Staphylococcus aureus - Plasminogen activation in vitro and prognostic markers in bacteraemic disease

Tomi Mölkänen

ACADEMIC DISSERTATION
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To my family
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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following four original publications. The publications are referred to in the text by Roman numerals I-IV.


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ABBREVIATIONS

AAA  abdominal aortic aneurysm
AIC  Akaike information criteria
APACHE II  Acute Physiological and Chronic Health Evaluation II
ARDS  acute respiratory distress syndrome
ASTA  antistaphylolysin
AUC  area under the curve
BSA  bovine serum albumine
CF-DNA  cell-free deoxyribonucleic acid
CI  confidence interval
CNS  central nervous system
CRP  C-reactive protein
CT  computed tomography
CWI  Charlson weighted index
DIC  disseminated intravascular coagulopathy
DNA  deoxyribonucleotide acid
ED  emergency department
ESR  erythrocyte sedimentation rate
Fas  human membrane protein, member of necrosis factor proteins
FasL  Fas ligand
sFas  soluble Fas
FDR  false discovery rate
GAPDH  glyceraldehyde-3-phosphate
HIV  human immunodeficiency virus
HNP  human neutrophil peptides
hs-CRP  high-sensitivity C-reactive protein
HUOH  Helsinki University Central Hospital
ICU  intensive care unit
IDU  injection drug user
IE  infective endocarditis
IL-1  interleukin-1
IL-6  interleukin-6
IMPDH  5´-monophosphate dehydrogenase
IQR  interquartile range
kDa  kilodalton
MODS  multiple organ dysfunction syndrome
MRI  magnetic resonance imaging
MRSA  methicillin-resistant Staphylococcus aureus
MSSA  methicillin-sensitive Staphylococcus aureus
OR  odds ratio
PA  plasminogen activator
PAI-1  plasminogen activator inhibitor-1
PAI-2  plasminogen activator inhibitor-2
PBS  phosphate-buffered saline
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>PCh</td>
<td>phosphocholine</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PCT</td>
<td>procalcitonin</td>
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<tr>
<td>PET</td>
<td>positron emission tomography</td>
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<tr>
<td>Pitt-score</td>
<td>Pitt bacteraemia score</td>
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<tr>
<td>plg</td>
<td>plasminogen</td>
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<tr>
<td>PTX3</td>
<td>pentraxin 3</td>
</tr>
<tr>
<td>PVL</td>
<td>Panton-Valentine leukocidin</td>
</tr>
<tr>
<td>RNR</td>
<td>ribonucleotide reductase</td>
</tr>
<tr>
<td>ROC</td>
<td>receiver operator characteristic</td>
</tr>
<tr>
<td>SAB</td>
<td>Staphylococcus aureus bacteraemia</td>
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<tr>
<td>SAK</td>
<td>staphylokinase</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SIRS</td>
<td>systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
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<tr>
<td>SOFA</td>
<td>sequential organ failure assessment</td>
</tr>
<tr>
<td>SPECT</td>
<td>single-photon emission computed tomography</td>
</tr>
<tr>
<td>SspA</td>
<td>Staphylococcus aureus serine protease A</td>
</tr>
<tr>
<td>SspB</td>
<td>Staphylococcus aureus staphopain B</td>
</tr>
<tr>
<td>suPAR</td>
<td>soluble urokinase plasminogen activator receptor</td>
</tr>
<tr>
<td>TAA</td>
<td>teichoic acid antibody</td>
</tr>
<tr>
<td>TEE</td>
<td>transesophageal echocardiography</td>
</tr>
<tr>
<td>TTE</td>
<td>transthoracic echocardiography</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue plasminogen activator</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase plasminogen activator</td>
</tr>
<tr>
<td>UTI</td>
<td>urinary tract infection</td>
</tr>
<tr>
<td>vWF</td>
<td>Von Willebrand factor</td>
</tr>
<tr>
<td>α-enolase</td>
<td>alpha-enolase</td>
</tr>
<tr>
<td>α2AP</td>
<td>alpha-2-antiplasmin</td>
</tr>
<tr>
<td>18F-FDG</td>
<td>18F-fluorodeoxyglucose</td>
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ABSTRACT

Introduction. *Staphylococcus aureus* is the second most frequent finding in blood cultures. *S. aureus* bacteraemia (SAB) is often (in 80% of cases) complicated by deep infection. The invasion mechanisms of *S. aureus* are incompletely understood. Several pathogens can bind plasminogen (plg) to the bacterial cell surface receptors. Binding of plg to these receptors enhances activation of plg by human plasminogen activators (PAs). The generated proteolytic plasmin activity on the bacterial surface gives pathogens the capacity to invade tissues. *S. aureus* produces PA staphylokinase (SAK), which can activate plg to active plasmin. On the *S. aureus* surface, the generated plasmin could turn the bacterium into an invasive proteolytic organism. Complicated SAB cases should be identified early to guide treatment decisions. The prognosis of SAB depends on the the underlying disease(s), immunosuppression, severity of sepsis and development of deep infection. C-reactive protein (CRP) is a marker of inflammation and organ dysfunction. Changes in CRP have been used to measure treatment response in systemic bacterial infections. Cut-off levels to guide treatment in SAB are unclear. Genetic factors account for up to 40% of the variation in basal CRP. Several single nucleotide polymorphisms (SNPs) in the CRP gene associate with variation of basal CRP. However, very little is known about how CRP gene SNPs impact CRP levels in serious systemic infections. Serum soluble urokinase plasminogen activator receptor (suPAR) is a new prognostic biomarker. Elevated suPAR levels have been observed in malignant and infectious diseases with poor prognosis. However, suPAR has shown only limited value in distinguishing bacterial causes from other causes of systemic inflammatory response syndrome (SIRS). The studies presented here aimed to elucidate potential invasive mechanism of *S. aureus* and to evaluate the value of CRP and suPAR in predicting complicated disease. The following studies were undertaken: 1) to identify the effect of *S. aureus* to plg activation by SAK and to characterize plg-binding proteins 2) to evaluate CRP and suPAR in identifying fatal outcome and presence of deep infections in SAB 3) to assess the determinants of maximal CRP levels in SAB.

Study bacteria, population and methods. In study I, clinical *S. aureus* isolates from Helsinki University Central Hospital were used in vitro. Radioactive labelling of plg was used to detect the binding of plg. Bacterial cell-wall proteins were isolated by affinity chromatography. Generation of plasmin activity was measured spectrophotometrically. Solubilised cell wall proteins were separated by electrophoresis and transferred to nitrocellulose membranes for protein blotting and visualization. N-terminal amino acid sequencing was performed to identify the cell wall proteins. Studies II-IV were based on 430 prospectively followed SAB patients from a multicentre study conducted in Finland from 1999 to 2002. In study III, a subset of 145 patients from Helsinki University Central
Hospital (HUCH) was included. In study IV, suPAR levels were analysed in 59 patients from HUCH. They stratified according to 1-month survival and presence of deep infection to three groups: 1) fatalities, 2) patients with a deep infection and 3) patients without deep infection. In studies II-IV, CRP levels and white blood cell (WBC) counts were measured using standard laboratory method. In Study III, DNA was extracted by the phenol chloroform extraction. DNA was quantitated by measuring the absorbance. The detection of SNPs was performed by the analysis of primer extension products from amplified genomic DNA CRP gene sequencing.

Results. *S. aureus* bacterial cells enhanced plg activation by SAK. Activation was not inhibited by alpha-2-antiplasmin (α2AP). The ability of bacteria to bind plg correlated with induced plasmin activity. Amino-acid sequencing revealed three tentative plg-binding proteins of *S. aureus*, inosine 5’-monophosphate dehydrogenase, alpha-enolase and ribonucleotide reductase. SAB patient fatalities could be distinguished from survivors by CRP levels already on day four. Cut-off values that predicted a 30-day fatal outcome were: CRP on day four >103mg/L (sensitivity 77%, specificity 55%), on day 14 CRP >61 mg/L (sensitivity 82%, specificity 80%) and WBC count on day 14 >8.6 x10⁹/L (sensitivity 77%, specificity 78%). CRP values that predicted deep infection were: on the day of the positive blood culture CRP >108 mg/L (sensitivity 77%, specificity 60%) and on day 14 CRP >22 mg/L (sensitivity 59%, specificity 62%). Absence of CRP decline was not prognostic of mortality or deep infection. A deep infection was found in 84% of patients. The maximal CRP during the first week was significantly elevated in patients who had A-minor allele of rs3091244. CRP gene SNPs were analysed and they did not affect the CRP levels at the time of positive blood culture or seven days after. No differences were observed in predisposing factors, underlying diseases and clinical manifestations related to CRP gene data. The presence of deep infection focus and A-minor allele were both significantly associated with the maximal CRP level during the first week of SAB. suPAR levels on day three were higher in fatalities than in survivors. This difference persisted for 10 days. Deep infection was not associated with suPAR levels. suPAR was a prognostic marker for fatal outcome starting from day three, while CRP in this study setup on day three was not.

Conclusions. Plg activation by SAK enhanced and protected against the inhibitory effect of α2AP when *S. aureus* cells or plg binding cell wall proteins were present. Three potential plg-binding proteins of bacterial cell surface were identified. In invasive SAB, the CRP level predicted both fatal outcome and presence of deep infection in the early phase of disease. SNPs of the CRP gene and deep infection were shown to determine the maximal CRP level in SAB. In SAB, serum suPAR level is a promising prognostic biomarker for fatal outcome.
1. INTRODUCTION

*Staphylococcus aureus* belongs to the human microbiota and most commonly colonizes anterior nares [1]. Dissemination from colonised sites results into various clinical infections, ranging from superficial skin and soft-tissue infections to severe invasive *S. aureus* bacteraemia (SAB) [2,3]. The invasive nature of SAB is substantiated by the common associated complications, in particular deep infection foci such as pneumonia, infective endocarditis (IE), deep-seated abscesses, urinary tract infection (UTI), osteomyelitis or meningitis [3-8]. SAB is observed more often in infants, in the elderly and in patients with severe underlying diseases. Advanced age, comorbidities and male gender have been shown to increase mortality [9-11]. Mortality due to SAB remains high and, according to recent publications, still over 20% [12-14]. It is therefore important to find rapidly patients who are at greatest risk of developing complications [15]. In addition to patient related factors, bacterial virulence factors also impact disease development and outcome [16,17]. Microbe-related mechanisms may contribute to the development of superficial *S. aureus* infection into a serious invasive infection. However, these mechanisms are still incompletely understood [18].

One important feature, the coagulative capacity of *S. aureus*, enables conversion of fibrinogen to fibrin. Accordingly, the coagulase test used in microbiology laboratories distinguishes *S. aureus* from other staphylococcal isolates. *S. aureus* secretes two coagulation-promoting enzymes, von Willebrand factor binding protein and coagulase, which both activate prothrombin non-proteolytically by forming a staphylothromin complex. This complex enables the protease enzyme to convert fibrinogen to fibrin [19]. In addition to this thrombogenic mechanism, *S. aureus* has the ability to digest fibrin clots. The majority of strains have a staphylokinase (SAK) gene and also produce the fibrinolytic enzyme SAK, the *S. aureus* plg activator enzyme [20-22]. Both of these mechanisms, coagulative and fibrinolytic, may facilitate bacterial pathogenesis.

Several invasive bacteria possess plg receptors. Cell-surface bound plg is activated to plasmin, which converts these bacteria into proteolytic organisms capable of invasion [23,24]. Theoretically, SAK could be a virulence factor, which could aid bacteria in invading and spreading throughout the body. However, SAK production has been shown to be associated with less complicated bacteraemia [25] rather than a feature favouring effective invasion. In contrast, SAK has been proposed to be an important factor for colonization [26] and to have a role in controlling biofilm formation, since SAK-induced plg activation has been proposed to prevent biofilm formation and to induce detachment of existing biofilms in *S. aureus* infection [27].
A prospective clinical trial on the antibiotic therapy of SAB was conducted between January and May 1999 and between January and August 2003 in five Finnish university and seven tertiary care hospitals, and comprised 430 patients [4]. The severity of underlying diseases, immunosuppressive treatment, severity of sepsis and complications like deep infectious foci all collectively determined prognosis in these SAB patients [4,12,28]. A deep infection focus was found in up to 80% of patients due to meticulous searching with imaging techniques [29]. Later, other studies have reported relatively high occurrence of deep foci in SAB [14]. The radiological investigations used in deep focus visualization, such as chest x-ray, computed tomography (CT) scans and even radionucleotide imaging techniques are sometimes expensive or difficult to perform and above all expose patients to radiation [30-32]. Therefore, biomarkers that predict the risk of development of complications might facilitate further investigations on the right patients at the right time [33].

C-reactive protein (CRP) is part of acute phase reaction and CRP levels typically rise rapidly in response to infection, inflammation or tissue destruction [34]. It has been widely used for decades in daily clinical practice in all age groups as a screening test for organic illness and as a sensitive indicator of disease severity and response to therapy in infections and inflammatory and ischaemic conditions [35-38]. In serious systemic bacterial infections, however, the results of studies on use of CRP in critically ill patients are not unanimous. One study concluded that CRP is a better marker of infection than temperature in critically ill patients [39], while another study claimed that CRP has only limited value in distinguishing sepsis from other inflammatory conditions and in predicting outcome [33]. In septic patients, CRP on the day of sepsis diagnosis was not a good marker of prognosis [40], but repeated CRP measurements were found useful on day three in predicting prognosis of patients with community-associated sepsis [41]. Specific studies on the use of CRP in predicting mortality and presence of deep infection in SAB are not available, and clear cut-off values might help in diagnosing patients with the most complicated disease.

Single nucleotide polymorphism (SNP) variations of the CRP gene have been shown to affect the basal CRP levels in subjects without any inflammatory processes [42,43]. In addition, studies on associations of CRP gene SNPs with risk for many diseases are available. However, it is uncertain whether CRP gene SNPs as such are risk factors for diseases. CRP gene SNPs have been studied as tentative risk factors for ischemic stroke [44], ischemic heart disease [45], depression [46] and pneumonia [47]. Studies on the association of polymorphism in the CRP gene on CRP levels in acute inflammatory situations are limited to one study on by-pass surgery, in which it was observed that low CRP levels were associated with CRP gene SNPs [48].
Urokinase plasminogen receptor (uPA) [49] is found on many cell surfaces. uPA may become soluble (suPAR) following cleavage and release. suPAR can be found in blood, serum, plasma, urine and cerebrospinal fluid. While the biological role of suPAR is unknown [50], as a prognostic marker it is better characterised. suPAR levels have been shown to correlate with fatal outcome or disease progression in cancer, human immunodeficiency virus (HIV), tuberculosis and malaria as well as in systemic inflammatory response syndrome (SIRS), type-2 diabetes, renal disease, ischemic stroke and coronary artery disease [51]. Even in the Danish population-based cohort study on Caucasian adults elevated suPAR levels were found to associate with the increased mortality [52]. Finally, two meta-analyses have summarised the value of suPAR in critically ill patients, and its prognostic value was shown to be significant [53,54].
2. REVIEW OF THE LITERATURE

2.1. 

2.1. Staphylococcus aureus

In 1882, Ogston described S. aureus as a cause of abscesses and bacteraemia [2], and even today it persists as a major pathogen causing infections in the healthy and the sick. This gram-positive, adaptable and dangerous bacterium belongs to the micrococcal family. In the laboratory, the gold pigmentation of colonies and coagulase, mannitol-fermentation, deoxyribonuclease and haemolysis tests distinguishes it from other staphylococci. It can be cultivated on regular agar plates, and tolerates transport and even drying well [55,56].

S. aureus produces vast amounts of different proteins [17]. The main components of cell wall are peptidoglycan and ribitol teichoic acid. Many other cell wall proteins bind covalently to peptidoglycan [57]. These cell wall proteins are immunogenic and bacteria protect these structures with capsule and protein A [58]. S. aureus on one hand appears to be a harmless commensal and on the other hand it may cause serious invasive infections. The bacterial pathogenetic mechanisms involved in invasive infections are only partly characterised [18]. In invasion, adhesion is essential as a first step and many adhesion molecules have been characterised, although the role of these is only partly known [59,60]. In addition to adhesion molecules, S. aureus produces many enzymes and other proteins that further aid the pathogenesis of this bacterium.

2.1.1. Surface-associated and secreted proteins of Staphylococcus aureus

S. aureus can express a wide array of both surface-associated and secreted proteins, which both play a role in virulence [17]. Altogether, there are 1354 predicted secreted proteins, of which only 41% have been identified. The function of less than half of these proteins is known [61].

