Coagulase-negative Staphylococci in Hematological Malignancy

"When I was young, I never dreamed of this.
I dreamed of colors and falling, among other things."
Neil Young

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Abstract

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Bacterial infections are common in hematological malignancy. Coagulase-negative staphylococci (CoNS) are among the most prevalent causes of bacteremia in patients with hematological malignancies.

In this thesis, different aspects of CoNS in hematological malignancy have been studied in four papers:

In paper 1, CoNS blood culture isolates from patients with hematological malignancies treated at the University Hospital of Örebro from 1980 to 2009 were revaluated for the presence of reduced sensitivity to glycopeptides. A high incidence of heterogeneous-intermediate glycopeptide resistance was observed and there was a trend towards increasing incidence of this phenotype over time.

In paper 2, the colonization pattern of CoNS among patients undergoing intensive chemotherapy for hematological malignancy was investigated. A successive homogenization and an accumulation of CoNS phenotypes mutually present in a majority of included patients were demonstrated.

In paper 3, a PCR method to determine the clinical significance of positive blood cultures of the CoNS species *Staphylococcus epidermidis* was evaluated. The test failed to discriminate bloodstream infection from blood culture contamination.

Finally, in paper 4, the long-term molecular epidemiology of *S. epidermidis* blood culture isolates from patients with hematological malignancies was studied with multilocus sequence typing. A predominance of sequence type 2 was demonstrated during the entire 30 year study period.

In conclusion, the results are consistent with that CoNS have established as important pathogens by its capacity to colonize the human skin, its ability to reside and spread in the hospital environment and its rapid adaptation to stressors such as antimicrobials.

Keywords: Coagulase-negative staphylococci, hematological malignancy, Staphylococcus epidermidis, health care-associated infection, antibiotic susceptibility, molecular epidemiology, bloodstream infection, bacteremia.

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Contents

List of original papers

1. Erik Ahlstrand, Karolina Svensson, Lennart Persson, Ulf Tidefelt and Bo Söderquist. **Glycopeptide resistance in coagulase-negative staphylococci isolated in blood cultures from patients with hematological malignancies during three decades.** Eur J Clin Microbiol Infect Dis 2011; 30:1349–1354.

2. Erik Ahlstrand, Lennart Persson, Ulf Tidefelt and Bo Söderquist. **Alteration of the colonization pattern of coagulase-negative staphylococci in patients undergoing treatment for hematological malignancy.** Eur J Clin Microbiol Infect Dis 2012; 31:1679-687.

3. Erik Ahlstrand, Anders Bäckman, Paula Mölling Lennart Persson, Ulf Tidefelt and Bo Söderquist. **Evaluation of a PCR method to determine the clinical significance of positive blood cultures with *Staphylococcus epidermidis* in patients with hematological malignancies.** Accepted for publication in APMIS.

4. Erik Ahlstrand, Bengt Hellmark, Karolina Svensson and Bo Söderquist. **Long-term molecular epidemiology of *Staphylococcus epidermidis* blood culture isolates from patients with haematological malignancies.** Submitted.

Abbreviations

ACME	Arginine catabolic element
Allo-SCT	Allogeneic stem cell transplant
AML	Acute myeloid leukemia
ANC	Absolute neutrophil count
ASCT	Autologous stem cell transplant
BSI	Bloodstream infection
CC	Clonal complex
CLL	Chronic lymphocytic leukemia
CoNS	Coagulase-negative staphylococci
CRBSI	Catheter-related bloodstream infection
ESBL	Extended spectrum β-lactamase
CT	Computer tomography
CVC	Central venous catheter
GIS	Glycopeptide intermediate staphylococci
GRD	Glycopeptide resistance detection
GvHD	Graft versus host disease
hGIS	Heterogeneous glycopeptide intermediate staphylococci
HSCT	Hematopoietic stem cell transplant
Ica	Intracellular adhesion
MALDI-TOF	Matrix-assisted laser desorption/ionization-time to flight
MASSC	Multinational association for supportive care in cancer
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
PCR	Polymerase chain reaction
PIA	Polysaccharide intercellular adhesion
PFGE	Pulsed field gel electrophoresis
PGA	Poly-γ-glutamic acid
PhP	PhenePlate
PSM	Phenol soluble modulin
SCT	Stem cell transplant
ST	Sequence type

Introduction

Bacterial infections in hematological malignancy

With the introduction of chemotherapy in the late 1940s, it first became possible to influence the natural course of disease in patients with hematological malignancies[1]. It was soon recognized that bacterial infections could lead to mortality even when the hematological malignancy was in remission[2]. Despite the development of supportive care, there is still considerable morbidity associated with bacterial infections in patients with hematological malignancies. For example, mortality in neutropenic fever can be as high as 10%, depending on the underlying diagnosis and co-morbid conditions[3]. Notably, in cases with confirmed bacteremia, the 7- and 30-day mortality is 6–17% and 15–32%, respectively[4-5]. Moreover, the use of antimicrobial therapies to treat these infections delays chemotherapy, prolongs hospitalization, and increases health care costs[3].

In a classic report published in 1966[6], Bodey and collaborators demonstrated that the most important risk factor for bacterial infections in patients with hematological malignancies is chemotherapy-induced neutropenia. Neutropenia, which is commonly defined as an absolute neutrophil count (ANC) below 0.5 x 10^9/L, is still considered the greatest risk factor for bacterial infections. The incidence and severity of bacterial infections are inversely related to the ANC and to the duration of neutropenia, with the highest incidence of infections seen for patients with ANC levels below 0.1 x 10^9/L. Patients undergoing intensive chemotherapy that induces neutropenia have a greater than 80% risk of developing fever during a treatment course[7]. In neutropenic fever, the bacterial etiology is identified in approximately 30% of all cases[8-9].

The underlying hematological malignancy has a pronounced influence on the risk of bacterial infections. In a Danish cohort of patients with hematological malignancies, acute myeloid leukemia (AML) was associated with the highest risk of bacteremia[5] (Figure 1). The differences in bacterial infection rates in patients with different hematological malignancies are most likely due to the influence of disease-specific factors, the intensity of chemotherapy schedules, patient age, and co-morbid conditions.

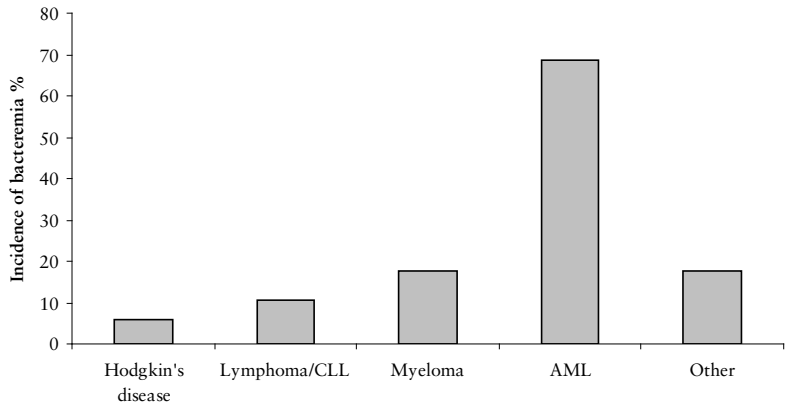

Figure 1. *The 1-year cumulative incidence of bacteremia in 1666 patients with different types of hematological malignancies. Adopted from Norgaard et al.*[5]*(CLL chronic lymphocytic leukemia, AML acute myeloid leukemia)*

Other well-established risk factors for bacterial infections in patients with hematological malignancies are the use of central venous catheters (CVCs) and immunomodulating therapies such as corticosteroids, mucositis, graft-versus-host disease (GVHD), hypogammaglobulinemia, and asplenia[10].

The etiology of bacterial infections in hematological malignancy

Bacterial infections in hematological malignancy are most commonly the result of translocation of gram-negative bacteria from the gastrointestinal tract or of gram-positive bacteria from the skin or oral cavity into the bloodstream. The most common sites of infection in hematological malignancy are primary bloodstream infection with or without CVC involvement, pneumonia, and skin or soft tissue infections. Urinary tract infections are relatively less frequent[11]. The term 'bloodstream infection' (BSI) is used interchangeably with 'bacteremia' and generally refers to the growth of bacteria in a blood culture from patients with signs of infection after contamination has been ruled out.

Several considerations should be kept in mind when interpreting reports on the epidemiology and etiology of bacterial infections in hematological malignancy. Studies based on microbiological findings tend to underestimate sources that lead to soft tissue and airway infections since these infections are typically culture-negative. Thus, many such studies focus solely on bacteremia[12]. Other considerations include how polymicrobial etiologies

are reported[12], the culturing rates[13], the use of antimicrobial prophylaxis[14], and 'referral bias' that leads to overestimation of actual infection rates at highly specialized units that fail to exclude cases in an external population[15]. Moreover, socioeconomic characteristics and regional climactic conditions influence the development of neutropenic fever. For example, *Pseudomonas aeruginosa* infections are reported more often in warmer climates[16]. For coagulase-negative staphylococci (CoNS) and other bacteria that commonly contaminate blood culture bottles, distinguishing between BSIs and bacterial contamination can have a pronounced influence on estimates of the infection rate[17].

