universität wien

DISSERTATION / DOCTORAL THESIS

Titel der Dissertation /Title of the Doctoral Thesis

„Role of Staphylococcus aureus cytolysins in human pulmonary infection“

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Doctor of Philosophy (PhD)

Wien, 2022 / Vienna 2022

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 794 685 490
Dissertationsgebiet lt. Studienblatt / field of study as it appears on the student record sheet:	Molekulare Biologie
Betreut von / Supervisor:	Dr. Dr. Eszter Nagy

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DEDICATION

To my Mom. Look at how far we've come. Thinking back of everything we've been through the only thing I can say is that I am as proud of you as you are of me.

To Philipp. You make me live my dreams and not my fears. Thank you for believing in me more than I believed myself.

To my friends. We cried together, and the sadness got reduced by half. We laughed together, and the happiness doubled. How does this math even work?

To my patients and their parents. You bring out the best in me. Your strength, your smiles and your gratitude mean more to me than I will ever be able to express.

Fig. 0.0. First gratitude letter addressing satisfactory medical service received by the PhD candidate. *Reprinted with permission of the author.*

ACKNOWLEDGEMENTS

This journey started in a foreign, intimidating country that is now my home, and the language that sounded so alien is now the one I dream in. I have an immense amount of people to thank for helping me navigate this exciting, not always straightforward and easy path – the one that I wouldn't ever be able to finish alone.

First and foremost, I would like to thank Ezter for giving me this opportunity and for welcoming me into the Arsanis family. Above all, I value your guidance, your support, patience and persistence. Being able to learn from you and your experience is a real privilege.

My huge gratitude goes to the whole Arsanis team: Zoltan - for trusting me with the clinical projects and helping me work and brainstorm through them. Lukas, Harald, Gabor and Vali - for the fruitful and interesting discussions. Manuel, Cili, Michele, Jacqui, Luis – thank you for your helping hands (and brains!), and for keeping an amazing atmosphere with your humor and smiles. Karin and Barbara, thank you for showing me the way around the lab and supporting me in my first attempts at German.

Our critical discussions of the lunch location (and timing!), radio station preferences of the individual labs, and the inhuman amounts of coffee consumed will all stay in my heart (some of them literally, in form of caffeine-induced tachycardia).

I would also like to thank the department of pediatric surgery (Martin, Valeria, Michal, Vicky, Sara, Gerold, Anton, Tobias and Indra), PICU (Christian, Thomas), as well as our small neuropediatric circle (Sharon and Laura) of the Klinik Donaustadt – you helped me navigate through the times of uncertainty and self-doubt.

My sincerest gratitude goes to my whole family and my friends that didn't get a chance to be mentioned before (Philipp, Andrea, Herbert, Melanie, Simona, Olga, Jens, Kristina).

Thank you for believing in me every step of the way.

Thank you for your support and patience.

Thank you for turning these years into an adventure.

ABSTRACT

S. aureus is one of the most notorious bacterial species causing a range of potentially life-threatening diseases, including ventilator-associated pneumonia (VAP), a complication of mechanical ventilation (MV). My work is aimed at achieving a better understanding of *S. aureus* VAP by investigating host- and pathogen-associated factors, and is constituted by four projects.

1. By collecting clinical and demographic data of 250 ICU-admitted and ventilated patients in a US hospital, we evaluated the epidemiology of *S. aureus* airway colonization and infection. VAP was diagnosed retrospectively using clinical data. Endotracheal aspirate (ETA) microbiology was used to determine airway colonization status and VAP causative pathogen. We found *S. aureus* to be the most common colonizing and VAP-associated species. Other significant findings included high progression rate to VAP among patients with heavy colonization in the trachea (defined semiquantitatively), and a 4-day delay between first detection of tracheal colonization and onset of VAP symptoms. This study was published in BMC Infectious Diseases in 2019.

2. Pore-forming toxins of *S. aureus* (α-hemolysin, or Hla, and 5 leukocidins) are implicated in human pulmonary infections pathogenesis. Using in-house developed ELISA and cell-based neutralization assays, we examined the serum samples of the patients recruited in the first project and of the healthy volunteers for the levels and repertoires of anti-staphylococcal antibodies. Latter were detected in all examined subjects, and healthy ones had significantly higher titers than ICU-admitted. Patients carrying *S. aureus* in the nose and/or trachea had approximately 2-fold higher anti-staphylococcal antibody titers compared to *S. aureus*-negative subjects. In a subset of patients values up to 10-fold higher than the mean were detected. These biologically attainable titers contextualize the relevance of the titer difference between colonized and non-colonized. We also noted the tendency of individual patients' immunoglobulin levels to reflect the toxin profile of the residing *S. aureus* strain, suggesting that the antibody presence is the consequence of a tight host-colonizer interplay.

3. Published data links exposure of *S. aureus* to sublytic antibiotic concentrations with increased bacterial toxicity. To further examine this effect, we cultured pre-selected clinical and laboratory *S. aureus* strains in a growth medium mimicking in-vivo low-nutrient and -iron conditions, which was supplemented with sublytic concentrations of antibiotics. Culture supernatants were then tested for cytotoxicity in the cell-based assays. Toxicity enhancement was observed across majority of the strains under the influence of antibiotics with anti-staphylococcal activity spectrum. This effect was proven to be an active bacterial response to the antibiotic insult. In the context of critically ill patient receiving antimicrobial prophylaxis or suboptimally dosed therapy, such behavior of *S. aureus* could contribute to infection onset or worsening.

4. As the timely detection of *S. aureus* is extremely important, we aimed to develop a point-of-care antibody-based diagnostic tool in a form of a latex-bead agglutination assay. Monoclonal antibodies targeting Staphylococcal protein A were used. The prototype test was capable of detecting *S. aureus* and had a bacterial density-dependent lower limit of detection of 2×10^7 CFU/ml. Although this sensitivity is not sufficient to reliably directly detect heavy colonization, this assay setup can be the first step towards developing a lateral flow test kit.

Our findings show that *S. aureus* remains a prominent nosocomial pathogen, and interventions aimed at reducing its burden and preventing staphylococcal infections are urgently needed. Possible measures include active surveillance of admitted patients for *S. aureus* carriage, potentially with the help of bedside diagnostic tool; correct choice and careful dosing of antibiotics to prevent antibiotic-induced toxicity enhancement, and augmentation of the standard therapy with pathogen- and toxin-specific immunotherapy, thus addressing the described hypogammaglobulinemia and limiting bacterial virulence by offsetting staphylococcal toxins.

KURZFASSUNG

S. aureus ist eine der berüchtigtsten Bakterienarten, die eine Reihe potenziell lebensbedrohlicher Krankheiten verursachen, darunter auch die beatmungsassoziierte Pneumonie (VAP), eine Komplikation der mechanischen Beatmung (MV). Meine Arbeit zielt darauf ab, ein besseres Verständnis von *S. aureus* VAP zu erlangen, indem ich wirts- und erregerassoziierte Faktoren untersuche, und besteht aus vier Projekten.

1. Durch die Erfassung klinischer und demografischer Daten von 250 auf der Intensivstation aufgenommenen und beatmeten Patienten in einem US-amerikanischen Krankenhaus haben wir die Epidemiologie der Kolonisierung und Infektion der Atemwege mit *S. aureus* untersucht. Die VAP wurde retrospektiv anhand klinischer Daten diagnostiziert. Mit Hilfe der Mikrobiologie des endotrachealen Aspirats (ETA) wurden der Status der Atemwegskolonisierung und der Erreger der VAP bestimmt. Wir stellten fest, dass *S. aureus* die häufigste kolonisierende und VAP-assoziierte Spezies war. Weitere signifikante Ergebnisse waren eine hohe Progressionsrate zu VAP bei Patienten mit starker Kolonisierung in der Luftröhre (semiquantitativ definiert) und eine Verzögerung von 4 Tagen zwischen dem ersten Nachweis einer Trachealkolonisierung und dem Auftreten von VAP-Symptomen. Diese Studie wurde 2019 in BMC Infectious Diseases veröffentlicht.

2. Porenbildende Toxine von *S. aureus* (α-Hämolysin oder Hla, und 5 Leukozidine) sind an der Pathogenese menschlicher Lungeninfektionen beteiligt. Mit eigenentwickelten ELISA- und zellbasierten Neutralisationstests untersuchten wir die Serumproben der im ersten Projekt rekrutierten Patienten und der gesunden Probanden auf den Gehalt und das Repertoire an Anti-Staphylokokken-Antikörpern. Letztere wurden bei allen Untersuchten nachgewiesen, wobei die gesunden Probanden deutlich höhere Titer aufwiesen als die auf der Intensivstation aufgenommenen. Patienten, die *S. aureus* in der Nase und/oder der Trachea hatten, wiesen im Vergleich zu *S. aureus*-negativen Probanden etwa 2-fach höhere Anti-Staphylokokken-Antikörper-Titer auf. Bei einer Untergruppe von Patienten wurden Werte festgestellt, die bis zu 10-fach über dem Mittelwert lagen. Diese biologisch erreichbaren Titer kontextualisieren die Relevanz des Titerunterschieds zwischen kolonisierten und nicht kolonisierten Personen. Wir stellten auch fest, dass die Immunglobulinwerte einzelner Patienten tendenziell das Toxinprofil des residenten *S. aureus*-Stammes widerspiegeln, was darauf hindeutet, dass das Vorhandensein von Antikörpern die Folge eines engen Zusammenspiels zwischen Wirt und Kolonisator ist.

3. Veröffentlichte Daten bringen die Exposition von *S. aureus* gegenüber sublytischen Antibiotika-Konzentrationen mit einer erhöhten bakteriellen Toxizität in Verbindung. Um diesen Effekt weiter zu untersuchen, haben wir vorselektierte klinische und Labor-*S. aureus*-Stämme in einem Wachstumsmedium kultiviert, das nährstoff- und eisenarme *In-vivo*-Bedingungen imitiert und mit sublytischen Antibiotikakonzentrationen angereichert wurde. Die Kulturüberstände wurden dann in zellbasierten Assays auf Zytotoxizität getestet. Bei der

Mehrzahl der Stämme wurde unter dem Einfluss von Antibiotika mit Anti-Staphylokokken-Aktivitätsspektrum eine Erhöhung der Toxizität beobachtet. Dieser Effekt erwies sich als eine aktive bakterielle Reaktion auf den antibiotischen Angriff. Bei kritisch kranken Patienten, die eine antimikrobielle Prophylaxe oder eine suboptimal dosierte Therapie erhalten, könnte ein solches Verhalten von *S. aureus* zum Ausbruch oder zur Verschlimmerung der Infektion beitragen.

4. Da der rechtzeitige Nachweis von *S. aureus* äußerst wichtig ist, wollten wir ein auf Antikörpern basierendes Point-of-Care-Diagnoseinstrument in Form eines Latexpartikeln-Agglutinationstests entwickeln. Es wurden monoklonale Antikörper gegen das Staphylokokkenprotein A verwendet. Der Prototyp-Test war in der Lage, *S. aureus* nachzuweisen und hatte eine von der Bakteriendichte abhängige untere Nachweisgrenze von 2×10^7 KBE/ml. Obwohl diese Empfindlichkeit nicht ausreicht, um eine starke Besiedlung zuverlässig direkt nachzuweisen, kann dieser Testaufbau der erste Schritt zur Entwicklung eines Lateral-Flow-Testkits sein.

Unsere Ergebnisse zeigen, dass *S. aureus* nach wie vor ein wichtiger nosokomialer Krankheitserreger ist und dass Maßnahmen zur Verringerung seiner Belastung und zur Verhinderung von Staphylokokkeninfektionen dringend erforderlich sind. Zu den möglichen Maßnahmen gehören die aktive Überwachung von eingewiesenen Patienten auf *S. aureus*-Träger, möglicherweise mit Hilfe eines bettseitigen Diagnoseinstruments; die richtige Auswahl und sorgfältige Dosierung von Antibiotika, um eine Verstärkung der antibiotikabedingten Toxizität zu verhindern, und die Ergänzung der Standardtherapie durch eine erreger- und toxinspezifische Immuntherapie, die der beschriebenen Hypogammaglobulinämie entgegenwirkt und die bakterielle Virulenz durch Kompensation der Staphylokokken-Toxine begrenzt.

LIST OF ABBREVIATIONS

AIP = autoinducing peptide

agr = accessory gene regulator

ARDS = acute respiratory distress syndrome

BAL = bronchoalveolar ravage

BSI = bloodstream infections

CFU = colony-forming unit

COPD = chronic obstructive pulmonary disease

CXR = chest X-ray

ETA = endotracheal aspirate

Hla = staphylococcal alpha-hemolysin

ICP = intracranial pressure

LLoD = lower level of detection

MSSA = methicillin-sensitive *S. aureus*

MRSA = methicillin-resistant *S. aureus*

MV = mechanical ventilation

NBAL = non-bronchoscopic bronchoalveolar lavage

PBP = penicillin-binding protein

PBS = protected specimen brush

POC = point-of-care

PSM = phenol-soluble modulins

PTSAg = pyrogenic toxin superantigens

PVL = Panton-Valentine leukocidin

RBC = red blood cells

SA-VAP = ventilator-associated pneumonia caused by *S. aureus*

SCC = staphylococcal chromosome cassette

SpA = staphylococcal protein A

SSTI = skin- and soft tissue infections

VAP = ventilator-associated pneumonia

WBC = white blood cell

I. INTRODUCTION

I.1 MICROBIOLOGY OF *STAPHYLOCOCCUS AUREUS*

Staphylococcus aureus is a member of Staphylococcaceae, a family of Gram-positive bacteria. It was one of the first microorganisms described: Staphylococci were first seen by Koch in 1878 in a pus specimen, and *S. aureus* was first differentiated as a species by Rosenbach in 1884, who observed phenotypic differences in the colonies of different members of the family [1]. *S. aureus* owes its name to its appearance under the microscope, where it forms representative grape-like clusters of the individual bacterial cells (from greek “staphylo” – grape, and “coccus” – grain), and to the typical golden-yellow pigmentation of the colonies (from Latin “aureus” – golden). This organism is resilient, able to survive in a variety of conditions including a wide pH range (4.8 – 9.4), temperature extremes as high as 60°C, high salt medium, and is resistant to drying [2]. When cultured, it is non-fastidious and grows readily in both aerobic and non-aerobic conditions. Most of the routinely used solid and liquid media support *S. aureus* growth, and additional selective media and biochemical tests allow further differentiation of this bacterial species from other Staphylococcaceae. On the solid agar, typical small, shiny, white or golden-colored colonies usually appear at 24-48 hours of incubation at 37°C. While assessment of colony morphology is possible with most rich general media, sheep blood agar offers an additional possibility of observing and evaluating strain hemolysis [3]. Microscopically, individual bacterial cells measure 0.7-1.2 μm in diameter, and are surrounded by a thick peptidoglycan wall representative of Gram-positive organisms. When dividing, the daughter cells are slow to separate from each other, owing to the formation of the characteristic eponymous clusters. The genome of *S. aureus* is represented by a single circular chromosome of 2.7–2.8 mbp in length complemented by accessory genetic material such as plasmids or prophages [4]. As with other pathogenic bacteria, accessory genome of *S. aureus* is significant for carrying a number of genes coding for additional virulence factors and antibiotic resistance [5]. These genes can be acquired and spread rapidly within a bacterial population by the means of horizontal gene transfer, making the accessory genome an important factor in propagation of clinically relevant features of *S. aureus*. Analysis of the *S. aureus* genome is commonly used in research and clinical practice both to define major lineages and evolutionary trends within the species, and to examine gene expression at a cell level.

Because of its outstanding resilience, quick adaptability to environmental challenges, and a remarkable set of virulence factors, *S. aureus* is one of the most successful human pathogens. It is widely distributed geographically, and is associated both with asymptomatic colonization and with a wide spectrum of diseases ranging from minor, self-limited conditions to life-threatening invasive infections. Disorders caused by *S. aureus* are often challenging to treat, not only because of high virulence of the bacterium, but also due to its ability to evade the immune system and its high rate of antibiotic resistance. Despite the progress made in the field of antimicrobial therapy in the recent years, *S. aureus*-caused infections remain common and claim thousands of lives yearly.

I.2. Molecular basis of *Staphylococcus aureus* virulence

Exceptional success of *S. aureus* as a human pathogen is achieved through its outstanding resilience, ability to survive environmental insults, and an impressive arsenal of the virulence factors, latter briefly summarized in **Fig. 1.1**. These factors can be broadly categorized into the cell surface-associated and secreted.

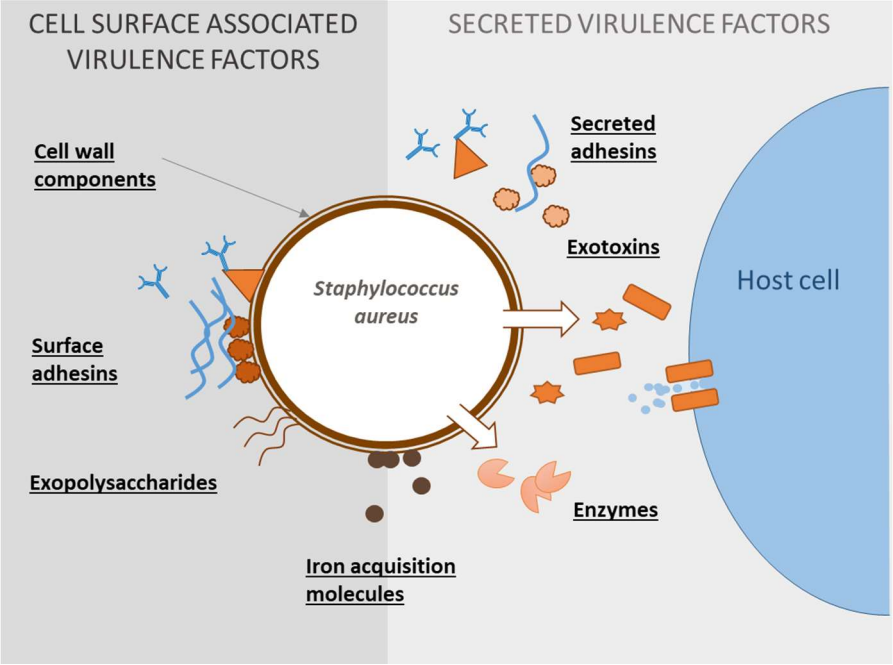

Figure 1.1. Schematic overview of S. aureus virulence factors. *Adapted from Staphylococci in Human Disease (p.66) by K. Crossley, 2nd edition. Brown shading: bacterial cell/molecules. Blue shading: host cell/molecules.*

Cell surface-associated virulence factors. Cell surface-associated virulence factors contribute to *S. aureus* disease establishment primarily by aiding colonization and immune evasion. They can be categorized into three broad groups: (1) components of the cell wall promoting colonization, inflammatory response and septic shock development [6–9]; (2) capsular exopolysaccharides restricting phagocytosis [10,11]; and (3) surface-bound adhesins comprising a group of 28 proteins [12] largely represented by the proteins mediating the adherence of the bacterial cells to the host extracellular matrix [13,14]. Available data links these virulence factors to the biofilm-related infections (e.g., endovascular or endoprosthesis-associated) [15].

One of the best-characterized members of the cell-surface associated virulence factors, and a focus of one of the projects comprising this thesis, is staphylococcal protein A (SpA). It is produced by the majority of the clinical *S. aureus* isolates, with ≥90% of the strains carrying the SpA gene [16,17]. This 42-kDa protein can be both membrane-bound and secreted, and is typically produced during the log phase [18,19]. Its principal function, and a major contribution to *S. aureus* virulence, is binding the host immunoglobulins at their F_c-portion. As the result of such binding the F_c-portion becomes inaccessible to the phagocytes, and the opsonization process is inhibited [20,21]. B-cells and antibodies play the key role in controlling the staphylococcal infections [22]; the inactivation of the host immunoglobulins by the SpA therefore accounts for one of the most important immune evasion mechanisms of *S. aureus*. Promoting the bacterial survival, in turn, may ultimately result in a more severe disease course through increased bacterial inoculum, higher concentrations of the virulence factors and enhancement of the inflammatory response. Apart from its antibody-binding activity, SpA also has a capacity to bind von Willebrand factor [23], which is thought to mediate adherence of the bacterium to the platelets; and to TNFR-1 which is implicated in *S. aureus* pneumonia and osteomyelitis pathogenesis [24,25].

Secreted virulence factors. Listed among the *S. aureus* extracellular virulence factors are secreted adhesins, thought to facilitate interaction between the host cells and the bacterium [26]; extracellular enzymes represented primarily by proteases [27]; as well an array of molecules forming a group of exotoxins. Additionally, some of the *S. aureus* virulence factors can be both membrane-bound and extracellularly expressed, as exemplified by protein A or the siderophores [28,29].

Exotoxins are commonly considered to be the cornerstone of *S. aureus* virulence. They can be broadly classified into staphylococcal pyrogenic toxin superantigens (PTSAg), exfoliative and pore-forming toxins [30]. PTSAg are a group of molecules notable for their potent immunostimulatory properties by stimulating proinflammatory cytokine

release. Best-described representatives of this group are the toxic shock syndrome toxin-1 and the staphylococcal enterotoxins, implicated in the pathogenesis of the toxic shock syndrome and staphylococcal food poisoning, respectively [31,32]. Exfoliative toxins of *S. aureus* are the cause of the staphylococcal scalded skin syndrome, with loss of superficial skin layer as its major manifestation [33]. The main focus of this project, however, is the third of the listed staphylococcal toxin groups – the pore-forming toxins. They can be further subdivided into three subgroups: (1) hemolysins; (2) leukocidins; (3) phenol-soluble modulins (PSM). As outlined by their name, the cytopathic potential of these toxins is represented by their ability to form pores in the membranes of the target cells. The importance of PSM as determinants of *S. aureus* virulence is well-recognized; they are thought to contribute to biofilm formation and dissemination of biofilm-associated infections. [34] Within the scope of the current thesis, however, the PSM will not be discussed in details, and our attention will be focused on the hemolysins and leukocidins.

Staphylococcal hemolysins and leukocidins are functionally homologous molecules with hemolytic and leukotoxic properties, respectively, and have been described to play a major role in pathogenesis of many human diseases [35]. Majority of these toxins are produced and secreted by the bacterium in the form of the monomers, with an exception of LukGH (discussed later) which forms a stable heterodimer. Upon binding of the monomer to the receptor on the host cell a polymerization step follows, resulting in a formation of a membrane bilayer-spanning pore (**Fig 1.1, Fig. 1.2**). The death of the host cell results from an uncontrolled efflux of the ions such as K^+, Ca^{2+}, as well as small molecules such as ATP, through the pore.

Antibiotic resistance. High rate of resistance against the antimicrobial agents is an important determinant of *S. aureus* success as a human pathogen. Antibiotic resistance is acquired via either the horizontal transfer of genetic elements coding for antibiotic resistance determinants, or the mutations in the core genome reducing the susceptibility to the antibacterial substances [36]. Acquisition of the resistance-determining genes leads to the expression of drug-inactivating enzymes, enzymes modifying the drug binding site, molecules involved in the drug efflux, or variant proteins capable of taking over the functions inhibited by an antibiotic. Antibiotic resistance gained via the core genome mutations is achieved by the alteration of the drug binding sites, upregulation of the efflux pumps, or reduced accessibility of the target molecule caused by the change in the cell composition [37].

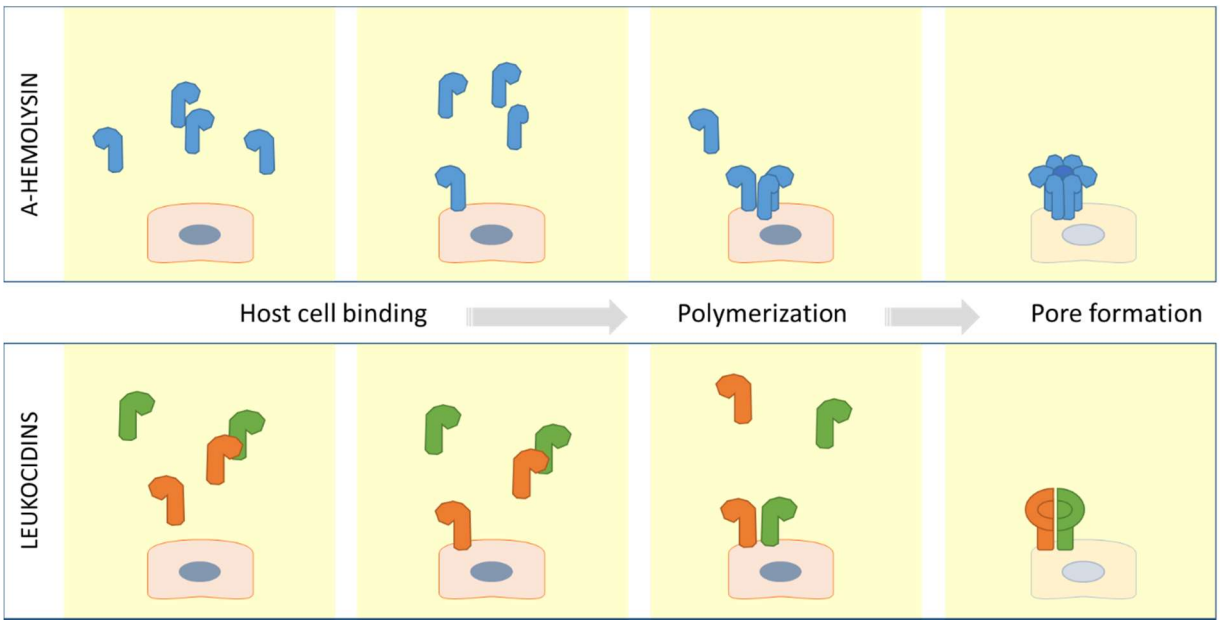

Figure 1.2. Schematic representation of *S. aureus* α-hemolysin (upper panel) and the leukocidins' (lower panel) mechanism of action. *Upon binding to the host cell, the toxin molecules polymerize to form a heptameric (α-hemolysin) or a heterodimeric (leukocidins) transmembrane pore.*

Among *S. aureus* isolates, a distinctive group of the strains resistant to most beta-lactam antibiotics is recognized. This resistance was originally described for methicillin, leading to the distinction between the methicillin-sensitive (MSSA) and methicillin-resistant *S. aureus* (MRSA). The target of beta-lactams is the penicillin-binding proteins (PBP), a family of the essential enzymes involved in the peptidoglycan synthesis. Binding of the drug molecule to PBP disrupts the cell wall production and causes lysis of the bacterial cell. MRSA produces a variant version of the bacterial enzyme, referred to as PBP2a, with an inaccessible beta-lactam binding site. The gene for this molecule is situated in staphylococcal chromosome cassette (SCC), a distinct family of mobile genetic elements [36]. Originally associated with hospital environment only, nowadays MRSA is also widely distributed in the community. Both hospital- and community-associated MRSA infections are common and remain challenging both clinically and epidemiologically [38,39]. Interestingly, there are some reports indicating that MRSA strains tend to be less virulent than MSSA [40]. The resistance profile of the MRSA is variable depending on the strain; however, most of the isolates display resistance against multiple antibiotic classes apart from the beta-lactam family, thus belonging to the class of the multidrug-resistant (MDR) bacteria [41]. Certain strains are even resistant against vancomycin, one of the most reliable antibiotics for the treatment of MRSA infections [42].

I.3. PORE-FORMING TOXINS OF *STAPHYLOCOCCUS AUREUS*

A-Hemolysin. Of the *S. aureus* hemolysins, by far the most prominent and the best characterized is the α-hemolysin (Hla), termed so for its ability to lyse rabbit red blood cells (RBC). Also referred to as alpha-toxin, this molecule is able to affect a large number of cells apart from the erythrocytes, and its pathogenic effect varies depending on the cell type and the species of the host. The gene for Hla is found in almost all of the *S. aureus* isolates, and is situated on the bacterial chromosome. Its primary amino acid sequence is highly conserved. Hla monomer is secreted as a water-soluble molecule with an atomic weight of 33 kDa. Upon contact with the receptor molecule, it oligomerizes into a heptamer, followed by the formation of a β-barrel amphipathic pore perforating the host cell lipid bilayer, which allows the flow of ions, ATP and small molecules. Subsequent death of the host cell results from depolarization. Although the pore formation is the most prominent cytopathic effect of this toxin, Hla is also known to affect the host organism by a number of secondary processes altering the metabolism and gene expression. These effects include but are not limited to altered cell proliferation, release of cytokines and inflammasome activation, cellular contractility dysfunction and increased platelet aggregation [43–46]. The receptor for Hla has been identified to be A-disintegrin and metalloprotease 10 (ADAM10), a zinc-dependent metalloprotease. This protein is expressed in high abundance on the surfaces of the susceptible cells. It is crucial not only for binding of the toxin, but also for its subsequent oligomerization and pore formation, and higher level of ADAM10 expression correlates with susceptibility of the cell to intoxication by Hla [47]. This explains the marked species specificity typical for Hla. For example, in contrast to rabbit RBC which are highly sensitive to lysis by the toxin, human erythrocytes require much higher concentrations of Hla for the lysis to occur [48]. This is because ADAM10 is found in greater abundance on the surface of rabbit RBC as compared to human erythrocytes. Other than the RBC, Hla is known to interact with a number of human cell types with varying effects depending on its concentration.

One of the most profoundly affected toxin targets in humans are epithelial cells, represented by pneumocytes, endothelial cells and keratinocytes. These cells form physical barriers and, as such, function as part of the innate immune system. Lysis of the epithelial cells by Hla disrupts their barrier function, which, in turn, paves the way for the bacterial invasion into the host tissues. Apart from barrier disruption, further manifestations of Hla activity on epithelial cells include stimulation of the cytokine release, modulation of the cell proliferation pathways, and alteration of the endothelial contractility and permeability. Other human cells susceptible to the intoxication by Hla

include platelets, where toxin action causes thrombocyte aggregation and formation of micro-thrombi, and immune cells including neutrophils, monocytes, and T-cells, which respond to the intoxication by release of the cytokines and inflammasome activation [49]. Cytopathogenic effects of Hla on different cell types act in a synergistic manner, contributing to the establishment of the staphylococcal disease [50]. In humans, the cytotoxic effect of Hla has been shown in a number of *in vitro* and *in vivo* studies. Published data indicates an especially important role of Hla in *S. aureus* sepsis, skin- and soft tissue- and pleuropulmonary infections. This has been supported by both animal experiments and human studies [51–54]. A considerable number of therapeutic and prophylactic efforts have been focusing on limiting the Hla activity.

Bicomponent leukocidins. Another prominent family of secreted exotoxins of *S. aureus* is the group of bicomponent leukocidins. This is a family of structurally similar, water-soluble molecules which, similarly to Hla, exert their cytolytic effect by forming pores in the membrane of a host cell. They owe their name to their ability to lyse white blood cells – an effect first described as early as 1894 by Van de Velde [55]. A total of six bicomponent leukocidins have been described to date; five of those are implicated in the human disease and will be discussed here. The β-barrel pore formed by the leukocidins is composed of extracellularly secreted water-soluble monomers - the so-called “F-component” and “S-component, which stands for “fast” and “slow” and refer to their chromatography elution profiles [56]. They are structurally and genetically similar, with a sequence homology of up to 80% between the individual S- and F molecules. The toxins are termed according to the names of their components, the S-subunits including HlgA, HlgC, LukE, LukS and LukG (LukA), and the F-subunits being HlgB, LukD, LukF, and LukH (LukB). Consequently, the following are the toxins in their oligomerized form: HlgAB and HlgCB, collectively referred to as gamma-hemolysins; LukSF (also referred to as Panton-Valentine leukocidin, or PVL), LukGH (also known as LukAB) and LukED. Of these, LukGH is notable since it is the only bicomponent leukocidin secreted as a heterodimer and not as a monomer. During the pore formation, the secreted F-component of the toxin binds its ligand on the host cell wall, followed by the recognition and recruitment of the S-component. The resulting dimers further oligomerize to form an octameric structure of the alternating S- and F-subunits. Subsequent conformational change results in the insertion of the stem domain into the host membrane and formation of a mature pore [57]. Similarly to the effects of Hla, the death of the host cell results from depolarization. Of the mentioned bicomponent leukocidins, three (HlgAB, HlgCB and LukGH) are produced by an overwhelming majority the clinical isolates [58]. LukED is also common, expressed by up to 87% of *S. aureus* strains [59]. LukSF, in contrast, is only seen in 2-3% of all human *S. aureus* isolates [60]; however, its frequency is reaching up to 90% in isolates responsible for

severe skin- and pulmonary staphylococcal disease [61]. Whereas the gamma-hemolysins and LukGH are part of the core genome of *S. aureus*, LukED is a part of one of the staphylococcal pathogenicity islands, and PVL gene is located in the genome of a temperate phage.

Ability to lyse the human cells is universal among the leukocidins. Evaluating additional pathogenic properties and dissecting the individual toxin's role in the disease remains a challenging field of *S. aureus* research. As noted previously, the effects of the individual molecules differ markedly among the species, which makes the development of a suitable and reliable animal model difficult and limits the degree to which the data obtained from the animal experiments can be generalized. A lot of the statements are thus relying on the results of the cell-based assays and the clinical data, including antibody response of the affected patients and characteristics of the disease caused by specific strains. Of the leukocidins, LukSF is by far the best studied toxin. The receptor for this toxin has been identified to be C5aR and C5L2, both being G-protein-coupled receptors found on the surface of the host immune cells [62]. As outlined previously, the proportion of the LukSF-positive *S. aureus* strains among the clinical isolates is low. In the recent years, however, there has been an emergence of largely LukSF-positive community-associated MRSA strains (CA-MRSA) [63]. Epidemiologically, strains expressing this toxin are associated with a more fulminant and invasive infection course, in particular of necrotizing pneumonia and severe skin- and soft tissue infections (SSTI) [64,65]. LukSF expression together with the combination of the global characteristics (increased production of virulence factors, antibiotic resistance and other alterations leading to enhanced bacterial fitness) are thought to contribute to the hypervirulence of these strains [66]. Not only the lysis of the host immune cells, but also the resulting release of potent proinflammatory mediators (interleukins, histamine and leukotrienes) promote the massive inflammation typical for the necrotizing pneumonia caused by the LukSF-positive *S. aureus* strains. This has been described using the mouse and the rabbit models of this disease, latter considered representative of the human disease [67].

Similarly to LukSF, dissecting the role of LukGH in human disease is challenging due to its limited activity against the rabbit and murine cells. In humans, the receptor for LukGH has been identified to be the CD11b receptor, expressed abundantly by PMNs, macrophages, monocytes, and dendritic cells [68]. *Ex vivo* studies have linked LukGH expression to the enhanced neutrophil killing and increased bacterial survival [68,69], as well as survival of endocytosis and escape from the human PMNs [70]. The data from the animal studies describes augmented virulence and increased bacterial burden in mice infected by LukGH-positive *S. aureus* strains [69]. These findings, however,

should be approached with caution and do not necessarily represent the disease mechanisms in humans as, as mentioned earlier, LukGH activity in murine cells is limited.

In comparison to LukSF and LukGH, gamma-hemolysins are less studied. HlgA targets CXCR1, CXCR2, and CCR2 proteins on the surface of the human cells, whereas HlgB binds C5aR [71]. As with other leukocidins, HlgAB and HlgCB expression render *S. aureus* more resistant against the killing by host PMNs through the lysis of the host immune cells. These toxins are also thought to contribute to *S. aureus* septic arthritis and intraocular infections, as demonstrated by the animal studies in mouse and rabbit models [72,73].

In the context of deciphering the role of bicomponent leukocidins using the animal models, LukED is a notable exception in this toxin family. The activity of LukED on the mouse and rabbit cells is comparable to that on the human cells, allowing a better insight in the toxin role in disease. Similarly to other leukocidins, LukED exerts its action via its ability to lyse the host white blood cells (WBC) [74]. It is targeting the CCR5 protein present on the cell surface of the macrophages, T-cells, and dendritic cells [75], and CXCR1 and CXCR2 proteins expressed by PMNs, monocytes and NK-cells [76]. A higher number of LukED-positive isolates are seen in patients suffering from impetigo, furunculosis and staphylococcal bloodstream infections (BSI) [77,78].

Dissection of the roles of individual leukotoxins in human *S. aureus* disease has been difficult, largely due to the lack of representative animal models and to the redundancy of individual toxins' functions. In general, the leukocidins' main mode of action is lysis of the host immune cells, which enhances bacterial survival, aids bacterial spread and contributes to the establishment of an invasive infection. The proinflammatory molecules released form the lysed cells further contribute to the local and systemic inflammation. Even at sublytic concentrations, leukocidins are able to elicit a proinflammatory response in the human PMNs [62,79,80], as well as macrophages and monocytes [81]. Similarly to Hla, leukocidins have been a target of pharmaceutical research, seeing their prominent role in human infection.

Regulation of toxin production. Production of *S. aureus* cytolytic toxins depends on a variety of factors, including growth phase, culture medium and environmental insults (e.g., antibiotics). Regulation of *S. aureus* toxin expression is complex and depends on several global regulatory systems. Of those, one of the best characterized is the accessory gene regulator, or *agr*, containing 4 individual *agr* genes and acting as the part of the *S. aureus* quorum-sensing system [82]. This system is activated in the late-log to stationary growth phase by sensing the critical concentration of the bacterial cell

density marker, the autoinducing peptide (AIP). *Agr* activity is also known to be affected by pH, presence of reactive oxygen species and exposure to host factors such as the serum. The effector molecule of *agr* is RNAIII, which, when produced, upregulates the production of Hla and leukocidins, and downregulates the production of surface-bound molecules including SpA [83,84]. Relevance of *agr* system in the human disease can be demonstrated using BSI as an example: in the early stages of an infection, concentration of AIP needed to activate *agr* cannot be achieved since the individual *S. aureus* bacterial cells are found in planktonic form in the bloodstream. This results in a decreased toxin production but an increased expression of surface-associated molecules. Latter aids bacterial attachment to the host tissues. Upon attachment and formation of bacterial clumps, or in case of engulfment of the bacterium by a phagocyte, local AIP concentration raises, thus activating *agr* and enhancing toxin expression while downregulating production of surface-associated molecules. This allows lysis of the host cells and tissues, and promotes bacterial invasion.

