From Department of Medicine, Huddinge
Karolinska Institutet, Stockholm, Sweden

STREPTOCOCCAL AND STAPHYLOCOCCAL TISSUE INFECTIONS: THERAPEUTIC CHALLENGES AND OPPORTUNITIES

Helena Bergsten

Karolinska Institutet

Stockholm 2021

All previously published papers were reproduced with permission from the publisher.
Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2021

ISBN 978-91-8016-377-4

Cover illustration: *Staphyloccus aureus* infecting and degrading the epithelium of an organotypic skin tissue model. Colors: Nile red (red, lipids), wheat germ agglutinin (green, carbohydrates), DAPI (blue, nucleic acid), bacteria (white, anti-*Staphylococcus aureus* antibody). By Helena Bergsten.

Streptococcal and Staphylococcal Tissue Infections: Therapeutic Challenges and Opportunities
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Helena Bergsten

The thesis will be defended in public at Karolinska Institutet, lecture hall 9Q Månen, Alfred Nobels Allé 8 floor 9, December 17th, 2021, at 09.00.

Principal Supervisor:
Anna Norrby-Teglund
Karolinska Institutet
Department of Medicine, Huddinge
Center for Infectious Medicine

Co-supervisor(s):
Mattias Svensson
Karolinska Institutet
Department of Medicine, Huddinge
Center for Infectious Medicine

Opponent:
Claire Poyart
Université de Paris
Groupe Hospitalo-Universitaire Centre
Department de Bactériologie

Examination Board:
Kristina Broliden
Karolinska Institutet
Department of Medicine, Solna
Division of Infectious Diseases

Åsa Melhus
Uppsala University
Department of Medical Sciences
Division of Infectious Medicine

Keira Melican
Karolinska Institutet
Department of Neuroscience
Center for the Advancement of Integrated Medical and Engineering Sciences

To science

POPULAR SCIENCE SUMMARY OF THE THESIS

The bacteria *Streptococcus pyogenes* and *Staphylococcus aureus* have been leading causes of human infections throughout history. After a century of research, no effective vaccines are available. For a few decades, we have had weapons against these bacteria. Antibiotics gave hope of a world where fever did not equal a death sentence. The development of antibiotic resistance is rapidly changing hope into worry about the future in a post-antibiotic era. But also currently, where antibiotic alternatives are available, and mortality has decreased, deaths still occur. Sequelae after infection are also common. Hence, improved therapeutics against bacterial infections are badly needed. Also, we need to know the efficacy of the treatments used today. Are expensive drugs effective? At what dose? Are bacteria in tissue killed by antibiotics? If not, how can we improve the antibiotic efficacy? Can we dampen bacterial pathogenicity, without promoting resistance, through interference with bacterial communication? Does lack of bacterial communication lead to increased persistence in tissue? These are the main questions assessed in this thesis.

Through culture of artificial human skin tissue, bacterial infection, and treatment, we have studied these questions. We found that 25g IVIG is enough to neutralize the explosive proliferation of T-cells induced by the toxins of *Streptococcus pyogenes*. We observed that the dose administered to patients correlated with the ability to neutralize these toxins. Our collaborators observed a trend of improved outcome in patients with necrotizing soft tissue infections caused by these bacteria when randomized to IVIG treatment, and IVIG treatment was an independent factor for survival in a large observational study.

When allowed to infect artificial human skin tissue, *Streptococcus pyogenes* forms biofilm, a molecular shield of protection. The standard treatment of penicillin and clindamycin was used in low, medium, and high doses and was only able to reduce the number of viable bacteria to a minor degree. When a low dose of rifampicin, another antibiotic, was added, the treatment outcome was better. Not only were more bacteria killed, but it also took longer time for the remaining bacteria in the biofilm to start to grow back, and the growth was decreased.

Staphylococcus aureus is a well-known producer of bacterial biofilm. This is regulated by a communication system, so that the individual bacteria can coordinate its behavior to the bacterial group. We used a strain that had a dysfunction in this system and studied the biofilm it formed. On plastic, the strain formed biofilm but was unable to disperse the biofilm, as the functional variant did after 9 hours of culture. In our tissue model, biofilm was formed irrespective of communication, but the communicating strain also dispersed the epithelium and invaded the deeper layers of the tissue. The dysfunctional variant continued to form biofilm on top of the intact skin tissue. This shows that drugs targeting this system would decrease the invasive properties of *Staphylococcus aureus* but not biofilm formation.

These studies illustrate the therapeutic challenges and opportunities of today, in tissue infections by *Streptococcus pyogenes* and *Staphylococcus aureus.*

ABSTRACT

Streptococcus pyogenes and *Staphylococcus aureus* have been leading causes of human infections throughout history. *S. pyogenes* is of the top-ten pathogens responsible for most death globally, 0.5 million deaths per year. *S. aureus* is carried asymptomatically by half the population at any point in time and *S. aureus* bacteremia is probably the most common life-threatening infection worldwide. These bacteria colonize us, cause mild self-limiting infections such as impetigo and pharyngitis but also rare grave conditions such as streptococcal/staphylococcal toxic shock syndrome (STSS) and necrotizing soft tissue infections (NSTI).

In STSS, patients are recommended to receive adjunctive intravenous immunoglobulin (IVIG) to dampen the mitogenic superantigen-response in T-lymphocytes. In NSTI, the benefit of IVIG treatment is unclear. The first randomized controlled trial of IVIG in NSTI by all microbiological etiologies showed no benefit, but the subgroup dominated by *S. pyogenes* and *S. aureus* infections indicated a trend of improved outcome. Here, we assessed plasma samples from these patients, demonstrating that a dose of 25g IVIG is effective at neutralizing toxins from most *S. pyogenes* strains. The neutralizing capacity of patient plasma correlated with the IVIG dose administered.

In NSTI, the antibiotic treatment recommendations include a β-lactam antibiotic such as penicillin, and a toxin-dampening antibiotic such as clindamycin. Using an organotypic 3D model of human skin, we treated *S. pyogenes* tissue infections with this standard treatment and observed only a minor effect on reduction of bacterial viability. When we added the antibiotic rifampicin as adjunctive treatment, we observed a significant reduction of bacterial viability and metabolism. Bacterial biofilm formation has been recognized as a complicating microbiological feature of *S. pyogenes* NSTI, and this could be the reason behind the treatment failure and high morbidity and mortality associated with the infections.

S. aureus biofilm formation is regulated by the Accessory gene regulator or Agr system. Using an Agr-silent mutant, we measured biofilm formation by methicillin-resistant *S. aureus* (MRSA). We observed impaired biofilm dispersal in the Agr-silent MRSA strain, resulting in sustained biofilm formation on polystyrene surfaces. When grown on collagen-coated surfaces, biofilm increased by both strains. In our skin tissue model, both isolates formed biofilm, but the Agr-silent strain did not affect the epithelial integrity while the Agr-signaling strain caused epithelial damage and disseminated into the deeper layers of the tissue.

Host-pathogen interactions are complicated due to the multitude of cells and molecules involved. In this thesis, we have studied bacterial pathogens in their natural habitat: near human cells. Although not as complex as real tissue, our model systems are relevant by mimicking important features of the clinical setting. Our research questions are clinical, and our setup is experimental.

LIST OF SCIENTIFIC PAPERS

I. **Bergsten, H.**, M. B. Madsen, F. Bergey, O. Hyldegaard, S. Skrede, P. Arnell, O. Oppegaard, A. Itzek, A. Perner, M. Svensson and A. Norrby-Teglund. Correlation Between Immunoglobulin Dose Administered and Plasma Neutralization of Streptococcal Superantigens in Patients With Necrotizing Soft Tissue Infections. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2020;71(7):1772-5.

II. **Bergsten, H.**, L. M. Palma Medina, M. Morgan, K. Moll, D. H. Skutlaberg, S. Skrede, T. Wajima, M. Svensson and A. Norrby-Teglund. Adjunctive Rifampicin Increases Antibiotic Efficacy in Group A Streptococcal Tissue Infection Models. *Antimicrobial agents and chemotherapy*. 2021;65(11):e0065821.

III. **Bergsten, H.**, S. M. Shambat, E. Kvedaraite, M. Svensson and A. Norrby-Teglund. Dysfunctional quorum sensing in a natural AgrC variant results in sustained biofilm by ST22 methicillin-resistant *Staphylococcus aureus*. *Manuscript*.

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. Emgard, J., **H. Bergsten**, J. K. McCormick, I. Barrantes, S. Skrede, J. K. Sandberg and A. Norrby-Teglund. MAIT Cells Are Major Contributors to the Cytokine Response in Group A Streptococcal Toxic Shock Syndrome. *Proceedings of the National Academy of Sciences of the United States of America*. 2019;116(51):25923-31.

II. Madsen, M. B., **H. Bergsten** and A. Norrby-Teglund. Treatment of Necrotizing Soft Tissue Infections: IVIG. *Advances in Experimental Medicine and Biology*. 2020;1294:105-25.

III. Siemens, N., B. Chakrakodi, S. M. Shambat, M. Morgan, **H. Bergsten**, O. Hyldegaard, S. Skrede, P. Arnell, M. B. Madsen, L. Johansson, J. Juarez, L. Bosnjak, M. Mörgelin, M. Svensson and A. Norrby-Teglund. Biofilm in group A streptococcal necrotizing soft tissue infections. *JCI Insight*. 2016;1(10):e87882.

IV. Lourda, M., M. Dzidic, L. Hertwig, **H. Bergsten**, L. M. Palma Medina, I. Sinha, E. Kvedaraite, P. Chen, J. R. Muvva, J. B. Gorin, M. Cornillet, J. Emgård, K. Moll, M. García, K. T. Maleki, J. Klingström, J. Michaëlsson, M. Flodström-Tullberg, S. Brighenti, M. Buggert, J. Mjösberg, K. J. Malmberg, J. K. Sandberg, J. I. Henter, E. Folkesson, S. Gredmark-Russ, A. Sönnerborg, L. I. Eriksson, O. Rooyackers, S. Aleman, K. Strålin, H. G. Ljunggren, N. K. Björkström, M. Svensson, A. Ponzetta, A. Norrby-Teglund and B. J. Chambers. High-dimensional profiling reveals phenotypic heterogeneity and disease-specific alterations of granulocytes in COVID-19. *Proceedings of the National Academy of Sciences of the United States of America*. 2021;118(40):e2109123118.

V. Mairpady Shambat, S., P. Chen, A. T. Nguyen Hoang, **H. Bergsten**, F. Vandenesch, N. Siemens, G. Lina, I. R. Monk, T. J. Foster, G. Arakere, M. Svensson and A. Norrby-Teglund. Modelling staphylococcal pneumonia in a human 3D lung tissue model system delineates toxin-mediated pathology. *Disease models & mechanisms*. 2015;8(11):1413-25.

CONTENTS

LIST OF ABBREVIATIONS

Agr	Accessory gene regulator
α-toxin/hla	α-hemolysin
CA-MRSA	Community associated methicillin-resistant *S. aureus*
CFU	Colony forming units
CRP	C-reactive protein
DNase	Deoxyribonuclease
emm	M-protein gene
GAS	Group A *Streptococcus*
GBS	Group B *Streptococcus*
HA	Hyaluronic acid
HBOT	Hyperbaric oxygen treatment
HLA	Human leukocyte antigen
IgG	Immunoglobulin G
IL	Interleukin
INFECT	Improving outcome of necrotizing fasciitis: elucidation of complex host pathogen signatures that dictate severity of tissue infection
INSTINCT	Immunoglobulin G for patients with necrotizing soft tissue infection: a randomised, blinded, placebo-controlled trial
IVIG	Intravenous immunoglobulin G
LRINEC	Laboratory Risk Indicator for Necrotizing Fasciitis
MRSA	Methicillin-resistant *Staphylococcus aureus*
NSTI	Necrotizing soft tissue infections (necrotizing fasciitis)
PBMC	Peripheral blood mononuclear cell
PSM	Phenol soluble modulin
PVL	Panton-Valentine leukocidin
SIRS	Systemic inflammatory response syndrome
SLO	Streptolysin O
SLS	Streptolysin S
SOFA	Sequential [Sepsis-related] Organ Failure Assessment
SpeA/B/C…	Streptococcal pyrogenic exotoxin A/B/C…
S. aureus	*Staphylococcus aureus*
S. pyogenes	*Streptococcus pyogenes*

STSS	Streptococcal/staphylococcal toxic shock syndrome
TSST-1	Toxic shock syndrome toxin-1
VRSA	Vancomycin-resistant *Staphylococcus aureus*
WHO	World Health Organization

1 LITERATURE REVIEW

TO CARRY A KILLER

All people carry bacteria, but most of them are harmless. What is frightening, is bacteria that act as "kind" commensals, but suddenly cause life-threatening infections. Several hundred thousand people in Sweden carry *Streptococcus pyogenes* (*S. pyogenes*) asymptomatically, and almost half the population carry *Staphylococcus aureus* (*S. aureus*) at any point in time. These are healthy people, at an increased risk for invasive bacterial infections.

> *Humanity has but three great enemies: fever, famine and war;*
> *of these by far the greatest, by far the most terrible, is fever.*
>
> Sir William Osler, 1896.

TYPICAL CASE REPORT

November 1st, a 42-year-old man walks into the emergency room at Danderyds Hospital north of Stockholm. For two days he has experienced increasing pain in his left foot, where he has had an eczema for a couple of weeks. He is previously healthy, except for a sore throat. Despite low inflammatory values (CRP 8), he is admitted for observation and the next morning the staff find him feverish with decreased consciousness and low blood pressure. He is given broad antimicrobial treatment and a total of six liters of fluids, but still, he is unable to keep adequate blood pressure, with a lactate value of eight. He is transferred to the intensive care unit via the operating room. An orthopedic surgeon makes an incision, removes necrotic tissue, and makes the diagnosis of necrotizing fasciitis. In wound cultures from his foot grows *S. aureus*. In blood cultures and cultures taken from the necrotic tissue grows *S. pyogenes*. He needs high doses of noradrenaline and dobutamine to keep blood flow to important tissues, his blood is taking 2.5 times longer than normal to coagulate, and he is sedated using propofol and morphine. He receives intravenous immunoglobulins and hydrocortisone. He is moved to Karolinska University Hospital and is treated with hyperbaric oxygen in a pressure chamber. November 7th the man is brought back to consciousness and on the 10th the wound is finally shut, after several surgeries. He still needs complex bandaging, oxygen treatment, pain, and sleep medications, and he lacks the strength to stand on his own feet for a couple of more weeks. But he is now a survivor of a necrotizing soft tissue infection.

INVASIVE INFECTIONS THAT CAUSE NECROSIS

Necrosis is a destructive way of cell death where damage leads to unregulated spread of cellular content. Necrotizing skin and soft tissue infections (NSTI), including necrotizing fasciitis, is the most severe form of bacterial-induced tissue pathology with unpredictable onset and rapid development into life-threatening conditions. NSTI is associated with high mortality (20-40%) and morbidity despite adequate antibiotic and organ-supporting treatment [1]. The infection is located deep in the fascia with rapid progressive tissue destruction and is often complicated by septic shock and multiorgan failure. Finding of necrosis at exploration of the affected area by a surgeon provides the diagnose of NSTI. Bacteria can be isolated from sterile sites as blood or tissue, or in some cases the causative agent is not identified. Factors that influence NSTI clinical presentation and course of outcome are host genetics, age, comorbidities, immunity, preceding

events (virus, trauma), bacterial port of entry, species and virulence, infection duration before treatment, and type of treatment. The diagnosis of NSTI is notoriously difficult. Some argue that 'pain out of proportion' is an early sign of NSTI, that CRP levels and LRINEC-scoring can aid in differentiating NSTI from milder infections [2]. Others argue that the most prevalent symptom (skin rash) is present in only around half of all NSTI patients (and all patients with milder infections) and that initial inflammatory values vary, making symptomatic/laboratory differentiation impossible [3]. The earliest report of NSTI was made within an outbreak of erysipelas [4]:

> *…[T]he erysipelas would quickly spread widely in all directions. Flesh, sinews and bones fell away in large quantities... Fever was sometimes present and sometimes absent... There were many deaths. The course of the disease was the same to whatever part of the body it spread. Many lost the arm and the entire forearm. If the malady settled in the sides there was rotting either before or behind…*
>
> Hippocrates, 5th century BC

MICROBIOLOGY OF NECROTIZING SOFT TISSUE INFECTIONS

NSTI is often placed in one of two categories. Type I NSTI affect either the thorax/abdomen/genital area and is caused by a polymicrobial infection including one or more anaerobic bacteria. Type II NSTI affect the extremities/head/neck-area and most commonly comprise of monomicrobial infections. Type II account for approximately half of NSTI cases and are the focus of this doctoral work. Beta-hemolytic *Streptococci* are the most common cause of monomicrobial NSTI and another common cause is *S. aureus*. The bacteria spread through direct and indirect contact between individuals and the most common way for bacteria to get access to the human body is through the throat or pre-existing wounds or cuts in the skin. Risk factors are diabetes mellitus, immunosuppression, obesity, malignancy, intravenous drug use and blunt trauma [5, 6].

HISTORY OF *STREPTOCOCCUS PYOGENES*

Theodor Billroth, an Austrian surgeon, described streptococcal erysipelas and wound infections in the 1860s. He observed "small organisms" (kettenkokken) organized in pairs or chains. In 1879, Louis Pasteur tried to resolve the problem of high rates of females dying of child-birth fever and stated: "Let the Academy allow me to draw the dangerous microbe I believe is responsible for puerperal fever". After this, he isolated "an organism made of grains in couples or chains" from a woman with puerperal fever. The name *Streptococcus pyogenes* was coined 1884 by Friedrich Julius Rosenbach, translating to "pus-forming round bacteria in chains". Two of three post-partum deaths were caused by *S. pyogenes* [7], that could spread easily in 19th century settings, and there was little physicians could do about the infections. Rebecca Lancefield discovered in 1919 that streptococci evoked protective, type-specific antibodies and classified them into groups [8], of which *pyogenes* belong to Lancefield group A (group A streptococci: GAS). In 1928 bacteriologist Alexander Fleming discovered penicillin and in the 1940s chemists Howard Florey and Ernst Chain succeeded in developing large-scale production of the drug (for which the trio was awarded the Nobel prize in 1945). The USA played a major role in the development, due to World War II, and priority for the miracle drug was given to military use. When penicillin became available to a larger audience,

the fear of bacteria decreased. There was a dramatic drop in *S. pyogenes* related conditions in the western world over the 20th century related in part to improved socioeconomic conditions, timely antibiotic treatment of streptococcal pharyngitis, and secondary prophylaxis for rheumatic fever [9]. In 1967, the American Surgeon General William H. Stewart travelled to the White House to deliver one of the most encouraging messages: the war on infectious disease is over. In the 1980s experimental infections (injections and inoculations onto skin) of *S. pyogenes* into human volunteers were performed on laboratory personnel, college student volunteers and investigators themselves [10]. In the late 1980s reports started to arise world-wide describing increased incidence and severity of *S. pyogenes* infections [9]. After years of steadily declining morbidity and mortality due to group A streptococcal infections young, previously healthy individuals fell victim of what the media termed "flesh eating bacteria". Since then, increased attention has been given to *S. pyogenes*. Invasive infections by *S. pyogenes* are notifiable and regulated in the The Swedish Law for Communicable Disease Control since 2004.

> *If you don't like bacteria, you are on the wrong planet.*
>
> Stewart Brand

STREPTOCOCCAL BURDEN OF DISEASE

S. pyogenes is a human pathogen, almost exclusively restricted to humans [11]. Healthy asymptomatic carriers of about 2-6% of the population function as reservoirs, most commonly in the respiratory tract [12-15]. School aged children, 5-15 years of age, are more often asymptomatic carriers: 10-25% [16-20]. There is a seasonal variation with increased levels of colonization and infections during winter months [21]. *S. pyogenes* cause a spectrum of diseases, ranging from superficial skin and throat infections such as impetigo to life-threatening toxic shock syndrome, necrotizing soft tissue infections, puerperal sepsis and additionally, there are autoimmune post-streptococcal sequelae such as rheumatic heart disease. Globally, there are over 616 million incident cases per year of streptococcal pharyngitis and more than 111 million cases of pyoderma [22]. The prevalence of severe streptococcal disease is at least 18,1 million cases, with 1,78 million new cases each year. An estimate is that there are at least 517 000 deaths each year due to severe *S. pyogenes* disease, making *S. pyogenes* one of the top-10 pathogens causing human death [22].

The greatest burden of disease is due to the post-streptococcal sequelae called rheumatic heart disease, with a prevalence of at least 15,6 million cases, with 282 000 new cases and 233 000 deaths each year. In low-income countries where living conditions are crowded, less hygienic, and there is low access to medical care and nutrition; the major severe diseases related to *S. pyogenes* are invasive streptococcal disease and acute rheumatic fever, rheumatic heart disease, and post-streptococcal glomerulonephritis. An autoimmune response to repeated throat infection with *S. pyogenes* result in acute rheumatic fever, and repeated episodes of rheumatic fever results in immune attack on the heart: rheumatic heart disease [23]. Throat and skin infection with *S. pyogenes* can lead to glomerulonephritis, where immune complex composition in the kidney's glomeruli post streptococcal disease cause non-reversible damage. In low-income countries, there is also high morbidity due to streptococcal impetigo and puerperal fever in pregnant and post-partum women. In high-income countries rheumatic fever

has become a forgotten disease, while tonsillopharyngitis and invasive streptococcal disease are of greatest public health importance [24]. Interestingly, a recent meta-analysis reported that asymptomatic carriage rate in children is higher in high-income countries [20]. The global burden of invasive *S. pyogenes* diseases is over 663 000 new cases and 163 000 deaths each year [22]. The most recent incident rate reported in Sweden is 6.5 cases per 100 000 inhabitants, an 8% increase since the year before [21]. Around 60% of invasive *S. pyogenes* infections occur in the gyneco-obstetrical sphere, for women between 18 and 40 years of age [25]. Group B *Streptococcus* (GBS) is more common in the female reproductive tract, but GAS/*S. pyogenes* causes more severe maternal infections [25]. *S. pyogenes* infections have also been associated with other disorders such as pediatric acute onset obsessive compulsory disorder [26] and acute guttate psoriasis [27].