2.1.1.1 Proteins and innate immunity

In one recent review Zecconi and Scali described S. aureus virulence-associated surface proteins and secreted proteins based on their role in evasion of first-line immune defence. This first-line immune defence (innate immunity) comprises three main components:

- Peptides and enzymes with antimicrobial properties
- Complement
- Phagocytosis
**S. aureus** produces in total eight virulence factors related to evasion of antimicrobial peptides, e.g. SAK and aureolysin against cathelicidin and peptidoglycan O-acetyltransferase resisting lysozyme. Seven virulence factors resist complement, of which SAK is one. In total, twelve other virulence factors, such as leukocidins A, B and Panton-Valentine (PVL), have antiphagocytic properties. Finally, twenty adhesins, e.g. plasmin-sensitive protein and fibronectin are important virulence factors [59].

### 2.1.1.2. Surface-associated proteins

In another review, Foster et al. described how surface-associated and cell wall-anchored proteins bound to peptidoglycan are classified into four families, based on the presence of motifs identified by structure-function analysis. Only eleven proteins remain structurally uncharacterised [60].

- The microbial surface components recognise adhesive matrix molecules, being the most abundant, and include e.g. fibronectin binding proteins and clumping factors.
- The near iron transporter motif family, comprising iron-regulated surface proteins.
- The three-helical bundle, including only protein A.
- Repeat domain family, comprising *S. aureus* protein G and plasmin sensitive protein.

### 2.1.1.3. Toxins

Poisonous substances produced by *S. aureus* are described as toxins. These toxins cause harm interfering with the host directly. Toxins do not include secreted surface-located proteins or proteins that resist host defense mechanisms inside bacteria. These toxins can be grouped into three categories [62]. 1) membrane-damaging toxins as receptor-mediated, such as alpha-toxin, PVL, leukocidin A/B, leukocidin D/E and gamma toxin or 2) non-receptor mediated, such as α-type phenol-soluble modulins [63] and 3) toxins that interfere with receptor function, such as enterotoxins [64], enterotoxin-like enterotoxins or toxic shock syndrome toxin [62].

### 2.1.1.4. Enzymes

By biochemical definition, many *S. aureus* secreted proteins are enzymes. These include proteases such as *S. aureus* serine protease A, *S. aureus* staphopain B cysteine protease and aureolysin (metalloproteinase) [62,65]. The roles of lipases and nucleases are unclear; however, nucleases are probably needed to degrade extracellular chromatin nets [66]. Coagulases staphylocoagulase and von Willebrand binding factor enable blood clotting. The complex formation of these coagulases with prothrombin leads to conversion of
fibrinogen to fibrin [67]. Fibrin clots on bacterial cells inhibit phagocytosis, favouring abscess formation [68], and in addition, increased adhesion to catheters [69]. *S. aureus* also produces the plasminogen (plg) activator SAK, which mediates fibrinolysis. Fibrinolysis mediated by SAK-activated plg has been demonstrated to lyse the fibrin network surrounding infection [27,70].

### 2.2. Plasminogen activation in fibrinolysis and in bacterial pathogenesis

#### 2.2.1. Fibrinolysis and invasion of cells

Activation of plg to plasmin is the main fibrinolytic mechanism in humans [71]. However, plasmin activity is also essential in physiological and pathophysiological invasion of human cells [72,73]. In fibrinolysis, plg is activated to plasmin by the serine proteases tissue-type plasminogen activator (tPA) and urokinase plasminogen activator (uPA). Endothelial cells produce tPA and uPA is produced by monocytes, macrophages and urinary epithelium [74,75]. The main activity of uPA has been observed in tissues and urine and the activity of tPA mainly intravascularly. Plg and plasmin are efficiently inhibited in circulation by the serine proteases plasminogen activator inhibitors (PAIs) 1,2 and alpha-2-antiplasmin (α2AP) [76]. These inhibitors control the half-life of tPA and uPA to 4-8 minutes [77]. The situation changes, however, when plg or plasmin binds to fibrin or to another target, and the inhibitory effect of plg activator inhibitors is lost locally [78].

Taken together, in fibrinolysis plg is activated to active plasmin by two serine proteases [74,75]. Plasmin activity in circulation is normally inhibited by three specific inhibitors [76]. However, when fibrinolysis is needed to dissolve blood clots, this inhibition is lost, and local fibrinolysis activates [78].

#### 2.2.2. Plasminogen activation in bacterial pathogenesis

A total of sixteen invasive bacterial species have been shown to interact with the plg system, based on *in vitro* experiments [79]. The role of plg interaction is best characterised in experiments *in vivo* with three pathogens, *Streptococcus pneumoniae*, group B streptococcus [80,81], and *Borrelia burgdorferi* [82,83].

Many bacteria utilise human plg activators for pathogenesis and invasion, but only three produce plg activators. *Yersinia pestis* produces *pla* [84,85], invasive streptococci streptokinase [86,87], and *S. aureus* SAK [20]. Bacteria may use these plg activators in several different ways in the pathogenesis of infection. Invasive streptococci can bind plg on the cell surface and then activate plg by streptokinase to plasmin [79,88]. This
mechanism may enable group A streptococci to penetrate pharyngeal cells [89], Group B and C streptococci, S. pneumoniae and S. mutans to degrade components of extracellular matrix and to invade host tissues [81,90,91]. Plg activation can also aid group B streptococci invasion of the central nervous system (CNS) and S. pneumoniae laminin degradation and migration through the lung epithelium [91]. Y. pestis has been shown to activate plasminogen by pla to facilitate in tissue-invasion [92]. Activation of plg by SAK in S. aureus infection has been demonstrated to promote at least local subcutaneous spreading of disease in skin infections [70,93]. In addition, studies have suggested a role for plg activation in immune evasion by group A streptococci, S. pneumoniae and S. aureus by complement C3/C3b degradation and for protection of group A streptococcus from macrophage killing [91,94-97].

To summarize, these studies support the view that the role of plg system in the bacterial pathogenesis is only partly characterised. Most studies on bacterial interaction with the plg system are in vitro studies [79]. Three bacteria produce bacterial plg activators [20,84,86]. The role of these activators in the tissue-invasion and in the bacterial pathogenesis, is best characterised in invasive streptococci [89], and Y. pestis [92]. In S. aureus, the role of SAK production and plg activation in the pathogenesis is partly resolved [70,93].

2.2.3. Bacterial plasminogen receptors

Plg-binding structures on the surface of many bacteria appear to be common among invasive pathogens. Glyceraldehyde-3-phosphate (GADH) binds plg on the surface of many gram-positive [98] and gram-negative bacteria [99]. Alphaenolase (α-enolase) is another well-characterised plg binding molecule found on both eukaryotic [100] and prokaryotic cells like, including streptococci [101], trichomonas [102], mycoplasma [103] and B. anthracis [104]. Furthermore, streptococcal M protein binds plg on streptococci [105]. Spirochetes, Borrelia and Leptospira also possess numerous plg receptors [79]. Interestingly, two of these plg receptors, GADPH and α-enolase, are both proteins better known as essential enzymes in glycolysis [106], which makes the presence of these intracellular proteins on the cell-surface even more intriguing but problematic to understand. Excretion of cytoplasmic proteins, however, is unlikely to result from cell lysis [107]. These enzymes probably have both intra- and extracellular functions representing a phenomenon called protein moonlightning. This protein moonlightning means that proteins possess many functions instead of only one. Protein moonlightning has been described as a single protein possessing multiple functions that are not result of splice variants, changes in transcription and resealing of genetic material, fusion of genes or consisting of many proteolytically cleaved protein fragments [108]. Recently, small
covalent changes in protein structure were described to partly explain protein moonlightning; these covalent changes were revealed to alter the biological function of proteins [109].

2.2.4. Staphylokinase

SAK is a 15.5 kilodalton (kDa) protein and it was first described in 1948 [110] as an activator of plasma protease. SAK production has been detected in 58% to 85% of S. aureus strains [25,111]. SAK is produced in the late exponential phase of the bacterial growth cycle. SAK is encoded by the SAK gene, which is under accessory gene agr regulation [112]. SAK comprises 136 amino acids and its three-dimensional structure has been described in detail [113,114]. Initial plg activation by SAK has been proposed to require a tiny amount of plasmin [115]. In circulation and tissues, this required amount of plasmin is available due to spontaneous low-level turnover of plg into plasmin. SAK has been suggested to form a 1:1 complex with plasmin, which would then non-proteolytically activate more plg to plasmin [115]. This activation is normally inhibited in circulation by alpha-2-antiplasmin (α2AP) [116]. In many studies, activation of plasminogen by SAK has been demonstrated to arise enhanced when components are bound in fibrin net [117] or within cell wall [118]. In addition, this activation has been found also to occur protected against the inhibitory effect of α2AP. In other bacteria, like in Y. pestis [85] and streptococci [119], secreted plg activators aid in invasion, but such mechanism has not been verified in S. aureus. In contrast, it has been suggested that plasminogen activation by staphylokinase could even reduce the severity of systemic S. aureus infection [120] and SAK production was even shown to be associated with less invasive infections in humans [93]. In other studies, SAK producing bacteria were demonstrated to favour enhanced subcutaneous spreading in skin infections [70] and to form less biofilms [27]. Finally, SAK has also been shown to interact with human neutrophil peptides and this interaction has been suggested to help bacteria at least to resist phagocytosis [121].

2.3. Staphylococcus aureus bacteraemia

2.3.1. Epidemiology

S. aureus is the second most common pathogen in the blood cultures from bacteraemia patients. This has been observed in several studies and in all age groups [122-124]. The overall Incidence of SAB was 26.1/100 000 population in a multinational study, and no evidence of an increase in incidence occurred during 2000-2008 [125]. In Finland, with a population of 5.45 million in 2013, S. aureus caused almost 1000 bacteraemia cases
annually [126,127], which comprised 13% of all bloodstream infections [128]. In Denmark, the incidence of SAB between years 1971 and 2000 doubled (from 14 to 31/100 000 population annually). Another study reported that the incidence of SAB had stabilised between 1995-2008 (23.4-21.8/100 000 population annually) [129], and that short-term mortality had improved from 27 % to 23 % during the study years. Increases in SAB incidence, hospital admissions and increases in invasive medical procedures have been suggested as key contributors to increases in health care-associated SAB acquisition [16]. Reflecting the same phenomenon, Cahill and Prendergast highlighted that wide medical advances [130] are associated with the increased amount of health-care associated endocarditis due to *S. aureus* [131]. In addition, the incidence of health-care associated SAB among haemodialysis patients is high; in worldwide studies incidence increased from over 3 000 to almost 18 000/ 100 000 person years [132-134].

SAB caused by methicillin-resistant *S. aureus* (MRSA) has remained for decades a primarily health care- associated infection, until community-associated MRSA epidemics emerged worldwide a decade ago [125,135]. However, in northern Europe the prevalence of MRSA has remained low [136,137]. In many countries, the high MRSA prevalence has increased the incidence of SAB due to MRSA. This has been shown to be an additional burden of disease on top of that caused by methicillin-sensitive *S. aureus* (MSSA) [138-140], although this is debatable and reports on replacement and additive effects exist [141].

Taken together, these epidemiological reports support the view that the incidence of SAB is not decreasing, in Finland or world-wide. Health care-associated SAB will probably remain frequent because of increased amount of hospital admissions and invasive medical procedures. In addition, MRSA will probably continue to increase burden of SAB disease.

**2.3.2. Predisposing factors**

**2.3.2.1. Colonization**

The connection between *S. aureus* nasal carriage and infection was first recognised in 1931 by Danbolt [142]. *S. aureus* colonises the skin and mucosal membranes in up to 36% of the population [143,144]. Among many possible body sites, nares are the primary niche for *S. aureus*. 20% of people are persistent carriers, 30% transient carriers and 50% non-carriers [145,146]. Nasal carriage has been shown to be a risk factor for *S. aureus* infection [147], and it is also well recognised as a source for bacteraemia [148,149]. This is supported by the observation that the strains both colonizing and causing infections are often genetically similar [143].
2.3.2.2. Age and gender

Both age and gender are shown to associate with the incidence of SAB. For unknown reasons, males have an approximately 1.5-fold higher SAB incidence than females [138,150]. The incidence of SAB is increased at both ends of the lifespan. Infants <1 years and people >60 years are more likely to get SAB than younger adults, and SAB incidence increases steadily with the increasing age [125,138,139].

2.3.2.3. Ethnicity

In addition of gender and age, ethnicity is also associated with the incidence of SAB. The incidence of invasive MRSA infection in the African-American population is over two times higher than that in Caucasian Americans [151,152]. Australian aboriginals, Maori-, and Pacific people have an up to 20 times higher incidence than people of European origin [153,154]. These differences are only partly explained by socioeconomic factors [153].

2.3.2.4. Underlying diseases

SAB rarely affects individuals with no underlying predisposing diseases, and many diseases and conditions have been associated with elevated risk for SAB. Cardiovascular diseases, chronic obstructive pulmonary disease, diabetes, malignancies, chronic renal failure, hepatic cirrhosis and HIV are all associated with an elevated risk for SAB [4,5,155-159]. Health-care associated SAB in HIV patients is related to low helper lymphocyte cell counts, and community-associated SAB with HIV positivity to intravenous drug abuse [160]. In older reports until the 1990s, the proportion of SAB patients with underlying diseases was high (>90%) [161,162]. Around the millennium, however, the situation has changed, probably due to increasing numbers of community-associated SAB; SAB patients have fewer underlying diseases than earlier [5,163,164].

2.3.2.5. Substance abuse

Intravenous drug use (IDU) causes a high risk for SAB, primarily due to direct injection of material into bloodstream. The high SAB risk is also due to the higher rate of *S. aureus* skin colonization than the normal population [165], to frequent skin-and soft-tissue infections [166] and living environments where *S. aureus* is easily transmitted between persons [167]. The proportion of IDU has varied from 2% to 30% in SAB studies [159,168,169]. The incidence of SAB among IDUs is as high as over 600 per 100 000 person years [170]. Both colonization of the drug user and contaminated drugs have been identified as a source of bacteremia [167,171,172].
Chronic alcoholism has been identified up to 15% of SAB patients \[159,173\]. Chronic alcoholism may increase both infection and SAB risk due to impaired general health, neglected dental hygiene and malnutrition \[174\]. In addition, chronic alcoholism may lead to compromised immunity via inhibition of neutrophil, macrophage and lung ciliary function \[173,174\].

2.3.3. Clinical disease

SAB diagnosis is based on positive blood culture. The clinical picture at the beginning of the disease is variable; the patient can present practically without symptoms or with signs of severe sepsis. Typically, a patient with SAB has symptoms such as chills, nausea or pain in the stomach, muscles or joints \[3\]. The most common symptom, fever, occurs in up to 96% of patients \[168,175\]. Without treatment for bacteraemia, the patient’s condition deteriorates and severe systemic inflammation symptoms such as hypotension, confusion or tachypnea may follow \[176\]. Studies have indicated that the most common acute complications such as septic shock or severe sepsis develop in 10-30% of patients \[159,177,178\] and any organ failure in 20-40% \[179,180\].

The prognosis of SAB improved at the beginning of the 1940s with the discovery of penicillin, which led to an increase in survival from 18% to 72\% \[181\]. However, resistance to penicillin was reported in 1942, soon after its first clinical use \[182\]. By the 1950’s, more than 50\% of \textit{S. aureus} strains in large hospitals had developed resistance to penicillin \[183,184\]. Methicillin was developed in 1959 to overcome the penicillin resistance, but MRSA was already observed in 1961 \[185\]. The US Food and Drug Administration approved vancomycin in 1958 \[186\]; resistance to vancomycin has taken four decades to develop. During the period of modern antibiotics (1950-1980) mortality due to SAB has varied in different studies from 25 to 50\% \[187-189\]. Before the millennium, prognosis was possibly improved and reported mortality fell to almost 20\%. During last two decades, reported mortality has been 14-30\% in both health care-associated and community-associated SAB \[190-192\].

2.3.4. Prognostic factors

Prognostic factors for mortality due to SAB consist of patient-related and clinical disease related factors. Prognostic factors with reported odds ratios are shown in Table 1. Other reported prognostic factors include hyperbilirubinaemia \[189\], thrombocytopenia \[193\], neutropenia \[10\], persisting bacteraemia \[169\] and acute renal failure \[159\]. Contrasting results of the prognostic significance of MRSA have been reported. Several studies have not been able to detect a difference in mortality between patients with SAB due to MRSA
or MSSA [194-198]. In some studies health care-associated origin of SAB has not impacted to mortality [6,164,168,199,200], while in one study mortality due to health care-associated SAB has been lower compared to mortality due to community-associated SAB [159].