Apart from the *agr*, the two-component systems were described to be involved in the regulation of staphylococcal virulence. They are exemplified by *sae* regulatory system, an *agr*-independent central downstream regulator with autoinductive properties to a number of virulence factors including Hla and LukSF [85]. In addition to the intrinsic bacterial mechanisms, this system is also influenced by environmental (pH and salt concentration in the medium) and the host factors [86]. The *Sar* regulator family, encoding DNA-binding molecules, is another system implicated in the regulation of toxin production. It can affect the gene expression directly (e. g., regulation of TSST-1), or indirectly through the interference with the *agr* system [87]. Additionally, there is a number of systems less characterized, with a less prominent involvement in the exotoxin production regulation, or with a minor role in it, which are not going to be discussed here [86]. In general, regulation of *S. aureus* toxin production is complex, involves the interplay of numerous systems, and is highly dependent on the environmental and host factors, as well as the self-regulating molecules produced by the bacterium itself. Recent reports have indicated that antibiotics may act as a factor up-regulating the production of *S. aureus* toxins, a phenomenon to be discussed in **Chapter IV**.

I.4. ROLE OF *STAPHYLOCOCCUS AUREUS* IN HUMAN DISEASES

Infections caused by *S. aureus*. The ubiquity of the microorganism, its remarkable arsenal of virulence factors, resilience and fast adaptability to the environmental insults have made *S. aureus* one of the most successful human pathogens. It is widely

distributed geographically and is associated with a variety of conditions, ranging from asymptomatic colonization to life-threatening infections. Diseases caused by *S. aureus* can be classified according to the affected organ or organ system, to antibiotic resistance profile of the bacterium, or according to whether it is community- or hospital acquired. All three classifications are important for predicting the course and severity of the infection and defining the best therapy approach.

When classifying *S. aureus* infections according to the affected organ or organ system, it is important to consider a significant overlap of the clinical scenarios, as isolated infections are known to spread: e.g., a localized *S. aureus* wound infection may progress into a bacteremia or osteomyelitis. The most common infections caused by *S. aureus* are the SSTI. This is a very heterogeneous group of disorders ranging from minor, self-limiting to life-threatening infections. They can be both community- and hospital-acquired. The more common, easier treated *S. aureus* SSTI presentations are exemplified by impetigo, folliculitis or furunculosis. These conditions are typically community-associated, are generally well-managed with topical antibiotics alone or incision and drainage, and only rarely do proceed to develop into an invasive infection. More serious presentations are complicated SSTIs which include cellulitis, abscesses, diabetic foot ulcers and wound infections. *S. aureus* is also commonly causing the surgical site infections. SSTI treatment failure may result in the further spread of the bacterium [88–90] and is a common source of the *S. aureus* bloodstream infections (BSI) [91,92]. The latter are characterized by high mortality rates and risks of long-term sequelae, as well as a high rate of recurrence [93,94]. Bacteremia, sepsis, disseminated intravascular coagulation (DIC) syndrome are examples of BSI manifestations. The following conditions can develop as a complication of this disease: (1) osteoarticular infections including acute hematogenous osteomyelitis and septic arthritis; (2) bacterial endocarditis; (3) pleuropulmonary and (4) device- and prosthesis-associated infections. Of special importance for the research projects discussed in the course of this dissertation are *S. aureus* pulmonary infections, which will be discussed in details in the following sections.

S. aureus infections are characterized by their marked geographic variability. The most common staphylococcal infections, SSTI, are estimated to occur at the rate of 32.1 to 48.1 per 1,000 people in the USA, with scarce data available from the developing countries. *S. aureus* BSI rate was described to be 10-30 per 100,000 person-years in the industrialized areas, and a wider range of 2.5-48 per 100,000 person-years reported in from the developing world [95]. *S. aureus* is also the most common cause of infective endocarditis, responsible for 16% to 34% of all cases, and of the osteoarticular infections. Because of the bacterium’s ability to form biofilm, it is

contributing to a significant number of device-associated infections, e.g. linked to prosthetic cardiac valves, intravenous catheters, cardiac devices or ventricular shunts. *S. aureus* pleuropulmonary infections, a primary focus of this thesis, can develop both independently and as the device- or a BSI-related complication [96], and will be discussed in details in the following sections. Reoccurrence is a common feature of *S. aureus* infections, especially those caused by MRSA. In the last years MRSA has established itself as a common isolate in *S. aureus* infections. Traditionally recognized as an exclusively hospital-associated pathogen, a subset of the recently emerged CA-MRSA strains are nowadays frequently seen colonizing healthy patients, and causing a variety of community-associated infections [97].

Colonization by *S. aureus*. Up to 30% of the whole population is colonized by *S. aureus* [98]. Although asymptomatic, *S. aureus* carriage is still clinically relevant. The bacterium is most commonly isolated from the anterior nares, the skin and the digestive tract. *S. aureus* carriage is known to put the patient at a higher risk of developing a subsequent staphylococcal infection [99,100]. Reports show strain concordance between the colonizing and infecting bacterial strains, suggesting that colonization niches may function as the infection source [99,101]. Eliminating nasal carriage has been described to reduce the rate of *S. aureus* infections in selected patient groups [102]. At the same time, there is some evidence suggesting that colonized patients may show a milder *S. aureus* infection course, which can partially be explained by the presence of protective pathogen-specific antibodies, induced by exposure of the host to the colonizing strain [98,103,104].

Immunity in *S. aureus* infection and colonization. Upon infecting the host, *S. aureus* induces a cascade of responses from both the innate and adaptive immune systems. The role of the innate immune system in the bacterium recognition and clearance is relatively well-established in both animal and human models: briefly, detection of the bacterial infection markers activates the pattern-recognition pathway, ultimately resulting in recruitment of phagocytes. Of the latter, neutrophils play the key role, as demonstrated by increased incidence of *S. aureus* infections in patients suffering from inborn or acquired defective neutrophil functionality [105]. The role of the adaptive immune system in *S. aureus* infection, on the other hand, is more complex and still poses many questions. It is activated with a relative delay, and relies largely on the functionality of the T- and the B-cells. The contribution of the T-cells to staphylococcal disease clearance is limited, as demonstrated by lack of increased susceptibility to the *S. aureus* infections in T-cell deficient mice or human subjects with absent or defective T-cell response [106,107]. B-cells, on the other hand, play a prominent role in combatting the *S. aureus* infection. Their principal function is secretion of antibodies,

which either directly neutralize the target bacterial virulence factors or promote phagocytosis by opsonizing the bacterial cells, in addition to their numerous immunostimulatory properties. These antibodies persist in the body as part of the antibody pool, protecting the host from the repeated infections. *S. aureus*-reactive antibodies are detected in the majority of the population, which emphasizes the ubiquity of the pathogen [108,109]. Despite the high prevalence of these antibodies, recurrent staphylococcal infections are extremely common. The role of the pre-existing *S. aureus*-reactive antibodies as an immunological correlate of the protection from- and susceptibility to a *S. aureus* infection remains to be fully elucidated. This will be discussed in more details in **Chapter III**. To counter the actions of the host immune system, *S. aureus* has developed a number of evasion mechanisms, many of which have been mentioned previously. Bacterial adhesion, biofilm formation, destruction of host tissue barriers, hindrance of the host immune system are exerted through a number of membrane-bound and secreted staphylococcal molecules [110–112]. These immune evasion mechanisms are ensuring bacterial persistence within the host, which introduces further difficulties in treatment of *S. aureus* infections and calls for development of newer and more effective treatment modalities.

I.5. *S. AUREUS* PULMONARY INFECTIONS

Bacterial pneumonia caused by *S. aureus* is not uncommon, and its incidence in the last decades shows no decrease despite the vigorous screening, prophylactic and therapeutic measures. As with other *S. aureus* infections, the presentation of staphylococcal pneumonia can range from a minor subacute illness to a fulminant, life-threatening condition. Both direct inoculation and hematogenous spread have been described as the mechanisms of the infection establishment. The infecting strain can originate from the patient's own bacterial flora, from another affected or colonized individual via direct contact or droplet spread, or from the surfaces of contaminated material (e.g., equipment or instruments in the healthcare facility setting). Staphylococcal pneumonia can be both community- and hospital-acquired. As far as the community-acquired pneumonia (CAP) is concerned, *S. aureus* is not a common causative pathogen, being responsible for approximately 3 to 5% of all cases. This proportion is even lower in patients receiving outpatient treatment, suggesting that most of the staphylococcal pneumonia cases require hospitalization [113]. Of special consideration is the subset of *S. aureus* CAP caused by previously mentioned CA-MRSA strains, the incidence of which has been estimated to be 0.51–0.64 cases per 100,000 [114]. Despite being infrequent, staphylococcal CAP remains a disease with a relatively poor prognosis [115]. Compared to the community setting, *S. aureus* is a

much more prominent cause of pulmonary disease in a hospital or healthcare environment.

Ventilator-associated pneumonia. Nosocomial pulmonary infections can be grouped into three distinct categories: healthcare-associated pneumonia (HCAP), hospital-associated pneumonia (HAP) and ventilator-associated pneumonia (VAP). HCAP is a broad term for the infections occurring in patients with a history of a recent hospital admission, residents of the long-term care facilities or nursing homes, and recent recipients of intravenous antibiotics, wound care, hemodialysis and chemotherapy. HAP refers to pneumonia occurring in patients admitted to the hospital, and a subset of pulmonary infections developing in the mechanically ventilated patients is referred to as VAP [116]. Regardless of the differences between the individual categories, all of the mentioned diseases are associated with increased mortality, morbidity and poorer health-economic outcomes including longer duration of hospitalization, higher rate of invasive therapeutic procedures and higher cost of care [117,118]. Research projects presented in this dissertation focus on VAP, one of the most feared of the *S. aureus* nosocomial infections. VAP is defined as pneumonia occurring 48 hours or more after intubation and initiation of mechanical ventilation (MV). It is almost exclusively limited to the patients admitted to the intensive care unit (ICU). This is an especially challenging patient cohort rendered vulnerable by, on one hand, the underlying disorders and comorbidities, and, on the other hand, by therapeutic measures including medications, sedation, invasive procedures and indwelling devices. Pneumonia is one of the most common nosocomial infections affecting these patients, with rates reported as high as 27% of the subjects. In the USA, its incidence is reported to be around 5 to 10 cases per 1000 admissions [119]. Due to a highly heterogeneous nature of the patient population, as well as the differences in VAP definitions (**see Chapter I.6**), the reported mortality rates of the disease are highly variable [120]. However, available data estimates the attributable mortality of VAP to be around 10% [121,122]. It also prolongs the hospital stay and greatly increases the care costs [123].

A number of factors are implicated in VAP pathogenesis. The source of the infecting bacterium can be either endogenous, coming from the patient's own flora, or exogenous, acquired from the ICU personnel, other patients and contaminated equipment or materials. Several factors aid bacterial persistence and promote invasive infection: (1) creation of an abnormal continuum between the upper and lower airways; (2) sedation, resulting in the loss of protective reflexes such as cough; (3) formation of the bacterial biofilm on the surface of the endotracheal tube; (4) mucosal lesions introduced in the course of intubation itself or manipulations with an endotracheal tube;

(5) common occurrence of the immunosuppressed state in the ICU-admitted population, either related to the underlying disease or to the administered therapy; (6) pooling and aspiration of the contaminated airway or gastric secretions. Risk factors for VAP development can be divided into modifiable and non-modifiable, and are summarized in the **Table 1.1.** Whilst the non-modifiable risk factors may aid narrowing down the patients most at risk of developing VAP, the modifiable help develop measures aimed at preventing the disease.

Non-modifiable	**Modifiable**
Male sex	Supine position
Age >60 years	Low intracuff pressure
ARDS	Antibiotic use
COPD	Failed subglottic aspiration
Coma	Bacterial colonization
Multi-organ system failure	Gastric over-distension
Head trauma	Prolonged sedation
Neurosurgery	Acid suppressive therapy
Thoracic surgery	Absence of antiseptic oral care
Reintubation	Use of contaminated equipment
Immunocompromised state	Cross-contamination through staff
History of smoking	Parenteral nutrition
Nutritional deficiencies	Delayed mobilisation
Tracheostomy	ICP monitor

Table 1.1. Non-modifiable and modifiable risk factors for VAP. ARDS = *acute respiratory distress syndrome; COPD = chronic obstructive pulmonary disease, ICP = intracranial pressure.*

Depending on how soon after the intubation the clinical signs of VAP are seen, an early- and late-onset VAP are often distinguished. Early-onset VAP develops ≤4 days, and late-onset - >4 days after MV initiation. Different pathogens have been implicated in the pathogenesis of the two disease categories, with the resulting differences in the first-line choice of the antibiotic therapy. As far as *S. aureus* is concerned, MSSA is typically described causing the early-onset VAP, and MRSA tends to cause pneumonia later in the course of MV [124]. However, taken into consideration the high rates of

colonization by the MRSA, as well as surge of CA-MRSA strains, nowadays this distinction is not necessarily relevant and may potentially delay the initiation of appropriate antimicrobial therapy [125].

Presence of the bacterial colonization (oropharyngeal, nasal and/or upper gastrointestinal) is an important risk factor in VAP development [126]. In case of *S. aureus*, colonization at the time of the admission has been linked to a 15-fold higher risk of subsequent *S. aureus* VAP [127]. Normally, potentially pathogenic bacteria carried by the patient are held at bay by the host immunity. For the infection to establish itself, a shift in the balance between the protective potential of the host and the pathogenic potential of the colonizing strain needs to happen. The the colonization-to-pneumonia progression, and the role of the cytolytic toxins in this process are depicted in **Figure 1.3**. Briefly, *S. aureus* colonizing the patient's airway produces Hla targeting the host epithelial cells, resulting in the destruction of the epithelial barrier, thus enabling bacterial invasion, and stimulation of the host inflammatory response. The WBC recruited to the site of the lesion are killed by the bicomponent leukocidins. Resulting breach in the epithelial barrier and an ineffective clearance of the invading bacteria by the phagocytes paves the way for an invasive infection.

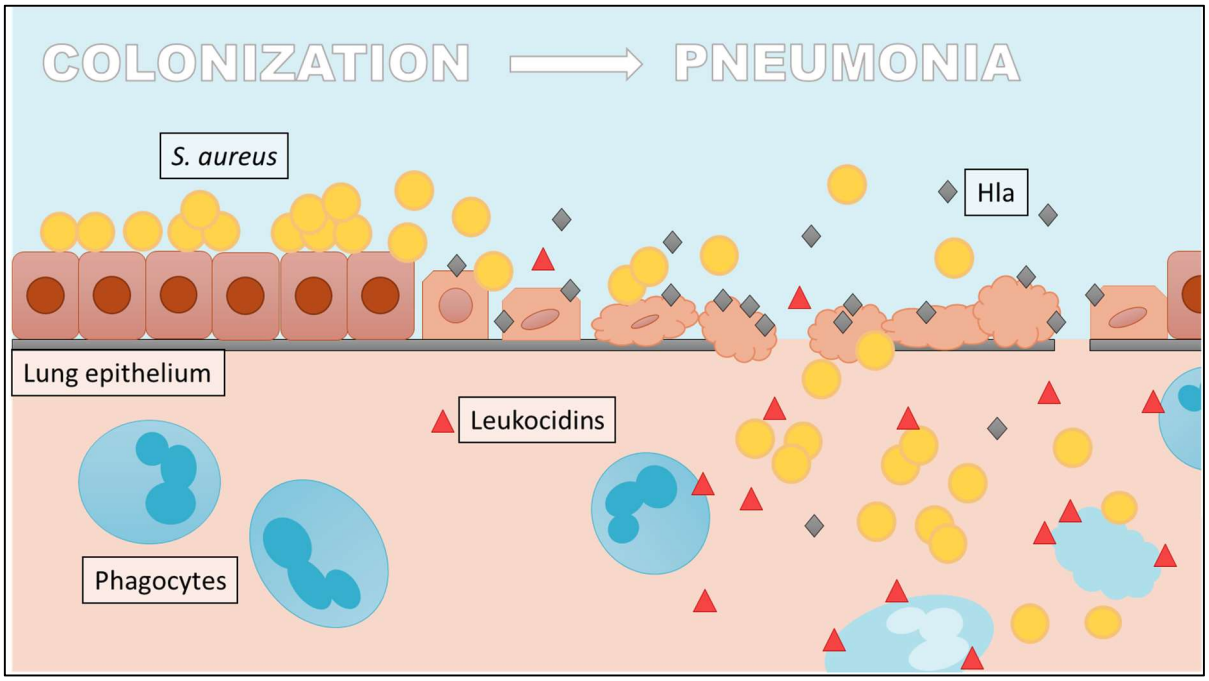

Figure 1.3. Progression of *S. aureus* colonization *to S. aureus* pneumonia. *Hla is depicted by gray diamonds, and leukocidins – by red triangles.*

Despite having considerable difference in geographic distribution, *S. aureus* remains one of the most common VAP pathogens universally [128]. Seeing the high mortality and morbidity of the disease, as well increased costs of care, better understanding of the disease pathogenesis, its early recognition and improved therapy modalities are urgently needed.

I.6. DIAGNOSIS, TREATMENT AND PREVENTION OF *S. AUREUS* VAP

Diagnosis of VAP. VAP remains a challenging disease to diagnose. On one hand, in a cohort of critically ill, multimorbid, often immunocompromised patients, pneumonia may be running an atypical course or mimicking another disorder. On the other hand, there is still no golden-standard guidelines to reliably diagnose this infection. Clinical, radiologic and microbiologic data are used to detect VAP.

Clinically, pneumonia should always be on a differential diagnosis list if an intubated patient develops signs of an infection, e.g. fever, high WBC counts, or purulent respiratory secretions. In addition to the clinical signs, presence of an infiltrate in a chest X-ray (CXR) should raise the suspicion of VAP in an absence of an infection focus elsewhere. This set of clinical and radiologic findings has been used to develop one of the oldest and the most universally accepted criteria for VAP diagnosis, outlined by Johanson et al in 1972 (further referred to as “Johanson criteria”) [129]. VAP is diagnosed by detecting the new or progressive infiltrate in the chest X-ray and at least two of the following: (1) fever >38°C, (2) leukocytosis or leukopenia, and (3) presence of purulent respiratory secretions. This set of criteria is the most encompassing and is characterized by a 69% sensitivity and 75% specificity [130]. Whilst chest X-ray (CXR) is a routine method of detecting pneumonia, one has to consider other causes for lung opacities such as ARDS, atelectasis, pulmonary hemorrhage or infarction, with an infectious focus residing elsewhere [131]. Additionally, prolonged intubation results in production of purulent respiratory secretions not necessarily indicative of pneumonia in many ventilated patients. Despite these caveats, Johanson criteria remain some of the most commonly used VAP diagnostic approaches. In the clinical studies this set of criteria is not infrequently modified to reflect the institution-specific preferences and limitations of the VAP diagnosis. A more recently developed clinical pulmonary infection score (CPIS) concentrates on defining the probability of a certain patient having VAP rather than explicitly diagnosing it. Five parameters are assessed: (1) body temperature, (2) leukocyte count, (3) volume and characteristics of tracheal secretion,

(4) PaO2/FiO2 values, and (5) presence of the pulmonary infiltrate. A numerical score is then derived from these clinical parameters; relation of the score to a cut-off value informs the clinician of the VAP probability. Despite being an expanded and supplemented set of criteria in comparison to Johanson criteria, CPIS does not seem to provide a massive diagnostic benefit. Its sensitivity has been estimated to be 72% to 77%, and the specificity was 42% to 85% [119]. Nowadays there is still no agreed standard of VAP diagnosis. Typically, this disease is diagnosed clinically using the Johanson criteria modified or expanded to reflect local clinical routine and epidemiology.

In the context of suspected VAP, the following respiratory samples can be used for microbiological analysis: endotracheal aspirate (ETA), bronchoalveolar lavage (BAL), non-bronchoscopic bronchoalveolar lavage (NBAL or mini-BAL), and protected specimen brush (PSB). Of these, BAL is the most invasive method of sample collection, as it involves rinsing the lung with saline under the bronchoscopic guidance. This procedure has associated risks, requires appropriate training and instrumentation, and is usually reserved for cases where visual documentation is needed in addition to the respiratory sample. NBAL and PSB, on the other hand, are both conducted “blind”, without bronchoscopic guidance. NBAL involves non-bronchoscopic lavage of the lung, and PSB is obtained by rubbing a sterile brush against the bronchial wall. Both methods require training, are moderately invasive (PSB less so than NBAL), and typically are not routinely available. ETA, on the other hand, is the most readily obtainable specimen, as it is the byproduct of subglottic suctioning - procedure performed in most ICUs as a part of routine patient care. It involves passing a suction line down the endotracheal tube to collect the pooled respiratory secretions. Being non-invasive, easily collectible, not requiring any special instruments or training, ETA is a preferred respiratory specimen in many institutions, used for both microbiological surveillance and diagnostic purposes.

Once the respiratory specimen is collected, the microbiological analysis is to be carried out. Currently, conventional microbiological culture is considered the “golden standard” for pathogen identification and characterization [132,133]. Bacterial species can be identified with the use of common (non-selective), selective and differential media, and by the use of the additional testing methods (e.g., biochemical, enzyme- or antibody-based assays). Antibiotic susceptibility of most isolates is assessed routinely. Gram-staining of the patient’s sample is another approach of pathogen identification frequently employed in the clinic. While cheap, fast and straightforward, its obvious drawback is inability to reliably assign the species to detected pathogen. Molecular diagnostic methods have been extensively developed and increasingly introduced into

the clinical practice in the recent years. Most of them are based on application of either polymerase chain reaction (PCR) or mass spectrometry technique. Both of those approaches are time-effective, with results available within a few hours after sample collection, which makes them appropriate in acute care setting. However, a number of drawbacks (e.g. cost of the equipment, reagents and maintenance, suboptimal quantification methods or sensitivity to contamination) limit their clinical use. The microbiological diagnostic modalities are discussed in more details in the **Chapter V**. For the pathogen to be considered responsible for VAP, it needs to reach a pre-defined threshold of the bacterial density in the patient sample. For PSB, a threshold of 10^3 colony-forming units (CFU) per milliliter, for BAL - 10^4 CFU/ml for BAL and for ETA - 10^5 CFU/ml is generally accepted [134]. Estimation of bacterial density can be quantitative (e.g., plating serially diluted specimens) or semi-quantitative (e.g., by checking presence of bacterial colonies in serially plated quadrants, a method described in **Chapter II**).

Treatment of *S. aureus* VAP. The mainstay of the bacterial VAP treatment remains antibiotic therapy, which needs to be initiated as soon as the disease is suspected. Since the microbiological analysis is delayed, it often means that the antimicrobials are administered empirically. The published guidelines, local epidemiological data and patient characteristics are used to guide the initial antibiotic choice [135]. Selected agents are typically broad spectrum and are often administered in the form of combination of several drugs. Since the ICU-setting is typically associated high rates of the MDR-organisms, considerations have to be made to account for the local bacterial resistance profile. An appropriate choice of initial therapy is challenging but important, as it has been shown to decrease VAP mortality [136]. In the areas with high MRSA rates, antibiotic regimen of choice needs to include appropriate agents such as vancomycin or linezolide. As soon as the results of the microbiological analysis are available, adjustments to the antibiotic regimen are to be made depending on the obtained antibiotic resistance profile and the condition of the patient. Presence of a clinical improvement favors early discontinuation of the antibiotic therapy, as this eliminates the risks and potential side effects of the antimicrobials, reduces the rates of the superinfection by the MDR pathogens, and does not increase VAP mortality [137,138]. In terms of the antibiotic choice for *S. aureus* VAP, a distinction needs to be made between the MSSA and MRSA isolates, and antibiotic susceptibility profile is to be defined for the individual strains. Beta-lactams are some of the most commonly used broad-spectrum antibiotics. For *S. aureus*, they are typically used for MSSA infections, but several studies have suggested their role in management of MRSA-caused diseases as well [185–187]. Apart from them, cephalosporins, carbapenems aminoglycosides, macrolides and fluoroquinolones are effective against most of the

MSSA isolates. Glycopeptides, lincosamines, streptogranins and sulfonamides are used in treatment of MRSA infections. The antibiotic classes of choice with their representative molecules are briefly summarized in the **Table 1.2**. Apart from the patient's characteristics (weight, underlying diseases, liver and/or kidney health, and presence of allergies) additional factors have to be kept in mind when guiding the therapy of suspected *S. aureus* lung infection. First, the bacterium's ability to form the biofilms may limit the antibiotic effectiveness, and rigorous hygienic measures need to be continued. Second, penetrability of the drug into the lung tissue has to be considered, and the dose adjusted according to it. This is especially relevant in the light of the recent findings of *S. aureus*' increase of virulence in response to the sublytic concentrations of certain drugs, further discussed in **Chapter IV**.

Airway delivery of the aerosolized antibiotics has been suggested as the means to increase the concentration of the drug in the lung tissue. To date, the efficacy of this approach is not defined, and there is a lot of conflicting evidence on the use of the inhaled antimicrobials. A recent meta-analysis by Zampieri et al suggests that the use of the nebulized antibiotics seems to increase the cure rates, although without reaching the level of statistical significance [139]. In case of *S. aureus*, however, the relevance of the data is limited, as most of the published data concentrates on VAP caused by the Gram-negative organisms such as *P. aeruginosa* or *A. baumannii*.

Mode of action	Antibiotic class	Example	Target isolate
Cell-wall active drugs	Beta-lactams	Methicillin	MSSA
	Cephalosporins	Cefazolin, cefepime	MSSA
	Carbapenems	Meropenem, ertapenem	MSSA
	Glycopeptides	Vancomycin	MRSA
Protein synthesis inhibitors	Aminoglycosides	Gentamycin	MSSA
	Macrolides	Erythromycin, azithromycin	MSSA
	Lincosamines	Linezolid	MRSA
	Streptogranins	Dalfopristin	MRSA
DNA synthesis inhibitors	Fluoroquinolones	Ciprofloxacin, levofloxacin	MSSA
RNA synthesis inhibitors	Rifamycins	Rifampin	MRSA
Antimetabolites	Sulfonamides	Sulfamethoxazole+ trimethoprim	MRSA

Table 1.2. Antibiotics used for treatment of *S. aureus* VAP. Most commonly used antimicrobial agents used in the treatment of MSSA and MRSA infections are listed.

Monoclonal antibodies have been increasingly introduced as an additional, relatively novel means of controlling infections in challenging or difficult to treat patient population, VAP patients included. Being administered alone or, more frequently, as an adjuvant therapy, the antibodies help opsonize the bacteria, neutralize their virulence factors and/or work synergistically with the antibiotics [140]. Monoclonal antibodies offer a number of advantages. First, they may help tackle the problem of MDR organisms, as immunoglobulins do not promote antibiotic resistance. Second, being an intrinsic part of the immune defense mechanisms, anti-infective antibodies are exceptionally well-tolerated and have a favorable side effect profile. Third, a lot of the bacterial pathogenic potential is exerted via the toxins, which can be bound and neutralized by the antibodies. In the ICU setting, high rates of the MDR pathogens may also translate into very limited antibiotic choice for a given patient, and the pathogen-specific antibodies' formulation may be a life-saving alternative or complement to the conventional therapy. In case of *S. aureus*, both its vast arsenal of extracellular toxins and its notorious resistance rates favor the use of monoclonal antibody therapy. Combining conventional antimicrobials and antibody therapy has shown synergistic effects and improved the survival rates in *S. aureus* pneumonia models as opposed to treatment with antibiotics alone. This favorable effect is thought to be achieved by reducing the inflammatory response and aiding the immune system in the pathogen clearance [140]. Not only therapeutic, but also prophylactic application of the monoclonal antibodies, discussed in the section below, is being increasingly studied and introduced in the clinical practice.

Prevention of *S. aureus* VAP. Seeing the devastating effects of VAP, a lot of efforts is concentrated around its prevention. Understanding of the disease pathogenesis and its risk factors helps develop the guidelines aimed at minimizing the VAP incidence. These measures, usually based on the local epidemiological data, make up a set of guidelines commonly referred to as the "VAP bundle". When implemented, these strategies were shown to reduce VAP incidence [141,142]. While varying across the countries and individual ICUs, the common strategies include but are not limited to: (1) keeping the patient in the semi-recumbent position; (2) regular subglottic suctioning; (3) oral care with an antiseptic agent like chlorhexidine; (4) maintaining the endotracheal cuff pressure at a >25mm Hg pressure; (5) early weaning trials and extubation. As outlined in the **Section I.4**, colonization by *S. aureus* has been linked to an increased risk of the subsequent infection. Many institutions therefore screen the patients for *S. aureus* carriage at the ICU admission. Most of the efforts, however, focus at the MRSA detection, as the initial therapy for a suspected lower respiratory tract infection in a MRSA-positive patient needs to include appropriate antimicrobial agents [143,144].

A number of published studies describe ventilator-associated tracheobronchitis (VAT) as an intermediate disease entity between the healthy state and/or asymptomatic colonization and the VAP. It is thought to affect between 1.4% and 18% of the ICU population [145–147] Briefly, it is defined as the condition fulfilling the VAP clinical criteria without the radiologic findings suggestive of pneumonia. Being considered a "precursor" of VAP, it could offer a possibility of intervention aimed at preventing VAP development. To date, however, VAT is still a relatively elusive clinical entity, and data on its clinical management and impact on VAP rates is conflicting, potentially due to ill-defined diagnostic criteria, or due to the reimbursement considerations (to date VAT, but not VAP, is reimbursed by most of the USA-based insurance companies) [148–150]. While being a challenging clinical entity and calling for extended research, VAT will not be discussed in details in this thesis, since the data collected in the course of the retrospective studies presented here is not sufficient for assessment of VAT.

Attempts to develop antibody-based means of *S. aureus* infection prevention, both in form of an active and a passive immunization, have been ongoing since as early as 1960 [151]. The following postulates, previously mentioned in the **Section I.4**, serve as the rationale for vaccine development: (1) anti-staphylococcal antibodies have been detected in the majority of the population, suggesting immunogenicity of the *S. aureus* virulence factors and their relevance in the *in vivo* environment; (2) higher titers of these antibodies have been described to have a protective effect in staphylococcal infection. Unfortunately, despite a robust approach to the vaccine development, as well as abundance of the published data on *S. aureus* microbiology, pathogenesis and host-pathogen interactions, none of the vaccines or monoclonal antibody (mAb) formulations tested to date have been successful in safely and effectively preventing staphylococcal infections in the clinical practice [152]. A number of reasons may explain this. First, majority of the vaccine candidates have been relying on a single staphylococcal molecule to elicit a protective response. *S. aureus*, being equipped with a large repertoire of the virulence factors, may be able to bypass neutralization of one of them and still exert its full pathogenic potential. Second, animal models used for the vaccine development are not ideal, and reaching the disease protection in animals does not necessarily translate into an equivalent protection in humans. Third, *S. aureus* is a bacterium characterized by an abundance of different strains with highly variable virulence profile and a varying degree of contribution of the individual virulence factors to the disease pathogenesis. This makes the development of a universal *S. aureus* vaccine extremely challenging. Finally, despite the years of extensive research, we still have a relatively limited understanding of the clinical correlates of protection, especially with regards to the levels of the antibodies and their targets [153]. For now, regrettably,

S. aureus remains a major human pathogen and a healthcare burden, with only a moderate progress being made in the field of its prevention and therapy.

I.7. OVERVIEW OF THE RESEARCH PROJECTS AND THESIS RATIONALE

Taking into consideration the marked variability of *S. aureus* epidemiological characteristics, we wanted to evaluate its prevalence in the ICU scene, both in terms of an asymptomatic bacterial carriage and a full-scale pulmonary infection. The progression of colonization to pneumonia was of particular interest, as its better understanding may help develop strategies of disease prevention. We aimed to assess the role of both the pathogen and the host in this process. From the pathogen side, we were interested in characteristics defining the success of the individual strains, and in factors influencing their toxin production – specifically, the antibiotics commonly employed in the ICU. From the side of the host, we focused on examining the serological characteristics of the critically ill population and evaluated the levels of immunoglobulins targeting *S. aureus* and its virulence factors in relation to the colonization state and the subsequent infection. Finally, seeing the high mortality and morbidity of the staphylococcal disease, and the potential complications following a delayed microbiological diagnosis of the disease, we addressed the often suboptimal traditional means of *S. aureus* detection by developing a point-of-care (POC) diagnostic tool.

Findings presented in this thesis are based on the four research projects, each discussed within a dedicated chapter. Epidemiology of the lower airway tract colonization and pulmonary infection in the ICU setting is described in **Chapter II** in form of an article previously published in BMC Infectious Diseases [154]. **Chapter III** focuses on the serological characteristics of the critically ill patients and their relationship with *S. aureus* colonization and infection. Data presented in **Chapter IV** describes the behavior of *S. aureus* – specifically, its production of the cytolytic exotoxins – upon its exposure to the sublytic concentrations of the antibiotics. Finally, in **Chapter V** we present a prototype of a point-of-care latex agglutination test aimed at a quick detection of *S. aureus* in the patient samples. Together, the projects presented in this thesis are aimed at expanding the understanding of the *S. aureus* pulmonary disease in the critically ill patients, and helping provide the clinical community with the means of predicting, preventing and combatting the staphylococcal infections.

II. ENDOTRACHEAL ASPIRATE BACTERIOLOGY AND VAP IN THE ICU

II.1 BACKGROUND

A retrospective observational study investigating the ETA bacteriology in a cohort of 250 critically ill, intubated and ventilated ICU patients was conducted by Arsanis GmbH (Vienna, Austria) and Lahey Hospital and Medical Center (MA, USA) between June 2014 and June 2015. Data obtained in the course of this study was used to address the prevalence of *S. aureus* lower respiratory tract colonization and pulmonary infection in the critically ill. The daily clinical records were used to retrospectively assess the patients for the presence of pulmonary infection according to modified Johanson criteria. ETA bacteriology data was then analyzed in the context of VAP and lower airway colonization. Our findings shed light on the ICU microbiological scene, and allow to evaluate the role of *S. aureus* in its context. Additionally, we were able to examine the effects of VAP on patients' outcomes, and to assess the role of the pathogens other than *S. aureus* in the pulmonary infections affecting the ICU patients.

Most important for the current thesis are, ultimately, the following findings: (1) colonizing and infecting pathogen were identical in the majority of recorded VAP cases; (2) *S. aureus* was the most prominent bacterial pathogen in the studied ICU population, accounting for the 56.8% of all isolated species, and detected in 21.2% of all VAP cases; (3) first detection of the bacterium occurred, on average, on the second day of MV, and heavy colonization of the lower airways by *S. aureus* was associated with higher chance of subsequent staphylococcal VAP. Importantly, there is a 4-day time window between the first detection of the bacterium and the onset of VAP symptoms, providing the clinicians with a possibility of therapy planning and implementation of preventative measures. The study presented in this chapter has been published in the BMC Infectious Diseases in August 2019, and the full-text version of the article is presented below.

Kabak et al. BMC Infectious Diseases (2019) 19:756
https://doi.org/10.1186/s12879-019-4367-7

BMC Infectious Diseases

RESEARCH ARTICLE Open Access

The utility of endotracheal aspirate bacteriology in identifying mechanically ventilated patients at risk for ventilator associated pneumonia: a single-center prospective observational study

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Abstract

Background: Ventilator-associated pneumonia (VAP) is a well-known, life-threatening disease that persists despite preventative measures and approved antibiotic therapies. This prospective observational study investigated bacterial airway colonization, and whether its detection and quantification in the endotracheal aspirate (ETA) is useful for identifying mechanically ventilated ICU patients who are at risk of developing VAP.

Methods: 240 patients admitted to 3 ICUs at the Lahey Hospital and Medical Center (Burlington, MA) between June 2014 and June 2015 and mechanically ventilated for > 2 days were included. ETA samples and clinical data were collected. Airway colonization was assessed, and subsequently categorized into "heavy" and "light" by semi-quantitative microbiological analysis of ETAs. VAP was diagnosed retrospectively by the study sponsor according to a pre-specified pneumonia definition.

Results: Pathogenic bacteria were isolated from ETAs of 125 patients. The most common species isolated was *S. aureus* (56.8%), followed by *K. pneumoniae*, *P. aeruginosa*, and *E. coli* (35.2% combined). VAP was diagnosed in 85 patients, 44 (51.7%) with no bacterial pathogen, 18 associated with *S. aureus* and 18 Gram-negative-only cases, and 5 associated with other Gram-positive or mixed species. A higher proportion of patients who were heavily colonized with *S. aureus* developed VAP (32.4%) associated with *S. aureus* compared to those lightly colonized (17.6%). The same tendency was seen for patients heavily and lightly colonized with Gram-negative pathogens (30.0 and 0.0%, respectively). Detection of *S. aureus* in the ETA preceded *S. aureus* VAP by approximately 4 days, while Gram-negative organisms were first detected 2.5 days prior to Gram-negative VAP. VAP was associated with significantly longer duration of mechanical ventilation and hospitalization regardless of microbiologic cause when compared to patients who did not develop VAP.

Conclusions: The overall VAP rate was 35%. Heavy tracheal colonization supported identification of patients at higher risk of developing a corresponding *S. aureus* or Gram-negative VAP. Detection of bacterial ETA-positivity tended to precede VAP.

Keywords: Ventilator-associated pneumonia, Endotracheal aspirate, *Staphylococcus aureus*, Gram-negative pathogens, Prospective study

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Background

Despite the approval of new antibiotics and the implementation of ventilator-associated pneumonia (VAP) prevention bundles, VAP remains a major concern and burden to the healthcare system, being associated with increased mortality, morbidity, hospital length of stay (LoS), and healthcare costs [1–4]. Several processes are implicated in VAP pathogenesis: aspiration of oropharyngeal secretions resulting from an altered state of consciousness, loss of natural protective mechanisms of the airways, and direct pathogen inoculation at the time of intubation [5]. Colonization of the airways by pathogens detected by routine bacterial cultures from endotracheal aspirate (ETA) may be useful to identify patients at increased risk for development of VAP [6].

