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BLOODSTREAM INFECTIONS IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES

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Bloodstream infections in patients with hematological malignancies

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"The person who takes medicine must recover twice, once from the disease and once from the medicine."

/William Osler, M.D.

To my beloved family and for my patients

ABSTRACT

Patients with hematological malignancies have an increased risk of infectious complications. These complications can be caused by disease-specific factors or be treatment-related. Bloodstream infections increase the risk of morbidity, mortality, have a negative impact on quality of life, and may lead to reductions in treatment intensity. Surveillance studies on infectious complications and new technologies in diagnosing bloodstream infections are two important fields in improving management of patients with hematological malignancies.

Paper I: This is a retrospective study of positive blood cultures from patients mainly treated with dose-intensive antitumoural treatment between 2002 and 2008. Bacterial distribution, bacterial resistance and mortality from 667 fever episodes are presented. Results are compared with historical, previous published, material from the same institution and setting. Subsequently, temporal trends from 1980 to 2008 could be analysed. In a setting with very low use of fluoroquinolone-prophylaxis it can be concluded that; the distribution of Gram-positive bacteremia is stable, the crude mortality remains low in an international perspective and acquired resistance is uncommon but a significant increase in ciprofloxacin resistance in *Escherichia coli* is observed. The five most common bacteria in the study are; *E. coli*, coagulase-negative staphylococci, viridans streptococci, *Klebsiella* spp., and *Staphylococcus aureus*.

Paper II: This is a retrospective study that investigated temporal trends in bloodstream infections in patients with chronic lymphocytic leukemia between 1988-2008. We find a decrease in positive blood cultures over time and speculate if this could be due to more effective antitumoural treatment in recent years. Moreover a bloodstream infection is, as intuitively foreseen, associated with worse prognosis. Dominating pathogens in the study are; *E. coli, Streptococcus pneumoniae, P. aeruginosa, S. aureus,* and viridans streptococci. Coagulase-negative staphylococcus, a common skin contaminant, is the most frequently detected bacteria.

Paper III: This is a prospective study of 33 patients with aggressive hematological malignancies in need of dose-intensive chemotherapy. One hundred thirty blood samples were collected at different time points during episodes with neutropenia and fever between 2013 and 2014. Conventional blood culture findings were compared with a method applicable also for unculturable bacteria, 16S rRNA amplicon sequencing. Sequencing yielded reads belonging to Proteobacteria (55.2%), Firmicutes (33.4%), Actinobacteria (8.6%), Fusobacteria (0.4%), and Bacteroidetes (0.1%). The results display a much broader diversity of bacteria in bloodstream infections than expected. Changes in the relative abundance in the sequence data after commencement of antibiotics could be suggestive for a new method for estimating antibiotic efficacy. Lastly, the results are indicative for translocation, especially of gut microbiota, playing an important etiological factor in fever episodes in the neutropenic host.

Paper IV: This is a prospective study of 9 patients with acute leukemia in which we applied shotgun metagenomics for 27 blood samples collected during episodes of neutropenia and fever between 2013 and 2014. Shotgun metagenomics can characterize DNAemia and reconstruct unculturable microbial communities, resistance markers and gene ontology. The study confirms the method's applicability in bloodstream infections demonstrating bacteria, viruses and fungi. Furthermore, the observed dynamics of microbe sequences during fever episodes as well as gene ontology makes this diagnostic approach appealing for exploring the fever episodes in this patient category.

LIST OF SCIENTIFIC PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I.   **Kjellander C**, Björkholm M, Cherif H, Kalin M, Giske CG
     Low all-cause mortality and low occurrence of antimicrobial resistance in
     hematological patients with bacteremia receiving no antibacterial prophylaxis: a
     single-center study.
     Eur J Haematol. 2012;88:422-30.

II. **Kjellander C**, Björkholm M, Källman O, Giske CG, Weibull CE, Löve TJ,
     Landgren O, Kristinsson SY
     Bloodstream infections in patients with chronic lymphocytic leukemia: a longitudinal
     single-center study.
     Ann Hematol. 2016;95:871-9

III. Gyarmati P, **Kjellander C**, Aust C, Kalin M, Öhrmalm L, Giske CG
     Bacterial Landscape of Bloodstream Infections in Neutropenic Patients via High
     Throughput Sequencing.
     PLoS One. 2015;10:e0135756.