**Table 1.** Prognostic factors with reported odds ratios for mortality due to *Staphylococcus aureus* bacteraemia. MRSA=methicillin-resistant *S. aureus*. MSSA=methicillin-sensitive *S. aureus*. OR= significant odds ratios for prognostic factors.

<table>
<thead>
<tr>
<th>Prognostic factor</th>
<th>OR</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &gt;65 years</td>
<td>1.6-2.3</td>
<td>[10,138,164,194,201]</td>
</tr>
<tr>
<td>Ultimately or rapidly fatal underlying diseases</td>
<td>2.7</td>
<td>[9]</td>
</tr>
<tr>
<td>Ultimately fatal underlying disease</td>
<td>1.02</td>
<td>[195]</td>
</tr>
<tr>
<td>Rapidly fatal underlying disease</td>
<td>3.38</td>
<td>[195]</td>
</tr>
<tr>
<td>Severe sepsis or septic shock</td>
<td>2.68-25.7</td>
<td>[159,199,202,203]</td>
</tr>
<tr>
<td>Delayed empiric antibiotic</td>
<td>2.15-3.8</td>
<td>[204,205]</td>
</tr>
<tr>
<td>SAB due to MRSA</td>
<td>1.69-2.6</td>
<td>[164,169,177,206,207]</td>
</tr>
<tr>
<td>Uneradicated infection focus</td>
<td>4.17</td>
<td>[196]</td>
</tr>
<tr>
<td>Deep infection focus:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pneumonia</td>
<td>5.8-6.46</td>
<td>[6,194]</td>
</tr>
<tr>
<td>Endocarditis</td>
<td>2.8-12.13</td>
<td>[207,208]</td>
</tr>
<tr>
<td>Meningitis</td>
<td>21.4</td>
<td>[8]</td>
</tr>
<tr>
<td>Unknown infection focus</td>
<td>4.1-8.67</td>
<td>[194,209-211]</td>
</tr>
<tr>
<td>Stroke associated with endocarditis</td>
<td>2.5-32</td>
<td>[212-214]</td>
</tr>
<tr>
<td>Health care-associated SAB</td>
<td>1.42-1.75</td>
<td>[10,215]</td>
</tr>
</tbody>
</table>

2.3.4.1. Underlying diseases

In the 1960’s, McCabe and Jackson created a widely-used classification system for categorising underlying diseases of patients to: healthy, non-fatal, ultimately- and rapidly fatal [216]. This classification has been successfully used in many studies, because it independently predicts fatal outcome [195,217-219]. In SAB studies, the risk of death was shown to increase incrementally among its three categories [195,219]. Underlying diseases associated with higher mortality in SAB include alcoholism [159], immunosuppression [159], diabetes [194], liver cirrhosis [9,10], cardiac disease [6,220], malignancies [9,221], renal failure and dialysis [10,159] and the presence of multiple comorbidities [5,222].
2.3.4.2. Severity of illness

The severity of illness is one of the established predictors of 30-day mortality in SAB [192]. Severe sepsis and septic shock in SAB is associated with a high overall mortality rate (between 40-80%) [192,200,203,223]. Severity of illness can be evaluated with different severity-of-disease scores, primarily used in intensive care units (ICU). These scores include:

- The Acute Physiological and Chronic Health Evaluation II (APACHE II), is designed to assess the severity of illness. In APACHE II score, severity of illness is utilised in addition to previous health status (including chronic illnesses) for patients admitted to ICUs [224].

- The sequential organ failure assessment score (SOFA) means that, on assessment of respiratory, cardiovascular, coagulative, hepatic, renal and neurological functions, SOFA points on a scale of 0-4 are given to each function [225].

- The Pitt bacteraemia score (Pitt-score), is a scoring system that predicts mortality due to sepsis in ICUs. Required clinical parameters are fever, presence of hypotension, need for mechanical ventilation, cardiac arrest and altered mental status [226].

- The multiple organ dysfunction score (MODS) uses simplified measures of physiological dysfunction in several organ systems with strong correlation to fatal outcome in ICU-treated patients [227].

These severity of disease scores have all predicted mortality in bacteraemia due to \textit{S. aureus} [6,218,222,228,229].

Specific conditions associated with higher risk for fatal outcome in SAB include altered mental status [230], need for mechanical ventilation [201] or ICU treatment and SAB occurring during ICU treatment [195,203,222].

2.3.4.3. Infection focus

Infection focus in SAB is important to identify because it can affect prognosis and the length of antibiotic treatment [231]. However, variable definitions of infection foci in the literature make comparison of different patient materials challenging. Studies on SAB have used three different classifications to describe the level of infection focus: 1) primary or secondary, 2) complicated or uncomplicated or 3) cutaneous or deep focus. Nolan
categorised infection foci as primary or secondary [232]. Primary foci included skin and soft-tissue infections [3,233,234], indwelling intravascular devices [162], pneumonia [235-237], urinary tract infection (UTI) [238,239], and unknown portal of entry [162,169]. Secondary infection foci are sometimes challenging to categorise strictly. However, clearly secondary infection foci are IE, deep-seated abscesses, meningitis (unless postoperative), septic arthritis without local trauma or surgery [155,194,232,240], and usually UTI if the same S. aureus strain causes both bacteraemia and UTI [162,241]. Pneumonia can be either a primary focus [235,236] as a result of aspiration or a secondary focus due to septic emboli from an infected tricuspid valve [240]. The presence of secondary infection focus, recurrence of SAB within three months, shock, acidosis, acute respiratory distress syndrome, disseminated intravascular coagulopathy, CNS involvement and evidence of autoimmune or embolic events have been definitions for complicated SAB [162,241-243].

Tan and File created a practical classification for infection foci. They categorised S. aureus infections as cutaneous and deep, which included bacteraemia, osteomyelitis, septic arthritis, deep-seated abscess, endocarditis, pneumonia, and foreign body or CNS infection [244]. The same kind of deep infection focus classification as Tan and File created, was used by our study group in previous articles [28].

2.4. Biomarkers and diagnosis of deep infection focus

Published data on use of erythrocyte sedimentation rate (ESR), white blood cell (WBC) count and CRP in predicting the presence of a deep infection focus in SAB is limited. Reported studies on the use of CRP in predicting deep infection focus other than IE, pneumonia, or surgery-related infections in SAB are not available.

In studies on predicting surgery-related infections but not specifying the causative microbes, high CRP but not elevated WBC count predicted (postoperative day two) development of a postoperative infection (median nine days postoperative) in patients who had underwent cardiac surgery [245]. In another study, both elevated CRP level and WBC count were found to be good markers for postoperative complications [246]. CRP >100 mg/L on postoperative day four was found predictive for infection in patients who had underwent post-lumbar instrumentation surgery, whereas greater elevation of leukocytes at day seven as compared to day four was not [247].

ESR is defined as the distance that a vertical column of anticoagulated blood has fallen in one hour. Although there have been many studies on the clinical use of ESR in the last several decades, its value and specificity in diagnosing any infections is still unclear [248]. Sensitivity was low (0.36) among patients with infection in general but specificity was high
ESR is moderately raised in hip prosthesis infection. It is useful in diagnostics, since symptoms, X-rays or scans do not distinguish infection from loosening. 25% of these infections were shown to be caused by \textit{S. aureus} [250]. Elevated ESR and leukocytosis were published to be markers of spinal bacterial abscess, but the causative microbes in this study were not specified [251]. In evaluating low-grade bone infection, ESR was found to have value [252]. However, when ESR was compared to CRP, ESR had lower sensitivity than the CRP acute-phase response [253].

A review article on emergency department use of biomarkers in diagnosing osteomyelitis concluded, that in patients with low-level suspicion of osteomyelitis, age-adjusted normal ESR and CRP <5 mg/L could be used to exclude osteomyelitis. However, in case the clinical suspicion is very strong, normal values should not be used to rule out the diagnosis. In addition, in this same article it was mentioned that WBC count is not helpful in diagnosing osteomyelitis [254]. In addition, one meta-analysis concluded that CRP appeared to have only limited value in diagnosing osteomyelitis and septic arthritis [255].

\section*{2.5. Deep infection focus in \textit{Staphylococcus aureus} bacteraemia}

\subsection*{2.5.1. Infective endocarditis}

Currently, \textit{S. aureus} is the most common causative microbe of IE in high-income countries, with a proportion of almost 30% [207,256]. The epidemiology of IE has changed radically in high-income countries. The major risk factor for IE, was earlier rheumatic heart disease, but it has been replaced by risk factors such as degenerative valve disease, diabetes, cancer, intravenous drug use, and congenital heart disease [131]. Rheumatic disease as a consequence of streptococcal pharyngitis is currently uncommon disease these days due to good availability of antibiotics and improved living standards [257]. Despite of these improvements, the incidence of IE has not decreased and is still 3-10/ per 100 000 people [256,258]. It was emphasised that the increased amount of staphylococcal IE could be a result of medical progress [259]. In hospitalised patients, the presence of long-term central venous catheter (CVC) and invasive procedures are known risk factors for SAB and in practice for IE [191,260]. In addition, extensive use of implemented material, indwelling catheters, pacemakers and prosthetic valves were all reported to increase risk of SAB and IE [261,262].

\textit{S. aureus} IE is usually acute and causes high fever in up to 90% of patients, often with no physical finding. When medical help is sought (on average in three days) [191,263], heart murmur can be heard in 90% of patients [264]. On admission, physical signs of peripheral
embolism include petechia or Janeway lesions; splenomegaly is occasionally seen [131]. IE may progress and local complications, such as myocardium and valve ring abscesses, and purulent pericarditis, might develop. In addition, extracardiac metastatic infections, such as vertebral osteomyelitis and peripheral abscesses are common [3].

Systemic septic thromboembolic complications occur in 25-50% of patients [234,265], mostly in left-sided IE, affecting the spleen, kidney, liver and CNS. Large vegetation (>10 mm) and IE in the mitral position are associated with elevated risk for embolization [234,265]. Less common right-sided IE (in 5-10% of patients) usually affects the tricuspid valve, which may cause respiratory symptoms as a result of pulmonary embolism together with signs of sepsis [266].

*S. aureus* prosthetic valve IE carries a poor prognosis (1-year mortality is >50%). One third of all cases are health-care-associated [267], and overall risk was found independent of valve position and material used [268,269]. *S. aureus* is a frequent cause of prosthetic valve IE occurring within 1-year after surgery. However, after this period the causative microorganisms were the same as those causing native valve IE [3].

Prevalence of IE in SAB has been 10-13% [169,270,271]. Echocardiography is indicated in most cases. Transthoracic echocardiography (TTE) is readily available and non-invasive. However, the sensitivity of TTE in diagnosing valvular IE is low (30-60%) [272,273]. However, a normal TTE was shown to rule out IE in health-care associated SAB, in high-risk cardiac conditions and in IDUs. Normal TTE might be acceptable to rule out IE in these SAB patients [274]. Transesophageal echocardiography (TEE) is more accurate and sensitive than TTE in visualising cardiac structures. In native valve IE, the sensitivity of TEE (95%) was higher compared to TTE (55%) [275,275,276].

### 2.5.2. Pneumonia

*S. aureus* is an uncommon (prevalence 1-11%) cause of community-associated pneumonia [277-279]. However, *S. aureus* causes one third of health-care associated pneumonia cases [235,236]. In health-care associated pneumonia, *S. aureus* first colonises mucous membranes followed by aspiration, which causes pneumonia. In general, pneumonia occurs in SAB in 6-34% of patients, and it may result from tricuspid valve endocarditis or septic pulmonary emboli. In addition, abscesses and empyema may also complicate pneumonia [155,157,280]. Community-associated pneumonia due to PVL toxin-producing *S. aureus* has emerged globally and causes necrotising, and often (in 56-63% of patients) fatal disease [135,281]. Chest X-ray to diagnose pneumonia has long been a fundamental diagnostic tool based on international recommendations [30]. Recently, lung
ultrasonography has been proposed to be even the first-line examination instead of chest X-ray [282]. Chest CT-scan has been the gold standard in diagnosing pneumonia due to its close to 100% sensitivity and specificity in diagnosing pneumonia [31]. However, its indications are limited due to ionization radiation exposure and availability. In diagnosing rare SAB related condition, septic pulmonary embolism, a CT-scan has been shown to be confirmative of diagnosis [283]

2.5.3. Deep-seated abscess

In earlier studies, the frequency of deep-seated abscesses (between 1-24%) [175,191,194,199,201,270,284] and its definition have varied. The variability is likely due to different definitions used in these studies. Infection was defined as superficial or deep [244]. Some studies included only psoas abscesses [191], while others only epidural abscesses [175,199] or abdominal abscesses [284].

2.5.4. Septic arthritis and osteomyelitis

S. aureus is the most common causative bacteria in septic arthritis, causing half of all cases [285,286]. Known risk factors are rheumatoid arthritis or osteoarthritis, joint prosthesis, low socioeconomic status, IDU, alcoholism, diabetes, previous intra-articular corticosteroid injection and cutaneous ulcer [287]. The frequency of septic arthritis in SAB varies from 1% to 24% [4,285,288]. Septic arthritis can be of non-haematogenous origin (caused by local injury or trauma) or iatrogenic (related to surgical-procedure), the latter origin being more frequent than the former [240,289]. Septic arthritis typically affects a single joint, most often the knee, the second most often the hip and the shoulder. The other joints are seldom involved, although any joint can be affected [290].

S. aureus is the primary pathogen in over half osteomyelitis cases [291]. The disease originates from haematogenous seeding, contiguous infection or is related to vascular insufficiency [292]. Vertebral column osteomyelitis commonly affects the intervertebral disc and surrounding soft tissues [293]. Bacteraemia may originate from another primary source, which then creates a secondary focus, spondylodiscitis, which serves as a continuous source of bacteraemia. Staphylococcal spondylodiscitis was found in 7% of 134 cases of bacteraemia by the ward physician and in 13% of 100 cases investigated by an infectious diseases specialist [294]. A Danish report concluded that 82% of spondylitis cases were due to SAB [295].

Radiographs are the initial imaging studies performed when bone and joint infections are suspected; the sensitivity and specificity are 50-75% and 75-83%, respectively. CT and
ultrasonography are not considered to be the primary imaging modalities for osteomyelitis. With magnetic resonance imaging (MRI), the earliest findings of osteomyelitis can be detected after two days in osteomyelitis. MRI also has a very good negative predictive value in ruling out osteomyelitis [283]. In addition, the use of modern techniques utilising radionucleotides has increased in diagnostics of patients suspected of difficult to diagnose musculoskeletal infections. Bone 67-Gallium imaging has diminishing importance due to more appropriate procedures, such as labelled leukocyte imaging and, in case of spinal infections, 18F-fluorodeoxyglucose (18F-FDG) imaging. Single-positron emission computed tomography/computed tomography (SPECT/CT) and positron emission tomography/computed tomography (PET/CT) have shown improved accuracy in the diagnostics of patients with suspected musculoskeletal infections [32].

2.5.5. Meningitis

*S. aureus* is unusual causative microbe of meningitis. Worldwide, it constitutes 0.3-8.8% of all cases of bacterial meningitis [296-298]. Mortality varies between 14-77%. Meningitis associated with SAB has a poorer prognosis than health care-associated *S. aureus* meningitis without SAB. Health care-associated *S. aureus* meniningitis are associated with neurosurgical procedures, cerebrospinal fluid shunt device placement or trauma [299-301].

2.6. Treatment of *Staphylococcus aureus* bacteraemia

The study subjects of this thesis do not address treatment of SAB. In brief, the standard treatment of SAB in countries with low MRSA prevalence, including Finland, is semisynthetic penicillin (cloxacillin) [4]. First-or second generation cephalosporins and clindamycin can be used, if the patient has a non-anaphylactic allergy to penicillin antibiotics [302]. Bacteriostatic clindamycin has good bone penetration; however, clindamycin is not recommended for treatment of endocarditis [157,303]. Vancomycin is used for treatment of MRSA SAB and for patients intolerant for beta lactam antibiotics [304,305]. The duration of parenteral therapy in uncomplicated SAB, typically central catheter related, is recommended to be 14 days [306,307]. Longer therapy durations (up to 4-6 six weeks) are recommended in patients with a deep infection focus, especially left-side endocarditis [3,308,309]. In a recent meta-analysis, rifampicin adjunctive therapy was shown to associate with trends towards reduced all-cause mortality and reduced clinical or bacteriological failure [310]. Rifampicin combination therapy may be associated with improved outcome in patients with a deep infection focus [311].
2.7. Biomarkers of infection

2.7.1. C-reactive protein (CRP)

In 1930, Tillet and Francis found C-reactive protein in the sera of pneumonia patients [312]. Later, it was shown that CRP production was not limited to streptococcal pneumonia but could also be detected in a variety of acute bacterial infections, including staphylococcal osteomyelitis, rheumatic fever, subacute bacterial endocarditis and lung abscesses [313-315].