HISTORY OF *STAPHYLOCOCCUS AUREUS*

1881 the Scottish surgeon Sir Alexander Ogston identified *Staphylococcus* as a causative agent of wound infection and named it for the grape-like clusters (*staphyle*-grape and *kokkos*-berry/seed in Greek) he observed under the microscope [28]. In 1884, German scientist Anton Rosenbach grew two strains of Staphylococcus, one white and one yellow. The yellow staph (due to staphyloxantine [29]) was named "golden": *Staphylococcus aureus* [30]. 1930 a coagulase-test made it possible to detect the plasma-coagulating enzyme secreted by *S. aureus*, the enzyme that separates it from most other stapylococci. 1941 a British policeman, seriously ill with *S. aureus* infection, was the first to be treated with penicillin. Before the community introduction of penicillin, the mortality rate of *S. aureus* bacteremia was reported as high as 80% [31]. In the end of the 1940s, benzylpenicillin had cured many staphylococcal infections, but penicillin-resistant outbreaks began to occur. In 1959, the new drug methicillin was introduced in Europe. Two years later, 1961, methicillin-resistant *S. aureus* (MRSA) was detected [32]. MRSA appeared first in hospitalized patients, and for three decades MRSA was contained to health care-settings. Though, in the 1990s reports started to arise about MRSA spreading in the community among otherwise healthy individuals [33, 34]. Also, these community-associated strains seemed to migrate into hospital settings [35], adding to the problem of traditional MRSA strains. Vancomycin is the usual compound used for MRSA treatment. In 2002, the first vancomycin-resistant *S. aureus* (VRSA) was described [36].

STAPHYLOCOCCAL BURDEN OF DISEASE

> *Thou art a boil, A plague sore, an embossed carbuncle, In my corrupted blood.*
>
> Shakespeare: King Lear

S. aureus has been a leading cause of human infections throughout history, responsible for a never-ending pandemic of human infections [37]. *S. aureus* bacteremia is probably the most common life-threatening infection worldwide [38]. Up to 30% of the human population are asymptomatically and permanently colonized with nasal, cutaneous or gastrointestinal *S. aureus*, and an additional 30% are non-permanent carriers [39]. Humans, dogs, cats, sheep, cattle, and poultry can host this highly successful pathogen. Although some researchers has explored the idea that *S. aureus* might only happen to be on the wrong place at the wrong time and attract blame due to their abundance [40], most researchers and physicians believe that *S. aureus* is a highly virulent bacterial pathogen causing community- and healthcare-acquired

infections. *S. aureus* is one of the most frequent pathogens isolated from the bloodstream of seriously ill patients in any hospital in the world [41-45], and despite the availability of antibiotics, the mortality of *S. aureus* bacteremia remains high [46]. Much like *S. pyogenes*, *S. aureus* infections range from superficial and self-limiting pyoderma to invasive necrotizing infections and toxic shock syndrome. *S. aureus* represent the main cause of infective endocarditis, septic arthritis, osteomyelitis and surgical wound contamination [47, 48]. It has a pronounced versatility and capacity to infect almost any type of tissue. Blood-borne septic metastasis facilitates its spread. *S. aureus* toxins cause the classical "food poisoning" gastrointestinal disease. Also, *S. aureus* cause pneumonia secondary to viral infection, aspiration, or mechanical ventilation [49-51]. Orthopedic surgeons are well-familiar with *S. aureus* infections of implanted materials, that are difficult to treat. A significant increase in hospitalizations associated with *S. aureus* was observed in all age groups in the US between 2001-2009 [52]. NSTI mortality is generally lower with *S. aureus* versus *S. pyogenes* [53]. The added burden of resistance differs between countries. In Europe, the proportion of MRSA ranges from 1% to 50% [54]. MRSA account for 44% of hospital associated infections, 41% of extra days of hospitalization and 22% of attributable extra deaths in the European Union [54]. It has been debated whether MRSA bacteremia causes higher mortality than methicillin-sensitive *S. aureus*. Vancomycin is an inferior alternative than β-lactams in the treatment of *S. aureus* infections with a deep focus. Two meta-analyses have found increased mortality risk associated with MRSA (1.93 and 2.03) but there is ongoing discussion about the methodological flaws of the studies [54].

SEPSIS, TOXIC SHOCK AND SUPERANTIGENS

NSTI are often associated with sepsis, which is a syndrome caused by a large variety of microbes. Sepsis is defined as the life-threatening organ dysfunction caused by a dysregulated host response to infection. These criteria were reassessed in 2016, resulting in the Sepsis-3 consensus definitions [55]. The new diagnostic criteria were developed due to the inadequate specificity and sensitivity of the systemic inflammatory response syndrome (SIRS) criteria used in previous definitions. A bedside clinical score called quickSOFA can be used to value the risk of sepsis: respiratory rate of 22/min or greater, altered mentation, or systolic blood pressure of 100 mm Hg or less. In the clinical setting organ dysfunction should be evaluated by an increase in the Sequential [Sepsis-related] Organ Failure Assessment (SOFA) score of 2 points or more, which is associated with an in-hospital mortality greater than 10% [55]. Septic shock, which is worse, was re-defined as a subset of sepsis associated with in-hospital mortality rates greater than 40% due to particularly profound circulatory, cellular, and metabolic abnormalities [55]. Patients with septic shock can be clinically identified by a vasopressor requirement and elevated serum lactate level in the absence of hypovolemia. A challenge with these criteria can be to identify patients that are actually infected, since other sources of organ dysfunction exist. Another study assessed fever in the emergency department and found that it predicts survival of patients with septic shock admitted to the intensive care unit, although not in the way one might imagine. More than half of patients had a body temperature below 38,3°C and in-hospital mortality was inversely correlated with temperature, decreasing on average >5% per °C increase. Patients with the lowest temperature had the highest mortality: 50%, in large because they received less timely and lower quality care [56].

The most fulminant expression of the spectrum of diseases caused by toxin-producing strains of *S. aureus* and *S. pyogenes* is staphylococcal/streptococcal toxic shock syndrome (STSS) which can present with different types of infections, most commonly necrotizing fasciitis (70%) [57]. First described in 1978, though similar descriptions exist as early as 1927, patients with STSS are in septic shock (including at least 3 dysfunctional organ systems) with fever of at least 39°C and in addition show specific signs of toxic influences on the skin and mucosal sites [58]. STSS occurs sporadically although some clusters of cases have been reported, with mortality rates varying between 30–80% [59-63]. A specific subcategory, the menstrual staphylococcal TSS, was associated to highly absorbent tampon use in the 1980s [64].

STSS is thought to be mediated by bacterial exotoxins acting as superantigens [65]. Superantigens are the most powerful T-cell mitogens ever discovered, hijacking the immune system, and activating up to 20% of all T-cells at low concentrations. This can be compared to regular antigens activating <0.01% of T-cells at much higher concentrations [66-69]. Superantigens bypass regular antigen processing, presentation and bind intact without prior cellular processing to major histocompatibility complex II on antigen presenting cells (APCs) and variable regions of the T-cell receptor (TCR) leading to polyclonal proliferation and a cytokine cascade (tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-2 and IL-6) and refractory shock [53, 70]. In total 19 distinct superantigens gene have been identified in *S. aureus* and most strains harbor one or more of them [71, 72]. Similarly, 14 distinct group A streptococcal genes encoding superantigens have been identified, and each strain generally harbor three to four [71, 73].

Streptococcal isolates from severe and non-severe cases induce similar mitogenic and cytokine responses [74]. However, there seem to be host variations in the responses to superantigens. Patients suffering from severe invasive cases of toxic shock and/or necrotizing fasciitis have been shown to have significantly higher frequencies of IL-2, IL-6, and TNF-α-producing cells in their circulation as compared to non-severe invasive cases [75]. It seems that patients with a propensity to produce higher levels of inflammatory cytokines in response to streptococcal superantigens develop significantly more severe systemic manifestations. Kotb et al. described that human leukocyte antigen (HLA) class II haplotypes conferred strong protection from severe systemic disease, whereas others increased the risk of severe disease [76]. Also, the HLA of the antigen presenting cell was shown to dictate the magnitude of the mitogenic T-cell response and the net cytokine release, rather than the Vβ-region of the T-cells [77]. Levels of anti-M1 bactericidal antibodies and of anti-streptococcal superantigen neutralizing antibodies in plasma have been reported to be lower in patients suffering from streptococcal infections than in matched healthy controls. However, the lower levels did not seem to modulate disease outcome [78]. There is much epidemiological evidence for superantigen involvement in invasive infections, but also serological and T-cell based studies [71]. The evidence of T-cell involvement has been supported by a trial of an anti-CD28 monocloncal antibody, acting as a superagonist that directly stimulate T-cells [79]. Six healthy young male volunteers were involved in a phase 1 clinical trial of this drug, and 90 minutes after receiving a single intravenous dose, all six had a systemic inflammatory response characterized by rapid induction of proinflammatory cytokines and unspecific sepsis-symptoms such as headache, myalgia, nausea, diarrhea, erythema, vasodilation and hypotension [79]. Within 12-16 hours after infusion, they became critically ill, with pulmonary

infiltrates and lung injury, and disseminated intravascular coagulation, and they were transferred to an intensive care unit, receiving cardiopulmonary support (including dialysis), high-dose methylprednisolone and anti-IL2-receptor antagonist antibody [79]. After 24 hours, their lymphocyte and monocyte levels decreased. The levels of cytokines such as INFγ, TNFα, IL-1β and IL-2 all increased rapidly and then decreased in 24 hours. Two patients developed cardiovascular shock and acute respiratory distress syndrome, requiring intensive organ support for 8-16 days [79]. All patients survived. All of them experienced generalized desquamation, a sign usually seen after STSS.

PATHOGENIC MECHANISMS

S. pyogenes and *S. aureus* produce a myriad of individual virulence factors that in different ways promote its survival. Some key virulence factors will be discussed below, which may be divided into surface-related and secreted factors (Table 1). Importantly, there is considerable heterogeneity among strains and the virulence factors they express.

> *Pathogenicity is, in a sense, a highly skilled trade, and only a tiny minority of all the numberless tons of microbes on the earth has ever involved itself in it; most bacteria are busy with their own business, browsing and recycling the rest of life. Indeed, pathogenicity often seems to me a sort of biological accident in which signals are misdirected by the microbe or misinterpreted by the host.*
>
> Lewis Thomas, The Medusa and the Snail

Surface associated factors are of importance for evasion of phagocytosis and adherence to host cells. *S. pyogenes* express an anti-phagocytic hyaluronic acid (HA) capsule, with HA similar to that of human connective tissue. This enables the bacteria to disguise as an immunological "self" and interact with CD44 enabling adherence to host cells [80, 81]. Typing of *S. pyogenes* is commonly based on the M protein, its major virulence and immunological determinant, protruding from the streptococcal surface like a strand of hair. Classic M-protein serological typing was largely replaced by sequence typing of the 5´ end of the M protein (*emm*) gene in the late 1990s. There is substantial overlap in common *emm* types found in invasive and pharyngeal isolates, but less overlap to common *emm* types found in skin isolates [24]. M-type 1 and 3 are strongly associated with invasive disease, which some argue is due to their procoagulant activity through stimulation of tissue factor [82]. M-protein bind complement factors [83], fibrinogen [84] and interact with C4b-binding protein [85]. M-protein can be cleaved off the bacterial surface by human or bacterial proteases and act pro-inflammatory through activation of neutrophils that cause release of heparin binding protein which induce vascular leakage [86] and there are also reports of superantigen activity [87]. One of the most successful clones, the M1T1 strain, possess the *emm1.0* allel of the M1 gene but has through loss and/or acquisition of phages evolved into a more virulent phenotype [88].

The most well-known surface associated factor of *S. aureus* is protein A. Staphylococcal protein A is a cell wall anchored protein released during bacterial growth composed of two regions with clear structural and functional differences: Region X (that connects to the cell wall) and the five immunoglobulin binding domains that bind human IgG so efficacious that it is used as a column substrate to purify antibodies [89]. This protects *S. aureus* from humoral immune responses and acts as a superantigen against B-cells [90]. Staphyloxanthin, the golden

pigment in *S. aureus*, functions as a virulence factor by serving as an antioxidant protecting the bacterium from neutrophil oxidative burst [29].

Secreted factors contribute to immune evasion mechanisms as well as growth and dissemination of the bacteria. Many virulence factors cause lysis of host cells, promote tissue invasion and destruction. There are also factors with the ability to specifically manipulate both innate and adaptive immune responses, including inhibition of complement activation, prevention of neutrophil function and recruitment, and inhibition of phagocyte function [89] *S. pyogenes* secrete two hemolysins, Streptolysin O and S (SLO and SLS) that form pores in the membranes of human erythrocytes [91, 92]. Hyaluronidase is an enzyme that facilitate the degradation of hyaluronic acid in connective tissue [93]. SpeB is a cysteine protease with many human substrates such as extracellular matrix proteins, antibodies, antimicrobial peptides; thereby promoting dissemination and immune evasion [94]. It can also cleave endogenous virulence factors and therefore, its secretion is tightly regulated.

Many *S. aureus* virulence factors are defined as toxins that are secreted from the bacteria and in a direct way damage host cells. α-toxin (α-hemolysin, hla) is a well-known, proinflammatory and beta-barrel forming toxin that causes cytolysis through pore formation [47]. Panton-Valentine leukocidin (PVL) is a bi-component, pore-forming toxin consisting of the LukS and LukF proteins [47]. It is non-hemolytic although highly cytotoxic toward human neutrophils, monocytes, and macrophages [95]. There is a strong epidemiological association among genetically diverse *S. aureus* strains carrying the PVL genes and disease outbreaks like fatal necrotizing pneumonia [96, 97]. Phenol soluble modulins (PSMs) are amphipathic peptides causing cell lysis without requiring specific receptor binding [47]. *S. aureus* also possess more toxin with superantigen activity, such as enterotoxins and toxic shock syndrome toxin-1. *S. aureus* also produce exoenzymes such as coagulase that clot blood plasma.

It should be noted that different strains produce different levels of each factor, and that distinguishing the role of specific factors can be challenging. Likely, different factors are involved in different processes of the pathogenesis [98].

Weckel et al. recently studied decidual infection by *S. pyogenes* and showed that SLO and speB are main factors in dissemination and that *S. pyogenes* limits the hosts immune response [25]. *S. pyogenes* invaded phagocytic cells in the first minutes of infection, and induced death in about half the stromal and immune cells in the decidua [25]. Both *S. aureus* and *S. pyogenes* can colonize humans without creating disease. The molecular basis for colonization is often described like that of infection. Schulz et al. used a humanized model to study how healthy adult human skin responds to colonizing MRSA and saw that adhesion to corneocytes induced a local inflammatory response in underlying skin layers, which recruited neutrophils that could regulate the bacterial population [99]. What determines our colonizers are not only our host cells and secreted factors but also who we are close to [100].

TABLE 1. MAJOR VIRULENCE FACTORS IN *STREPTOCOCCUS PYOGENES* AND *STAPHYLOCOCCUS AUREUS*.

Action/mechanism	*S. pyogenes*	*S. aureus*
Adherence/colonization	Capsule M protein Fibronectin-binding protein Collagen-binding proteins Lipoteichoic acid	Fibronectin binding protein A and B Fibrinogen binding proteins; Efb, EAP, Clf A and B Collagen binding protein Elastin binding protein Extracellular adherence protein (EAP)
Anti-phagocytic	Capsule M protein M-like protein C5a peptidase Streptococcal inhibitor of complement (SIC) Streptococcal cystein protease (SpeB) Cell envelope proteinase (SpyCEP)	Capsule Protein A (SpA) Clumping factor A (Clf A) Extracullular fibrinogen binding protein (Efb) Staphylococcal complement inhibitor (SCIN) Staphylokinase Chemotaxis inhibitory protein (CHIPS) Extracellular adherence protein (EAP)
Dissemination	DNase Hyaluronidase Plasminogen-binding proteins Streptokinase	
Systemic toxicity and pro-inflammatory activity	Streptolysins O and S Superantigens: SpeA-M, SmeZ, SSA Peptidoglycan Lipoteichoic acid CpG DNA	α-haemolysin (α-toxin) γ-haemolysin Panton-Valentine leukocidin Leukocidin E/D and M/F Superantigens: staphylococcal enterotoxins A-E, G-Q and TSST-1 Expholiative toxin A and B (SEA, SEB) Peptidoglycan Lipoteichoic acid CpG DNA Protein A
Inhibition of leukocyte chemotaxis	C5a peptidase SpyCEP	Chemotaxis inhibitory protein (CHIPS) Extracellular adherence protein (EAP)

NO AVAILABLE VACCINES

There is currently no vaccine available for the prevention of *S. pyogenes* infection, despite over a century of research and careful scrutiny of many promising targets [101]. Lancefield's studies in the 1950s indicated M-protein as a vaccine candidate as sera containing serotype-specific antibodies protected against re-infection [102]. However, to protect against a large proportion of epidemiologically significant strains, a vaccine would need to be relatively complex. Some M proteins elicit both protective antibodies and antibodies that cross-react with human tissues, hindering attempts to develop an M protein-based vaccine. Sequencing of M-proteins revealed that tissue–cross-reactive epitopes were localized to repeating amino acid sequences. Hence, an M-protein vaccine would need to cover the different serotypes of clinical relevance while avoiding the regions associated with tissue cross-reactive antibody development. New molecular techniques have allowed the previous obstacles to be largely overcome and currently a 26-valent M-protein-based *S. pyogenes* vaccine is under clinical investigation [103]. Shaw et al. investigated protein antigens targeting a broad range of *S. pyogenes* disease presentations

Vaccine approaches to *S. aureus* thus far have proven unsuccessful when progressed to clinical trials and it has been proposed that *S. aureus* cannot be hindered by pre-existing antibodies as long as protein A bind human IgG. Although, it may be feasible to utilize vaccine-based strategies as adjuvant infectious treatment to limit the severity of infection [104]. Recently, a four-component vaccine consisting of two capsular polysaccharides conjugated to tetanus toxoid, mutated α-toxin, and clumping factor A has been developed and has completed phase one clinical trials [105]. Others believe we need to consider the route of vaccination, suggesting intranasal administration is more efficacious [106].

ANTIBIOTIC TREATMENT AND RESISTANCE

The World Health Organization (WHO) classifies antibiotic resistance as one of the greatest threats to global health, food security and development [107]. Antibacterial resistance is defined as the reduction or loss of bacteriostatic or bactericidal efficacy of an antimicrobial agent at doses that would normally exert its therapeutic effect [107]. Infections by antibiotic resistant bacteria in Europe more than doubled between 2007 and 2015, and the burden of disease is now comparable to that of influenza, tuberculosis and human immunodeficiency virus (HIV) combined [108]. The prevalence of multi-resistant bacteria in the community can increase fast [109].

> *It has been demonstrated that a species of penicillium produces in culture a very powerful antibacterial substance which affects different bacteria in different degrees. Generally speaking, it may be said that the least sensitive bacteria are the Gram-negative bacilli, and the most susceptible are the pyogenic cocci.*
>
> Alexander Fleming

Penicillin remains an appropriate antibiotic treatment in infections by *S. pyogenes* since these bacteria are known to be uniformly susceptible to all β-lactam antibiotics [110]. However, there are reports on the emergence of antibiotic resistance in *S. pyogenes*. Alarmingly, strains with decreased sensitivity to penicillin were recently described, consistent with the first step towards

developing penicillin resistance [111]. Resistance has been described in *S. pyogenes* against commonly used antibiotics such as erythromycin, tetracycline, clindamycin, and fluroquinolone [16, 112-114]. During an outbreak of scarlet fever in Beijing, the strains were resistant to erythromycin in 96% of cases and 79% to clindamycin [112]. In asymptomatic Korean children, rates of resistance were 51% towards erythromycin and 34% against clindamycin 2002, a marked increase from 1995 [16].

In addition to reports on developing resistance, a previous finding of susceptible yet viable *S. pyogenes* in 74% of infected patient tissue, despite prolonged antibiotic therapy, demonstrated an antibiotic treatment failure at the tissue site [115]. This opened a novel line of research focusing on improved antimicrobial strategies against bacterial resistance phenotypes at the tissue site. In patients with toxic shock or a deep tissue focus, the recommendation is that a β-lactam is combined with a drug capable of suppressing toxin production, most often clindamycin but also linezolid is an alternative [70, 116]. Clindamycin reduces SLO activity and DNase Sda1 *in vivo*, whereas subinhibitory concentrations are suboptimal [117]. Also, there is reduced expression of penicillin-binding proteins and diminished susceptibility to β-lactams when *S. pyogenes* reaches the stationary growth phase. This is known as the Eagle-effect [118].

Contrary to the naivety of *S. pyogenes*, *S. aureus* is on the WHO list of priority pathogens for which new antibiotics are badly needed [119]. In *S. aureus* infections, choice of antibiotics includes penicillinase-resistant isoxazolyl-penicillins, clindamycin, vancomycin, linezolid or daptomycin [120]. *S. aureus* has a pronounced capacity to acquire and express antibiotic resistance. It has acquired resistance to virtually all antibiotics [46]. Methicillin-resistant *S. aureus* (MRSA) has become an inclusive common term used to describe *S. aureus* strains that are typified by resistance to most β-lactam antibiotics with the exception of some modern cephalosporin classes of β-lactam compounds [89]. MRSA constitute a large clinical problem worldwide [34, 37]. 20-80% of all S. aureus infections worldwide can be attributed to MRSA strains, depending on the country reporting [107]. The WHO report that MRSA infections result in more hospital days to resolve the infection, an increase in sepsis outcomes and increased duration in intensive care units [107]. MRSA, once known as "the hospital superbug", now seems to recirculate from reservoirs in the community, such as daycares, prisons, dorm rooms, or locker rooms, referred to as community-associated *S. aureus* [33-35, 121]. Unlike hospital-associated MRSA, this type is typically resistant only to β-lactam antibiotics [34, 37]. The emergence of MRSA in the community has several clinical implications: treatment failure and increasing costs, as the alternative vancomycin is inherently less efficacious [37, 43, 122, 123]. Methicillin resistance in *S. aureus* is carried by the genetic element SCCmec, of which there are four different types [124]. Intrinsic bacterial resistance to β-lactam antibiotics is associated with altered production of penicillin binding proteins [125]. It is likely that virulent CA-MRSA strains can arise from insertion of SCCmec (especially type IV) into a virulent MSSA strain through horizontal gene transfer [51]. Some argue that what makes *S. aureus* a dangerous pathogen is the combination of antibiotic resistance and high virulence [121]. CA-MRSA have been reported in severe fatal infections that only rarely are associated with *S. aureus* [51, 121], which indicates that CA-MRSA has a virulence potential that exceeds that of traditional hospital associated MRSA strains. Sweden has low prevalence of MRSA, but occasional outbreaks occur [126]. Recently, MRSA was found in 64% of wild

hedgehogs in Sweden [127]. When resistance increases in society, more people will learn how to cope with the stress of carrying a resistant bacterium [128].