In the case of VAP radiological and clinical signs, an ETA sample demonstrating high bacterial burden is considered confirmatory of microbial etiology according to recently issued guidelines from the Infectious Diseases Society of America [7]. *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Escherichia coli* are the most common pathogens associated with VAP [5]. These microorganisms are often multi-drug resistant (MDR) [8] and/or highly virulent, making early detection particularly important for initiating the appropriate treatment regimen [9–11].

The majority of reports focuses on the analysis of microbiologically confirmed VAP cases; less data are available on frequency and timing of airway colonization, and semi-quantitative culture methods, as well as their value for identification of patients at increased risk of developing VAP. The purpose of this study was to analyze semi-quantitatively cultured, serially collected ETA samples to better understand the association of bacterial colonization with progression to VAP in mechanically ventilated patients. We hypothesized that patients heavily colonized with pathogenic bacterial species in the trachea were more likely to develop culture-positive VAP during their ICU stay, and that heavier burden of colonization is associated with a higher risk of developing a bacterial VAP. VAP incidence in relation to bacterial airway colonization was examined as the primary outcome of the study. Secondary outcomes that were evaluated included the duration of mechanical ventilation (MV), ICU and hospital LoS, as well as all-cause mortality in patients with and without VAP.

Methods

Study population and data collection

A total of 250 mechanically ventilated patients admitted to two medical ICUs (MICU) and one surgical ICU (SICU) at the Lahey Hospital & Medical Center (Burlington, MA, USA) between June 2014 and June 2015 were included in this prospective observational study. Exclusion criteria included age below 18 years, and an expected length of MV of less than 48 h as judged by the treating physician (e.g., admission to the ICU from the operating theatre just for weaning, or moribund condition of the patient). Subjects exited the study upon extubation, tracheostomy, transfer to another facility, death, or initiation of "comfort measures only" protocol. Basic demographic data and the medical history were collected upon ICU admission from the patient directly, from the relatives with the help of collateral history taking, and/or from the available medical records. Hospital and ICU LoS, length of MV was recorded at the end of hospitalization, and diagnostic parameters, such as body temperature, white blood-cell count, and chest X-ray (CXR) readings were recorded daily during the ICU stay. Additionally, local epidemiology data on antibiotic susceptibility of the bacterial isolates, as well as empiric and prescribed antibiotic regimens were recorded.

All patient-related data were received in de-identified form. Informed consent was waived, since only leftover materials (otherwise discarded) were used, and no additional intervention or change in treatment plan was implemented. CXR was assessed by two independent, trained observers; in the case of non-unanimous judgement, the opinion of a senior radiologist was used as decisive. ETA samples were to be collected daily as part of the standard of care.

ETA samples were cultured and analyzed in the clinical microbiology laboratories of the Lahey Hospital and Medical Center. Bacterial species were determined by standard methods. Local rate of MDR pathogen isolation was not assessed separately for the ICUs included in the study. However, upon the commencement of the study global susceptibility rates of all isolates in the Lahey Hospital and Medical Center for the year preceding the study was provided. Multi-drug resistance was determined by demonstrating resistance to multiple antibiotic classes, with the conventional microbiological methods and Microscan® panels (latter used for all organisms except *P. aeruginosa*, where a disc diffusion test was employed), and/or detection of ESBL or carbapenemase activity. Alpha-hemolytic *Streptococcus* spp., apathogenic *Neisseria* spp., *Bacteroides* spp., *Fusobacterium* spp., *Spirochaetes*, and *Candida* spp. were considered as normal respiratory flora (NRF) [12]. Semi-quantitative microbiological analysis of ETA samples (SQ-ETA) was performed; samples were streaked onto appropriate agar culture plates in four consecutive quadrants. Presence of 5 or less colonies in the first quadrant corresponded to 1+ (rare), of 6 and more colonies in the first quadrant to 2+ (few), any amount of colonies in the first and second quadrants to 3+ (moderate), and in three consecutive or all 4 quadrants corresponded to 4+ (many). SQ-ETA

data were used to categorize bacterial burden as light (1+ and 2+) or heavy (3+ and 4+). Heavy colonization corresponded to approximately $\geq 10^5$ CFU/mL of ETA [13]. Purulent ETA samples were detected by light microscopy and defined as > 10 polymorphonuclear cells per low power field. Pathogenic bacterial species recovered from the ETA were shipped to the sponsor, and additional analyses (species confirmation and MRSA status assignment by genetic tests) were performed as described previously [14].

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and the local regulations; and was approved by the Lahey Hospital and Medical Center Institutional Review Board.

VAP diagnosis

Modified criteria outlined by Johanson et al. [15] were used for retrospective assignment of VAP by the sponsor and was defined by the presence of a new or progressive infiltrate determined by CXR, and at least two of the following clinical findings: (1) fever or hypothermia, (2) leukocytosis or leukopenia, and (3) presence of purulent respiratory secretions, all appearing > 2 days after initiation of MV. For this study, patients were defined as "progressing" to VAP if they had bacterial colonization and one or more episodes of VAP at any time during the study period. A bacterial pathogen was considered a potential causative agent of VAP if isolated from the ETA on the same day or within the 2 days preceding or following the detected of VAP clinical signs. In this study, both heavy and light ETA bacterial burden were accepted for determination of VAP bacterial etiology for the purpose of a more comprehensive overview. In selected patients, upon orders of the treating physicians, bronchoalveolar lavage (BAL) and/or non-bronchoscopic bronchoalveolar lavage (NBAL) was performed during the ICU stay. In order to avoid potential bias in this study, however, results of these tests were not included in the analysis, as they were not carried out routinely, were not limited to cases of suspected pneumonia (e.g. lung malignancy, interstitial lung disease) and were performed in less than 10% of the study population. No other nosocomial infections other than VAP was systematically assessed or recorded in this study.

Ventilator-associated tracheobronchitis (VAT) was not assessed as part of this study, as the authors believe that the retrospective diagnosis of VAT, in absence of documented radiological findings as in case of VAP, is most accurate when corroborated by the treating clinicians, which conflicted with the design of the study.

Antibiotic use in the studied ICUs

Selective digestive decontamination (SDD) was not administered as part of routine patient care. Upon suspected VAP, cefepime and vancomycin was administered empirically; if a patient was found to be negative for *S. aureus*, vancomycin was removed from the regimen, and, depending on recovered bacterial flora, cefepime was continued or changed to ceftriaxone or cefazolin. In case of cephalosporin allergy levofloxacin was administered.

Statistical analysis

Reported variables were grouped into continuous (e.g. age, MV duration, hospital LoS), and categorical values (e.g., gender, underlying disorders, all-cause mortality). Continuous variables were represented as mean with standard deviation, whereas categorical variables as percentage. Continuous variables were tested with Student's two-tailed unpaired t-test; categorical ones were organized into contingency tables and tested with Fisher's exact test. Statistical tests were performed with the Prism® 6.07 (GraphPad) software package. Level of statistical significance was set at p value ≤0.05.

Results

Patient demographics and ICU characteristics

Out of the 250 patients enrolled, 241 were mechanically ventilated for > 2 days; one additional patient was excluded due to missing clinical information crucial for analysis (Additional file 3: Fig. S1**,** Additional file 7). The average age among the remaining 240 patients was 64 years, and a slight male predominance was seen. The mean LoS and MV duration were 20 and 9 days, respectively. We addressed the potential differences in patient characteristics between those admitted to SICU and MICU, respectively. Significant differences were observed: patients in the SICU were hospitalized and ventilated significantly longer, but displayed significantly lower mortality rates compared to those in the MICU. Additionally, patients with respiratory failure were significantly more likely to be admitted to the MICU, whereas subjects requiring an emergency surgery or having a history of recent trauma – to the SICU, as expected by the ICU profiles. Other characteristics, including most frequently observed comorbidities, history of smoking, alcohol abuse, use of antibiotics and of immunosuppressive medications, did not differ significantly among the groups. VAP was detected in approximately one-third of the patients irrespective of the ICU type (Table 1).

Few clinical indicators such as clinical pulmonary infection score (CPIS) and disease severity scores could not be analyzed as some of the clinical parameters required for their calculation were not available, as they

Table 1 Patient demographics

Baseline variables*	All population ($n = 240$)	MICU ($n = 138$)	SICU ($n = 102$)	p value
Age, yr	63.9 ± 14.8	62.5 ± 14.7	65.9 ± 14.7	0.0752
BMI	30.2 ± 9.5	30.1 ± 9.3	30.4 ± 9.7	0.7875
Hospital LoS	19.6 ± 13.3	16.5 ± 10.4	23.8 ± 15.4	< 0.0001
Duration of MV	9.1** ± 5.1	8.5 ± 5.1	9.8** ± 5.1	0.0482
Male, % (*n*)	58.8 (141)	58.0 (80)	59.8 (61)	0.7923
All-cause mortality, % (*n*)	31.3 (75)	39.9 (55)	19.6 (20)	0.0062
SICU, % (*n*)	42.5 (102)	N/A	N /A	N/A
Emergency surgery, % (n)	30.4 (73)	8.0 (11)	60.8 (62)	< 0.0001
Trauma, % (*n*)	9.2 (22)	2.9 (4)	17.6 (18)	0.0001
Hypertension, % (*n*)	50.4 (121)	48.6 (67)	52.9 (54)	0.5165
Diabetes, % (*n*)	27.5 (66)	29.0 (40)	25.5 (26)	0.5627
Asthma, % (*n*)	7.5 (18)	8.0 (11)	6.9 (7)	0.8088
COPD, % (*n*)	17.1 (41)	16.7 (23)	17.6 (18)	0.8636
Chronic heart failure, % (*n*)	16.3 (39)	19.6 (27)	11.8 (12)	0.1146
Malignancy, % (*n*)	23.8 (57)	21.0 (29)	27.5 (28)	0.2837
Previous smoker, % (*n*)	26.7 (64)	26.1 (36)	27.5 (28)	0.8829
Current smoker, % (*n*)	18.3 (44)	21.7 (30)	13.7 (14)	0.1303
Alcohol abuse, % (*n*)	23.3 (56)	25.4 (35)	20.6 (21)	0.4416
Antibiotics in the last 90 days, % (*n*)	43.3 (104)	34.1 (47)	46.1 (47)	0.1774
Antibiotics upon admission, % (*n*)	15.0 (36)	15.9 (22)	13.7 (14)	0.7161
Immunoactive drugs***, % (*n*)	22.1 (53)	24.6 (34)	18.6 (19)	0.3450
Admission due to sepsis, % (*n*)	17.1 (41)	19.6 (27)	13.7 (14)	0.2982
Admission due to respiratory failure or hypoxia, % (*n*)	27.9 (67)	44.2 (61)	5.9 (6)	< 0.0001
VAP, % (*n*)	35.4 (85)	31.9 (44)	40.2 (41)	0.2193
S. aureus VAP, % (*n*)	7.5 (18)	5.8 (8)	9.8 (10)	1.000
Gram-negative VAP, % (*n*)	7.5 (18)	6.5 (9)	8.8 (9)	0.6215

*Results are presented as mean ± standard deviation for numerical, and as percentage with an absolute count (n) for categorical variables. For comparison of MICU and SICU groups, p-values were determined by unpaired two-tailed t-test for numerical data and two-tailed Fisher's exact test for categorical data. N/A = not applicable. **1 patient excluded since MV start date was not recorded. ***Including corticosteroids and chemotherapy; upon admission and/or in the last 90 days

were not recorded for every patient, or were recorded at different time points during the ICU stay.

Bacterial pathogens isolated from the ETA samples

Upon study commencement the most recent, yearly assessed global rates of local bacterial susceptibility to the antibiotics were provided, summarized and adapted for the purposes of the study to the organisms most commonly isolated from the ETA in Additional file 1: Table S1. ETA sampling frequency was approximately 2 out of every 3 MV days in the overall ICU population studied. The ETA microbiological culture results for patients were grouped into three main categories: yielding *S. aureus* ($n = 71$), Gram-negative bacteria (18 species; $n = 75$), or displaying no bacterial growth or only NRF ($n = 115$). 35.0% of the study subjects (84/240) yielded a single pathogen throughout the study, whereas 12.9% had two, and 4.2% yielded three or more pathogens. The most common bacterial species in ETAs was *S. aureus*, isolated from 29.6% of the study patients (71/240), and 56.8% (71/125) of patients with pathogen-positive ETAs (Additional file 9). 40.8% (29/71) of *S. aureus* positive patients carried MRSA. This was similar to the global MRSA rate (38%) reported for Lahey Hospital and Medical Center in the year preceding the study (Additional file 1: Table S1). The three most commonly detected Gram-negative pathogens, *K. pneumoniae*, *P. aeruginosa* and *E. coli,* were present in ETA samples of 18.3% (44/240), and 15 other Gram-negative species were detected in 22.1% (53/240) of patients (Table 2). 47.8, 43.8, and 42.7% of *S. aureus*, *K. pneumoniae*, and *P. aeruginosa* respectively were detected in the ETA by day 2 of MV. *E. coli*, *S. maltophilia*, *E. cloacae*, and *E. aerogenes* appeared later in the course of MV, with only

Table 2 Bacterial pathogens isolated from ETA

Bacterial species	*n*	% of all subjects (*n* = 240)	% of subjects carrying pathogen in ETA (*n* = 125)
Staphylococcus aureus	71	29.6	56.8
MRSA*	29	12.1	23.2
MSSA*	43	17.9	34.4
Klebsiella pneumoniae	17	7.1	13.6
Pseudomonas aeruginosa	14	5.8	11.2
Escherichia coli	13	5.4	10.4
Haemophilus influenzae	9	3.8	7.2
Enterobacter cloacae	7	2.9	5.6
Enterobacter aerogenes	6	2.5	4.8
Stenotrophomonas maltophilia	6	2.5	4.8
Serratia marcescens	5	2.1	4.0
Klebsiella oxytoca	4	1.7	3.2
Diphtheroid	4	1.7	3.2
Burkholderia cepacia	3	1.3	2.4
Moraxella spp.	3	1.3	2.4
Achromobacter xylosoxidans	2	0.8	1.6
Streptococcus pneumoniae	2	0.8	1.6
Acinetobacter baumannii	2	0.8	1.6
β-hemolytic Group F Streptococci	2	0.8	1.6
Proteus mirabilis	2	0.8	1.6
Serratia rubidaea	1	0.4	0.8
Acinetobacter lwoffii	1	0.4	0.8
Alcaligenes denitrificans	1	0.4	0.8
Streptococcus agalactiae	1	0.4	0.8
β-hemolytic Group C Streptococci	1	0.4	0.8
Citrobacter freundii	1	0.4	0.8

*Two subjects carried both MRSA and MSSA simultaneously. One of the 71 patients with *S. aureus* positive ETA excluded as the isolate was not tested for antibiotic susceptibility

17–33% of these species isolated within the first two MV days (Fig. 1).

ETA microbiology during VAP events

Eighty-five of the 240 study patients experienced VAP during MV (Additional file 8). In 41 (48.2%) of these patients, potential pathogenic bacteria were recovered from the ETA samples at the time of VAP diagnosis, ±2 days (Fig. 2A). *S. aureus* was isolated from 18 patients; however, in 7 of these cases Gram-negative pathogens, and in one case *S. pneumoniae* were also isolated (Fig. 2B, Additional file 2: Table S2, Additional file 4: Fig. S2, Additional file 5: Fig. S3). Twenty-seven patients (65.8%) had at least one Gram-negative pathogen, the most common ones being *P. aeruginosa*, *E. coli*, and *S. maltophilia* (Fig. 3 and Additional file 2: Table S2).

Both medical and surgical ICU had similar proportions of *S. aureus* and Gram-negative VAP cases (Table 1). In 51.8% of the VAP patients, no culturable bacteria (other than NRF) were detected in the ETA (Fig. 2A).

Association of airway colonization with subsequent pneumonia

Patients yielding ETA samples with *S. aureus* and Gram-negative (pooled) organism were divided into heavily or lightly colonized. Seventy-one of the 240 patients had at least one ETA sample positive for *S. aureus*: 37 were heavily colonized and 34 were lightly colonized. 12 of the 37 heavily colonized patients (32.4%) developed VAP associated with *S. aureus* during the VAP-relevant period, compared to only 6 out of 34 lightly colonized patients (Fig. 4). The difference between progression to

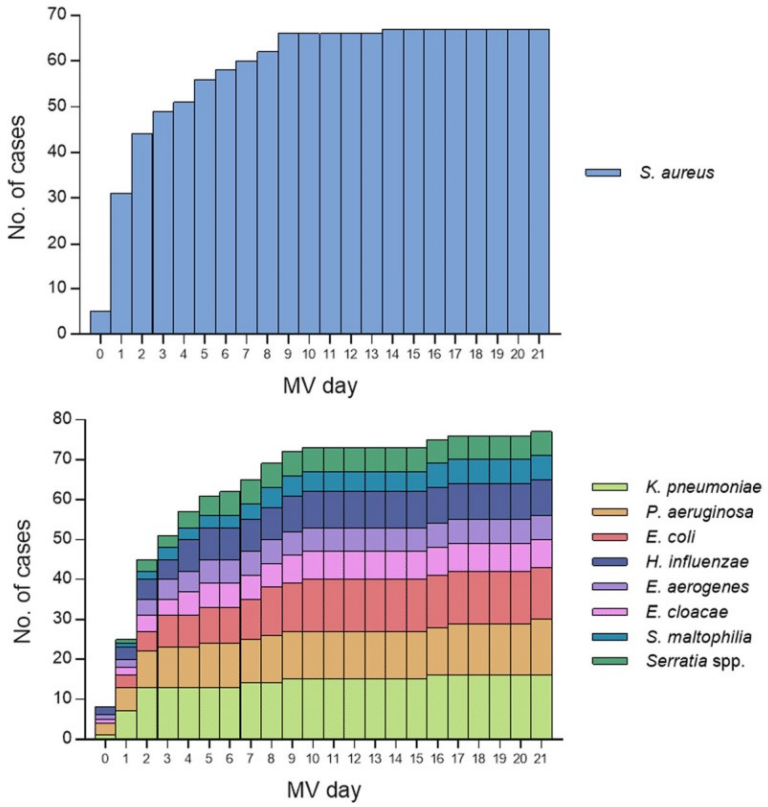

Fig. 1 First detection of *S. aureus* and the most common Gram-negative pathogens in ETA samples. 5 patients were excluded (no ETA collected during the first 3–5 days of MV or no MV start date recorded)

VAP from heavily colonized (32.4%) compared with lightly colonized (17.6%) did not reach statistical significance, possibly due to low sample size ($p = 0.1808$). Notably, out of the 18 total VAP cases with *S. aureus* present 15 yielded at least one ETA with only *S. aureus* at any time during the VAP-relevant period.

Of the 71 patients yielding *S. aureus* positive ETAs, 41 had MSSA, 27 MRSA, 2 had both, and for 1 patient the isolate was not received and its methicillin resistance could not be assessed. 48.6% (18/37) of the *S. aureus* from ETAs with heavy colonization were MSSA, and 67.6% (23/34) with light colonization were MSSA. Patients heavily colonized only with MSSA progressed to VAP in 44.4% (8/18) of cases, while only 23.5% of patients heavily colonized with MRSA did so (4/17, $p = 0.289$). In total, MSSA was implicated in 11 out of 18 VAP cases with *S. aureus* present, and 7 out of 10 where only *S. aureus* was isolated during the whole VAP-relevant period. Notably, in all *S. aureus* VAP patients colonizing and VAP strains had the same methicillin resistance status.

The first isolation of *S. aureus* in ETA occurred early during MV (median: 2 days) (Fig. 1), and no temporal difference was observed between MRSA and MSSA carrying patients. In patients who later developed *S. aureus*-only VAP (9 patients: 10 total, and 1 excluded due to insufficient ETA coverage prior to VAP event), detection of *S. aureus* in the ETA preceded VAP clinical symptoms by a median value of 4 days. Of these 9 patients, only 1 did not have an ETA positive for *S. aureus* prior to the *S. aureus*-only VAP event (Fig. 5**,** Additional file 4: Fig. S2).

Out of the 75 patients with ETA samples colonized with any Gram-negative pathogen, 60 were heavily colonized and 15 were lightly colonized. Eighteen of the 60 Gram-negative heavily colonized progressed to Gram-negative VAP (30.0%), while none (0.0%) of the 15 lightly colonized did so ($p = 0.0156$) (Fig. 3). In 94.4% (17/18) of Gram-negative VAP cases patients were colonized with at least one of the species causing the infection. On average, Gram-negative organisms were first detected in the ETA 2.5 days (median value) before the onset of VAP.

Impact of VAP on duration of ventilation, hospital stay and mortality

Regardless of presence of ETA colonization, patients with at least one VAP episode required MV support and hospital care for twice as long (mean 13.0 and

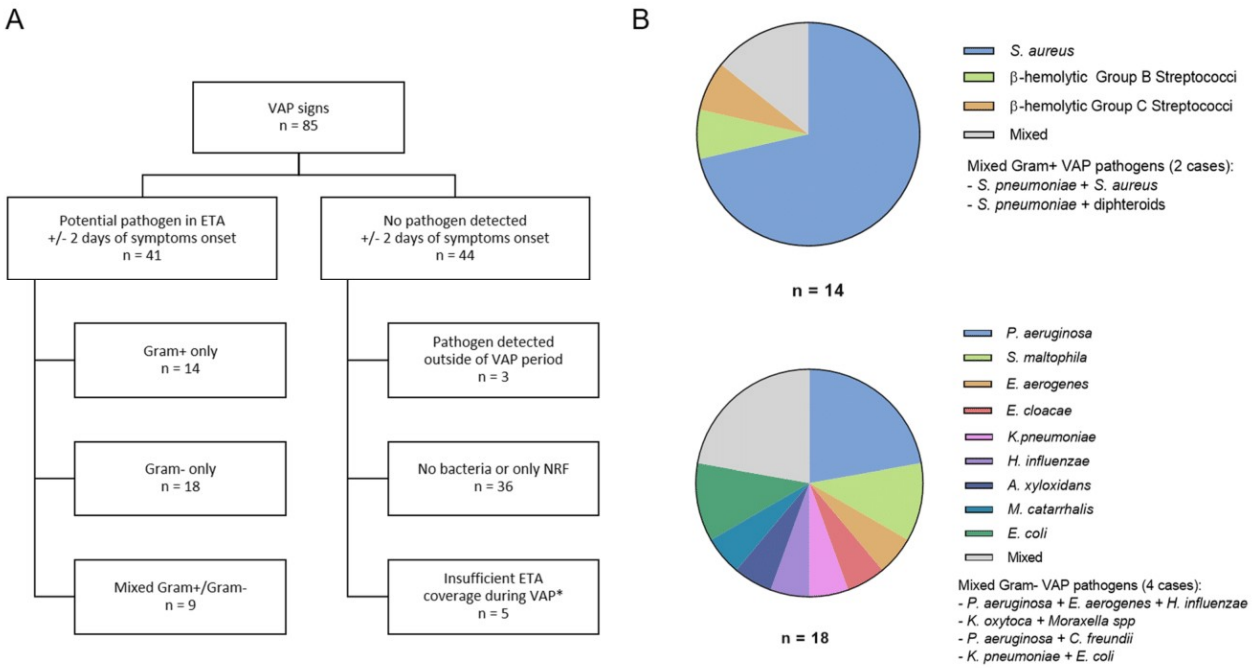

Fig. 2 ETA bacteriology and its association with VAP. **a**: Bacteriological characteristics of ETA samples obtained in the VAP relevant-period (defined as day where VAP was diagnosed ±2 days). *not sufficient ETA sample coverage: ETA samples obtained on < 50% of days during VAP-relevant period and not yielding any pathogens. **b:** Gram-positive and Gram-negative species isolated from ETAs in VAP-relevant period. All cases where multiple bacterial species were isolated are listed individually

16.6 days, respectively) as those who did not develop VAP (mean 6.9 and 25.1 days) (Fig. 6A). This trend was seen for all VAP patients regardless of the type of ICU (medical or surgical) (Fig. 6B-C), pathogen presence and causative bacterial species (Fig. 7A-D), with the exception of the Gram-negative VAP group, where no significant difference in hospital LoS was seen. Although statistical significance was not reached, notable observations regarding all-cause mortality were made. Whereas overall mortality rate between VAP and non-VAP patients were similar (Fig. 6A), an increase of approximately 10% was seen in VAP patients whenever a

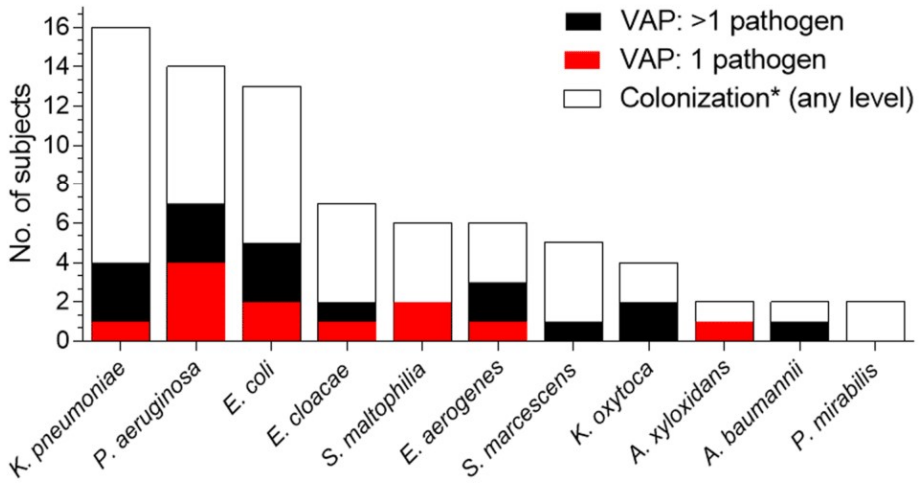

Fig. 3 Role of Gram-negative organisms in VAP and colonization**.** *Does not exclude VAP-positive subjects with no pathogens isolated in VAP-relevant period

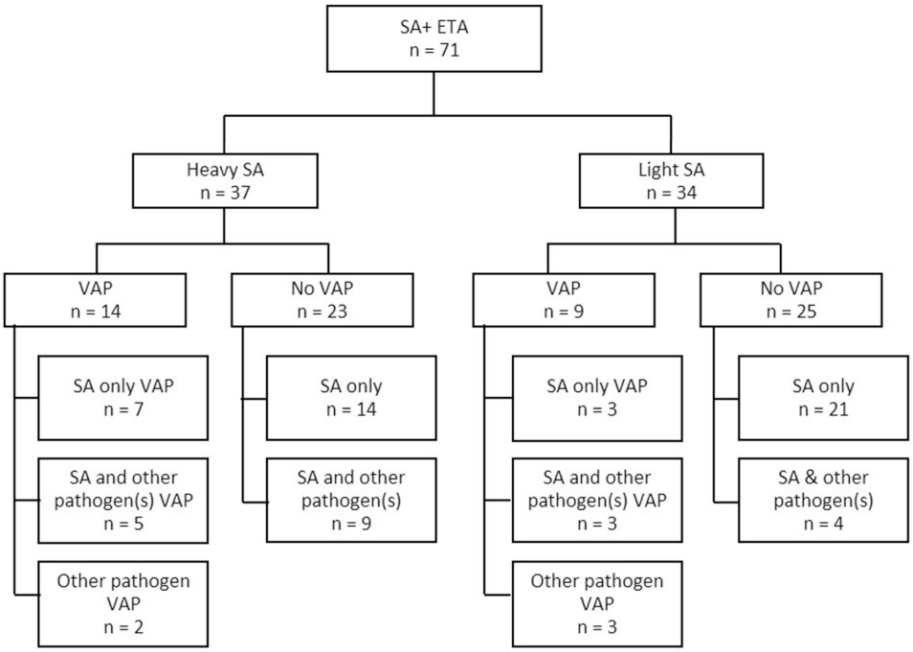

Fig. 4 VAP in patients demonstrating a heavy or low *S. aureus* burden in ETA. SA = *S. aureus*

pathogen was implicated (Fig. 7B-D). This was not true for the bacteria-negative/NRF VAP group, where VAP was paradoxically associated with a 10% decrease in mortality.

Interestingly, a temporal difference was observed between bacterial pathogen-positive VAP peaking in November–December, and bacterial pathogen-negative ones in the January–February period (Additional file 6: Fig. S4).

Discussion

In this study, we investigated the microbiology of upper airway colonization in ICU-admitted mechanically ventilated patients. We noted significant differences in MV duration, hospital LoS and mortality between the patients admitted to the surgical or medical ICUs. Similar observations on mortality rates have been reported [16]. Importantly, there were no significant differences in

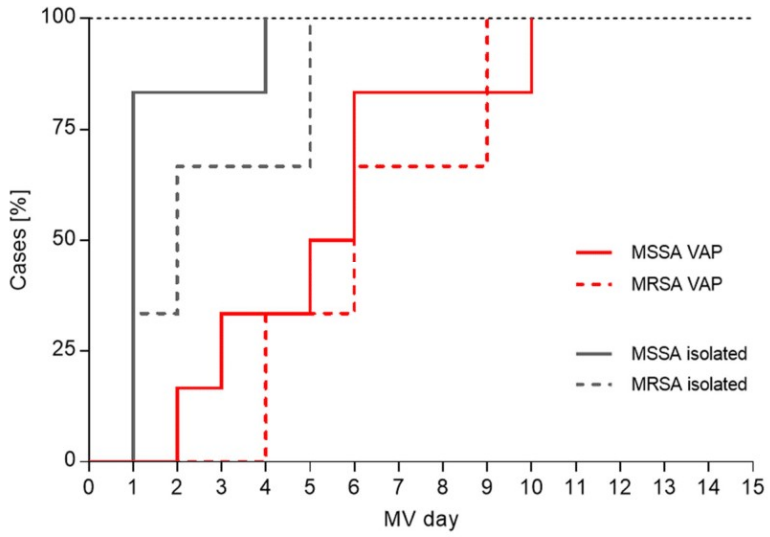

Fig. 5 First detection of *S. aureus* lower airway colonization and progression to *S. aureus* VAP. Cumulative curves of first *S. aureus* ETA colonization detection and first day of *S. aureus* monomicrobial VAP diagnosis shown against MV days. One of 10 *S. aureus* VAP subjects was excluded: no ETA was collected during the first 5 days of MV

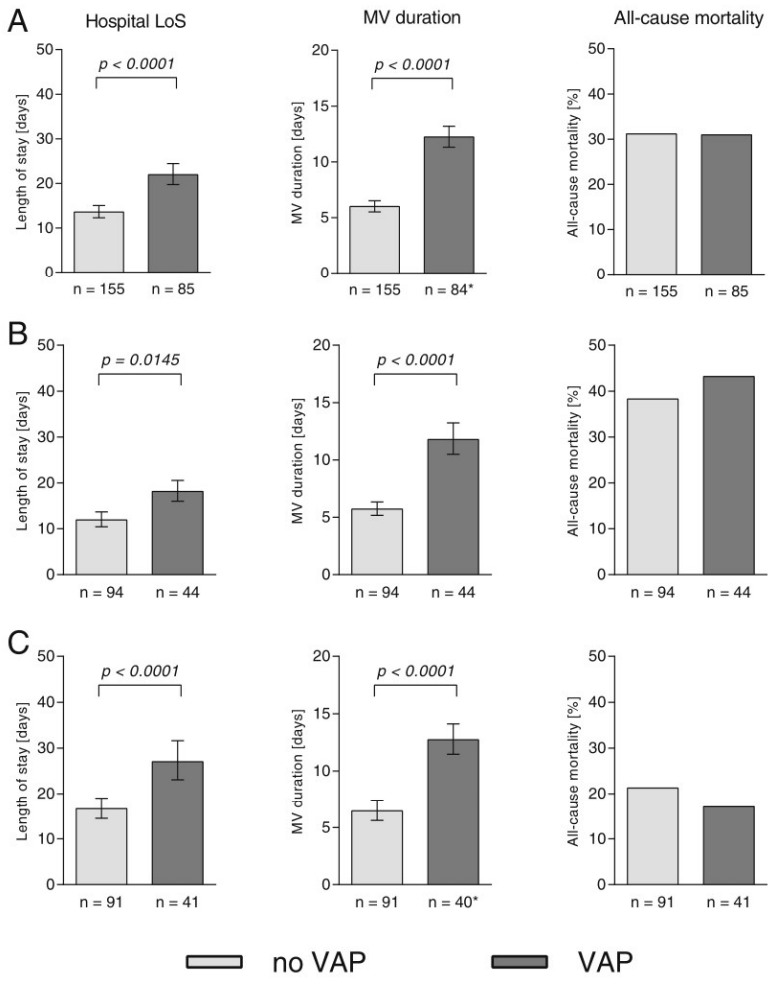

Fig. 6 Impact of VAP on hospital LoS, duration of MV, and all-cause mortality in study population. **a:** All subjects; **b:** subjects admitted to the medical ICUs; **c:** subjects admitted to the surgical ICU. Geometric mean +/− 95% confidence interval is shown. Significance, calculated by two-tailed unpaired t-test (hospital LoS, MV duration) or with Fisher's exact test (mortality) is indicated when *p*-value is < 0.05. *1 patient excluded since MV start date was not recorded

basic demographic parameters (age, gender, BMI, etc.) and in the clinical variables (comorbidities, smoking and alcohol history, etc.). This supports the generalization of our findings on the airway bacterial colonization and VAP incidence to the studied patient population. Approximately one third of all patients developed VAP. When assigning a pathogen to a VAP event, a two-day period prior to the clinical diagnosis has been suggested as clinically appropriate [17]. Patients categorized by ETA bacteriological analysis results as *S. aureus*-positive, yielding Gram-negative organisms, or no bacterial growth/NRF, all developed VAP at about the same rate, 25, 24, and 31%. The principal pathogens implicated in VAP as shown in this study are consistent with universally reported species [9, 10, 18], as is the proportion of VAP without bacterial association [2].

S. aureus was the most prominent individual pathogenic species isolated. Higher prevalence of *S. aureus* VAP in the United States compared to Europe has been widely published [19]. The *S. aureus* tracheal colonization rate (~ 30%) from this study was similar to that reported 5 years earlier in the same ICUs [14]. Data on the temporal pattern and onset of *S. aureus* tracheal colonization is limited; however, available studies report both MSSA and MRSA colonization appearing early during the MV period [20–22]. There was a tendency for a higher MSSA-to-MRSA ratio in VAP patients when compared to the ratio of patients colonized without pneumonia, which is consistent with our previous observation, and is in line with reports of MRSA isolates characterized with higher persistence, but lower virulence [14, 23]. Nearly half of the *S. aureus*-positive ETA samples were detected within the

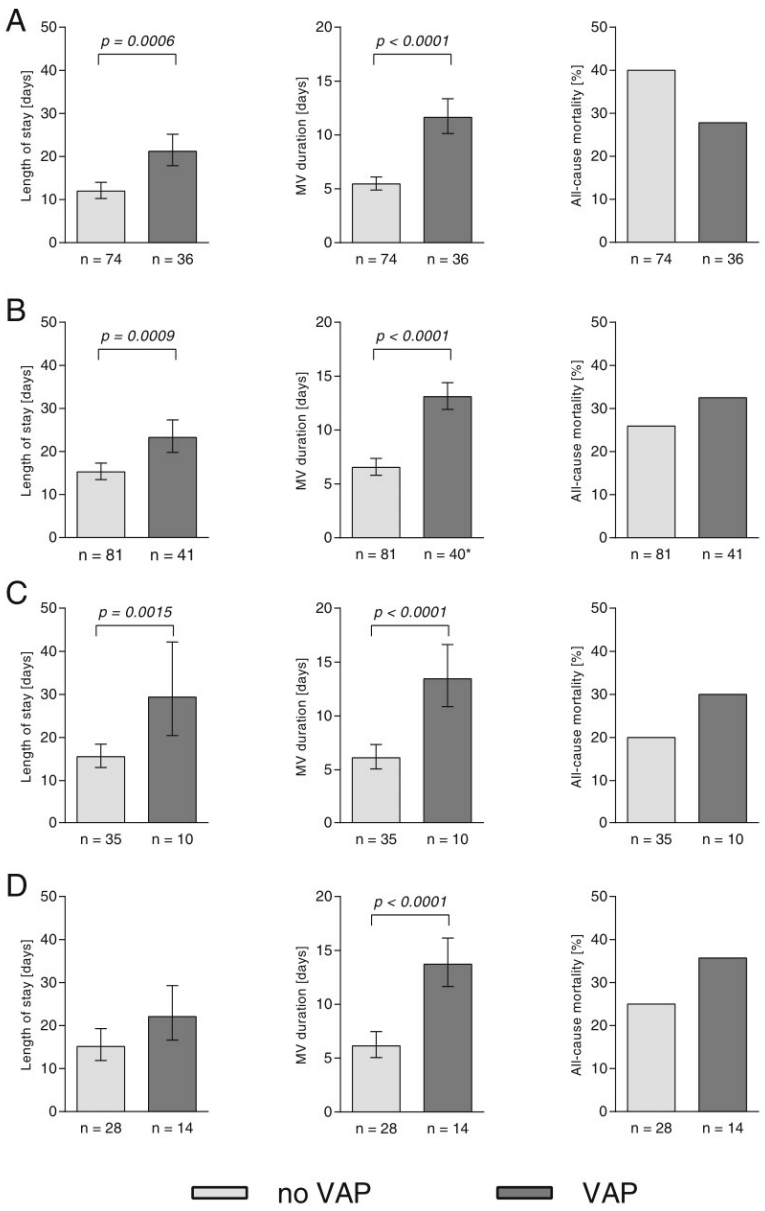

Fig. 7 Impact of VAP caused by different pathogens on hospital LoS, duration of MV, and all-cause mortality. **a:** Subjects with no bacteria recovered or only NRF in the ETA. 5 patients with insufficient ETA coverage during VAP, and 3 patients colonized with bacterial pathogens outside of VAP-relevant period, are excluded; **b:** subjects with any pathogenic species in the ETA; **c:** subjects positive for *S. aureus* in ETA, monomicrobial only; **d:** subjects positive for Gram-negative species in ETA, monomicrobial only. Geometric mean +/− 95% confidence interval is shown. Significance, calculated by two-tailed unpaired t-test (hospital LoS, MV duration) or with Fisher's exact test (mortality) is indicated when p-value is < 0.05. *1 patient excluded since MV start date was not recorded

first two days of mechanical ventilation, irrespective of methicillin resistance.