Despite these considerations, some general trends in the epidemiology of bacterial infections in hematological patients are evident. During the early era of modern hematology in the 1950s and 1960s, gram-negative bacteria such as *Escherichia coli*, *Klebsiella* species, and *Pseudomonas aeruginosa* were the primary causative microorganisms. Later, during the 1980s and 1990s, there was a shift towards gram-positive bacteria as the causative microorganisms. The reasons behind this shift are not fully understood, although increased use of CVCs[18], quinolone prophylaxis, the use of high-dose cytarabine in the consolidation of acute leukemia, and the introduction of omeprazol[19] have been associated with this development. Recently, several groups have reported on single center experiences that document a rebound towards gram-negative bacteremia[20]. This may be due to increasing resistance to quinolones[21], reduced use of quinolone antimicrobial prophylaxis, the emergence of multi-drug resistant gram-negative bacteria such as ESBL-producing *Enterobacteriacaea*[22], and perhaps to less aggressive chemotherapy schedules that cause less mucositis and thereby reduce the risk of α-streptococcal bacteremia[19]. Recent reports on the etiology of BSI in hematological patients show that gram-positive bacteria account for 46–67% and gram-negative bacteria for 27–51% of infections, with the dominating species being CoNS in 15–35% and *Escherichia coli* in 8–26% of infections (Table 1).

	Wisplinghof, 2003[23]	Kjellander, 2012[4]	Gudiol, 2012[22]
Location	49 hospitals, USA	Stockholm, Sweden	Barcelona, Spain
Patients	Neutropenic cancer pat	Hematology, excl SCT	Hematology, incl SCT
Ab prophylaxis	Not defined	None	None
Gram-positive bacteria	487 (67%)	407 (51%)	125 (46%)
Staph aureus	98 (13%)	55 (7%)	14 (5%)
CoNS	252 (35%)	117 (15%)	50 (18%
Streptococci	73 (10%)	126 (16%)	29 (11%
Enterococccus species	50 (7%)	72 (9%)	26 (10%)
Other	14 (2%)	37 (5%)	6 (2%)
Gram-negative bacteria	199 (27%)	349 (44%)	138 (51%)
Escherichia coli	58 (8%)	141 (18%)	71 (26%)
Klebsiella species	43 (6%)	78 (10%)	32 (12%)
Pseudomonas species	29 (4%)	42 (5%)	32 (12%)
Other	30 (4%)	88 (11%)	3 (1%)
Anaerobes	38 (5%)	38 (5%)	8 (3%)
Total	724	794	271

Table 1. Bacteria isolated from the blood cultures of neutropenic patients with cancer and hematological malignancies.

Treatment and prophylaxis of bacterial infections in neutropenic patients

Neutropenic patients with hematological malignancies generally lack an adequate inflammatory response to bacterial infections. As a consequence, there may be few or none of the classical signs and symptoms of bacterial infection. Focal signs of infection are often lacking, and fever is often the only sign of a serious infection[24].

Neutropenic fever is most often defined as a temperature ≥38.5°C (in American publications, ≥101°F=38.3°C) or >38°C on two occasions in combination with an ANC <0.5 or an ANC that is expected to decrease to <0.5 during the next 48 hours.

Risk assessment of patients presenting with neutropenic fever

The outcomes of patients with neutropenic fever are variable and range from transient fever episodes with spontaneous recovery to complicated courses with prolonged hospital stays and mortality. The most commonly reported risk factors for a complicated course include:

- Profound neutropenia (ANC <0.1)
- Anticipated neutropenia >7 days
- Hemodynamic instability
- Mucositis
- Abdominal pain, diarrhea, or vomiting
- Neurological symptoms or mental status change
- CVC
- Pulmonary infiltrate
- Hepatic insufficiency
- Renal insufficiency
- Previous pulmonary disease
- Acute leukemia

Based on these risk factors, a number of tools have been developed to predict the outcomes of patients and to divide patients into high- and low-risk groups for complications. Of these tools, the MASSC assessment score[25] is the most widely used. This score has been validated for identifying subgroups of patients at low and high risk of complications or death[26].

Clinical and laboratory investigations in neutropenic fever

Initial patient assessment includes a thorough history and physical examination. The physician must keep in mind that the relative lack of inflammation makes clinical signs sparse.

Laboratory tests should include a compete blood count, including differential leukocyte count, as well as an analysis of basic blood chemistry and liver enzymes. Blood markers of inflammation such as C-reactive protein are generally less informative in neutropenic patients than in non-neutropenic patients[27].

Prior to antimicrobial therapy, a minimum of two sets of blood cultures should be obtained, each set consisting of one draw of blood divided into 2 blood culture bottles (1 bottle for aerobic and 1 bottle for anaerobic culture).

Ideally, each blood cultures should use ≥20 mL of blood since the inoculated blood volume correlates with the probability of detecting bacteremia[28]. It is recommended that one culture set be drawn from every CVC lumen plus one from a peripheral vein[29]. There is no consensus regarding whether additional cultures should be obtained routinely. However, stool, urine, skin, and respiratory specimens should always be obtained when the patient has symptoms indicative of involvement of the corresponding organ.

Lastly, chest radiography or chest CT scan should be performed in the initial workup of patients with respiratory signs or symptoms.

Treatment of neutropenic fever

After adequate cultures are obtained, the standard of care is rapid initiation of empirical broad-spectrum antimicrobial therapy. This concept was introduced following a clinical trial conducted by Schimpff in 1971[30] that demonstrated a marked reduction in mortality associated with *Pseudomonas aeruginosa* infection when empirical antibiotics were administrated.

The aim of the initial antimicrobial therapy is to provide non-toxic broad coverage of the most likely pathogens, with an emphasis on pathogens that are potentially life threatening when untreated. Initial antimicrobial therapy should have broad gram-negative coverage that includes *Pseudomonas* species and should cover the most virulent gram-positive pathogens, including *S. aureus*. In general, initial coverage of anaerobic bacteria, CoNS, enterococci species, and fungi is unnecessary due to their lower pathogenic potential.

Several antimicrobial regimens have been proposed that fulfill these criteria. A meta-analysis of studies comparing monotherapy with β-lactam antibiotics to combinations of aminoglycosides and β-lactams found similar survival rates between groups but a lower risk of adverse events and morbidity in patients treated with monotherapy[31]. Notably, subgroup analyses indicate that combination therapy has a protective effect for patients who are in septic shock[32]. Therefore, monotherapy with intravenous anti-pseudomonal β-lactam agents, such as a third generation cephalosporin, piperacillin/tazobactam, or a carbapenem, is recommended as first-line therapy in high-risk patients without specific localized symptoms and other guiding signs[29] (Figure 2). The addition of an aminoglycoside could be considered in patients presenting with shock. Among the cephalosporins, ceftazidime and cefepime have been recommended for first-line use, although the emergence of extended spectrum β-lactamase (ESBL)-producing gram-negative bacteria has decreased the usage of cephalosporins in recent years. Moreover, there is evidence that the activity of ceftazidime against α-streptococci has decreased[33] and, interestingly, a meta-analysis of studies of

cefepime in neutropenic patients demonstrated increased overall mortality in patients treated with cefepime compared with other agents.[34] These findings have decreased the use of the drug.

Two randomized trials have studied the benefits of adding vancomycin to the initial empiric antibiotic therapy[35-36]. Although there was no difference in early mortality in gram-positive bacterial infections, there was substantially higher toxicity in vancomycin-treated patients. Therefore, the addition of vancomycin is not recommended except in cases with cultures that are positive for methicillin-resistant microbial and/or suspected catheter-related infections.

After antibiotic treatment is initiated, the therapy should be modified continuously based on the clinical response to treatment, the duration of fever, the results of blood cultures, the appearance of localized infection signs, and the development of ANC (Figure 2).