IV. Gyarmati P, **Kjellander C**, Aust C, Song Y, Öhrmalm L, Giske CG
     Metagenomics analysis of bloodstream infections in patients with acute leukemia
     and therapy-induced neutropenia.
     Sci Rep. 2016;6:23532

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LIST OF ABBREVIATIONS

16sRNA	16S ribosomal RNA
ALL	Acute lymphoblastic leukemia
alloSCT	Allogeneic stem cell transplantation
AML	Acute myeloid leukemia
ANC	Absolute neutrophil count
autoSCT	Autologous stem cell transplantation
BP	Base pair
BSI	Bloodstream infection
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CoNS	Coagulase negative staphylococci
DDD	Daily defined dose
ECDC	European Centre for Disease Prevention and Control
ESBL	Extended-spectrum β-lactamase
HCK Solna	Karolinska University Hospital Solna, hematology ward
HL	Hodgkin lymphoma
IFI	Invasive fungal infection
Karolinska Solna	Karolinska University Hospital Solna
MALDI-TOF MS	Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
MRSA	Methicillin-resistant *S. aureus*
NGS	Next generation sequencing
NHL	Non-Hodgkin lymphoma
OTU	Operational taxonomic units
PCR	Polymerase chain reaction
PE	Paired end
SCT	Stem cell transplantation
SLL	Small lymphocytic lymphoma
TKI	Tyrosine kinase inhibitor
VRE	Vancomycin-resistance enterococci

1 INTRODUCTION

Hematological malignancies, as a group, are the fourth most common cancer in Sweden. Yearly, approximately 2,400 patients are diagnosed, which is 7% of all diagnosed malignancies; a proportion comparable with other developed countries (1, 2). Hematological malignancies are responsible for approximately 8% of cancer-related deaths in Europe (3). Patients with hematological malignancies have increased risk for infectious complications attributed to inherent disease-, host-, and therapy-related factors. Also, serious infectious complication as the direct cause of death is a common manifestation of an end-stage, often refractory, hematological malignancy (4). In high-risk settings, such as after allogeneic stem cell transplantation (alloSCT), the cumulative incidence of death attributed to infection has significantly decreased over the years. However, infectious-related death within a year still remains at 1% for those transplanted between 1999 and 2001 (5). In low-income countries the risk of infection related death after antitumoural treatment is even higher (6). In addition, an infectious complication is an important cause of morbidity causing dose reduction and treatment delays, negatively effecting outcome (7).

The view of infectious complications and causative pathogens, in bloodstream infections (BSI) in particular, have changed over the last decades due to changes in antitumoural treatments, use of indwelling catheters, antimicrobial prophylaxis, and vaccination. BSI can be caused by bacteria, viruses and fungi. Sepsis is characterised by a life-threatening organ dysfunction caused by a dysregulated host response to infection (8). Microbial invasion of the bloodstream or the release of microbial products results in the deleterious host response. Sepsis ranks among top ten causes of death (9). Delayed initiation or delayed specific coverage of pathogens decreases the survival rate of patients with sepsis several-fold (10-12).

Knowledge of the local panorama of infectious complications and antibiotic resistance are essential parts of management of hematological malignancies. Today, effective treatment of suspected infectious complication is hampered due to limitations of diagnostic methodologies. For example; results of blood cultures might take days to receive and can only detect culturable bacteria and polymerase chain reaction (PCR) for pathogen detection in blood is associated with several problems regarding interpretation and accuracy, to mention a few (13).

Genome information derived from next generation sequencing (NGS) has revealed important information for hematological malignancies and will pave the way for personalized medicine (14). NGS offers, in the context of microbiology, means for identifying viable, dead and viable but unculturable bacteria, fungi and viruses. Moreover, NGS can retrieve information about resistance markers, virulence, antimicrobial treatment effect, new pathogens, and host-microbiome interactions, and hopefully unravel fever episodes where traditional microbial diagnostics have failed (15).

Management of suspected fever and prevention of infectious complications will doubtless also in the future be a challenge, and hopefully, a field of continuing improvement regarding the care of patients with hematological malignancies.

