(6917):846-52.

(267) Kumar R, Clermont G, Vodovotz Y, Chow CC. The dynamics of acute inflammation. J Theor Biol 2004 Sep 21;230(2):145-55.

(268) Barton GM. A calculated response: control of inflammation by the innate immune system. J Clin Invest 2008 Feb;118(2):413-20.

(269) Angelone DF, Wessels MR, Coughlin M, Suter EE, Valentini P, Kalish LA, et al. Innate Immunity of the Human Newborn Is Polarized Toward a High Ratio of IL-6/TNF-[alpha] Production In Vitro and In Vivo. Pediatric Research 2006;60(2).

(270) Kloos W, Schleifer KH. Staphylococcus. In: Bergey`s Manual of Systematic Bacteriology. Editors: Sneath PHA., Mair NS:, Sharpe ME., Holt JG Baltimore: Williams & Wilkins; 1986.

(271) Kloos WE, Musselwhite MS. Distribution and persistence of Staphylococcus and Micrococcus species and other aerobic bacteria on human skin. Appl Microbiol 1975 Sep;30(3):381-5.

(272) Otto M. Virulence factors of the coagulase-negative staphylococci. Front Biosci 2004 Jan 1;9:841-63.

(273) Otto M. *Staphylococcus epidermidis* - the 'accidental' pathogen. Nat Rev Micro 2009 Aug;7(8):555-67.

(274) Vadyvaloo V, Otto M. Molecular genetics of *Staphylococcus epidermidis* biofilms on indwelling medical devices. Int J Artif Organs 2005 Nov;28(11):1069-78.

(275) Vuong C, Otto M. *Staphylococcus epidermidis* infections. Microbes and Infection 2002 Apr;4(4):481-9.

(276) Arciola CR, Gamberini S, Campoccia D, Visai L, Speziale P, Baldassarri L, et al. A multiplex PCR method for the detection of all five individual genes of ica locus in *Staphylococcus epidermidis*. A survey on 400 clinical isolates from prosthesis-associated infections. J Biomed Mater Res A 2005 Nov 1;75(2):408-13.

(277) Costerton JW, Stewart P, Greenberg E. Bacterial biofilms: a common cause of persistent infections. Science 1999;284:1318-22.

(278) Elder MJ, Stapleton F, Evans E, Dart JK. Biofilm-related infections in ophthalmology. Eye (Lond) 1995;9 (Pt 1):102-9.

(279) Rupp ME, Ulphani JS, Fey PD, Mack D. Characterization of *Staphylococcus epidermidis* polysaccharide intercellular adhesin/hemagglutinin in the pathogenesis of intravascular catheter-associated infection in a rat model. Infect Immun 1999 May;67(5):2656-9.

(280) Stoll BJ, Hansen N, Fanaroff AA, Wright LL, Carlo WA, Ehrenkranz RA, et al. Late-Onset Sepsis in Very Low Birth Weight Neonates: The Experience of the NICHD Neonatal Research Network. Pediatrics 2002 Aug 1;110(2):285-91.

(281) Caputo GM, Archer GL, Calderwood SB, DiNubile MJ, Karchmer AW. Native valve endocarditis due to coagulase-negative staphylococci. Clinical and microbiologic features. Am J Med 1987 Oct;83(4):619-25.

(282) Mylonakis E, Calderwood SB. Infective endocarditis in adults. N Engl J Med 2001 Nov 1;345(18):1318-30.

(283) von Eiff C, Peters G, Heilmann C. Pathogenesis of infections due to coagulase-negative staphylococci. Lancet Infectious Diseases 2002;2(11):677-85.

(284) Diekema DJ, Pfaller MA, Schmitz FJ, Smayevsky J, Bell J, Jones RN, et al. Survey of infections due to Staphylococcus species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997-1999. Clin Infect Dis 2001 May 15;32 Suppl 2:S114-S132.

(285) Biavasco F, Vignaroli C, Varaldo PE. Glycopeptide resistance in coagulase-negative staphylococci. Eur J Clin Microbiol Infect Dis 2000 Jun;19(6):403-17.

(286) Kotilainen P, Nikoskelainen J, Huovinen P. Emergence of ciprofloxacin-resistant coagulase-negative staphylococcal skin flora in immunocompromised patients receiving ciprofloxacin. Journal of Infectious Diseases 1990 Jan 1;161(1):41-4.

(287) Kozitskaya S, Cho SH, Dietrich K, Marre R, Naber K, Ziebuhr W. The bacterial insertion sequence element IS256 occurs preferentially in nosocomial *Staphylococcus epidermidis*

isolates: association with biofilm formation and resistance to aminoglycosides. Infect Immun 2004 Feb;72(2):1210-5.

(288) Klingenberg C, Sundsfjord A, Ronnestad A, Mikalsen J, Gaustad P, Flaegstad T. Phenotypic and genotypic aminoglycoside resistance in blood culture isolates of coagulase-negative staphylococci from a single neonatal intensive care unit, 1989-2000. J Antimicrob Chemother 2004 Nov 1;54(5):889-96.

(289) Schmitz FJ, Fluit AC, Gondolf M, Beyrau R, Lindenlauf E, Verhoef J, et al. The prevalence of aminoglycoside resistance and corresponding resistance genes in clinical isolates of staphylococci from 19 European hospitals. J Antimicrob Chemother 1999 Feb;43(2):253-9.

(290) Marshall SA, Wilke WW, Pfaller MA, Jones RN. *Staphylococcus aureus* and coagulase-negative staphylococci from blood stream infections: frequency of occurrence, antimicrobial susceptibility, and molecular (mecA) characterization of oxacillin resistance in the SCOPE program. Diagn Microbiol Infect Dis 1998 Mar;30(3):205-14.

(291) Klingenberg C, Aarag E, Rønnestad A, Sollid JE, Abrahamsen TG, Kjeldsen G, et al. Coagulase-negative staphylococcal sepsis in neonates: Association between antibiotic resistance, biofilm formation and the host inflammatory response. The Pediatric Infectious Disease Journal 2005;24(9).