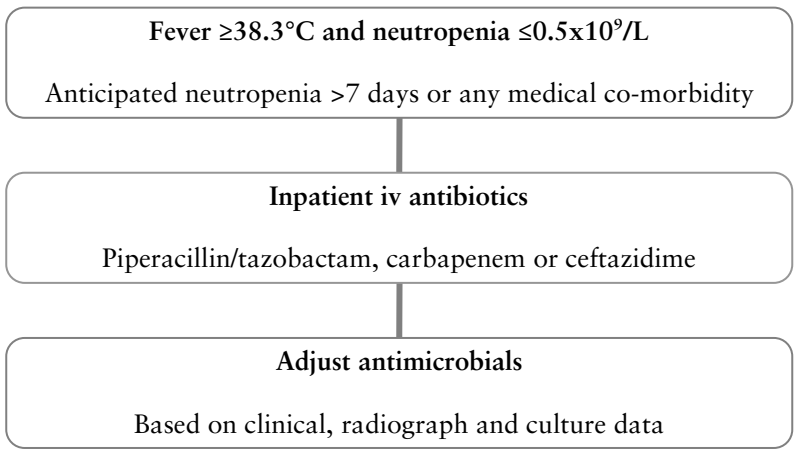

Figure 2. Initial management of fever and neutropenia in high-risk patients[29].

Antimicrobial prophylaxis

The efficacy of antimicrobial prophylaxis in chemotherapy-induced neutropenia has been debated for years. Since the introduction of fluoroquinolones in the 1980s, fluoroquinolone prophylaxis has been used due to its broad-spectrum effects against gram-negative pathogens and its relatively low toxicity. Studies with fluoroquinolone prophylaxis have demonstrated a reduction in febrile events and documented infections; the latter is mostly due to a reduction in gram-negative bacterial infections. On the other hand, individual studies have failed to show any survival benefit for anti-

microbial prophylaxis, and concerns have been raised about emerging resistance to fluoroquinolones and other antibiotics driven by the use of fluoroquinolone prophylaxis[21]. It remains unclear whether the appearance of fluoroquinolone-resistant clones alone has any bearing on prophylaxis efficacy[37].

A meta-analysis of 14 placebo-controlled trials of fluoroquinolone prophylaxis found that it conferred a pronounced survival benefit[38]. Specifically, the meta-analysis found that fluoroquinolone prophylaxis decreased relative all-cause mortality by 48% and infection-related mortality by 62%. Patients in the included studies were mainly high-risk patients treated for acute leukemia who were receiving (hematopoietic stem cell transplant) HSCT. There are some concerns about the validity of the results since the treatment effect was generally smaller in studies reporting adequate allocation concealment and double-blinding compared with studies in which the methods were unclear. Moreover, the effect of treatment, i.e. mortality, was larger in smaller studies, indicating a publication bias (Figure 3).

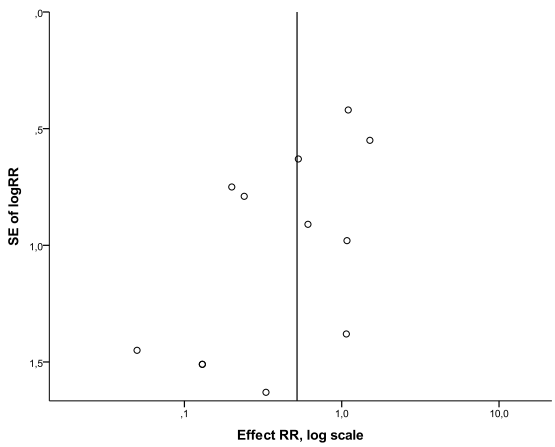

Figure 3. Funnel plot of all-cause mortality in fluoroquinolone prophylaxis trials. The vertical line indicates the average effect of death (relative risk, RR 0.52). There is an asymmetry, with fewer studies in the lower right area compared to the lower left area, indicating a publication bias. Adopted from the meta-analysis by Gafter-Gvili et al.[38]

Thus, the benefits of fluoroquinolone prophylaxis remain controversial. Low-risk patients, who are mainly patients with solid tumors or lymphoma, probably do not benefit from antimicrobial prophylaxis. However,

fluoroquinolone prophylaxis can be considered for high-risk patients such as those with acute leukemia and HSCT recipients.

Aciclovir prophylaxis is indicated to prevent herpes virus infections in patients undergoing chemotherapy for acute leukemia and during HSCT. There is one report that this strategy may also reduce the risk of bacterial infections in this patient group[39].

Intravascular devices

Intravascular devices are essential in the management of patients with hematological malignancies. These devices are indispensable for chemotherapy, transfusion therapy, and parental nutrition. The types of intravascular devices used most frequently are long-term CVCs and subcutaneous ports. Long-term CVCs are surgically implanted. While a tunneled portion extends out through the skin, subcutaneous ports placed under the skin have a membranous septum that can be accessed with a needle through intact skin. Unfortunately, it is common for these devices to become infected[40], leading to catheter-related bloodstream infection (CRBSI). Most infections in long-term CVCs emanate from the catheter hub[41]. The dominant etiologic organisms in patients with hematological malignancies are CoNS, *E. coli*, *S. aureus,* and *P. aeruginosa*[42-43].

CRBSI should be suspected when there is inflammation and purulence around the insertion site and in cases of fever and blood cultures that are positive for CoNS or *S. aureus* in the absence of another potential focal source of infection. Due to the lack of a clear definition of CRBSI that does not involve removal and culture of the catheter, the exact incidence of these infections is difficult to asses[44]. The most common definition of CRBSI without catheter removal is the growth of the same bacteria in cultures of blood drawn from peripheral blood and in blood from the catheter hub, where the growth of bacteria is detected in blood from the catheter hub at least 2 hours earlier than in peripheral blood[45].

Coagulase-negative staphylococci

CoNS are common colonizers of human skin and mucous membranes, where they constitute an important part of normal flora. CoNS rarely cause disease in healthy individuals but are increasingly recognized to cause infections in immunocompromised hosts and in patients with indwelling medical devices. The staphylococci are a family of gram-positive cocci divided into *S. aureus* and CoNS. In the past, the division was based on the ability of *S. aureus* to form yellow carotenoid pigment, while the division today is determined using the coagulase test. There are currently at least 40

known species of CoNS[46], approximately half of which were isolated from human sources[46]. Of these, at least ten have repeatedly been identified as having pathogenic potential in humans (Table 2). The predominant species that causes infections in hematological malignancy is *S. epidermidis*, followed by *S. haemolyticus*.

Human pathogens	**Case reports of infection in humans**
S. epidermidis	*S. auricularis*
S. haemolyticus	*S. carnosus*
S. lugdunensis	*S. cohnii*
S. warneri	*S. pasteuri*
S. capitis	*S. pettenkoferi*
S. caprae	*S. saccharolyticus*
S. simulans	*S. xylosus*
S. schleiferi	*S. sciuri*
S. saprophyticus	*S. intermedius*
S. hominis	

Table 2. CoNS species with pathogenic potential in humans.

The CoNS genome includes a core genome that encompasses approximately 80% of all genes[47-48]. These genes are well conserved among different strains of the same species and encode essential metabolic and regulatory proteins as well as so-called housekeeping genes. The accessory genome consists of a myriad of mobile (or once mobile) genetic elements that in most cases are integrated into the 2.5-million base pair chromosome. The mobile genetic elements consist of pathogenicity islands, bacteriophages, chromosomal cassettes, genomic islands, and plasmids that can potentially be transferred between strains. These elements are in *S. aureus* and, to a lesser extent, in CoNS and are known to carry genes that encode virulence factors. These elements are thought to play crucial roles in the pathogenesis and adaptation of the staphylococci to different environments and hosts. In many CoNS species, for example in *S. epidermidis* and *S. haemolyticus,* the genome is highly dynamic, whereas other species, such as *S. lugdunensis,* have more conserved genomes. This has possible clinical implications in that *S. lugdunensis* has a lower potential for developing antimicrobial resistance[49].

Pathogenesis of coagulase-negative staphylococcal infections

The pathogenesis of CoNS infections in patients with hematological malignancy is not fully understood. CoNS lack many of the virulence factors that are important in the pathogenesis of infections caused by *S. aureus*. Traditionally, CoNS bacteremia has been assumed to be of endogenous etiology i.e. to be derived from patients' endogenous flora. In contrast, recent studies suggest that most of these infections are hospital-acquired and are due to transmission between hospitalized patients[50-52].

The routes by which CoNS invade the bloodstream are still being debated. Field studies have been performed in a wide range of study populations, and it seems likely that different and overlapping mechanisms have relatively greater or lesser importance in infection, depending on the setting.

The dominating theory has been that CoNS strains that colonize patients' skin migrate on the external surfaces of CVCs into the bloodstream and adhere to the intravascular CVC elements by biofilm formation (Figure 4). Therefore, CoNS bacteremia has often been referred to as being catheter-related. As a consequence, attempts to prevent CoNS bacteremia have generally focused on CVC insertion procedures and on disinfecting the skin around the CVC insertion site[53-54].

An alternative hypothesis states that CoNS bacteremia in hematological malignancy is mainly caused by invasion of CoNS-colonizing mucosal surfaces secondary to chemotherapy-, radiation-, or sepsis-induced mucosal injuries (Figure 4). This theory is supported by studies demonstrating the molecular relatedness of CoNS that colonize mucosal surfaces and bloodstream isolates[55] as well as by the recent finding that poor periodontal status is associated with CoNS bacteremia[56].