2 BACKGROUND

Hematological malignancies can be subdivided in numerous ways; for practical reasons and in regard of the patient population under study the following major subgroups are presented: acute myeloid (AML) and lymphoblastic leukemia (ALL), chronic myeloid (CML) and lymphocytic leukemia (CLL), and aggressive-, indolent non-Hodgkin lymphoma (NHL) and Hodgkin lymphoma (HL) (Figure 1). Epidemiology is presented in Figure 2.
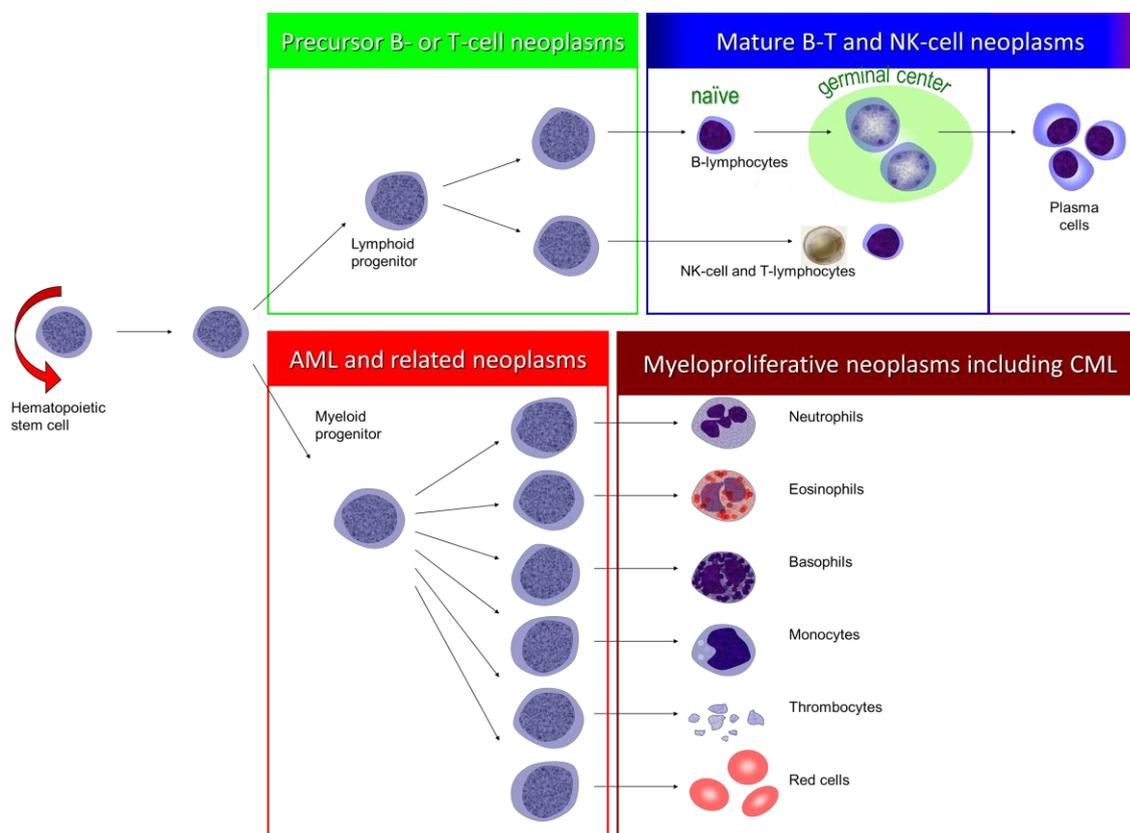

Figure 1. Hematological malignancies, adapted from Dr Dinesh Bhurani (16).

2.1 ACUTE LEUKEMIAS

Acute leukemia is characterized by the neoplastic proliferation of precursors to myeloid and lymphoid blood cells, respectively. The immature blast cells accumulate in the bone marrow and suppress normal hematopoiesis resulting in anemia and thrombocytopenia. Blast cells may or may not enter the bloodstream (leading to either high or low leukocyte counts) and can infiltrate other organs. Common manifestations of leukemia are fatigue (related to anemia), bleeding (due to thrombocytopenia) and infection due to secondary effects on the immune system.