(292) Casey AL, Lambert PA, Elliott TS. Staphylococci. Int J Antimicrob Agents 2007 May;29 Suppl 3:S23-S32.

(293) Katayama Y, Ito T, Hiramatsu K. A new class of genetic element, staphylococcus cassette chromosome mec, encodes methicillin resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother 2000 Jun;44(6):1549-55.

(294) Fuda C, Suvorov M, Vakulenko SB, Mobashery S. The basis for resistance to beta-lactam antibiotics by penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus*. J Biol Chem 2004 Sep 24;279(39):40802-6.

(295) Classification of staphylococcal cassette chromosome mec (SCCmec): guidelines for reporting novel SCCmec elements. Antimicrob Agents Chemother 2009 Dec;53(12):4961-7.

(296) Tsubakishita S, Kuwahara-Arai K, Baba T, Hiramatsu K. Staphylococcal cassette chromosome mec-like element in Macrococcus caseolyticus. Antimicrob Agents Chemother 2010 Apr;54(4):1469-75.

(297) Tsubakishita S, Kuwahara-Arai K, Sasaki T, Hiramatsu K. Origin and molecular evolution of the determinant of methicillin resistance in staphylococci. Antimicrob Agents Chemother 2010 Oct;54(10):4352-9.

(298) Ahlstrand E, Svensson K, Persson L, Tidefelt U, Söderquist B. Glycopeptide resistance in coagulase-negative staphylococci isolated in blood cultures from patients with

hematological malignancies during three decades. European Journal of Clinical Microbiology & Infectious Diseases 2011 Nov 1;30(11):1349-54.

(299) Raad I, Alrahwan A, Rolston K. *Staphylococcus epidermidis*: emerging resistance and need for alternative agents. Clin Infect Dis 1998 May;26(5):1182-7.

(300) Scheifele DW, Bjornson GL, Dyer RA, Dimmick JE. Delta-like toxin produced by coagulase-negative staphylococci is associated with neonatal necrotizing enterocolitis. Infect Immun 1987 Sep 1;55(9):2268-73.

(301) von Eiff C, Jansen B, Kohnen W, Becker K. Infections associated with medical devices: pathogenesis, management and prophylaxis. Drugs 2005;65(2):179-214.

(302) Stoll BJ, Gordon T, Korones SB, Shankaran S, Tyson JE, Bauer CR, et al. Early-onset sepsis in very low birth weight neonates: A report from the National Institute of Child Health and Human Development Neonatal Research Network. The Journal of Pediatrics 1996 Jul;129(1):72-80.

(303) Stoll BJ, Gordon T, Korones SB, Shankaran S, Tyson JE, Bauer CR, et al. Late-onset sepsis in very low birth weight neonates: A report from the National Institute of Child Health and Human Development Neonatal Research Network. The Journal of Pediatrics 1996 Jul;129(1):63-71.

(304) Isaacs D. A ten year, multicentre study of coagulase negative staphylococcal infections in Australasian neonatal units. Archives of Disease in Childhood - Fetal and Neonatal Edition 2003 Mar 1;88(2):F89-F93.

(305) van den Hoogen A, Gerards LJ, Verboon-Maciolek MA, Fleer A, Krediet TG. Long-term trends in the epidemiology of neonatal sepsis and antibiotic susceptibility of causative agents. Neonatology 2010;97(1):22-8.

(306) Healy CM, Palazzi DL, Edwards MS, Campbell JR, Baker CJ. Features of invasive staphylococcal disease in neonates. Pediatrics 2004 Oct;114(4):953-61.

(307) Rastogi A, Luken JA, Pildes RS, Chrystof D, LaBranche F. Endocarditis in neonatal intensive care unit. Pediatric Cardiology 1993 Jul 1;14(3):183-6.

(308) Salzman MB, Isenberg HD, Shapiro JF, Lipsitz PJ, Rubin LG. A prospective study of the catheter hub as the portal of entry for microorganisms causing catheter-related sepsis in neonates. Journal of Infectious Diseases 1993 Feb 1;167(2):487-90.

(309) Blot SI, Depuydt P, Annemans L, Benoit D, Hoste E, De Waele JJ, et al. Clinical and economic outcomes in critically ill patients with nosocomial catheter-related bloodstream infections. Clinical Infectious Diseases 2005 Dec 1;41(11):1591-8.

(310) Richard A. The "Ins and Outs" of neonatal sepsis. The Journal of Pediatrics 2003 Jul;143(1):3-4.

(311) Malik A, Hui CPS, Pennie RA, Kirpalani H. Beyond the complete blood cell count and C-reactive protein: A systematic review of modern diagnostic tests for neonatal sepsis. Arch Pediatr Adolesc Med 2003 Jun 1;157(6):511-6.

(312) de Silva GDI, Kantzanou M, Justice A, Massey RC, Wilkinson AR, Day NPJ, et al. The ica Operon and Biofilm Production in Coagulase-Negative Staphylococci Associated with Carriage and Disease in a Neonatal Intensive Care Unit. J Clin Microbiol 2002 Feb 1;40(2):382-8.

(313) Hanna R, Raad II. Diagnosis of catheter-related bloodstream infection. Curr Infect Dis Rep 2005 Nov;7(6):413-9.

(314) Fanaroff AA, Korones SB, Wright LL, Verter J, Poland RL, Bauer CR, et al. Incidence, presenting features, risk factors and significance of late onset septicemia in very low birth weight infants. The National Institute of Child Health and Human Development Neonatal Research Network. Pediatr Infect Dis J 1998 Jul;17(7):593-8.

(315) Maayan-Metzger A, Linder N, Marom D, Vishne T, Ashkenazi S, Sirota L. Clinical and laboratory impact of coagulase-negative staphylococci bacteremia in preterm infants. Acta Paediatr 2000 Jun;89(6):690-3.