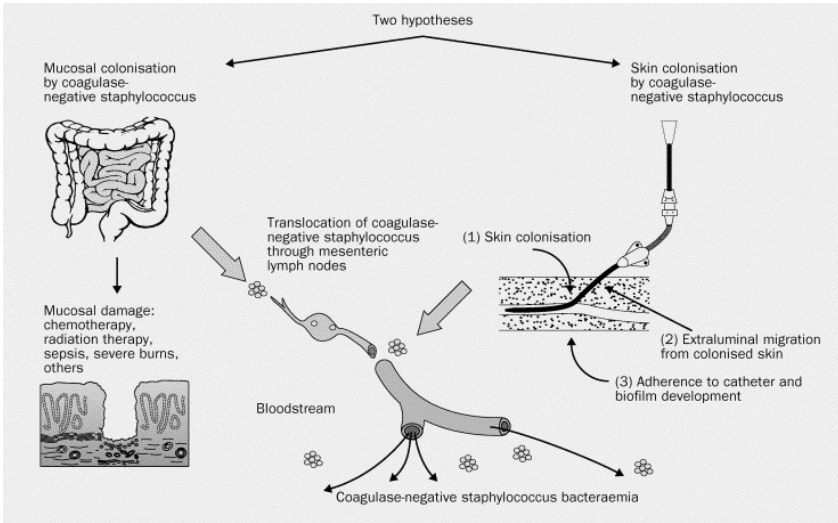

Figure 4. Possible sources of CoNS bacteremia. Reprinted from Costa et al.[55] with permission from Elsevier.

A third proposed mechanism is that CoNS from the patient's skin flora or from the health care environment invade the bloodstream through CVC hub colonization. The latter can be a consequence of manipulation of the CVC or can be secondary to contaminated infusates or blood products. This theory is consistent with the observation that red blood cell transfusion therapy is associated with catheter-related bacteremia in patients in intensive care units[57].

After penetrating the skin or mucosal epithelial barrier, CoNS has several mechanisms for evading ingestion by neutrophils. The most important mechanism is biofilm formation, which is discussed in detail below, but production of exopolymers such as poly-γ-glutamic acid (PGA) also plays a role in CoNS evasion of neutrophil phagocytosis[58].

Virulence factors of coagulase-negative staphylococci

Colonization

The innate ability of CoNS to colonize human skin and mucous membranes is recognized as an important factor in its pathogenesis, as most known CoNS virulence factors originally played roles in its commensal life on human skin[59]. The population density of CoNS on human skin ranges from 10^1 to $10^6/cm^2$, with the highest densities observed in moist areas. *S. epidermidis* is widely distributed over the body, whereas other species tend

to colonize specific parts of the body. For example, *S. haemolyticus* is found mostly in areas with apocrine glands such as the axillae, while *S. capitis* is most commonly found on the head[60].

Several factors contribute to the ability of these bacteria to colonize human skin. CoNS can tolerate acidic pH, lipids, and high-salt concentrations. The presence of the arginine catabolic mobile element (ACME), which encodes enzymes that converting arginine into carbon dioxin and ammonia, may favor the tolerance of CoNS to low pH environments[61]. Furthermore, the expression of PGA by *S. epidermidis* may aid survival in highly osmotic environments[58].

Biofilm formation

Biofilms, as opposed to planktonic cells, are communities of microorganisms that adhere to each other and to surfaces via an extracellular biochemical matrix. Unfortunately, biofilms not only have the capacity to colonize organic surfaces, but they can also adhere to virtually every type of implanted medical device[46]. Many stressors induce biofilm formation, including increased temperature, osmotic shock, low oxygen tension, changes in the availability of nutrients, and exposure to ethanol or antibiotics[62]. The main component of the CoNS biofilm matrix is of carbohydrate origin and is built up by polysaccharide intercellular adhesin (PIA), which is synthesized by enzymes encoded by the *ica* (intracellular adhesin) operon. When conditions are unfavorable for PIA synthesis, as well as in *ica*-negative strains, *S. epidermidis* can also produce a proteinaceous intercellular biofilm. For example, there can be transcription of the *aap* gene, which encodes the biofilm-forming accumulation-associated protein Aap[63].

Biofilm formation involves several stages (Figure 5), and the process is partially controlled by quorum-sensing systems, which are interbacterial communication mechanisms that are dependent on population density. The Agr quorum-sensing system is the most important such system in staphylococci and is encoded by the *agr* gene locus[62]. The formation of biofilm begins with bacterial adhesion to biomaterial surfaces. Adhesion is initially mediated by nonspecific factors; later, bacterial production of adhesins facilitates binding to the host extracellular matrix[59]. In subsequent steps, the bacteria produce extracellular matrix. There are also alterations in gene expression that lead to metabolic inactivity and to a slower growth rate[62]. In the final stage of biofilm formation, bacteria detach from the biofilm and metastasize to distant sites, a process involving so-called phenol-soluble modulins (PSMs), which include the *S. epidermidis* δ-toxin[59].

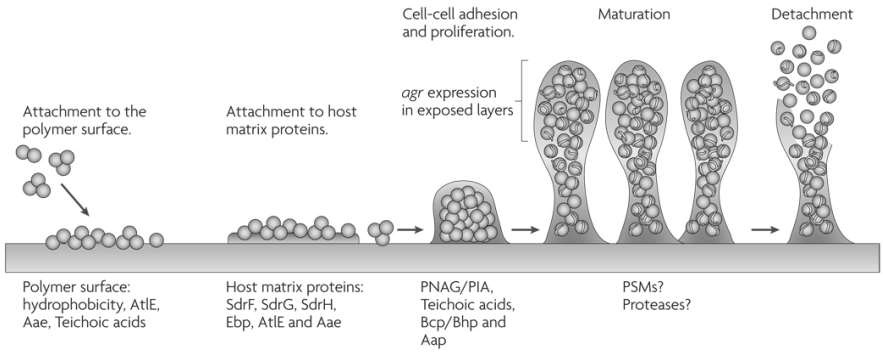

Figure 5. Stages of biofilm development in Staphylococcus epidermidis. Reprinted by permission from Macmillan Publishers Ltd. [Nature Rev Microbiol][59], copyright 2010.

Bacteria in biofilm are more resistant to immune defenses such as phagocytosis and antimicrobial peptides[64]. Moreover, bacteria in biofilms can tolerate antibiotics and disinfectants at 10- to 1000-times higher concentrations than genetically identical planktonic cells[62]. The mechanisms behind these properties of biofilm are not entirely understood but are thought to involve the following:

- Slow-growing bacterial cells[65]
- Reduction of the effect of antibiotics by the extracellular matrix[66]
- Changes in gene expressions as part of the stress response[64]

Toxin production

CoNS generally lack many of the exotoxins that are important in *S. aureus* pathogenesis. One exception is *S. lugdunensis*, which produces several toxins, including hemolysins. *S. epidermidis* can produce δ-toxin, a potent cytolytic toxin, although *in vitro* experiments do not indicate that δ-toxin plays a crucial role in *S. epidermidis* immune evasion, since production is very low[67]. However, δ-toxin has antimicrobial activity and has been proposed to be important for counteracting rival bacterial colonizers on the skin[68].

Antibiotic resistance

CoNS have developed widespread resistance to antimicrobial agents. There are some species-specific differences, and antimicrobial resistance is more pronounced in *S. haemolyticus*, which may reflect the extreme plasticity of its genome[69].

Most notably, approximately 70% of health care-associated CoNS isolates are resistant to the first-line antibiotic against staphylococci, methicillin (isoxazolyl penicillin)[70]. Methicillin resistance is conferred by a mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*). This element contains the *mecA* gene, which encodes a penicillin-binding protein, PBP2a, that has reduced affinity for methicillin.

In addition to methicillin resistance, CoNS have acquired resistance to a wide range of other antibiotics, including rifampicin, fluoroquinolones, aminoglycosides, clindamycin, erythromycin, tetracycline, and sulfamethaxole[46].

Since the mid-1980s, there have been reports of rare cases of CoNS that are resistant to glycopeptides[71-72]. By nomenclature, pure glycopeptide resistance in staphylococci is accomplished by the acquisition of the *van* gene cluster[73]. More commonly, susceptibility to glycopeptides is reduced in staphylococci by a different mechanism. Although it is not well characterized, this mechanism involves a thickening of the cell wall that counteracts glycopeptide binding[74]. Such strains show intermediate resistance to glycopeptides according to conventional minimum inhibitory concentration (MIC) testing, in which case they are referred to as glycopeptide-intermediate staphylococci (GIS). Alternatively, strains may have a conventional vancomycin MIC below the breakpoint, but subpopulations of bacteria can grow in the presence of >4 µg/mL vancomycin; in this case, they are termed heterogeneous intermediate staphylococci (hGIS)[75]. Experiments show that GIS and hGIS are genetically identical and that the GIS phenotype develops under vancomycin pressure; therefore, the presence of hGIS may predict the presence of GIS[73].