2.7.1.1. Biological function of CRP

The discovery of CRP led to the identification of many other molecules whose production is enhanced in inflammatory processes; these proteins are collectively called acute-phase proteins. The expression, structure and function of CRP, the major acute phase protein, has since been solved, as reviewed [316]. CRP is known to have calcium-dependent specific binding to phosphocholine (PCh), an important component of most cell membranes, including bacterial and fungal polysaccharides. PCh is thought to be the most important ligand of CRP and responsible for major biological functions. The reason for this is believed to be its wide distribution in many pathogens and in necrotic and damaged eukaryotic cells [316]. In addition, ligand-complexed CRP can also activate the classical pathway of human complement [317] and recruit phagocytic cells [318,319]. These functions, namely the ability to activate complement and to facilitate phagocytosis, place CRP as an integral element of the innate immunity [316].

2.7.1.2. Synthesis of CRP

The liver is the primarily source of CRP production during the acute-phase response to cytokines released by macrophages and adipocytes. A large (even up 1000-fold) and rapid increase in CRP concentration within 24-48 hours, followed by an equally rapid decrease are characteristics for CRP. The circulating CRP concentration is determined by the synthesis rate, and CRP production directly reflects the strength of the inflammatory process stimulating it [320]. The main inducer of the CRP gene is interleukin 6 (IL-6), while interleukin 1 (IL-1), complement-activated products and glucocorticoids augment the effect of IL-6 [316,317,319].
2.7.1.3. CRP gene

The human CRP gene is located in the first chromosome between 1q21 and 1q23 and consists of 2 exons and 1 intron. CRP expression is regulated mainly at the transcriptional level by IL-6, while IL-1, complement and glucocorticoids enhance its effect [321,322]. Factors associated with genes account for up to 40% of the basal CRP level variations; more than 100 single nucleotide polymorphisms (SNPs) residing in the noncoding regions around the CRP gene have been characterised [42,43]. In addition, SNPs in the genes of CRP promoters e.g. IL1 and IL-6 have been shown to impact basal CRP variation [323]. In addition, CRP gene SNPs tentatively associate e.g. with the risk of diabetes [324], ischaemic stroke [325,326], reduced arterial compliance [327], cognitive decline after cardiac surgery [328], ischaemic heart disease [329,330] and susceptibility to infections [327,331].

Seven SNPs across the CRP gene in survey have been identified as functional [332]. SNPs within the CRP gene were genotyped in large American general population cohort. This study revealed that two SNPs rs3093058 and rs3091244 were associated with increased baseline levels of serum CRP. Two other SNPs rs1205 and rs2808630 were associated with decreased levels of baseline serum CRP [333]. Six bin tag SNPs association with CRP levels was studied in another large study. Study indicated highly significant association of SNPs rs3093059 and rs3091244 with baseline CRP levels [334]. Triallelic SNP rs3091244 is within promoter region of the gene and it is functionally most important, both rare T and A alleles associate with high CRP levels [332-334]. In addition, tentative associations of this functionally important SNP with many diseases have been studied extensively. In all studies shown in Table 2 elevated baseline CRP levels were shown to associate with minor A-allele carriage.
Table 2. Association observed between the CRP gene SNP rs3091244 A-minor allele with diseases. Association = +, lack of association = -

<table>
<thead>
<tr>
<th>Disease</th>
<th>+/-</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolic syndrome</td>
<td>+</td>
<td>[335,336]</td>
</tr>
<tr>
<td>Reduced carotid artery elasticity</td>
<td>+</td>
<td>[337]</td>
</tr>
<tr>
<td>Fatigue in breast cancer patients</td>
<td>-</td>
<td>[338]</td>
</tr>
<tr>
<td>Increased prevalence of coronary artery disease</td>
<td>-</td>
<td>[339]</td>
</tr>
<tr>
<td>Elevated activity of ankylosing spondylitis (AS)</td>
<td>+</td>
<td>[340]</td>
</tr>
<tr>
<td>Risk for developing AS</td>
<td>+</td>
<td>[341]</td>
</tr>
<tr>
<td>Low dispositional optimism (only in obesity)</td>
<td>+</td>
<td>[342]</td>
</tr>
<tr>
<td>Ischaemic stroke</td>
<td>+</td>
<td>[343]</td>
</tr>
<tr>
<td>Abdominal aortic aneurysm (AAA) and greater AAA diameter</td>
<td>+</td>
<td>[344]</td>
</tr>
<tr>
<td>Tumorigenesis especially in rectal cancer</td>
<td>+</td>
<td>[345]</td>
</tr>
</tbody>
</table>

2.7.1.4. High-sensitivity CRP

High-sensitivity CRP (hs-CRP) assays measures CRP in the low range, from 0.5- mg/L to 10 mg/L [346]. Compared to standard CRP, hs-CRP can detect more precisely baseline CRP levels. Baseline CRP elevations may indicate low but persistent inflammation [347]. CRP elevations over 2 mg/L are associated with “metabolic inflammatory” states, including cardiac ischaemia, smoking, and other low level non-infectious inflammatory conditions such as uraemia [347,348]. Elevated baseline CRP levels were also associated with an increased risk for lung cancer, and possibly breast, ovarian and colorectal cancers [349].

2.7.1.5. CRP and infectious diseases

Elevated CRP levels generally indicate infection, inflammation or trauma. It is not a specific diagnostic marker for diagnosing infection in these conditions and between-patient variation in CRP levels can be remarkable in seemingly similar conditions [36]. Rather, CRP should be used to monitor disease progression in individual patients. Pneumonia and UTIs are among the most frequent diseases in the general population, and CRP may be useful in
assessing severity or a possible bacterial cause. In severe life-threatening conditions, such as meningitis, CRP is used to differentiate bacterial infection from viral [37,39]. Although a viral infection such as influenza can elevate CRP to a relatively high level, an extremely high CRP elevation (>500 mg/L) was shown to associate with an over 80% likelihood of bacterial infection [350]. The usefulness of CRP in following SAB has not been studied but there is data on its use in other severe bacterial infections.

2.7.1.6. CRP in pneumonia

In community-associated-pneumonia, CRP was shown to be an independent predictor of disease severity [351,352]. However, the use of CRP use as a prognostic marker in pneumonia has also been questioned [353,354]. While the role of CRP as a prognostic marker in pneumonia seems to be unclear, it can be useful in distinguishing between bacterial and viral respiratory tract infections. In lower respiratory tract infections, it has been suggested that CRP could be used to assess the need for antibiotics: CRP level <20 mg/L no antibiotics usually needed, 21-50 mg/L no antibiotics in most cases, 51-99 mg/L antibiotics in most cases and >100 mg/L prescribing antibiotics is usually needed [355,356]. Thus, in accordance, with a narrative-review of primary care point-of-care testing, CRP was shown to distinguish relatively well whether patients with respiratory tract infection needed antibiotics or not [357]. However, in the very acute initial phase of the disease (e.g. streptococcal sepsis) CRP level should be interpreted with caution. In patients with a very acute disease onset, CRP levels may be at low-level at presentation due to slow kinetics of CRP [320].

2.7.1.7. CRP in infective endocarditis

In patients, who had undergone a cardiac operation, CRP level elevations were reported in all patients postoperatively and peak values were reached at 72 hours after the operation. In addition, a progressive decline in CRP levels was seen in those without postoperative infection. The difference in CRP levels between patients with infection (including patients with prosthetic valve IE) and those without were significant and of prognostic value in evaluating the response to therapy and in predicting outcome [358]. Serial CRP measurements as a predictor of IE outcome were evaluated in a study. In this study 19.5% of all patients had S. aureus IE. The adjusted OR for poor outcome at one week was 10.3 (95% confidence interval, OR 2.2-49.4) for patients with CRP levels in the highest tertile (>122 mg/L) vs. the lowest tertile (1-69 mg/L). In addition, a slow percentage decline in CRP level in the first week indicated poor clinical outcome [359]. In an IE study 32% of patients had S. aureus IE. In this study, CRP levels measured on admission were evaluated in predicting poor clinical outcome. The primary endpoint, poor clinical outcome, was defined
as death or serious complications occurring during the hospital stay. Infectious complications comprised of septic arthritis, osteomyelitis, meningitis, visceral abscess, mycotic aneurysm, peripheral emboli, septic pulmonary infarction and intracranial infarction or haemorrhage. The study showed that on admission, CRP levels were significantly higher in patients with poor clinical outcome than patients who survived and did not develop serious complications during hospitalisation. [360]. Later, one more study including also S. aureus IE patients showed, that increasing preoperative CRP reliably predicted poor outcome in surgically treated IE patients (OR 18.15, 95% CI: 1.5-223.60) [361].

In diagnosing IE, elevated CRP values have been shown to correlate with the presence of IE with mixed etiologies, [362-364], and a normal CRP was useful in excluding IE [365]. In a study, which compared patients with bacteraemia of mixed aetiologies, including S. aureus, it was found out that both procalcitonin (PCT) and CRP were not significant in differentiating patients with IE from those with bacteraemia only [366]. In diagnosing IE, the value of CRP was later evaluated in a meta-analysis, (based on 6 studies). This analysis did not support the use of CRP to rule out IE [367]. Finally, in a recent study evaluating the diagnosis of lead-dependent IE, the second most common causative microbe was S. aureus, and CRP levels were found to have a significant relationship with possible lead-dependent IE [368].

2.7.1.8. CRP in urinary tract infection

CRP may assist a clinician in differential diagnosis between cystitis and pyelonephritis. Clear cut-off values, however, are not so well established. Sandberg et al. studied women with symptomatic UTI and found CRP >30 mg/L to confirm the diagnosis of pyelonephritis in 94% of non-pregnant patients, in 91% of pregnant patients. In patients with cystitis, 5% had CRP above this limit [369]. In a large study on patients on rapid diagnostics of UTI, good concordance of CRP with clinical pyelonephritis diagnosis was observed [370]. Consistent with these studies, Agrawal et al. observed that in adult patients (>16 years) the mean CRP was significantly higher in patients with UTI infection as compared to controls [371]. In a Korean study on febrile UTI with 325 patients, bacteraemia was detected in 106 patients. Bacteraemic patients had a higher mean CRP than patients with negative blood cultures. However, in multivariate analysis, CRP was not associated with bacteraemia [372]. In detecting fatal outcome, CRP was not prognostic for 30-day mortality in febrile UTI patients [373], but was prognostic for mortality in pyelonephritis patients with emergency nephrectomy due to infection [374]. The diagnostic value of leukocytosis, CRP, pyuria, urine cultures and duration of time before arrival to hospital in detecting kidney abscess were evaluated in 213 patients who underwent CT scan. 50 patients were found to have an
abscess, nevertheless, not any risk factor or diagnostic parameter, including CRP, could predict the presence of an abscess [375].

2.7.1.9. CRP in meningitis

In bacterial meningitis, the serum CRP level is typically elevated while in viral meningitis it is usually low [376-379]. CRP has been shown to have a good diagnostic accuracy in distinguishing bacterial meningitis from viral meningitis if used as an adjunct to lumbar puncture [380]. A cut-off value of >50 mg/L was proposed for adults combined with cerebrospinal fluid pleocytosis. For children under 6 years, a cut-off value of >20 mg/L was proposed to distinguish bacterial from viral meningitis. However, the accuracy was low in patients with symptoms less than 12 h [377]. CRP levels were observed to be higher in bacterial meningitis than aseptic meningitis in a small study of 32 patients [379].

2.7.2. White blood cell count

The white blood cell (WBC) count is frequently measured biomarker in many infections. In recent review, Honda and Takayuki described use of WBC count and neutrophil left shift as markers of bacterial infection. Neutrophil left shift means that there are more than usually immature forms of neutrophils in the blood. In bacterial infection leukocytes are consumed in large amounts and WBC count reflects both consumption and production of neutrophils in the blood marrow. Unchanged WBC count means that consumption and production are in balance. During bacterial infection changes in WBC count are dynamic and reflect the seriousness of bacterial infection [381].

Up to fifth of all patients had no leukocytosis in bacteraemic pneumococcal pneumonia at presentation [382]. WBC counts showed no differences between groups of patients who had either bacterial, viral or mixed viral-bacterial pneumonia [383]. In diagnosing upper UTI, blood WBC count is usually not needed for diagnosis. However, in predicting UTI in urolithiasis patients, blood WBC count was significantly different between urine culture negative and positive patients [384]. Whereas WBC count was not significantly different between patients with urosepsis, severe urosepsis and uroseptic shock following percutaneous nephrolithotomy [385]. WBC count was significantly prognostic and diagnostic biomarker for infection and combination of WBC count and PCT had better diagnostic and prognostic value for infection than PCT alone in ICU patients [386]. WBC counts measured on admission were significantly higher in patients with poor clinical outcome than patients who survived and did not develop serious complications during hospitalisation in an IE study [360]. WBC count was reported as prognostic also in initially
healthy individuals. Leucocytosis (>9.0 leukocytes/ml) was a significant predictor in Normative Aging Study population for increased future mortality in men [387].

To summarize, these studies support the view that WBC count in pneumonia is a diagnostic supplement at most [382,383]. Blood WBC count can be useful in urolithiasis [384,385], but blood WBC count is normally not needed in diagnosis of upper UTI. Furthermore, WBC count is useful or at least additive to other biomarkers in predicting infection or prognosis in ICU patients [386]. Leukocytosis could also be future prognostic marker in healthy men [387].

2.8. Other potential biomarkers in *Staphylococcus aureus* bacteraemia

2.8.1. Soluble urokinase plasminogen activator (suPAR)

suPAR has been reported as a promising prognostic marker. Poor outcome and disease progression in cancer, HIV, tuberculosis, malaria, SIRS, diabetes, renal disease, ischemic stroke and coronary artery disease correlated with elevated suPAR levels [51]. The urokinase plg activator receptor, a well-described versatile protein [49], is found on many cell surfaces and soluble suPAR can be detected in blood, serum, plasma, urine and cerebrospinal fluid. However, biological function of suPAR is not clear [50,388]. Elevated suPAR levels in *S. pneumoniae* bacteraemia on admission were prognostic for in-hospital mortality [389]. Elevated suPAR levels during the first 1-4 days in *S. pneumonia*, β-haemolytic streptococci and *Escherichia coli* bacteraemia predicted increased 30-day in-hospital mortality [390]. In addition, low levels of suPAR may be a positive predictor for overall survival in patients with sepsis of mixed aetiology [391].

Two meta-analyses have evaluated the potential use of suPAR in critically ill intensive care patients, as a prognostic marker for in-hospital mortality and as a diagnostic marker for infection [53,54]. In the study of Backes et al., suPAR had a low diagnostic value in differential diagnosis of infection in patients with SIRS [53]. On the other hand, the prognostic value of suPAR was significant and complementary to other biomarkers and clinical classifications in the study of Donadello et al. [54]. In conclusion, these studies emphasised that elevated suPAR predicts mortality in sepsis, but is not diagnostic for infection in SIRS patients.

2.8.2. Soluble apoptosis biomarkers

Apoptosis, or active gene-directed cell death, is both a physiological and pathologic phenomenon [392]. When it is triggered by death-receptor pathway markers, measurable
concentrations of soluble Fas protein (human membrane protein, member of necrosis factor proteins) and Fas protein ligand (both studied as apoptosis biomarkers) are produced. Increased levels of soluble Fas protein were detected in patients with sepsis and multiple organ dysfunction syndrome [393]. Soluble Fas protein may be a therapeutic target in preventing post traumatic hyperinflammation [394]. In patients with bacteraemia, including 24% patients with SAB, apoptosis biomarkers were found not to be prognostic, but they correlated with high SOFA score in patients with bacteraemia [395].