(316) Makhoul IR, Sujov P, Smolkin T, Lusky A, Reichman B. Epidemiological, clinical, and microbiological characteristics of late-onset sepsis among very low birth weight infants in Israel: a national survey. Pediatrics 2002 Jan;109(1):34-9.

(317) Bjorkqvist M, Soderquist B, Tornqvist E, Sjoberg L, Fredlund H, Kuhn I, et al. Phenotypic and genotypic characterisation of blood isolates of coagulase-negative staphylococci in the newborn. APMIS 2002 Apr;110(4):332-9.

(318) Auriti C, Maccallini A, Di LG, Di C, V, Ronchetti MP, Orzalesi M. Risk factors for nosocomial infections in a neonatal intensive-care unit. J Hosp Infect 2003 Jan;53(1):25-30.

(319) Gastmeier P, Geffers C, Schwab F, Fitzner J, Obladen M, Ruden H. Development of a surveillance system for nosocomial infections: the component for neonatal intensive care units in Germany. J Hosp Infect 2004 Jun;57(2):126-31.

(320) Clark R, Powers R, White R, Bloom B, Sanchez P, Benjamin DK, Jr. Nosocomial infection in the NICU: a medical complication or unavoidable problem? J Perinatol 2004 Jun;24(6):382-8.

(321) Rønnestad A, Abrahamsen TG, Medbo S, Reigstad H, Lossius K, Kaaresen PI, et al. Late-onset septicemia in a Norwegian national cohort of extremely premature infants receiving very early full human milk feeding. Pediatrics 2005 Mar 1;115(3):e269-e276.

(322) Persson E, Trollfors B, Brandberg LL, Tessin I. Septicaemia and meningitis in neonates and during early infancy in the Goteborg area of Sweden. Acta Paediatr 2002;91(10):1087-92.

(323) Avila-Figueroa C, Goldmann DA, Richardson DK, Gray JE, Ferrari A, Freeman J. Intravenous lipid emulsions are the major determinant of coagulase-negative staphylococcal bacteremia in very low birth weight newborns. Pediatr Infect Dis J 1998 Jan;17(1):10-7.

(324) Sweeney B, Puri P, Reen DJ. Modulation of immune cell function by polyunsaturated fatty acids. Pediatr Surg Int 2005 May;21(5):335-40.

(325) Yu WK, Li WQ, Li N, Li JS. Influence of acute hyperglycemia in human sepsis on inflammatory cytokine and counterregulatory hormone concentrations. World J Gastroenterol 2003 Aug;9(8):1824-7.

(326) Schutze GE, Hall MA, Baker CJ, Edwards MS. Role of neutrophil receptors in opsonophagocytosis of coagulase-negative staphylococci. Infect Immun 1991 Aug;59(8):2573-8.

(327) Haase B, Faust K, Heidemann M, Scholz T, Demmert M, Troger B, et al. The modulatory effect of lipids and glucose on the neonatal immune response induced by *Staphylococcus epidermidis*. Inflamm Res 2011 Mar;60(3):227-32.

(328) Fleer A, Gerards LJ, Aerts P, Westerdaal NA, Senders RC, van DH, et al. Opsonic defense to *Staphylococcus epidermidis* in the premature neonate. J Infect Dis 1985 Nov;152(5):930-7.

(329) Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol 2003;21:335-76.

(330) Massey RC, Horsburgh MJ, Lina G, Hook M, Recker M. The evolution and maintenance of virulence in *Staphylococcus aureus*: a role for host-to-host transmission? Nat Rev Micro 2006 Dec;4(12):953-8.

(331) Lee YM, Almqvist F, Hultgren SJ. Targeting virulence for antimicrobial chemotherapy. Curr Opin Pharmacol 2003 Oct;3(5):513-9.

(332) Salyers AA WD. Virulence factors that promote colonization. Bacterial Pathogenesis: A Molecular Approach.Washinton DC: American Society for Microbiology; 1994. p. 30-46.

(333) Heilmann C, Hussain M, Peters G, Gotz F. Evidence for autolysin-mediated primary attachment of Staphylococcus epidermidis to a polystyrene surface. Mol Microbiol 1997 Jun;24(5):1013-24.

(334) Heilmann C, Thumm G, Chhatwal GS, Hartleib J, Uekotter A, Peters G. Identification and characterization of a novel autolysin (Aae) with adhesive properties from *Staphylococcus epidermidis*. Microbiology 2003 Oct;149(Pt 10):2769-78.

(335) Heilmann C, Schweitzer O, Gerke C, Vanittanakom N, Mack D, Gotz F. Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. Mol Microbiol 1996 Jun;20(5):1083-91.

(336) Tormo M, Knecht E, Götz F, Lasa I, Penadès JR. Bap-dependent biofilm formation by pathogenic species of *Staphylococcus*: evidence of horizontal gene transfer? Microbiology 2005 Jul 1;151(7):2465-75.

(337) Nilsson M, Frykberg L, Flock JI, Pei L, Lindberg M, Guss B. A Fibrinogen-Binding Protein of *Staphylococcus epidermidis*. Infect Immun 1998 Jun 1;66(6):2666-73.

(338) Arrecubieta C, Lee MH, Macey A, Foster TJ, Lowy FD. SdrF, a *Staphylococcus epidermidis* surface protein, binds type I collagen. Journal of Biological Chemistry 2007 Jun 29;282(26):18767-76.

(339) Arrecubieta C, Toba FA, von Bayern M, Akashi H, Deng MC, Naka Y, et al. SdrF, a *Staphylococcus epidermidis* surface protein, contributes to the initiation of ventricular assist device driveline-related Infections. PLoS Pathog 2009 May 1;5(5):e1000411.

(340) McCrea KW, Hartford O, Davis S, Eidhin DN, Lina G, Speziale P, et al. The serine-aspartate repeat (Sdr) protein family in *Staphylococcus epidermidis*. Microbiology 2000 Jul 1;146(7):1535-46.