Coagulase-negative staphylococcal infections

Prevention

To date, prevention of CoNS infections in hematological malignancy has focused on hygiene measures related to CVC use[46]. Hygienic interventions reduce the incidence of CVC-related infections, including CoNS in intensive care units[76]. However, rigorous infection control interventions that were more stringent than standard infection control procedures failed to demonstrate a reduction of CoNS infections in hemato-oncological patients[77]. Risk factors of catheter-related CoNS infections in hematological malignancy include the type of CVC, the duration of catheterization, and the number of CVC manipulations, but not the anatomical site of CVC insertion[46, 78]. A small study of cancer patients who were randomized to use minocycline/rifampicin-coated long-term catheters or non-coated catheters

showed a significant reduction in catheter-related infections in general and an absence of catheter-related CoNS infections in the intervention group[79].

Clinical presentation

The symptoms and signs of a CoNS BSI include classical signs of sepsis, such as fever and hypotension[80]. The clinical course is often less dramatic than in bacteremia due to differing etiologies, although fulminant sepsis and mortality can occur[81]. Infective endocarditis and septic embolization are rare manifestations of CoNS bacteremia in hematological malignancy[81]. As CoNS bacteremia is often a part of a complicated medical course in severely ill patients, the crude mortality is high and does not differ from the mortality of bacteremia caused by other organisms in neutropenic patients[23]. In a recent Swedish study, the 7-day and 30-day mortality rates were 6% and 15%, respectively[4]. The mortality attributed to CoNS bacteremia varies in different study populations, but is estimated to be 10–15%[80, 82].

Management

In BSIs caused by methicillin-sensitive strains, an intravenous β-lactam is the preferred treatment. When a methicillin-resistant CoNS strain is cultured, as is often the case, the first line therapy is a glycopeptide, usually vancomycin. Intravenous treatment should ideally continue for at least the duration of neutropenia (until ANC $\geq 0.5 \times 10^9$/L) or for 10–14 days[29].

In CoNS CRBSIs with severe sepsis or with signs of extraluminal infection, short-term CVCs and long-term CVCs should be removed, and systemic therapy should continue for 5–7 days. However, in uncomplicated episodes involving long-term CVCs, the use of systemic antibiotic therapy and antibiotic lock therapy for 2 weeks may allow the catheter to remain in place[45].

In the rare case of a CoNS strain with reduced vancomycin sensitivity or in patients with vancomycin intolerance, newer antibiotic agents are preferred, such as linezolid, daptomycin, or cephalosporins with activity against methicillin-resistant staphylococci (e.g. ceftobiprole and ceftarolin)[83].

Aims

To improve the methods used to prevent and treat CoNS infections in hematological malignancy, increased knowledge of the epidemiology and pathophysiology of these infections is needed. Furthermore, better diagnostic methods and new treatment options need to be developed for these infections.

The aims of the thesis were as follows:

- To investigate the emergence of reduced sensitivity to glycopeptides in CoNS isolates from patients with hematological malignancies

- To study the colonization patterns of CoNS and the relationship between colonizing and bacteremic isolates in hematological malignancy

- To evaluate new methods for the diagnosis of CoNS bacteremia

- To describe the long-term molecular epidemiology of CoNS infections in hematological malignancy

Methods

PhenePlate System

The PhenePlate (PhP) System (PhPlate AB, Stockholm, Sweden) is a fingerprinting tool that performs bacterial phenotyping based on multiple bacterial biochemical characteristics. Previous studies demonstrated that this system has a satisfactory ability to identify phenotypically-related isolates of CoNS[84-85]. Compared to molecular typing[86], PhenePlate has the advantage of being less laborious and costly. Results have shown a high concordance with genotyping methods such as PFGE[85] and randomly-amplified polymorphic DNA (RAPD) analysis[87]. The system uses 96-well plates, each with four sets of 22 different dehydrated reagents: fructose, galactose, mannose, D-xylose, L-arabinose, D-ribose, maltose, lactose, sucrose, trehalose, turanose, melizitose, mannitol, xylitol, D-arabinose, glycerol, deoxyribose, β-methyl-glucoside, arbutin, urea, ornitin, and arginine. Two wells contain negative controls. Color indicators visually reflect changes in pH that are the result of bacterial metabolism of the substrates. The 620-nm absorbance values for each reaction are read after 16, 40, and 64 hours using a microplate reader. The mean values are calculated for each reaction, the results for different isolates are compared pair-wise, and the similarity between each pair is calculated as a correlation coefficient. The correlation coefficients are clustered using the unweighted pair group method with arithmetic mean (UPGMA), yielding a dendrogram. Isolates with a correlation coefficient ≥0.975 are regarded as belonging to the same PhP type[88].

In paper 2, we analyzed CoNS that were colonizing patients' nares, throat, and axillae as well as CoNS-causing bacteremia. A total of 2047 CoNS isolates were analyzed using the PhP system.

ID32 Staph

Paper 1 used API®/ID32 Staph (BioMérieux, Marcy-l'Etoile, France) for species-level identification of CoNS. API®/ID32 Staph is a phenotyping method based on characterization of the bacterial metabolism of 25 substrates. It is a standard method that is used for routine species identification of CoNS. According to recent publications, the correct identification rate of this method is in the range of 80–86%[89-90].

In paper 1, API®/ID32 Staph identified 387 isolates to species level *S. epidermidis*. In paper 4 these isolates were re-evaluated and MALDI-TOF identified 9 of the isolates as species that were different than *S. epidermidis*.

MALDI-TOF

Matrix-assisted laser desorption/ionization-time to flight mass spectrometry, or MALDI-TOF MS, has recently been introduced for rapid species-level identification of CoNS. MALDI-TOF identification is based on the ionization of bacterial proteins by laser pulses. Bacteria are placed in an electromagnetic field that causes the bacteria to 'fly' according to the degree of ionization. A characteristic spectrum is recorded based on the time of flight (TOF) information. The computer compares the recorded spectrum with a database of unique spectra for different bacterial species and lists the best matches according to species[91].

MALDI-TOF is superior to standard phenotypic and genotypic species-level identification methods for CoNS. Therefore, MALDI-TOF is now considered a reference method for CoNS species identification[90]. Papers 3 and 4 describe species-level identification of CoNS using MALDI-TOF.

Disk diffusion test

The agar disk diffusion test is a standard method for antimicrobial resistance screening. This method is not suitable for large antibiotic molecules, such as glycopeptides. The bacteria are swabbed on agar plates, and a filter-paper disk impregnated with the antibiotic to be tested is placed on the surface of the agar. This creates a zone of growth inhibition around the disk that is measured after 24 hours of culture.

Papers 2 and 4 describe the use of the disk diffusion test to determine the antibiotic resistance profiles of CoNS isolates. In paper 1, the breakpoints used for defining antibiotic resistance were those of the Swedish Reference Group for Antibiotics. In paper 4, the breakpoints were those recommended by the European Committee on Antimicrobial Susceptibility Testing.

E-test

The Epsilometer test or E-test (BioMérieux, Marcy l'Etoile, France) is an antimicrobial susceptibility test that uses a plastic strip containing an antibiotic gradient that is laid on top of a bacterial culture plate. The E-test has greater precision than the disc diffusion test because it uses a gradient. The drug diffuses into the agar and, after 24 hours of incubation, there is an elliptical zone of inhibition. The strip is printed with an exponential scale showing the MIC, and the point on the scale at which the elliptical zone of inhibition meets the strip is the MIC reading for that antibiotic. The E-test generally gives higher MIC values for vancomycin than does broth microdilution for CoNS[92].

In paper 1, the E-test was used as a standard method for determining the MICs of CoNS.

Macro method E-test

In general, staphylococci expressing the hGIS/GIS phenotype of reduced susceptibility to vancomycin cannot be detected with standard MIC methods such as disk diffusion and the E-test[73]. The gold standard for detecting hGIS/GIS is population analysis, a method that is both time-consuming and costly. Compared to population analysis, the macro method E-test has a >95% sensitivity and specificity for discriminating vancomycin-sensitive *S. aureus* and *S. epidermidis* from hGIS/GIS[75]. Compared to standard antibiotic susceptibility testing methods, the macro method uses a thicker bacterial inoculum of 2.0 McFarland on a richer medium (Brain Heart Infusion agar) and is read after 48 hours. The test is based on the slower growth rate of hGIS/GIS compared with vancomycin-sensitive isolates.