Antibiotic research and development have steadily decreased in the last twenty years, with only seven new antibiotics launched from 2009–2012. Although, a range of approved antibiotics to treat MRSA infections have been implemented only recently including established classes such as fluoroquinolones and cephalosporins, but also novel compound classes such as peptide mimics, oxadiazoles, and diaminopyrimidines [129, 130]. In the case of vancomycin-resistant *S. aureus* effective alternatives include daptomycin, linezolid, tigecycline, trimethoprim-sulfamethoxazole or a 5th generation β-lactam (e.g. ceftaroline or ceftobiprole) [131].

PERSISTENCE

Notably, treatment recommendations are based solely on *in vitro* susceptibility and do not take into account potential resistance phenotypes at the tissue site. Regarding invasive infections, most research has focused on bacterial toxin-mediated tissue pathology [47, 95, 97, 132-135]. However, recent attention has been given to certain subpopulations of bacterial cells demonstrating an increased antibiotic tolerance at the tissue site, known as persisters [136-138]. Bacterial persisters are different from antibiotic-resistant mutants in that their antibiotic tolerance is non-heritable and reversible [136]. The persistence phenomenon demonstrates that a bacterium that is susceptible to an antibiotic *in vitro* might still have means to survive *in vivo* despite of antibiotic therapy. Potential mechanisms behind persistence are biofilm formation, small colony variants and intracellular survival. Contrary to antibiotic resistant clones, persistent clones are not able to grow in the presence of antibiotics [131]. Andreoni et al. observed persistence in a patient with NSTI, reporting that the clindamycin concentration was 10xMIC in necrotic tissue at day 0 and day 2, while the bacterial load was 10^6 viable CFU/g tissue at day 0 and $>10^3$ at day 2 [117]. Even in the healthy tissue, $>10^3$ viable CFU/g tissue was found at day 0.

Traditionally *S. pyogenes* and *S. aureus* have been considered extracellular bacteria, and they are treated with extracellular antibiotics such as penicillin. However, *S. pyogenes* has been found to survive within phagocytes [115]. M-protein has been show to mediate survival of intracellular *S. pyogenes* in neutrophils [139, 140] and macrophages [141]. *S. pyogenes* was also shown to internalize into epithelial cells [142], and even at higher rates then GBS [143]. However, no intracellular replication was observed. Also *S. aureus* is increasingly being recognized as an opportunistic intracellular pathogen [121].

Small-colony variants are characterized by slow growth in small colonies approximately one-tenth the size of the parent strain [144]. Small-colony variants emerge because of stress due to nutrient limitation or exposure to sublethal concentrations of antibiotics resulting in genetic mutations or metabolic variations.

BIOFILM - BUGS IN A SHIELD OF PROTECTION

Biofilm formation is an ancient mode of growth where bacteria grow in a protected manner. The bacteria can alter their genetic expression from planktonic “swimmers” to biofilm “stickers” in minutes [145], and adhere to biological and non-biological surfaces. There, the

bacteria aggregate and produce an extracellular matrix consisting of carbohydrates, lipids and extracellular DNA. A lawn of growth is formed and when reaching a certain tension, tower formation is initiated and the bacterial growth rises to form a mature biofilm [146]. This protects the bacteria from antibiotics, immune attacks, mechanical assault and provides growing bacteria with nutrients [145]. The opposite of biofilm mode of growth is planktonic growth, formerly thought to dominate the bacterial life cycle. Now, biofilm is increasingly being recognized as the dominating way of bacterial existence. Classical biofilm-associated infections are chronic infections such as diabetic wound ulcers and infections of implanted materials: hip replacement implants, mechanical heart valve infections and catheters. Some argue that biofilm formation and antimicrobial resistance are deeply interconnected and do not function independently of each other [147]. Waters et al. recently explored the idea that research into biofilm and persister phenotypes has converged, arguing that biofilm cells, persisters and stationary phase cells are quite similar [148]. Biofilm formation provides a mechanical barrier and bacteria inside a biofilm are in an altered metabolic state with a decreased susceptibility towards most antibiotics. The antibiotic concentration needed to inhibit or eradicate biofilm can be a thousand times higher than that needed for planktonic bacteria. Concentrations that high are not achievable in humans, leaving biofilm associated infections with limited prospects for clinical cure [149]. Hence, there is an unmet need to develop and include parallel approaches that target *S. aureus* biofilm infections [150, 151]. Some antibiotics are more efficacious towards biofilms. The research field of staphylococcal biofilm is massive, and biofilm is a well-known microbiological factor in many, or all, staphylococcal infections.

Streptococcal biofilm has been described *in vitro* and *in vivo* [152, 153] but it's implications for disease are not fully understood. Our research group identified streptococcal biofilm formation in the soft tissue of 32% of patients tested during acute NSTI [154]. This underscores the limitations of *in vitro* bacterial antibiotic susceptibility assays as they fail to consider the properties of bacteria in tissues. We hypothesize that bacterial persistence, because of antibiotic failure at the tissue site, is a key determinant of disease severity as it results in continued replication, sustained toxin production [132] and pathology locally. SpeB has been implicated in streptococcal biofilm dispersal, and some argue that it leads to increased disease severity [153]. Other research groups have also identified biofilm as a mechanism for treatment failure [155]. Baldassarri et al. investigated 289 *S. pyogenes* strains and found that 90% had the ability to form biofilm [155].

QUORUM SENSING - BACTERIAL CONVERSATION

> *If a bacterium is trying to infect you, it won't secrete alone, because your immune system will block it. Bacteria will hide until they can all act together and make an impact.*
>
> Bonnie Bassler

In order to communicate cell-cell, bacteria use a mechanism called quorum sensing [156]. Depending on signals such as cell density, bacteria can organize behavior like exotoxin production or biofilm formation to strategize pathogenesis [157]. Species-specific auto-inducing peptides (AIPs) are produced during the exponential growth phase of the bacteria, upon reaching a threshold cell density where a genetic locus gets auto-activated. Of particular interest for my project: transcription of many virulence factors in *S. aureus* is regulated by a

gene complex called Accessory gene regulator, Agr, which is the main staphylococcal system for quorum sensing using the regulatory molecule RNAIII [158]. Toxins and enzymes are generally positively regulated by this system, in contrast to cell surface molecules that are repressed [159]. In 2011, Cheung et al. [160] demonstrated that Agr functionality is critical for *S. aureus* disease and indicated that an adaptation of the *agr* regulon contributed to the evolution of the highly pathogenic CA-MRSA.

Streptococcal quorum sensing is mediated by several systems that can be categorized into four groups: regulator gene of glucosyltransferase, Sil, lantibiotic systems and LuxS/AI-2 [161]. Our previous research has implicated that neither CovR/S, SpeB nor capsule are required for biofilm formation in the tissue setting [154]. Instead Nra/RofA,which are negative and positive regulators of genes in the FCT-region (fibronectine-binding, collagen-binding, T-pilus), were indicated as biofilm controllers in *S. pyogenes*.

TREATMENT OF NECROTIZING SOFT TISSUE INFECTIONS

Historical depictions of NSTI were mainly recorded in wartime reports of battle injuries. Treatment approaches originate from those used in wars: infection control by surgical debridement of necrotic tissue, also referred to as source control [162]. Antibiotics and intensive care are other corner stones in the treatment of NSTI. Time to antibiotics and surgery are considered the most critical treatment factors [6]. Delay of surgery has been shown to be an independent risk factor for mortality, and studies stress the importance of surgical debridement and early amputation of infected limbs. However, an aggressive surgical approach increases the risk of severe disability and impaired quality of life [163]. Antibiotic treatment is empirical meaning that it is based on etiological studies, clinical trials & observational studies until the causative agent is isolated and the resistance pattern identified. In some cases, NSTI treatment includes hyperbaric oxygen and intravenous immunoglobulin.

HYPERBARIC OXYGEN TREATMENT

Hyperbaric oxygen treatment (HBOT) was first used for decompression sickness, also called divers disease, in 1937. In 1961, Willem Hendrik Brummelkamp made a seminal finding: hyperbaric conditions inhibit anaerobic infections [164]. Oxygen is necessary for cellular metabolism, promotion of host defenses and tissue repair. Leukocytes bactericidal abilities are enhanced, collagen formation is stimulated, and superoxide dismutase levels increase (resulting in increased tissue survival) [165]. Vasoconstriction decreases tissue edema and efficacy of certain antibiotics is enhanced [166]. HBOT has shown proinflammatory effects such as decreasing plasma levels of TNF-α in animal infection models and production of reactive oxygen species by neutrophils in sepsis [167]. The effects of HBOT were studied in the 1960s indicating that HBOT inhibits the growth of many aerobic bacteria *in vitro* [168]. HBOT was implemented as adjuvant treatment in NSTI and the outcome in case-reports and observational studies have been varying [1, 169-175]. A recent systematic review failed to locate relevant clinical evidence to support or refute the effectiveness of HBOT in the management of NSTI [176]. Clinical trials are needed to define the role of HBOT in NSTI treatment.

INTRAVENOUS IMMUNOGLOBULIN G

Polyspecific intravenous immunoglobulin G (IVIG) is comprised of pooled IgG antibodies from the serum of thousands of donors and is commonly used as IgG replacement therapy in immunocompromised patients [177]. Adjuvant IVIG treatment has been suggested in invasive infections by *S. pyogenes* as a means of limiting the effects of bacterial toxins in regard to septic shock and tissue damage [178]. The clinical efficacy of IVIG has also been proposed in a variety of diseases associated with either viral or microbial infections [179-182]. IVIG has three known mechanisms of action: opsonization of bacteria, binding of specific (Fab-) parts to toxins/superantigens that inhibit their activation of T-cells and anti-inflammatory effects due to anti-cytokine autoantibodies, replacement of soluble immune components and binding of unspecific (Fc-) parts to Fc-receptors [183]. IVIG have been found to efficiently neutralize streptococcal superantigens and to mitigate subsequent tissue damage (reviewed in [70]).

Through *in vitro* studies IVIG has been shown to neutralize *S. pyogenes* superantigenicity, enhance bacterial killing, systemic clearance of bacteria and neutrophil infiltrate into infected tissues [184]. IVIG has been shown to enhance the ability of patient plasma to neutralize bacterial mitogenicity and reduce T cell production of IL-6 and TNF-α [185, 186]. Staphylococcal superantigens have been demonstrated to require higher doses of IVIG in order to achieve protective titers [187, 188]. The clinical evidence for the use of IVIG in invasive disease by *S. pyogenes* or *S. aureus* comes mainly from observational studies and case reports. Several studies have reported beneficial effects [116, 186, 189-191]. However, some studies found no impact of IVIG [192, 193]. A trial comparing IVIG to placebo in invasive infections has been greatly anticipated [194]. Darenberg *et al.* randomized patients with STSS to IVIG or placebo, but the trial was stopped prematurely after the inclusion of only 21 patients because of slow patient recruitment [187]. A recent advance was the randomized trial by Madsen *et al.* that observed no beneficial effect of IVIG versus placebo in NSTI by all microbial etiologies. However, a trend of benefit towards IVIG was observed in the subgroup dominated by *S. pyogenes* and *S. aureus* infections. Potential biases include that 40% of placebo-treated patients had been treated with IVIG prior to inclusion in the study (compared to 16% in the IVIG-group) and the rate of *S. pyogenes*-infections in the placebo group was low (15% compared to 38% in the IVIG-group). A meta-analysis of IVIG in clindamycin treated streptococcal TSS reported a mortality reduction of 33.7% to 15.7% [195]. Also in sepsis, effects of IVIG treatment are unclear. This meta-analysis suggest that the patients most likely to benefit from IgM-enriched IVIG therapy are those with Gram-negative septic shock [196].

INNOVATIVE THERAPEUTIC STRATEGIES

New anti-infectious agents are badly needed, and researchers are looking beyond classic chemical inhibition of growth and means of bacterial killing. Inhibiting bacterial virulence without inhibiting growth is a way of reducing the risk of resistance development.

Let's start with *S. aureus* virulence factor inhibitors. For *S. aureus*, α-toxin is an obvious target for therapeutic blocking. A monoclonal antibody aimed at α-toxin was shown to increase survival in a mouse pneumonia model [197]. The authors suggested a combination of virulence targeting and classical antibiotic treatment to increase survival and reduce the

duration of treatment, possibly reducing resistance development. This antibody has been under clinical trials and was shown to be safe [198] but failed to reduce the frequency of ventilator-associated pneumonia in intensive care treated patients colonized with *S. aureus* [199]. François et al. report that 18% of the antibody-treated and 26% of the placebo-treated patients developed *S. aureus* pneumonia, a relative risk reduction of 32%, but with a wide confidence interval of -7.5% to 57% [199]. Despite this, monoclonal antibodies still represent a promising therapeutic option to be evaluated.

Both vaccination against α-toxin and α-toxin antisera resulted in a reduction of skin lesions and dermonecrosis in a murine model of *S. aureus* infection [200]. Another group created a crossreactive antibody that bind both α-toxin, γ-hemolysin and PVL [201]. This antibody increased survival in murine models, infected both intranasally and intravenously. A monoclonal Fab fragment antibody with high affinity to α-toxin was tested in eye-infections of rabbits caused by *S. aureus* [202]. The authors were concerned that the full antibody would have limited penetration into the corneal tissue. The area of the corneal erosion was reduced, and this was enhanced when it was used in combination with a compound that increase the ocular permeability. Baicalin is a naturally occurring compound from a traditional Chinese medicinal herb which has shown to be effective in disrupting α-toxin oligomerization which prevents its pore assembly. This was shown to reduce hemolysis and increase mice survival in a *S. aureus* pneumonia model [203]. Anti-platelet adhesive drug Ticagrelor has been shown to block α-toxin mediated platelet injury and resulting thrombocytopenia, and to protect mice in a lethal *S. aureus* sepsis model [204]. It has also been tested as adjunctive therapy in a patient with promising result [204]. The Agr system is an attractive target for *S. aureus* anti-virulence agents. This is discussed in further detail in the discussion-part of this thesis.

PSMs have been targeted since they are present in almost all *S. aureus* strains and involved in dissemination. In animals treated with anti-PSMβ, reduced dissemination to organs including the liver, spleen and lymph nodes were seen from a biofilm-associated infection by *S. epidermidis* on a catheter [205]. Targeting the transporter of PSMs (phenol-soluble modulin transporter) could lead to their cytosolic accumulation, bacterial membrane damage and abnormal cell division [206]. Protein A is another *S. aureus* virulence factor that could be targeted. Its IgG binding activity protects against opsonophagocytic killing and acts as a B-cell superantigen. Deleting protein A reduces mortality from 60% to 25% in a mouse model [207]. Also, vaccination with this mutant was able to raise antibodies that blocked against infection by a virulent MRSA strain [207]. Others have used anti-protein A antibodies to deliver gold nanorods to selectively kill *S. aureus* using photothermal therapy [208]. In a mouse model, this treatment resulted in a significant reduction (73%) of CFU.

PVL is an additional factor to inhibit through targeted therapy. Using a humanized mouse model, Tseng et al. could show that a PVL knockout strain had decreased lesion size [209]. However, many hospital-associated MRSA strains lack PVL and directed therapy would in those cases be ineffective. Superantigens such as the staphylococcal enterotoxins could also be targets for treatment. Vaccinating mice against one enterotoxin developed high cross-specific antibody titers against other enterotoxins [210]. Also, multivalent vaccination

resulted in higher titers against each superantigen. Using monoclonal antibodies against SEB, Varshney et al. showed that human PBMC proliferation could be reduced with 50% [211]. Using cholesterol synthesis inhibitors, Liu et al. could show that biosynthesis of staphyloxanthine was blocked and that this increased the susceptibility to bacterial clearance in human blood and in a mouse infection model [212].

Bacteriophages have been explored as a possible treatment against MRSA infection [89] and there are specific centers for phage therapy for example in Tbilisi, Georgia [213]. However, phages are generally highly specific for bacterial strains and thus can exhibit lower efficiency within strains of the same species [214]. Attempts using phages placed on the surface of implanted material have shown promise: CFU counts were lower than on control implants, the infection cleared earlier, however, the phage-coating did not completely block MRSA infection of the implant [215].

Nanomedicine strategies have emerged as a promising therapy that show great promise in the field of MRSA biofilm infections [216]. Nanoparticles themselves can be cytotoxic for bacteria, can enhance the efficacy of current antibiotics by protecting them from detection and degradation or provide a means of targeted delivery to the microorganism to maximize the local concentration of agent and bactericidal effect [217, 218]. Also, nanopatterning and modification of surfaces and implants at the nanoscale can interfere with bacterial adherence, colonization, and biofilm formation. However, despite the wealth of research studies available, few of the promising preclinical studies have translated into clinical trials.

Metal-containing nanoparticles (NPs) such as silver, zinc, gold etc. are used to eradicate or inhibit the bacterial growth. Bacterial cell membrane disruption occurs when the positively charged ions of the NP bind to negatively charged parts of the bacterial membrane. When the NP is internalized, it results in DNA damage and cell death. However, these chemotherapeutic effects are equally damaging to eukaryotic cells, inducing gravely toxic injuries to the kidneys, liver, and spleen of experimental mice [219]. In contrast, cationic anti-microbial peptides (AMPs), derived from host cells, represent natural antibiotic compounds aimed at pathogens, produced by eukaryotic cells. These are pore-forming compounds that intracellularly also inhibit bacterial protein and cell wall synthesis [220]. However, their stability and activity at physiological conditions is poor, also, they are vulnerable to protease degradation and cytotoxic against red blood cells [220].

RNA interference in eukaryotic cells using oligonucleotides is well-established, but now this is also being considered in bacterial infections [221] with solutions such as RNAi, transcription factor decoys, clustered regularly interspaced short palindromic repeats (CRISPR) and aptamers (single-stranded nucleic acids, 20-100 nucleotides of RNA/DNA). By encoding *S. pyogenes* CRISPR *cas9*-system in MRSA it was possible to elicit a 10^4-fold reduction in viable colonies *in vitro* [222]. However, it is challenging to deliver negatively charged nucleic acids across both the cell wall and membrane of gram-positive bacteria, such as MRSA. Others are trying to reinvigorate or repurpose previously approved molecules through screening of approved drugs or adding NPs to improve the efficacy of existing therapeutics [223]. Chitosan is a linear polysaccharide, obtained from chitin in crustaceans or fungi [224]. It is especially attractive to use for nano-encapsulation of drugs since it has

been found to exhibit its own antimicrobial effects through its positive charge, and it is described to be biodegradable and non-toxic [225]. Additional ways of creating spherical vesicles for drug delivery is using liposomes and solid lipid NPs [226]. There are some advantages: flexibility in size, composition, charge, lamellarity, and the pre-existing record of clinical approval. However, they are unstable and unwanted burst drug release occur. Another way of drug delivery are synthetic polymer nano-carriers [227].

For *S. pyogenes*, less novel anti-virulence agents are in development, due to the relative effectiveness of existing antibiotic treatments. However, there are a few novel concepts to be explored. Bioengineered nanoparticles have been shown to inhibit the cytotoxicity of SLO in *S. pyogenes* and α-toxin in *S. aureus*, a type of broad spectrum neutralizing and absorbing agent [228]. One of the most potent bacterial cytolytic toxins know is SLS. *S. pyogenes* SLS-deficient mutants produced fewer necrotic lesions and lead to less weight loss in a mice model [229]. This was also seen in another work where both necrotic skin ulcers and dissemination to circulation was diminished by deleting SLS [230]. Also, the SLS toxin impaired phagocytic clearance and promoted epithelial cell cytotoxicity. Dale et al. inhibited SLS through an antibody produced in rabbits following stimulation with a synthetic peptide containing amino acid residues of the putative SLS propeptide [231]. SLS is non-immunogenic, and no neutralizing antibodies are evoked during natural infection. These antibodies completely neutralized the hemolytic activity of the toxin in vitro. However, the SLS antisera did not opsonize *S. pyogenes*.

Pinho-Ribeiro et al. recently described that *S. pyogenes* through nociceptor activation inhibit recruitment of neutrophils [232]. Mediated through bacterial SLS, nociceptors release a neuropeptide that has anti-chemotactic effects. This neuron-mediated suppression of host defense could be prevented by botulinum neurotoxin A and other antagonists. It is plausible that the optimal treatment of invasive infections is neurological. Intrathecal injection of neurotoxin efficiently blocked pain and hyperalgesia but had no effect on dermonecrotic lesions, abscess size or body weight loss. Using dual transcriptome RNA-sequencing of a primate NSTI-model Kachroo et al. described bacterial upregulation of virulence genes and altered metabolic genes, as well as host inflammation and defense processes [233]. This work identified 5 new pathogen genes as therapeutic targets. Xu et al. discovered a series of antivirulence agents against S. pyogenes that were able to inhibit gene expression of multiple virulence factors and protect mice in infection models [234]. Sortase A has been targeted with an inhibitor [235], however its efficacy remains to be shown. The sortase enzymes pose interesting targets for antivirulence strategies due to their catalyzation of virulence factors attachment to the cellular membrane, which is a transpeptidation reaction.