A major focus of this study was to evaluate whether differences in semi-quantitative bacterial ETA colonization could support the identification of patients who would more likely progress to VAP with the colonizing bacteria. We found that high ETA bacterial burden increases the likelihood of progression to bacterial VAP, as demonstrated by a significantly higher proportion of patients heavily colonized by Gram-negative bacteria progressing to VAP (30.0%) compared to those lightly colonized (0%). For *S. aureus* this difference did not reach statistical significance, although heavily colonized patients still progressed to *S. aureus* VAP more frequently (32.4%) than lightly colonized (17.6%). The detection of *S. aureus* in ETA preceded the onset of VAP by approximately 4 days, offering clinicians time to identify and characterize the bacterium and apply prophylactic measures. For

Gram-negative bacteria, however, this period was shorter, only 2.5 days. Although antibiotic intervention is not recommended as prophylaxis of VAP and has shown to be ineffective [24], the early choice of appropriate antibiotic therapy is expected to reduce LoS, MV duration, and mortality [25, 26]. Moreover, several pathogen-specific non-antibiotic approaches (such as monoclonal antibodies) are being evaluated in preemptive settings, mainly targeting *S. aureus*, but also MDR Gram-negative pathogens [27, 28]. Identifying patients at increased risk of pneumonia allows to focus clinical trial study populations on those patients that would most likely benefit from preemptive intervention.

We observed that heavy colonization by *S. aureus* based on the bacterial load in the ETA was associated with numerically higher VAP rate (32.4%) compared to light colonization (17.6%), when the ETA was also positive during the VAP event. However, similarly high rate (31.3%) of VAP was detected among those patients whose ETA samples were negative for potential bacterial species before VAP diagnosis. This was a surprising finding, and calls for possible explanations. For this study, only ETA samples were considered in assigning a bacterial pathogen to a VAP episode, and the information on deep respiratory sample (such as bronchoalveolar lavage or a protected specimen brush) microbiology was not included. This constitutes one of the limitations of the study, and may explain some of VAP episodes with no causative bacterial species identified. At the same time, apart from technical reasons (e.g., low bacterial inoculum size due to successful antibiotic therapy, suboptimal sampling), this may also be linked to non-bacterial pathogens such as viruses or fungi, not routinely cultured organisms (such as *Legionella pneumophila*), as well as pathogens not covered by the standard microbiological culture methods [5]. Some reports state that as many as 56% of the pathogens causing VAP were not identified by the standard microbiological methods, and suggested a higher complexity of VAP microbiology than currently thought [29]. Additionally, non-infectious lung diseases, and diseases with secondary lung involvement mimicking pneumonia need to be considered. A critically ill, multimorbid patient with signs of infection and chest X-ray infiltrate, but negative bacterial culture of airway secretions presents a diagnostic challenge, and requires a careful diagnostic workup by the treating physician to consider other conditions with clinical presentation similar to VAP [30]. Microbiologically non-confirmed VAP remains under-represented in the literature and needs to be addressed by further research, including.

In one fifth of all monomicrobial VAP cases, pathogens were present at low abundance (1+ or 2+ SQ-ETA) during the VAP-relevant period. Along with the scarce presence of bacterial cells in the respiratory tract, such results can also be explained by suboptimal or difficult sampling, or by the low number of viable bacterial cells as a result of antibiotic therapy, which is frequent in the ICU population. There is no consensus on how to interpret such cases. While targeted antibiotic therapy for VAP is only recommended after confirmatory qualitative respiratory sample with bacterial counts at or above diagnostic threshold [7], lower bacterial burden associated with VAP in certain patients (high clinical suspicion, deteriorating patient, absence of other infection source) cannot be ignored.

Other significant observations included hospital length of stay, time on mechanical ventilation, mortality, and time to onset of VAP. Importantly, significant differences in hospital LoS and MV duration were observed between patients with and without VAP, regardless of the detectability of bacterial pathogens in ETA samples. No significant differences were seen in all-cause mortality as a consequence of VAP, although there was a trend for approximately 10% higher mortality when an ETA bacterial pathogen was detected, and a 10% lower mortality when no bacterial cause was identified. Previous studies on bacterial VAP also reported approximately two-fold increase in MV duration and hospital LoS and significant mortality difference of ~10% (attributable mortality) [1, 3, 31]. We observed a temporal difference between the bacterial pathogen-positive and -negative VAP groups, with the former being detected in autumn, and the latter peaking in the winter period, indicating possible seasonality of both groups.

The limitations of the present study need to be acknowledged. First, although data and samples were prospectively collected, diagnosis of VAP was assigned retrospectively by the sponsor based on generally accepted criteria and not by the treating physicians' diagnosis. Second, the relatively small sample size, confined to a single medical center, limits the generalization of our data to other centers and ICUs. Moreover, analysis of the contribution of individual bacterial species other than *S. aureus* to VAP is limited due to low case number.

Conclusions

Currently available VAP prevention measures, while effective [4] are suboptimal and may be time consuming for nursing staff [32]. Antibiotic therapy has not demonstrated an effect on VAP prevention and therefore it is not recommended. Continuous SQ-ETA surveillance cultures from the start of mechanical ventilation for those patients expected to be ventilated for more than 2 days to identify bacterial pathogens may be useful to identify patients at high risk for bacterial VAP infection. Moreover, tracheal colonization precedes the clinical signs and symptoms of pneumonia by several days on

average providing a window of opportunity for VAP prevention. We found that approximately every third patient with heavy *S. aureus* or Gram-negative colonization in SQ-ETA progressed to VAP with a corresponding pathogen. At the same time, only every sixth patient lightly colonized with *S. aureus* progressed to VAP, and no patient with Gram-negative light colonization did so. This study provides prospective data to support ongoing efforts to identify high risk bacterial colonized patient populations to target and focus pneumonia preventive measures [33].

Additional files

Additional file 1: Table S1. Antibiotic susceptibility of pathogenic bacterial species isolated in Lahey Clinic**.** Global antibiotic susceptibility rates are depicted for the bacterial species most commonly (≥2.5% of all ETA species) isolated from the ETA. Data was collected in the period between 01.01.2013 and 31.12.2013; only 1 isolate per patient and only the first isolate was considered. (DOCX 15 kb)

Additional file 2: Table S2. Species isolated from ETA samples of patients with mixed Gram-positive and Gram-negative VAP episodes. (DOCX 13 kb)

Additional file 3: Figure S1. Disposition of the patients included in the analysis. (PDF 181 kb)

Additional file 4: Figure S2. Patients with *S. aureus* monomicrobial VAP episodes. *S. aureus* burden dynamics (shown as SQ-ETA readout) and VAP clinical diagnosis days with VAP-relevant period highlighted. Only those days when ETA was obtained and analyzed are shown on X-axis. (PDF 80 kb)

Additional file 5: Figure S3. Patients with *S. aureus* mixed infection VAP episodes. *S. aureus* and other pathogens are indicated as SQ-ETA readout and VAP clinical diagnosis days with VAP-relevant period highlighted. Only those days when ETA was obtained and analyzed are shown on X-axis. (PDF 74 kb)

Additional file 6: Figure S4. Temporal distribution of VAP cases during the study period. **A:** Number of cases detected shown against corresponding time period. **B:** Number of cases normalized against number of recruited patients in the corresponding time period. Study commenced in the middle of June 2014; therefore VAP cases were counted at intervals from 17th of the month 16th of the subsequent month. In the December–January period no bacterial VAP cases were observed, despite active recruitment of patients in the study. (PDF 22 kb)

Additional file 7: Demographic characteristic of the study population. (PDF 584 kb)

Additional file 8: Daily observations, VAP diagnosis and ETA culture results. (PDF 109 kb)

Additional file 9: Methicillin resistance of the received *S. aureus* isolates. The data for the entire study population presented. (PDF 34 kb)

Abbreviations

BAL: Bronchoalveolar lavage; CFU: Colony-forming unit; CXR: Chest X-ray; ETA: Endotracheal aspirate; ICU: Intensive care unit; LoS: Length of stay; MDR: Multi-drug resistant; MICU: Medical intensive care unit; MRSA: Methicillin-resistant *Staphylococcus aureus*; MSSA: Methicillin-sensitive *Staphylococcus aureus*; MV: Mechanical ventilation; NBAL: Non-bronchoscopic bronchoalveolar lavage; NRF: Normal respiratory flora; SA: *Staphylococcus aureus*; SICU: Surgical intensive care unit; SQ-ETA: Semi-quantitative endotracheal aspirate; VAP: Ventilator-associated pneumonia; VAT: Ventilator-associated tracheobronchitis

Acknowledgements

The authors would like to thank Donald Craven for his guidance throughout the study; Jawad Rashid, Caitllin Scopa, Ahsan Waqas, Maryam Khan, Syed Saquib, Carol Herbert for their assistance with data acquisition; Curtis Bakal for assisting with chest X-ray interpretation; Manuel Zerbs for his support with microbiological analysis, as well as all participating healthcare specialists for their critical review and input to the manuscript.

Author contributions

EK contributed to data analysis and interpretation, drafting the article, and its revision prior to submission. JH contributed to hypothesis, design and conduct of the study ZM contributed to the hypothesis and design of the study, and data interpretation. LS, VS, MG and CS contributed to data analysis and manuscript preparation EN contributed to the hypothesis and design of the study; data analysis, drafting the article, and revision. All authors have read and approved the final submitted manuscript.

Funding

Grant support for the work was received from the FFG (Österreichische Forschungsförderungsgesellschaft) Basisprogramm. The funding source had no role in the study design, data collection, analysis and interpretation of the results and manuscript preparation.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and the local regulations, and was approved by Lahey Hospital and Medical Center Institutional Review Board. Informed consent was waived, since only leftover materials, otherwise discarded, were used, and no additional intervention or change in treatment plan was implemented.

Consent for publication

Not applicable.

Competing interests

E. K., Z. M., L. S., V. S., E. N. and C.S. were employees and shareholders of Arsanis, Inc. (USA), the sponsor of this study, a biotechnology company that developed monoclonal antibody-based products targeting bacterial infections.

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Received: 9 February 2019 Accepted: 7 August 2019
Published online: 29 August 2019

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Publisher's Note

III. ANTISTAPHYLOCOCCAL ANTIBODIES IN THE ICU POPULATION

III.1. INTRODUCTION

Due to the ubiquity of *S. aureus* and its ability to cause both infection and colonization - persistent as well as transient - anti-staphylococcal immunoglobulins are detected in the majority of the population. These antibodies appear in serum early in the childhood and remain detectable throughout the individual's life [155]. Typically, presence of the pathogen-specific immunoglobulins is associated with protection against the infection or its more severe course. Even though most of the population is equipped with *S. aureus*-specific antibodies, the bacterium remains implicated in a range of diseases, and is an especially prominent cause of recurrent infections. On one hand, available data supports *S. aureus*-specific antibodies having host-protective properties: low levels of the anti-staphylococcal immunoglobulins have been described to predispose patients to ensuing *S. aureus* infections, with invasive disease seen in the bearers of the lowest titers [156]. Conversely, high antibody levels are associated with a more favorable outcome of the infection, and a lower incidence of staphylococcal sepsis and its serious complications [157,158]. On the other hand, patients colonized by *S. aureus* tend to display increased pathogen-specific titers expected to be protective [159], but are still at a higher risk of a *S. aureus* infection than the non-colonized [160]. At the same time, several reports suggest that patients carrying the bacterium tend to have better clinical outcomes of these infections [98,103], suggesting a potential protective role of the host immunoglobulins. The role of these circulating antibodies in the disease progression therefore remains to be fully elucidated. This is especially relevant for the critically ill patients, where *S. aureus*, as demonstrated in **Chapter II**, is one of the most common pathogens, responsible for a multitude of life-threatening infections. As described previously, VAP is a disease characterized by detrimental effects on the patients' outcomes [161–163]. Both nasal and tracheal colonization by the bacterium are known risk factors for the staphylococcal disease [154,164]. In the ventilated patients *S. aureus* isolated from the ETA was shown to be originating from the nasal cavity [165], and was associated with a subsequent pulmonary infection [166]. Exact mechanisms implicated in the progression of nasal to tracheal colonization are not fully understood, and the role of the strain characteristics, as well as the host *S. aureus*-specific immunoglobulins, in this process remains unclear. Despite our lack of knowledge in this field, numerous *S. aureus*-targeting vaccine candidates have relied on the induction of the protective antibodies by the host, or on their supplementation.

Specifically, staphylococcal pore-forming toxins (Hla and leukocidins) are an attractive target for immunotherapy and prophylaxis, as these molecules are thought to significantly contribute to *S. aureus* infection establishment [49,167], and the presence of intrinsic or supplemented toxin-specific antibodies was described to be protective [152,168–170]. Taking into consideration the conflicting evidence regarding the protective effect of the host anti-staphylococcal antibodies, gaining deeper insights into the host-bacterium interactions can help develop and improve the prophylactic and therapeutic modalities targeting *S. aureus*. In this project, we examined the endogenous anti-staphylococcal antibodies in a cohort of the ICU patients described in **Chapter II** and the healthy volunteers, and evaluated them in the context of *S. aureus* carriage status, characteristics of the obtained isolates and the subsequent staphylococcal infection.

III.2. MATERIALS AND METHODS

Study population and sample collection. Clinical records, nasal swabs, plasma and ETA samples were collected from 250 patients admitted to the ICU in Lahey Hospital and Medical Center and mechanically ventilated for >2 days, the same population described in **Chapter II** and the corresponding article [154]. Plasma samples were collected at the time of the subject study entry, and analyzed at Arsanis in whole cell-, culture supernatant- and toxin ELISA, as well as toxin neutralization assays described in the following sections. ETA samples were taken in an aseptic manner daily as standard of care, and nasal swabs were performed from both nostrils upon enrollment. Both samples were then processed using the standard laboratory methods in the Lahey Hospital and Medical Center. Isolated *S. aureus* strains were shipped to and analyzed by Arsanis. Due to the observational nature of the study, use of leftover sample material only, and lack of study-specific interventions, requirement of informed consent was waived. The study was approved by the Institutional Review Board of the Lahey Hospital and Medical Center. For the healthy controls, leftover pre-ASN100 administration serum samples of 52 healthy volunteers recruited in the course of the ASN100 (antibody mixture neutralizing six staphylococcal toxins) Phase I study (EudraCT#2015-003144-39) were used. The subjects' demographics are available in the corresponding publication [171].

Whole cell (WC), culture supernatant (CS) and toxin ELISA with human serum/plasma. For WC and CS ELISA, *S. aureus* TCH1516 (USA300) strain mutant lacking *spa* and *sbi* genes was grown in the RPMI medium supplemented with 1% casamino acids to the mid-log phase (defined by OD_{600nm} 0.5). For CS ELISA,

supernatant was collected by centrifugation of the bacterial culture, sterile filtered and diluted 150-fold in phosphate-buffered saline (PBS). For WC ELISA, bacterial pellet was washed twice and resuspended in PBS to yield an OD_{600nm} of 0.5, and then diluted 150-fold in PBS. For *S. aureus* toxin ELISA, coating with a 4 µg/mL in 0.1 M $NaCO_3$ of in-house produced recombinant Hla, HlgA, HlgB, HlgC, LukS, LukF, LukE LukD and LukGH was used. Diluted CS, whole bacterial cell suspension, and recombinant toxin dilutions were applied to Maxisorp plates (Nunc, Thermo Scientific) for an overnight coating at 4°C. After a blocking step, coated plates were incubated with plasma or serum samples serially diluted in PBS at room temperature for 2 hours, followed by detection with a goat F(ab´)2 anti-human IgG (H+L) HRP conjugate (Southern Biotech). Normal human serum (NHS, source: PAA/GE-Healthcare) was included as control in all experiments. Color reaction was developed using TMB substrate. Final values for WC and CS ELISA were obtained by correcting the lowest measured signal in the linear range (defined as OD 0.1 – 1.8 at 450 nm) by dilution factor. For toxin ELISA, patient plasma EC_{50} values were determined and normalized against normal human serum (NHS, source: PAA/GE-Healthcare).

***In vitro* toxin neutralization assays with human serum/plasma.** The toxin-neutralizing capacity of the subject's serum or plasma was assessed in an *in vitro* assay using A549 lung epithelial cells (human lung carcinoma ATCC CCL-185) for Hla, differentiated HL-60 (human myeloid leukemia cell line, ATCC CCL-240TM) for LukGH and LukSF, and human PMNs for HlgAB, HlgCB and LukED. Briefly, $5x10^3$ A549 cells per well in F12K medium (Gibco) supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich) were seeded 12-16 hours before the cytotoxicity assay. HL-60 cells were cultured in RPMI-1640 medium supplemented with 10% FCS (Sigma-Aldrich), L-Glutamine (Invitrogen) and Penicillin-Streptomycin (Gibco), further referred to as "PMN medium", and differentiated with N,N-dimethylformamide for at least 3 days. Successful differentiation was confirmed by flow cytometry. PMN cells were isolated from the fresh whole blood coming from healthy donors (obtained from Red Cross or in-house healthy volunteers) using Percoll Plus (GE Healthcare) gradient centrifugation, and their viability was confirmed with Trypan blue (Thermo Fisher Scientific) exclusion. Differentiated HL-60 cells and isolated PMN cells were suspended in PMN medium and added to 96-well plates at a density of $2.5x10^4$ cells per well. Serially diluted patients' plasma or serum was preincubated for 30 minutes with Hla diluted in F12K medium supplemented with 10% FCS, LukGH or equimolar mixtures of S- and F- components of LukSF, HlgAB, HlgCB and leukocidins in PMN medium, and applied to the cells. Plates were then incubated at 37°C, 5% CO_2 for 6 (A549 cells) or 4 hours (differentiated HL-60 and PMN cells). Cell viability was measured using the Cell Titer-Glo® Luminescent Cell Viability Assay Kit (Promega)

according to manufacturer's instructions on a Synergy™ HT Multi-Mode Microplate Reader (BioTek). After IC_{50} determination final values were expressed as percent cell death inhibition relative to the NHS.

Determination of IgG and albumin content in serum/plasma samples. The concentration of total IgG in the patient samples was measured using the commercially available IgG (Total) Human ELISA Kit (Invitrogen) as described in the kit protocol. Commercially available Human Albumin ELISA Kit (Abcam) was used to determine the albumin content in the patient samples according to the manufacturer's instructions. The readout was obtained using the Synergy™ HT Multi-Mode Microplate Reader (BioTek), and the final values were calculated based on the provided standard curve equations for the individual assays.

Microbiologic analysis of *S. aureus* isolates. Methicillin-resistance (MRSA status) of the received isolates was determined by differential agar plating. PCR was used for confirmation of MRSA status (detection of *mecA*) and for determination of LukSF positivity (detection of *lukSF-PV* genes). Spa- and MLST typing were performed according to the standard methods. For the hemolysis intensity evaluation, isolates were plated on sheep blood agar plates and incubated for 16-24 hours at 37°C. Cleared zones (complete hemolysis) was assessed macroscopically by four independent observers and the readout was expressed as an average semiquantitative (0 for no hemolysis to 4 for largest clearance area) score.

Preparation of bacterial supernatants. *S. aureus* isolates were pre-plated on the blood agar plates. One colony of the selected strain was added to the sterile RPMI supplemented with 1% Casamino acids (RPMI-CAS) and grown at 37°C, shaking, for 8 hours or until the stationary phase was reached (defined as OD_{600nm} change of <0.03 within 1 hour). Bacteria were pelleted by centrifigation, and the supernatants collected, sterile-filtered and stored at -80°C.

Assessment of toxin content in *S. aureus* supernatants. Hla and LukGH content in the *S. aureus* supernatants was quantified using the in-house developed ELISA. Briefly, 96-well plates (Nunc Maxisorp) were coated overnight at 4°C with 1 µg/ml Hla- and LukGH-specific monoclonal antibody diluted in PBS + Ca/Mg. After a blocking step, 5- and 50-fold diluted *S. aureus* supernatants and serially diluted recombinant toxin dilution were added (100 µl/well) and incubated at RT for 1 hour. As detection reagents, biotinylated ASN-1 monoclonal antibody (in-house developed antibody binding to Hla and S-components of HlgAB, LukSF and LukED) was used for Hla, and hyperimmune mouse serum for LukGH, followed by an application of the secondary antibody (streptavidin-HRP conjugate and anti-mouse-HRP, respectively). Colour

reaction was developed using TMB substrate. Hla and LukGH content were determined using an equation calculated from the standard curve.

Analysis of *S. aureus* supernatant toxicity. Plates with A549 and PMN cells were prepared as described above. Serially diluted supernatants in the sterile cell medium were applied to the cells and incubated at 37°C, 5% CO_2 for 6 (A549 cells) or 4 hours (PMN, HL-60 cells). Cell viability was measured using the Cell Titer-Glo® Luminescent Cell Viability Assay Kit (Promega), and results were expressed as percentage of cell viability in supernatant-treated relative to mock-treated wells.

Data analysis and statistics. Obtained raw data were analyzed using Prism 6 GraphPad software. EC50 and IC50 values were determined using non-linear regression plotting. Differences between the groups were analyzed by Mann-Whitney and Student's t-test. Correlation between age and serum characteristics was evaluated using the linear regression, with R-squared values examined. P values below 0.05 were considered significant. Correlation was assessed by Pearson correlation coefficient; r values are reported.

III.3. RESULTS

Patients colonized with *S. aureus* display higher anti-staphylococcal antibody titers than noncolonized subjects. Of the 250 patients included in the study, 9 were ventilated for less than two days, and one did not have clinical data sufficient for the purposes of the current project. From further 6 patients, plasma samples were not available or not collected (**Fig. S3.1**). Of the 234 patients included in the final dataset, 91 (38.9%) had *S. aureus* (nasal or ETA-derived) at any time during the study. Seventy-two subjects (30.8%) were nasally colonized, with majority of those (52 patients, 72.2%) also carrying the bacterium in the ETA (**Fig. S3.1**). To investigate how the bacterial presence correlated with the patients' anti-staphylococcal antibody profile, we determined the patients' plasma antibody levels against WC and CS, and their toxin neutralization capacity. Higher antibody titers were observed in *S. aureus* positive patients regardless of colonization site. With an exception of LukSF, geometric mean antibody titer and neutralization capacity stayed below 100% (defined by NHS neutralizing capacity and marked by "100" on the **Fig. 3.1**), even for the colonized patients (**Fig. 3.1**). Notably, significantly higher Hla neutralizing titers were observed in the cohort of the nasally and tracheally colonized subjects, as opposed to those who were only colonized in the nose. This suggests the importance of this toxin in the nasal-to-tracheal colonization progression. When comparing subjects who did not harbor *S. aureus* in the ETA, we observed significant differences between patients with and

without nasal colonization in neutralizing antibody titers against almost all examined toxins.

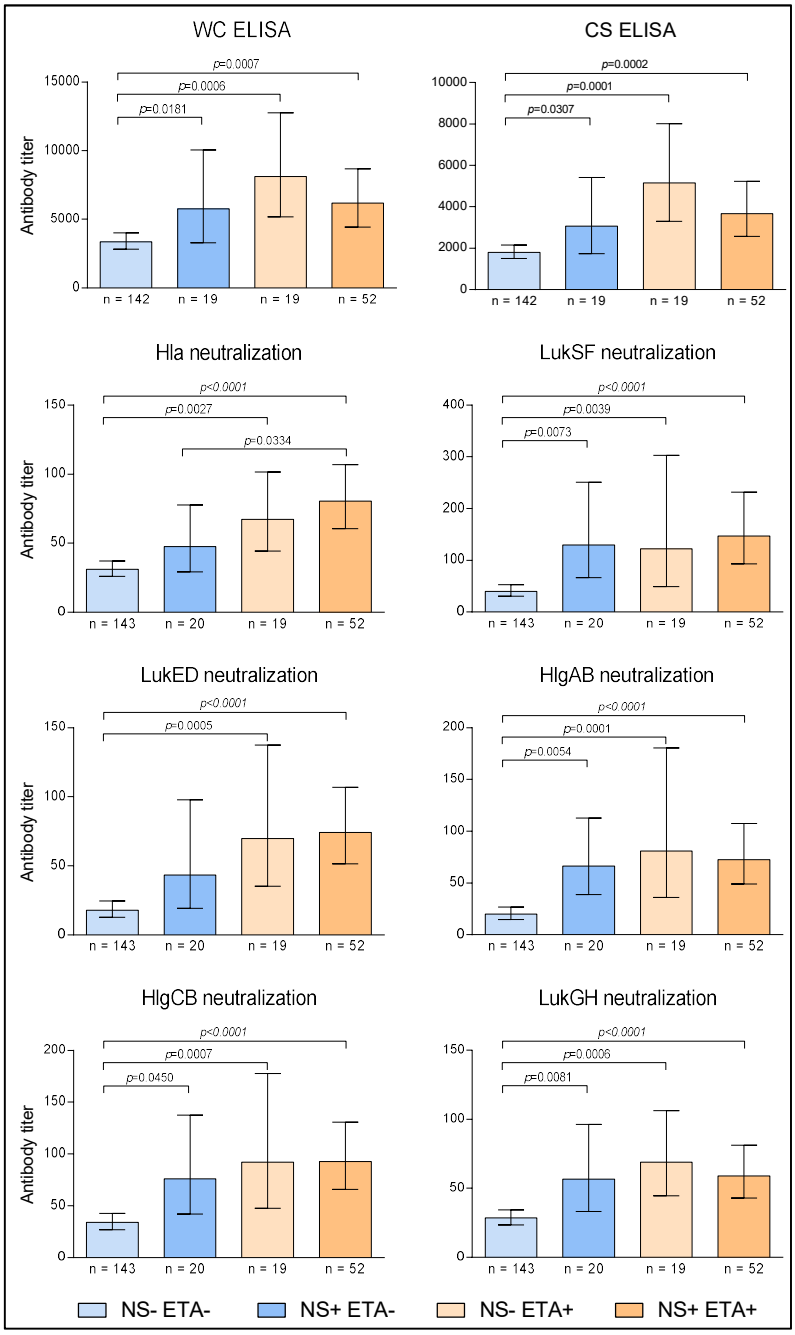

Fig. 3.1. Neutralization potential of the serum of patients colonized by *S. aureus* nasally and/or tracheally. *Antibody titer is expressed as % neutralization by the NHS. NS refers to the status of the nasal colonization by S. aureus (NS+: nasally colonized, NS-: not colonized nasally); ETA refers to whether S. aureus was isolated from the tracheal aspirate (ETA+: S. aureus was isolated; ETA-: S. aureus was not isolated). Geometric mean with 90% confidence interval is plotted.*

Importance of Hla in progression from nasal to tracheal colonization is supported by hemolysis intensity, supernatant toxicity and toxin quantification data. By macroscopically assessing the clearance zones around the colonies of nasal colonizing strains on sheep blood agar, we found that bearing a highly hemolytic *S. aureus* isolate in the nose was associated with higher likelihood of subsequent positivity for *S. aureus* in the ETA. In fact, 50% (9/18) of patients whose nasal isolate had hemolysis score below 1 did not develop ETA colonization. This percentage was 20% (2/10) if nasal isolate's hemolysis was between 1 and 2; 12% (3/25) – if between 2 and 3, and only 9% (3/33) – if above 3 (**Fig. S3.2**). To establish the link between hemolysis intensity and toxin production, supernatants were generated from the obtained nasal *S. aureus* strains. Concentrations of Hla and LukGH in the supernatants were determined. We observed that the hemolysis score correlated well with the amounts of Hla but not LukGH (**Fig. 3.2A**), indicating that macroscopic assessment of hemolysis intensity is accurately reflecting the levels of Hla expression in a low iron, *in-vivo* mimicking condition. Toxin concentrations (both Hla and LukGH), in turn, correlated with supernatants' toxicity in cell-based assays (**Fig. S3.3**).

Next, by using spa- and MLST-typing data of the *S. aureus* isolates, we created a collection of nasal strains spreading to the trachea or limited to the nose only. ETA isolate with the same spa- and MLST type as the nasal isolate of an individual patient was considered to be nasally derived. Analysis of the supernatants obtained from the tested *S. aureus* isolates has demonstrated significantly higher Hla content in supernatants of nasal strains that were later detected in the ETA than of those that were not. Statistical difference was not reached for the toxicity analysis in A549 assay (possibly due to a wide range of activities among a relatively low number of samples), although a noticeably higher mean EC_{50} of nasal strains migrating to the trachea was evident. Such difference has not been seen for LukGH, emphasizing the role of Hla in the tracheal colonization process (**Fig. 3.2B**).

Host antibody repertoire correlates with the toxin production profile of the residing *S. aureus* strain. When toxin neutralizing titers were compared to the hemolysis intensity of the nasal *S. aure*us strains, we observed that higher hemolysis score was associated with increased Hla neutralizing capacity of the host. Notably, patients harboring highly hemolytic nasal isolates also had significantly higher antibody titers against LukED, HlgAB and HlgCB compared to patients colonized with weakly hemolytic strains (**Fig. 3.3**).

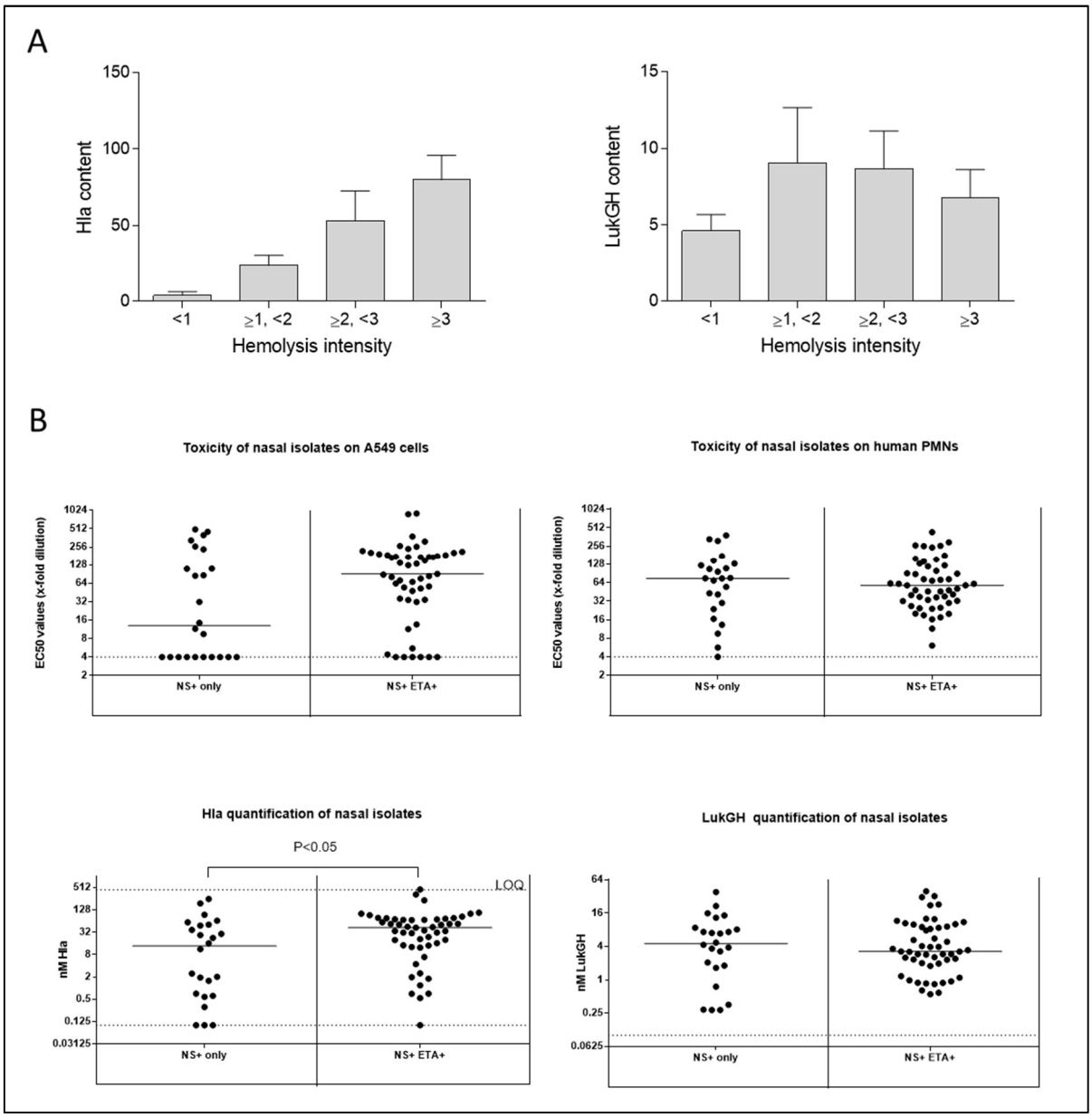

Fig. 3.2. Characteristics of the *S. aureus* nasal isolates. *A: Correlation between macroscopically assessed hemolysis intensity of the isolates and the toxin production (Hla and LukGH) determined by ELISA. B: Differences in bacterial supernatant toxicity and toxin production between nasally colonized patients with and without subsequent S. aureus detection in the ETA. NS+ only = patients with nasal colonization only. NS+ ETA+ = patients with nasal and tracheal colonization. LOQ = limit of quantification.*

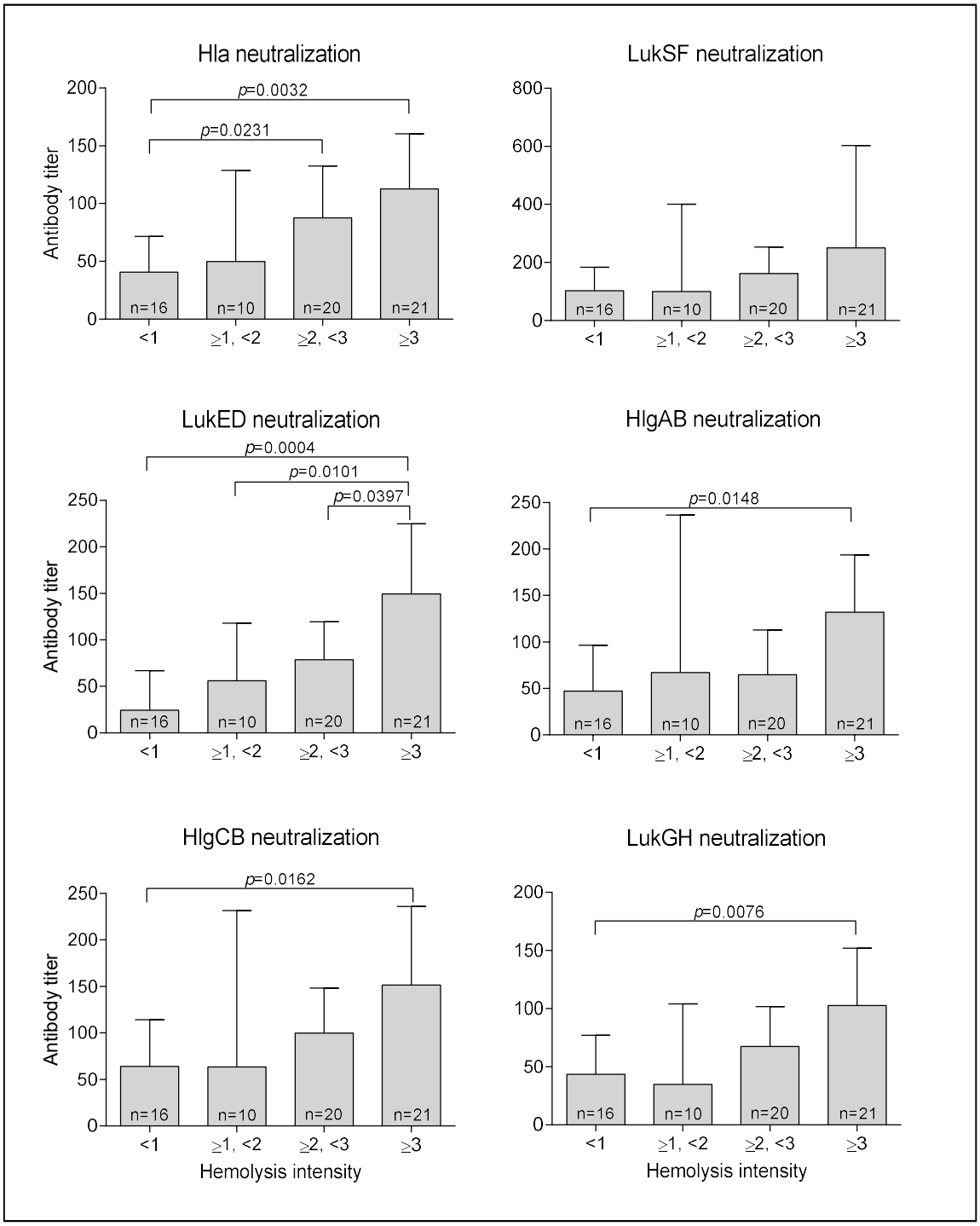

Fig. 3.3. Hemolysis intensity of the *S. aureus* isolates and anti-toxin antibody titers of the corresponding hosts. *Plotted are geometric mean with 95% confidence interval; "n" on the columns denotes the number of patients analyzed. In case of multiple isolates per patient, only one with highest hemolysis score was considered.*

Absence of the differences in the neutralizing capacity against LukSF in these groups may be explained by relatively rare *lukSF* gene carriage (~5%) in *S. aureus* isolates in the analysed population. Indeed, when patients were further categorized based on *lukSF*-positivity of the isolates, a significantly higher neutralizing capacity of the plasma against LukSF, as well as LukED, HlgAB and HlgCB, but not against Hla or LukGH, was observed in patients with *LukSF*-positive strains (**Fig. 3.4**).