Paper 1 describes the use of the macro method E-test for hGIS detection.

GRD E-test

The Glycopeptide Resistance Detection (GRD) E-test (BioMérieux, Marcy l'Etoile, France) is a new tool for detecting hGIS/GISA. It uses a standard inoculum of 0.5 McFarland and, like the Macro method E-test, it uses rich media (Mueller Hinton agar with 5% blood) and is read after 48 hours. The GRD E-test strip consists of a double-sided gradient of vancomycin and teicoplanin on a calibrated plastic strip. This method exploits both the slow growth of hGIS and the more pronounced resistance of hGIS to teicoplanin. The GRD E-test was validated for *S. aureus* and has a sensitivity of 89% and a specificity of 95% for detecting hGIS[93].

Paper 1 describes the use of the GRD E-test to detect S. *epidermidis* and *S. haemolyticus* hGIS. The GRD E-test identified a considerably higher proportion of hGIS compared to the macro method E-test.

Real-time PCR

Real-time PCR or quantitative PCR is a PCR-based method that both detects and quantifies a targeted DNA molecule. PCR consists of a series of repeated temperature changes, termed cycles. In each cycle, a heat-stable DNA polymerase amplifies a selected DNA region. The polymerase is targeted to the sequence to be copied by short DNA primers that hybridize to the DNA template at the beginning and end of the desired DNA sequence. The PCR is typically repeated for 25–40 cycles, resulting in logarithmic amplification of the selected gene[94].

In real-time PCR, a DNA probe with a florescent reporter tag is added to the PCR reaction. The probe comprises DNA that is complementary in sequence to the amplified DNA sequence. The fluorescent reporter is detectable by a laser only after the probe has hybridized with its complementary sequence. The number of cycles at which the fluorescence exceeds the threshold is called the threshold cycle.

In paper 3, DNA primers to amplify the *hld* gene were synthesized along with a probe with a fluorescent reporter tag. After purifying the DNA from bacterial isolates and from blood samples from patients with hematological malignancies, the *hld* gene was detected and quantified by real-time PCR. The results were reported as cycle threshold values.

Multilocus sequence typing

The multilocus sequencing typing (MLST) scheme for *S. epidermidis*[95] is a genetic typing method based on the sequences of polymorphisms of fragments of seven housekeeping genes. These fragments range from 412 to 465 base pairs. MLST is highly discriminatory and can be used to elucidate relationships between strains and to identify ancestral genotypes[96]. MLST is now considered the method of choice for investigating long-term genetic relationships within *S. epidermidis* populations[97]. The population structure of *S. epidermidis* as described by MLST is composed of a major and highly diverse lineage, the clonal complex 2 (CC2) lineage, which can be divided further into two subdivisions or clusters, CC2-I and CC2-II[98]. In previous studies, one sequence type (ST), termed ST2, CC2, cluster CC-I, has repeatedly been shown to represent the majority of methicillin-resistant *S. epidermidis* that cause health care-associated infections[99-104].

Paper 4 describes the use of MLST for genotyping 374 *S. epidermidis* isolates. Genomic DNA for PCR was extracted, and MLST was performed using primer sequences to amplify the 7 housekeeping genes *arC, aroE, gtr, mutS, pyrR, tpiA*, and *yqiL* as described by Thomas et al.[95] In addition, alternative primers were used to analyze *aroE*[105] and *tpiA*[106]. Nucleotide sequences were determined by direct automated sequencing, and the sequences were compared with the MLST database (http://www.mlst.net) to determine sequence types and clonal complex identity.

Results and discussion

Paper 1

Due to widespread methicillin-resistance in CoNS, vancomycin is typically used as empirical treatment of CoNS infections. The presence of staphylococci with reduced sensitivity to vancomycin has been associated with glycopeptide antibiotic treatment failure both in *S. aureus*[107-108] and in CoNS[109-110]. Since glycopeptide resistance in staphylococci is most commonly expressed as the hGIS/GIS phenotype, these bacteria are difficult to detect with standard susceptibility testing methods. Moreover, incremental increases in glycopeptide MIC values below the breakpoint for resistance, a phenomenon termed MIC creep, has been described for *S. aureus*[108].

In paper 1 we investigated whether an increase in glycopeptide MIC values over time for CoNS could be observed at our institution. All stored blood isolates of the two dominating species of CoNS in hematological malignancies, *S. epidermidis* and *S. haemolyticus*, that were isolated from patients with hematological malignancies who were treated at the University Hospital of Örebro from 1980 to 2009 were revaluated for the presence of reduced sensitivity to glycopeptides. Three methods were used to detect glycopeptide resistance: the standard E-test, the macro E-test, and the GRD E-test.

The MICs for glycopeptides as evaluated by the standard E-test were unchanged during the study period. However, a high incidence of hGIS was observed with both the macro E-test and the GRD E-test, and there was a trend towards an increasing incidence of hGIS during the last decade of the study period (Figure 6).

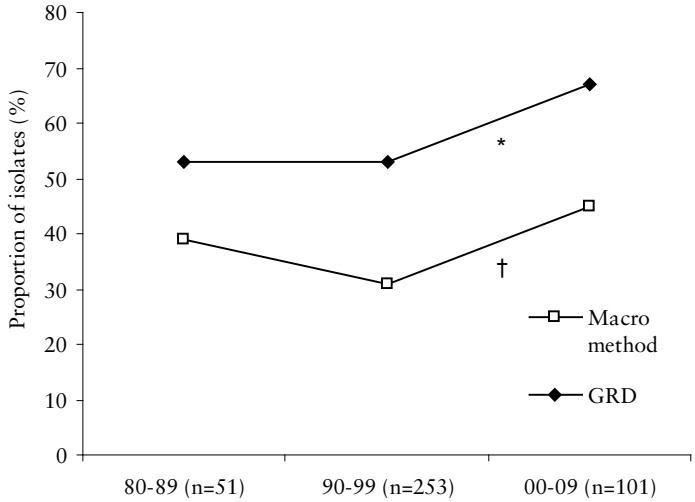

*Figure 6. The proportion of hGIS as determined by the macro method E-test and the GRD E-test over three decades (1980–2009). *p=0.027, †p=0.018.*

The study included the vast majority of isolates from patients with hematological malignancies during the study period. However, isolates that had not been stored at the time when the blood cultures were positive were excluded. In many cases, these isolates were duplicate cultures from the same infectious episode but could also be isolates that were recognized as contaminated cultures by the laboratory at the time of a positive blood culture. In this respect, there was a possible selection bias in that only isolates that were known to cause bacteremia were stored during the first period, since CoNS were not recognized as having pathogenic potential during this period. This may in turn exaggerate the findings of antibiotic resistance during the first decade, since resistance typically is more pronounced in isolates that cause confirmed infections[111]. Moreover, we were not able to assess whether the analyzed CoNS isolates represented true BSIs or contaminations.

In paper 4, the 387 isolates identified at the species level as *S. epidermidis* by API®/ID32 Staph were reevaluated using MALDI-TOF. Nine isolates were identified as a different species using MALDI-TOF compared to API®/ID32 Staph. Since MALDI-TOF can be considered a reference method for species determination in CoNS, the specificity of the API®/ID32 Staph is less than 100%.

The standard E-test generally results in 1- to 1.5-times higher MIC values compared to the gold standard method, broth micro dilution. There-

fore susceptibility testing would ideally be performed with broth micro dilution. The GRD E-test resulted in a high proportion of hGIS, since the breakpoint for teicoplanin MIC was relatively low (≥8). The test is not systematically validated for CoNS, and it is likely that the test gave raise to false positive hGIS as used in the study.

The results would ideally have been analyzed with a more robust statistical method that analyzed data for every year during the study period instead of grouping the years into decades. One such statistical method is logistic regression analysis in which the categorical binary outcome (hGIS or not hGIS) is the dependent variable and the blood culture year is the independent variable (Table 3).

Method	Odds ratio	*p*	95% Confidence Interval
Macro	1.04	0.007	1.01–1.08
GRD	1.06	0.001	1.02–1.09

Table 3. Logistic regression analysis with hGIS as the dependent variable and the sample collection year (1980–2009) as the independent variable. The odds ratio of detecting hGIS increased on average 1.05 times each year of the study period and by approximately 1.6 times over a ten-year period (1.05^{10}).

There is evidence that even a modest increase in vancomycin MIC may predict treatment failure on vancomycin in staphylococci infections[112]. Since the results indicate a successive emergence of glycopeptide resistance, there is a need for continuous monitoring of glycopeptide sensitivity in CoNS.