Using secreted streptokinase *S. pyogenes* can induce plasmin, the central enzyme of the fibrinolysis system [236]. Streptokinase-mediated plasminogen activation and protease activity on the cell surface is critical for dissemination, and thus could be targeted [237]. This was done by Sun et al. who tested 55,000 small molecules and report that they found several compounds that can downregulate both streptokinase and other important virulence factors of *S. pyogenes*. This results in enhanced granulocyte phagocytosis and killing as well as protection in a mice infection model [238]. 5-Dodecanolide is a compound with antivirulence

and antibiofilm potential, present in fresh fruit in low abundance, commonly synthesized and used as a flavoring agent. It was recently shown to inhibit *S. pyogenes* synthesis of hyaluronic acid, hemolysin, proteases, surface hydrophobicity and biofilm [239]. Similarly, a compound called fukugiside, from Garcinia travancorica, also has antivirulence and antibiofilm properties against *S. pyogenes* [240]. The CRISPR-Cas system, today a widely used gene-editing tool, origins as a prokaryotic immune system. Hynes et al. identified a phage-encoded protein with anti-CRISPR-Cas activity in a virulent phage of *Streptococcus thermophilus* [241]. This protein inhibited *S. pyogenes* Cas9 and resulted in immune evasion. Tu et al. reviewed the functions of small RNAs in S. pyogenes regulatory networks and pathogenicity, which could be exploited as therapeutic targets [242].

2 RESEARCH AIMS

The overall aim of my doctoral studies is to further our understanding regarding the pathogenesis and therapeutic options in severe invasive infections caused by the Gram-positive bacteria *Streptococcus pyogenes* and *Staphylococcus aureus*. A particular focus is the tissue persistence and it's underlying mechanisms, related to therapeutic interventions. My studies will explore bacterial behavior and the effects of treatments in patient samples and artificial skin.

Determine the efficacy of immunotherapeutic strategies used in severe tissue infections.

A particular focus is on adjunctive IVIG-therapy.

Determine the efficacy of antimicrobial strategies used in severe tissue infections.

A particular focus is on efficacy against bacterial biofilm.

Characterize the development of antibiotic resistance phenotypes at the tissue site in relation to pathogenic events.

A particular focus is biofilm by MRSA and its control by the Agr system.

3 MATERIALS AND METHODS

ETHICAL CONSIDERATIONS

INFECT
All studies were conducted in accordance with the Helsinki Declaration [3]. Patient enrolment and sample analyses were approved by the regional Ethical Review Board at the National Committee on Health Research Ethics in Copenhagen (Ref 1151739), the regional ethics committee in Gothenburg (Ref 930-12), the regional ethics committee Vest, Norway (Ref 2012/2227), and the regional Ethics Committee in Stockholm (Ref 2012/2110-31/2).

INSTINCT
The trial was approved by the Regional Ethics Committee of the Capital Region (Ref 30113713), Denmark, and the Danish Medicines Agency and was externally monitored by the Good Clinical Practice Unit, Copenhagen University Hospital [243].

The clinical material collected in the INFECT-project was pseudonymized at collection and I have not been in direct contact with patients or sensitive patient data during my studies. However, I need to consider the milieu in where my research samples were collected. All patients or, if impossible, next of kin have been provided with written information including the complete voluntarism of their contribution before leaving written consent. All tissue material collected consisted of tissue that was clinically indicated to be removed. Since NSTI are associated with high mortality and morbidity, it's easy to see the benefits of this research. In INFECT, no treatment intervention is included. Some people, after losing tissue, function or a close one, to this terrible disease, might feel anger towards science. Why did we not perform clinical studies of therapeutic strategies sooner, so that their outcome would've been higher? From time to time, I believe, researchers lose precious time while observing. Regulations and financing are important determinants of what studies are performed, but it is also important to have a solid scientific evidence base before moving into clinical trials. When randomizing patients to placebo versus a treatment believed to be superior to placebo, we treat people differently and hope for outcome to be higher in one group. The idea of randomized placebo-controlled trials is unethical, although they need to be performed. This is since the alternative, to not know, is worse for humanity and will result in more people not getting the treatment believed to be the best.

When a trial cannot answer the question it was supposed to answer, due to lack of quality in study design, it can be considered unethical. In INSTINCT, an attempt was made to further our knowledge on adjuvant treatment with IVIG in NSTI. The study had several strengths, but also limitations. An efficacious drug could be considered non-significant when used in a low dose in a clinical trial. This could be considered unethical. In INSTINCT, a dose of 25g IVIG was used, which is lower than that used in previous studies reporting a positive effect. This drug is a biological product and has a high price. Before the trial, treatment protocols in Copenhagen stated that all patients with NSTI should be treated with 25g IVIG. Now we know that 25g IVIG given to all NSTI patients does not influence outcome in a significant way, but that there is a trend of benefit in the subgroup dominated by *S. pyogenes* and *S. aureus* infections. This

subgroup should therefore be studied further, and samples collected was used in my follow-up study, which provided additional answers.

These studies could not have been performed without blood donations from healthy volunteers. These donations are given in a pseudo-anonymous fashion through buffy coats, decay products, from the blood donation reception at the Karolinska University Hospital. However, in some periods, for example during the summer vacations, there is no blood available from this source. During these times, there were occasional possibilities to sample blood from healthy volunteers at the research facilities, or in other words, colleagues. This is the opposite of anonymous donations, and several ethical considerations need to be addressed. Of most importance is the question of peer-pressure, and issues of integrity. However, the volunteers are well-acquainted with the research performed and this is not a rarity in research environments. In this research, the immune cells are used to study the function of bacteria and treatments against bacteria. The research question is not focused on the immune cells from the donor. Hence, we are not studying our colleagues, we use their blood at rare occasions when other options are not available to enable studies on human cells, which greatly enhances the clinical relevance of obtained data. Considering the high morbidity and mortality of these infections, there is a clear benefit gained from the studies.

My studies are based on tissue-modelling in which I use cell-lines and primary cells isolated from volunteer donors. If a donor would regret his/her donation later, it would be a complex task to collect all infected tissue-models since it has been a widely used technique in our lab and results are stored in many boxes in our biobank. Also, for publication ethical reasons results should not be destroyed within a period of time after publication. The cells are anonymized, but with modern technology one could find genetic information in these cells and discover things about the donors. That could lead to an ethical conflict. We have no plans of studying our donors and try to identify ethical conflicts when we plan future analysis of samples. Studies using human cells and tissue modelling are not only more relevant due to the complexity of molecular interactions in host-pathogen interactions, but also spare animal lives and suffering. Animal models are important for humanity, without them we would lead lives colored by more disease, less high efficacy treatments and more low efficacy treatments. However, when possible, one should aim to use models that does not cause suffering and death of animals. In this thesis work, no animals have been harmed.

When working with BSL2 pathogens, radioactive material and biobanks, it is important to remember that there are forces in the world who could try to gain access to your material to use for vicious purposes. In everyday life, it could easily be forgotten, and one could happen to open the door for someone, one did not want to let in.

Finally, this research is important, and one of the worst things one could do, is to quit science and stop trying to make the world a better, more enlightened place.

BACTERIAL STRAINS

In experimental microbiology, it is of importance to study bacteria that have caused severe disease clinically. The bacterial strains used in these studies have all been isolated from patients with either NSTI or other biofilm-associated infections. I had the advantage of working in the

INFECT consortium that during five years collected the largest cohort of NSTI patients ever described [3]. From that collection, many bacterial isolates were available. Great care has been taken to minimize passage of the strains in the laboratory. Determination of minimum inhibitory concentration (MIC) was performed by broth microdilution assay or agar gradient diffusion.

PATIENT SAMPLES

Patient samples (blood, plasma, surgical biopsies) were available from NSTI patients in the INFECT and INSTINCT cohort [3]. This allowed the study of IVIG-related neutralization of streptococcal superantigens in patient plasma. In INSTINCT, plasma was collected from patients before and after treatment with IVIG [243].

PROLIFERATION ASSAY

Human peripheral blood mononuclear cells were purified from buffy coats of healthy volunteers and used in proliferation assays as previously detailed [244]. A method using 3H-coupled Thymidine was chosen. This method is sensitive, robust, and high throughput. Also, the aim of the study was clear and uncomplicated, making more complex methods unnecessary. However, this required a specific permit, course, radioactive waste-handling and the method did constitute a minor health risk for the laboratory staff.

BIOFILM ASSAYS

To measure formation of bacterial biofilm and investigate how it is affected, bacteria are cultured in liquid media in wells with different conditions. Most commonly polystyrene 96 well plates are used. This setting is easy to reproduce and manipulate. Different protocols for measuring biofilm formation in polystyrene 96 well plates have been developed.

First, we designed a protocol based on the publication by Lembke et al [152]. In this protocol *S. pyogenes* strains are grown in BHI media and stained with safranin. The biofilm is read in the microplate reader at 495 nm, optical density values above 0.05 are considered positive. The microplate reader measure safranin binding in the center of the well, and at times the biofilm could be decreased in thickness in the center due to the PBS washing. The plate could be used uncoated or coated with different proteins such as fibronectin, collagen I, collagen IV.

When the ST22 MRSA-biofilm project was initiated, it was obvious that *S. aureus* did not grow as well as *S. pyogenes* in BHI media, even though overnight cultures contain higher amounts of CFU. When BHI was supplemented with 1% glucose it increased biofilm formation to the same ODs as *S. pyogenes* biofilm and therefore this media was used throughout the project.

An investigation around the strong biofilm produced by the *S. pyogenes* strain 2028 on collagen coated plates was initiated and it was of importance to measure small differences in biofilm formation, then the protocol was changed to include more washing steps and an additional step was added to elute the stain from the biofilm. This method was more time consuming but had less standard deviation. This protocol used crystal violet instead of safranin. Also, another collaborator recommended using yet another protocol using crystal violet. This protocol was less time consuming, but when compared to the safranin-staining protocol, several differences

were found. We also measured biofilm grown in minimal medium that had been exposed to human cells in the skin tissue model. In the end, we decided to not use these protocols in my studies. Several researchers report [145], as observed by us, that bacterial biofilm is highly dependent on the growth conditions: what nutrients and adherence-molecules are available, the timing of measurement, the inoculum, the mechanical stress, and dye included in the protocol. This made us even more certain that we had to measure biofilm in a biologically relevant system.

ORGANOTYPIC 3D TISSUE MODELS

Experimental research that aims to understand the pathophysiology of bacterial infections require models that recapitulate the human conditions. This has stimulated biologist and engineers to develop in vitro organotypic models mimicking human tissues [245]. The models contain, in varying degrees, cell types present in real tissue in combination with different extracellular matrix components. Animal studies, particularly mouse models, that can be troublesome in several aspects. Ethically, animal studies are always problematic. Also, *S. pyogenes* and *S. aureus* are primarily human pathogens, and many secreted factors and surface associated molecules are human specific [246]. Mouse cells are resistant to several staphylococcal toxins, including superantigens and PVL. Also, many drugs that perform well in preclinical animal studies have failed in humans due to toxicity and/or poor efficacy. This work has been done in a group with much experience in tissue culture [132, 154, 247-250]. We work with multilayered multicellular organotypic 3D tissue models with an air-liquid interface, sharing several features with the microenvironment of real tissue. However, the tissue models are not as complex as real tissue. Also, NSTI is localized in the deep soft tissues, a depth not resembled by the skin tissue models. The tissue models recapitulate important features of real skin, with a protective epithelium and a dermal layer, but as a model of NSTI, they are simplistic. Also, the model used in this work lacked immune system. It is also possible to make these models immunocompetent by including immune cells, however this was not done in the work described in this thesis. Recently, advances in the use of pluripotent stem cells have made it possible to generate tissue models developed in the laboratory, so called organoids. However, the cells used in this work are primary *in vitro* immortalized cells, hence, the term organotypic models are a more fitting description. Despite immortalization, the cells used have not lost important structural features and framework proteins such as E-cadherin and claudin-1 can be found in appropriate compartments of the model [247]. Previous studies have shown that the tissue models are susceptible to bacterial virulence factors and the flexibility of these *in vitro* systems make them highly valuable tools [132, 154, 248-250]. Mairpady Shambat et al. described that IVIG could hinder toxin-related injury in these tissue models [132]. In this doctoral work, the tissue models are used for the first time to do antimicrobial drug testing.

CONFOCAL MICROSCOPY

The infected skin tissue models are snap-frozen using liquid nitrogen. The tissue was cryosectioned and immunofluorescence staining was performed as previously described [154]. The stained sections were visualized using a Nikon A1R confocal microscope (Nikon Instruments).

MICROCALORIMETRIC ASSAY

To study antimicrobial treatment efficacy, we utilized a new development allowing for more quantitative measurements of metabolism in real time. Microcalorimetric measurements were carried out using the CalScreener® according to the manufacturer's instructions (Symcel AB, Sweden). The individual heat flow (μW) per insert was recorded at 42 second intervals for 120 hours.

MINIMUM BIOFILM ERADICATION CONCENTRATION

Minimum biofilm eradication concentration (MBEC) was determined by a modification of the broth microdilution assay [251]. Biofilm was formed by overnight incubation in a microtiter plate using Tryptic Soy Broth containing 1% glucose as growth medium. After rinsing with sterile PBS, antibiotics were added in Mueller-Hinton-F broth in two-fold serial dilutions [252]. Following overnight incubation and PBS wash, fresh broth without antibiotics was added. The biofilms were disintegrated by sonication and the resulting suspensions were transferred to a fresh microtiter plate and incubated overnight. MBEC was determined as the lowest concentration of antimicrobial agent resulting in no growth evident by visual assessment.

4 RESULTS AND DISCUSSION

I. CORRELATION BETWEEN IMMUNOGLOBULIN DOSE ADMINISTERED AND PLASMA NEUTRALIZATION OF STREPTOCOCCAL SUPERANTIGENS IN PATIENTS WITH NECROTIZING SOFT TISSUE INFECTIONS.

Polyspecific IVIG has been suggested as adjunctive therapy in STSS due to its ability to neutralize bacterial superantigens and dampening inflammation. IVIG was recently assessed in the INSTINCT study; a randomized, placebo-controlled clinical trial of NSTI [253]. The results showed no statistically significant differences in outcome between the placebo and IVIG-treated groups. However, a favorable trend was noted in the subgroup of patients that are associated with a higher rate of *S. pyogenes* or *S. aureus* infections. There are unresolved issues with regards to IVIG use, including the optimal dosage. Here, we assessed *S. pyogenes* toxin-triggered T-cell proliferation in PBMCs through a proliferation assay. Analyses of plasma collected pre- and post-administration of IVIG from patients with *S. pyogenes* NSTI demonstrated a negative correlation between IVIG dose and toxin-triggered T-cell proliferation ($r=-0.67$, $p<0.0001$). Low dose (25g) IVIG was sufficient to neutralize streptococcal superantigenic activity in most strains. However, 25g was borderline effective towards some strains, hence we found it reasonable to propose a dosage regimen of 0.5g/kg bodyweight day 1, followed by fixed doses of 25g daily for 1 to 2 additional days. We also present the *emm* type and superantigen gene profile of 29 *S. pyogenes* strains included in the INFECT cohort. In addition, we screened the strains for mitogenic T-cell activity (present in 27 of 29 strains) and determined their dose-response to IVIG. All mitogenic strains were neutralized >90% by the low dose 0.5 mg/ml IVIG.

The cytokine storm induced by superantigens, resulting in STSS, is rare and rapid. The patient arrives at the hospital in a pre-septic state, and the clinical condition quickly deteriorates. This is where IVIG is most likely to be effective, and this is where one would like to randomize patients to receive treatment or placebo. This is also where it is most difficult to find time to give out study information, get patient consent, include patients in trials and randomize them to receive different treatments. The INSTINCT study was a major advancement in the field, showing that it is possible to do large-scale randomized trials in NSTI [243]. When trials of rare diseases include several clinical sites, and every site only include a few cases, it affects the quality of the trial, and this was not done here, which is a strength. However, since the infections are rare, NSTI by all microbiological etiologies were included, even though *S. pyogenes* is only the causal agent in a third of cases. INSTINCT showed that IVIG should not be given to all patients with NSTI, because there is no benefit. However, there are pathophysiological differences between pathogens, and this was not accounted for. Also, the fact that 40% of the placebo-treated patients had received IVIG prior to inclusion in the study, illustrates the difficulties of studying rare and rapid diseases: it is only possible to collect the cohort at a major referral center, but once the patients get there, many have already received the treatment. Despite this, there was a trend towards benefit of three doses of IVIG in the subgroup dominated by *S. pyogenes* and *S. aureus*. Would the trial have shown benefit of IVIG if it would have been performed not in Denmark but in Sweden, where IVIG is usually administered later in the disease? It is possible. In the study by Darenberg et al. (randomized

controlled study of IVIG in STSS in Sweden) the mortality was 3.6-fold higher in the placebo group, however not statistically significant, due to the small size of the cohort, 21 patients [187]. It's also possible that IVIG administration is not effective, if not provided within the first few hours of the cytokine storm. On the other hand, presence of high bacterial load, bacterial virulence factors including toxins, and cytokines have been detected up to 20 days after diagnosis in studies of patient tissue biopsies, which would motivate IVIG also at later time points [115]. Our study demonstrated that 25g IVIG resulted in a sufficient neutralization of the mitogenic T-cell response to most *S. pyogenes* superantigens. The lack of placebo-treated patients infected with *S. pyogenes* in INSTINCT made us include a comparator cohort from INFECT (larger observational study), and the results clearly demonstrate the lack of patients susceptible to a superantigen response in INSTINCT. Also, a negative correlation between IVIG administered and toxin-triggered T-cell proliferation was observed. Additionally, in the analysis of *S. pyogenes* NSTI within INFECT, Bruun et al. identified IVIG as an independent factor for 90-day survival [254].

In another recent observational study, Peetermans et al. studied IVIG given to patients with STSS requiring extracorporeal membrane oxygenation and observed a mortality rate lower than expected [255]. Tarnutzer et al. performed an experimental study and observed that IVIG decreased several *S. pyogenes* virulence factors *in vitro*: hemolytic SLO and Sda1 activity was completely abolished, SpyCEP activity was significantly reduced, while SpeB was not influenced [256]. Also, IVIG treatment was shown to reduce the disease severity in a murine necrotizing fasciitis model, but the bacterial load was not influenced [256]. Additionally, after IVIG treatment, patient sera showed elevated titers of antibodies specific for SLO and Sda1, which resulted in reduced SLO and Sda1 activity [256]. It is clear that IVIG inhibit *S. pyogenes* virulence *in vitro* at several levels. A reason why its clinical efficacy has been more unclear could be the tissue penetrance. Tarnutzer et al. observed six times lower IgG concentrations in the skin compared to the serum, demonstrating the difficulty of delivering large molecules to the site of infection is NSTI [256]. If superantigens and other virulence factors that can be inhibited by IVIG are important at the tissue site, it might be difficult to deliver enough molecules where they're needed and make a difference in NSTI. However, much of the pathogenesis relating to the cytokine storm and septic shock is thought to take place in the patient's blood. Therefore, it is still probable that IVIG can be beneficial in *S. pyogenes* NSTI. This should be studied in a clinical trial.

II. ADJUNCTIVE RIFAMPICIN INCREASES ANTIBIOTIC EFFICACY IN GROUP A STREPTOCOCCAL TISSUE INFECTION MODELS.

Biofilm formation at the tissue site has been as a complicating feature of patients with *S. pyogenes* NSTI. Also, viable bacteria have been isolated from patients despite prolonged antibiotic therapy [115]. In this study, we assessed antibiotic combinations against bacterial biofilm formed in a 3D organotypic skin tissue model to try to improve antimicrobial efficacy at the tissue site. We found that the standard treatment of benzylpenicillin and clindamycin only reduced the bacterial load with about 10-fold, despite high concentrations (500 x MIC). When adjunctive rifampicin was added, a 100-fold reduction was seen. Compared to the standard treatment, adjunctive rifampicin increased the antibiotic efficacy of bacterial clearance with 97.5% versus 93.9% ($p=0.006$). Also, microcalorimetric measurements revealed that adjunctive rifampicin resulted in decreased metabolic activity and prolonged time

to regrowth for all clinical *S. pyogenes* strains tested ($p<0.05$). In addition, a case report describes a case of a persistent *S. pyogenes* tissue infection where the patient receives rifampicin treatment.

The role of rifampicin in staphylococcal biofilm infections is well defined [257], but the role in streptococcal biofilm infections is not. The tissue penetrating abilities of rifampicin are well known, the systemic bioavailability of rifampicin is 90-100% on an empty stomach, and there is no rationale for intravenous administration unless there is an inability to swallow or evidence of poor enteral uptake [257].

Adjunctive rifampicin was recently tested in the ARREST trial of *S. aureus* bacteremia [38]. After 12 weeks of treatment, 17% vs. 18% suffered severe adverse events such as treatment failure or death. No benefit of adjunctive rifampicin was seen, even though 40% of participants had an initial deep infection focus on an implanted vascular device, native or prosthetic heart valve, native or prosthetic bone or joint, deep tissue infection or abscess (including vertebral bone or disc or other bone infection, epidural or intraspinal empyema, infected intravascular thrombus, brain infection). 17% in the rifampicin-group had antibiotic adverse events and 6% had drug interactions vs. 10% and 2% in the placebo-group. The authors concluded that adjunctive rifampicin still could have benefits long-term but that it does complicate other drug treatment. It cannot be excluded that a clinical trial of adjunctive rifampicin in *S. pyogenes* NSTI would give the same results. However, the data is promising enough to support a clinical trial. The timing of rifampicin administration could be discussed, for NSTI patients, typically treated in the intensive care unit, rifampicin is likely be introduced in the post-acute phase, to reduce interactions with other drugs. Especially cases complicated by bacterial persistence despite comprehensive surgical debridement and treatment with β-lactams and clindamycin.