(341) Shahrooei M, Hira V, Stijlemans B, Merckx R, Hermans PWM, Van Eldere J. Inhibition of *Staphylococcus epidermidis* biofilm formation by rabbit polyclonal antibodies against the SesC protein. Infect Immun 2009 Sep 1;77(9):3670-8.

(342) Vuong C, Kocianova S, Voyich JM, Yao Y, Fischer ER, DeLeo FR, et al. A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. J Biol Chem 2004;279(52):54881-6.

(343) Vuong C, Voyich JM, Fischer ER, Braughton KR, Whitney AR, DeLeo FR, et al. Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. Cell Microbiol 2004 Mar;6(3):269-75.

(344) Mack D, Fischer W, Krokotsch A, Leopold K, Hartmann R, Egge H, et al. The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. J Bacteriol 1996 Jan 1;178(1):175-83.

(345) Rohde H, Burdelski C, Bartscht K, Hussain M, Buck F, Horstkotte MA, et al. Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. Mol Microbiol 2005 Mar;55(6):1883-95.

(346) Hussain M, Herrmann M, von Eiff C, Perdreau-Remington F, Peters G. A 140-kilodalton extracellular protein is essential for the accumulation of *Staphylococcus epidermidis* strains on surfaces. Infect Immun 1997 Feb 1;65(2):519-24.

(347) Christner M, Franke GC, Schommer NN, Wendt U, Wegert K, Pehle P, et al. The giant extracellular matrix-binding protein of *Staphylococcus epidermidis* mediates biofilm accumulation and attachment to fibronectin. Mol Microbiol 2010 Jan;75(1):187-207.

(348) Williams RJ, Henderson B, Sharp LJ, Nair SP. Identification of a fibronectin-binding protein from *Staphylococcus epidermidis*. Infect Immun 2002 Dec 1;70(12):6805-10.

(349) Kocianova S, Vuong C, Yao Y, Voyich JM, Fischer ER, DeLeo FR, et al. Key role of poly-gamma-DL-glutamic acid in immune evasion and virulence of *Staphylococcus epidermidis*. J Clin Invest 2005 Mar;115(3):688-94.

(350) Li M, Lai Y, Villaruz AE, Cha DJ, Sturdevant DE, Otto M. Gram-positive three-component antimicrobial peptide-sensing system. Proceedings of the National Academy of Sciences 2007 May 29;104(22):9469-74.

(351) Lai Y, Villaruz AE, Li M, Cha DJ, Sturdevant DE, Otto M. The human anionic antimicrobial peptide dermcidin induces proteolytic defence mechanisms in staphylococci. Mol Microbiol 2007 Jan;63(2):497-506.

(352) Yao Y, Sturdevant DE, Otto M. Genomewide analysis of gene expression in *Staphylococcus epidermidis* biofilms: Insights into the pathophysiology of S. epidermidis biofilms and the role of Phenol-Soluble Modulins in formation of biofilms. Journal of Infectious Diseases 2005 Jan 15;191(2):289-98.

(353) Mehlin C, Headley CM, Klebanoff SJ. An Inflammatory Polypeptide Complex from *Staphylococcus epidermidis*: Isolation and Characterization. The Journal of Experimental Medicine 1999 Mar 15;189(6):907-18.

(354) Cogan NG. Effects of persister formation on bacterial response to dosing. Journal of Theoretical Biology 2006 Feb 7;238(3):694-703.

(355) Arciola CR, An YH, Campoccia D, Donati ME, Montanaro L. Etiology of implant orthopedic infections: a survey on 1027 clinical isolates. Int J Artif Organs 2005 Nov;28(11):1091-100.

(356) Rupp ME, Archer GL. Coagulase-negative staphylococci: pathogens associated with medical progress. Clin Infect Dis 1994 Aug;19(2):231-43.

(357) Verhoef J, Fleer A. *Staphylococcus epidermidis* endocarditis and *Staphylococcus epidermidis* infection in an intensive care unit. Scand J Infect Dis Suppl 1983;41:56-64.

(358) Warren JW. Catheter-associated urinary tract infections. Int J Antimicrob Agents 2001 Apr;17(4):299-303.

(359) Stoll BJ, Hansen N. Infections in VLBW infants: studies from the NICHD Neonatal Research Network. Semin Perinatol 2003 Aug;27(4):293-301.

(360) Hausner M, Wuertz S. High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. Appl Environ Microbiol 1999 Aug;65(8):3710-3.

(361) Parsek MR, Singh PK. Bacterial biofilms: an emerging link to disease pathogenesis. Annu Rev Microbiol 2003;57:677-701.

(362) Cerca F, Andrade F, Franca A, Andrade EB, Ribeiro A, Almeida AA, et al. *Staphylococcus epidermidis* biofilms with higher proportions of dormant bacteria induce a lower activation of murine macrophages. J Med Microbiol 2011 Jul 28.

(363) Foster TJ. Immune evasion by staphylococci. Nat Rev Microbiol 2005 Dec;3(12):948-58.

(364) Knobloch JK, Von OH, Horstkotte MA, Rohde H, Mack D. Biofilm formation is not necessary for development of quinolone-resistant "persister" cells in an attached *Staphylococcus epidermidis* population. Int J Artif Organs 2008 Sep;31(9):752-60.

(365) Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. Lancet 2001 Jul 14;358(9276):135-8.

(366) Gotz F. *Staphylococcus* and biofilms. Mol Microbiol 2002;43:1367-78.

(367) Mack D, Horstkotte MA., Rohde H, Knobloch J. Coagulase-negative staphylococci. In: Biofilms, infections and Antimicrobial Therapy. Editors: JL, Rupp M, Finch RG. Boca Raton: CRC Press; 2006. p. 109-53.