2.8.3. Pentraxin 3

Pentraxin 3 (PTX3) is an acute phase protein. The of PTX3 as a diagnostic marker for infection and as a prognostic marker for mortality in critically ill patients was recently reviewed by Liu et al. [396]. Based on the evaluation of seven studies on critically ill patients with SIRS, PTX3 levels in patients with bacteraemia were found significantly higher than those in blood-culture negative patients. However, PTX3 levels were not significantly different in patients with gram-negative bacteraemia compared to those with gram-positive bacteraemia. As a prognostic marker for mortality, PTX3 levels measured on days 1-4 were significantly higher in patients with fatal outcome (both in 28-30 days and in 90 days) compared to survivors. PTX3 is complementary, not superior, to the other commonly used biological markers CRP and PCT [396]. In one study including SAB patients, PTX3 was found to be an independent prognostic marker in sepsis and showed better prognostic value than CRP [397]. Another recent study evaluated PTX3 values upon admission and it revealed good prognostic value of fatal outcome in patients with confirmed bacteraemia. However, the proportion of SAB in patients in this study was not mentioned [398].

2.8.4. Procalcitonin

PCT, a glycoprotein and a precursor of calcitonin, [399] is a useful marker in distinguishing serious infection from non-infectious diseases [400,401]. Originally, very high concentrations of PCT were measured in paediatric patients who had severe bacterial infections. Low levels were seen in patients who had viral infections, local bacterial colonization or infections without sepsis. In this same study, PCT levels were higher in severely burned children with infectious complications than those without an infection [400]. Later studies on adult SIRS patients also demonstrated that PCT levels have a significant predictive value for sepsis [402] and bacteraemia [403]. PCT appears to be valuable in discriminating blood contamination and clinical infection when coagulase-negative staphylococci were cultured from blood [404]. In addition, there seems to be some predilection of PCT for gram negative bacteraemia, since higher PCT levels were
detected in bacteraemia due to gram-negative bacteria than gram-positive bacteraemia [405].

In guiding antibiotic treatment, PCT may be effective. However, as reviewed recently, cost-effectiveness, variable kinetics, and concerns related to the diagnostics of different infection foci and pathogens were found to limit the utility of PCT [406]. In a general emergency department (ED) patient population, evidence-based support for PCT in guiding antibiotic therapy was inadequate [407]. However, another study found that PCT had prognostic value of moderate accuracy in ED patients in identifying septic patients with fatal outcome [408]. A recent ICU study found that a combination of CRP, PCT and sepsis related SOFA-score together was better than those used separately in diagnosing sepsis as early as possible [409]. Finally, a meta-analysis of 21 studies on pneumonia and use of PCT concluded that elevated PCT was a risk factor for death in both health care-associated and community-associated pneumonia [410].

2.8.5. Cell-free DNA

The presence of cell-free DNA (cf-DNA) in human blood was first detected in 1984 [411]. Circulating cf-DNA levels are related to necrosis, apoptosis and cell lysis [412,413].

cf-DNA levels are elevated in septic patients [414]. In patients with septic shock, cf-DNA levels are significantly compared to patients with severe sepsis [415]. The diagnostic accuracy of cf-DNA is equal with PCT but better than CRP in identifying infection in febrile patients. Normal cf-DNA levels excluded infection with 97% specificity in febrile patients, and high concentrations of cf-DNA are prognostic of fatal outcome [416]. In one study on bacteraemic patients, which also included SAB patients, cf-DNA was demonstrated to be an independent prognostic marker for increased mortality [417]. In another study in sepsis patients, cf-DNA demonstrated independent prognostic value of mortality in patients with severe sepsis [418].

2.9. Specific biomarkers in *Staphylococcus aureus* infections

2.9.1. Teichoic acid and antistaphylolysin

The teichoic acid antibody (TAA) assay and antistaphylolysin (ASTA) are among the oldest serological tests in detecting *S. aureus* infection. Both have been frequently used in clinical practice to distinguish complicated from uncomplicated SAB [419,420]. TAA titre elevations 1:2-1:4 were suggestive of *S. aureus infection*; a titer of 1:8 was particularly suggestive of *S. aureus* endocarditis [420,421]. TAA response to infection can be expected in the first 1-
2 weeks [420], which makes it more useful in diagnosing the cause of a chronic rather than an acute infection [422]. Deep infection raised TAA levels in 80%-91% of patients with a verified deep infection. However, TAA response was also detected in 68% of patients with uncomplicated disease and even in 44% of healthy persons [423-425].

ASTA was the first available diagnostic test for routine use, but is considered unreliable in diagnosing deep infection due to its low sensitivity [426]. Positive ASTA titers ≥2.0 IU/ml were detected in 32-62% of patients with a deep infection, including IE [427]. In dermatological patients, ASTA has been used because as it correlates better with the skin barrier function rather than with the actual stage of infection [428]. Although, TAA and ASTA have been widely studied and widely used in diagnosing S. aureus infection, no serological test is positive in all SAB patients or able to differentiate complicated from uncomplicated bacteraemia [429]. Due to poor serological response, TAA and ASTA were found not helpful in predicting presence of deep infections in SAB [29].
3. AIMS OF THE STUDY

The main aims were to elucidate the plasminogen activation by *Staphylococcus aureus* and to study prognostic markers in *Staphylococcus aureus* bacteraemia.

The specific aims were to identify

I  the effect of *Staphylococcus aureus* and its surface proteins on plasminogen activation by staphylokinase and to characterise potential *Staphylococcus aureus* plasminogen receptors *in vitro*

II the predictive value of C-reactive protein in identifying fatal outcome and deep infections in *Staphylococcus aureus* bacteraemia

III the effect of CRP-gene polymorphisms on CRP levels in *Staphylococcus aureus* bacteraemia

IV the prognostic value of suPAR in *Staphylococcus aureus* bacteraemia
4. MATERIALS AND METHODS

4.1. Study population and bacteria

Study I comprised of five *S. aureus* isolates from bacteraemic patients, five from patients with a superficial infection and one laboratory strain (*Newman*). The clinical isolates were obtained from the Laboratory of Bacteriology, Division of Clinical Microbiology, HUCH Laboratory Diagnostics, Helsinki University Central Hospital, Helsinki, Finland.

Studies II-IV were based on a previous prospective randomised multicenter study of 430 patients where standard treatment with or without a fluoroquinolone (trovafloxacin or levofloxacin) were compared. Patients with a positive blood culture for *S. aureus*, were collected consecutively in five university and seven central hospitals from 1999 to 2002. The exclusion criteria were: age under 18 years, imprisonment, pregnancy, breastfeeding, epilepsy, bacteraemia in the previous 28 days, polymicrobial bacteraemia (≥3 microbes), allergy to any quinolone antibiotic, previous tendinitis during a fluoroquinolone therapy, prior fluoroquinolone use for more than 5 days before randomization, positive culture for *S. aureus* only from a central intravenous catheter, meningitis, neutropaenia (<0.5×10⁹ cells/L) or failure to provide informed consent. Patients with MRSA bacteraemia and strains resistant to any fluoroquinolone were excluded.

In Study III the patient population was part of the whole 430 patient population from the Study II and included patients from one center, Helsinki University Hospital, as the permission for genetic testing was asked only from these patients. Blood samples for genetic testing samples were obtained from 151 of 158 patients from the study site. Blood samples from three patients could not be obtained for further analysis and three patients were rejected due insufficient DNA yield. In total, 145 patients were included in this study from Helsinki University Central Hospital.

Study IV included a total of 59 patients from Helsinki University Central Hospital (from the whole 430- patient population). The patients were selected in consecutive numerical order such that they belonged to one of three groups: 1) death in 28 days, 2) deep infection, 3) no deep infection. The patients were required to have serum samples from the first 10 days after positive blood culture for *S. aureus* (with the exception of patients with fatal outcome). The objective was to include at least 15 patients in each group.
4.2. Study designs

Study I was a basic research study designed to clarify the possible role of *S. aureus* cells to SAK plg activation and characterize plasminogen binding structures of *S. aureus*.

Studies II-IV were based on data collected in a prospective study where blood samples for CRP and WBC count were measured on the day of positive blood culture and every other day thereafter throughout the first week and subsequently twice a week during the hospital stay and at 28 days. Clinical characteristics, underlying diseases and predisposing factors were stratified per 30-day mortality and presence of deep infection.

Six common SNPs of the CRP gene were genotyped in blood samples to assess the possible impact of genetic polymorphisms on complicated disease course and CRP response (III). First week CRP levels and distribution and characteristics of underlying diseases were stratified per genotype distribution.

Study IV used the prospectively collected serum samples for suPAR analyses on day three, four and ten, and one month after the first positive *S. aureus* blood culture. The mean interval from the blood culture to the first suPAR sampling was three days (standard deviation 1.1; range 2–5 days). Characteristics and underlying diseases were stratified according to fatal outcome at one month and presence of deep infection. Median suPAR levels on days 3, 4, 10 and 30 were compared between these groups to determine whether elevated suPAR levels could predict fatal outcome or presence of deep infection in SAB.

4.3. Laboratory methods

4.3.1. Methods in Study I

4.3.1.1. Bacterial cultivation and SAK phenotypic status

Bacteria were grown in Todd- Hewitt broth overnight and pelleted by centrifugation. The bacterial pellets were washed twice with phosphate-buffered saline (PBS) and suspended in the same buffer containing bovine serum albumin (BSA). SAK producer status of *S. aureus* was determined by measuring the SAK activity in the spent overnight culture medium. In brief, plg, spent culture medium from overnight cultures and chromogenic substrate S-2251 (Chromogenix) were incubated. The cleavage of the S-2251 was measured spectrophotometrically (Ultrospec II, LKB/Wallac).
4.3.1.2. Iodogen method and plg binding

For plg binding experiments, plg was labelled with carrier-free Na$^{125}$I by the Iodogen method. In brief, the Iodogen oxidant (Sigma) solution was coated on the walls of the glass reaction tube. Radioiodination was then initiated by the addition of plg and Na$^{125}$I and terminated by removal of the reaction mixture. Plg binding was determined by incubating bacteria with radioactive labelled plg. The bacterial cells were pelleted and washed twice and radioactivity associated with bacteria was quantified with an γ-counter 1270 Rackgamma II (LKB/Wallac).

4.3.1.2. Lysostaphin treatment and isolation of plg binding proteins

Isolation of *S. aureus* surface proteins started with lysostaphin treatment. In brief, in lysostaphin treatment bacterial suspension was incubated with recombinant lysostaphin (Applied Microbiology, Inc., New York, N.Y.), ribonuclease and deoxyribonuclease (Sigma) in the presence phenylmethylsulfonyl fluoride (Sigma) and ethylmaleimide (Sigma). Intact bacterial cells were removed by centrifugation, and the supernatants were incubated at 80°C to stop the enzyme activity. Proteins interacting with plg were isolated by passing lysostaphin digest through a plg-Sepharose column. After that, the column was washed with PBS. The bound proteins were eluted by epsilon-aminocaproic acid (Fluka Chemie AG). Possible trace amounts of plasmin were inactivated with aprotinin (Sigma).

4.3.1.3. Measuring activation of bacteria-bound and soluble plg by SAK

Activation of plg was detected by incubating plg, SAK, α2AP, and chromogenic substrate S-2251 in the presence of different concentrations of intact bacteria. In addition, solubilized *S. aureus* cell wall proteins, were used instead of intact bacteria. The cleavage of S-2251 was measured spectrophotometrically. The quantity of plg bound to bacteria was determined by incubating radioactive labelled plg solution with *S. aureus* cells. Subsequently, cells were washed and the radioactivity associated with bacteria was counted. Plasmin activation was performed by incubating equal amounts of bacteria-bound or soluble plg, as judged by the radioactivity, with SAK in the presence of S-2251. The cleavage of s-2251 was measured spectrophotometrically.

4.3.1.4. Bacterial surface proteins ligand blotting

Lysostaphin digest or eluted proteins from Sepharose (Pharmacia)-plg affinity column were run on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [430] in order to separate the plg-binding proteins. Proteins were then transferred electrophoretically to nitrocellulose membrane (Amersham, Hybond-C extra) and stained
to visualize the transferred proteins. The membrane was then destained and saturated with defatted milk powder in buffer solution and treated with plg. After several washes, the membrane was treated with rabbit anti-human plg dilution. The membrane was washed again and incubated in solution of affinity-purified antibodies to rabbit (immunoglobulin G) IgG (Jackson Immunoresearch Laboratories, Westgrove, PA, USA). Nitrocellulose membrane bound plg was revealed by 1,3,4,6-tetrachloro-3α,6α-diphenylglycouracil treatment of the membrane.

The cell wall proteins isolated by the affinity chromatography were separated by electrophoresis using SDS-PAGE gels. The protein bands were excised from the gel, washed, reduced, and digested with sequencing-grade trypsin. The resulting peptides were solubilized and filtered. The peptides were separated in a reversed phase high performance liquid chromatography system. After elution and detection of the peptides, individual peptides were sequenced by an automatic amino acid sequencer, Procise 494A (Perkin Elmer, Applied Biosystems Division, Foster City, USA). Peptide separation and sequencing were done in Protein Chemistry Unit and Institute of Biomedicine, Biomedicum, Helsinki, Finland and Laboratory of Protein Chemistry, Institute of Biotechnology, University of Helsinki, Helsinki, Finland, respectively.

4.3.2. Methods in Studies II-IV

4.3.2.1 CRP concentrations and white blood cell counts (Studies II-IV)

Serum CRP concentrations and WBC counts were measured in study sites by standard laboratory methods. The serum or plasma (plasma instead of serum was used after 18.3.2002) was analysed by automatic immunoturbidimetric method using analysers 917 or Modular PP-analyser (Hitachi Ltd, Tokyo, Japan) and Tina-quant CRP reagents (Roche Diagnostics, Tina-quant CRP). For both methods, the normal value of CRP concentration was <10mg/L. In Study III leukocytosis was determined if WBC count was over 12×10⁹ cells/L.

4.3.2.2 DNA extraction, sequencing and genotyping (Study III)

Deoxyribonucleic acid (DNA) extraction, sequencing and genotyping were done in cooperation with the National Institute of Health and Welfare, the Unit of Public Health Genomics, Helsinki, Finland. DNA was extracted by the phenol chloroform method. DNA was quantified by measuring absorbance at 260 nm and by Pico Green fluorescence assay (PicoGreen dsDNA Quantitation Kit, Molecular Probes, Carlsbad, USA). SNPs were detected by analysing primer extension products from amplified genomic DNA. For detection of
SNPs, Sequenom MassArray system with the homogeneous Mass Extension reaction (Sequenom, San Diego, USA) was used. MassArray design software was used to design PCR and extension reactions. For these PCR and extension reactions, 10 ng of template DNA was used. In all samples, six common SNPs of the CRP gene (rs27954521, rs3091244, rs1800947, rs1130864, rs1205 and rs3093075) were genotyped. The six SNPs selected were haplotype bin tagging SNPs. They covered the whole gene and large parts of the untranslated regions of the 5’ and 3’ ends of the gene.

4.3.2.3. suPAR analytics (Study IV)

suPAR levels were measured in the Clinical Research Centre, Copenhagen University Hospital, Hvidovre, Denmark. The suPARnostic™ kit (ViroGates, Copenhagen, Denmark) was used to measure the suPAR levels. The kit consists of catching monoclonal antibody pre-coated plates and a horseradish peroxidase labelled detection monoclonal antibody. Sample and detection antibody in dilution buffer in duplicates were added to the pre-coated plate and incubated for 1 h. Following washing substrate was added for 20 min and the reaction was stopped with sulphuric acid. The plates were measured at 450 nM with a reference distance of 630 nM.

4.4. Statistics

The statistical program used in Studies II-IV was SPSS® (SPSS Inc., Chicago, IL, USA); version 14.0 in Study III, 17.0 in Study IV, and 20.0 in Study II.

In Studies II-IV, normality of continuous variables was tested by Kolmogorov-Smirnov and Shapiro-Wilks tests. Differences between skewed variables were assessed by the Kruskal-Wallis H test and parametric tests were chosen when possible.

Chi-square test or Fisher’s exact test was used when appropriate to analyse the associations between categorical variables. For skewed variables, heterogeneity of groups was assessed with the Kruskal-Wallis H test. Odds ratios with 95% confidence intervals (CI) were determined to estimate the significance of differences between the groups. All tests were two-tailed and the level of significance was considered p<0.05. Interquartile range (IQR), was defined as the difference between the third and the first quartile. Receiver operating characteristics (ROC) curves analyses were performed to find cut-off values of CRP and WBC in predicting fatal outcome and presence of deep infection focus (II). Cox regression analysis, adjusted with predictors of fatal outcome, was calculated to determine hazard ratios for CRP and WBC count cut-off values predicting mortality. Odds ratios (OR)
for factors associated with the presence of deep infection were analysed by binary logistics regression analysis.