2.1.1 Acute myeloid leukemia

AML is the most common form of acute leukemia in the adult, constituting 80% of leukemias in this group. This is contrast to the situation for children with acute leukemia, where <10% are AML (17). The majority of patients with AML receive dose-intensive chemotherapy resulting in profound immunosuppression: median time of neutropenia (absolute neutrophil count (ANC) $<0.5\times10^9$/L) is approximately 10 days (18). Prognosis is dependent on patient-related factors like age, performance status and comorbidities as well as diseases-related factors like cytogenetic aberrations. In general, children and elderly (>60 years) individuals have a poorer prognosis with overall survival of

approximately 65% and <13%, respectively (19, 20). Treatment is often divided into an induction phase, and a consolidation phase. The induction phase aims to eradicate the malignant cells, to induce a so-called complete remission, and is successful in this in over 60-90% in adult patients <60 years. A complete remission is often defined as ≤5% blast in the bone marrow with recovery of ANCs and thrombocytes. Consolidation treatment aims to reduce risk for relapse, which is substantial. This can be done through chemotherapy alone or in conjunction with autologous stem cell transplantation (autoSCT) or alloSCT. The cytotoxic regime given with stem cell transplantation mandates stem cell rescue to renew hematopoietic cell. Stem cell can either be the patient's own stem cells (autologous stem cells) or originate from a matched donor (allogeneic stem cells).
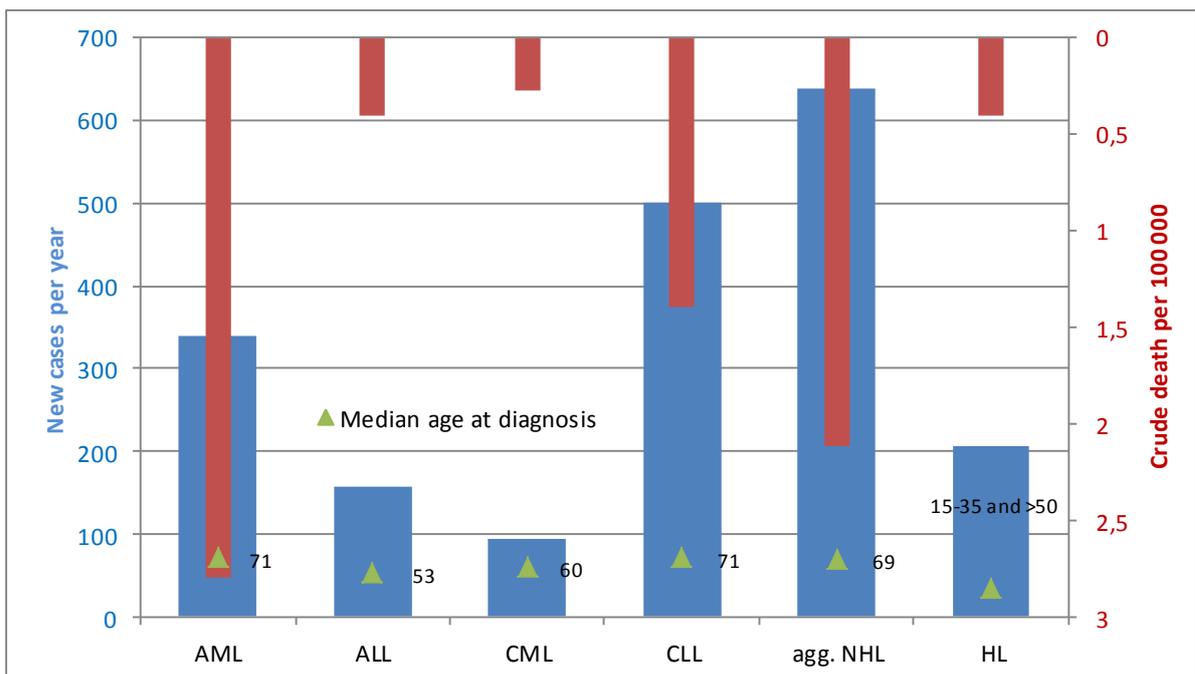

Figure 2. Epidemiological differences in hematological malignancies. Incidence and median age for adults were retrieved from National quality registries for respective diagnosis and estimated deaths per year were extracted from National Cancer Institute's (United States of America) database. Only for aggressive NHL (agg. NHL) death rate was retrieved form The National Board of Health and Welfare <Diffuse large-b-cell lymphoma>. AML (21, 22), ALL (23, 24), CML (25, 26), CLL (27, 28), agg. NHL (29, 30) and HL (31, 32).