(368) Gristina AG. Biomaterial-Centered Infection: Microbial Adhesion Versus Tissue Integration. Science 1987 Sep 25;237(4822):1588-95.

(369) Veenstra GJ, Cremers FF, van Dijk H, Fleer A. Ultrastructural organization and regulation of a biomaterial adhesin of *Staphylococcus epidermidis*. J Bacteriol 1996 Jan 1;178(2):537-41.

(370) Gross M, Cramton SE, Götz F, Peschel A. Key role of teichoic acid net charge in *Staphylococcus aureus* colonization of artificial surfaces. Infect Immun 2001;69:3423-6.

(371) Gerke C, Kraft A, Süssmuth R, Schweitzer O, Götz F. Characterization of theN-Acetylglucosaminyltransferase Activity Involved in the Biosynthesis of the *Staphylococcus epidermidis* Polysaccharide Intercellular Adhesin. Journal of Biological Chemistry 1998 Jul 17;273(29):18586-93.

(372) Bateman A, Holden MTG, Yeats C. The G5 domain: a potential N-acetylglucosamine recognition domain involved in biofilm formation. Bioinformatics 21(8):1301-3.

(373) Qin Z, Yang X, Yang L, Jiang J, Ou Y, Molin S, et al. Formation and properties of in vitro biofilms of ica-negative *Staphylococcus epidermidis* clinical isolates. J Med Microbiol 2007 Jan;56(Pt 1):83-93.

(374) Hennig S, Nyunt Wai S, Ziebuhr W. Spontaneous switch to PIA-independent biofilm formation in an ica-positive *Staphylococcus epidermidis* isolate. International Journal of Medical Microbiology 2007 Apr 16;297(2):117-22.

(375) Rohde H, Burandt EC, Siemssen N, Frommelt L, Burdelski C, Wurster S, et al. Polysaccharide intercellular adhesin or protein factors in biofilm accumulation of *Staphylococcus epidermidis* and *Staphylococcus aureus* isolated from prosthetic hip and knee joint infections. Biomaterials 2007 Mar;28(9):1711-20.

(376) Chokr A, Watier D, Eleaume H, Pangon B, Ghnassia JC, Mack D, et al. Correlation between biofilm formation and production of polysaccharide intercellular adhesin in clinical isolates of coagulase-negative staphylococci. Int J Med Microbiol 2006 Oct;296(6):381-8.

(377) Conrady DG, Brescia CC, Horii K, Weiss AA, Hassett DJ, Herr AB. A zinc-dependent adhesion module is responsible for intercellular adhesion in staphylococcal biofilms. Proceedings of the National Academy of Sciences 2008 Dec 9;105(49):19456-61.

(378) Schommer NN, Christner M, Hentschke M, Ruckdeschel K, Aepfelbacher M, Rohde H. *Staphylococcus epidermidis* uses distinct mechanisms of biofilm formation to interfere with phagocytosis and activation of mouse macrophage-like cells 774A.1. Infect Immun 2011 Jun;79(6):2267-76.

(379) Rohde H, Bartscht K, Hussain M, Buck F, Horstkotte MA, Knobloch JKM, et al. The repetitive domain B of the accumulation associated protein Aap mediates intercellular adhesion and biofilm formation in *Staphylococcus epidermidis*. Int J Med Microbiol 2004;294:128.

(380) Stevens NT, Tharmabala M, Dillane T, Greene CM, O'Gara JP, Humphreys H. Biofilm and the role of the ica operon and aap in *Staphylococcus epidermidis* isolates causing neurosurgical meningitis. Clin Microbiol Infect 2008 Jul;14(7):719-22.

(381) Vandecasteele SJ, Peetermans WE, Merckx R, Rijnders BJ, Van EJ. Reliability of the ica, aap and atlE genes in the discrimination between invasive, colonizing and contaminant *Staphylococcus epidermidis* isolates in the diagnosis of catheter-related infections. Clin Microbiol Infect 2003 Feb;9(2):114-9.

(382) Kogan G, Sadovskaya I, Chaignon P, Chokr A, Jabbouri S. Biofilms of clinical strains of Staphylococcus that do not contain polysaccharide intercellular adhesin. FEMS Microbiol Lett 2006 Feb;255(1):11-6.

(383) Teufel P, Gotz F. Characterization of an extracellular metalloprotease with elastase activity from *Staphylococcus epidermidis*. J Bacteriol 1993 Jul 1;175(13):4218-24.

(384) Dubin G, Chmiel D, Mak P, Rakwalska M, Rzychon M, Dubin A. Molecular Cloning and Biochemical Characterisation of Proteases from *Staphylococcus epidermidis*. Biological Chemistry 2001 Nov 1;382(11):1575-82.

(385) Ohara-Nemoto Y, Ikeda Y, Kobayashi M, Sasaki M, Tajika S, Kimura S. Characterization and molecular cloning of a glutamyl endopeptidase from *Staphylococcus epidermidis*. Microbial Pathogenesis 2002 Jul;33(1):33-41.

(386) Kong KF, Vuong C, Otto M. Staphylococcus quorum sensing in biofilm formation and infection. International Journal of Medical Microbiology 2006 Apr 6;296(2-3):133-9.

(387) Hunt SM, Werner EM, Huang B, Hamilton MA, Stewart PS. Hypothesis for the role of nutrient starvation in biofilm detachment. Appl Environ Microbiol 2004 Dec;70(12):7418-25.

(388) Cerca N, Jefferson KK, Oliveira R, Pier GB, Azeredo J. Comparative antibody-mediated phagocytosis of *Staphylococcus epidermidis* cells grown in a biofilm or in the planktonic state. Infect Immun 2006 Aug 1;74(8):4849-55.

(389) Stuyt RJ, Kim SH, Reznikov LL, Fantuzzi G, Novick D, Rubinstein M, et al. Regulation of *Staphylococcus epidermidis*-induced IFN-gamma in whole human blood: the role of endogenous IL-18, IL-12, IL-1, and TNF. Cytokine 2003 Jan 21;21(2):65-73.