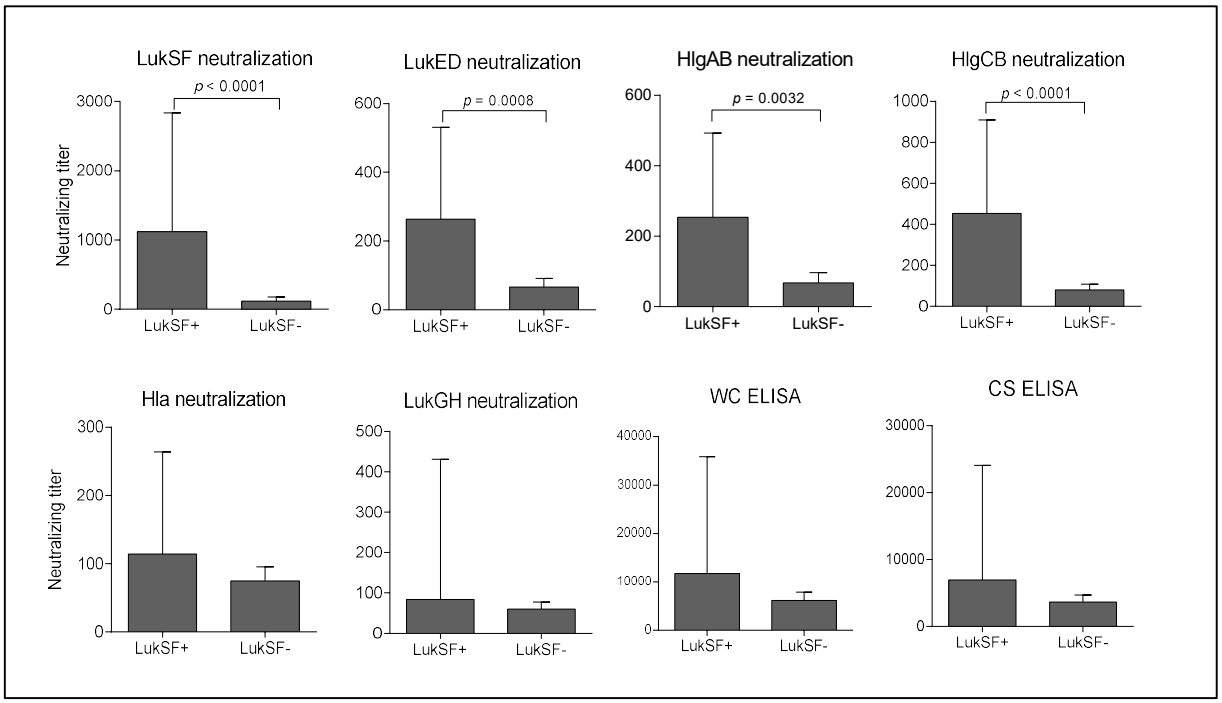

Fig. 3.4. Expression of LukSF by *S. aureus* isolates and anti-toxin antibody titers of the hosts. *Plotted are geometric mean values with 95% confidence interval. LukSF+: patients harboring LukSF-producing S. aureus strains (n = 7). LukSF-: patients harboring S. aureus negative for LukSF (n = 65). In case of multiple strains, patient was assessed in the LukSF-positive category as long as at least one of the isolates had the LukSF gene.*

Next, we investigated whether antibody titers differ between the groups of patients with MRSA or MSSA carriage. We found that MRSA-bearing patients had higher neutralizing titers against all toxins except Hla and LukGH. This was, however, explained by higher proportion of *lukSF*-positive isolates among MRSA: 6 patients with *lukSF*-positive MRSA strains were detected in our study, as opposed to only one with *lukSF*-positive MSSA. Indeed, when isolates were further subcategorized on the basis of their *LukSF*-genes carriage, antibody titers of patients carrying *LukSF*-negative

MRSA strains were not different from those having *LukSF*-negative MSSA (**Fig. S3.4, S3.5**).

Average antibody titers of *S. aureus* positive populations are manifold lower than the highest levels measured. To better understand the relevance of the observed higher titers in the colonized population and their link to the subsequent *S. aureus* infection, we separated the study population into three groups according to their colonization and infection status - *S. aureus* ETA-positive subjects with and without VAP, and *S. aureus* ETA-negative patients - and compared their antibody levels to the highest detected levels measured in the study. In comparison to the median values of *S. aureus* positive population, maximal detected titers were up to 5-fold higher by ELISA, and up to 10-fold by toxin neutralization assays. Relative to this range, differences between *S. aureus* positive VAP and non-VAP categories, and *S. aureus*-negative group were negligible. We can put our findings into a perspective by examining the values obtained from subject P053 (**Fig. 3.5A**). This patient was admitted in the ICU because of a documented *S. aureus* bacteremia, and is one of the few subjects in whom the responsible pathogen is defined at the time of the enrollment. The clinical data of this and other individual patients in the study, including those with the highest titers of anti-*S. aureus* antibodies, are available in the supplementary material of the publication presented in **Chapter II**. Already at the time of the admission the antibody levels of P053 in the WC ELISA were approximately 5-fold, and in the CS ELISA – 10-fold higher than the mean values of the patients with *S. aureus* VAP. Among the individual toxins, P053 was characterized by the nearly 5-fold higher neutralization titers against Hla than the *S. aureus* VAP patients' mean (**Fig. 3.5A)**. This offers one an insight into the potentially attainable antibody levels during an active infection. Unfortunately, P053 is the only patient with *S. aureus* infection already at the time of enrollment rather than developing one later, which limits our observations. Ideally, serum samples of the patients taken during an active *S. aureus* infection (e.g., *S. aureus* VAP) could be tested for the antistaphylococcal antibody titers and compared to those at the time of study enrollment. Unfortunately, because of the retrospective diagnosis of VAP, and limitations introduced by the study protocol (serum samples collected at enrollment and partially at study exit only, lack of insight into the detailed clinical course of the individual patients, observational nature of the study) this question should be addressed by the potential follow-up studies.

Another notable finding is a considerable variability among the antibody titers against the individual virulence factors (**Fig. 3.5B**). In this context, successful neutralization of single toxins by the host does not necessarily provide the patient with sufficient protection from the damage by other molecules. Seeing the amount of the virulence

factors *S. aureus* is equipped with, this may be one of the reasons why staphylococcal diseases remain recurrent and exceedingly difficult to treat and prevent.

Lower anti-*S. aureus* antibody titers in the ICU population are associated with lower total IgG and albumin levels. Geometric means of the neutralizing titers against most of the examined toxins in the ICU population failed to reach the level of the control (pooled NHS), even when only *S. aureus*-positive patients were considered. This was unlikely to be stemming from the reduced neutralizing capacity of the toxin-specific antibodies, since a strong correlation between levels of antibodies in toxin ELISA and their neutralizing capacity could be demonstrated (**Fig. S6**). To address this observation, we compared the plasma samples of the ICU patients to the sera of 52 healthy volunteers recruited in our Phase I study. Importantly, we confirmed the identical performance of the two sample types (plasma and serum) in the planned assays by testing the plasma and serum samples from the same healthy volunteers side by side (data not shown). Significantly lower neutralizing titers in the ICU population have been confirmed against all toxins except LukSF in comparison to the Phase I (healthy) population (**Fig. 3.6A**). To differentiate whether our findings were caused by a global or specific immunoglobulin deficit, we determined the total IgG concentrations in the critically ill. The ICU admitted population showed a marked hypogammaglobulinemia: only 31.7% had IgG within the normal range, compared to 92.3% of healthy subjects. (**Fig. 3.6B**) Importantly, statistical significance between the neutralizing antibody titers in healthy and ICU-admitted population was lost upon normalization of the titers to the total IgG concentration (**Fig. S3.7**). In addition to hypogammaglobulinemia, we observed lower albumin counts in the ICU population (**Fig. 3.6C**). Importantly, the two examined groups were not matched demographically; mean age constitutes one of the most considerable differences between the ICU and the Phase II subjects (64 and 31 years, respectively). To address the potential influence of the increasing age on the total immunoglobulin content and the serum neutralizing capacity against the individual toxins, we plotted the measured values against the age of the critically ill. No correlation between the examined parameters could be established, with maximal measured R-squared value of 5.3 (data not shown).

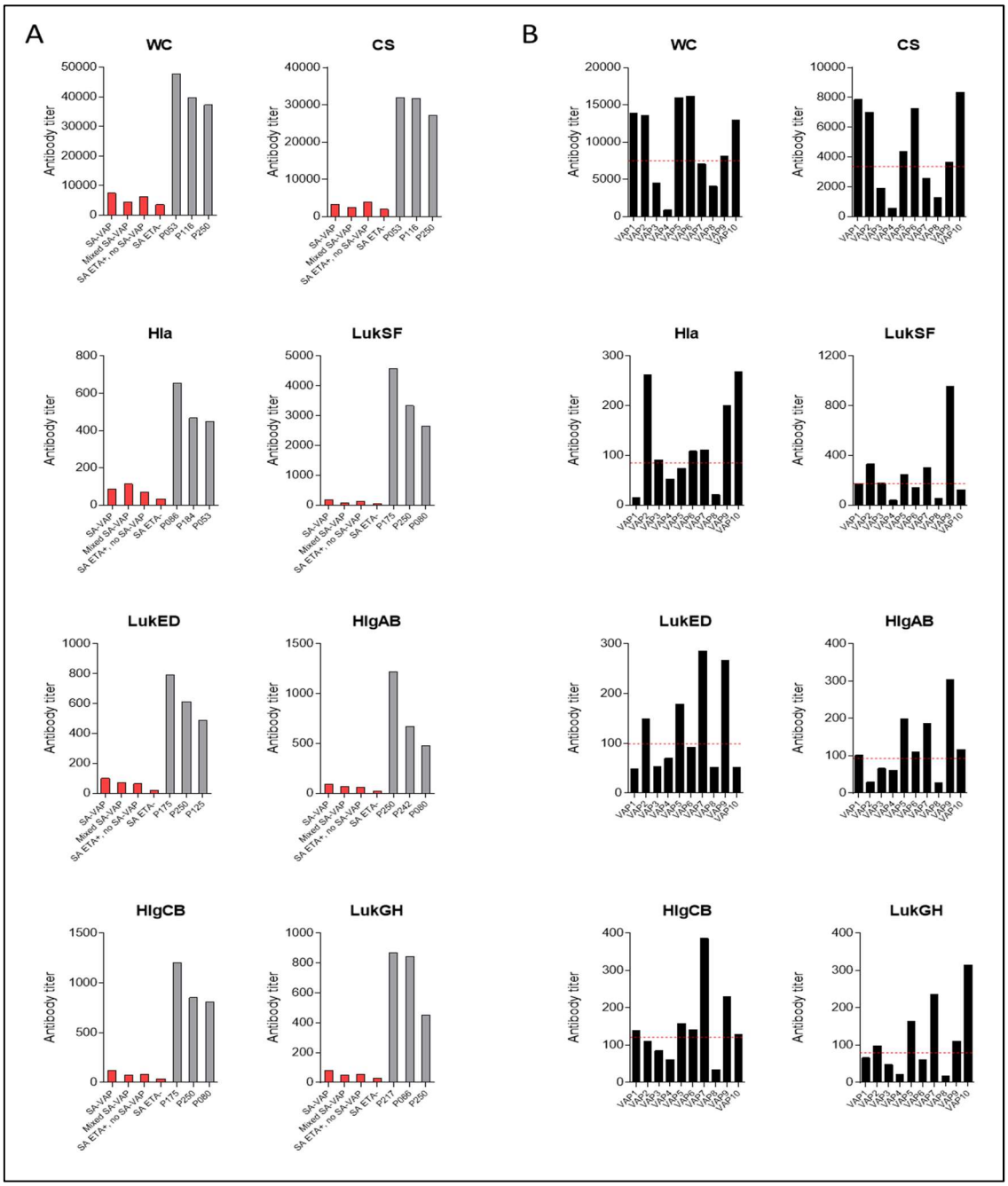

Fig. 3.5. Anti-*S. aureus* antibody titers in the ICU-admitted patients. A: *Mean values of the patient categories are shown in red. Individual patients' values are shown in gray. SA-VAP = Patients with S. aureus VAP. Mixed SA-VAP = patients with VAP caused by S. aureus and other bacterial species. SA-ETA+, no SA-VAP = patients positive for S. aureus in the ETA, but not developing SA-VAP. SA-ETA- = Patients negative for S. aureus in the ETA.* **B**: *Anti-S. aureus antibody titers in patients with SA-VAP. Mean values for SA-VAP group are shown with red dotted line.*

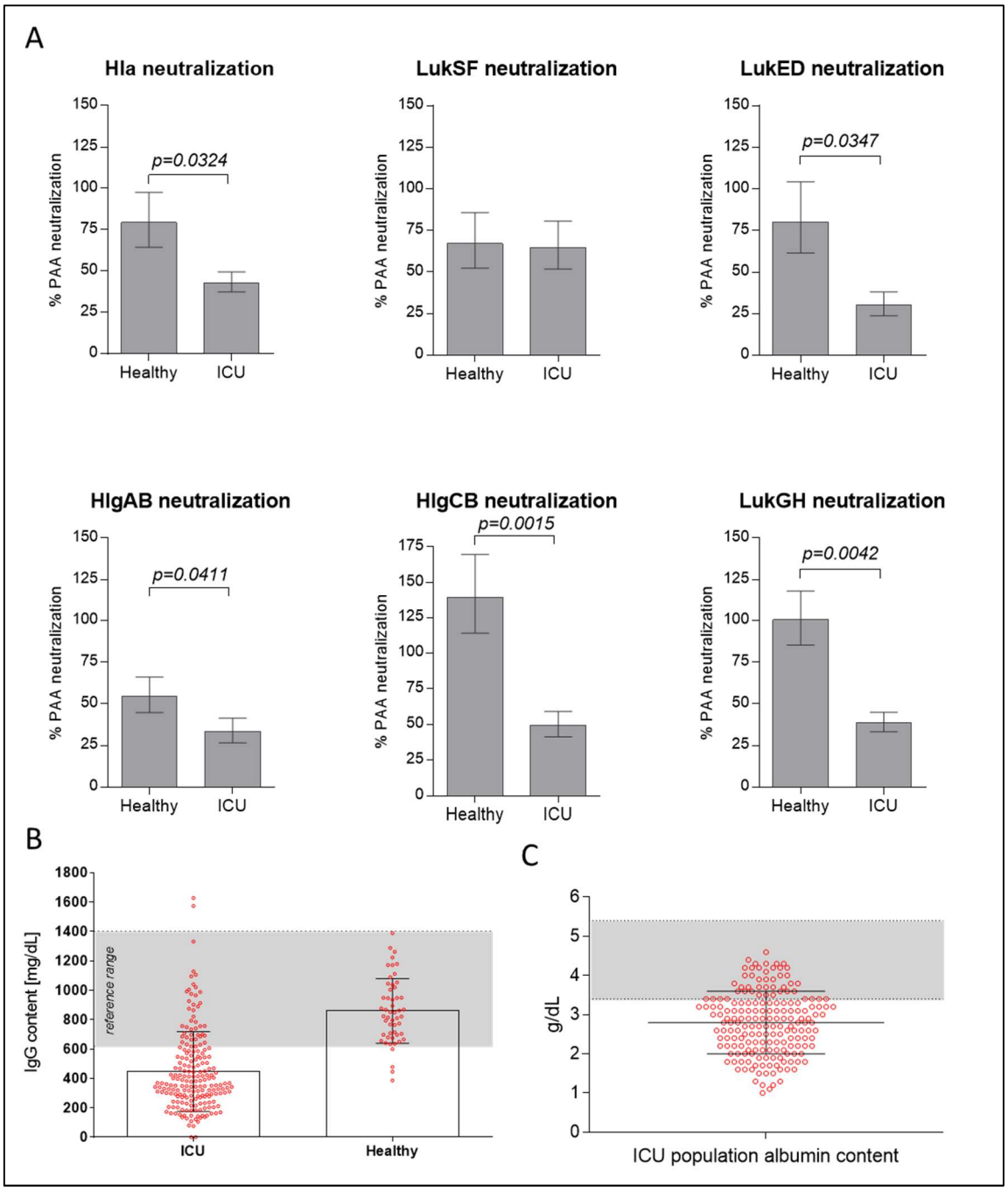

Fig. 3.6. Serological differences between ICU-admitted and healthy population. A: *Serum/plasma neutralization potential of the S. aureus pore-forming toxins. Plotted is geometric mean and 95% confidence interval.* **B**: *IgG levels in ICU and healthy population. Reference range is shown in gray. Plotted is mean with standard deviation.* **C**: *Albumin content in the ICU population. Reference range is shown in gray. Plotted is mean with standard deviation. PAA = pooled normal human serum (PAA/GE-Healthcare).*

III.4. DISCUSSION

Nasal *S. aureus* carriage predisposes for the subsequent tracheal colonization. In this project, a previously described cohort of ICU-admitted and mechanically ventilated critically ill patients was examined. In accordance with the universally reported rate [98], *S. aureus* was detected in the nasal swabs of approximately one-third of the examined population. Nasally colonized subjects were more likely to harbor *S. aureus* in their tracheal aspirates: this was demonstrated by 72.2% of nasally colonized patients developing tracheal colonization, as opposed to only 11.7% of those not nasally colonized. Published data has demonstrated matching molecular identity of the nasal and tracheal isolates [165], suggesting that the lower airway colonization originated from the nasal niche. In our study, similarly, the nasal-to-tracheal spread was confirmed by demonstrating same molecular identity of the isolates obtained from the nasal swab and from the ETA. Tracheal colonization by *S. aureus* is a known risk factor for the staphylococcal pulmonary infection [160,172]. In this context, screening the patients for nasal carriage of *S. aureus* is a reliable, easy and cost-effective way of identifying patients likely to develop tracheal colonization during their ICU stay.

Hla plays an important role in nasal-to-tracheal spread of colonization. Immunoglobulins against all of the tested *S. aureus* antigens could be detected in both the ICU-admitted and the healthy population. Presence of these antibodies supports the importance of the staphylococcal toxins in the establishment and progression of the infection, and confirms that these virulence factors are produced by the bacterium *in vivo*. Of the examined toxins, Hla is thought to play an especially important role in the bacterial invasion. A previously published report linked high hemolysis intensity of the MSSA strains to subsequent VAP development [173]. This current study complements this observation by showing that Hla activity is contributing to the nasal-to-tracheal spread of the bacterium. The following findings support this conclusion: (1) correlation between the higher hemolysis score of the nasal *S. aureus* isolates and the subsequent ETA positivity, and (2) presence of significantly higher Hla-neutralizing antibodies in the sera of nasally and tracheally colonized patients, as opposed to only nasally colonized. Both findings link the increased toxin production in situ to a higher antigen exposure and thus to increased specific antibody levels. The accuracy of macroscopic hemolysis assessment was demonstrated by the good correlation with Hla - but not LukGH - content in the bacterial culture supernatants of the respective strains. Concentrations of these toxins were, in turn, shown to correlate with the supernatant toxicity in cell-based assays.

Taken together, these findings lead us to conclude that assessment of the hemolysis intensity helps estimate the concentrations of Hla produced by the strain, both *in vitro* and *in vivo*. Latter is supported by demonstrating significantly higher Hla-neutralizing antibody titers in patients nasally and tracheally colonized (i.e., bearing more hemolytic isolates) as opposed to only nasally colonized. Importantly, this was not observed with the other toxins examined in this study. One of the most probable explanation for our observation is the increased direct lysis of the epithelial cells by the toxin, which results in loss of the barrier function. This, in turn, allows an unhindered spread of the bacterium in the host's body. Additional toxin activity, its interaction with other molecules, or other virulence factors may also be contributing the nasal-to-tracheal spread of colonization. The exact mechanism of this process, as well as the role of Hla in it remains to be addressed by future research.

Antibody titers of individual patients reflect the characteristics of the colonizing strain. Detecting increased Hla-neutralizing antibody titers in patients harboring the highly hemolytic isolates, as well as the overall higher immunoglobulin titers against all of the tested staphylococcal antigens may seem counter-intuitive since, based on the published data, presence of pre-existing antibodies targeting *S. aureus* is expected to help eliminate the bacterium and provide protection against the ensuing infection [104,174]. However, several findings allow us to speculate that a complex host-pathogen interplay is responsible for the observed increased levels of immunoglobulins, and the measured antibody titers are the result of being colonized with a particular strain, rather than a factor preventing colonization and/or infection in the first place.

Higher hemolysis intensity of the nasal isolate translated into increased neutralizing antibody titers against all toxins except LukSF. This can be explained by the fact that the leukocidins, being subject to the global regulation by *agr*, are co-expressed with Hla, and the higher hemolysis implies higher levels of the aforementioned leukocidins. Naturally, one may also attribute the increased titers of anti-LukED, -LukGH, -HlgAB and –HlgCB immunoglobulins to the cross-reactivity of the antibodies primarily targeting Hla, although one must take into consideration a comparatively low sequence homology between Hla and these toxins. Absence of correlation between hemolysis score and plasma LukSF neutralizing titers is linked to a relatively low (~5%) frequency of LukSF-positive isolated in our study. Significantly higher neutralizing titers against all toxins but Hla and LukGH were seen in patients yielding LukSF-positive isolates, compared to those harboring LukSF-negative ones. Hla and LukGH are less structurally similar to LukSF, as opposed to other leukocidins, indicating probable cross-reactivity of LukSF-neutralizing antibodies. Finally, maximally attainable titers

were much higher than those measured in the colonized population (discussed in the next section), which questions the protection level provided by the immunoglobulins detected in the *S. aureus*-bearing subjects.

Increased antibody titers seen in the colonized patients may be insufficient to protect from *S. aureus* infection. Patients carrying *S. aureus* demonstrated higher levels of circulating anti-staphylococcal antibodies measured in ELISA or neutralization assays. These antibodies, however, did not seem to translate into a better protection against *S. aureus* infection, as shown by increased VAP rates in this cohort. To date, however, there has not been defined a level or a composition of the *S. aureus*-specific immunoglobulins deemed protective. The maximal titers measured in our study were manifold higher than the mean values obtained from the colonized patients. This may suggest that an approximately two-fold difference between the colonized and non-colonized population, as well as colonized VAP-free and those with VAP, could be irrelevant. While the highest titers seen in our study in no way define levels needed to prevent subsequent infection, they give an impression of biologically attainable values. Interestingly, the bearer of the highest measured titers (P053) was a patient with a confirmed *S. aureus* bacteremia. Other patients whose individual values are reported had no data of a confirmed *S. aureus* systemic infection, and none of them had a *S. aureus* VAP. Patients developing *S. aureus* VAP show marked variability in the neutralizing titers against the single virulence factors. Even very high neutralizing antibody titers against the individual toxins may still be insufficient to prevent a staphylococcal infection. Absence of a uniform antibody coverage of the tested staphylococcal toxins could be stemming from the differences in the individual responses to the same antigen, or from the diversity of infecting and/or colonizing *S. aureus* strains. Because of this heterogeneous, incomplete antigen coverage, extensive offsetting of one toxin does not reliably prevent the infection, as the non-neutralized virulence factors continue to exert tissue damage and promote bacterial invasion. Apart from the suboptimal antibody profile, generally lower levels of anti-staphylococcal immunoglobulins in the ICU population may contribute to the increased infection susceptibility. Compared to the healthy subjects, critically ill showed significantly lower *S. aureus*-specific antibody levels against all tested antigens except LukSF. Importantly, the statistical significance disappeared when the obtained antibody titers were normalized against the individual total IgG count, suggesting that the hypogammaglobulinemia in the critically ill patients is global, and a selective deficiency of *S. aureus*-specific antibodies is unlikely.

Hypogammaglobulinemia is characteristic for the ICU population. Low IgG levels seen in the ICU admitted population is not uncommon. The pathogenesis of this

condition is thought to be multifactorial, and represents a global antibody deficit rather than absence of the antibodies targeting a specific antigen. Hypogammaglobulinemia has been associated with poor outcomes of the infectious diseases: low antibody titers are independent predictors of mortality in patients with sepsis [175,176] and CAP [177]. Interventions aimed at the restoration of this deficit have been employed, using both the intravenous immunoglobulins [178] and the specific antibody formulations [179]. IgG deficit is known to be caused by a range of diseases, and may also result from the increased antibody consumption, antibody loss, or massive hemodilution [180,181]. In our study, hypogammaglobulinemia was associated with a hypoalbuminemia. This could point both to an independent depletion of both the antibodies and albumin, as well as to the loss of the plasma as a whole. The latter can be caused by the increased loss of blood and its components, reduced production of the plasma proteins, and/or an aggressive volume expansion intervention resulting in hemodilution.

It needs to be noted that the two groups that were examined for immunoglobulin and albumin values were not matched demographically and/or epidemiologically. Most noticeably, the mean age of the healthy volunteers was considerably lower than that of the ICU population (31 versus 64 years old). Whereas the reference ranges for the normal IgG values are defined for adult population independent of the age, there is conflicting data on how the antibody content changes throughout life. Both age-dependent decrease and increase in the IgG levels have been described [182,183]. In our study, however, the link between the age and the IgG content or the antibodies' neutralizing capacity could not be established, suggesting that the observed difference is not stemming from the generally older subjects admitted to the ICU. The exact causes of this hypogammaglobulinemia in the critically ill, therefore, remains to be determined. Naturally, low antibody values alone are not sufficient to fully explain the increased susceptibility of the ICU patients to the staphylococcal infections. One needs to take into consideration other factors, e.g. the pre-existing conditions, reduced level of consciousness and the resulting loss of protective reflexes, invasive procedures disrupting the host barriers, use of the immunomodulators, immunoparalysis associated with the critical illness, and the hypervirulent and/or multidrug-resistant microbes found in the hospital environment.

This study allowed us to gain valuable insights into the serological correlates of *S. aureus* colonization and infection in the critically ill population, and to better understand the process of the staphylococcal infection establishment from perspective of the host. That being said, a number of limitations needs to be acknowledged. First, in spite of the convincing yet indirect evidence regarding the role of Hla in progression from the nasal to tracheal colonization, the exact mechanism of this process is to be dissected

and needs to be addressed by the future research. Second, because of the retrospective nature of the study, only limited clinical data was available for analysis, including that of the patients presenting with the highest *S. aureus* antibody titers. With an exception of P053 with a recorded MRSA bacteremia, it is not known whether patients suffered from any *S. aureus* infection other than VAP, and no diseases other than staphylococcal VAP were assessed. It is therefore unknown why some patients display such high antibody titers, and whether they are a factor protective against the staphylococcal infection, or the consequence of a previous or an active *S. aureus* disease. Third, the ICU patients and the healthy subjects were stemming from the geographically distinct areas with differing *S. aureus* epidemiology, and, as mentioned previously, were not matched demographically or epidemiologically. Although we used the universally accepted reference ranges of total IgG and serum albumin values, one needs to interpret the results of the comparison with care. Ideally, a study comparing the antibody levels of the critically ill subjects with a matched cohort should follow our findings up.

To sum up, we show that both total immunoglobulin levels and specific *S. aureus* antibody titers were deficient in the ICU population. Even the increased staphylococcal antibody levels measured in the colonized subjects were considerably lower than maximal titers attained in the study. These observations do not comprehensively explain the pathogenesis of the *S. aureus* infections in the critically ill, which remains a multifactorial process. Apart from the serological characteristics of the host, the following factors may contribute to the disease establishment and progression: (1) attributes of the infecting *S. aureus* strain, i.e. its virulence or means of immune evasion; (2) presence of the indwelling devices which serve as an entry port for the bacterium and promote formation of staphylococcal biofilm; (3) underlying conditions of the host influencing the effectiveness of the immune response; (4) administration of the immunosuppressive therapy; (5) inappropriate or inadequate antibiotic therapy; (6) impaired function of the immune system elements other than immunoglobulins (e.g., innate system cellular components, cytokines, T-cells). While most of these factors are difficult to modify, there is a possibility to correct the lack of anti-staphylococcal immunoglobulins by administration of *S. aureus*-targeting antibody formulations, specifically those targeting staphylococcal pore-forming toxins. Not only does their neutralization limit the direct cytopathic effect on the host tissues, it also enhances bacterial clearance by preventing the lysis of the host immune cells by the leukocidins. Over the years, numerous attempts to develop such therapeutic or prophylactic modalities have been made. One example is the ASN100, a formulation of two monoclonal antibodies collectively binding five staphylococcal toxins (Hla, HlgAB, HlgCB, LukED, LukGH and LukSF) developed by Arsanis GmbH [171]. It has been

investigated as an add-on to the conventional therapy to prevent the development of *S. aureus* VAP in the ICU-admitted and ventilated patients (ClinicalTrials.gov identifier NCT02940626). One of the distinguishing characteristics of this antibody cocktail is its ability to broadly and uniformly neutralize five of the cytolytic toxins. In this way, its therapeutic or prophylactic administration allows to compensate the deficient intrinsic immunoglobulin titers against single toxins. Although the phase II clinical trial of ASN100 was discontinued due to its projected failure to meet its primary endpoints (among other reasons, potentially because of the suboptimal study design), it demonstrated an novel approach of offsetting the multiple *S. aureus* virulence factors at once to improve the disease outcome [170]. A number of other antibody-based agents are being currently developed and investigated along with other novel modalities to combat the staphylococcal disease. Unfortunately, success has been moderate at best. As the medical community continues to rely on antibiotic therapy against an increasingly resistant pathogen, the medical need for the new therapeutic and prophylactic options grows.

IV. ENHANCEMENT OF *S. AUREUS* TOXICITY UPON EXPOSURE TO ANTIBIOTICS

IV.1. BACKGROUND

Both in the outpatient and inpatient environment, antibiotics are widely used for the treatment of the bacterial infections [184], and are also routinely administered as part of medical, pre-, peri- or postoperative prophylaxis, and as a treatment of some non-infectious disorders, although the latter is relatively rare nowadays [185]. In the ICU, antibiotics are the most commonly prescribed, often life-saving medications [186]. However, their excessive, indiscriminate and/or inappropriate use remains a major concern, as it worsens the patients outcomes and increases the antibiotic resistance rates [187,188]. This has led to the development of the antibiotic stewardship programs, aimed at optimizing and guiding the antimicrobial therapy. In terms of the antibiotic stewardship, ICU is an especially challenging environment, as the patients often harbor MDR organisms and may be in need of a prompt antimicrobial therapy before the causative pathogen is identified. It is therefore not surprising that the empirically administered antibiotic is often not the drug of choice against the responsible bacterium, is dosed incorrectly or is applied to the organism resistant against it. As mentioned in the **Chapter I.6**, late or inappropriate antimicrobial therapy is associated with worsened patient outcomes. This is especially relevant in case of a suspected *S. aureus* infection, as up to 60% of all isolates in the USA are methicillin-resistant [189], and the first-line empiric antibiotic regimens often include agents not effective against the MRSA. The antibiotics commonly used in the treatment of *S. aureus* infections have been summarized in the **Chapter I.6**.

Recent reports indicate that antimicrobials may have a role in regulating *S. aureus* virulence. Depending on the drug class, different antibiotics may increase or decrease exotoxin production by the bacterium [190]. Exposure to sub-lytic concentrations of the cell-wall active antibiotics such as beta-lactams results in increased Hla and LukSF production in both MSSA and MRSA strains. Protein synthesis inhibitors such as linezolid or clindamycin, on the other hand, have been shown to decrease the production of the aforementioned exotoxins [191–193]. Hla production was also shown to decrease in response to aminoglycosides and erythromycin, increase in response to fluoroquinolones and remain unchanged after treatment with glycopeptides [192]. With an exception of LukSF, data on the modulation of other leukocidins' production remains limited. The mechanisms of virulence modulation are not completely understood. It is assumed that decrease of toxin production in response to clindamycin

and linezolid is exerted by its mode of action, i.e. inhibition of ribosomal translation. This is supported by the fact that there has been little to no decrease in the levels of the toxin mRNA with a concomitant decrease of the final protein concentration [194,195]. Increase in the exotoxin production resulting from the exposure to the beta-lactams can be explained both by protein release upon bacterial cell wall lysis, and by the upregulation of the toxin expression on the gene level [193]. Several systems have been implicated in the observed response. The best-characterized mechanism of enhancing bacterial virulence suggests that PBP1 is inhibited by beta-lactams resulting in the modulation of *sarA* and *rot* global virulence regulators, which, in turn, triggers cell-wall turnover readjustment and upregulation of the toxins expression [190]. This virulence increase may have direct implications on the clinical management of staphylococcal infections. Improper antibiotic choice or its inappropriate dosing can result in increased toxin production and worsen patient outcomes. At the same time, appreciating the bacterial response to certain antibiotic classes can help selecting the effective drug with the potential to reduce the toxin production by the bacterium. Understanding the phenomenon of antibiotic-associated *S. aureus* virulence modulation could allow the clinicians to employ the anticipatory approach when administering the therapy. The potential increase of the toxin production could be curbed by appropriate supportive measures, or by administration of the toxin-specific agents like monoclonal antibodies.

Certain aspects remain underrepresented in the published studies. Among the strains examined in the literature, a considerable focus lies on the LukSF-producing MRSA strains, with a relatively little attention given to MSSA and the LukSF-negative isolates. Moreover, standard laboratory media for bacterial cultivation are often used, which fails to approximate the *in vivo* conditions. Finally, there is a considerable lack of data on the expression of the toxins other than Hla and LukSF. In the presented project, we address these issues by investigating the effect of the sublytic antibiotic concentrations on the toxin production by clinically-relevant *S. aureus* strains in the *in vivo*-mimicking conditions.

IV.2. Materials and methods

Bacterial strains and growth conditions. Eight *S. aureus* strains were selected for the experiments. To address the heterogeneity of the isolates encountered in the clinical practice, both MRSA and MSSA were included. Of each, two of LukSF-positive and –negative strains were selected. Of the MRSA, prototype strains of the epidemiologically relevant clonal lineages (USA100, USA200, USA300 and USA400)

were included. The same could not be done for the MSSA isolates because of the marked strain heterogeneity and the absence of well-defined global lineages. We included a LukSF-negative laboratory reference strain (Newman), as well as a livestock-derived MSSA H19, notable for expressing a variant LukGH toxin. Of the LukSF-positive MSSA, clinical isolates CC5 A960420 and CC121 HT20040111, were included. The strains used are summarized in **Table 4.1**. Bacterial growth medium used in the experiments included RPMI liquid medium (Gibco) supplemented by 1% casamino acids (further referred to as RPMI-CAS), as well as RPMI or RPMI-CAS supplemented by human albumin (further referred to as RPMI-Alb or RPMI-CAS-Alb, respectively).

Strain ID	Resistance profile	LukSF profile
USA400 MW2	MRSA	positive
USA300 TCH1516	MRSA	positive
USA200	MRSA	negative
USA100	MRSA	negative
Newman	MSSA	negative
H19	MSSA	negative
CC5 A960420	MSSA	positive
CC121 HT20040111	MSSA	positive

Table 4.1. Characteristics of the S. aureus strains used in the experiments.

Selected *S. aureus* strains were pre-plated on the sheep blood agar plates. Overnight cultures were prepared by suspending one bacterial colony in 50mL of RPMI-CAS, and incubating for at least 8 hours at 37°C, shaking at 200 rpm. To obtain the bacterial supernatants, overnight cultures were diluted 1:100 in the fresh medium and allowed to grow at 37°C, shaking at 200 rpm. The antibiotics were either added to the fresh medium before initiating the incubation, or spiked in into the bacterial suspension at the pre-defined time points after the start of incubation. Bacterial growth was documented by measuring the optical density at the 600nm wavelength (OD_{600nm}) at regular time intervals. The culture was considered to have reached the stationary phase after 8 hours of the incubation.

MIC determination. MIC values for the antibiotics used in the study (oxacillin, vancomycin, cefepime) were determined according to the standard protocol [196] with a single modification of using the RPMI-CAS as the growth medium. Briefly, an overnight culture of selected *S. aureus* strain in RPMI-CAS was diluted 1:100 in fresh medium, allowed to incubate (37°C, shaking at 200 rpm) until it reached the mid-log phase, defined as an OD_{600nm} 0.5 and corresponding to the concentration of

approximately $2x10^8$ CFU/mL, and placed on ice. The bacterial suspension was then diluted to yield a final concentration of $5x10^5$ CFU/mL, controlled by track dilution method on the sheep blood agar plates, and added to the fresh medium containing selected antibiotics in the predefined concentration range, followed by incubation (37°C, shaking at 200 rpm) for 24 hours. MIC was then determined as the lowest antibiotic concentration completely inhibiting the bacterial growth as assessed by naked eye.