The treatment of a patient with a severe infection is usually decided by an infectious disease specialist. Commonly, a set dosage is used, that is above the minimum inhibitory concentration (MIC) for most strains. The bacterial viability in treated tissue is not determined in routine clinical practice. Infectious disease specialists calculate risks and recommend empirical treatment that is likely to inhibit bacteria in their planktonic state. The results of this study clearly show that bacterial susceptibility in tissue is different from that in blood. Potentially could several treatments used today only be effective in blood, and our immune system could account for all the bacterial clearance in tissue. In this study, bacterially infected tissue was treated with high doses of antibiotics for several days, and they were still able to grow back when the antibiotic pressure was removed. This method of determining treatment efficacy in tissue could be used more broadly, but there are technical limitations. The method is not high throughput, instead it requires weeks of care in a laboratory and well-timed infection, treatment, and processing. The amount of data per hour spent in the laboratory is strikingly low. However, it does provide important insight into a phenomena that could be applicable to additional fields: tissue antibiotic resistance phenotypes.

Paradoxically, increased ambition in the treatment of antibiotic resistance phenotypes in tissue could drive development of antibiotic resistance. It is possible that antibiotic combination therapy and adjunctive treatments increase in the coming years, increasing the environmental antibiotic pressure and driving antibiotic resistance. It is important to perform clinical trials to

know that added treatment also provide a benefit, and not only selection for resistant phenotypes.

III. DYSFUNCTIONAL QUORUM SENSING IN A NATURAL AGRC VARIANT RESULTS IN SUSTAINED BIOFILM BY ST22 METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*.

The alarming increase of methicillin-resistant *Staphylococcus aureus* (*MRSA*) worldwide, including an emergence of highly virulent community associated strains, is a major concern. Recently, natural variants of the rapidly disseminating MRSA clone of sequence type 22 (ST22), with starkly different phenotypes, were identified in community associated strains [258]. The phenotypic variants were separated only by a single amino acid substitution in AgrC, a component of its quorum sensing system resulting in defective Agr signaling in one of the strains. Here, we sought to investigate whether this Agr inactivation also made these variants to differ with respect to biofilm formation. Through culture on polystyrene surfaces and infection of an organotypic skin tissue model we observed differences between the strains. Initial biofilm formation by both strains on polystyrene was dispersed after 9 hours by the Agr signaling strain that then became biofilm negative. The Agr-silent strain continuously formed biofilm at late time points (up to 72 hours). Collagen coating of the polystyrene surface increased biofilm formation by both strains and decreased the differences between the strains. In the skin tissue model, both isolates formed biofilm but only the Agr-signaling variant disrupted the epithelium and disseminated into deeper tissue layers. It seems like dysfunctional quorum sensing in strains of the Agr-silent phenotypes with cysteine at position 223 of AgrC resulted in sustained biofilm formation while the Agr-signaling strains instead are more invasive.

This work shows that stimulating Agr would be a way of dispersing *S. aureus* biofilm. Agr-agonists could be combined with toxin-dampening agents and used to treat biofilm-associated infections. However, most people focus on inhibiting the virulence properties of *S. aureus*, and what better way than inhibiting Agr?

Inhibiting Agr signaling would decrease *S. aureus* virulence, while not being bacteriostatic or bactericidal, which would not drive resistance as much as classical antibiotics do. Also, Agr-targeting is likely to be more effective than inhibiting single virulence factors since it controls transcription of many *S. aureus* factors. In addition, it would preserve the commensal bacteria. Sully et al. described an inhibitor targeting AgrA that was dubbed savarin (*S. aureus* virulence inhibitor) [259]. Savarin was identified through screening for Agr-blocking molecules, that did not affect *Staphylococcus epidermidis*. The authors could show that savarin treatment resulted in decreased tissue injury and reduced bacterial burden upon treatment. Another group inhibited Agr using a naturally isolated substance named Solonamide B from *Photobacterium halotolerans* [260]. Bacterial growth was not impaired but a reduction in hemolysis and neutrophil lysis were shown. Also, Solonamide B did not elicit cytotoxicity for human PBMCs [261]. ω-hydroxyemodin (OHM) is another compound shown to inhibit Agr [262]. OHM is isolated from *Penicillium restrictum* and, without affecting bacterial growth, it reduced lesion size as well as bacterial burden when used in treatment of infected mice. OHM did not elicit cytotoxicity against eukaryotic cells (kidney, alveolar and hepatocytes).

Perhaps one of these treatments will be available to prescribe in *S. aureus* infections soon. If so, it could dampen the epithelial damage and tissue invasion in skin infections, but biofilm is likely to form irrespective of Agr-inhibition, and it will be important to have an awareness of the potential of increased biofilm formation.

5 CONCLUSIONS

The treatment of bacterial tissue infections caused by *S. pyogenes* and *S. aureus* is more complicated than some may think. The threat of antibiotic resistance is a major concern. Also, the treatment available today is not good enough. There are challenges to overcome, such as the life-threatening toxic effects of superantigens, but also persistence and biofilm formation at the tissue site. However, there are therapeutic opportunities available today with efficacy against these complicating features of infection.

KEY FINDINGS

- *Administration of 25g IVIG result in S. pyogenes superantigen-neutralizing activity in patient plasma.*
- *Adjunctive rifampicin treatment enhances clearance of S. pyogenes in an in vitro tissue infection model.*
- *Dysfunctional quorum sensing in ST22 MRSA results in sustained biofilm formation and reduced invasive capacity.*

CLINICAL ASPECTS: TAKE HOME MESSAGE

There are knowledge gaps and a lack of tools today within the field of infectious diseases/microbiology, relating to antibiotic resistance phenotypes in tissue during acute infections. Treatment regimens are focused mainly on minimum inhibitory concentrations (MIC). Some NSTI patients are treated with a carbapenem antibiotic in single therapy since they are broad spectrum drugs. However, penicillin and clindamycin would be a better treatment option to inhibit *S. pyogenes*, the causal agent in a third of NSTI cases. Also, an addition of IVIG and rifampicin is likely to be beneficial. When only considering MIC values, one treats the infectious setting in blood but disregard the situation in the tissue. In a patient that has an NSTI focused to an extremity (OR 7.8 for *S. pyogenes* [3]), has an age below 61, are previously healthy and has septic shock – there are reasons to suspect *S. pyogenes* as causal agent. The adjuvant treatments aimed at *S. pyogenes* should not be wasted on non-*S. pyogenes*/non-*S. aureus* cases due to the cost, development of resistance, and development of disbelief in their effectiveness. NSTI of the abdomen or anogenital area are likely to be caused by polymicrobial type I NSTI (OR 8.0 [3]), rarely *S. pyogenes*. When it comes to *S. aureus* infections, their basal mode of growth is biofilm formation, and this is only disrupted when signaling through Agr occurs. Agr inactivation unable epithelial disruption and tissue dissemination. Clindamycin and rifampicin are recognized as anti-biofilm agents and are frequently used in suspected biofilm-related *S. aureus* infections today. It is likely that biofilm is present in additional infections and that these compounds would improve the outcome in these infections, however this needs to be tested and balanced against the potential of resistance development. IVIG does inhibit the superantigens of *S. aureus*, but the dosage required has not been tested and there are some indications that it could be higher than that for *S. pyogenes*.

6 POINTS OF PERSPECTIVE

> *Without effective human intervention, epidemics and pandemics typically end only when the virus or bacteria has infected every available host and all have either died or become immune to the disease.*
>
> Alan Huffman

S. pyogenes and *S. aureus* are on us all the time. They infect us repeatedly without causing long-lasting immunity. The burden of disease is massive, making effective human interventions badly needed. In tissue infections by *S. pyogenes* and *S. aureus*, future studies on therapeutic options are required:

Firstly, the efficacy of 25g IVIG in *S. pyogenes* NSTI needs to be tested in a clinical trial.

Secondly, the efficacy of adjunctive rifampicin in *S. pyogenes* biofilm-associated tissue infections (such as NSTI) should be tested in a clinical trial.

Thirdly, Agr-targeting in *S. aureus* shows promise as a way of decreasing tissue pathology, preserving epithelial integrity, decreasing bacterial dissemination with the side-effect of also enhancing biofilm formation. Opposingly, Agr-stimulation could help disperse biofilm in biofilm-related infection. Drugs with Agr-action should be tested in relevant model systems.

Other substances could be as or more effective than the drugs tested in this doctoral work, using the same mechanism. Perhaps there are less costly ways of neutralizing superantigens than IVIG? Reglinski and Sriskandan recently affinity-purified pathogen reactive antibodies from IVIG preparations and found antibodies that recognize cell wall antigens of *S. pyogenes* and *S. aureus* [263]. Treatment with these preparations resulted in higher levels of neutrophil bacterial uptake. This type of boosted-IVIG preparations could be more effective and less costly. Also, monoclonal antibodies aimed at superantigens, or T-cell proliferation could to the same thing. However, monoclonal antibodies could be more expensive than IVIG, hence defeating the purpose of development. Also, they would not have the polyspecific effects that IVIG has, such as anti-cytokine antibodies and Fc-receptor-interactions.

Perhaps other antibiotics such as linezolid, macrolides or tetracyclines are as effective as adjunctive rifampicin against streptococcal biofilm in tissue. This could be tested in our model system; however, candidates should be screened in assays such as microcalorimetric assays which are more high throughput before being tested in the tissue models. When anti-biofilm agents enter the market, also these substances could be tested in our model system to view their efficacy on biofilm in tissue. Of course, the skin tissue model is designed to reflect the skin and soft tissue setting, while our lung tissue model is designed to mimic lung tissue. Host cells have different receptors and other characteristics depending on their origin, and a model should always try to imitate the tissue in focus of the study.

Bacterial clearance after antibiotic treatment of *S. aureus* infection in the skin tissue model should be assessed using the ST22 AgrC variants. This will reveal the effect of Agr-silencing in tissue under treatment. One can speculate that the Agr-silent isolate should be more difficult to treat due to sustained biofilm formation, however this is not known. Also, one should challenge the AgrC variants against each other in coinfections.

Another important aspect is patient stratification and individualized medicine. However, this requires improved diagnostics. Today, even the diagnosis of (untyped) NSTI is difficult to make. However, there have been some progress. Recently, Palma Medina et al. described thrombomodulin as a unique biomarker for detection of necrotizing soft tissue infections [264]. If confirmed, it may be used as a diagnostic tool. Also, all patients do receive individualized antimicrobial therapy as soon as the microbiological diagnostics are done. However, it can take days to get the full resistance profile on an isolate, and that is time that some patients don't have. While waiting for the diagnostics, patients are usually treated with broader treatment than needed, to be on the safe side, and some receive treatments such as IVIG even though there is low risk of a superantigen-producing causal agent (*S. pyogenes*/*S. aureus*). This can decrease the in-hospital confidence in these drugs, and the cost can make people question their use. Improved diagnostics are vital for the development in the field of acute infectious diseases. Soon, we will know the causal agent, its resistance profile and virulence factors at the first encounter with the patient. This will revolutionize the field, decrease usage of broad-spectrum antibiotics, facilitate usage of adjunctive therapeutics such as IVIG and rifampicin and facilitate randomized controlled trials. That in turn, will further accelerate our understanding of acute infectious diseases and the effectiveness of treatments.

Another diagnostic tool that is missing today is a biomarker for biofilm. In our previous studies, we identified biofilm in over 30% of patients with NSTI caused by *S. pyogenes*. If this is the incidence of biofilm in *S. pyogenes* NSTI, this is the number of patients that should be treated. Performing a trial on all patients with *S. pyogenes* NSTI could mask a benefit in the subgroup of patients with actual biofilm infections. However, we believe that biofilm is more frequent and without a promising biomarker in development, we need to start with a trial on all *S. pyogenes* NSTI with anti-biofilm agents such as rifampicin, to gain more knowledge and potentially improve the outcome in *S. pyogenes* NSTI.

An initial plan for this thesis work was to include a study on the efficacy of HBOT on *S. pyogenes*-infected skin tissue models. Logistically, this was complicated. To be able to study HBOT, hyperbaric pressure and high oxygen concentrations are required. One needs to be educated in safety procedures when working with pressure and high concentrations of oxygen (fire hazard). There was no pressure chamber at KI allocated for research (patient chambers cannot be used for research). We were able to collect one pressure chamber that was found to lack appropriate safety gear, one that was too large for the lab and one that was well-suited for the studies except that it could only be borrowed for a short period of time. A few months ago, an experimental chamber was placed in a laboratory at KI which could facilitate future studies in this area. A study of specific interest would be infection of immunocompetent skin tissue models containing live neutrophils, with and without HBOT. It would be possible to determine treatment efficacy through bacterial counts and to isolate the neutrophils for characterization. This could be done in the presence or absence of antibiotics.

Though media coverage on NSTI and STSS has decreased, cases still occur [3, 265], and both *S. aureus* and *S. pyogenes* strains seem to circle in the community in 10-year intervals, possibly due to immune status [266, 267]. In addition, most of the burden of disease is in the less developed part of the world, invisible to western media.

One thing is certain: humans will continue to live with these terrible infections and related conditions until vaccines are developed and distributed globally. Until then, we should treat the infections properly. That includes awareness of the therapeutic challenges and opportunities today, and continued scientific endeavors.

7 ACKNOWLEDGEMENTS

I would like to take the opportunity to thank Karolinska Institutet for the opportunity to conduct my doctoral studies. I'm grateful for the scientifically stimulating and international work environment. As a teenager in the Karolinska summer research school 2011, you inspired me to pursue science and medical training. As an MD PhD candidate 2021, I feel empowered to take on the world's medical challenges and you have made that happen.

This work would not have been possible without the financial support from the funding agencies, for which I'm very grateful. Thank you for funding our studies.

I want to thank the patients and their families, who in times of despair found it in their heart to consent to inclusion in our studies. I hope you are proud of what we have done.

I want to thank the blood donors, who regularly and altruistically donate time and blood.

All the studies in this thesis were performed at the Center for Infectious Medicine. This is a truly special place, filled with ambitious young scientists (the CIMers) from different corners of the world, working day and night to answer scientific questions. I'm so grateful to have shared this journey with all of you.

First, I would like to thank my supervisor Anna Norrby-Teglund. You are brilliant. Not only do you know everything in the field of streptococci, but you are still curious and have many great ideas. Additionally, you are strategical and well-formulated. I've felt safe under your supervision, confident that you always know what to do. You navigate everything from clinical studies to big datasets like it's your backyard and you always consider the patients. In addition, you are fun, kind, sophisticated, hardcore, and you have class. It has been a privilege to be your PhD student. Thank you for believing in me. Also, thank you for always encouraging me to take part in meetings and collaborations, I've felt unobstructed.

Secondly, I want to thank my co-supervisor Mattias Svensson. You have developed an important niche at KI with immunological studies in organotypic tissue. You are positive and have a way of seeing the larger picture. You consider the people around you, which not all at KI do, thank you for that. You are also a fantastic baker, thank you for all the cakes.

Kerstin Nilsson, thank you for being my mentor. Thank you for your calm and wisdom, reminding me about scientific quality, my medical background, and of what is important.

Present and past group members:

Johanna Emgård, thank you for the most exciting part of my doctoral studies. Persuading you to do secret pilot experiments was not easy, but it went better than I could have imagined. Thank you for showing me Jerusalem. Thank you for teaching me about MAITs. Who knows if the future contain more crazy experiments or not, but working with you was a bliss.

Laura Palma Medina, thank you for the way you plunged into tissue modelling and biofilm studies. It has been a pleasure working with you, in every way. Thank you for explaining computer modelling. Thank you for your optimism, our discussions, and all the music.

Sriktanth Mairpady Shambat, thank you for supervising my bachelor's and master's thesis and teaching me how to work with *S. aureus* host-pathogen interactions. Your scientific enthusiasm is radiant. Thank you for letting me follow your every step. Egle Kvedaraite, thank you for your optimism and insightful questions, you inspire. Kirsten Moll, thank you for organizing our group and leading technical advancements. Victoria Vassen, thank you for bringing molecular techniques into the group. Majda Dzidic, thank you for teaching me about granulocytes. Magdalini Lourda, your dedication is inspiring, thank you for including me in the neutrophil team and thanks for our crazy pilot that we need to follow up some day. Puran Chen, thank you for introducing me to CIM and tissue modelling. Johanna Snäll, thank you for teaching me confocal microscopy. Julia Uhlmann, thank you for being so open and positive, and thank you for leaving me your things, such as the poster-case. Takeaki Wajima, thank you for inspiring with your accurate measurements. Muhammad Afzal, thank you for being a nice colleague. Nikolai Siemens, thank you for pursuing studies of *S. pyogenes*. Bhavya Chakrakodi, it was a pleasure meeting you, thank you for sharing stories from India.

CIMers: Cell module 1: Caroline Boulouis. Olga Rivera Ballesteros, Marco Loreti, Marina Garcia. The Jungle office: Ebba Sohlberg, Takuya Sekine, Marta Butrym, Curtis Cai, thank you for always making me feel at home in the office. Inga Rodahl, thank you for your energy. The green lab crew: Anna Rao, Efthymia Kokkinou, Luca Mazzurana. The big lab crew: Emma Ringqvist, Virginia Stone, Sebastian Kapell. Kimia Maleki, you crazy person, thank you for bringing a laid-back attitude to CIM, and your vegan conviction inspire. Elza Evren, Laura Hertwig, Tiphaine Parrot, Jonna Bister, Tyler Sandberg, Martin Ivarsson, Kim Blom, Benedikt Strunz, Alvaro Haroun-Izquierdo, Quirin Hammer, Jakob Theorell, Alvin Brodin, Demi Brownlie, Iva Flipovic, Mira Akber, Wanda Christ, Christ Tibbitt, Emma Patey, Anne Marchalot, Lena Berglin, Angelica Cuapio Gomez, Jagadeeshwara Rao Muuva, Natalie Sleiers, Marina Garcia, Julia Niessl, Whitney Weigel, Tobias Kammann, David Wullimann, Yu Gao, Arlisa Alisjahbana, Pouria Momayyezi, Elli Mouchtaridi, Archilleas Fardellas, Chris Stamper. CIM support team: Elisabeth Henriksson, Eve-Marie Rowe Kärkäs, Christina Hammarstedt, Lena Radler, Margit Ekström, thank you for making CIM run smoothly. Johan Sandberg, thanks for our adventures in Israel, had a blast. Marcus Buggert and group, you are all so nice, and I'm impressed with your seemingly smooth machinery of high-quality research. Niklas Björkström, thank you for your advice, we're all so proud of your work here at CIM. Andrea Ponzetta, thank you for showing me tricks in Excel. Benedict Chambers, thank you for your laughter and dedication to immunology. Jenny Mjösberg, thank you for being an inspiration to us all through high-quality publications and awards. Sara Gredmark Russ: thank you for showing that it's possible to be a clinical clinican and have a research group, you inspire. Jonas Klingström, thanks for our discussions during the pandemic. Malin Flodström-Tullberg, thank you for your continued efforts to explain and extinguish type 1 diabetes. Hans-Gustaf Ljungren, thank you for founding CIM and your continuous efforts to

develop us. Also, thank you: Myriam Aouadi, Kalle Malmberg, Susanna Brighenti, Jakob Michaelsson, Nicole Marquardt, Tim Willinger.

INFECT-consortium: Steinar Skrede, Ole Hyldegaard, Martin Brun Madsen, Marina Morgan, Per Arnell, Andreas Itsek, Anders Rosén, Trond Bruun, Suba Nookala, Francois Vandenesch, Francois Bergey. Thank you for overcoming obstacles and working translationally together to improve the outcomes of NSTI. Also, Per and Anders – thank you for thinking about my safety. HBOT people: Michael Nekludov, Anders Kjellberg, Peter Kronlund, Folke Lind, thanks for trying.

I would like to thank Örebro Univeristy, where I went to medical school, for giving me the foundational tools to be a great physician, encouraging research and empowering me to change what is not yet perfect. Especially my mentor Kerstin Nilsson, but also Godfried M. Roomans, Ulf Tidefelt, Bo Söderquist, Torbjörn Norén, Eva Särndahl, Jens Schollin and Mattias von Beckerarth.

The clinic of Infectious Diseases at Universitetssjukhuset Örebro: Martin Widlund, Henrik Eliasson and Rebecca Borg. Thank you for trusting, supporting me and giving me my first experiences as a graduated physician. The clinic of Infectious Diseases at Danderyds Hospital: Magnus Hedenstierna, Anna Westman, Jesper Ericsson, Patrik Gille-Johnsson, Carin Scholander, Johan Ursing, Maria Norrby. Thank you for your burning interest in infectious diseases and for valuing clinical work. The clinic of Infectious Diseases at Karolinska Univeristy Hospital: Kristoffer Strålin, Jonas Sundén-Cullberg, Anna Färnert, Sara Gredmark-Russ. Thank you for making research and the clinic feel close. The emergency department at Södersjukhuset (Stockholm Southern Hospital): Clara Strömberg, Ali Al-Khiat, Ruth Kalvelage, Sepehr Zanjani, Maria Elfsson, David Stigsson, Lisa Forkman, Hani Rafiq, Christian Duré. Thank you for helping me remember everything I forget and saving me in stressful situations. Thank you for letting me come back time and time again to work at the largest emergency department in Scandinavia. Your eternal AT-läkare. The clinic of Infectious Diseases at Södersjukhuset: Hampus Nordqvist, Anders Håkansson. Thank you for highlighting *S. pyogenes* NSTI. AT-organization at Södersjukhuset: Fredrik Larsson, Lina Sjödin, Christina Almkvist Osterman. Thank you for supporting me in my efforts to combine research and clinical work. AT-colleagues Kati Johansson, Sara Török, Emil Kronblad, Adrian Kimiae, Olle Elfwén, Gutav Lindmark, thank you for lunches, after works, support.

My former teachers and supporters: Lorentz Persson, Svetlana Slavin, Thomas Aidehag, Claes Folkesson. Thank you for all you did, that you didn't have to do. You changed my life.