(390) Megyeri K, Mandi Y, Degre M, Rosztoczy I. Induction of cytokine production by different Staphylococcal strains. Cytokine 2002 Aug 21;19(4):206-12.

(391) Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, Davidson MG, et al. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. The Lancet 2006 Mar 4;367(9512):731-9.

(392) Degnan BA, Palmer JM, Robson T, Jones CE, Fischer M, Glanville M, et al. Inhibition of human peripheral blood mononuclear cell proliferation by *Streptococcus pyogenes* cell extract is associated with arginine deiminase activity. Infect Immun 1998 Jul 1;66(7):3050-8.

(393) Podbielski A, Pohl B, Woischnik M, Korner C, Schmidt KH, Rozdzinski E, et al. Molecular characterization of group A streptococcal (GAS) oligopeptide permease (opp) and its effect on cysteine protease production. Mol Microbiol 1996 Sep;21(5):1087-99.

(394) Otto M, Götz F. ABC transporters of staphylococci. Research in Microbiology 2001 Apr;152(3-4):351-6.

(395) Goering RV, McDougal LK, Fosheim GE, Bonnstetter KK, Wolter DJ, Tenover FC. Epidemiologic Distribution of the Arginine Catabolic Mobile Element among Selected Methicillin-Resistant and Methicillin-Susceptible *Staphylococcus aureus* Isolates. J Clin Microbiol 2007 Jun 1;45(6):1981-4.

(396) Diep B, Stone G-G, Basuino L, Graber C, Miller A, Etages, et al. The Arginine Catabolic Mobile Element and Staphylococcal Chromosomal Cassette mec Linkage: Convergence of Virulence and Resistance in the USA300 Clone of Methicillin Resistant *Staphylococcus aureus*. The Journal of Infectious Diseases 2008 Jun 1;197(11):1523-30.

(397) Miragaia M, de Lencastre H, Perdreau-Remington F, Chambers HF, Higashi J, Sullam PM, et al. Genetic Diversity of Arginine Catabolic Mobile Element in *Staphylococcus epidermidis*. PLoS ONE 2009 Nov 6;4(11):e7722.

(398) Pan ES, Diep BA, Charlebois ED, Auerswald C, Carleton HA, Sensabaugh GF, et al. Population dynamics of nasal strains of methicillin-resistant *Staphylococcus aureus*--and their relation to community-associated disease activity. J Infect Dis 2005 Sep 1;192(5):811-8.

(399) Tenover FC, McDougal LK, Goering RV, Killgore G, Projan SJ, Patel JB, et al. Characterization of a strain of community-associated methicillin-resistant *Staphylococcus aureus* widely disseminated in the United States. J Clin Microbiol 2006 Jan;44(1):108-18.

(400) Gonzalez BE, Martinez-Aguilar G, Hulten KG, Hammerman WA, Coss-Bu J, Avalos-Mishaan A, et al. Severe Staphylococcal sepsis in adolescents in the era of community-acquired methicillin-resistant *Staphylococcus aureus*. Pediatrics 2005 Mar;115(3):642-8.

(401) Miller LG, Perdreau-Remington F, Rieg G, Mehdi S, Perlroth J, Bayer AS, et al. Necrotizing fasciitis caused by community-associated methicillin-resistant *Staphylococcus aureus* in Los Angeles. N Engl J Med 2005 Apr 7;352(14):1445-53.

(402) Diep BA, Otto M. The role of virulence determinants in community-associated MRSA pathogenesis. Trends Microbiol 2008 Aug;16(8):361-9.

(403) Montgomery CP, Boyle-Vavra S, Daum RS. The Arginine Catabolic Mobile Element Is Not Associated with Enhanced Virulence in Experimental Invasive Disease Caused by the Community-Associated Methicillin-Resistant *Staphylococcus aureus* USA300 Genetic Background. Infect Immun 2009 Jul 1;77(7):2650-6.

(404) Barbier F, Lebeaux D, Hernandez D, Delannoy AS, Caro Vr, Francois P, et al. High prevalence of the arginine catabolic mobile element in carriage isolates of methicillin-resistant *Staphylococcus epidermidis*. J Antimicrob Chemother 2011 Jan 1;66(1):29-36.

(405) Miller LG, Diep BA. Clinical practice: colonization, fomites, and virulence: rethinking the pathogenesis of community-associated methicillin-resistant *Staphylococcus aureus* infection. Clin Infect Dis 2008 Mar 1;46(5):752-60.

(406) Pi B, Yu M, Chen Y, Yu Y, Li L. Distribution of the ACME-arcA gene among meticillin-resistant *Staphylococcus haemolyticus* and identification of a novel ccr allotype in ACME-arcA-positive isolates. J Med Microbiol 2009 Jun 1;58(6):731-6.

(407) Mack D, Siemssen N, Laufs R. Parallel induction by glucose of adherence and a polysaccharide antigen specific for plastic-adherent *Staphylococcus epidermidis*: evidence for functional relation to intercellular adhesion. Infect Immun 1992 May;60(5):2048-57.

(408) Mack D, Riedewald J, Rohde H, Magnus T, Feucht HH, Elsner HA, et al. Essential functional role of the polysaccharide intercellular adhesin of *Staphylococcus epidermidis* in hemagglutination. Infect Immun 1999 Feb;67(2):1004-8.

(409) Hanssen AM, Kjeldsen G, Sollid JU. Local variants of Staphylococcal cassette chromosome mec in sporadic methicillin-resistant *Staphylococcus aureus* and methicillin-resistant coagulase-negative Staphylococci: evidence of horizontal gene transfer? Antimicrob Agents Chemother 2004 Jan;48(1):285-96.

(410) Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, et al. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol 1985 Dec 1;22(6):996-1006.

(411) Wayne P. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. 12th Informational Supplement. 2002.