Growing *S. aureus* in the presence of antibiotics and supernatant collection. Overnight *S. aureus* culture was produced as described before, and diluted 1:100 in the fresh bacterial medium. Antibiotic of choice was either added to the fresh medium before commencing the incubation, or added to the culture at a predefined time point (3 or 4 hours after the incubation start). Concentration of the antibiotics was based on the previously determined MIC values, and usually corresponded to 1/2, 1/4 or 1/8 of the MIC. Bacterial suspension with no added antibiotic was used as a control. The cultures were then incubated at 37˚C shaking at 200 rpm. Bacterial growth dynamics was recorded by measuring the optical density of the bacterial suspension at the 600nm. The cultures were placed on ice after 8 hours of incubation and spinned down in the centrifuge. The supernatants were collected, sterile-filtered using 0.1 μm Millipore sterile syringe filters (Millex) and stored at -80°C. Bacterial density was controlled by track-dilution method on the sheep blood agar plates: serial 10-fold dilutions of the suspension were performed in the 96-well plates to produce a total of 8 suspensions. 10 μl of the 4 last dilutions were plated onto blood agar plates (in triplicates), and the plates were incubated for 24 hours at 37˚C. The number of the CFU in each strip, corrected by the dilution factor, was then used to calculate the original bacterial density. To address the potential effect of antibiotics on the cell survival in the subsequent cell-based viability assays, sterile RPMI-CAS supplemented with antibiotics of choice was incubated for 8 hours, checked for absence of bacterial contamination by plating on the sheep blood agar plates, sterile-filtered and stored at -80°C. For positive-control solutions used in the cell killing assays, in-house produced Hla and LukGH recombinant toxins in the concentration of 100 nM were spiked in into the sterile antibiotic-containing medium.

Assays with human albumin. For the experiments involving the use of human albumin, commercially available solution of pooled human donor-derived albumin for medical use with the concentration of 20g/L was used (Albiomin, Biotest). To address for the possible presence of donor-derived immunoglobulins, we measured the concentration of IgG molecules in Albiomin using the commercially available IgG (Total) Human ELISA Kit (Invitrogen) according to the kit protocol. The albumin solution

was then cleared of donor-derived immunoglobulins by spA-coated resin. Briefly, the resin was added to the chromatography column, washed with 100 mM Tris (pH8.0) buffer and drained under gravity. Albumin solution was then added to the column, allowed to elude, and collected to be used for the preparation of the bacterial growth medium. Prior to adding the solution to the resin, an aliquot of the original albumin formulation was collected and stored. This sample and the immunoglobulin-depleted albumin solution were controlled for the presence of human IgG using the previously mentioned commercially available IgG (Total) Human ELISA Kit (Invitrogen). For the experiments involving the use of the buffer-exchanged albumin solution, the immunoglobulin-depleted albumin was added to a 10 kDA molecular weight cut-off dialysis cassette (Thermo Fisher), immersed in the PBS and dialyzed overnight at 4°C.

Testing the supernatant toxicity. Hla-sensitive A549 lung epithelial cells, LukGH- and LukSF-sensitive differentiated HL-60 (human myeloid leukemia cell line, ATCC CCL-240TM), and HlgAB-, HlgCB- and LukED-sensitive human PMNs were used for assessing the toxicity of produced *S. aureus* supernatants. The cell viability assays described in **Chapter III.2** were modified to omit the addition of the human serum. For the A549 assays, the cells were seeded at a density of $5x10^3$ A549 cells per well in F12K medium (Gibco) supplemented with 10% FCS (Sigma-Aldrich) and incubated for 12-16 hours. HL-60 cells were cultured in PMN medium (described in **Chapter III.2**), and differentiated with N,N-dimethylformamide for 3 days. Differentiation was confirmed by flow cytometry. PMN cells were isolated from fresh whole blood from the healthy donors using Percoll Plus (GE Healthcare) gradient centrifugation, with a subsequent confirmation of the cell viablitiy by Tryphan blue (Thermo Fisher Scientific) exclusion staining. Differentiated HL-60 or PMN cells were added to the 96-well plates at a density of $2.5x10^4$ cells/well. In separate 96-well plates, serial dilutions of the supernatants in the corresponding medium (F12K with 10% FCS for A549 assays, and PMN medium for HL-60 or PMN assays) were prepared and added to the plate (A549) or mixed with the cell suspension (HL-60/PMN). Twofold and threefold dilutions were used for A549 and HL-60/PMN assays, respectively. Following this, the plates were incubated at 37°C with 5% CO_2 for 6 hours (A549 assay) or 4 hours (HL-60/PMN). For the blank controls, cell-free wells were filled with assay medium only. For the mock controls, cells were treated with toxin-free assay medium. Cell viability was assessed using the Cell Titer-Glo® Luminescent Cell Viability Assay Kit (Promega) according to the manufacturer's instructions. Cell survival was expressed as percentage of the surviving cells relative to the mock control.

Preparing the bacterial lysates. To assess the presence of the intracellularly detained toxins, we prepared lysates of *S. aureus* TCH1516 and Newman strains. The

1:100 dilution of the overnight bacterial culture in the RPMI-CAS was incubated at 37°C, shaking at 200 rpm for 8 hours. The suspension was then placed on ice and centrifuged. Bacterial supernatant was sterile-filtered using the 0.1 µm Millipore sterile syringe filters (Millex) and stored at -80°C. Bacterial pellet was divided into 7ml aliquots, washed twice in ice-cold PBS and resuspended in 7ml or 3.5ml of PBS supplemented with a protease inhibitor cocktail (Roche). Bacterial cells were then lysed using 20 µg lysostaphin (Sigma Aldrich) pro pellet at 37°C for 45 minutes. Lysis effectiveness, defined as no growth or <10 solitary colonies at the 10^6-fold dilution of the bacterial lysate, was confirmed by plating the lysates on the sheep blood agar plates. The lysate preparations were then spinned down; the soluble fraction was collected, sterile filtered using the 0.1 µm sterile syringe filters and stored at -80°C.

Data analysis. Collected data was analyzed using the Prism 6 GraphPad software. Data from the cell-based toxicity assays was plotted using non-linear regression, and supernatant toxicity was expressed by the EC50 values.

IV.3. RESULTS

Exposure of *S. aureus* to certain antibiotics results in the increase of supernatant toxicity. To rule out the influence of the antibiotics on cell survival in the cell-based assays, we applied the antimicrobials in the concentrations used in the subsequent experiments to the A549, HL-60 and PMN cells, allowed to incubate for the assay-defined time period, and demonstrated that the cell viability was not affected. However, when the recombinant toxins were added to the sterile culture medium with the same antibiotic concentrations, toxin concentration-dependent cell killing was demonstrated (data not shown). The preselected *S. aureus* strains were then incubated with the clinically relevant antibiotics (vancomycin, oxacillin, cefepime) at the MIC, 1/2 MIC, 1/4 MIC and 1/8 MIC concentrations. Supernatants collected from the cultures displaying growth dynamics similar to the no-antibiotic control (i.e., reaching the stationary phase with no significant delay) were then tested in the cell-based viability assays. We observed an up to 5.5-fold increase of the supernatant toxicity when *S. aureus* was grown with oxacillin and cefepime, but not when grown in the presence of vancomycin. The magnitude of the observed effect varied greatly among the strains, and was typically largest with the half-MIC antibiotic concentration. This toxicity increase was seen with both MRSA and MSSA strains. Importantly, increase of the leukocidin-mediated supernatant toxicity was also observed in the LukSF-negative strains (**Fig. 4.1**).

To evaluate whether the exposure to the antibiotics with no anti-staphylococcal activity is able to influence the resulting supernatant toxicity, *S. aureus* strains TCH1516 and Newman were incubated in the presence of clinically relevant concentrations of metronidazole, an antibiotic of choice for the treatment of anaerobic infections. No influence on the bacterial growth was seen, and no effect on the resulting supernatant toxicity could be demonstrated (**Fig. 4.2**).

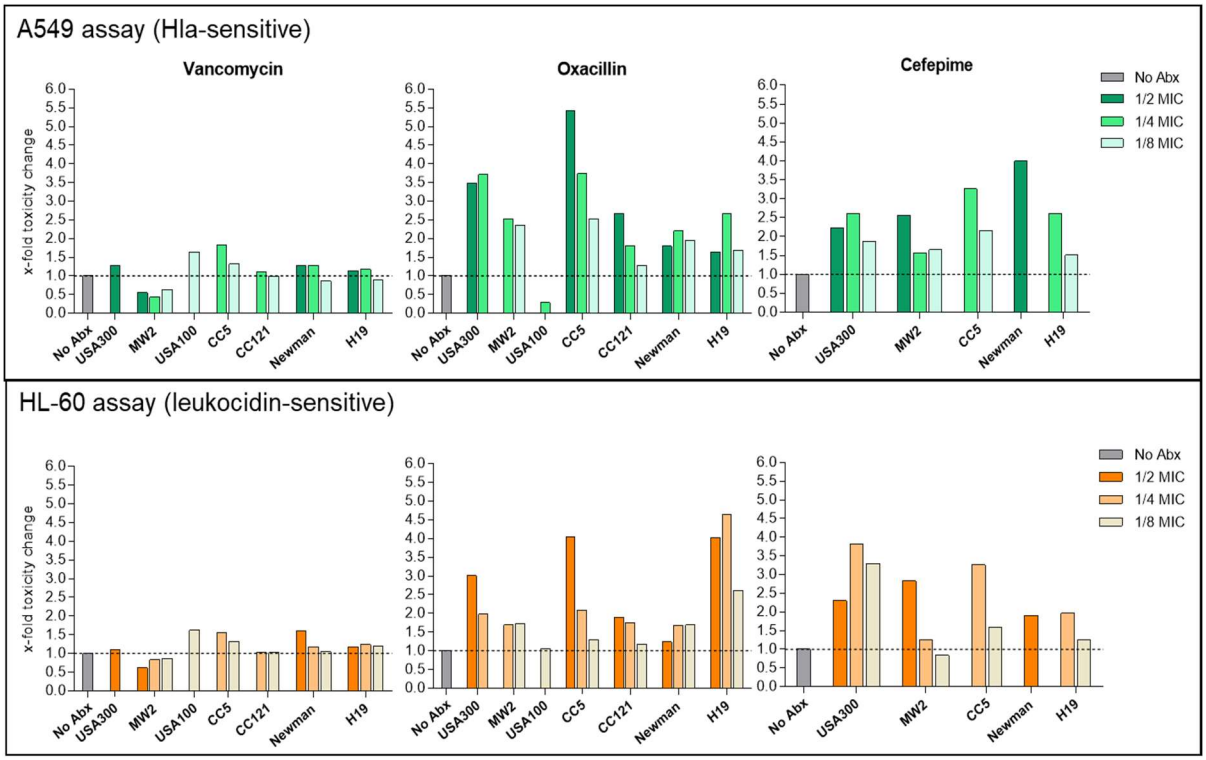

Fig. 4.1. Toxicity of supernatants of S. aureus grown in presence of sub-lytic antibiotic concentrations. *Data expressed as fold change of the EC50 values relative to the no-antibiotic control. Only supernatants collected from the bacterial cultures incubated in the same conditions as the no-antibiotic control (i.e. no influence of the antibiotics on the bacterial growth) were tested. No Abx = S. aureus grown in RPMI-CAS without addition of antibiotics.*

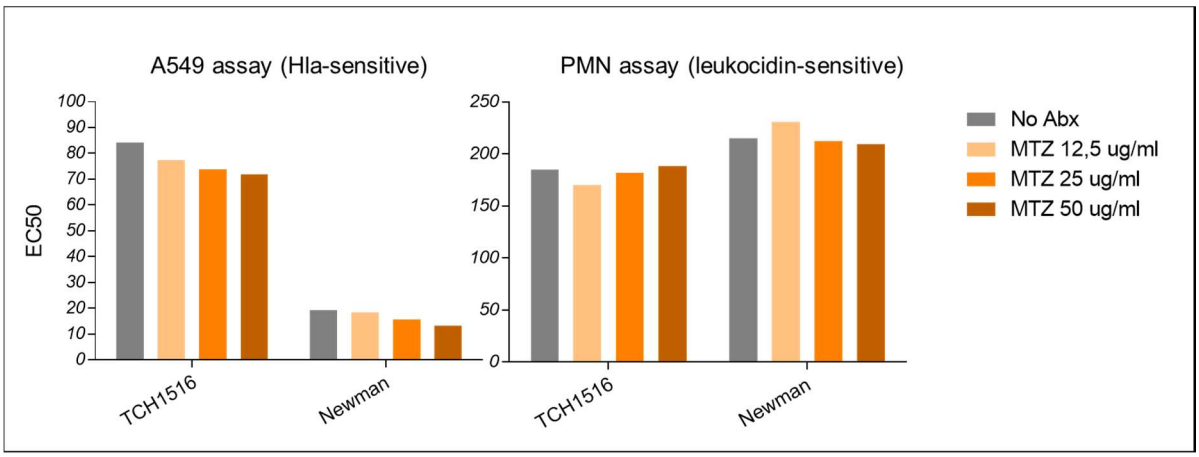

Fig. 4.2. Toxicity of supernatants of *S. aureus* grown in the presence of irrelevant antibiotic (metronidazole). *Data expressed as EC50 values. MTZ = metronidazole, No Abx = S. aureus grown in RPMI-CAS without addition of antibiotics.*

Addition of the antibiotic to the bacterial culture at different growth phases influences the degree of toxicity increase. In the clinical setting, at the time when the *S. aureus* infection symptoms are detected, microbiological samples typically contain high bacterial density. This means that the antibiotic therapy is often commenced with the bacterium in the advanced growth phase. We tried to simulate these conditions by allowing *S. aureus* to grow without antibiotics, and adding the antimicrobial to the bacterial culture 3 or 4 hours after the start of the incubation. For these experiments, we only selected the first-line agents employed for a suspected *S. aureus* infection, beta-lactams represented by oxacillin, and vancomycin. Delayed introduction of the antibiotics into the bacterial culture allowed us to increase the tolerated antibiotic concentrations up to 4-fold compared to the pre-defined MIC values. The bacterial growth dynamics were not affected. Adding the antimicrobial agents 4 hours following commencing the incubation translated into the supernatant toxicity levels similar to those of the control supernatants for the most strains (**Fig. S4.1**). Exposure of the bacterium to the antibiotic 3 hours after the beginning of culturing, however, resulted in toxicity comparable to or slightly higher than the no-antibiotic control. At the same time, the bacterial density of the suspensions with the antibiotic added 3 hours after incubation start was higher and closer to the no-antibiotic control values, as opposed to lower bacterial densities measured in the cultures where the antibiotic was present from the beginning of the bacterial culturing. These effects were the most prominent in the cultures treated with oxacillin (**Fig. 4.3**). Importantly, no change in supernatant toxicity was seen when strains were exposed to vancomycin (**Fig. S4.1**).

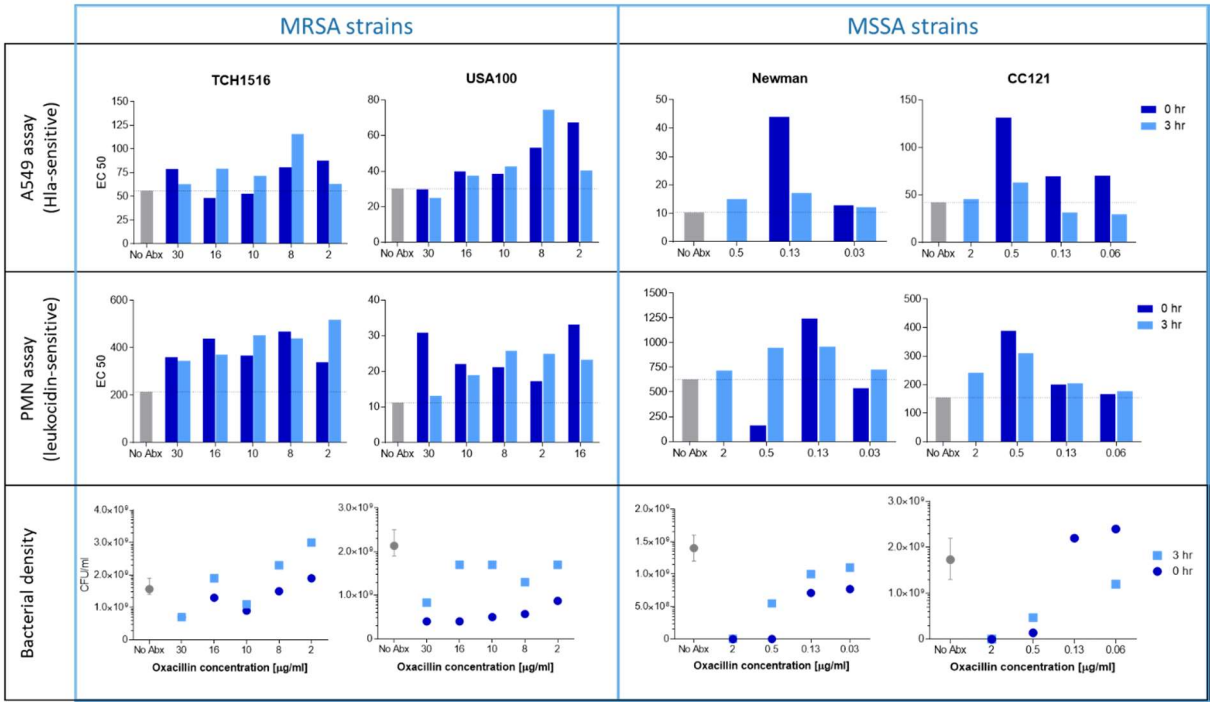

Fig. 4.3. Influence of the timing of oxacillin addition on the supernatant toxicity and resulting CFU counts of *S. aureus*. *"0 hr" = supernatants obtained from the bacterial cultures where oxacillin was at the start of the incubation; "3 hr" = oxacillin added to the S. aureus culture 3 hours after commencing the incubation. No Abx = S. aureus grown in RPMI-CAS without addition of antibiotics.*

Supplementing RPMI-CAS with human albumin allows the simulation of low-iron, high-protein *in vivo* environment. RPMI-CAS medium simulates the low-iron *in vivo* milieu the bacterium is typically exposed to. To further approximate the culture conditions to the serum environment encountered within the infected host, we supplemented the bacterial growth medium with albumin in the physiological serum concentration (40 mg/ml). Since a commercially available solution of the pooled donor-derived albumin was used, it was assessed for the presence of donor immunoglobulins, and found to contain them in the concentration of approximately 400 ng/ml. The donor-derived antibodies were then removed from the solution as outlined before; successful clearance was confirmed in a total human IgG ELISA. For simplicity reasons, "albumin" will be used to denote IgG-depleted albumin solution from here on. To investigate whether albumin could be used as an alternative amino acid course instead of the casamino acids, *S. aureus* TCH1516 and Newman strains were grown in the RPMI-CAS, RPMI-CAS and RPMI supplemented with 40 mg/ml albumin (RPMI-CAS-Alb and RPMI-Alb, respectively). Whereas *S. aureus* grown in RPMI-CAS and

RPMI-CAS-Alb showed similar growth dynamics, use of RPMI-Alb resulted in *S. aureus* failing to increase in bacterial density over 0.5 OD_{600nm}, the value corresponding to the mid-log phase of bacterial growth, for over 4 hours (**Fig. 4.4**). To rule out the interference of the Albiomin buffer with the bacterial growth seen with the use of RPMI-Alb, we performed buffer exchange to PBS using the dialysis method. Bacterial growth dynamics in the RPMI-CAS supplemented by buffer-exchanged albumin was identical to that where albumin in Albiomin commercial buffer was used (data not shown). Notably, supplementing the medium with albumin allowed us to increase the concentrations of the antibiotics used for the subsequent preparation of *S. aureus* supernatants: depending on the strain, MIC values of oxacillin or vancomycin in RPMI-CAS-Alb were increased 2- to 4-fold. RPMI-CAS-Alb was therefore used as the medium of choice for mimicking the low-iron, high protein *in vivo* environment.

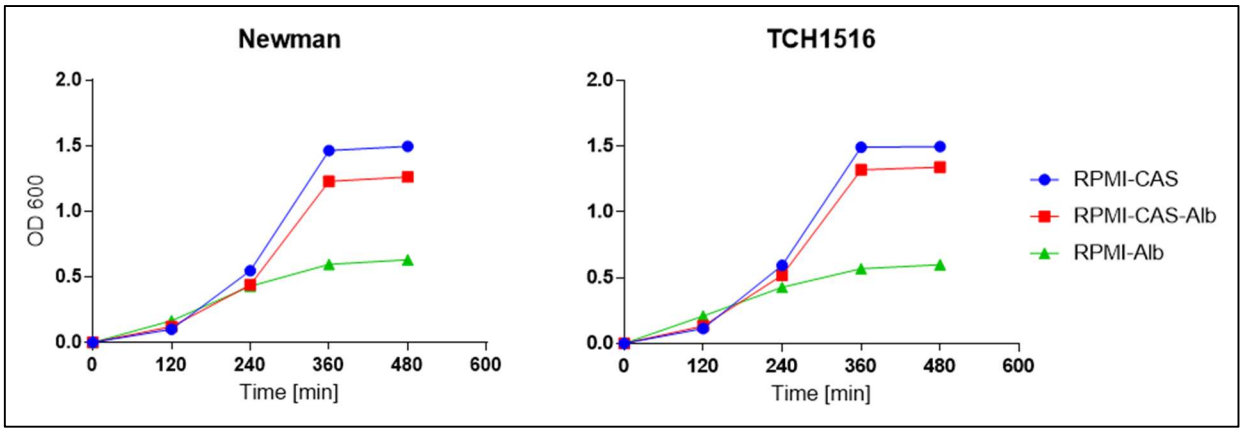

Fig. 4.4. Influence of human albumin in the medium on the *S. aureus* growth. *RPMI-CAS = RPMI supplemented with 1% casamino acids; RPMI-CAS-Alb = RPMI supplemented with 1% casamino acids and 40mg/ml immunoglobulin-depleted human albumin; RPMI-CAS-Alb = RPMI supplemented with 40mg/ml immunoglobulin-depleted human albumin.*

Increase of the supernatant toxicity upon antibiotic exposure is observed when *S. aureus* is grown in the albumin-rich medium. To examine the potential effect of the growth medium on the toxicity increase upon antibiotic treatment, we exposed *S. aureus* TCH1516 grown in RPMI-CAS and RPMI-CAS-Alb to oxacillin and vancomycin and collected the resulting supernatants. We included both the cultures where antibiotic was present at the start of the incubation and those where it was added 3 hours after incubation was commenced. In general, use of the albumin-supplemented growth medium resulted in bacteria tolerating higher antibiotic concentrations without affecting the growth dynamics.

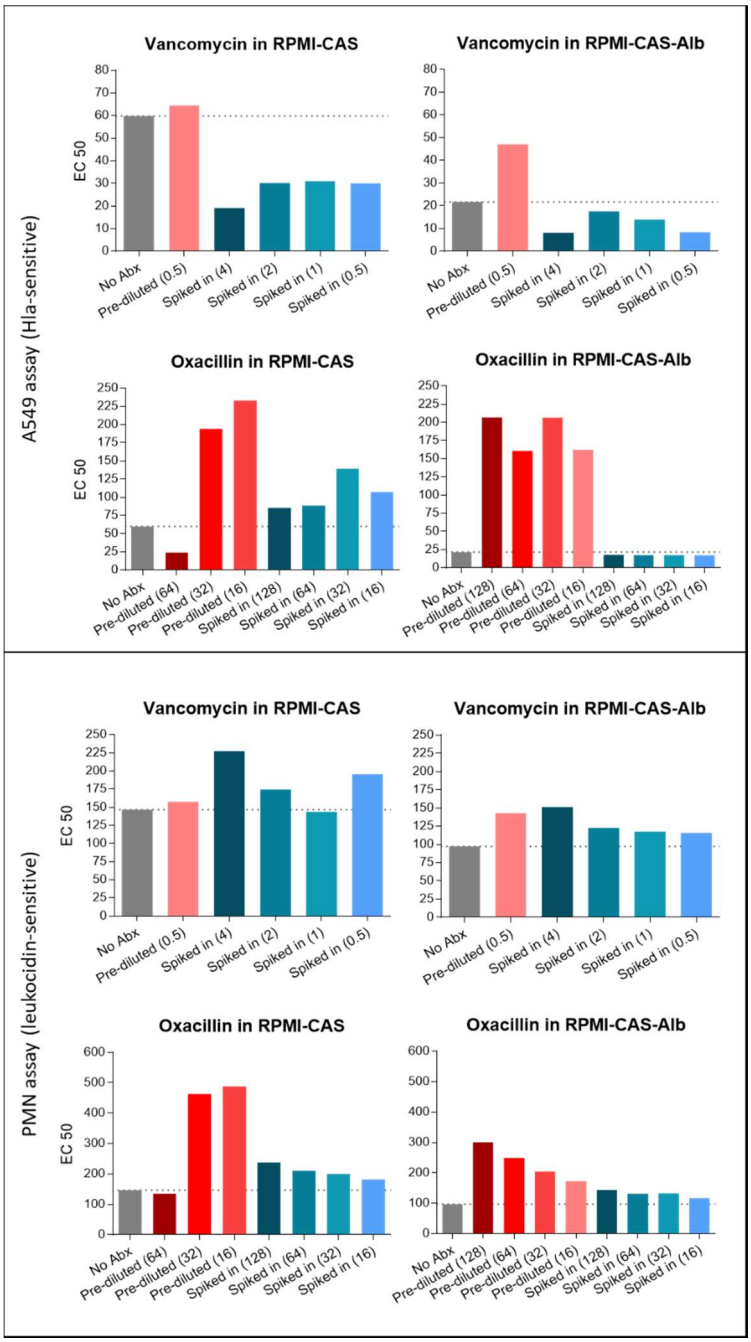

Fig. 4.5. Influence of antibiotics and albumin in the bacterial growth medium on *S. aureus* TCH1516 supernatant toxicity. *No Abx = S. aureus grown without antibiotics. Pre-diluted = antibiotics were present in the bacterial suspension at the start of the incubation. Spiked in = antibiotics added to the bacterial suspension 3 hours after initiation of incubation. Antibiotic concentrations are shown in parenthesis and are expressed in µg/ml. RPMI-CAS = RPMI supplemented with 1% casamino acids; RPMI-CAS-Alb = RPMI supplemented with 1% casamino acids and 40mg/ml immunoglobulin-depleted human albumin.*

Similarly to the previous experiments, exposure of *S. aureus* to oxacillin resulted in the increase of supernatants' toxicity, both in the A549 and in PMN assays. Such increase could not be demonstrated when vancomycin was added to the bacterial suspension. In fact, exposure of *S. aureus* to vancomycin 3 hours after the incubation start seemed

to reduce the supernatant toxicity approximately two-fold. Importantly, these observations were made for *S. aureus* grown in both RPMI-CAS and RPMI-CAS-Alb. The absolute toxicity of the supernatants was lower when the bacterium was grown in the presence of albumin. Addition of oxacillin to *S. aureus* 3 hours after starting the incubation resulted in a mild to absent change in toxicity in both RPMI-CAS and RPMI-CAS-Alb media, compared to its marked enhancement when it was present in the culture at the start point (**Fig. 4.5**).

Increased supernatant toxicity is the result of an active bacterial response. The observed toxicity modulation was the strongest when the cell-wall-active antibiotics represented by oxacillin were used. One may hypothesize that increased concentrations of the cytolytic toxins in the bacterial supernatant is the result of their leakage through the bacterial cell wall rendered more permeable by the antimicrobial action. To address this and to assess the potential amounts of the intracellularly retained toxins, we tested the Hla- and leukocidin-mediated toxicity of the stationary-phase bacterial cell lysates. It was found to be up to 40 times lower than the toxicity of the corresponding culture supernatant. The toxicity of the lysate concentrated to half the original volume was approximately twice as high as of that of the full original volume, indicating the concentration of the toxins upon volume reduction. These effects were observed for both Newman and TCH1516 strains (**Fig. 4.6**).

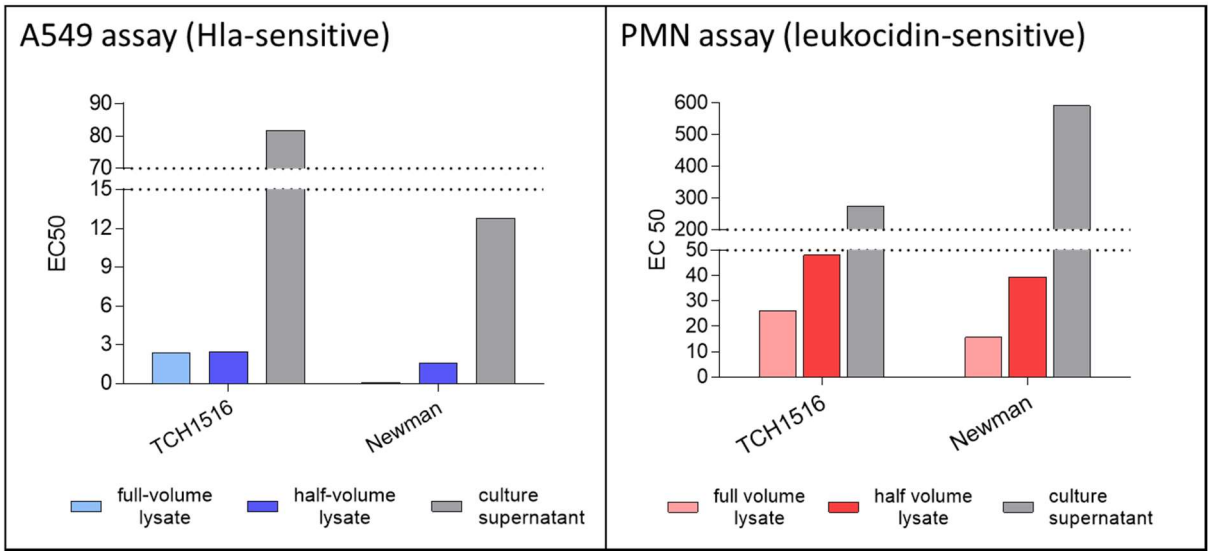

Fig. 4.6. Toxicity of *S. aureus* bacterial cell lysates in PMN and A549 assays. *"full-volume lysate" = bacterial pellet resuspended in the original volume during the lysis step; "half-volume lysate" = bacterial pellet resuspended in half of the original volume during the lysis step; "culture supernatant" = supernatant of the S. aureus stationary culture.*

IV.4. Discussion

In the face of the increasing antibiotic resistance, the choice of the antibiotic therapy has to be informed and ideally pathogen-tailored. Unfortunately, because of the nature of the underlying pathologies of the critically ill patients, antimicrobial therapy in the ICU setting often needs to be started immediately, and is therefore often administered empirically. This introduces the possibility of applying non-effective or non-first-choice agents. Apart from the antimicrobial stewardship considerations, this raises concern about the modulation of the bacterial toxicity as the result of the antibiotic exposure. Our observations demonstrate how *S. aureus* virulence changes when sub-lytic drug concentrations are employed. Specifically, the cell-wall active antibiotics represented by oxacillin seemed to affect the supernatant toxicity the most, and the effect was less pronounced with vancomycin. Our observations support the published data on *S. aureus* toxicity modulation in response to antibiotics: literature available to date, however, focuses mainly on LukSF and, to a much lesser degree, on Hla. The data on other leukocidins remains scarce. Whereas Hla is produced by an overwhelming majority of the clinically relevant strains, LukSF is only present in approximately 5% of them, and is potentially overrepresented in the published studies in comparison to other bicomponent leukocidins. We therefore included both LukSF-positive and LukSF-negative strains for our experiments, and found that leukocidin-mediated toxicity of the supernatants is increased in response to the antibiotic exposure regardless of whether the strain expressed LukSF. This suggests that other leukocidins (LukGH, HlgAB, HlgCB, LukED) are upregulated in fashion similar to Hla or LukSF. Since one of the possible mechanisms of the antibiotic-dependent toxicity modulation involves enhancement of *agr* activity, uniform modulation of the pore-forming cytotoxins' expression is not surprising. Still, to our knowledge this is the first report of a non-LukSF dependent increase of leukocidin activity upon antimicrobial exposure.

The degree of the overall toxicity change varies among the strains, and is dependent on the bacterial growth medium, timing of the antibiotic addition, as well as its amount. Highest well-tolerated antibiotic concentration (i. e., not influencing the bacterial growth dynamics) and presence of the drug at the beginning of the incubation led to the highest observed increase of the supernatant toxicity, suggesting that this response is dependent on the magnitude of the environmental insult experienced by the bacterium. Supporting this notion, delayed addition of the antibiotic resulted in little to no toxicity change. This may be explained by the lower amount of the drug molecules per bacterial

cell in the advanced stages of incubation. Such behavior of the bacterium is likely an active adaptation to the environmental offenses, as shown by testing the stationary phase bacterial lysates. The amount of the intracellularly retained toxins, accounting for approximately 3.5% or less (Hla), or 9% or less (leukocidins) of the supernatant toxicity, is low and therefore unable to explain the up to 5.5-fold increase in toxicity. Naturally, one cannot rule out the contribution, albeit minor, of the intracellular toxins passively leaking through an antibiotic-treated cell wall, to the overall toxicity enhancement. However, it is unlikely to be a defining factor. Further supporting the notion of active antibiotic-induced virulence modulation is the lack of change in toxicity upon exposure of *S. aureus* to the therapeutic doses of non-relevant antibiotics (in our case, metronidazole). Published data supports our hypothesis of the *S. aureus* toxicity upregulation being an active stress response of the bacterium. Toxin gene expression was shown to increase after treatment with beta-lactams [193], and active transcription of the genes (specifically, Hla and LukSF) could be demonstrated [192,197]. Antibiotic-associated upregulation of *S. aureus* toxicity is thought to be mediated via the activation of the bacterial SOS system with a subsequent upregulation of the SaPI1 and virulence increase [198,199], and/or PBP1-mediated modulation of *sarA* and *rot* regulators of toxicity [191]. Notably, the published reports rely on the standard laboratory media (CCY, TSB) to investigate the antibiotic-associated toxicity change. These media are optimal for bacterial growth, but do not approximate the conditions encountered by the bacterium *in vivo*. To address this, as well as to investigate the influence of the different bacterial media on the supernatant toxicity, we simulated the low-iron, high-protein *in vivo* conditions by supplementing the RPMI-CAS by immunoglobulin-depleted human albumin. We observed a lower overall toxicity of the RPMI-CAS-Alb supernatants compared to those obtained from the PRMI-CAS-grown *S. aureus*. The underlying mechanism remains to be elucidated, although some published data suggests that albumin may decrease the bacterial virulence by capturing free iron in the medium and thus restricting its supply [200]. The antibiotic-associated toxicity increase was reliably seen in both RPMI-CAS and RPMI-CAS-Alb. Regardless of the medium, this effect was pronounced with oxacillin and less so with vancomycin. The degree of toxicity change upon antibiotic exposure, however, was lower when bacteria were grown in presence of albumin. This could be explained by the binding of the antibiotic to the albumin molecules with subsequent decrease of their effective concentration. Such binding was described both for beta-lactams and vancomycin [201,202]. This could also be responsible for MIC concentrations increase upon supplementation of the bacterial growth medium with albumin.

The toxicity modulation observed in the RPMI-CAS-Alb suggests that this behavior is not restricted to the standard laboratory media, and could also be expected in a more

in vivo-like environment. The use of RPMI-CAS-Alb also allowed us to tackle one of the major limitations of the project: comparatively low antibiotic concentrations used. Standard doses of the antimicrobials administered in the clinical practice typically translate into concentrations considerably higher than the MIC values. For example, the recommended trough concentrations of vancomycin should not be less than 10 µg/ml, which is a concentration 5- to 10-times higher than MIC values calculated for the majority of the strains used in the study. Actual concentrations of oxacillin to which the bacterium may be exposed *in vivo* are more difficult to outline because of a relatively short half-life of the antibiotic and the dosing regimen dictated by time above the MIC value rather than achievement of a predefined therapeutic concentration like in case with vancomycin. The use of RPMI-CAS-Alb allowed us to apply up to 4-fold higher amounts of antibiotics to the bacterium without considerable effect on its growth dynamics. Whilst in the experiments with the MRSA strains the recommended therapeutic concentrations of oxacillin are achievable, this is not the case for MSSA strains.

Although it is difficult to define which antibiotic concentration the bacterium is exposed to *in vivo*, and whether the observed toxicity modulation happens in the context of the clinical staphylococcal colonization or infection, our data raises additional concerns about the antimicrobial therapy in the ICU. Whereas, for example, an out-patient setting could accommodate choosing an antimicrobial based on the local epidemiology, or withholding the therapy until the microbiology results were available, in the critical care setting the treatment often needs to be started immediately. Additionally, the responsible pathogen is not always readily identifiable (e.g., infections caused by opportunistic or multi-resistant pathogens in multi-morbid or immunocompromised patients). This commonly leads to “blind” initiation of empirical broad-spectrum antibiotic therapy, potentially translating into administration of the ineffective or non-first-choice antibiotics to the patient colonized by *S. aureus* or harboring a staphylococcal infection. Moreover, the optimal dosing of the antimicrobials is sometimes difficult to reach, as exemplified by patients with abnormal hepatic or renal functions requiring an individualized, organ function-tailored drug administration. Half-life of the anti-staphylococcal antibiotics may also be decreased in the some patient cohorts, such as children, burn victims, or patients suffering from cystic fibrosis [190]. Additionally, antibiotic tissue penetration is markedly different depending on the infection site and host comorbidities Combined, these factors could lead to antibiotics reaching sub-lytic concentrations in sites with *S. aureus* presence. On one hand, upregulation of the toxin production may translate into worsening the patient outcome by exacerbating an already existing infection. Increased quantities of Hla and leukocidins could contribute to augmented destruction of host epithelial and immune

cells recruited to the infection site. Combined, this may result in not only extensive tissue damage, but also an aggravated inflammation response following an increased cytokine release. On the other hand, it could be hypothesized that administration of the antibiotics prophylactically or to tackle non-*S. aureus* infections could promote production of exotoxin by a colonizing *S. aureus* strain (e.g., in the nasophynx or on the skin) and potentially trigger the conversion of asymptomatic colonization into an infection. Naturally, these scenarios should be addressed in the future research as the current data involving the *in vitro* experiments is extremely scarce. The limitations of our study also need to be taken into consideration: first, only planktonic-state *S. aureus* cultures were examined. Second, our primary focus remained on Hla and leukotoxins, and other important virulence factors of *S. aureus* were not investigated. Third, a very limited selection of the antibiotics was tested. Finally, although effort was made to simulate the *in vivo* environment by using RPMI-CAS-Alb, and clinical setting - by delayed introduction of the antibiotic into the bacterial culture, further research involving *in vivo* experiments or clinical data is needed to gather evidence of *S. aureus* toxicity increase upon antibiotic exposure within the host, and to examine whether such increase is relevant on the organism level. Even without convincing clinical and/or *in vivo* data, our findings once again emphasize the importance of carefully chosen and planned antibiotic therapy. Not only can an inappropriate treatment promote antibiotic resistance and cause adverse effects, it also has a potential to render the bacterium more virulent by enhanced toxin production. As described in the previous chapter, critically ill patients are especially vulnerable in face of *S. aureus* infections. While this predisposition is multifactorial, these patient cohort has low levels of circulating antibodies – specifically those targeting *S. aureus* bicomponent cytotoxins. Antibiotic-induced upregulation of these toxins may thus prove detrimental for these subjects. Our data supports the use and development of novel or adjunct antimicrobial agents. For example, *agr* system of *S. aureus* is involved in a variety of pathogenic processes such as exotoxin production or formation of biofilm, and thus offers an attractive target for development of therapeutics. Since the antibiotic-induced toxicity enhancements of *S. aureus* seems to be *agr*-dependent, such agents could help prevent or minimize this bacterial response. Currently there is a number of *agr*-targeting molecules in development showing promise in the animal studies [203]. Therapeutic monoclonal antibodies is another modality that could be employed to offset the excessive toxin production by the bacterium. Among its advantages are the favorable adverse effect profile, possibility of the use as an adjunct therapy and its relative cost-effectiveness. In the context of the critically ill population use of antibodies targeting staphylococcal toxins may not only tackle the potential antibiotic-induced virulence enhancement, but also increase the deficient immunoglobulin levels described earlier.