Paper 2

In order to develop strategies for the prevention of CoNS infections, the reservoirs and common transmission routes of CoNS must be better understood. It is generally agreed that the pathogenesis of CoNS infections involves CoNS strains that colonize the skin and mucous membranes[55]. The commensal flora of CoNS is relatively stable over time among healthy individuals[60]. In the community, there is extensive heterogeneity in this flora, and antibiotic resistance, such as methicillin resistance, is seldom detected[111]. However, hospital admissions[113-114], antibiotic treatment, and chemotherapy[113, 115] have all been associated with alterations in commensal flora.

Given the ubiquitous presence of CoNS on human skin and mucous membranes, the colonization patterns and therapy-related alterations of commensal flora have been difficult to describe; accordingly, only a few studies have investigated this topic prospectively[50, 116]. One major obstacle

has been that conventional genetic typing methods, including pulsed-field gel electrophoresis (PFGE), are expensive and labor-intensive considering the large amount of bacterial isolates that have to be investigated. Notably, previous phenotypic methods, such as the antibiogram, suffer from a low discriminatory capacity[117].

The aims of paper 2 were to prospectively describe the dynamics of the colonization patterns of CoNS and to try to characterize the relationship between colonizing and invasive CoNS isolates in patients undergoing treatment for hematological malignancies. Fourteen newly diagnosed patients with either multiple myeloma or acute leukemia were included in the study. Patients were repeatedly sampled for CoNS from skin and mucous membranes before, during, and after intensive chemotherapy. From each sample, 12 colonies of CoNS were sub-cultured. In addition, isolates of CoNS were collected in positive blood cultures from the included patients. All of the CoNS isolates were subsequently analyzed using the PhenePlate System. A total of 2047 isolates were analyzed.

All of the included patients developed pronounced alterations in CoNS flora during chemotherapy treatment. These alterations included a successive phenotypic homogenization and an accumulation of CoNS phenotypes that were present in several patients who were treated at different times. These changes were most notable in multiple myeloma patients before and after autologous stem cell transplant (ASCT). Moreover, among the multiple myeloma patients, the accumulated CoNS isolates had a multidrug-resistant phenotype that persisted several months after therapy ended. A few positive blood cultures with CoNS were obtained. Of these, many isolates were phenotypically identical to the CoNS isolates colonizing patients' skin and mucosa.

One mechanism that could explain the observed homogenization and accumulation of antibiotic-resistant CoNS isolates, at least in part, is that antimicrobial prophylaxis selects resistant CoNS isolates. The results would have been easier to analyze if a control group of patients had been included in the study. Such a control group would ideally be a patient group that was hospitalized for longer periods without receiving antimicrobial therapy. Moreover, in light of the results, it would have been most interesting to analyze colonizing CoNS on the hands of the health care personnel. Concerning the methodology, genotyping methods were unfeasible due to the large number of isolates analyzed. However, a limited number of isolates selected by PhP could have been analyzed with, for instance, MLST to improve the reproducibility and generalizability of the results.

To conclude, the results showed that a combination of hospital admissions, chemotherapy, and antimicrobial therapy is associated with alterations in the commensal flora. These alterations included gradual phenotypic homogenization and an accumulation of multidrug-resistant CoNS. These results are concordant with the theory that CoNS isolates acquired in the hospital environment cause invasive disease in patients with hematological malignancies.

Paper 3

Because of the ubiquitous prevalence of *S. epidermidis* on the human skin, growth of *S. epidermidis* in blood cultures may reflect either a true BSI or culture contamination. The most widely used microbiological criterion for discriminating a *S. epidermidis* BSI from a contamination is the number of positive blood culture sets compared with the total number of sets inoculated. Even though *S. epidermidis* more often grow in all obtained blood culture sets in presumed true BSIs compared with incidents classified as contaminations, there is a high degree of overlap, resulting in a low positive predictive value[118]. The positive predictive value can be improved by blood culturing methods that reflect the bacterial load. For example, short blood-culture incubation time to positivity has repeatedly been associated with true bacteremia, even though the threshold times have been varyiable[119-120].

In paper 3 a quantitative PCR method was developed for the detection and quantification of *S. epidermidis* DNA in blood samples. The *hld* gene was chosen to identify *S. epidermidis* DNA. As a hypothesis-generating study, 30 previously collected blood samples from patients with hematological malignancies were analyzed. The samples were obtained simultaneously with *S. epidermidis*-positive blood cultures. The aim was to investigate whether the detection and quantification of *S. epidermidis* DNA in blood could distinguish *S. epidermidis* BSIs from blood culture contaminations in patients with hematological malignancies.

Control samples showed that the *hld* gene is potentially specific for *S. epidermidis*. Episodes of positive blood cultures of *S. epidermidis* were classified as BSIs or contaminations by three criteria that used microbiological and clinical data: 1) clinical assessment, 2) CDC criteria for health care-associated BSI[121], and 3) Herwaldt criteria for CoNS BSI in neutropenic patients[122]. The *hld* gene was detected in 16 of 30 clinical blood samples with 26 to 38 PCR cycles. Overall there was no association of the detection and quantification of *hld* and episodes interpreted as BSIs with any of the three definitions (Figure 7).

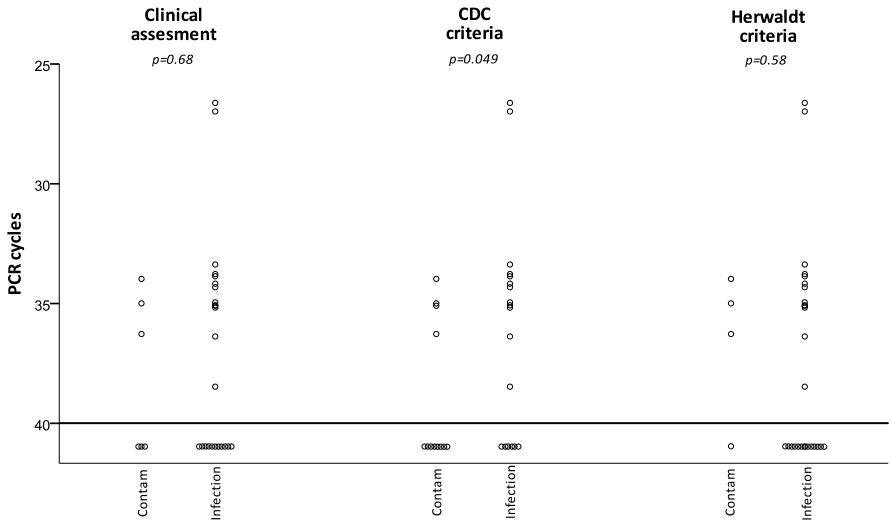

Figure 7. Cycle threshold values of hld PCR in 30 episodes of S. epidermidis-positive blood cultures in patients with hematological malignancies. The PCR was repeated for 40 cycles, and PCR-negative episodes are binned under the cut-off line. Episodes were classified as infection or blood culture contamination using three different criteria.

The results could potentially have been influenced by the long storage times of the blood samples. However, it is not likely that the main result would have been different had the storage time been shorter since *hld* was detected both in episodes interpreted as BSIs and as contaminations. The main disadvantage of the study design, as in any study in the field, is the lack of a clear definition of a significant *S. epidermidis* BSI.

In conclusion, the *hld* PCR failed to distinguish *S. epidermidis* BSIs from blood culture contaminations.

Paper 4

In paper 1, a marked variation in the incidence of *S. epidermidis*-positive blood cultures was observed in patients treated at the Division of Hematology at the Örebro University Hospital in 1980–2009. A corresponding incidence variation was observed for CoNS-positive blood cultures in the entire Örebro County (Figure 8).

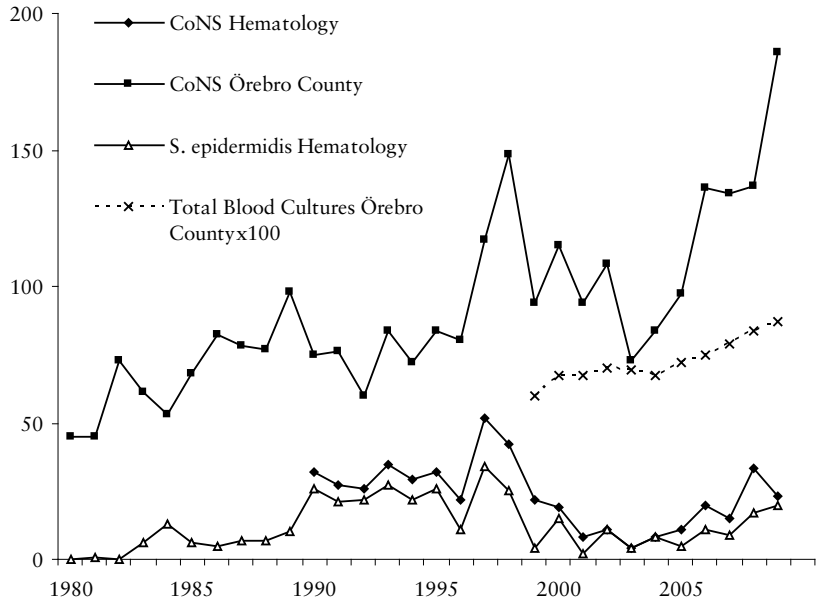

Figure 8. The incidence of blood cultures that were positive for CoNS in Örebro County, for CoNS at the Division of Hematology, Örebro University Hospital, and for S. epidermidis in patients with hematological malignancies at Örebro University Hospital in the indicated years. The total number of blood cultures in Örebro County in 1999-2009 is shown as well.