(412) Predari SC, Ligozzi M, Fontana R. Genotypic identification of methicillin-resistant coagulase-negative staphylococci by polymerase chain reaction. Antimicrob Agents Chemother 1991 Dec 1;35(12):2568-73.

(413) Oliveira DC, de LH. Multiplex PCR strategy for rapid identification of structural types and variants of the mec element in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 2002 Jul;46(7):2155-61.

(414) Klingenberg C, Ronnestad A, Anderson AS, Abrahamsen TG, Zorman J, Villaruz A, et al. Persistent strains of coagulase-negative staphylococci in a neonatal intensive care unit: virulence factors and invasiveness. Clin Microbiol Infect 2007 Nov;13(11):1100-11.

(415) Sadovskaya I, Faure S, Watier D, Leterme D, Chokr A, Girard J, et al. Potential use of poly-N-acetyl-beta-(1,6)-glucosamine as an antigen for diagnosis of staphylococcal orthopedic-prosthesis-related infections. Clin Vaccine Immunol 2007 Dec;14(12):1609-15.

(416) Zahringer U, Lindner B, Inamura S, Heine H, Alexander C. TLR2 - promiscous or spesific? A critical re-evaluation of a receptor expressing apparent broad specificity. Immunobiology 2008;213(3-4):205-24.

(417) Sadovskaya I, Vinogradov E, Flahaut S, Kogan G, Jabbouri S. Extracellular Carbohydrate-Containing Polymers of a Model Biofilm-Producing Strain, *Staphylococcus epidermidis* RP62A. Infect Immun 2005 May 1;73(5):3007-17.

(418) Sidorczyk Z, Zych K, Toukach FV, Arbatsky NP, Zablotni A, Shashkov AS, et al. Structure of the O-polysaccharide and classification of *Proteus mirabilis* strain G1 in Proteus serogroup O3. Eur J Biochem 2002 Mar;269(5):1406-12.

(419) Fure H, Nielsen EW, Hack CE, Mollnes TE. A neoepitope-based enzyme immunoassay for quantification of C1-inhibitor in complex with C1r and C1s. Scand J Immunol 1997 Dec;46(6):553-7.

(420) Wolbink GJ, Bollen J, Baars JW, ten Berge RJM, Swaak AJG, Paardekooper J, et al. Application of a monoclonal antibody against a neoepitope on activated C4 in an ELISA

for the quantification of complement activation via the classical pathway. Journal of Immunological Methods 1993 Jul 6;163(1):67-76.

(421) Mollnes TE. Analysis of in vivo complement activation. In: Weir's Handbook of Experiental Immunology.Editors: Herzenberg LA, Weir DM Boston: Blackwell scinence; 1997.

(422) Mollnes TE, Lea T, Harboe M. Detection and quantification of the terminal C5b-9 complex of human complement by a sensitive enzyme-linked immunosorbent assay. Scand J Immunol 1984 Aug;20(2):157-66.

(423) Mollnes TE, Lea T, Froland SS, Harboe M. Quantification of the terminal complement complex in human plasma by an enzyme-linked immunosorbent assay based on monoclonal antibodies against a neoantigen of the complex. Scand J Immunol 1985 Aug;22(2):197-202.

(424) Mollnes TE, Videm V, Christiansen D, Bergseth G, Riesenfeld J, Hovig T. Platelet compatibility of an artificial surface modified with functionally active heparin. Thromb Haemost 1999 Sep;82(3):1132-6.

(425) Granslo H, Gammelsrud KW, Fredheim EA, Flaegstad T, Klingenberg C. Coagulase-negative staphylococci--biofilm and antibiotic resistance. Tidsskr Nor Laegeforen 2008 Dec 4;128(23):2746-9.

(426) Klingenberg C, Glad GT, Olsvik R, Flaegstad T. Rapid PCR detection of the methicillin resistance gene, *mecA*, on the hands of medical and non-medical personnel and healthy children and on surfaces in a neonatal intensive care unit. Scand J Infect Dis 2001;33(7):494-7.

(427) Hedin G. *Staphylococcus epidermidis*--hospital epidemiology and the detection of methicillin resistance. Scand J Infect Dis Suppl 1993;90:1-59.

(428) Shore AC, Rossney AS, Brennan OM, Kinnevey PM, Humphreys H, Sullivan DJ, et al. Characterization of a novel arginine catabolic mobile element (ACME) and staphylococcal chromosomal cassette mec composite island with significant homology to *Staphylococcus epidermidis* ACME type II in methicillin-resistant *Staphylococcus aureus* genotype ST22-MRSA-IV. Antimicrob Agents Chemother 2011 May;55(5):1896-905.

(429) Cafiso V, Bertuccio T, Santagati M, Campanile F, Amicosante G, Perilli MG, et al. Presence of the ica operon in clinical isolates of *Staphylococcus epidermidis* and its role in biofilm production. Clin Microbiol Infect 2004 Dec;10(12):1081-8.

(430) Keil LB, Jimenez E, Guma M, Reyes MD, Liguori C, DeBari VA. Biphasic response of complement to heparin: fluid-phase generation of neoantigens in human serum and in a reconstituted alternative pathway amplification cycle. Am J Hematol 1995 Dec;50(4):254-62.

(431) Schouten M, Wiersinga WJ, Levi M, van der Poll T. Inflammation, endothelium, and coagulation in sepsis. J Leukoc Biol 2008 Mar;83(3):536-45.

(432) Zimecki M, Artym J, Kocieba M, Weber-Dabrowska B, Borysowski J, Gorski A. Prophylactic effect of bacteriophages on mice subjected to chemotherapy-induced immunosuppression and bone marrow transplant upon infection with *Staphylococcus aureus*. Med Microbiol Immunol 2010 May;199(2):71-9.

(433) Sauer M, Altrichter J, Kreutzer HJ, Logters T, Scholz M, Noldge-Schomburg G, et al. Extracorporeal cell therapy with granulocytes in a pig model of Gram-positive sepsis. Crit Care Med 2009 Feb;37(2):606-13.