V. DIAGNOSTIC TOOL DEVELOPMENT

II.1. PROJECT RATIONALE

Identification and characterization of bacterial species in patient-derived samples serves several functions. First, in a symptomatic subject it allows to define the causative organism, aiding the choice of correct antibiotic regimen. Second, identification of carrier state is helpful in assigning the individual to an at-risk group for subsequent infection. Third, pooled data on colonizing or infecting bacterial species shapes local microbiologic landscape, and is therefore useful in infection control. Diagnostic methods commonly used in the clinical practice include microbiological culture, Gram-staining, PCR and mass spectrometry (MS). Of those, conventional bacteriological culture remains the mainstay of the microbiological analysis. While reliable, versatile and cheap, it does have several drawbacks. It is labor-intensive, requires trained personnel, and typically requires 24-48 hours for the readout to be achieved. Gram-staining of the patient sample, on the other hand, is quick, cheap and may provide guidance for a subsequent choice of the antibiotic regimen. However, it depends on the sample quality, is non-specific and fails to provide the detailed information on the infecting or colonizing bacterium. In the recent years, methods based on PCR were increasingly employed in the clinical practice. Its advantages include a relatively quick time to the result (as low as 2-3 hours, depending on the system used), quantification possibility, and potential automatization [204]. PCR is particularly useful for detecting fastidious bacteria or viruses. Unfortunately, its high running costs and the need for trained staff somewhat limit its use in the clinical practice [205–209]. MS-based tools have recently been adapted to the clinical practice; the most prominent of those is the matrix-assisted laser resorption/ionization-time of flight (MALDI-TOF) system [210]. It is characterized by a very short time between specimen collection and microbial species identification (typically less than 1 hour, and as low as 10 min.), high accuracy of the readout, possibility of bacterial burden quantification and simplicity of sample preparation and device operation. Its major drawback, however, is very high cost of the equipment, which limits its use to well-funded or highly specialized centers. Additionally, result interpretation is automated and database-dependent, suggesting the readout may be influenced by the software and the database quality [211]. Diagnostic tools are being continuously developed and improved. Some examples of the novel techniques include next-generation sequencing (NGS) [212], microarray and digital PCR [213]. The most

common microbiological diagnostic entities, as well as their advantages and drawbacks, are summarized in **Table 5.1**.

Method	Time to result	Advantages	Disadvantages	POC
Microbiological culture	24-48 hours	Robust; Low operational cost; Quantification possible	Long duration; Trained personnel needed	No
Gram-stain of patient sample	<1 hour	Quick; Sensitive; Low operational cost; Semi-quantitative assessment possible	Non-specific; Trained personnel needed	No
qPCR	2-6 hours	Relatively quick; Specific; Sensitive; Quantification possible	High equipment and operational cost; Genotypic and phenotypic discordances	Possible
MALDI-TOF	<1 hour	Very quick; Operationally simple; Specific	High equipment cost; Database-dependent	No

Table 5.1. Most commonly used methods to detect *S. aureus* in the patient samples. *Expected time from sample collection to receiving results is indicated. POC = point-of-care test.*

One common characteristic of the listed diagnostic modalities is that none of those can be performed outside of the microbiological laboratory, which introduces additional delay of the definitive diagnosis and start of the antibiotic therapy. This is especially problematic in the critical care setting, where a quick microbiological diagnosis may markedly improve the patient outcomes. A point-of-care (POC) test is well-suited for rapid pathogen identification, for example, in the ICU or in the primary care facility. It is defined by the following characteristics: (1) the test is performed in the rooms where patient is treated (e.g., in-patient wards, out-patient clinic, emergency room etc); (2) its measurement system is easy to operate; (3) investigation is done in the context of direct patient care; (4) results show therapeutic relevance for patients at risk; and (5) investigation can be performed by untrained medical personnel [214]. Examples of POC tests include devices used for analyzing blood glucose, hemoglobin, occult blood in stool, or pregnancy or toxicological tests. In the field of infectious diseases, the rapid strep-test is an example of POC test successfully employed in the primary care. Advantages of POC tests include immediate result availability, improved workflow of the personnel, portability and accessibility of the testing in the small or rural hospitals, and ease of specimen and device handling. All of these may contribute to improved

patient outcome [215], emphasizing the clinical need for an informative, fast and cost-effective point-of-care diagnostic tools [216]. In case of *S. aureus*, no quick, cheap and reliable POC test is available currently, and most of the novel diagnostic modalities to date were focusing on detecting MRSA [217]. To address this unmet medical need, we developed a latex agglutination assay capable of detecting *S. aureus*. In the positive latex agglutination assay, binding between the antibody-coated latex particles and the target antigen results in the formation of "clumps" (**Fig. 5.1**) discernible with the naked eye. Developed test is thought to serve as a proof-of-concept assay for the subsequent development of the POC tool (e.g., a lateral flow test) to be used in the clinical practice.

II.2. METHODOLOGY

Choice of antibody and *S. aureus* strain. The principle of the *S. aureus* agglutination assay is summarized in **Fig. 5.1.** For our agglutination assay development, SpA was chosen as an antigen for *S. aureus* detection; as a detecting antibody, an in-house developed and produced SpA-specific antibody was selected (Patent No. PCT/EP2016/058238). In its native form, it binds SpA molecule both with the F_{ab} portion (preferred binding), as well as with F_c portion (unwanted binding). To limit the latter, we included the in-house produced version of the anti-SpA antibody with QRF mutation (Lys274Gln, His435Arg, Tyr436Phe) which prevents SpA binding to the F_c but not F_{ab}. As a negative control, an anti-respiratory syncytial virus (RSV) antibody, both in native form and with a QRF mutation, were used.

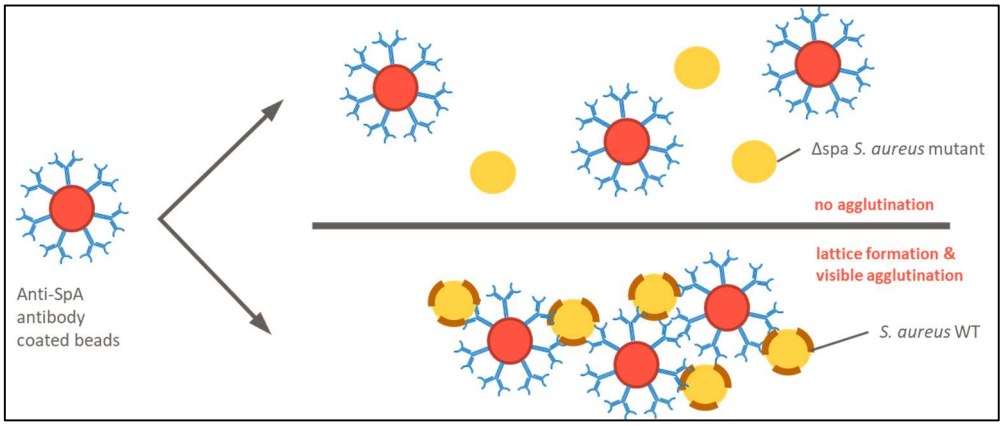

Figure 5.1. Principle of *S. aureus* latex agglutination assay. *Upper panel: in absence of bacterium, or when spa-lacking mutant is present, antigens are not bound by the antibodies and latex bead mixture remains homogenous. Lower panel: in presence of S. aureus latex beads are binding the antigen, forming "clumps" visible with naked eye.*

Three *S. aureus* strains were included in the experiments: (1) *S. aureus* TCH1516, known to produce high SpA levels; (2) *ΔspA* mutant of *S. aureus* TCH1516; (3) clinical *S. aureus* isolate from the Lahey study (**Chapter II** and **III**) characterized by low SpA production (determined previously in-house in the experiments unrelated to the current thesis).

Production of antibody-coated bead suspension. Polybead polystyrene red dyed microspheres (Polysciences, Cat.N. 15711-15) were diluted to the concentration of 2% in PBS + 0.1% BSA + 0.05% Tween-20, then coated with antibody of choice in 400 µg/ml antibody solution (20 µg/µl bead) by incubating the mixture at 37°C for 60min with 400 rpm shaking. After coating, the beads were spinned down in the centrifuge (4000 rpm for 3 min), and excess coating solution was removed by pipetting. After resuspending the bead pellet in PBS + 0.1% BSA + 0.05% Tween-20, the solution was transferred to 4°C, and could be used for up to 7 days for the reactions.

Preparation of bacterial suspension. To ensure the optimal SpA content in the suspensions used for testing, mid-log cultures were prepared, since, as mentioned in the **Chapter I.3**, this molecule is produced in the log to early stationary growth phase. The bacterial strain of choice was plated on sheep blood agar plates and incubated overnight at 37°C and 5% CO_2. For the overnight bacterial culture, 1 colony from the plate was inoculated in 20 mL RPMI-CAS and incubated overnight at 37°C, shaking at 200 rpm. Overnight cultures were then diluted 100-fold in RPMI-CAS and incubated at 37°C and 200 rpm until the mid-log phase (defined by optical density of 0.5 at 600 nm) was reached. The suspension was then placed on ice and spinned down. Using the pre-determined bacterial density at the mid-log (equivalent to $5x10^9$ CFU/ml) the bacterial pellet was resuspended in PBS to yield a solution with concentration of 2×10^{10} CFU/ml. Ten-fold serial dilutions of this suspension in PBS were carried out to generate samples for use with the assay. For the experiments including the use of human immunoglobulins, anti-RSV antibody was additionally introduced to the bacterial suspensions to yield concentrations of 250 µg/ml, 800 µg/ml and 2000 µg/ml. The final bacterial densities of the used suspensions were controlled by track dilution method as described in **Chapter IV.2**.

ETA liquefaction and homogenization. Selected ETA samples were retrieved and thawed. Care was taken to make the selection representative and include samples with varying color, consistency and homogeneity. To liquefy them, Sputasol (Oxoid, cat. #SR0233) working solution was prepared according to manufacturer's instructions.

Equal volumes of Sputasol and ETA were combined, placed into a heating block heated to 37°C, and incubated with intermittent vortexing (once every 3-5 minutes) for 15 minutes. To homogenize the liquefied samples, cca. 500-700 µl of ETA was placed into the tube compatible with Precellys bead homogenizer, and 1.4 mm Precellys zirconium beads were added. Device was run at 6500 rpm (thrice for 5 seconds with 5 second interval). Liquefied and homogenized samples were used immediately, or stored at -80°C.

Determination of the immunoglobulin content in the ETA samples. The total IgG content of the ETA samples was determined by the commercially available IgG (Total) Human ELISA Kit (Invitrogen). Briefly, after the plate coating step and preparation of the dilutions for the standard curve, ten ETA liquefied and homogenized samples with varying physical characteristics were selected (color, viscosity, *S. aureus* colonization status). Four 5-fold serial dilutions of the ETAs in PBS were performed starting from 10000-fold; the dilutions were then transferred to the plates and processed according to the manufacturer's instructions. The final immunoglobulin concentrations were extrapolated in the Prism 6 GraphPad software using the standard curve.

Agglutination reaction. To test the agglutination, 10 µl of the antibody-coated latex bead suspension was mixed with 10 µl of the antigen-containing sample. Each of the liquids was placed as a drop onto a glass slide; the reagents were then mixed together with a clean disposable pipette tip. By gently rocking the glass slide, presence of agglutination was evaluated by the naked eye. An example of reaction outcome is demonstrated in the **Fig. 5.2**. Evaluation was done within the first 5-10 minutes, as dried suspension may form patterns that could be mistaken for clumps, resulting in a false positive readout.

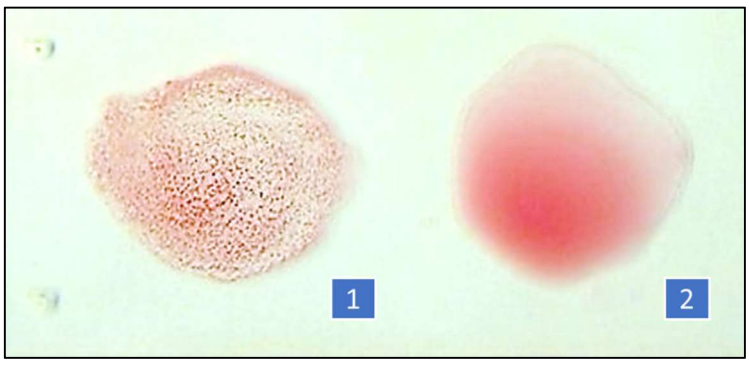

Figure 5.2. Examples of a positive and a negative latex agglutination assay.
1: Positive reaction. Clumps are seen in the reaction mixture.
2. Negative reaction. The reaction mixture is homogenous, no clumps are to be identified.

II.3. RESULTS

Agglutination is seen with the bacterial pellet, but not supernatant or mid-log culture. As the first step in this project, we demonstrated absence of the non-specific agglutination between *S. aureus* and the uncoated native bead solution. Two to three single colonies of pre-plated TCH1516 wild-type and *ΔspA* TCH1516 were mixed in to a 2% slurry uncoated beads to form a homogenous mixture with no signs of agglutination. Similarly, upon applying the bacterial suspension, supernatant and the bacterial pellets of TCH1516 wild-type and *ΔspA* TCH1516 to the uncoated bead mixture failed to elicit an agglutination reaction. The beads were then coated with anti-SpA antibodies. Clear agglutination was seen upon mixing the coated latex beads with wild-type TCH1516 bacterial pellet, but not its supernatant or the midlog culture. No reaction was seen in any of *ΔspA* TCH1516 (pellet, supernatant or midlog suspension).

QRF-mutation is needed to avoid unwanted antibody binding. Clear agglutination was seen upon applying the TCH1516 bacterial pellet to the anti-SpA-QRF-coated bead mixture. Upon mixing the same bacterial sample with anti-SpA wild type antibody-coated latex beads the agglutination, albeit present, was substantially weaker (**Fig. 5.3**).

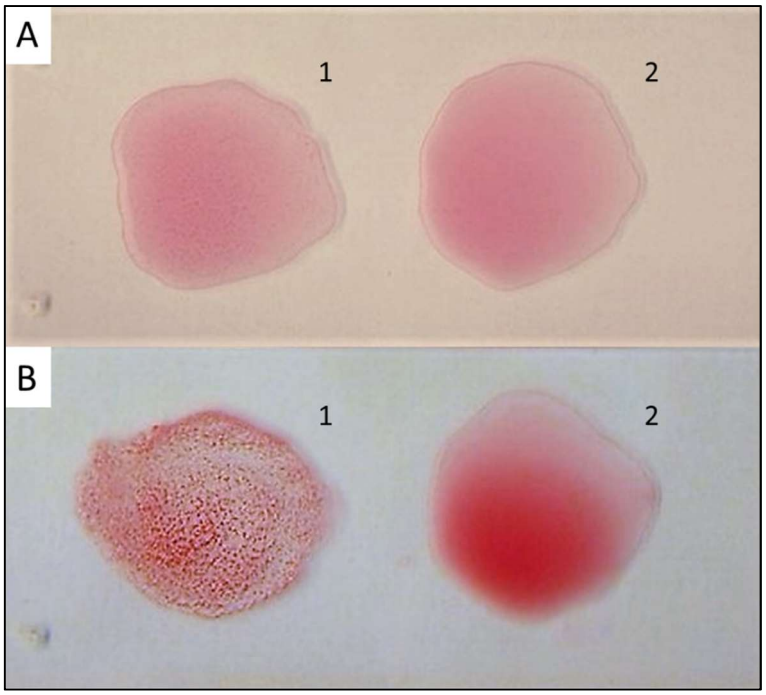

Figure 5.3. Agglutination assay using beads coated with wild-type anti-SpA antibody and anti-SpA antibody with QRF mutation. *1 = bacterial pellet of wild-type TCH1516 used as the sample; 2 = ΔspA TCH1516 bacterial pellet used as a sample. A: beads coated with wild-type anti-SpA antibody. B: beads coated with anti-SpA antibody with QRF mutation.*

In the experiments involving the application of the bacterial pellet to the beads coated with the control (anti-RSV) antibody, beads coated with wild-type immunoglobulin showed minimal agglutination (data not shown; minimal effect could not be captured due to the camera's technical limitations). No reaction could be elicited with QRF-mutated control antibody beads. Similarly to the previous experiments, no agglutination was seen in any of the assays involving the *ΔspA* TCH1516.

Assay lower limit of detection (LLoD) is approximately 2 x 10^7 CFU/sample. To assess the sensitivity of the latex agglutination assay, we prepared 10-fold dilution of *S. aureus* TCH1516 and clinical *S. aureus* isolate characterized by low SpA expression, starting from the concentration of 2 x 10^{10} CFU/ml. Agglutination could be observed with both strains at the concentrations of 2 x 10^8 and 2 x 10^7 CFU per 10 µl sample (corresponding to 2 x 10^{10} CFU/ml and 2 x 10^9 CFU/ml, respectively), although the agglutination with the clinical strain was somewhat weaker than with TCH1516. At the concentrations of 2 x 10^6 CFU per sample or less no positive reaction could be elicited. No agglutination was observed with the negative control (*ΔspA* TCH1516). LLoD was therefore determined to be at the cutoff of 2 x 10^7 CFU per sample.

Presence of the immunoglobulins influences the assay sensitivity. Since SpA binds the immunoglobulins at the F_c portion, we tested whether presence of immunoglobulins in the sample influences the assay sensitivity. We found that supplementation of the bacterial suspension with IgG increases the LLoD of the assay: no agglutination could be elicited with the 2 x 10^7 CFU per sample or lower if the samples were supplemented with immunoglobulins. Notably, the agglutination reaction was weaker with higher concentrations of the IgG added into the sample (**Table 5.2**).

CFU / sample	No IgG added	250 µg/ml IgG (2.5 µg/sample)	800 µg/ml IgG (8 µg/sample)	2000 µg/ml IgG (20 µg/sample)
	Agglutination present?			
2 x 10^8	YES	YES	YES	YES, weak
2 x 10^7	YES	NO	NO	NO
2 x 10^6	NO	NO	NO	NO

Table 5.2. Influence of IgG presence in the sample on the assay sensitivity. *Wild-type TCH1516 was used in all experiments. "Sample" refers to the 10 µl sample volume used in the assay.*

ETA contains significant amounts of IgG, and *S. aureus* positive ETA samples did not elicit a positive agglutination reaction. Immunoglobulins were detected in all of the pre-selected liquefied and homogenized ETA samples. The IgG content ranged from 143 µg/ml (ETA#9) to 670 µg/ml (ETA#10), and its average was estimated

to be around 330 µg/ml. Considerable differences between the IgG content of the individual samples were detected (**Fig. 5.4**).

The ETA samples were then tested in the agglutination assay with the anti-SpA-QRF antibody-coated beads. No agglutination could be elicited in the assays with *S. aureus*-negative ETAs, demonstrating the absence of the non-specific agglutination. We then included tested ETA samples with light and heavy *S. aureus* colonization (corresponding to 50 CFU and 5 x 10^3 CFU per 10 µl sample, respectively). Similarly, no agglutination could be elicited, potentially since the LLoD of the test was not reached.

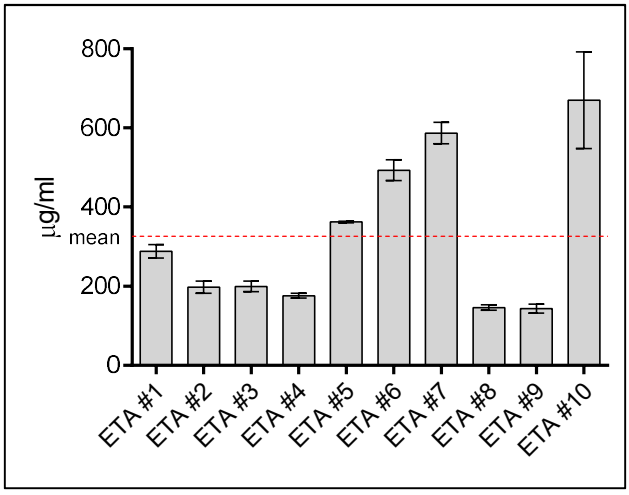

Fig. 5.4. IgG content in the ETA samples. *Plotted: average of 2 repeat measurements and range. Average of all tested ETA samples is depicted with the dotted red line.*

II.4. Discussion

In this project, we developed a latex agglutination assay aimed at the quick detection of *S. aureus*. Taken into consideration the heterogeneity of *S. aureus* clinical isolates, we selected SpA as the antigen of choice. On one hand, it is specific to the bacterium, thus minimizing the cross-reactivity with other bacterial species. On the other hand, published studies report ≥90% of the isolates carrying the *spa* gene [16,17], making it an appropriate target molecule. SpA is an important component of *S. aureus* immune evasion mechanisms and is known to bind the F_c portion of the host immunoglobulins. In the context of the latex agglutination assay, such binding may interfere with the clump formation because of the excess of the SpA binding sites. This effect is demonstrated by markedly weaker agglutination in the assay where wild-type anti-SpA antibody was used to coat the beads, as opposed to antibody with the QRF mutation. QRF mutation is therefore essential for the assay development, as it decreases the unwanted binding and markedly improves the readout.

For the assay to be relevant in the clinical setting, it needs to function at the bacterial densities expected to be seen in a biological sample. We have previously quantified the bacterial density of the heavily and lightly colonized ETA samples [154,218]. In the context of the agglutination assay, a 10 µl sample of the lightly colonized respiratory sample would be expected to contain approximately 50 *S. aureus* CFU, and heavily colonized - 5 x 10^3 CFU. The lower limit of detection of the agglutination assay at 2 x 10^7 CFU per sample is markedly higher than the typical bacterial densities measured in the ETA. The assay in the current form is therefore not yet appropriate for use with the clinical samples. This is supported by the absence of agglutination with the *S. aureus*-colonized aspirates. Another limitation of the developed assay is the influence of the immunoglobulin presence on the assay sensitivity. ETA is a sample characterized by IgG presence with the concentration of up to 670 µg/ml. ETA IgG content was approximately 1/20 to 1/10 of the normal serum IgG concentrations. The source of these immunoglobulins in the aspirate is unclear: it could either be produced and/or accumulated locally, or originate from the serum mixing in with the tracheal secretions as the result of mechanical trauma to the airway or inflammation. To simulate a clinical sample containing host-derived antibodies, we used the bacterial suspensions supplemented with the immunoglobulins at the concentrations based on those measured in the ETA. Additionally, taking into consideration the findings described in **Chapter III**, we included the samples supplemented with 2000 µg/ml IgG (i.e., approximately 1/10 of the healthy serum IgG concentration) to account for potentially lower measured IgG content in the ETA because of global hypogammaglobulinemia in the examined ICU population. Already at the lowest concentrations of the supplemented IgG, the sensitivity of the assay was reduced. Such effect could be explained by SpA binding to the immunoglobulins in the sample, thus reducing the number of potential binding sites for the bead-bound detection antibody. In the ETA sample, it is unknown how much of the *S. aureus*-produced (either membrane-bound or secreted) SpA is already bound to the host immunoglobulins, either at the F_c portion of the antibodies as part of bacterial immune evasion mechanisms, or at the F_{ab} portion as part of the recognition of the bacterium by the host. Naturally, this adds a complexity layer to the assay development, and presence of the host immunoglobulins and immunoglobulin-SpA complexes should be taken into account for the subsequent steps. Possible solutions could include, for example, pre-treatment of the sample to remove the host antibodies or dissociate them from the SpA, or exploring other diagnostic modalities to adapt the developed assay to.

In general, ETA could be a suitable sample for POC test development, as it did not demonstrate any non-specific agglutination (e.g. due to the matrix effect of the sample). However, it does present several challenges and disadvantages to be accounted for.

Previously discussed presence of the host-derived IgG is one of them. Second, one needs to acknowledge the marked heterogeneity of the samples in terms of their copiousness, viscosity and color. This influences not only the physical and biological characteristics of the aspirate, but also its ease of its handling. For example, very viscous, clumpy ETA may be difficult to pipette and to measure the volume appropriate for the assay. Current protocol of ETA processing is labor-intensive and not suitable for POC use. Next steps of the assay development may include addressing this limitation, e. g., by introducing an ETA-liquefying assay buffer, or by selecting an assay format more permissive of the non-homogenous reagent. Apart from that, ventilated patients often undergo airway lavage upon ETA retrieval if the tracheal secretions are thick and viscous. This may dilute the sample and affect the accuracy of the readout. In the context of a POC assay, correcting for dilution is possible, but, once again, is relatively labor-intensive and thus not ideal.

Our latex agglutination assay was able to test the patient samples for *S. aureus* presence. In its current form, it presents the hypothetical user with the major drawbacks of high LLoD and interference of the host-derived antibodies with the assay sensitivity. Further steps should therefore focus on addressing these limitations. In general, it is expected that such assay can be used for quick assessment of bacterial presence in the endotracheal aspirate. In the clinical practice, however, additional information is useful, as demonstrated by the projects within this thesis described before. Not only the presence of *S. aureus*, but also the resistance profile of the isolate (MRSA or MSSA), as well as the bacterial density, are important for therapy planning and assessment of the risk of subsequent *S. aureus* VAP. We therefore propose to use the latex agglutination assay for further development of a more informative POC diagnostic tool in the form of a lateral flow assay. Since such tool does not require trained personnel, it could be implemented both as a quick diagnostic in case of a suspected infection, and as a screening tool, e.g. in the facilities with a known high prevalence of *S. aureus*, or in patients at an especially high risk for *S. aureus* infection. Ideally, the final product should contain three tests assessing the following: (1) *S. aureus* presence; (2) MRSA presence; (3) presence of heavy colonization by the *S. aureus*. An example of such assay, as well as possible readouts, are presented in **Fig. 5.5**.

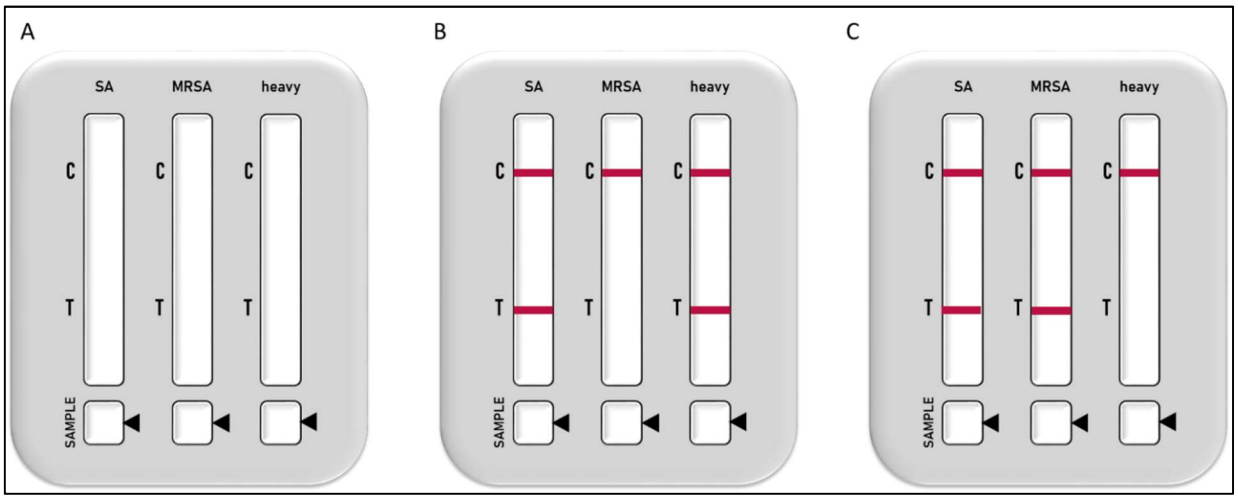

Fig. 5.5. Example of the lateral flow POC diagnostic tool. *Squares labelled by "sample" and marked with arrowhead are areas where the sample is to be added. Tests from left to right: "SA" = presence of S. aureus in the sample; "MRSA" = presence of MRSA in the sample; "heavy" = presence of heavy colonization by S. aureus in the sample. "C" refers to control; "T" refers to test. Upon sample addition, stripe appearing at C represents a valid test. Stripe at T denotes a positive result. A: example of the quick diagnostic tool with no sample added. B: simulated test result of a patient heavily colonized by MSSA. C: simulated test result of a patient lightly colonized by MRSA.*

VI. CONCLUSIONS

Better understanding of *S. aureus* epidemiology and its disease pathogenesis, continuous therapy improvement, and the search for the novel diagnostic modalities are key to combatting this major human pathogen. In the projects presented within the scope of this dissertation, we examined the following: (1) prevalence and epidemiological characteristics of *S. aureus* in the critically ill population; (2) serological characteristics of the population colonized or infected by *S. aureus*; (3) behavior of the bacterium upon exposure to the routinely used antibiotics. Additionally, we worked on a proof-of-concept diagnostic test as a first step in developing a POC diagnostic kit for a quick bedside detection of *S. aureus* in biological samples.

By analyzing the microbiological data from a tertiary medical center in a comprehensive manner, we demonstrated a remarkably high prevalence of *S. aureus* in the critically ill patients, both as a colonizing and as an infecting pathogen. Our findings are in line with the international and country-specific data (on our case, the USA). This, on one hand, makes the analyzed dataset representative, and, on the other hand, once again emphasizes the consistently major role of this bacterium in the global public health. Such prominence in spite of the effective antimicrobials and rigorous hygienic measures is multifactorial. In our project, we touch on some of the reasons for *S. aureus* prevalence: colonization of one-third of the population, high rates of antibiotic resistance, multiple immune system evasion mechanisms, wide array of virulence factors and adaptability to the external stress factors. This bacterium is considered part of the normal human flora; still, colonization by *S. aureus* puts patients at an increased risk of a subsequent infection, as demonstrated by the link between colonization and subsequent VAP in the ICU-admitted patients (investigated in **Chapter II**). Routine screening for staphylococcal colonization is performed upon admission in many centers. Findings presented in our project support this practice: early identification of patients at risk helps correctly predict a causative pathogen in a symptomatic patient, or guide the decolonization and/or specific preventative measures. In general, staphylococcal colonization is a more dynamic process than commonly thought of. Patients carrying *S. aureus* in the nose tend to develop tracheal colonization. This process seems to be Hla-driven, as demonstrated by higher hemolysis scores, higher toxin concentrations in the bacterial supernatants, as well as increased Hla-reactive antibody titers in patients displaying both nasal and tracheal colonization. It is not known whether the bacteria producing more Hla generally tends to occupy more niches at all times, or whether Hla production enables *S. aureus* to spread towards the lower airway (either actively or passively, being inoculated into the trachea in the course of

the invasive medical procedures) and thrive there. Tracheal colonization, in turn, is an important predisposing factor for developing staphylococcal VAP, a feared complication of the mechanical ventilation. It seems that, among other factors, increased bacterial density in the trachea is increasing the likelihood of subsequent VAP development. Therefore, defining the bacterial burden and its regular monitoring, together with evaluating the hemolysis intensity of the clinical isolates could prove helpful in estimating the risk of staphylococcal VAP in ventilated patients. ETA assessment should ideally be carried out daily: as demonstrated in **Chapter II**, first detection of *S. aureus* in the trachea precedes VAP diagnosis by 4 days on average, and microbiological culture typically takes about 48 hours to completion, so even an eventual diagnostic delay would provide the clinicians with an appropriate time interval for planning and implementation of the anti-infective measures. Even if the patient remains asymptomatic, identification of *S. aureus* tracheal colonization could inform the clinicians about a potential VAP pathogen, and prompt actions aiming at preparing for a possible infection (e.g., planning the antimicrobial therapy according to the antibiogram and the patient's organ functions, or ordering the antibiotics not stocked in the hospital pharmacy) or preventing it. Latter is routinely done regardless of the patient's colonization status with the help of the VAP bundle. While typically institution-specific, several components of this bundle are almost invariably present across the globe. Among those are semi-recumbent positioning of the patient, oral care, daily subglottic suctioning and regular sedation assessments and spontaneous breathing trials. Daily aspiration of the subglottic secretions translates into a readily available microbiological sample that can be used for screening purposes. Sedation "vacations" with frequent extubation attempts are especially relevant for prevention of *S. aureus* infections, seeing how the bacterium, potentially brought into the lower airway in the course of intubation, is known for forming biofilms on devices. Unfortunately, there are currently no specific measures aimed at preventing *S. aureus* VAP. However, dissecting the mechanisms implicated in pathogenesis of this disease and, particularly, those driving the transition from colonization to infection, could help develop such strategies. Since the spread of nasal colonization to tracheal seems to be Hla-driven, and tracheal colonization is a risk factor for *S. aureus* VAP, one such approach could involve neutralizing the toxin or minimizing its production. Application of the monoclonal antibodies targeting staphylococcal toxins, including Hla, could be investigated as a means of limiting the colonization spread and/or colonization-to-infection transition. Another point for consideration is a conscious approach to the antibiotic therapy (e.g., prophylactic, aiming at non-staphylococcal infections or administered in the course of the selective digestive decontamination) to avoid enhancing toxin production by the bacterium and possibly triggering the colonization-

to-infection transition. These approaches remain theoretical and have to be considered as the follow-up projects, although multiple clinical trials involving the monoclonal antibodies are currently running. Naturally, *S. aureus* infections can also occur in non-colonized individuals. This, however, is relatively rare, as demonstrated by our data, and planning the specific preventative efforts in this population is more challenging.

Unfortunately, *S. aureus* VAP remains associated with high mortality and worsened clinical and economic outcomes despite stringent hygienic measures and availability of effective antimicrobials. Even if the environmental risk factors are minimized (e.g., by strict adherence to the VAP bundle recommendations and implementation of hygienic measures), ICU-admitted and ventilated patients are an especially vulnerable group in face of this nosocomial infection. While this predisposition is multifactorial and determined by the plethora of host-, environment- and pathogen-associated factors, low levels of the *S. aureus*-specific antibodies, described in **Chapter III**, are likely to be contributing to it. On one hand, these patients demonstrate overall low titers of immunoglobulins reactive against *S. aureus* surface and secreted antigens (assessed in whole-cell and supernatant ELISA, respectively). On the other hand, marked variability in the antibody titers against specific staphylococcal toxins introduce another level of complexity: even if the patient is equipped with sufficient antibody titers against several toxins, the bacterium can still bypass the body's protection by producing its multitude of the virulence factors. Importantly, patients colonized by *S. aureus* typically show high levels of anti-staphylococcal antibodies, which creates a paradox: these patients are most at risk for a subsequent VAP despite the fact that the higher antibody titers should be protective. Data presented in this thesis, however, shed light on this contradiction: in comparison to the maximal levels measured in the study, increase of the titers in the colonized patients is very mild and may be physiologically non-relevant. Instead, composition of the immunoglobulins in the patients carrying *S. aureus* seems to mirror the characteristics of the residing strain, as demonstrated by the concordance of Hla-neutralizing titers and the bacterial hemolysis score. Until now, an anti-staphylococcal titer deemed protective has not been defined. This could, however, be a basis for the follow-up studies, as such data could prove helpful for developing new antibody-based therapeutic and prophylactic modalities. Importantly, all of the investigated cytotoxins are produced *in vivo*, as demonstrated by universally detected neutralizing antibodies against all of them. This implies that therapies based on monoclonal antibodies should ideally be offsetting as many of *S. aureus* virulence factors as possible. One mentioned example of such therapy, ASN100, has regrettably been discontinued in Phase II clinical trial, but it does demonstrate an antibody formulation capable of neutralizing multiple staphylococcal toxins.