In Study II, ROC curve analysis for 30-day mortality and the presence of deep infection was calculated to determine cut-off values for CRP and WBC count. The area under the curve (AUC) was calculated for each ROC. The cut-off points for general optimal tests were chosen to optimise the rate of true positives whilst minimising the rate of false positives. Cox regression analysis adjusted with characteristics, predisposing factors and severity of illness, was performed to determine hazard ratios of CRP and WBC cut-off values for 30-day mortality. Adjusted ORs for factors associated with the presence of deep infection focus were analysed by binary logistic regression. Both multivariable analyses included significant covariates (p<0.2) by the forward selection method using the Akaike information criteria (AIC) [431].

In Study III, genotypic frequencies within SNP loci were compared to values expected from the Hardy-Weinberg proportion. Associations between explanatory variables and outcome were calculated by linear regression univariate analysis. Multivariate regression analysis in studies III and IV were performed such that clinically meaningful and significant (P<0.05) variables associated in univariate analysis were all included in the model and were included in order of strength of their association with the outcome. Contribution to variance and possible collinearity with variables in the model was assessed when a new variable was added. False discovery rate analysis (FDR) [432] was applied for the multiple comparisons in Study III.

### 4.4. Definitions

Infection foci were definite if verified by radiological, bacteriological or pathological investigations but regarded as suspected if based on clinical findings only. However, both definite and suspected infection foci were pooled together in analyses. Deep infection included: pneumonia, endocarditis, deep-seated abscess, septic arthritis, osteomyelitis, septic thrombophlebitis, mediastinitis, UTI, CVC infection, foreign body infection, verified bacteraemia and CNS infection. In addition, a deep-seated abscess was defined as parenchymal, lung, peritoneal, subphrenic, gynaecological and pericardial abscess or pleural empyema. IE was defined using the modified Duke criteria as definite or possible by echocardiographic, clinical and pathological data [433]. CVC infection was defined according to the guidelines of the Infectious Diseases Society of America [434]. Severity of underlying diseases and prognosis were described as healthy, nonfatal, ultimately fatal or rapidly fatal using the criteria of McCabe and Jackson [216]. Bacteraemia was defined as
CA when the positive blood culture was received within 48 hours of hospital intake and there was no preceding hospitalization in seven days. SAB was health-care associated when positive blood culture was received ≥48 hours after hospital admission or the patient had been in haemodialysis within the past two months or had lived in a long-term care facility. [433]. Sepsis was defined as severe sepsis when hypotension, hypoperfusion or organ failure were detected as described [435]. The time to defervescence was measured in days until the axillary temperature was ≤37.5°C. Altered mental status was defined as unconsciousness or severe confusion. IDUs were patients who had injected drugs intravenously 6-month prior randomization.

4.5. Ethical statement

Study I comprised experiments only with bacterial strains originating from patients with bacteraemia or with superficial infections; patient data was not included. The protocol for Studies II-IV was approved by the ethics committees of all study sites, including the Ethics Committee of Helsinki University Hospital and the Finnish National Agency for Medicines. Written informed consent was obtained from all patients or their representatives. However, severely ill patients were also included without signed consent, because they were presumed to benefit from the study medication. A signed informed consent was taken in all cases afterwards by the patient or by a representative.
5. RESULTS

5.1. *Staphylococcus aureus* and activation of plasminogen by staphylokinase (Study I)

5.1.1. Bacteria and plasminogen activation

It was first shown that plasmin activity was effectively inhibited by α₂AP in a reaction solution containing only plg, SAK and α₂AP. When bacteria cells were added to the reaction solution, this led to a dose-dependent increase in plasmin activity, which was revealed by the cleavage of chromogenic substrate s-2251 (Study I; Fig.1.A). This enhancing effect of intact *S. aureus* bacterial cells was demonstrated with all strains, including both SAK producers and non-producers. There is a correlation between the capacity of bacteria to bind plg and the enhancing effect on plg activation (Study I; inset of Fig.1.A). It was also shown that only plg bound to bacterial cells, not free plg, was activated by SAK and exogenous SAK was necessary for the activation to occur (Study I; Fig.1.B).

5.1.2. Cell wall proteins and plasminogen activation

At a concentration of 50-200 μg/ml, cell-wall solubilised plg-binding components enhanced plg activation and eliminated α₂AP-dependent inhibition of active plasmin (Study I; hatched bars in Fig. 2.). The enhancing effect and ability to resist α₂AP dependent inhibition of the cell-wall containing solution were shown to stop after passage through a plg affinity column (Study I; dark grey bars in Fig. 2). Proteins eluted from the column were able to protect and enhance again plg activation to plasmin in the presence of α₂AP. When the ability of eluted proteins and whole lysostaphin were compared, 25 μg/ml plg-binding proteins were as effective as 100-200 μg/ml of whole lysostaphin digest in generating plasmin activation.

5.1.3. *Staphylococcus aureus* plasminogen binding proteins

Plg-binding proteins eluted from a Sepharose-plg affinity chromatography column were analysed by SDS-PAGE. Major 59-, 56-, and 39-kDa, and minor 45- and 43-kDa bands were detected. Plg binding to bands was further studied and visualised bands are shown (Study I; Fig. 3.). The affinity of plg for the 59-, 56- and 43-kDa proteins was strong, while the affinity for the 39- and 45-kDa proteins was barely detectable. Peptide sequences were then compared to protein databases. Homologies between the 59-, 56-, and 43-kDa proteins and known *S. aureus* proteins were revealed. The 59-kDa protein corresponded to 5’-monophosphate dehydrogenase (IMPDH), the 56-kDa protein with α-enolase and 43-
kDa protein with ribonucleotide reductase β-chain. This enabled reliable identification of major plg-binding proteins. The 45-kDa protein band was too faint for identification and the 39-kDa protein did not match any known protein.

5.2. Predictive value of CRP in identifying fatal outcome and deep infections in *Staphylococcus aureus* bacteraemia (Study II)

5.2.1. 30-day mortality and prognostic factors

In Study II, 53 out of a total 430 *S. aureus* bacteraemia patients had a fatal outcome within 30 days. In univariate analysis, age >60 years (n=43/53, 81%, OR 5.0, p<0.0001), immunosuppressive treatment (n=16/53, 30%, OR 3.5, p<0.0001), chronic alcoholism (n=11/53, 21%, OR 2.4, p<0.032), chronic renal failure (n=14/53, 26%, OR 2.6, p=0.010), malignancy (n=13/53, 25%, OR 2.1, p=0.038), ultimately or rapidly fatal underlying disease (n=31/53, 59%, OR 4.6, p<0.0001), severe sepsis during the first three days (n=12/53, 23%, OR 4.1, p=0.001), any deep infection focus (n=51/53, 96%, OR 6.5, p=0.002), pneumonia (n=34/53, 64%, OR 3.9, p<0.0001) and endocarditis (n=17/53, 32%, OR 2.6, p=0.006) were all significantly associated with 30-day mortality. Health care-associated infection, presence of a foreign body, diabetes, liver disease, deep-seated abscess, osteomyelitis, foreign body infection and septic arthritis were not significantly associated. Mortality among IDUs was lower (n=1/53, 1.9%, OR 0.15, p=0.029) than non-users.

In multivariate analysis, prognostic factors for 30-day mortality in SAB were: age >60 years (HR 4.2, p <0.0001), chronic alcoholism (HR 2.7, p=0.017), chronic renal failure (HR 2.3, p=0.037), ultimately or rapidly fatal underlying disease (HR 2.1, p=0.033), pneumonia (HR2.0, p<0.033) and endocarditis (HR 2.5, p=0.004).

5.2.2. Deep infection and associated factors

In 352 (82%) patients, a deep infection focus was found during the 30-day surveillance period. However, in three days starting after the positive blood culture result, a majority of all deep foci were evident and found in 325 (75%) patients. Deep infection foci in order of prevalence were: deep-seated abscess in 185 (43%), osteomyelitis in 141 (33%), pneumonia in 152 (35%), infection of foreign body in 79 (18%), IE in 74 (17%), and septic arthritis in 56 (13%) patients. In univariate analysis, a significantly associated risk factor for the presence of deep infection focus was IDU (OR 11, p=0.002); male gender, chronic renal failure and malignancy were not associated. Health care-associated origin of SAB (OR 0.48,
p=0.006) and presence of foreign body (OR 0.50, p=0.010) lowered the risk for deep infection focus.

5.2.3. CRP levels and outcome

The mean CRP level was reached its maximum at the time the positive blood culture was collected (Study II; Fig 1.A). Starting from the day four after positive blood culture, the mean CRP level in patients with fatal outcome differed from those in survivors. The difference remained significant during the one-month observation period. CRP levels stratified according to one-month mortality are shown (Study II; Fig 1.B). At the earliest, CRP levels on day four predicted mortality; the median in deceased patients was 127 mg/L (IQR, 97 mg/L) and 91 mg/L (IQR 96 mg/L, p=0.005) in survivors. CRP concentrations and WBC counts at the time of positive blood culture did not predict 30-day mortality as analysed by ROC curve analyses. Day four CRP >103 mg/L was a significant predictor of fatal outcome, but WBC count >8.0 x10⁹/L was not (Table 3). On day seven, CRP >66mg/L and WBC count >9.8 x10⁹/L and on day 14 CRP >61 mg/L and WBC count >8.6 x10⁹/L were significant in predicting mortality (Table 3). ROC curve analyses predicting 30-day mortality using CRP levels and WBC counts on day four and seven (Figure 1) and on day 14 (Figure 2) are shown.

Table 3. Cut-off values for predicting 30-day mortality for CRP and WBC count on days four, seven and 14 in Staphylococcus aureus bacteraemia according to ROC analysis. AUC= area under the curve in ROC analysis. The p-value for odds ratio (OR) is shown.

<table>
<thead>
<tr>
<th></th>
<th>CRP</th>
<th>WBC count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Four days</td>
<td>Seven days</td>
</tr>
<tr>
<td>AUC (95%)</td>
<td>0.65 (0.55-0.78)</td>
<td>0.68 (0.58-0.81)</td>
</tr>
<tr>
<td>cut-off</td>
<td>103 mg/L</td>
<td>66 mg/L</td>
</tr>
<tr>
<td>sensitivity</td>
<td>77%</td>
<td>73%</td>
</tr>
<tr>
<td>specificity</td>
<td>55%</td>
<td>55%</td>
</tr>
<tr>
<td>OR (95%, CI)</td>
<td>2.6 (1.4-4.8)</td>
<td>2.6 (1.3-5.1)</td>
</tr>
<tr>
<td>p-value</td>
<td>0.004</td>
<td>0.005</td>
</tr>
</tbody>
</table>
Figure 1. ROC curve analysis of CRP levels (continuous line) and WBC count (dotted line) for day four (left panel) and for day seven predicting 30-day mortality in 430 Staphylococcus aureus bacteraemia patients (right panel).
Figure 2. ROC curve analysis of CRP levels (continuous line) and WBC count (dotted line) for day 14 predicting 30-day mortality in 430 *Staphylococcus aureus* bacteraemia patients.

The prognostic value of WBC count improved when both low (<4.5 x10⁹/L) and high (above reference limit) WBC counts were combined as abnormal WBC count. In univariate analyses, odds ratios (OR, 95% CI) were as follows: fall of CRP <50% in 14 days (OR 5.7, 95% 2.5-13.1; p=0.0001) and fall of CRP <50% between days 7-14 (OR 8.5, 95% CI 2.5-29.2; p <0.0001), day four WBC count <4.5 or >10.3 x10⁹/L (OR 2.0, 95% CI 1.1-3.7; p=0.024) and day seven WBC count <4.5 or >9.8 x10⁹/L (OR 5.8, 95% CI 2.6-12.8; p <0.0001).

Table 4 shows significant prognostic factors for 30-day mortality due to SAB as analysed by multivariate analysis (comprising characteristics, predisposing factors and severity of illness in patients Study II; Table 1). Table 5 shows significant prognostic factors for 30-day mortality as analysed by multivariate analysis (comprising factors listed in the Study II; Table 1) and cut-off values for CRP levels and WBC counts (listed in Study II; Table 2). The predictive power of multivariate analysis with cut-off values for CRP levels, WBC counts and decline of CRP <50% appeared superior (Table 5) compared to the analysis without (Table 4).
Table 4. Significant prognostic characteristics, predisposing factors and severity of illness, calculated by multivariate analysis, for 30-day mortality in 430 SAB patients. Cut-off values for CRP levels and WBC counts were excluded.

<table>
<thead>
<tr>
<th>Significant prognostic factor</th>
<th>Hazard ratio and 95% CI</th>
<th>Multivariate analysis p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &gt;60 years</td>
<td>4.2 (2.0-8.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Chronic alcoholism</td>
<td>2.7 (1.2-6.1)</td>
<td>0.017</td>
</tr>
<tr>
<td>Chronic renal failure</td>
<td>2.3 (1.1-5.1)</td>
<td>0.037</td>
</tr>
<tr>
<td>Ultimately or rapidly fatal</td>
<td>2.1 (1.1-4.0)</td>
<td>0.033</td>
</tr>
<tr>
<td>disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pneumonia</td>
<td>2.0 (1.1-3.7)</td>
<td>0.033</td>
</tr>
<tr>
<td>Endocarditis</td>
<td>2.6 (1.4-4.9)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Model overall predictive power: -2 log likelihood 547.718, chi-square 100.102, P <0.0001.

Table 5. Significant prognostic characteristics, predisposing factors and severity of illness, calculated by multivariate analysis, for 30-day mortality in 430 SAB patients. Cut-off values for CRP levels and WBC counts were included.

<table>
<thead>
<tr>
<th>Significant prognostic factor</th>
<th>Hazard ratio and 95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP &gt;103 mg/L on day four</td>
<td>3.5 (1.2-10.3)</td>
<td>0.024</td>
</tr>
<tr>
<td>CRP &gt;61 mg/L on day 14</td>
<td>3.6 (1.1-10.3)</td>
<td>0.039</td>
</tr>
<tr>
<td>WBC &gt;8.6 x10⁹/L on day 14</td>
<td>8.2 (2.9-23.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Age &gt;60 years</td>
<td>3.6 (1.1-11.5)</td>
<td>0.029</td>
</tr>
<tr>
<td>Ultimately or rapidly fatal</td>
<td>6.5 (2.4-17.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pneumonia</td>
<td>8.1 (2.5-25.8)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Model overall predictive power: -2 log likelihood 169.320, chi-square 92.437, p<0.0001.
5.2.4. Predictors of deep infection foci

The mean CRP level in patients with and without a deep infection focus are shown Figure 3. CRP levels were significantly higher already on the day of positive blood culture in patients with deep infection compared to patients without and also during the 30-day surveillance period.

**Figure 3.** Mean CRP concentrations in 351 *Staphylococcus aureus* bacteraemia patients with a deep infection focus (divot bars) and in 79 *Staphylococcus aureus* bacteraemia patients with no deep infection focus (large grid bars).

ROC curve analysis for CRP levels and WBC count gave cut-off values for predicting the presence of a deep infection focus. CRP cut-off values on the day one, seven and 14 were significant in predicting a deep infection focus whereas WBC count was significant only on day 7 (Figure 4) and (Table 6).
Figure 4. ROC curve analysis of CRP levels and WBC count for predicting presence of any deep infection focus in 430 *Staphylococcus aureus* bacteraemia patients during 30-day surveillance period.
Table 6. Cut-off values for predicting deep infection for CRP and WBC count on days one, four, seven and 14 in Staphylococcus aureus bacteraemia according to ROC curve analysis. AUC=area under the curve in ROC curve analysis. The p-value for odds ratio (OR) is shown.