(434) Ovstebo R, Brandtzaeg P, Brusletto B, Haug KB, Lande K, Hoiby EA, et al. Use of robotized DNA isolation and real-time PCR to quantify and identify close correlation between levels of Neisseria meningitidis DNA and lipopolysaccharides in plasma and cerebrospinal fluid from patients with systemic meningococcal disease. J Clin Microbiol 2004 Jul;42(7):2980-7.

(435) Buttery JP. Blood cultures in newborns and children: optimising an everyday test. Arch Dis Child Fetal Neonatal Ed 2002 Jul;87(1):F25-F28.

(436) Hackett SJ, Guiver M, Marsh J, Sills JA, Thomson AP, Kaczmarski EB, et al. Meningococcal bacterial DNA load at presentation correlates with disease severity. Arch Dis Child 2002 Jan;86(1):44-6.

(437) Stout RD, Li Y, Miller AR, Lambe DW, Jr. Staphylococcal glycocalyx activates macrophage prostaglandin E2 and interleukin 1 production and modulates tumor necrosis factor alpha and nitric oxide production. Infect Immun 1994 Oct;62(10):4160-6.

(438) Giese MJ, Mondino BJ, Glasgow BJ, Sumner HL, Adamu SA, Halabi HP, et al. Complement system and host defense against staphylococcal endophthalmitis. Investigative Ophthalmology & Visual Science 1994 Mar 1;35(3):1026-32.

(439) Heinzelmann M, Herzig DO, Swain B, Mercer-Jones MA, Bergamini TM, Polk HC, Jr. Phagocytosis and oxidative-burst response of planktonic *Staphylococcus epidermidis* RP62A and its non-slime-producing variant in human neutrophils. Clin Diagn Lab Immunol 1997 Nov;4(6):705-10.

(440) Johnson GM, Lee DA, Regelmann WE, Gray ED, Peters G, Quie PG. Interference with granulocyte function by *Staphylococcus epidermidis* slime. Infect Immun 1986 Oct;54(1):13-20.

(441) Lutz HU, Fumia S, Schurtenberger C, Alaia V. Opinion paper: Stimulation of complement amplification or activation of the alternative pathway of complement? Mol Immunol 2007 Sep;44(16):3862-5.

(442) van Bronswijk H, Verbrugh HA, Heezius HC, Renders NH, Fleer A, van der Meulen J, et al. Heterogeneity in opsonic requirements of *Staphylococcus epidermidis*: relative

importance of surface hydrophobicity, capsules and slime. Immunology 1989 May;67(1):81-6.

(443) Kelly-Quintos C, Kropec A, Briggs S, Ordonez CL, Goldmann DA, Pier GB. The role of epitope specificity in the human opsonic antibody response to the staphylococcal surface polysaccharide poly N-acetyl glucosamine. J Infect Dis 2005 Dec 1;192(11):2012-9.

(444) Conway Morris A, Kefala K, Wilkinson TS, Dhaliwal K, Farrell L, Walsh T, et al. C5a Mediates Peripheral Blood Neutrophil Dysfunction in Critically Ill Patients. Am J Respir Crit Care Med 2009 Jul 1;180(1):19-28.

(445) Huber-Lang M, Sarma VJ, Lu KT, McGuire SR, Padgaonkar VA, Guo RF, et al. Role of C5a in multiorgan failure during sepsis. J Immunol 2001 Jan 15;166(2):1193-9.

(446) Huber-Lang MS, Younkin EM, Sarma JV, McGuire SR, Lu KT, Guo RF, et al. Complement-induced impairment of innate immunity during sepsis. J Immunol 2002 Sep 15;169(6):3223-31.

(447) Ward PA. Role of the complement in experimental sepsis. J Leukoc Biol 2008 Mar;83(3):467-70.

(448) Chenoweth DE, Hugli TE. Demonstration of specific C5a receptor on intact human polymorphonuclear leukocytes. Proc Natl Acad Sci U S A 1978 Aug;75(8):3943-7.

(449) Riedemann NC, Guo RF, Bernacki KD, Reuben JS, Laudes IJ, Neff TA, et al. Regulation by C5a of neutrophil activation during sepsis. Immunity 2003 Aug;19(2):193-202.

(450) Riedemann NC, Guo RF, Hollmann TJ, Gao H, Neff TA, Reuben JS, et al. Regulatory role of C5a in LPS-induced IL-6 production by neutrophils during sepsis. The FASEB Journal 2003 Dec 19.

(451) Brekke OL, Christiansen D, Fure H, Fung M, Mollnes TE. The role of complement C3 opsonization, C5a receptor, and CD14 in *E. coli*-induced up-regulation of granulocyte and monocyte CD11b/CD18 (CR3), phagocytosis, and oxidative burst in human whole blood. J Leukoc Biol 2007 Jun;81(6):1404-13.

(452) Lappegard KT, Christiansen D, Pharo A, Thorgersen EB, Hellerud BC, Lindstad J, et al. Human genetic deficiencies reveal the roles of complement in the inflammatory network: lessons from nature. Proc Natl Acad Sci U S A 2009 Sep 15;106(37):15861-6.

(453) Brekke OL, Christiansen D, Fure H, Pharo A, Fung M, Riesenfeld J, et al. Combined inhibition of complement and CD14 abolish E. coli-induced cytokine-, chemokine- and growth factor-synthesis in human whole blood. Mol Immunol 2008 Aug;45(14):3804-13.

(454) Wang L, Han G, Wang R, Chen G, Xu R, Xiao H, et al. Regulation of IL-8 production by complement-activated product, C5a, in vitro and in vivo during sepsis. Clinical Immunology 2010 Oct;137(1):157-65.

Paper 1

Paper 2

Paper 3

Appendix

ISBN xxx-xx-xxxx-xxx-x