If a ventilated patient displays symptoms suggestive of VAP, empirical antibiotic therapy is often initiated. Therapy regimens are usually defined according to the local epidemiological and microbiological data, and tend to account for the most common bacterial pathogens. It is still administered “blindly” and may contain antibiotics that the microorganism is resistant against. Regardless of whether the causative pathogen is identified, correct dosing of an antimicrobial is frequently challenging, especially in the critically ill, where the underlying diseases or co-administered medications may influence the drug metabolism. Clinician’s expertise and antibiotic stewardship considerations may similarly affect therapeutic decisions and lead to a suboptimal or insufficient dosing. Finally, patients may be receiving therapy for non-staphylococcal infections with no consideration of *S. aureus* colonization or concomitant infection. An inappropriate choice of an antimicrobial, or its delayed administration have been associated with worsened patient outcomes, possibly due to disease progression and therapy side effects. Along with that, in **Chapter IV** we describe a potentially relevant increase of toxin production by *S. aureus* in response to the sublytic concentrations of antibiotics. Stimulation of *S. aureus* into producing larger toxins quantities may potentially lead to both a more detrimental course of an infection, and to triggering a transition of the colonization to bacterial invasion. Importantly, since this is an active bacterial response to antibiotic stress, only agents effective against *S. aureus* seemed to trigger this effect. This emphasizes the importance of correct dosing and consideration of the infected organ and presence of *S. aureus* colonization in cases, where an antibiotic with anti-staphylococcal activity spectrum is administered for treatment of non-*S. aureus* infections. In case of two equally effective and safe antibiotics available, a concerned treating physician could consider choosing one, exposure to which does not result in a marked toxicity enhancement. For example, our data would favor inclusion of vancomycin, and support a very careful dosing of beta-lactam antibiotics to make sure that the effective concentration in the affected organ is manifold above the MIC for the bacterium. Alternatively, one can consider offsetting the increased toxin concentrations after antibiotic exposure by concomitant administration of neutralizing antibody formulations, or agents modulating *agr* activity. None of such drugs are currently widely available; however, numerous promising clinical trials are being run. Naturally, we do not yet know whether this bacterial behavior is relevant in the in vivo situation, or whether enhancement of toxin production may indeed trigger the transition of staphylococcal colonization to an infection. Further research could address the limitations of our project by testing *S. aureus* in non-planktonic form, expanding the antibiotic selection and collecting *in vivo* data.

To administer the antibiotic therapy early and appropriately, quick and accurate identification of *S. aureus* is essential, especially considering a four-day delay between

the first detection of the bacterium in the trachea and the onset of VAP symptoms. Currently available diagnostic methods are not ideal, and commonly present the clinicians with either a delayed or an equivocal result. E.g., if ETA assessment is not carried out daily, routinely or regularly, a 48 hour delay typically experienced with the conventional microbiological culture can translate into an empirical start of antibiotic therapy. A quick, user-friendly, bedside diagnostic tool could therefore be helpful for *S. aureus* identification, either as a part of routine screening or of a targeted diagnostic workup. Ideally, such kit should become a standard member of the classical point-of-care tests' array, alongside with rapid Strep-A, rotavirus or RSV test. The latex agglutination assay presented in the **Chapter V** is the first step towards the development of such diagnostic kit. It successfully identified samples containing *S. aureus*, and showed no false-positives with samples not containing this bacterium. Additionally, we optimized the assay to prevent the binding of the latex-bound antibodies at the unwanted site of the SpA molecule. The major drawback of the test in its current form, however, is its low sensitivity, because of which it is not suitable for the clinical use. Additionally, interference of the host immunoglobulins and a labor-intensive sample preparation process further limit its applicability. It may, however, serve as a proof-of-concept assay and as a basis for its further development into a lateral flow assay. This format is suggested because of its simplicity, convenience and accessibility. Here, the materials involved in a currently tedious ETA treatment step could be incorporated in the assay buffer. Additional tests, such as detection of heavy colonization or methicillin resistance, should ideally be part of the test.

In addition to the limitations discussed in the individual chapters, one needs to recognize the caveats applicable to the projects in general. First, our findings on the *S. aureus* epidemiology and the levels of anti-staphylococcal antibodies in the critically ill population are based on the data and samples obtained from a single center in the USA, and are not generalizable to the global population. Despite being one of the most prevalent pathogens in the world, *S. aureus* is still characterized by marked geographical differences in its prevalence, epidemiology and resistance profiles. Similarly, demographics of the critically ill population vary greatly depending on the geographic location, specialization and type of the ICU. The serological characteristics of the ICU-admitted patients in other centers may therefore deviate from the values reported here. Whereas the normal ranges of albumin and total IgG are pre-defined, no such range is available for the anti-staphylococcal antibodies. Our conclusions about the overall lower titers in the critically ill therefore stem from the comparison with a geographically and demographically different group, which translates into another limitation of the study. That being said, the reported differences between the healthy and the critically ill patients remain thought-provoking. Ideally, a follow-up study

comparing the matched patient- and healthy subject groups could help further expand and dissect our findings. The demonstrated increase of the toxin production by *S. aureus* in response to the sub-lytic antibiotic concentrations is universally seen in both laboratory and clinical strains and is reproduced in the *in vivo*-mimicking conditions. Its relevance in the clinical setting, however, remains to be demonstrated. The follow-up studies could include, for example, series of the *in vivo* experiments or clinical data analysis to evaluate whether suboptimal dosing indeed results in a more aggressive disease course, or whether it is able to promote the colonization-to-infection shift. Even though our findings could not be corroborated by the clinical data, the presented *in vitro* data supports efforts to achieve a timely, correct and optimally dosed antibiotic therapy.

In general, the relevance and practicality of the projects presented in this thesis can be demonstrated by a following hypothetical clinical scenario: a 72-year old male patient is admitted to the ICU because of a congestive heart failure. His medical history is remarkable for a poorly controlled hypertension and a stage 3a chronic renal failure. Upon admission to the ICU nasal swabs, among others, are collected and sent to the laboratory for screening for *S. aureus* colonization. He is intubated and ventilated, and is given appropriate therapy for the underlying disorder. Local VAP bundle, including semi-recumbent positioning, daily subglottic suctioning, oral care with chlorhexidine, sedation “vacation” and regular spontaneous breathing trials is followed stringently. Endotracheal aspirates are therefore collected daily, and their routine microbiological assessment is established. Although the PCR equipment is available in the institution, according to the hospital’s policy it is reserved for selected cases, and routine screening is carried in the form of a conventional microbiological culture for the financial reasons. According to the same policy, antibiograms are not routinely done on the screening samples. Two days after the admission, the patient’s nasal swabs appear to be positive for *S. aureus*. Aware of the link between the hemolysis intensity and the tracheal colonization, the treating physician pays a visit to the microbiological laboratory and, together with the laboratory technicians, sees that the isolate is highly hemolytic. This informs the doctor of an increased likelihood of a subsequent tracheal colonization and subsequent VAP. The ETA collected on the day of the intubation, however, only shows growth of the normal respiratory flora. It remains inconspicuous for two more days. ETA collected on the third day post-intubation, however, shows heavy growth of *S. aureus*, confirming the previous concern of the nasal-to-tracheal colonization spread. Since the delay with the conventional microbiological culture is 48 hours, by the time the physician receives the information, the patient is already intubated for 5 days. Knowing that the first detection of the *S. aureus* precedes VAP symptoms by approximately 4 days, the doctor orders an expanded antibiogram for an

optimal planning of the eventual antibiotic therapy. On the next day, the isolate is characterized as an MSSA susceptible to the beta-lactams, cephalosporins and vancomycin. The physician then checks with the local antibiotic stewardship recommendations and writes down the treatment plan in case the patient starts displaying symptoms. The next morning, indeed, he presents with a 39.2°C fever. A blood count shows a leukocytosis of 16,000 cells per microliter, and the ETA obtained during the daily subglottic suctioning is grossly purulent. Seeing that the patient is showing symptoms of VAP, a chest X-ray is ordered, which shows diffuse opacities in the right lung field. With the diagnosis confirmed, the treating physician obtains the diagnostic respiratory sample. Since the definitive diagnosis is delayed by approximately 48 hours with the conventional culture, and several hours if PCR is used, empirical antibiotic therapy needs to be initiated. Thankfully, the plan is already available from the previous day, and can be administered immediately. As the anti-staphylococcal agent of choice oxacillin is selected. Taking into consideration the 3a renal failure, a suggestion is made to decrease the dosing regimen. However, the treating physician remembers that exposure of *S. aureus* to sub-MIC concentrations of the antibiotics may enhance the toxin production by the bacterium. S/he confirms that no rigid recommendations concerning oxacillin dose reduction in kidney failure exist, and that the patient's kidney functions are massively improved after diuretic therapy. S/he then decides to apply the regular dose and monitor for side effects. The result of the PCR testing of the ETA is available in the evening of the same day and confirms presence of methicillin-sensitive *S. aureus*. Seeing an unequivocal result of the testing, a decision is made not to proceed with the invasive sample collection such as PSB or BAL. The patient shows steady improvement and is fever-free on the fourth day after initiation of the antibiotic therapy. Because of VAP, he needs respiratory support for twelve days after the VAP diagnosis, but manages to make an uneventful recovery and be transferred to the floor unit. Although satisfied with the outcome, the treating physician realizes that there are a number of opportunities for improvement of this patient's care. There is a need for specific prophylactic measures which can be applied upon detecting the lower airway colonization, so that the ICU stay would not be prolonged by VAP. Similarly, specific therapeutic agents targeting *S. aureus* are required, with a possibility of administering them as soon as VAP symptoms are detected. Taking into consideration the involvement of bicomponent cytotoxins in the staphylococcal colonization and infection, as well as hypogammaglobulinemia reported in the ICU population, monoclonal antibody formulation targeting *S. aureus* toxins would be an ideal treatment modality. It has a favorable side effect profile and does not promote antibiotic resistance, i.e. it can be administered without waiting for identification of the causative pathogen. Furthermore, to optimize the VAP-therapy, the

delay until the responsible microorganism is identified should be made shorter. A bedside test giving the information on presence of *S. aureus*, its resistance profile and bacterial density would be ideal to optimize the treatment planning. Collectively, these data and tools should improve the approach to any ICU patient with *S. aureus* colonization or a suspected staphylococcal VAP.

Taken together, our findings deepen our understanding of the *S. aureus* human infection, and pave the way to improving its clinical management. Once again, we emphasize the prevalence and the impact of the staphylococcal disease despite the preventive strategies and effective antimicrobials, and encourage continuous clinical research and development of the novel therapeutic, prophylactic and diagnostic modalities. We shed light on the host-pathogen interactions during infection and colonization, and demonstrate how suboptimal therapy may increase staphylococcal virulence. We contribute to the combat against the bacterium by initiating the development a point-of-care diagnostic tool for quick detection of *S. aureus*. The data presented in this thesis provides the basis for further research and has, on the long run, the potential to affect the lives and health of thousands of patients affected by the staphylococcal infections.

VII. SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TO CHAPTER II, ENDOTRACHEAL ASPIRATE BACTERIOLOGY

The supplementary files for the article “The utility of endotracheal aspirate bacteriology in identifying mechanically ventilated patients at risk for ventilator associated pneumonia: a single-center prospective observational study” are available online under https://bmcinfectdis.biomedcentral.com/articles/10.1186/s12879-019-4367-7#Sec15

SUPPLEMENTARY TO CHAPTER III, ANTISTAPHYLOCOCCAL ANTIBODIES IN THE ICU POPULATION

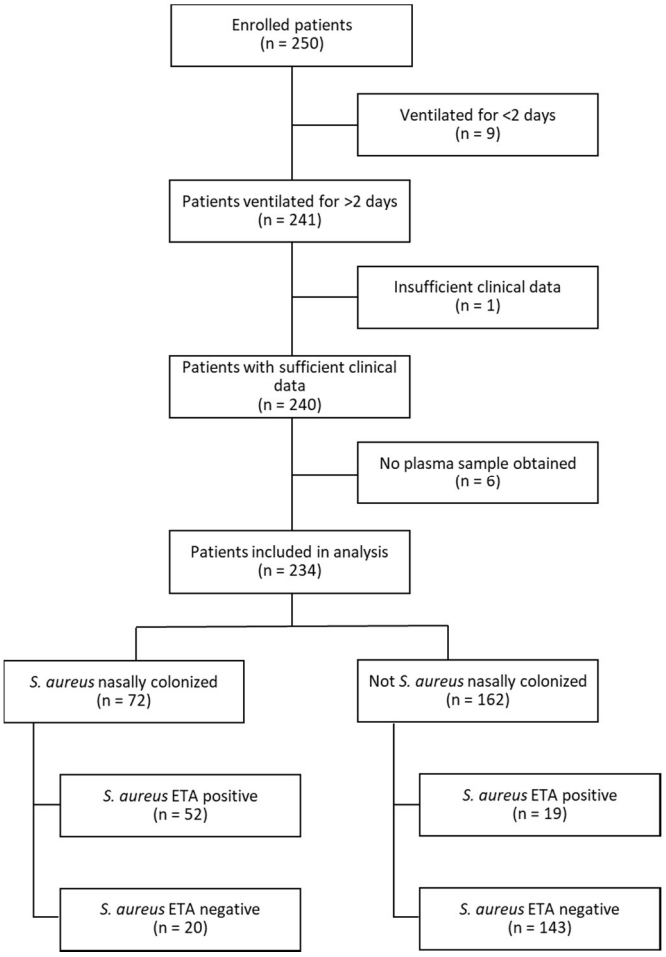

Fig. S3.1. Disposition of the study patients included in the study, and their nasal and tracheal colonization by *S. aureus*.

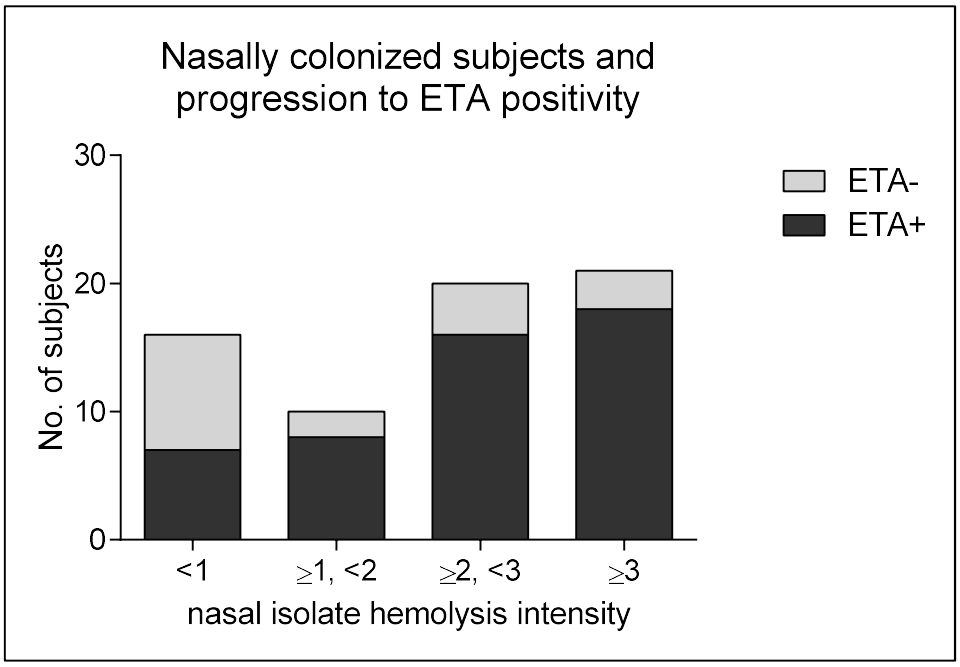

Fig. S3.2. Association of the hemolysis intensity of the nasal *S. aureus* isolates with the subsequent ETA colonization. *ETA-: patients remaining negative for S. aureus colonization in the tracheal aspirate throughout the whole study. ETA+: patients who had at least one ETA with S. aureus colonization throughout the study.*

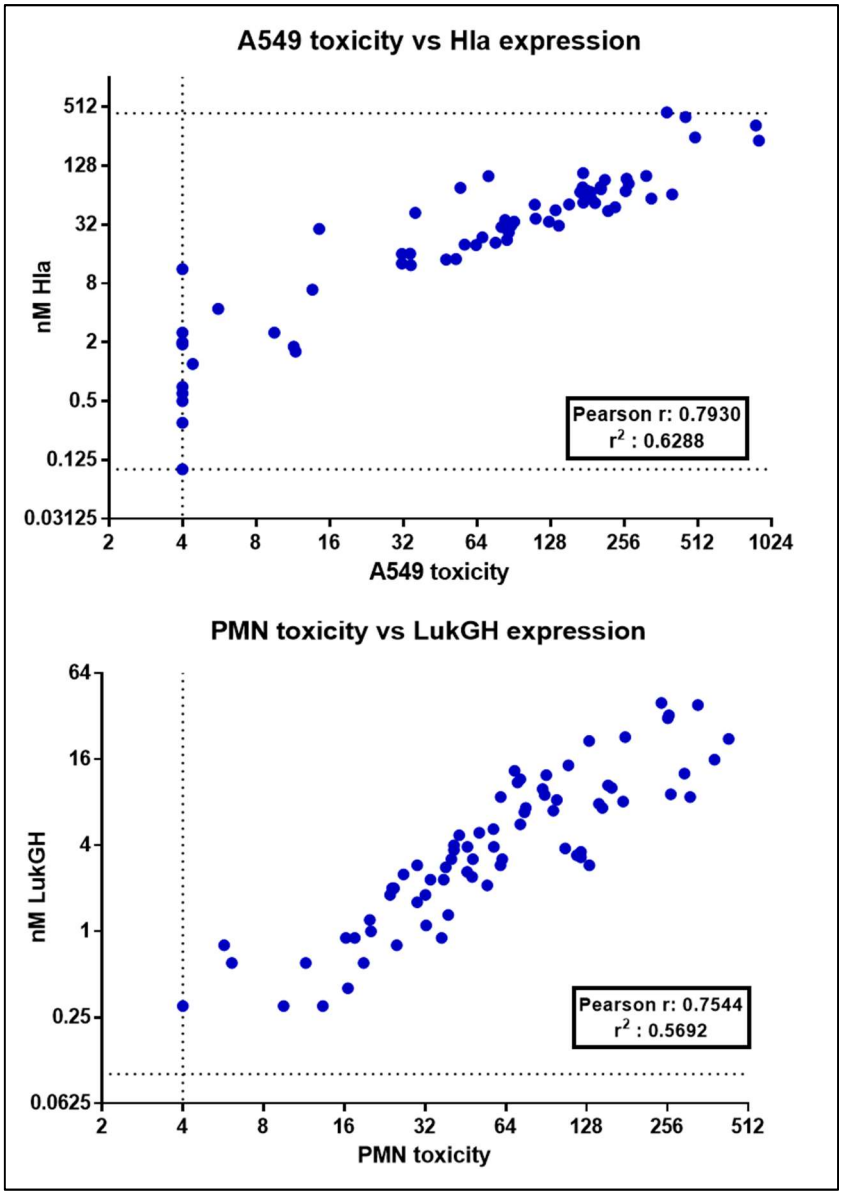

Fig. S3.3. Relationship between toxin concentrations measured in the bacterial supernatants and their toxicity in the cell-based assay. *Toxicity of Hla and LukGH were assessed in the A549 and PMN assays, respectively. Detection limits of the assay are shown by the dotted lines. Supernatant toxicity is expressed as EC50 values.*

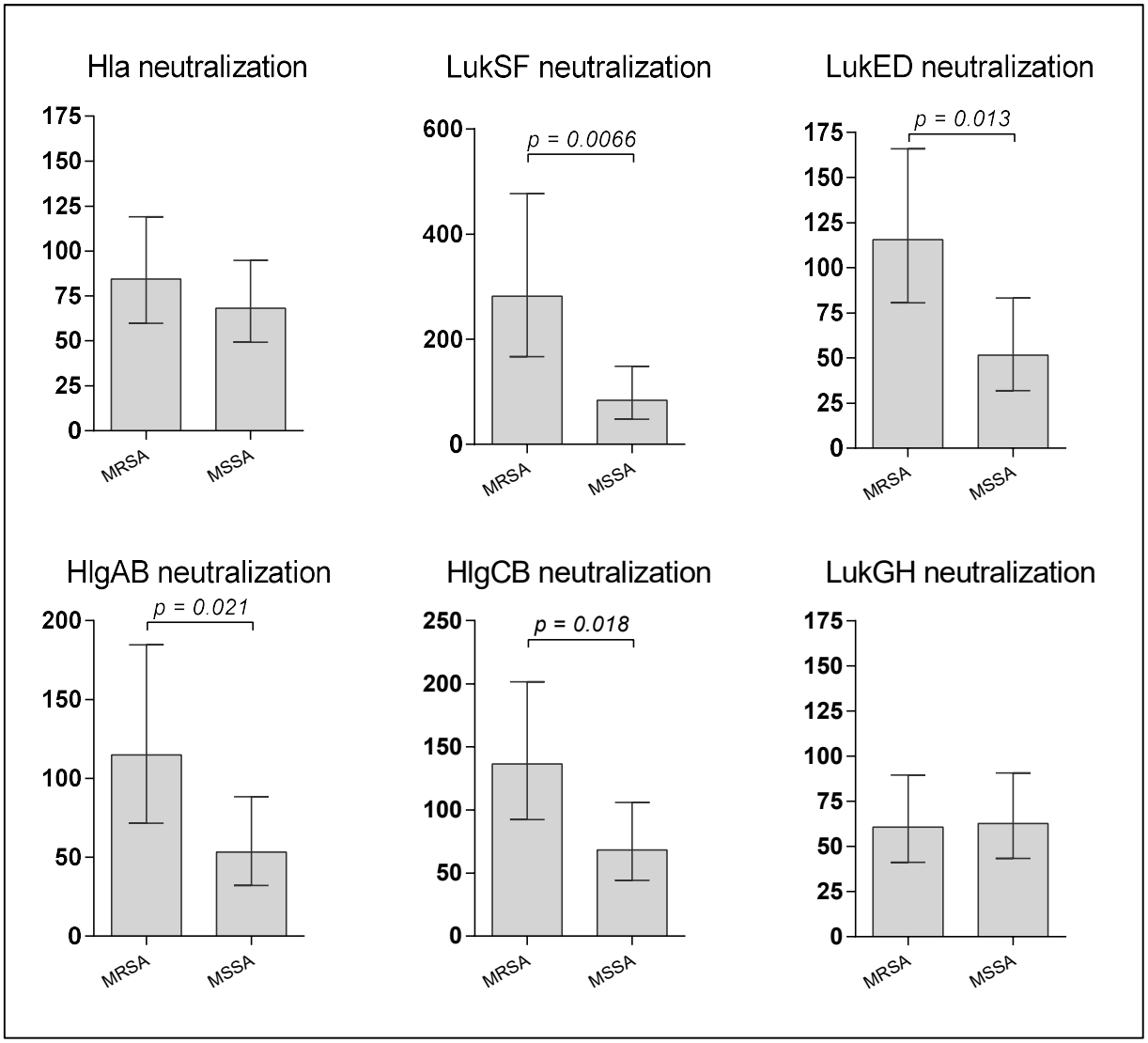

Fig. S3.4. Anti-toxin antibody titers in patients colonized with MRSA and MSSA. *Plotted is geometric mean values and 95% confidence intervals. 29 patients were MRSA-colonized, and 40 patients were MSSA-colonized.*

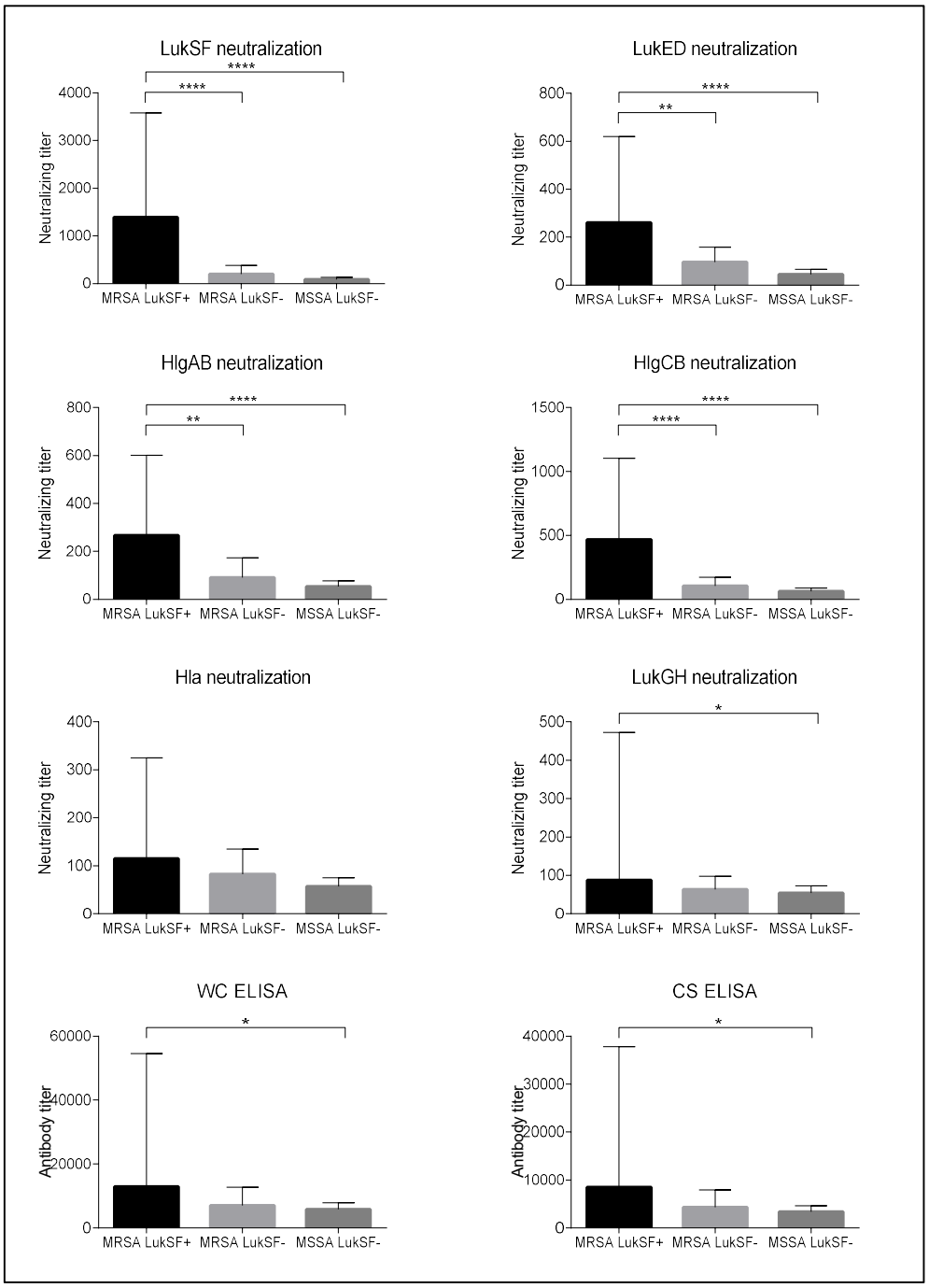

Fig. S3.5. Anti-toxin antibody titers in patients colonized with MRSA and MSSA, LukSF-stratified. *MRSA LukSF+: MRSA isolates positive for LukSF gene (n = 6). MRSA LukSF-: MRSA isolates negative for LukSF gene (n = 18). MSSA LukSF-: MSSA isolates negative for LukSF gene (n = 57). For 5 MRSA and 2 MSSA isolates no LukSF-gene status could be identified. Single LukSF-positive MSSA was not included. Plotted is geometric mean values and 95% confidence intervals.*

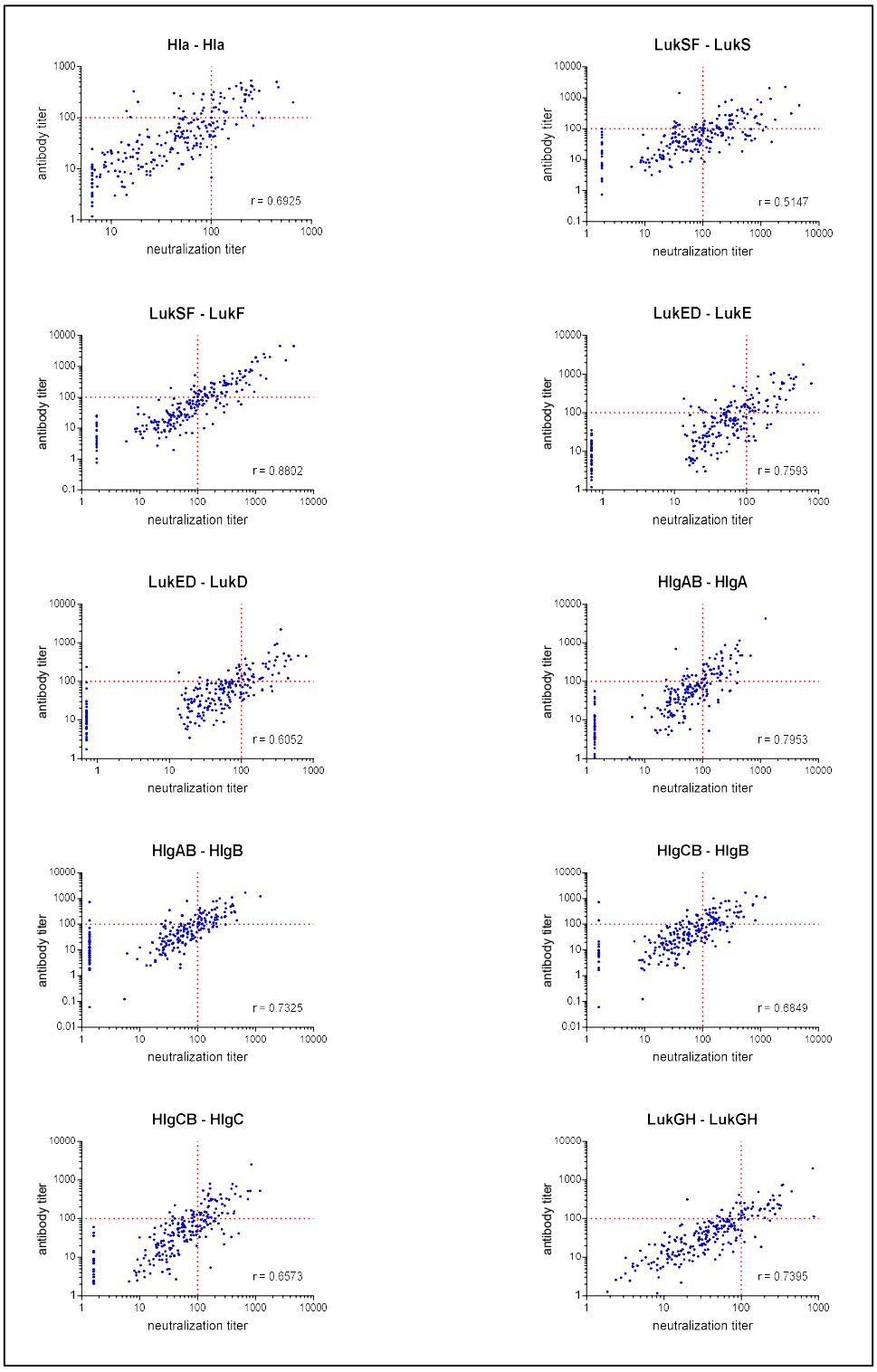

Fig. S3.6. Correlation between anti-toxin antibody titers and serum neutralizing capacity. *In the individual graphs' heading, the first word denotes the toxin tested in the neutralization assay, and the second refers to toxin tested in ELISA.*

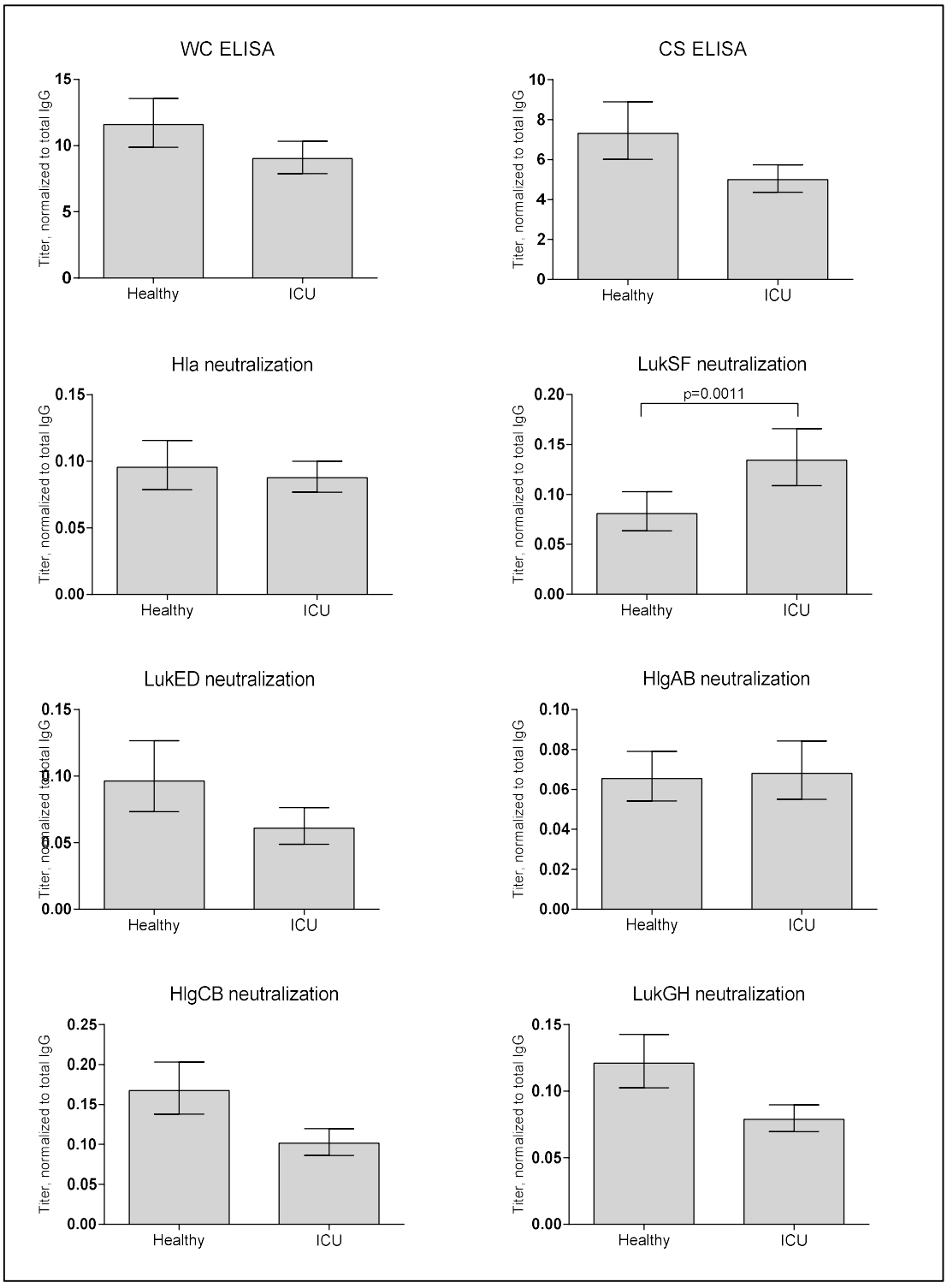

Fig. S3.7. *S. aureus*-specific antibody titers and neutralization capacity of ICU and healthy population after correction to total IgG levels. *Geometric mean and range are plotted.*

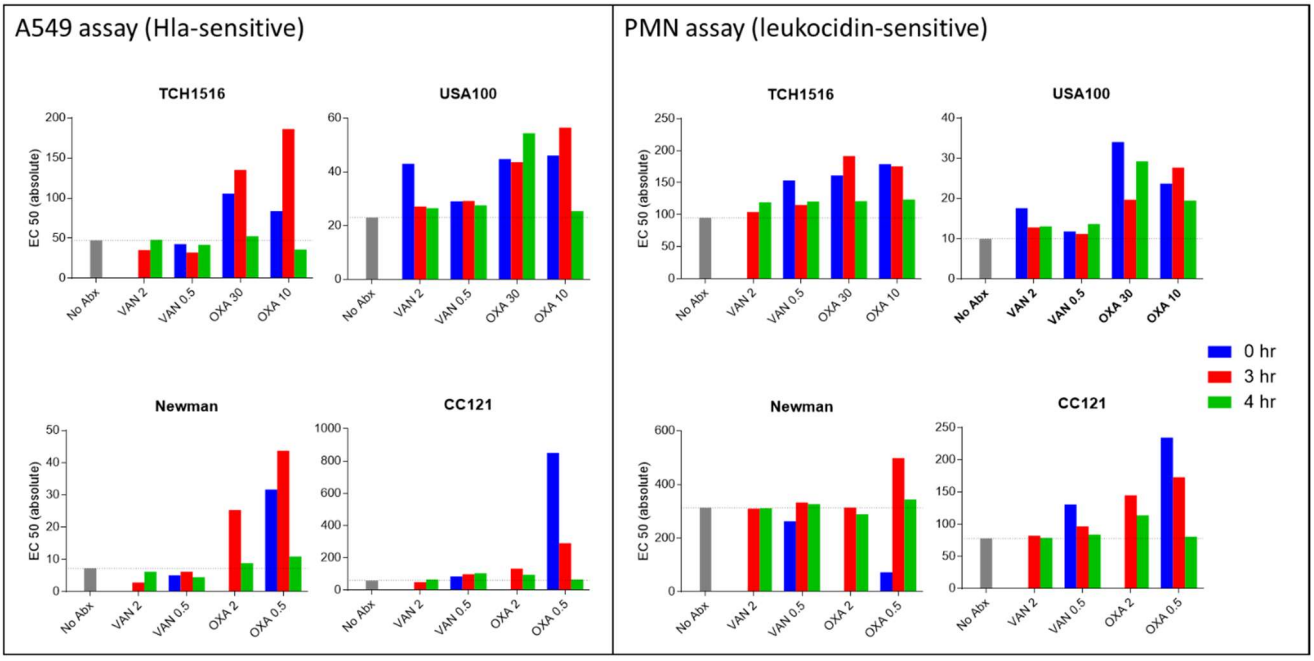

Fig. S4.1. Influence of timepoint of antibiotic addition on the toxicity of the *S. aureus* supernatants. *EC50 values are plotted; supernatants obtained from the bacteria grown with no antibiotics (= "No Abx") are shown as control. Legend refers to the timing of the antibiotic addition relative to the start of the incubation. VAN = vancomycin, OXA = oxacillin. The number after the antibiotic abbreviation refers to the antibiotic concentration expressed in µg/ml (i.e., VAN 2 = 2 µg/ml vancomycin).*

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