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC (95% CI)</td>
<td>0.74 (0.67-0.81)</td>
<td>-</td>
<td>0.75 (0.68-0.81)</td>
<td>0.70 (0.64-0.77)</td>
</tr>
<tr>
<td>cut-off</td>
<td>108 mg/L</td>
<td>-</td>
<td>44 mg/L</td>
<td>22 mg/L</td>
</tr>
<tr>
<td>sensitivity</td>
<td>77%</td>
<td>-</td>
<td>68%</td>
<td>59%</td>
</tr>
<tr>
<td>specificity</td>
<td>60%</td>
<td>-</td>
<td>67%</td>
<td>68%</td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td>4.9 (2.9-8.2)</td>
<td>3.4 (2.0-5.6)</td>
<td>5.7 (3.2-10)</td>
<td>4.4 (2.4-8.1)</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>WBC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC</td>
<td>0.55 (0.46-0.63)</td>
<td>-</td>
<td>0.65 (0.58-0.72)</td>
<td>0.56 (0.49-0.63)</td>
</tr>
<tr>
<td>P</td>
<td>0.236</td>
<td>-</td>
<td>p &lt;0.0001</td>
<td>0.153</td>
</tr>
<tr>
<td>cut-off</td>
<td>not determined</td>
<td>8.0 x10^9/L</td>
<td>8.5 x10^9/L</td>
<td>7.25 x10^9/L</td>
</tr>
<tr>
<td>sensitivity</td>
<td>-</td>
<td>-</td>
<td>59%</td>
<td>44%</td>
</tr>
<tr>
<td>specificity</td>
<td>-</td>
<td>-</td>
<td>62%</td>
<td>75%</td>
</tr>
<tr>
<td>OR (95%, CI)</td>
<td>2.2 (1.4-3.7)</td>
<td>2.4 (1.4-4.0)</td>
<td>2.1 (1.2-3.8)</td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>0.002</td>
<td>0.001</td>
<td>0.010</td>
<td></td>
</tr>
</tbody>
</table>

In multivariate analysis of deep infection focus, prognostic factors included: IDU, male gender, chronic renal failure, malignancy, health care-associated SAB and foreign body. Prognostic factors and cut-off values for CRP levels, WBC counts and falls of CRP <50% predicting presence of deep infection focus (listed in original article) were included in multivariate analysis (Study II; Table 3). Significant prognostic factors from multivariate analysis are listed in Table 7.
Table 7. Prognostic factors for deep infection focus in 430 SAB patients. The p-value for multivariate analysis is shown. IDU= intravenous drug use.

<table>
<thead>
<tr>
<th>Predictor of deep infection focus</th>
<th>Odds ratio and 95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDU</td>
<td>11.9 (1.5-93.2)</td>
<td>0.018</td>
</tr>
<tr>
<td>Day one CRP &gt;108 mg/L</td>
<td>2.6 (1.3-4.9)</td>
<td>0.005</td>
</tr>
<tr>
<td>Day 14 CRP &gt;22 mg/L</td>
<td>3.9 (1.6-9.5)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

5.3. Genetic polymorphism of the CRP gene and a deep infection focus determine maximal serum CRP level in Staphylococcus aureus bacteraemia (study III)

5.3.1. Characteristics and underlying diseases

145 SAB patients with permission for genetic analyses from Helsinki University Hospital were included in Study III. The majority of patients (66%) were male and mean age was 52±17 years. In 60% of patients, infection was hospital-associated, 47% had a prosthetic or intravascular device, 25% had been in previous surgery, 21% had CVC, 19% had a history of intravenous drug abuse, 6% had used corticosteroids ≥1 month, 6% were alcohol abusers, 10% needed immunosuppressive therapies, 22% had coronary artery disease, 26% were diabetics, 26% had chronic lung disease, 18% had chronic renal failure, 3% had hepatic cirrhosis, 6% had autoimmune disease, 16% had malignancy, 4% were HIV positive and in 68% McCabe´s classification was healthy or nonfatal. All diagnosed deep infection foci during the 3-month surveillance period are shown in Table 8.

Table 8. All deep infection foci diagnosed in 3-month surveillance period

<table>
<thead>
<tr>
<th>Infection foci</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deep infection</td>
<td>122 (84)</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>62 (43)</td>
</tr>
<tr>
<td>Any deep abscess</td>
<td>71 (49)</td>
</tr>
<tr>
<td>Osteomyelitis</td>
<td>60 (41)</td>
</tr>
<tr>
<td>Any foreign body</td>
<td>29 (20)</td>
</tr>
<tr>
<td>Endocarditis</td>
<td>28 (19)</td>
</tr>
<tr>
<td>Septic arthritis</td>
<td>19 (13)</td>
</tr>
<tr>
<td>Mediastinitis</td>
<td>10 (7)</td>
</tr>
</tbody>
</table>
5.3.2. CRP gene single nucleotide polymorphisms

CRP gene SNPs genotypic distribution followed the Hardy-Weinberg equilibrium. Two of the selected bin tagging SNPs rs3091244 and rs2794521 originated from the promoter region, rs1205 and rs3093075 from 3´ flanking region, rs1800947 from exon 2 and rs1130864 and from 3´ UTR region. All six SNPs were found to be in strong linkage disequilibrium (LD) by Haplovie 3.2 Program. In full LD was A-minor allele of rs3091244 with rs3093075 and T-minor allele of rs3091244 with rs1130864. Within one haplotype block were all other SNPs, except rs1205.

5.3.3. CRP levels and single nucleotide polymorphisms

Maximal CRP levels during the first week of SAB were found to be significantly higher in patients who carried the minor allele of rs3091244 or the T-minor allele rs3093075 compared to patients who were not carriers of these minor alleles (Table 9). Strong LD between these two SNPs explained similar results. CRP levels at the time of the blood culture or at day seven were not affected by any SNPs analysed in additive and recessive models. CRP levels on the day of the positive blood culture, on day seven and maximal CRP levels during the first week were stratified by additive and recessive genotypes of rs3091244 (Study III; Table 2).

<table>
<thead>
<tr>
<th>Maximal CRP mg/L during the first week (IQR)</th>
<th>Genotype distribution of rs3091244 (n)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>182 (167)</td>
<td>TT (21)</td>
<td>Additive model p=0.023</td>
</tr>
<tr>
<td>193 (157)</td>
<td>TC (55)</td>
<td></td>
</tr>
<tr>
<td>299 (202)</td>
<td>TA (4)</td>
<td></td>
</tr>
<tr>
<td>163 (102)</td>
<td>CC (28)</td>
<td></td>
</tr>
<tr>
<td>280 (188)</td>
<td>CA (8)</td>
<td></td>
</tr>
<tr>
<td>179 (148)</td>
<td>TT+TC+CC (104)</td>
<td>Recessive model p=0.004</td>
</tr>
<tr>
<td>282 (169)</td>
<td>TA+CA (12)</td>
<td></td>
</tr>
</tbody>
</table>

Table 9. Maximal CRP levels during the first week in SAB patients stratified according to A-minor allele distribution of SNP rs3091244. The p-value for Kruskal Wallis H test is shown. n=number of patients. IQR=Interquartile range.
In addition, when demographic characteristics, underlying diseases and predisposing factors were stratified by the A minor allele of rs3091244, no differences in distribution of these were detected between A-minor allele carriers and non-carriers (Study III; Table 3).

5.3.4. Maximal CRP levels

Demographic and clinical factors that could impact maximal CRP levels (age, gender, body mass index, surgical intervention, deep infection focus, intravenous drug use and carrier status of rs3091244 A-minor allele) were analysed in univariate and multivariate analysis. Significant factors affecting maximal CRP level in univariate analysis were the presence of any deep infection focus and SNP rs3091244 A-minor allele carriage. Associations of any other SNPs analysed in recessive and additive models were not detected with maximal CRP levels. The adjusted model indicated that the presence of deep infection and A-minor allele explained 21.7% of the maximal CRP variation in SAB during the first week (Study III; Table 4).

All CRP gene SNPs were analysed in additive and recessive models. No associations with time to defervescence, leukocytosis, amount of deep infection foci, CVC-associated infection or mortality were detected.

5.4. Elevated soluble urokinase plasminogen activator receptor (suPAR) in predicting mortality in Staphylococcus aureus bacteraemia (study IV)

5.4.1. suPAR levels in Staphylococcus aureus bacteraemia

A total of 59 S. aureus bacteraemia patients were divided into three groups based on 30-day survival and on verified presence of a deep infection focus. All patients who died had a deep infection focus. The patients with fatal outcome were older and had ultimately or rapidly fatal disease more often than survivors (Study IV; Table 1).

When patients were stratified according to 30-day outcome, suPAR levels in fatalities were higher at days three, four and 10 after the first positive blood culture compared to suPAR levels in survivors. No difference was found when suPAR levels in patients with no deep infection focus were compared to levels in patients with a deep infection focus (Table 10).
Table 10. Median suPAR levels in fatalities as compared to survivors and in survivors with or without deep infection. Data is shown as median suPAR levels ng/mL (range), n= number of patients. The p-value for the Mann-Whitney U-test is shown.

<table>
<thead>
<tr>
<th></th>
<th>Fatalities</th>
<th>Survivors</th>
<th>p-value</th>
<th>No deep infection</th>
<th>Deep infection</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 3</td>
<td>12.3 (5.7-64.6) n= 19</td>
<td>8.4 (3.7-17.6) n=40</td>
<td>0.002</td>
<td>9.2 (3.7-17.6) n=15</td>
<td>7.9 (5.0-16.3) n=25</td>
<td>0.319</td>
</tr>
<tr>
<td></td>
<td>Day 4</td>
<td>11.5 (4.7-20.6) n=18</td>
<td>7.1 (2.5-15.2) n=40</td>
<td>0.001</td>
<td>7.3 (3.4-14.9) n=15</td>
<td>6.6 (2.5-15.2) n=25</td>
</tr>
<tr>
<td></td>
<td>Day 10</td>
<td>11.8 (6.1-17.8) n=16</td>
<td>6.5 (2.3-17.3) n=40</td>
<td>&lt;0.0001</td>
<td>7.5 (2.3-14.9) n=14</td>
<td>6.1 (3.0-17.3) n=20</td>
</tr>
</tbody>
</table>

5.4.2. suPAR as a prognostic marker in *Staphylococcus aureus* bacteraemia

In ROC curve analysis, the prognostic value of suPAR was evaluated against CRP as a prognostic factor for fatal outcome. On day three, the area under the curve (AUC) for suPAR was 0.754 (95% CI, 0.615-0.894; p=0.003) and the AUC for CRP was 0.596 (95% CI, 0.442-0.75; p=0.253), (Figure 6). ROC curve analysis gave a cut-off value of 9.25 ng/mL for suPAR on day three with a sensitivity of 0.79, a specificity of 0.68 and a positive likelihood ratio of 2.5. When patient characteristics and underlying diseases were analysed by suPAR cut-off 9.25 ng/mL, suPAR was >9.25 mg/mL in 64% of patients >65 years, in 64% having ultimately or rapidly fatal disease, in 46% having renal failure and in 36% with a CVC.
In univariate analysis, the factors predicting mortality in 30-days and odds ratios were calculated: age $>$65 years, OR 5.2 (95% CI, 1.6-17.4; $p=0.011$), suPAR >9.25 ng/mL, OR 7.8 (95% CI, 2.2-28.2; $p=0.002$), ultimately or rapidly fatal underlying disease, OR 6.5 (95% CI, 1.9-22.2; $p=0.002$), presence of cerebral symptoms on the first three days, OR 4.1 (95% CI, 1.1-15.3; $p=0.042$). In multivariate analysis, suPAR >9.25 ng/mL was analysed only with one factor (presence of cerebral symptoms), while other significant factors were omitted from the model. This was due to the significant collinearity of suPAR with age $>$65 years and presence of ultimately or rapidly underlying disease. In binary logistic regression analysis, adjusted with the presence of cerebral symptoms during the first three days in the beginning of the SAB, suPAR >9.25 ng/mL was associated with increased risk (OR 8.0; 95% CI, 2.1-30.5; $p=0.002$) for fatal outcome in 30 days in SAB.
6. DISCUSSION

6.1. Enhanced activation of bound plasminogen on *Staphylococcus aureus* by staphylokinase

In our study, the SAK-plasmin complex activating plg to plasmin was demonstrated to enhance and protect against the inhibitory effect of α₂AP when complex was bound either to *S. aureus* cells or to plg-binding cell wall components. Purified cell wall components were shown to bind plg and were identified as *S. aureus* IMPDH, α-enolase and rRNR; these proteins might be *S. aureus* plg receptors.

Our finding is consistent with earlier experiments showing that *S. aureus* bound to plasmin is effectively protected against the inhibitory effect of α₂AP [436]. Enhanced activation of the SAK-plasminogen complex has been shown to take place when the complex is bound to fibrinogen [437]. We now observed that enhanced activation of plg occurs on the bacterial cell surface in direct correlation with bacterial plg binding capacity. We could demonstrate that binding of plg to *S. aureus* rendered plg more sensitive to SAK-mediated activation and this reaction occurred only when bacterial cells were present. Binding-induced conformational changes in the plg molecule have been suggested as the mechanism responsible for enhanced activation [436]. Consistent with this theory, many studies have shown a strong fibrin-specific activity of SAK [438-440].

SAK exhibits weak affinity to plg, but strong affinity for plasmin, which in theory is formed spontaneously in small amounts from plg. On the other hand, the SAK-plasmin complex, rather than the SAK-plg complex, activates plg to plasmin [116,441,442]. In our experiment, however, the plasmin activity of plg was eliminated with aprotinin preincubation. This was necessary to reduce the possibility of SAK-plasmin complexes, which are responsible for the enhanced activation as opposed to bacterial cells or purified cell wall proteins interacting with plg.

Solubilised cell wall proteins, like whole bacterial cells, were shown to abrogate the α₂AP-mediated inhibition of plasmin and enhanced activation of plg by SAK. After treatment with immobilised plg, however, both the ability to resist α₂AP and to enhance activation could be stopped. This demonstrated that the plg-binding ability of *S. aureus* is at least partly mediated by cell-surface proteins. The 59-, 56-, and 43-kDa proteins with strong plg-binding capacity were identified as staphylococcal IMDPH, α-enolase and RNR, respectively.
IMPDH is a nicotinamide adenine dinucleotide dependent enzyme that controls *de novo* synthesis of guanine nucleotides. IMPDH is an important target enzyme for a number of immunosuppressive agents and has provided potential therapeutic targets for the treatment of viral and parasitic diseases [443-445]. In addition, IMPDH has been suggested to be a potential biomarker in organ transplantation [446]. RNR is a known mediator of deoxyribonucleotide biosynthesis, which is required for DNA synthesis in all living cells. RNR could be a target for anti-proliferative therapeutic compounds in malignancies and also in parasitic, viral and bacterial diseases [447]. Both IMPDH and RNR are intracellular enzymes and have not been described earlier to have extracellular function. However, the excretion of cytoplasmic proteins in general is a known phenomenon in bacteria and eukaryotes and it is not likely due to cell lysis [107].

The extracellular location of intracellular glycolytic enzyme α-enolase has been described earlier in *S. pneumonia*. Consistent with our study, α-enolase is also expressed as a plg-binding molecule on the surface of many bacteria, including *S. pneumoniae* [448], *Mycoplasma fermentans* [103] and *Bacillus anthracis* [104]. In *Vibrio parahaemolyticus*, enolase has been shown to bind plasminogen [449]. Surface-displayed α-enolase also bound fibronectin in *Lactobacillus* [450]. In addition, enolase like protein was observed to bind plasminogen on the outer membrane of *Pseudomonas aeruginosa* [451]. In *Lactobacillus plantarum*, enolase was characterised as a collagen-binding molecule [452].

Another intracellular glycolytic enzyme, glyceraldehyde-3-phosphate (GAPDH), has been shown to function not only as a transferrin receptor on *S. aureus* surface but also as a plg-binding molecule [453]. In addition, in streptococci groups A, B and C, in gram-negative bacteria and in spirochetes, GADPH is an important plg receptor [79]. Accordingly, the glycolytic enzymes GADPH and α-enolase might have dual functions as glycolytic intracellular enzymes and also as bacterial surface plg receptors, representing a phenomenon known as moonlighting [454].

Limitations of our study include the possibility that some bacterial cells during overnight cultivation or lysostaphin digest may break. This may lead to release of intracellular enzymes together with cell surface proteins as complex. These complexes could be falsely interpreted as cell surface proteins. If so, the proteins from the Sepharose-plg would also contain intracellular enzymes associated with the cell wall proteins. However, all eluted proteins analysed by both SDS-PAGE and ligand blotting were shown to interact with plg. In addition, protein patterns of lysostaphin digest were not detected to differ depending on the presence or absence of 20% sucrose during digest. Sucrose was used during lysostaphin digest to prevent bacterial cells break-down.
According to our hypothesis, Staphylococcal plg-binding structures along with SAK production could form a system that enables bacteria to produce cell-surface α2AP-protected protease activity. This could theoretically facilitate bacterial invasion and act as a virulence factor in invasive SAB. In contrast, other studies on *S. aureus* and SAK have found out that plg activation by SAK might reduce the severity of systemic infection [120]. *S. aureus* SAK production seemed to be associated with uncomplicated local infections in humans rather than with invasive systemic infections. SAK production was also shown to enhance only local subcutaneous spreading in skin infections [70] and to lead to less biofilm formation [27]. Most *S. aureus* strains causing infections to humans can produce SAK [22], whereas SAK producing *S. aureus* strains from veterinary infections are rare [70]. For unknown reasons SAK production appears to be an adaptation mechanism for *S. aureus*. Peetermans et al. described how SAK production enhances local spreading rather than systemic invasion. In their experiments, they demonstrated that SAK-mediated plasmin activity outside of abscess rapidly diminishes. This could be due to low bacterial and fibrin concentrations outside of an abscess [70]. However, SAK production as virulence factor of *S. aureus*, even after these studies, remains partly resolved.