My friends: Victoria Rådström, my best friend and colleague. Thank you for all those nights in the library. Thank you for early mornings in the car to our internships. Thank you for never forgetting to celebrate an advancement. Thank you for all emotional support and advice. Thank you for not complaining enough when I'm late. Thank you for choosing to be a pediatrician. Linnea Björk, my oldest friend and critic. Thank you for all the laughter. Thank you for keeping me on the ground. Alexandra Timmerman, thank you for bringing life to

Örebro. I'm proud of you for becoming a surgeon. Anna Båtelsson, my role-model and rockstar anesthesiologist. Thank you for your laid back view on life. Miléne Fernandez, thank you for dragging me out to dance, swim and work out. Thank you for your never ending interest in emergency medicine, you inspire. Sussan Alipour and Felicia Forss, thank you for ignoring my nerdiness. Linn Klöfver, thank you for inspiring me to go after both science and music. Emilie Laurent, Andrea Höglund, Elin Ronqvist, Rebecca Petersson, My Strömberg, Frida Jädersten, thank you for engilding my life with unconcerned celebrations, travel and always reminding me to love life. Also Sofia Sigra, Elin Sandberg, Oskar Enoksson, Johan Ringlander, Anna Dimic, thank you for making life in Örebro exciting.

My family: The single most important reason that I'm writing this book today is because my parents helped me get there. You taught me to value science and knowledge. You taught me to not overthink but just do. You taught me to not loose myself in work. You taught me that I'm important and that my opinion matter. You supported me in every endeavor, every setback, and every celebration. My dad, Pär Bergsten, you taught me mathematics and physics at early age, you provided books on science and movies on science fiction. You taught me to have goals in life and to plan for success. Thank you for always supporting me. My mum, Elisabeth Bergsten, you taught me to never give up, just take a break from time to time. You taught me about health and the foundation of well-being. You have done everything and a little bit more than what could ever be expected of a parent. Thank you for always supporting me. Julia Bergsten, my sister, and friend, thank you for looking up to me, asking me about everything. I'm so impressed and proud of all that you have achieved. No one understands me like you do. Thank you for always supporting me. Agne Nyström, thank you for always helping with everything, you could actually be accused of helping out too much. Thank you for building the ceiling for the wedding, thank you for picking me up as a child when others forgot to. Ruth Nystöm, Ingela and Boen Östervall, Malin and Mikael Axenstig, Mats and Inger Nyström, Rebecka and Kristoffer, Hillevi and Elliot, Charlotta Bergsten and Ove. Thank you for always welcoming me, focusing on the good things in life. My grandparents who left us too soon: Britta and Bert Bergsten, Klas Nyström. We miss you. My new family: Gabriella Manieri and Niclas, Alexander and Tove, Birgitta Tallroth, other Manieris, thank you for welcoming me into the family and always asking about my research.

Sebastian, my husband, my love, my soulmate: Thank you for patience, listening to my monologues without having any scientific education. Thank you for understanding, I don't know how you do it. Thank you for taking care of everything that I don't have the energy to do in stressful periods, or ever. Thank you for not complaining when I'm working too much. Thank you for supporting me in all crazy endeavors. Thank you for carrying me when I'm broken down in pieces. Thank you for all the amazing things we have done together.

Additionally, I want to thank other people who have seen, encouraged, and believed in me. Those of you who were not mentioned: thank you!

8 REFERENCES

[1] Jallali N, Withey S, Butler PE. Hyperbaric oxygen as adjuvant therapy in the management of necrotizing fasciitis. Am J Surg 2005;189:462-6.
[2] Borschitz T, Schlicht S, Siegel E, Hanke E, von Stebut E. Improvement of a Clinical Score for Necrotizing Fasciitis: 'Pain Out of Proportion' and High CRP Levels Aid the Diagnosis. PLoS One 2015;10:e0132775.
[3] Madsen MB, Skrede S, Perner A, Arnell P, Nekludov M, Bruun T, et al. Patient's characteristics and outcomes in necrotising soft-tissue infections: results from a Scandinavian, multicentre, prospective cohort study. Intensive Care Med 2019;45:1241-1251.
[4] Descamps V, Aitken J, Lee MG. Hippocrates on necrotising fasciitis. Lancet 1994;344:556.
[5] Lamb L, McDonald W, Scudamore C, Tan L, Lynskey N, Sriskandan S. The effect of trauma on invasive group A streptococcal disease. Lancet 2015;385 Suppl 1:S60.
[6] Green RJ, Dafoe DC, Raffin TA. Necrotizing fasciitis. Chest 1996;110:219-29.
[7] Gergis H, Barik S, Lim K, Porter W. Life-threatening puerperal infection with group A streptococcus. J R Soc Med 1999;92:412-3.
[8] Lancefield RC. THE ANTIGENIC COMPLEX OF STREPTOCOCCUS HAEMOLYTICUS : I. DEMONSTRATION OF A TYPE-SPECIFIC SUBSTANCE IN EXTRACTS OF STREPTOCOCCUS HAEMOLYTICUS. J Exp Med 1928;47:91-103.
[9] Stevens DL, Tanner MH, Winship J, Swarts R, Ries KM, Schlievert PM, et al. Severe group A streptococcal infections associated with a toxic shock-like syndrome and scarlet fever toxin A. N Engl J Med 1989;321:1-7.
[10] Leyden JJ, Stewart R, Kligman AM. Experimental infections with group A streptococci in humans. J Invest Dermatol 1980;75:196-201.
[11] Vela AI, Villalón P, Sáez-Nieto JA, Chacón G, Domínguez L, Fernández-Garayzábal JF. Characterization of Streptococcus pyogenes from Animal Clinical Specimens, Spain. Emerg Infect Dis 2017;23:2013-2016.
[12] Begovac J, Bobinac E, Benic B, Desnica B, Maretic T, Basnec A, et al. Asymptomatic pharyngeal carriage of beta-haemolytic streptococci and streptococcal pharyngitis among patients at an urban hospital in Croatia. Eur J Epidemiol 1993;9:405-10.
[13] Christenson B, Sylvan SP, Noreen B. Carriage of multiresistant Streptococcus pneumoniae among children attending day-care centres in the Stockholm area. Scand J Infect Dis 1997;29:555-8.
[14] Agerhäll M, Henrikson M, Johansson Söderberg J, Sellin M, Tano K, Gylfe Å, et al. High prevalence of pharyngeal bacterial pathogens among healthy adolescents and young adults. APMIS 2021.
[15] Hedin K, Bieber L, Lindh M, Sundqvist M. The aetiology of pharyngotonsillitis in adolescents and adults - Fusobacterium necrophorum is commonly found. Clin Microbiol Infect 2015;21:263.e1-7.
[16] Kim S, Lee NY. Epidemiology and antibiotic resistance of group A streptococci isolated from healthy schoolchildren in Korea. J Antimicrob Chemother 2004;54:447-50.
[17] Rick AM, Zaheer HA, Martin JM. Clinical Features of Group A Streptococcus in Children With Pharyngitis: Carriers versus Acute Infection. Pediatr Infect Dis J 2020;39:483-488.
[18] Shaikh N, Leonard E, Martin JM. Prevalence of streptococcal pharyngitis and streptococcal carriage in children: a meta-analysis. Pediatrics 2010;126:e557-64.

[19] Ozturk CE, Yavuz T, Kaya D, Yucel M. The rate of asymptomatic throat carriage of group A Streptococcus in school children and associated ASO titers in Duzce, Turkey. Jpn J Infect Dis 2004;57:271-2.
[20] Oliver J, Malliya Wadu E, Pierse N, Moreland NJ, Williamson DA, Baker MG. Group A Streptococcus pharyngitis and pharyngeal carriage: A meta-analysis. PLoS Negl Trop Dis 2018;12:e0006335.
[21] Carlander A, Byfors S. Invasive group A streptococcal disease, seasonal report 2016-2017. Public Health Agency of Sweden 2017.
[22] Carapetis JR, Steer AC, Mulholland EK, Weber M. The global burden of group A streptococcal diseases. Lancet Infect Dis 2005;5:685-94.
[23] Carapetis JR, McDonald M, Wilson NJ. Acute rheumatic fever. Lancet 2005;366:155-68.
[24] Steer AC, Law I, Matatolu L, Beall BW, Carapetis JR. Global emm type distribution of group A streptococci: systematic review and implications for vaccine development. Lancet Infect Dis 2009;9:611-6.
[25] Weckel A, Guilbert T, Lambert C, Plainvert C, Goffinet F, Poyart C, et al. Streptococcus pyogenes infects human endometrium by limiting the innate immune response. J Clin Invest 2021;131.
[26] Sigra S, Hesselmark E, Bejerot S. Treatment of PANDAS and PANS: a systematic review. Neurosci Biobehav Rev 2018;86:51-65.
[27] Telfer NR, Chalmers RJ, Whale K, Colman G. The role of streptococcal infection in the initiation of guttate psoriasis. Arch Dermatol 1992;128:39-42.
[28] Ogston A. Micrococcus Poisoning. J Anat Physiol 1882;17:24-58.
[29] Clauditz A, Resch A, Wieland KP, Peschel A, Gotz F. Staphyloxanthin plays a role in the fitness of Staphylococcus aureus and its ability to cope with oxidative stress. Infect Immun 2006;74:4950-3.
[30] Rosenbach FJ. Mikro-organismen bei den Wund-Infections-Krankheiten des Menschen. Weisbad. Bergmann. 1884.
[31] Skinner DKC. Significance of bacteremia caused by staphylococcus aureus. Arch Intern Med 1941;68(5):851.
[32] Barber M. Methicillin-resistant staphylococci. J Clin Pathol 1961;14:385-93.
[33] Gorak EJ, Yamada SM, Brown JD. Community-acquired methicillin-resistant Staphylococcus aureus in hospitalized adults and children without known risk factors. Clin Infect Dis 1999;29:797-800.
[34] Herold BC, Immergluck LC, Maranan MC, Lauderdale DS, Gaskin RE, Boyle-Vavra S, et al. Community-acquired methicillin-resistant Staphylococcus aureus in children with no identified predisposing risk. JAMA 1998;279:593-8.
[35] Carleton HA, Diep BA, Charlebois ED, Sensabaugh GF, Perdreau-Remington F. Community-adapted methicillin-resistant Staphylococcus aureus (MRSA): population dynamics of an expanding community reservoir of MRSA. J Infect Dis 2004;190:1730-8.
[36] Chang S, Sievert DM, Hageman JC, Boulton ML, Tenover FC, Downes FP, et al. Infection with vancomycin-resistant Staphylococcus aureus containing the vanA resistance gene. N Engl J Med 2003;348:1342-7.
[37] Chambers HF. The changing epidemiology of Staphylococcus aureus? Emerg Infect Dis 2001;7:178-82.
[38] Thwaites GE, Scarborough M, Szubert A, Nsutebu E, Tilley R, Greig J, et al. Adjunctive rifampicin for Staphylococcus aureus bacteraemia (ARREST): a multicentre, randomised, double-blind, placebo-controlled trial. Lancet 2018;391:668-678.
[39] Sakr A, Bregeon F, Mege JL, Rolain JM, Blin O. Staphylococcus aureus Nasal Colonization: An Update on Mechanisms, Epidemiology, Risk Factors, and Subsequent Infections. Front Microbiol 2018;9:2419.

[40] van Wamel WJ. Staphylococcus aureus infections, some second thoughts. Curr Opin Infect Dis 2017.
[41] Ryan KJ, Ray CG, Ahmad N, Drew WL, Plorde JJ, Sherris Medical Microbiology, McGraw-Hill Medical, International edition, 5th ed, 2010.
[42] French K, Evans J, Tanner H, Gossain S, Hussain A. The Clinical Impact of Rapid, Direct MALDI-ToF Identification of Bacteria from Positive Blood Cultures. PLoS One 2016;11:e0169332.
[43] van Hal SJ, Jensen SO, Vaska VL, Espedido BA, Paterson DL, Gosbell IB. Predictors of mortality in Staphylococcus aureus Bacteremia. Clin Microbiol Rev 2012;25:362-86.
[44] Laupland KB, Church DL. Population-based epidemiology and microbiology of community-onset bloodstream infections. Clin Microbiol Rev 2014;27:647-64.
[45] DeLeo FR, Diep BA, Otto M. Host defense and pathogenesis in Staphylococcus aureus infections. Infect Dis Clin North Am 2009;23:17-34.
[46] Lowy FD. Antimicrobial resistance: the example of Staphylococcus aureus. J Clin Invest 2003;111:1265-73.
[47] Otto M. Staphylococcus aureus toxins. Curr Opin Microbiol 2014;17:32-7.
[48] Lowy FD. Staphylococcus aureus infections. N Engl J Med 1998;339:520-32.
[49] Adem PV, Montgomery CP, Husain AN, Koogler TK, Arangelovich V, Humilier M, et al. Staphylococcus aureus sepsis and the Waterhouse-Friderichsen syndrome in children. N Engl J Med 2005;353:1245-51.
[50] de Bentzmann S, Tristan A, Etienne J, Brousse N, Vandenesch F, Lina G. Staphylococcus aureus isolates associated with necrotizing pneumonia bind to basement membrane type I and IV collagens and laminin. J Infect Dis 2004;190:1506-15.
[51] Mongkolrattanothai K, Boyle S, Kahana MD, Daum RS. Severe Staphylococcus aureus infections caused by clonally related community-acquired methicillin-susceptible and methicillin-resistant isolates. Clin Infect Dis 2003;37:1050-8.
[52] Suaya JA, Mera RM, Cassidy A, O'Hara P, Amrine-Madsen H, Burstin S, et al. Incidence and cost of hospitalizations associated with Staphylococcus aureus skin and soft tissue infections in the United States from 2001 through 2009. BMC Infect Dis 2014;14:296.
[53] McCormick JK, Yarwood JM, Schlievert PM. Toxic shock syndrome and bacterial superantigens: an update. Annu Rev Microbiol 2001;55:77-104.
[54] Köck R, Becker K, Cookson B, van Gemert-Pijnen JE, Harbarth S, Kluytmans J, et al. Methicillin-resistant Staphylococcus aureus (MRSA): burden of disease and control challenges in Europe. Euro Surveill 2010;15:19688.
[55] Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA 2016;315:801-10.
[56] Sunden-Cullberg J, Rylance R, Svefors J, Norrby-Teglund A, Bjork J, Inghammar M. Fever in the Emergency Department Predicts Survival of Patients With Severe Sepsis and Septic Shock Admitted to the ICU. Crit Care Med 2017;45:591-599.
[57] Stevens DL. Invasive group A streptococcus infections. Clin Infect Dis 1992;14:2-11.
[58] Defining the group A streptococcal toxic shock syndrome. Rationale and consensus definition. The Working Group on Severe Streptococcal Infections. JAMA 1993;269:390-1.
[59] Geller JL, Hackner D. Diffuse venous thromboemboli associated with IVIg therapy in the treatment of streptococcal toxic shock syndrome: case report and review. Ann Hematol 2005;84:601-4.
[60] Davies HD, McGeer A, Schwartz B, Green K, Cann D, Simor AE, et al. Invasive group A streptococcal infections in Ontario, Canada. Ontario Group A Streptococcal Study Group. N Engl J Med 1996;335:547-54.
[61] Zavala S, Arias M, Legua P. Fulminant streptococcal toxic shock syndrome. J R Coll Physicians Edinb 2018;48:33-35.

[62] Arnholm B, Lundqvist A, Strömberg A. High-dose immunoglobulin life-saving in invasive group A streptococcal infection. Report of eleven cases with only one fatality. Lakartidningen 2004.
[63] Shalaby T, Anandappa S, Pocock NJ, Keough A, Turner A. Lesson of the month 2: toxic shock syndrome. Clin Med (Lond) 2014;14:316-8.
[64] Davis JP, Chesney PJ, Wand PJ, LaVenture M. Toxic-shock syndrome: epidemiologic features, recurrence, risk factors, and prevention. N Engl J Med 1980;303:1429-35.
[65] Kotb M. Bacterial pyrogenic exotoxins as superantigens. Clin Microbiol Rev 1995;8:411-26.
[66] Choi YW, Kotzin B, Herron L, Callahan J, Marrack P, Kappler J. Interaction of Staphylococcus aureus toxin "superantigens" with human T cells. Proc Natl Acad Sci U S A 1989;86:8941-8945.
[67] Marrack P, Kappler J. The staphylococcal enterotoxins and their relatives. Science 1990;248:705-11.
[68] Webb SR, Gascoigne NR. T-cell activation by superantigens. Curr Opin Immunol 1994;6:467-75.
[69] Li H, Llera A, Malchiodi EL, Mariuzza RA. The structural basis of T cell activation by superantigens. Annu Rev Immunol 1999;17:435-66.
[70] Lappin E, Ferguson AJ. Gram-positive toxic shock syndromes. Lancet Infect Dis 2009;9:281-90.
[71] Commons RJ, Smeesters PR, Proft T, Fraser JD, Robins-Browne R, Curtis N. Streptococcal superantigens: categorization and clinical associations. Trends Mol Med 2014;20:48-62.
[72] Bohach GA, Fast DJ, Nelson RD, Schlievert PM. Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses. Crit Rev Microbiol 1990;17:251-72.
[73] Hackett SP, Stevens DL. Streptococcal toxic shock syndrome: synthesis of tumor necrosis factor and interleukin-1 by monocytes stimulated with pyrogenic exotoxin A and streptolysin O. J Infect Dis 1992;165:879-85.
[74] Chatellier S, Ihendyane N, Kansal RG, Khambaty F, Basma H, Norrby-Teglund A, et al. Genetic relatedness and superantigen expression in group A streptococcus serotype M1 isolates from patients with severe and nonsevere invasive diseases. Infect Immun 2000;68:3523-34.
[75] Norrby-Teglund A, Chatellier S, Low DE, McGeer A, Green K, Kotb M. Host variation in cytokine responses to superantigens determine the severity of invasive group A streptococcal infection. Eur J Immunol 2000;30:3247-55.
[76] Kotb M, Norrby-Teglund A, McGeer A, El-Sherbini H, Dorak MT, Khurshid A, et al. An immunogenetic and molecular basis for differences in outcomes of invasive group A streptococcal infections. Nat Med 2002;8:1398-404.
[77] Nooh MM, Nookala S, Kansal R, Kotb M. Individual genetic variations directly effect polarization of cytokine responses to superantigens associated with streptococcal sepsis: implications for customized patient care. J Immunol 2011;186:3156-63.
[78] Basma H, Norrby-Teglund A, Guedez Y, McGeer A, Low DE, El-Ahmedy O, et al. Risk factors in the pathogenesis of invasive group A streptococcal infections: role of protective humoral immunity. Infect Immun 1999;67:1871-7.
[79] Suntharalingam G, Perry MR, Ward S, Brett SJ, Castello-Cortes A, Brunner MD, et al. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. N Engl J Med 2006;355:1018-28.
[80] Cunningham MW. Pathogenesis of group A streptococcal infections. Clin Microbiol Rev 2000;13:470-511.
[81] Cywes C, Wessels MR. Group A Streptococcus tissue invasion by CD44-mediated cell signalling. Nature 2001;414:648-52.

[82] Bryant AE, Hayes-Schroer SM, Stevens DL. M type 1 and 3 group A streptococci stimulate tissue factor-mediated procoagulant activity in human monocytes and endothelial cells. Infect Immun 2003;71:1903-10.
[83] Horstmann RD, Sievertsen HJ, Knobloch J, Fischetti VA. Antiphagocytic activity of streptococcal M protein: selective binding of complement control protein factor H. Proc Natl Acad Sci U S A 1988;85:1657-61.
[84] Carlsson F, Sandin C, Lindahl G. Human fibrinogen bound to Streptococcus pyogenes M protein inhibits complement deposition via the classical pathway. Mol Microbiol 2005;56:28-39.
[85] Thern A, Stenberg L, Dahlback B, Lindahl G. Ig-binding surface proteins of Streptococcus pyogenes also bind human C4b-binding protein (C4BP), a regulatory component of the complement system. J Immunol 1995;154:375-86.
[86] Herwald H, Cramer H, Mörgelin M, Russell W, Sollenberg U, Norrby-Teglund A, et al. M protein, a classical bacterial virulence determinant, forms complexes with fibrinogen that induce vascular leakage. Cell 2004;116:367-79.
[87] Påhlman LI, Olin AI, Darenberg J, Morgelin M, Kotb M, Herwald H, et al. Soluble M1 protein of Streptococcus pyogenes triggers potent T cell activation. Cell Microbiol 2008;10:404-14.
[88] Aziz RK, Kotb M. Rise and persistence of global M1T1 clone of Streptococcus pyogenes. Emerg Infect Dis 2008;14:1511-7.
[89] Kane TL, Carothers KE, Lee SW. Virulence Factor Targeting of the Bacterial Pathogen Staphylococcus aureus for Vaccine and Therapeutics. Curr Drug Targets 2018;19:111-127.
[90] Goodyear CS, Silverman GJ. Death by a B cell superantigen: In vivo VH-targeted apoptotic supraclonal B cell deletion by a Staphylococcal Toxin. J Exp Med 2003;197:1125-39.
[91] Bhakdi S, Tranum-Jensen J, Sziegoleit A. Mechanism of membrane damage by streptolysin-O. Infect Immun 1985;47:52-60.
[92] Higashi DL, Biais N, Donahue DL, Mayfield JA, Tessier CR, Rodriguez K, et al. Activation of band 3 mediates group A Streptococcus streptolysin S-based beta-haemolysis. Nat Microbiol 2016;1:15004.
[93] Egesten A, Olin AI, Linge HM, Yadav M, Morgelin M, Karlsson A, et al. SpeB of Streptococcus pyogenes differentially modulates antibacterial and receptor activating properties of human chemokines. PLoS One 2009;4:e4769.
[94] Norrby-Teglund A, Kotb M. Host-microbe interactions in the pathogenesis of invasive group A streptococcal infections. J Med Microbiol 2000;49:849-52.
[95] Vandenesch F, Lina G, Henry T. Staphylococcus aureus hemolysins, bi-component leukocidins, and cytolytic peptides: a redundant arsenal of membrane-damaging virulence factors? Front Cell Infect Microbiol 2012;2:12.
[96] Vandenesch F, Naimi T, Enright MC, Lina G, Nimmo GR, Heffernan H, et al. Community-acquired methicillin-resistant Staphylococcus aureus carrying Panton-Valentine leukocidin genes: worldwide emergence. Emerg Infect Dis 2003;9:978-84.
[97] Diep BA, Otto M. The role of virulence determinants in community-associated MRSA pathogenesis. Trends Microbiol 2008;16:361-9.
[98] Olsen RJ, Musser JM. Molecular pathogenesis of necrotizing fasciitis. Annu Rev Pathol 2010;5:1-31.
[99] Schulz A, Jiang L, de Vor L, Ehrström M, Wermeling F, Eidsmo L, et al. Neutrophil Recruitment to Noninvasive MRSA at the Stratum Corneum of Human Skin Mediates Transient Colonization. Cell Rep 2019;29:1074-1081.e5.
[100] Tazi A, Plainvert C, Anselem O, Ballon M, Marcou V, Seco A, et al. Risk Factors for Infant Colonization by Hypervirulent CC17 Group B Streptococcus: Toward the Understanding of Late-onset Disease. Clin Infect Dis 2019;69:1740-1748.