In paper 4, one of the aims was to clarify whether the observed incidence variation could be explained by specific *S. epidermidis* genotypes that occurred temporarily. Furthermore, since little is known about the long-term relatedness of clinical *S. epidermidis* isolates, an additional aim was to study the long-term molecular epidemiology of *S. epidermidis* isolates at our institution.

The same *S. epidermidis* isolates that were collected in 1980–2009 and used in the study described in paper 1 were analyzed by MLST and by standard antibiotic susceptibility testing.

The results showed that a majority of the isolates, 361/373 (97%), belonged to clonal complex 2. The 373 isolates included 45 sequence types. The predominant ST was ST2 (243/373; 65%). ST2 is a well-known ST type that causes infections in a wide range of populations worldwide and has been the most reported genotype in several studies[96, 99, 101-103, 123], especially in those describing BSI and catheter-related infections[102]. The second

most frequent ST was ST215 28/373 (8%). ST215 is reported to cause a wide range of infections in Sweden and Norway[100, 124].

Methicillin resistance was detected in 78% of all isolates. For the two predominating STs, ST2 and ST215, the proportion of methicillin resistance was 95% and 86% were multi-drug resistant.

Regarding the observed incidence variation of *S. epidermidis*-positive blood cultures, STs other than the two most prevalent STs, ST2 and ST215, were isolated at a relatively constant level during the study period (Figure 9). In contrast, ST2 and ST215 showed marked incidence variations, and these two STs jointly accounted for most of the incidence peak of *S. epidermidis*-positive blood cultures during the 1990s (Figure 9).

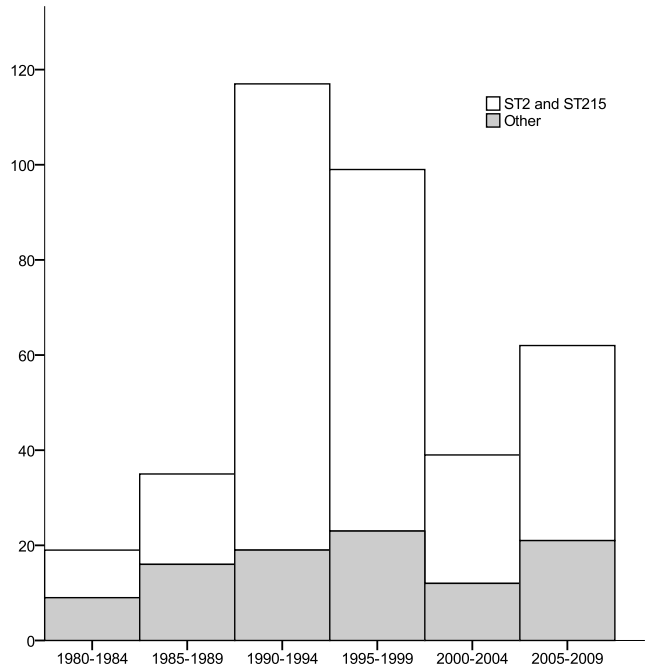

Figure 9. The incidence of S. epidermidis-positive blood cultures in the indicated time periods. Each bar represents a five-year period. The relative proportions of ST2/ST215 and other STs are shown using shading.

Because of the retrospective study design, we could not include *S. epidermidis* isolates that were not stored at the time of diagnosis. These isolates mainly represented multiple positive cultures from the same infection epi-

sode or isolates with growth in only one blood culture set that were interpreted as contamination at the time of diagnosis. Compared to later decades, fewer isolates were identified during the 1980s. This may indicate a selection bias in that a smaller proportion of *S. epidermidis* isolates may have been stored during the earliest time periods. Moreover, with the study design, it was not possible to assess whether the analyzed *S. epidermidis* isolates had caused true BSIs or were blood culture contaminants.

The data do not support the notion that transiently-occurring MLST genotypes caused the variation in the incidence of positive blood cultures. Since incidence peaks were caused by the most prevalent STs, ST2 and ST 215, these variations could be a consequence of transiently-occurring virulence traits of ST2 and ST215. However, it was more likely due to undetermined changes in patient care or in the health care facility. Because of the long-term dominance of the two STs, i.e. ST2 and ST215, these results provide additional evidence that CoNS infections in hematological malignancy are caused by genetically-related CoNS strains that are acquired in the health care facility.

General discussion and future directions

By nature, CoNS are harmless commensal bacteria on the skin that do not cause disease in humans. However, modern therapies, including chemotherapy, antimicrobial prophylaxis, and the use of indwelling devices such as CVCs, have turned CoNS into important pathogens in hematological malignancy. It seems that the success CoNS as pathogens in this population is due mainly to factors that normally sustain the commensal lifestyle rather than to aggressive virulence factors. The multiplicity of human skin flora and the high genetic recombination rate of many CoNS species make it difficult to study the pathogenesis of CoNS infections in hematological malignancy.

In papers 2 and 4, we provide additional evidence that CoNS infections are caused by genetically- and phenotypically-related CoNS strains that are acquired in the hospital environment. These CoNS strains generally possess a high degree of antibiotic resistance and have the capacity to maintain in the hospital milieu. Previous reports have indicated that such strains remain in health care facilities as colonizers of the hospital staff, but this needs to be confirmed in controlled studies. Common preventive measures for CoNS infections include rigorous hygienic routines and skin disinfection. However, eradicating commensal CoNS strains on the skin may allow re-colonization of potentially more harmful CoNS strains from the hospital environment. Further studies are needed to evaluate the best way of prevent these infections.

The diagnosis of a CoNS BSI in hematological malignancy is constrained by frequent CoNS culture contamination and by the lack of a clear definition of CoNS BSI. The diagnosis has recently been made easier by the use of culturing methods based on the bacterial load in blood. In study 3 we tried to develop this concept further by quantitative measurement of CoNS DNA in blood. Even though our study has limitations, it is not likely that quantitative PCR will improve BSI diagnosis. One way to improve the diagnosis would be to reevaluate bacterial markers of invasiveness. Since biofilm production is believed to be the most important virulence factor of CoNS, previous studies have mainly used biofilm production as a marker of invasive disease. Unfortunately, these attempts have not been successful, since not all isolates associated with disease produce this factor. Either the ideal marker of a true BSI is yet to be discovered or it could be that infection involves a combination of bacterial factors interacting with host factors and requires a more complex approach.

In general, CoNS can develop resistance to a wide variety of antibiotics. CoNS that cause invasive disease or CoNS collected in hospital environments have higher rates of resistance than commensal isolates, as described in paper 2. In paper 1, we describe the high prevalence of hGIS among blood culture isolates of CoNS. Although vancomycin is still effective in most cases of CoNS bacteremia, a high proportion of hGIS may indicate a emerging vancomycin resistance of clinical significance. Therefore, there is a need to evaluate new therapies for CoNS bacteremia. Of the newer agents already in use, linezolid is less attractive for use in patients with hematological malignancies due to its bone marrow toxicity. Daptomycin, like vancomycin, has a mode of action that affects bacterial cell wall synthesis, and there are reports indicating that daptomycin is less effective for the hGIS phenotype with its thickened cell wall. In this respect, the new-old concept of treating methicillin-resistant gram-positive bacteria with β-lactam is promising in light of its documented low toxicity in patients with hematological malignancies. Methods for preventing biofilm formation show promise as novel therapeutic strategies. Vaccination against the biofilm protein SesC decreases *S. epidermidis* biofilm formation *in vivo*[125]. *In vitro,* bacteriophage[126] and agr inhibitor-coated catheters have been shown to prevent biofilm formation[127].

Even though CoNS are common etiologies of bacterial infections in immunocompromised hosts, our knowledge of CoNS basic pathogenesis and epidemiology is limited. The main results of this thesis are consistent with that CoNS have established as important pathogens by:

- its natural capacity to colonize the human skin and mucous membranes
- its ability to reside in the hospital environment
- its rapid adaptation to different environments and stressors including antimicrobials
- its successful spread of genetically related and virulent strains in the hospital environment

We are only beginning to understand the complex interactions between CoNS, both as a harmless colonizer and invasive pathogen, and its human hosts.

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