Staphylococcus epidermidis - virulence factors and innate immune response

Contents

LIST OF PAPERS .......................................................................................................................... 3

ABBREVIATIONS .......................................................................................................................... 4

1. INTRODUCTION .......................................................................................................................... 5
   1.1 Clinical relevance of the thesis ............................................................................................. 5
   1.2 Innate immune system .......................................................................................................... 6
       1.2.1 Recognition - Pathogen recognition receptors (PRRs) ................................................. 10
       1.2.2 Effector - Antimicrobial peptides (AMPs) ................................................................. 11
       1.2.3 Effector - Complement .................................................................................................. 13
       1.2.4 Effector - Leukocytes .................................................................................................... 20
       1.2.5 Effector - Cytokines ...................................................................................................... 22
       1.2.6 Antibodies in neonates ................................................................................................ 26
       1.2.7 Inflammation ................................................................................................................ 27
   1.3 Staphylococcus epidermidis .................................................................................................... 29
       1.3.1 General characteristics of Staphylococcus epidermidis .............................................. 29
       1.3.2 S. epidermidis - clinical significance ........................................................................... 29
       1.3.3 S. epidermidis – antibiotic resistance .......................................................................... 30
       1.3.4 S. epidermidis infections in neonates ........................................................................... 31
       1.3.5 Virulence factors – general ......................................................................................... 33
       1.3.6 Biofilm .......................................................................................................................... 37
       1.3.6 The Arginine Catabolic Mobile Element (ACME) ...................................................... 43

2. AIMS OF THE THESIS .............................................................................................................. 46

3. MATERIAL AND METHODS ..................................................................................................... 47
   3.1 Materials ............................................................................................................................... 47
       3.1.1 Bacterial collection ....................................................................................................... 47
       3.1.2 Study group and blood donors ..................................................................................... 48
       3.1.3 Ethical considerations .................................................................................................. 48
   3.2 Methods ............................................................................................................................... 49
List of papers

Paper I


Paper II


Paper III


Appendix

### Abbreviations

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

<table>
<thead>
<tr>
<th>Function</th>
<th>Premature</th>
<th>Term</th>
<th>&lt;1 year</th>
<th>1-2 years</th>
<th>2-5 years</th>
<th>5-10 years</th>
<th>10-20 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barriers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathogen recognition</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complement</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytokine production</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phagocytosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 lipoteichoic 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,
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 thrombicidins 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-stable 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).
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 three 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 O2 in the cell to produce toxic chemicals such as hydroxyl radicals (OH⁰), 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 hyporesponsiveness 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.

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

(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 Micrococcaceae. 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 beta lactam 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 enterocolitis (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*

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

<table>
<thead>
<tr>
<th>Component</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIA</td>
<td>Cytokine secretion</td>
</tr>
<tr>
<td>Peptidoglycan</td>
<td>Component of cell wall, induce cytokine secretion</td>
</tr>
<tr>
<td>Lipopetides</td>
<td>Component of cell wall, induce cytokine secretion</td>
</tr>
</tbody>
</table>

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 deacetylase) which encodes a surface enzyme that deacetylate the monomers after export.
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 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
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 its 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. epidermidis* 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 were 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

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

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

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 \( \sim 10^8 \) 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, Malmö, 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 \( \mu \)g ml\(^{-1}\) for 2 h at 37 °C), and H\(_2\)O\(_2\) (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 NaIO₄-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~ 10⁸ CFU/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

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

* C1rs-C1inhinhibitor 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% H₂O₂) 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 paraformaldehyde (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 NH₄Cl, 0.01 M NaHCO₃). 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 H₂SO₄ 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
- $\chi^2$ 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


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


(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.


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


(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.


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


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


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


111
Paper 2
Paper 3