**6.2. Predictive value of CRP in identifying fatal outcome and deep infections in *Staphylococcus aureus* bacteraemia**

CRP level and WBC count are common biomarkers followed in patients with SAB, however, cut-off values for predicting mortality or disease complications from deep infection focus are unknown. A clear definition of these values could assist in allocating resources, both diagnostic and therapeutic, to the SAB patients who need them most.

In our study, we observed that by the fourth day after the first positive blood culture, CRP and (by day 14) WBC count were higher in patients with fatal outcome than survivors. Furthermore, already from the day of the positive blood culture, CRP level and (from day four) WBC count were higher in patients with a deep infection focus than patients without. Abnormal WBC count (also including low WBC count) and CRP level both predicted mortality; CRP starting from day four and abnormal WBC count from day seven. After adjustment with prognostic and predisposing clinical factors, however, only CRP on day four and 14 remained prognostic. Abnormal WBC count was prognostic only on day 14. Day four CRP >103 mg/L as a predictor of 30-day fatal outcome was consistent with other studies, as CRP on day one in critically ill patients with suspicion of sepsis did not predict in-hospital mortality [40,455]. Consistent with our study, a study on critically ill South Korean patients found that high CRP levels on day one were not predictors of poor
outcome. Interestingly, in this same study a decline in CRP (>36%) compared between days 1-2 and 5-7 was predictive for reduced mortality [456]. In our study, we did analyse fall of CRP <50% as a predictor of mortality. In our univariate analysis, a fall of CRP <50% in seven and in 14 days were significant predictors of fatal outcome, but were not significant in multivariate analysis. This was probably because the fall of CRP <50% was not independent of CRP levels as a marker of mortality and only the most significant predictors remained significant. In contrast to our results, in one large Japanese study CRP >150 mg/L measured on admission was an independent predictor of 30-day in-hospital mortality [457]. In these studies, however, the patient populations had microbiologically heterogenous infections or the causative microbes were undefined; our study population included only S. aureus bacteraemia patients. In these studies, the patients were also more seriously ill than in our study and were in need of ICU treatment. These differences could partly explain the different results; the prognostic cut-off value of CRP might be microbe and disease specific.

Mortality in our study was only 12% as compared to 14-23% in other SAB studies [159,207,284,458]. Limitation of our study may be the use of clinical drug research study population [4]. We may have missed critically ill patients due to recruitment problems. These patients may have been rejected due to very rapid disease progression and fatal outcome. In addition, without antibiotic study inclusion criteria more patient would have been included. Possible reasons for rather low mortality could be that patients with MRSA were not included and patients were treated with effective antibiotics from the beginning. In our prospective patient material, all patients received infectious disease specialist consultation, which is one key factor in lower mortality and in finding deep infection foci [459]. Possible limitation of our study was also the use of multivariate analysis for predicting mortality. Mortality was low and relatively small number of fatalities may have limited the power of the multivariate analysis. CRP and WBC count are also only predictive biomarkers, not real clinical determinants of prognosis, such as age or severity of disease. In addition, CRP and WBC count are not independent of each other, which may have caused certain collinearity. However, forward selection in multivariate analysis using Akaike information criteria may have mitigated the risk of collinearity. While mortality in SAB has not improved dramatically during last 10 years, treatment strategies have changed, suggesting that CRP cut-off levels from our study could still be valid today.

CRP on day one was not prognostic for fatal outcome and this is consistent with the finding that CRP gene SNP rs3091244 A-minor allele and a deep infection focus determine the maximal CRP level in SAB (Study III). Possible limitation of our study is that CRP gene SNPs were not analysed in all 430 SAB patients. In genetic analysis of 430 SAB patients we could have detected associations of CRP gene SNPs also with low CRP levels or other clinical
parameters. CRP gene analysis of all 430 patients could have helped to understand better individual variation in clinical course of SAB. In univariate analysis, lack of CRP decline in one or two weeks were predictive for fatal outcome. However, in final multivariate-adjusted analysis, they appeared to be non-significant. In multivariate analysis, number of patients was relatively small, which may have influenced the result. In an individual level, lack of decline of CRP might still bear some importance as a warning signal but it cannot be used as a sign of deep infection focus.

In predicting the presence of deep infection focus, CRP was diagnostic in adjusted analysis starting from the day of positive blood culture. Day seven WBC count was predictive of deep infection in univariate analysis, but was not significant in adjusted multivariate analysis. Serological tests, teichoic acid antibody (TAA) assay and ASTA are among the oldest and most frequently used tests in clinical practice to distinguish complicated from uncomplicated SAB [419,420], but low specificity limits their use [423,425]. TAA and ASTA were observed to be not prognostic in SAB [111]. ESR has good sensitivity but low specificity in diagnosing a deep infection focus [295,460]. Other markers for diagnosing a deep infection focus specifically in SAB are not well characterised. PCT was reported to be useful in diagnosing infection in general [401]. PTX3 is nonspecific marker of inflammation with low diagnostic value for sepsis [396]. As a marker of infection in febrile patients, cf-DNA was equally good as PCT but better than CRP in diagnosing an infection in patients with fever of unknown origin, localized infection and sepsis or septic shock [416]. Apoptosis markers were not able to distinguish patients who had fatal outcome, although they appeared predictive for high SOFA score in bacteraemia study [395].

6.3. Genetic polymorphism of the CRP gene and CRP level in Staphylococcus aureus bacteraemia

We analysed the associations between six CRP gene SNPs with CRP levels in 145 SAB patients during the acute phase of disease. CRP gene SNPs have been shown to independently influence variations in basal CRP levels [332] However, studies on CRP gene SNPs associations with CRP levels in SAB or in any other infectious disease are not available.

We observed that the A-minor allele rs3091244 in the CRP gene promoter region is associated with a higher maximal CRP level during the first week of SAB. However, this minor allele had no effect on CRP levels on the day of the positive blood culture or on day seven. This finding is in contradiction with the finding of the Study II. In Study II, mean CRP levels were at the highest level on the day of the positive blood culture and declined thereafter (in 430 SAB patients). Whereas in Study III, in 12 patients with the A-minor allele
of rs3091244 allele, maximal CRP response was recorded later, few days after day one. CRP levels in the beginning of the SAB may have more variable elements than maximal CRP during the first week. Severity of illness in patients may vary at the moment the blood culture is collected, and CRP in these patients may reach maximum levels few days after day one. However, no associations were detected in analyses of predisposing factors, underlying diseases and clinical manifestations with data comprising all CRP gene SNPs. In our study, CRP gene SNP partly determined maximal CRP levels in SAB. This genetic determination of higher maximal CRP suggests that extremely high CRP levels in individual patient should be interpreted with caution. Our finding partly explains why extremely high CRP is not necessarily prognostic for mortality or for the presence of deep infection.

A deep infection focus was significantly associated with higher CRP levels and in multivariate analysis both A-minor allele rs3091244 and verified a deep infection focus determined the maximal CRP level during the first week. Factors which might have had influence on maximal CRP: age, gender, body mass index, surgical intervention and intravenous drug abuse were included but found non-significant in adjusted analysis. However, limitation of this analysis may have been the relatively small number of patients, and that severity of illness was not included.

None of the studied CRP gene SNPs had an effect on mortality. Consistent with our findings, CRP gene SNPs were associated only with elevated basal CRP levels but had no association with increased mortality [461]. In contrast to our study, SNP rs2794521 was associated with increased mortality due to *S. pneumonia* bacteraemia but not due to bacteraemia caused by *E. coli* or α-haemolytic streptococci [47]. The only other study on CRP gene SNPs in infectious diseases observed a reduced risk (HR 0.5; 95% CI 0.3-0.9) of incident pneumonia but higher CRP level with rs3093058 in black people [462].

A small number of patients may have influenced the results. In the original clinical drug research study [4] 1226 SAB patients were identified and 430 included. In our study, 145 patients from HUCH were included and 12 were carriers of rs3091244 A-minor allele. Multivariate analysis of the relatively small number patients may have reduced the power of the analysis. However, use of FDR analysis may have improved the reliability of multiple comparisons. The use of clinical drug research patient material as such may have led to bias in the selection of patients. Patients may have been excluded due to recruitment problems among critically ill with rapid disease course and fatal outcome.

In our study, the A-minor allele of rs3091244 partly determined maximal CRP levels in SAB and. This same minor allele was modestly associated with elevated basal CRP levels at the populational level [463], and in acute coronary syndrome patients [464]. Association of this
SNP minor allele with CRP response in subjects with metabolic syndrome has been shown [465], but this association is unlikely with diseases such as acute maculodegenerative disease [466] or stroke [467]. This minor allele is also not associated with risk of abdominal aortic aneurysm (AAA) or high CRP levels [468], however, this allele was later found to be related to the increased incidence of AAA among general population and to correlate with higher CRP levels and increased AAA diameter [344]. In breast cancer patients, the same A-minor allele of rs3091244 is associated with higher basal CRP levels but not with increased risk for fatigue, which is a common symptom in breast cancer patients [338]. In ankylosing spondylitis, higher CRP levels are associated with rs3091244 A-allele in patients with low disease activity as compared to patients without this allele [340], and in another publication with elevated risk for ankylosing spondylitis [469].

6.4. suPAR as a prognostic factor in *Staphylococcus aureus* bacteraemia

suPAR levels were evaluated in SAB patients and they were significantly higher in patients with a fatal outcome than survivors in 30-day surveillance period. Day three, four and 10 suPAR levels were higher in fatalities than in survivors, whereas suPAR levels in patients with a deep infection focus did not differ from those without a deep infection focus. In contrast to suPAR, CRP on day three was not prognostic for fatal outcome. suPAR and CRP levels were not evaluated on the positive blood culture day. However, in the entire 430 SAB material (Study II), CRP levels on the positive blood culture day and on day three were evaluated, and found non-prognostic for mortality. Consistent with our study, high suPAR levels appeared to be prognostic for fatal outcome in other bacteraemia studies on *Streptococcus* (pneumonia and β-haemolytic) and *E. coli* [389,390]. In critically ill septic patients, low suPAR levels are a positive predictor of overall survival [391]. In our study, we observed that suPAR levels on day 10 could predict mortality, which is consistent with findings of suPAR being prognostic in serial measurements in septic ICU patients up to 10-14 days [470,471].

In our study, presence of a deep infection focus was not associated with elevated suPAR levels. Publications on suPAR and diagnosis of deep infection in SAB are not available. In *Streptococcus pneumonia* bacteraemia study, patients with deep infection were not included [389] and in a report on pneumonia and meningitis and use of suPAR, the impact of a deep infection focus on suPAR levels was not analysed [472]. Deep infection focus is very common in SAB (84% prevalence in our study), whereas mortality due to SAB is generally lower (about 20%) [159,207,284,458]. Our small pilot study suggests that suPAR might not be a suitable diagnostic marker for detecting a deep infection focus. As a
diagnostic marker for infection, however, suPAR is useful when measured simultaneously with PCT. This combination could improve the efficiency of sepsis diagnosis, and the combination of plasma suPAR and APACHE II score could lead to more accurate mortality prediction [473].

Elevated suPAR levels are recognised as a marker of poor prognosis in HIV, tuberculosis, S. pneumonia bacteraemia and malaria [389,474-476]. In our study, suPAR >9.25 ng/mL was predictive of mortality in SAB with a specificity of 0.68 and sensitivity of 0.79. In HIV, suPAR >6 ng/ml is associated with high two-year mortality [477]. In tuberculosis, elevated suPAR levels associated with mortality, but the highest median levels reported were rather low 3.17 ng/mL [475]. In malaria, the highest median levels were slightly higher (7.9 ng/mL). In S. pneumonia bacteraemia, even higher cut-off values (10-10.3 ng/mL) were prognostic [389,472]. In critically ill patients, in (ICU) sepsis and in ventilator associated pneumonia predictive cut-off value for 28-day mortality was >12.9 ng/mL [478]. In another ICU study, suPAR level >12 ng/mL independently predicted mortality and suPAR levels were shown to remain stable for 10 days within survivors and non-survivors [479].

Although suPAR seems to predict mortality quite reliably in many kinds of infectious diseases, individual levels should be interpreted with caution. Even in our relatively small patient group, overlapping between groups of survivors and non-survivors was seen. This could be partly explained by the use of serum instead of plasma, since suPAR levels in serum are higher than in plasma, although suPAR concentrations in serum and plasma correlate well [480]. In addition, a number of ELISA methods to measure suPAR levels are available. Use of different ELISA methods in studies may partly explain differences in suPAR levels. One commonly used ELISA assay has been suPARnostic kit. This kit is validated to measure suPAR levels between 0.6 and 22 ng/mL. Whereas, another ELISA method can detect suPAR levels down to 15 pg/ml of suPAR [481]. In previous reports, suPAR levels in women are higher than men [52]. However, in our small study we could not detect any differences according to gender. Levels of suPAR increase with age [482] and we observed this in our analysis. In patients >65 years high suPAR levels (>9.25 ng/mL) were clearly more common compared to patients <65 years (Study IV, Table 3). Limitation of the Study IV was the small number of patients. In multivariate analysis number of covariates was limited due to insufficient number of patients. In addition, due to strong linkage, we could not determine the relationship between suPAR with age and fatal underlying diseases in the same multivariate analysis.

suPAR could possibly be used in predicting risk for poor outcome in SAB. Certain high suPAR level could help to allocate more resources to SAB patient at the greatest risk of dying. Such cut-off value due in SAB could be 9.25 ng/ml. However, low suPAR levels should not
be interpreted as an impetus for a reduction in therapeutic intensity. CRP could help in clinical-decision making in SAB. CRP levels greater than 100 mg/L on days 1-4 should lead to meticulous search, drainage and surgical treatment of deep infections and assessment of antibiotic therapy. CRP greater than 20 mg/L after two weeks of treatment should help raise clinical suspicion of deep infection at the latest.
7. SUMMARY AND CONCLUSIONS

The effect of *S. aureus* on plg activation and prognostic factors in *S. aureus* bacteraemia can be summarised as follows:

I. An enhancing effect of both *S. aureus* bacterial cells and surface proteins on plg activation by SAK was discovered. In an analysis of *S. aureus* cell-surface proteins, three plg-binding proteins were revealed as inosine 5'-monophosphate dehydrogenase, alpha-enolase and ribonucleotide reductase. In conclusion, we demonstrated that *S. aureus* enhanced plg activation by SAK. Furthermore, we could identify three previously unidentified tentative plg receptors of *S. aureus*.

II. In 430 SAB patients, predictive values of CRP in identifying fatal outcome and deep infections were determined. On day four, CRP >103 mg/L and on day 14, CRP >61 mg/L and WBC count >8.6 x10⁹/L appeared prognostic of 30-day mortality in adjusted multivariate analysis. On the day of the positive blood culture, CRP >108 mg/L and on day 14 CRP >22 mg/L predicted the presence of deep infection. Compared to WBC count, CRP levels were superior in predicting mortality and presence of deep infection in SAB. These precise cut-off values of CRP might help diagnose patients with the complicated SAB.

III. In 145 *S. aureus* bacteraemia patients, the effect of CRP gene SNP on CRP levels in SAB was identified. The SNP rs3091244 A-minor allele was found to be a significant predictor of maximal CRP level in SAB. CRP gene SNPs were not associated with predisposing factors, underlying diseases, clinical outcomes, presence of deep infection or with the CRP levels at the time of the positive blood culture or one week after that. Together with a deep infection focus, carriage of the rs3091244 A-minor allele carriage was found to determine the maximal CRP during the acute phase in SAB.

IV. The prognostic value of suPAR in 66 *Staphylococcus aureus* bacteraemia patients was identified. Day three suPAR levels >9.25 ng/mL predicted fatal outcome, and the difference in suPAR levels was observed up to 10 days after the onset of the disease. suPAR appeared not to be helpful in raising a suspicion of a deep infection.
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