[101] Reglinski M, Lynskey NN, Choi YJ, Edwards RJ, Sriskandan S. Development of a multicomponent vaccine for Streptococcus pyogenes based on the antigenic targets of IVIG. J Infect 2016;72:450-9.
[102] Lancefield RC. A Serological Differentiation of Human and Other Groups of Hemolytic Streptococci. J Exp Med 1933;57:571-95.
[103] McNeil SA, Halperin SA, Langley JM, Smith B, Warren A, Sharratt GP, et al. Safety and immunogenicity of 26-valent group a streptococcus vaccine in healthy adult volunteers. Clin Infect Dis 2005;41:1114-22.
[104] Olaniyi R, Pozzi C, Grimaldi L, Bagnoli F. Staphylococcus aureus-Associated Skin and Soft Tissue Infections: Anatomical Localization, Epidemiology, Therapy and Potential Prophylaxis. Curr Top Microbiol Immunol 2017;409:199-227.
[105] Giersing BK, Dastgheyb SS, Modjarrad K, Moorthy V. Status of vaccine research and development of vaccines for Staphylococcus aureus. Vaccine 2016;34:2962-2966.
[106] Stegmiller NP, Barcelos EC, Leal JM, Covre LP, Donatele DM, de Matos Guedes HL, et al. Intranasal vaccination with adjuvant-free S. aureus antigens effectively protects mice against experimental sepsis. Vaccine 2016;34:3493-9.
[107] Antimicrobial Resistance: Global Report on Surveillance. Available at: http://apps.who.int/iris/bitstream/10665/112642/1/9789241564748_eng.pdf?ua=1.
[108] Cassini A, Hogberg LD, Plachouras D, Quattrocchi A, Hoxha A, Simonsen GS, et al. Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. Lancet Infect Dis 2018.
[109] Kaarme J, Riedel H, Schaal W, Yin H, Nevéus T, Melhus Å. Rapid Increase in Carriage Rates of Enterobacteriaceae Producing Extended-Spectrum β-Lactamases in Healthy Preschool Children, Sweden. Emerg Infect Dis 2018;24:1874-1881.
[110] Zwart S, Sachs AP, Ruijs GJ, Gubbels JW, Hoes AW, de Melker RA. Penicillin for acute sore throat: randomised double blind trial of seven days versus three days treatment or placebo in adults. BMJ 2000;320:150-4.
[111] Vannice KS, Ricaldi J, Nanduri S, Fang FC, Lynch JB, Bryson-Cahn C, et al. Streptococcus pyogenes pbp2x Mutation Confers Reduced Susceptibility to beta-Lactam Antibiotics. Clin Infect Dis 2020;71:201-204.
[112] Yang P, Peng X, Zhang D, Wu S, Liu Y, Cui S, et al. Characteristics of group A Streptococcus strains circulating during scarlet fever epidemic, Beijing, China, 2011. Emerg Infect Dis 2013;19:909-15.
[113] Montes M, Tamayo E, Orden B, Larruskain J, Perez-Trallero E. Prevalence and clonal characterization of Streptococcus pyogenes clinical isolates with reduced fluoroquinolone susceptibility in Spain. Antimicrob Agents Chemother 2010;54:93-7.
[114] Ayer V, Tewodros W, Manoharan A, Skariah S, Luo F, Bessen DE. Tetracycline resistance in group a streptococci: emergence on a global scale and influence on multiple-drug resistance. Antimicrob Agents Chemother 2007;51:1865-8.
[115] Thulin P, Johansson L, Low DE, Gan BS, Kotb M, McGeer A, et al. Viable group A streptococci in macrophages during acute soft tissue infection. PLoS Med 2006;3:e53.
[116] Carapetis JR, Jacoby P, Carville K, Ang SJ, Curtis N, Andrews R. Effectiveness of clindamycin and intravenous immunoglobulin, and risk of disease in contacts, in invasive group a streptococcal infections. Clin Infect Dis 2014;59:358-65.
[117] Andreoni F, Zurcher C, Tarnutzer A, Schilcher K, Neff A, Keller N, et al. Clindamycin Affects Group A Streptococcus Virulence Factors and Improves Clinical Outcome. J Infect Dis 2017;215:269-277.
[118] Eagle H. The paradoxically retarded bactericidal activity of penicillin at high concentrations in vitro and in vivo. J Clin Invest 1948;27:531.

[119] Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis 2018;18:318-327.
[120] Gould IM, Cauda R, Esposito S, Gudiol F, Mazzei T, Garau J. Management of serious meticillin-resistant Staphylococcus aureus infections: what are the limits? Int J Antimicrob Agents 2011;37:202-9.
[121] Otto M. Basis of virulence in community-associated methicillin-resistant Staphylococcus aureus. Annu Rev Microbiol 2010;64:143-62.
[122] Soriano A, Martinez JA, Mensa J, Marco F, Almela M, Moreno-Martinez A, et al. Pathogenic significance of methicillin resistance for patients with Staphylococcus aureus bacteremia. Clin Infect Dis 2000;30:368-73.
[123] Choo EJ, Chambers HF. Treatment of Methicillin-Resistant Staphylococcus aureus Bacteremia. Infect Chemother 2016;48:267-273.
[124] Katayama Y, Ito T, Hiramatsu K. A new class of genetic element, staphylococcus cassette chromosome mec, encodes methicillin resistance in Staphylococcus aureus. Antimicrob Agents Chemother 2000;44:1549-55.
[125] Georgopapadakou NH, Smith SA, Bonner DP. Penicillin-binding proteins in a Staphylococcus aureus strain resistant to specific beta-lactam antibiotics. Antimicrob Agents Chemother 1982;22:172-5.
[126] Gideskog M, Melhus Å. Outbreak of Methicillin-resistant Staphylococcus aureus in a Hospital Center for Children's and Women's Health in a Swedish County. APMIS 2019;127:181-186.
[127] Bengtsson B, Persson L, Ekström K, Unnerstad HE, Uhlhorn H, Börjesson S. High occurrence of mecC-MRSA in wild hedgehogs (Erinaceus europaeus) in Sweden. Vet Microbiol 2017;207:103-107.
[128] Wiklund S, Örtqvist Å, Berlin A, Stamm C, Broliden K. Experiences and consequences of living with extended-spectrum β-lactamase-producing bacteria: A qualitative study. Am J Infect Control 2018;46:1394-1399.
[129] Russo A, Concia E, Cristini F, De Rosa FG, Esposito S, Menichetti F, et al. Current and future trends in antibiotic therapy of acute bacterial skin and skin-structure infections. Clin Microbiol Infect 2016;22 Suppl 2:S27-36.
[130] Gonzalez-Ruiz A, Seaton RA, Hamed K. Daptomycin: an evidence-based review of its role in the treatment of Gram-positive infections. Infect Drug Resist 2016;9:47-58.
[131] Huemer M, Mairpady Shambat S, Brugger SD, Zinkernagel AS. Antibiotic resistance and persistence-Implications for human health and treatment perspectives. EMBO Rep 2020;21:e51034.
[132] Mairpady Shambat S, Chen P, Nguyen Hoang AT, Bergsten H, Vandenesch F, Siemens N, et al. Modelling staphylococcal pneumonia in a human 3D lung tissue model system delineates toxin-mediated pathology. Dis Model Mech 2015;8:1413-25.
[133] Lina G, Piemont Y, Godail-Gamot F, Bes M, Peter MO, Gauduchon V, et al. Involvement of Panton-Valentine leukocidin-producing Staphylococcus aureus in primary skin infections and pneumonia. Clin Infect Dis 1999;29:1128-32.
[134] Gillet Y, Issartel B, Vanhems P, Fournet JC, Lina G, Bes M, et al. Association between Staphylococcus aureus strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. Lancet 2002;359:753-9.
[135] Voyich JM, Otto M, Mathema B, Braughton KR, Whitney AR, Welty D, et al. Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant Staphylococcus aureus disease? J Infect Dis 2006;194:1761-70.
[136] Jayaraman R. Bacterial persistence: some new insights into an old phenomenon. J Biosci 2008;33:795-805.

[137] Maisonneuve E, Gerdes K. Molecular mechanisms underlying bacterial persisters. Cell 2014;157:539-48.
[138] Peng C, Tao X, Wenhong Z, Ying Z. Molecular mechanisms of bacterial persistence and phenotypic antibiotic resistance. Yi Chuan 2016;38:859-871.
[139] Staali L, Bauer S, Morgelin M, Bjorck L, Tapper H. Streptococcus pyogenes bacteria modulate membrane traffic in human neutrophils and selectively inhibit azurophilic granule fusion with phagosomes. Cell Microbiol 2006;8:690-703.
[140] Staali L, Morgelin M, Bjorck L, Tapper H. Streptococcus pyogenes expressing M and M-like surface proteins are phagocytosed but survive inside human neutrophils. Cell Microbiol 2003;5:253-65.
[141] Hertzen E, Johansson L, Wallin R, Schmidt H, Kroll M, Rehn AP, et al. M1 protein-dependent intracellular trafficking promotes persistence and replication of Streptococcus pyogenes in macrophages. J Innate Immun 2010;2:534-45.
[142] Molinari G, Talay SR, Valentin-Weigand P, Rohde M, Chhatwal GS. The fibronectin-binding protein of Streptococcus pyogenes, SfbI, is involved in the internalization of group A streptococci by epithelial cells. Infect Immun 1997;65:1357-63.
[143] Greco R, De Martino L, Donnarumma G, Conte MP, Seganti L, Valenti P. Invasion of cultured human cells by Streptococcus pyogenes. Res Microbiol 1995;146:551-60.
[144] Proctor RA, von Eiff C, Kahl BC, Becker K, McNamara P, Herrmann M, et al. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat Rev Microbiol 2006;4:295-305.
[145] Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol 2004;2:95-108.
[146] Paharik AE, Horswill AR. The Staphylococcal Biofilm: Adhesins, Regulation, and Host Response. Microbiol Spectr 2016;4.
[147] McCarthy H, Rudkin JK, Black NS, Gallagher L, O'Neill E, O'Gara JP. Methicillin resistance and the biofilm phenotype in Staphylococcus aureus. Front Cell Infect Microbiol 2015;5:1.
[148] Waters EM, Rowe SE, O'Gara JP, Conlon BP. Convergence of Staphylococcus aureus Persister and Biofilm Research: Can Biofilms Be Defined as Communities of Adherent Persister Cells? PLoS Pathog 2016;12:e1006012.
[149] Revest M, Jacqueline C, Boudjemaa R, Caillon J, Le Mabecque V, Breteche A, et al. New in vitro and in vivo models to evaluate antibiotic efficacy in Staphylococcus aureus prosthetic vascular graft infection. J Antimicrob Chemother 2016;71:1291-9.
[150] Pletzer D, Hancock RE. Antibiofilm Peptides: Potential as Broad-Spectrum Agents. J Bacteriol 2016;198:2572-8.
[151] Bhattacharya M, Wozniak DJ, Stoodley P, Hall-Stoodley L. Prevention and treatment of Staphylococcus aureus biofilms. Expert Rev Anti Infect Ther 2015;13:1499-516.
[152] Lembke C, Podbielski A, Hidalgo-Grass C, Jonas L, Hanski E, Kreikemeyer B. Characterization of biofilm formation by clinically relevant serotypes of group A streptococci. Appl Environ Microbiol 2006;72:2864-75.
[153] Connolly KL, Roberts AL, Holder RC, Reid SD. Dispersal of Group A streptococcal biofilms by the cysteine protease SpeB leads to increased disease severity in a murine model. PLoS One 2011;6:e18984.
[154] Siemens N, Chakrakodi B, Shambat SM, Morgan M, Bergsten H, Hyldegaard O, et al. Biofilm in group A streptococcal necrotizing soft tissue infections. JCI Insight 2016;1:e87882.
[155] Baldassarri L, Creti R, Recchia S, Imperi M, Facinelli B, Giovanetti E, et al. Therapeutic failures of antibiotics used to treat macrolide-susceptible Streptococcus pyogenes infections may be due to biofilm formation. J Clin Microbiol 2006;44:2721-7.
[156] Rutherford ST, Bassler BL. Bacterial quorum sensing: its role in virulence and possibilities for its control. Cold Spring Harb Perspect Med 2012;2.

[157] Castillo-Juarez I, Maeda T, Mandujano-Tinoco EA, Tomas M, Perez-Eretza B, Garcia-Contreras SJ, et al. Role of quorum sensing in bacterial infections. World J Clin Cases 2015;3:575-98.
[158] Le KY, Otto M. Quorum-sensing regulation in staphylococci-an overview. Front Microbiol 2015;6:1174.
[159] Tegmark K, Morfeldt E, Arvidson S. Regulation of agr-dependent virulence genes in Staphylococcus aureus by RNAIII from coagulase-negative staphylococci. J Bacteriol 1998;180:3181-6.
[160] Cheung GY, Wang R, Khan BA, Sturdevant DE, Otto M. Role of the accessory gene regulator agr in community-associated methicillin-resistant Staphylococcus aureus pathogenesis. Infect Immun 2011;79:1927-35.
[161] Jimenez JC, Federle MJ. Quorum sensing in group A Streptococcus. Front Cell Infect Microbiol 2014;4:127.
[162] Lamb LE, Sriskandan S, Tan LK. Bromine, bear-claw scratch fasciotomies, and the Eagle effect: management of group A streptococcal necrotising fasciitis and its association with trauma. Lancet Infect Dis 2015;15:109-21.
[163] Hansen MB, Rasmussen LS, Garred P, Bidstrup D, Madsen MB, Hyldegaard O. Pentraxin-3 as a marker of disease severity and risk of death in patients with necrotizing soft tissue infections: a nationwide, prospective, observational study. Crit Care 2016;20:40.
[164] Brummelkamp WH. The importance of administration of oxygen under atmospheric positive pressure in the treatment of gas phlegmon. Ned Tijdschr Geneeskd 1961;105:2430-2.
[165] Park MK, Myers RA, Marzella L. Oxygen tensions and infections: modulation of microbial growth, activity of antimicrobial agents, and immunologic responses. Clin Infect Dis 1992;14:720-40.
[166] Kolpen M, Mousavi N, Sams T, Bjarnsholt T, Ciofu O, Moser C, et al. Reinforcement of the bactericidal effect of ciprofloxacin on Pseudomonas aeruginosa biofilm by hyperbaric oxygen treatment. Int J Antimicrob Agents 2016;47:163-7.
[167] Grimberg-Peters D, Buren C, Windolf J, Wahlers T, Paunel-Gorgulu A. Hyperbaric Oxygen Reduces Production of Reactive Oxygen Species in Neutrophils from Polytraumatized Patients Yielding in the Inhibition of p38 MAP Kinase and Downstream Pathways. PLoS One 2016;11:e0161343.
[168] Kaye D. Effect of hyperbaric oxygen on aerobic bacteria in vitro and in vivo. Proc Soc Exp Biol Med 1967;124:1090-3.
[169] Devaney B, Frawley G, Frawley L, Pilcher DV. Necrotising soft tissue infections: the effect of hyperbaric oxygen on mortality. Anaesth Intensive Care 2015;43:685-92.
[170] Elander J, Nekludov M, Larsson A, Nordlander B, Eksborg S, Hydman J. Cervical necrotizing fasciitis: descriptive, retrospective analysis of 59 cases treated at a single center. Eur Arch Otorhinolaryngol 2016.
[171] Escobar SJ, Slade JB, Jr., Hunt TK, Cianci P. Adjuvant hyperbaric oxygen therapy (HBO2)for treatment of necrotizing fasciitis reduces mortality and amputation rate. Undersea Hyperb Med 2005;32:437-43.
[172] Hansen MB, Rasmussen LS, Svensson M, Chakrakodi B, Bruun T, Madsen MB, et al. Association between cytokine response, the LRINEC score and outcome in patients with necrotising soft tissue infection: a multicentre, prospective study. Sci Rep 2017;7:42179.
[173] Jorgensen NP, Hansen K, Andreasen CM, Pedersen M, Fuursted K, Meyer RL, et al. Hyperbaric Oxygen Therapy is Ineffective as an Adjuvant to Daptomycin with Rifampicin Treatment in a Murine Model of Staphylococcus aureus in Implant-Associated Osteomyelitis. Microorganisms 2017;5.
[174] Massey PR, Sakran JV, Mills AM, Sarani B, Aufhauser DD, Jr., Sims CA, et al. Hyperbaric oxygen therapy in necrotizing soft tissue infections. J Surg Res 2012;177:146-51.

[175] Shupak A, Shoshani O, Goldenberg I, Barzilai A, Moskuna R, Bursztein S. Necrotizing fasciitis: an indication for hyperbaric oxygenation therapy? Surgery 1995;118:873-8.
[176] Levett D, Bennett MH, Millar I. Adjunctive hyperbaric oxygen for necrotizing fasciitis. Cochrane Database Syst Rev 2015;1:Cd007937.
[177] Buckley RH, Schiff RI. The use of intravenous immune globulin in immunodeficiency diseases. N Engl J Med 1991;325:110-7.
[178] Norrby-Teglund A, Muller MP, McGeer A, Gan BS, Guru V, Bohnen J, et al. Successful management of severe group A streptococcal soft tissue infections using an aggressive medical regimen including intravenous polyspecific immunoglobulin together with a conservative surgical approach. Scand J Infect Dis 2005;37:166-72.
[179] Newburger JW, Takahashi M, Burns JC, Beiser AS, Chung KJ, Duffy CE, et al. The treatment of Kawasaki syndrome with intravenous gamma globulin. N Engl J Med 1986;315:341-7.
[180] Kaveri SV, Mouthon L, Kazatchkine MD. Immunomodulating effects of intravenous immunoglobulin in autoimmune and inflammatory diseases. J Neurol Neurosurg Psychiatry 1994;57 Suppl:6-8.
[181] Hall PD. Immunomodulation with intravenous immunoglobulin. Pharmacotherapy 1993;13:564-73.
[182] Ogawa M, Ueda S, Anzai N, Ito K, Ohto M. Toxic shock syndrome after staphylococcal pneumonia treated with intravenous immunoglobulin. Vox Sang 1995;68:59-60.
[183] Basma H, Norrby-Teglund A, McGeer A, Low DE, El-Ahmedy O, Dale JB, et al. Opsonic antibodies to the surface M protein of group A streptococci in pooled normal immunoglobulins (IVIG): potential impact on the clinical efficacy of IVIG therapy for severe invasive group A streptococcal infections. Infect Immun 1998;66:2279-83.
[184] Sriskandan S, Ferguson M, Elliot V, Faulkner L, Cohen J. Human intravenous immunoglobulin for experimental streptococcal toxic shock: bacterial clearance and modulation of inflammation. J Antimicrob Chemother 2006;58:117-24.
[185] Norrby-Teglund A, Kaul R, Low DE, McGeer A, Andersson J, Andersson U, et al. Evidence for the presence of streptococcal-superantigen-neutralizing antibodies in normal polyspecific immunoglobulin G. Infect Immun 1996;64:5395-8.
[186] Kaul R, McGeer A, Norrby-Teglund A, Kotb M, Schwartz B, O'Rourke K, et al. Intravenous immunoglobulin therapy for streptococcal toxic shock syndrome--a comparative observational study. The Canadian Streptococcal Study Group. Clin Infect Dis 1999;28:800-7.
[187] Darenberg J, Ihendyane N, Sjölin J, Aufwerber E, Haidl S, Follin P, et al. Intravenous immunoglobulin G therapy in streptococcal toxic shock syndrome: a European randomized, double-blind, placebo-controlled trial. Clin Infect Dis 2003;37:333-40.
[188] Mairpady Shambat S, Haggar A, Vandenesch F, Lina G, van Wamel WJ, Arakere G, et al. Levels of alpha-toxin correlate with distinct phenotypic response profiles of blood mononuclear cells and with agr background of community-associated Staphylococcus aureus isolates. PLoS One 2014;9:e106107.
[189] Linner A, Darenberg J, Sjolin J, Henriques-Normark B, Norrby-Teglund A. Clinical efficacy of polyspecific intravenous immunoglobulin therapy in patients with streptococcal toxic shock syndrome: a comparative observational study. Clin Infect Dis 2014;59:851-7.
[190] Adalat S, Dawson T, Hackett SJ, Clark JE, In association with the British Paediatric Surveillance U. Toxic shock syndrome surveillance in UK children. Arch Dis Child 2014;99:1078-82.
[191] Cavazzuti I, Serafini G, Busani S, Rinaldi L, Biagioni E, Buoncristiano M, et al. Early therapy with IgM-enriched polyclonal immunoglobulin in patients with septic shock. Intensive Care Med 2014;40:1888-96.

[192] Mehta S, McGeer A, Low DE, Hallett D, Bowman DJ, Grossman SL, et al. Morbidity and mortality of patients with invasive group A streptococcal infections admitted to the ICU. Chest 2006;130:1679-86.
[193] Kadri SS, Swihart BJ, Bonne SL, Hohmann SF, Hennessy LV, Louras P, et al. Impact of Intravenous Immunoglobulin on Survival in Necrotizing Fasciitis With Vasopressor-Dependent Shock: A Propensity Score-Matched Analysis From 130 US Hospitals. Clin Infect Dis 2017;64:877-885.
[194] Valiquette L, Low DE, Chow R, McGeer AJ. A survey of physician's attitudes regarding management of severe group A streptococcal infections. Scand J Infect Dis 2006;38:977-82.
[195] Parks T, Wilson C, Curtis N, Norrby-Teglund A, Sriskandan S. Polyspecific intravenous immunoglobulin in clindamycin-treated patients with streptococcal toxic shock syndrome: a systematic review and meta-analysis. Clin Infect Dis 2018.
[196] Norrby-Teglund A, Haque KN, Hammarstrom L. Intravenous polyclonal IgM-enriched immunoglobulin therapy in sepsis: a review of clinical efficacy in relation to microbiological aetiology and severity of sepsis. J Intern Med 2006;260:509-16.
[197] Hua L, Hilliard JJ, Shi Y, Tkaczyk C, Cheng LI, Yu X, et al. Assessment of an anti-alpha-toxin monoclonal antibody for prevention and treatment of Staphylococcus aureus-induced pneumonia. Antimicrob Agents Chemother 2014;58:1108-17.
[198] Yu XQ, Robbie GJ, Wu Y, Esser MT, Jensen K, Schwartz HI, et al. Safety, Tolerability, and Pharmacokinetics of MEDI4893, an Investigational, Extended-Half-Life, Anti-Staphylococcus aureus Alpha-Toxin Human Monoclonal Antibody, in Healthy Adults. Antimicrob Agents Chemother 2017;61.
[199] François B, Jafri HS, Chastre J, Sánchez-García M, Eggimann P, Dequin PF, et al. Efficacy and safety of suvratoxumab for prevention of Staphylococcus aureus ventilator-associated pneumonia (SAATELLITE): a multicentre, randomised, double-blind, placebo-controlled, parallel-group, phase 2 pilot trial. Lancet Infect Dis 2021;21:1313-1323.
[200] Kennedy AD, Bubeck Wardenburg J, Gardner DJ, Long D, Whitney AR, Braughton KR, et al. Targeting of alpha-hemolysin by active or passive immunization decreases severity of USA300 skin infection in a mouse model. J Infect Dis 2010;202:1050-8.
[201] Rouha H, Badarau A, Visram ZC, Battles MB, Prinz B, Magyarics Z, et al. Five birds, one stone: neutralization of α-hemolysin and 4 bi-component leukocidins of Staphylococcus aureus with a single human monoclonal antibody. MAbs 2015;7:243-54.
[202] Caballero AR, Foletti DL, Bierdeman MA, Tang A, Arana AM, Hasa-Moreno A, et al. Effectiveness of Alpha-toxin Fab Monoclonal Antibody Therapy in Limiting the Pathology of Staphylococcus aureus Keratitis. Ocul Immunol Inflamm 2015;23:297-303.
[203] Qiu J, Niu X, Dong J, Wang D, Wang J, Li H, et al. Baicalin protects mice from Staphylococcus aureus pneumonia via inhibition of the cytolytic activity of α-hemolysin. J Infect Dis 2012;206:292-301.
[204] Ulloa E, Uchiyama S, Gillespie R, Nizet V, Sakoulas G. Ticagrelor Increases Platelet-Mediated Staphylococcus aureus Killing Resulting in Clearance of Bacteremia. J Infect Dis 2021.
[205] Wang R, Khan BA, Cheung GY, Bach TH, Jameson-Lee M, Kong KF, et al. Staphylococcus epidermidis surfactant peptides promote biofilm maturation and dissemination of biofilm-associated infection in mice. J Clin Invest 2011;121:238-48.
[206] Chatterjee SS, Joo HS, Duong AC, Dieringer TD, Tan VY, Song Y, et al. Essential Staphylococcus aureus toxin export system. Nat Med 2013;19:364-7.
[207] Kim HK, Cheng AG, Kim HY, Missiakas DM, Schneewind O. Nontoxigenic protein A vaccine for methicillin-resistant Staphylococcus aureus infections in mice. J Exp Med 2010;207:1863-70.

[208] Shokri R, Salouti M, Zanjani RS. Anti protein A antibody-gold nanorods conjugate: a targeting agent for selective killing of methicillin resistant Staphylococcus aureus using photothermal therapy method. J Microbiol 2015;53:116-21.
[209] Tseng CW, Biancotti JC, Berg BL, Gate D, Kolar SL, Müller S, et al. Increased Susceptibility of Humanized NSG Mice to Panton-Valentine Leukocidin and Staphylococcus aureus Skin Infection. PLoS Pathog 2015;11:e1005292.
[210] Bavari S, Ulrich RG, LeClaire RD. Cross-reactive antibodies prevent the lethal effects of Staphylococcus aureus superantigens. J Infect Dis 1999;180:1365-9.
[211] Varshney AK, Wang X, Cook E, Dutta K, Scharff MD, Goger MJ, et al. Generation, characterization, and epitope mapping of neutralizing and protective monoclonal antibodies against staphylococcal enterotoxin B-induced lethal shock. J Biol Chem 2011;286:9737-47.
[212] Liu CI, Liu GY, Song Y, Yin F, Hensler ME, Jeng WY, et al. A cholesterol biosynthesis inhibitor blocks Staphylococcus aureus virulence. Science 2008;319:1391-4.
[213] Fadlallah A, Chelala E, Legeais JM. Corneal Infection Therapy with Topical Bacteriophage Administration. Open Ophthalmol J 2015;9:167-8.
[214] Mattila S, Ruotsalainen P, Jalasvuori M. On-Demand Isolation of Bacteriophages Against Drug-Resistant Bacteria for Personalized Phage Therapy. Front Microbiol 2015;6:1271.
[215] Kaur S, Harjai K, Chhibber S. In Vivo Assessment of Phage and Linezolid Based Implant Coatings for Treatment of Methicillin Resistant S. aureus (MRSA) Mediated Orthopaedic Device Related Infections. PLoS One 2016;11:e0157626.
[216] Hibbitts A, O'Leary C. Emerging Nanomedicine Therapies to Counter the Rise of Methicillin-Resistant Staphylococcus aureus. Materials (Basel) 2018;11.
[217] Singh R, Lillard JW, Jr. Nanoparticle-based targeted drug delivery. Exp Mol Pathol 2009;86:215-23.
[218] Wong IY, Bhatia SN, Toner M. Nanotechnology: emerging tools for biology and medicine. Genes Dev 2013;27:2397-408.
[219] Chen Z, Meng H, Xing G, Chen C, Zhao Y, Jia G, et al. Acute toxicological effects of copper nanoparticles in vivo. Toxicol Lett 2006;163:109-20.
[220] Han HM, Gopal R, Park Y. Design and membrane-disruption mechanism of charge-enriched AMPs exhibiting cell selectivity, high-salt resistance, and anti-biofilm properties. Amino Acids 2016;48:505-22.
[221] Yanagihara K, Tashiro M, Fukuda Y, Ohno H, Higashiyama Y, Miyazaki Y, et al. Effects of short interfering RNA against methicillin-resistant Staphylococcus aureus coagulase in vitro and in vivo. J Antimicrob Chemother 2006;57:122-6.
[222] Bikard D, Euler CW, Jiang W, Nussenzweig PM, Goldberg GW, Duportet X, et al. Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials. Nat Biotechnol 2014;32:1146-50.
[223] Thangamani S, Mohammad H, Younis W, Seleem MN. Drug repurposing for the treatment of staphylococcal infections. Curr Pharm Des 2015;21:2089-100.
[224] Jarmila V, Vavríková E. Chitosan derivatives with antimicrobial, antitumour and antioxidant activities--a review. Curr Pharm Des 2011;17:3596-607.
[225] Kong M, Chen XG, Xing K, Park HJ. Antimicrobial properties of chitosan and mode of action: a state of the art review. Int J Food Microbiol 2010;144:51-63.
[226] Pattni BS, Chupin VV, Torchilin VP. New Developments in Liposomal Drug Delivery. Chem Rev 2015;115:10938-66.
[227] Mohammadi-Samani S, Taghipour B. PLGA micro and nanoparticles in delivery of peptides and proteins; problems and approaches. Pharm Dev Technol 2015;20:385-93.
[228] Chen Y, Chen M, Zhang Y, Lee JH, Escajadillo T, Gong H, et al. Broad-Spectrum Neutralization of Pore-Forming Toxins with Human Erythrocyte Membrane-Coated Nanosponges. Adv Healthc Mater 2018;7:e1701366.

[229] Betschel SD, Borgia SM, Barg NL, Low DE, De Azavedo JC. Reduced virulence of group A streptococcal Tn916 mutants that do not produce streptolysin S. Infect Immun 1998;66:1671-9.
[230] Datta V, Myskowski SM, Kwinn LA, Chiem DN, Varki N, Kansal RG, et al. Mutational analysis of the group A streptococcal operon encoding streptolysin S and its virulence role in invasive infection. Mol Microbiol 2005;56:681-95.
[231] Dale JB, Chiang EY, Hasty DL, Courtney HS. Antibodies against a synthetic peptide of SagA neutralize the cytolytic activity of streptolysin S from group A streptococci. Infect Immun 2002;70:2166-70.
[232] Pinho-Ribeiro FA, Baddal B, Haarsma R, O'Seaghdha M, Yang NJ, Blake KJ, et al. Blocking Neuronal Signaling to Immune Cells Treats Streptococcal Invasive Infection. Cell 2018;173:1083-1097.e22.
[233] Kachroo P, Eraso JM, Olsen RJ, Zhu L, Kubiak SL, Pruitt L, et al. New Pathogenesis Mechanisms and Translational Leads Identified by Multidimensional Analysis of Necrotizing Myositis in Primates. mBio 2020;11.
[234] Xu Y, Ma Y, Sun H. A novel approach to develop anti-virulence agents against group A streptococcus. Virulence 2012;3:452-3.
[235] Wójcik M, Eleftheriadis N, Zwinderman MRH, Dömling ASS, Dekker FJ, Boersma YL. Identification of potential antivirulence agents by substitution-oriented screening for inhibitors of Streptococcus pyogenes sortase A. Eur J Med Chem 2019;161:93-100.
[236] Sun H, Ringdahl U, Homeister JW, Fay WP, Engleberg NC, Yang AY, et al. Plasminogen is a critical host pathogenicity factor for group A streptococcal infection. Science 2004;305:1283-6.
[237] McArthur JD, Cook SM, Venturini C, Walker MJ. The role of streptokinase as a virulence determinant of Streptococcus pyogenes--potential for therapeutic targeting. Curr Drug Targets 2012;13:297-307.
[238] Sun H, Xu Y, Sitkiewicz I, Ma Y, Wang X, Yestrepsky BD, et al. Inhibitor of streptokinase gene expression improves survival after group A streptococcus infection in mice. Proc Natl Acad Sci U S A 2012;109:3469-74.
[239] Valliammai A, Selvaraj A, Sangeetha M, Sethupathy S, Pandian SK. 5-Dodecanolide inhibits biofilm formation and virulence of Streptococcus pyogenes by suppressing core regulons of virulence. Life Sci 2020;262:118554.
[240] Nandu TG, Subramenium GA, Shiburaj S, Viszwapriya D, Iyer PM, Balamurugan K, et al. Fukugiside, a biflavonoid from Garcinia travancorica inhibits biofilm formation of Streptococcus pyogenes and its associated virulence factors. J Med Microbiol 2018;67:1391-1401.
[241] Hynes AP, Rousseau GM, Lemay ML, Horvath P, Romero DA, Fremaux C, et al. An anti-CRISPR from a virulent streptococcal phage inhibits Streptococcus pyogenes Cas9. Nat Microbiol 2017;2:1374-1380.
[242] Tu Y, Jia X, Yang R, Peng X, Zhou X, Xu X. Genetic Regulation of Streptococci by Small RNAs. Curr Issues Mol Biol 2019;32:39-86.
[243] Madsen MB, Hjortrup PB, Hansen MB, Lange T, Norrby-Teglund A, Hyldegaard O, et al. Immunoglobulin G for patients with necrotising soft tissue infection (INSTINCT): a randomised, blinded, placebo-controlled trial. Intensive Care Med 2017;43:1585-1593.
[244] Darenberg J, Ihendyane N, Sjölin J, Aufwerber E, Haidl S, Follin P, et al. Intravenous immunoglobulin G therapy in streptococcal toxic shock syndrome - an European randomized double-blind placebo-controlled trial. Clin. Infect. Dis. 2003;37:333-340.
[245] Svensson M, Chen P. Human Organotypic Respiratory Models. Curr Top Microbiol Immunol 2018.
[246] Loffler B, Hussain M, Grundmeier M, Bruck M, Holzinger D, Varga G, et al. Staphylococcus aureus panton-valentine leukocidin is a very potent cytotoxic factor for human neutrophils. PLoS Pathog 2010;6:e1000715.

[247] Nguyen Hoang AT, Chen P, Juarez J, Sachamitr P, Billing B, Bosnjak L, et al. Dendritic cell functional properties in a three-dimensional tissue model of human lung mucosa. Am J Physiol Lung Cell Mol Physiol 2012;302:L226-37.
[248] Chella Krishnan K, Mukundan S, Alagarsamy J, Hur J, Nookala S, Siemens N, et al. Genetic Architecture of Group A Streptococcal Necrotizing Soft Tissue Infections in the Mouse. PLoS Pathog 2016;12:e1005732.
[249] Siemens N, Kittang BR, Chakrakodi B, Oppegaard O, Johansson L, Bruun T, et al. Increased cytotoxicity and streptolysin O activity in group G streptococcal strains causing invasive tissue infections. Sci Rep 2015;5:16945.
[250] Nguyen Hoang AT, Chen P, Bjornfot S, Hogstrand K, Lock JG, Grandien A, et al. Technical advance: live-imaging analysis of human dendritic cell migrating behavior under the influence of immune-stimulating reagents in an organotypic model of lung. J Leukoc Biol 2014;96:481-9.
[251] Performance standards for antimicrobial susceptibility testing, Clinical and Laboratory Standards Institute, Twenty-second informational supplement. Document M100-S22, CLSI, Wayne, PA, 2012.
[252] Media preparation for EUCAST disk diffusion testing and for determination of MIC values by the broth microdilution method, Version 6.0, January 2020. Available at: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/2020_manuals/Media_preparation_v_6.0_EUCAST_AST.pdf.
[253] Madsen MB, Hjortrup PB, Hansen MB, Lange T, Norrby-Teglund A, Hyldegaard O, et al. Immunoglobulin G for patients with necrotising soft tissue infection (INSTINCT): a randomised, blinded, placebo-controlled trial. Intensive Care Med 2017.
[254] Bruun T, Rath E, Madsen MB, Oppegaard O, Nekludov M, Arnell P, et al. Risk Factors and Predictors of Mortality in Streptococcal Necrotizing Soft-tissue Infections: A Multicenter Prospective Study. Clin Infect Dis 2021;72:293-300.
[255] Peetermans M, Wan RYY, Camporota L, Barrett NA, Retter A. Use of Intravenous Immunoglobulins in Patients with Suspected Toxin-Mediated Shock Requiring Extracorporeal Membrane Oxygenation. Shock 2020;54:209-212.
[256] Tarnutzer A, Andreoni F, Keller N, Zurcher C, Norrby-Teglund A, Schupbach RA, et al. Human polyspecific immunoglobulin attenuates group A streptococcal virulence factor activity and reduces disease severity in a murine necrotizing fasciitis model. Clin Microbiol Infect 2019;25:512 e7-512 e13.
[257] Zimmerli W, Sendi P. Role of Rifampin against Staphylococcal Biofilm Infections In Vitro, in Animal Models, and in Orthopedic-Device-Related Infections. Antimicrob Agents Chemother 2019;63.
[258] Mairpady Shambat S, Siemens N, Monk IR, Mohan DB, Mukundan S, C. KK, et al. A point mutation in AgrC determines cytotoxic or colonizing properties associated with phenotypic variants of ST22 MRSA strains. Sci Rep 2016;11:31360.
[259] Sully EK, Malachowa N, Elmore BO, Alexander SM, Femling JK, Gray BM, et al. Selective chemical inhibition of agr quorum sensing in Staphylococcus aureus promotes host defense with minimal impact on resistance. PLoS Pathog 2014;10:e1004174.
[260] Nielsen A, Månsson M, Bojer MS, Gram L, Larsen TO, Novick RP, et al. Solonamide B inhibits quorum sensing and reduces Staphylococcus aureus mediated killing of human neutrophils. PLoS One 2014;9:e84992.
[261] Baldry M, Kitir B, Frøkiær H, Christensen SB, Taverne N, Meijerink M, et al. The agr Inhibitors Solonamide B and Analogues Alter Immune Responses to Staphylococccus aureus but Do Not Exhibit Adverse Effects on Immune Cell Functions. PLoS One 2016;11:e0145618.
[262] Daly SM, Elmore BO, Kavanaugh JS, Triplett KD, Figueroa M, Raja HA, et al. ω-Hydroxyemodin limits staphylococcus aureus quorum sensing-mediated pathogenesis and inflammation. Antimicrob Agents Chemother 2015;59:2223-35.

[263] Reglinski M, Sriskandan S. Treatment potential of pathogen-reactive antibodies sequentially purified from pooled human immunoglobulin. BMC Res Notes 2019;12:228.
[264] Palma Medina LM, Rath E, Jahagirdar S, Bruun T, Madsen MB, Strålin K, et al. Discriminatory plasma biomarkers predict specific clinical phenotypes of necrotizing soft-tissue infections. J Clin Invest 2021;131.
[265] Balayla J, Gil Y, Mattina J, Al-Shehri E, Ziegler C. Streptococcal Toxic Shock Syndrome After Insertion of a Levonorgestrel Intrauterine Device. J Obstet Gynaecol Can 2019;41:1772-1774.
[266] Schlievert PM, Davis CC. Device-Associated Menstrual Toxic Shock Syndrome. Clin Microbiol Rev 2020;33.
[267] Ikebe T, Murai N, Endo M, Okuno R, Murayama S, Saitoh K, et al. Changing prevalent T serotypes and emm genotypes of Streptococcus pyogenes isolates from streptococcal toxic shock-like syndrome (TSLS) patients in Japan. Epidemiol Infect 2003;130:569-72.

ABOUT THE AUTHOR

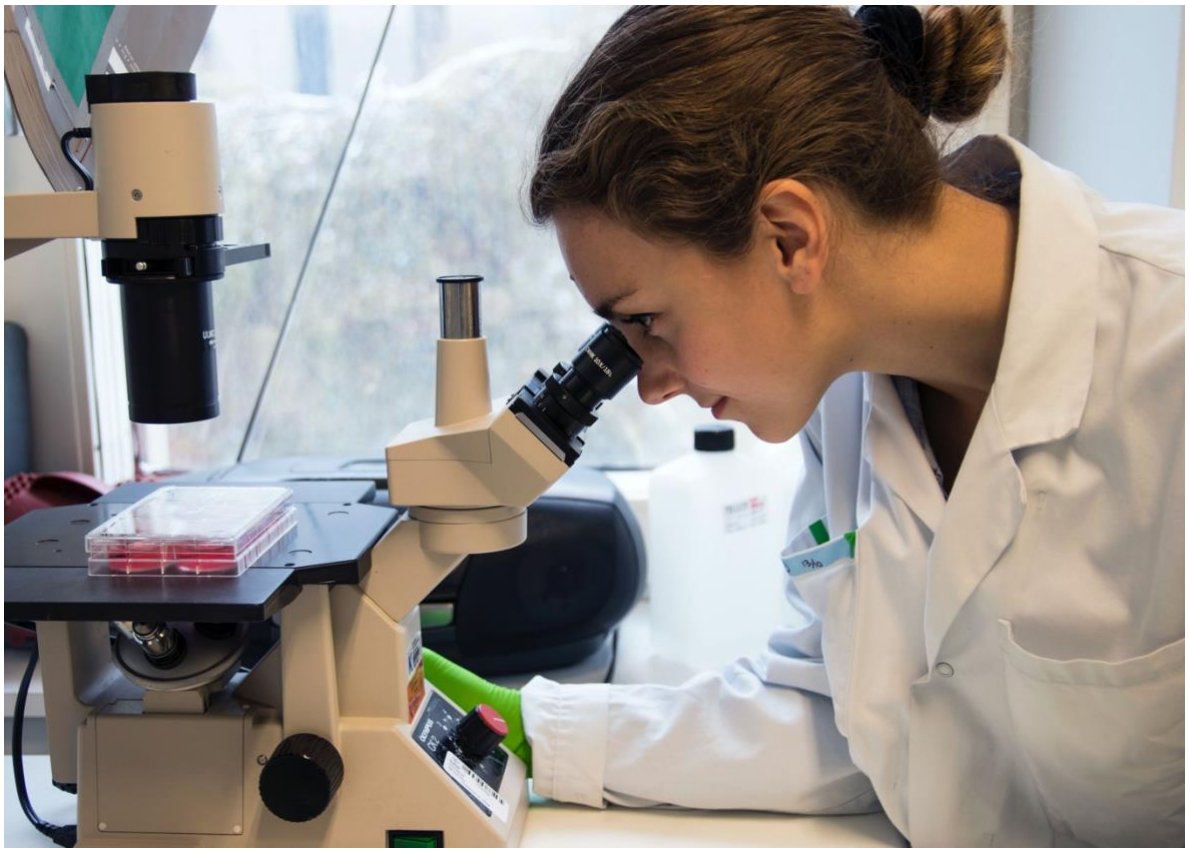

Photo: Linn Johansson

Helena Bergsten, M.D.

Helena participated in the Summer Research School for high school students at Karolinska Institutet 2011. Three years later, she came to the laboratory of Professor Anna Norrby-Teglund as a summer student to work on *Staphylococcus aureus* infections in organotypic tissue models. She returned to the laboratory whenever she could, started to work on *Streptococcus pyogenes* infections as well, and in 2016 she was admitted as a doctoral student.

Helena earned her medical degree in 2017 at Örebro University. She has been working as a junior physician at the clinic of Infectious Diseases at Örebro University Hospital followed by Danderyds Hospital after which she completed her medical internship at Södersjukhuset (Stockholm Southern Hospital). She received her license to practice medicine in 2021 and has since then been practicing at the emergency clinic at Södersjukhuset.

During these years, Helena has been performing advanced experimental research at the Center for Infectious Medicine (CIM) under the supervision of Anna Norrby-Teglund and Mattias Svensson, while also working clinically, pursuing a career as a physician-scientist.

On December 17th, 2021, Helena will defend her doctoral thesis at Karolinska Institutet.