2.1.2 Acute lymphoblastic leukemia

ALL is a clonal disease characterized by B-or T-cell origin. Leukemia is the most common malignancy in children (approximately 25% of all new cases) and the majority (90%) of pediatric leukemias are ALL. Even though remission rates of 80% (only slightly lower than for children) can be achieved in the adult setting the long term survival is only about 10%, which is in sharp contrast to the children setting (33). Bad prognostic markers are age (usually above 35 years), white blood count (usually greater than 30×10^9/L) and some cytogenetic aberrations. Approximately 30% of ALL carry the 9;22 chromosome translocation associated with poor prognosis that is also commonly (>90%) found in CML. Treatment constitutes of several antitumoural agents for intravenous and intrathecal administration. Also in ALL, high-risk patients can be subjects for alloSCT.

2.2 CHRONIC LEUKEMIAS

Chronic leukemias are commonly divided into CML and CLL, although other rare forms exist. Great progress in management has been made in the last decades (34, 35).

2.2.1 Chronic myeloid leukemia

CML is a clonal disease characterized by proliferation of mature granulocytes (neutrophils, eosinophils and basophils) and their precursors. The prevalence of CML is increasing due to introduction of effective treatment with tyrosine kinase inhibitors (TKI) (36). CML can be divided into three subgroups: chronic phase, accelerated phase and blastic phase. Patients in the two latter groups have the worst prognosis. According to the Swedish population-registry patients at diagnosis belong to corresponding group in 93%, 5% and 2%, respectively (37). For chronic phase CML standard therapy is lifelong TKIs. Interestingly, reports on discontinuation of TKI treatment with very good treatment free progression results are now appearing (38). For those patients that cannot tolerate or achieve a good response on TKI in chronic phase (and some in low risk accelerated phase) as well as those in blastic phase, alloSCT offers the best option for long-term survival (39). Patients in need of quick blast reduction, for example before alloSCT, are offered AML–like treatment. Patients with CML in chronic phase do not have an increased risk for infections (40).

2.2.2 Chronic lymphocytic leukemia

CLL originate from mature lymphocytes, most commonly B-lymphocytes. The natural history of CLL varies considerably: less than 30% of CLL patients never progress and die from an unrelated cause (41). Prognosis can be estimated with regard to disease stage, blood counts and cytogenetic abnormalities. Antitumoural management has constantly changed the last 25 years, and is still dynamic (42). Treatment today consists of chemotherapeutic agents (purine analogs and alkylating agents) and monoclonal antibodies (alemtuzumab, rituximab, ofatumumab and obinutuzumab). Recently cellular signaling pathway inhibitors have been approved, the Bruton tyrosine kinase inhibitor ibrutinib, phosphatidylinositol-3 kinase delta inhibitor idelalisib and the Bcl-2 inhibitor venetoclax, that substantially change the options for refractory, relapsed, comorbid, or elderly CLL patients. New check point inhibitors and engineered T-cells are in advances clinical trials. For physically fit patients with refractory CLL or with a high risk disease (i.e del17p or TP53 mutations) alloSCT might be an option if they relapse after a kinase inhibitor and respond to subsequent treatment. The pathogenesis of infections in CLL patients is multifactorial, and is related to inherent immune defects and therapy-related immunosuppression (43).

2.3 LYMPHOMAS

Lymphomas are a diverse group of malignant neoplasms that can be divided in indolent and aggressive NHL and HL (Figure 1). NHLs are derived from B cell progenitors, T cell progenitors, mature B cells, mature T cells, or (rarely) natural killer cells. HL arises from a neoplastic B cell that is surrounded by inflammatory cells.

2.3.1 Indolent non-Hodgkin lymphoma

Indolent NHLs represent a big heterogeneous group of insidious lymphomas often presenting with lymphadenopathy, hepatosplenomegaly, or cytopenia. Typical examples of NHL are follicular lymphoma, CLL/small lymphocytic lymphoma (SLL) and splenic marginal zone lymphoma.

CLL/SLL is considered the same entity but with different manifestation (the latter predominantly in lymphoid tissue outside the bone marrow compartment). There has been major improvement in therapeutic options but careful monitoring of asymptomatic patients remains the golden standard of management. Cure is often not possible but long time survival is common and risk of infectious complications varies widely (44).

2.3.2 Aggressive non-Hodgkin lymphoma

About half of NHLs are aggressive (45). If untreated, patients with aggressive NHL will succumb within weeks to months. Patient history often reveals typical associated night sweats, weight loss, fever and a rapid growing mass; and an elevated level of lactate dehydrogenase is often observed. Examples of aggressive lymphomas are diffuse large B cell lymphoma (25-30% of all lymphomas), Burkitt lymphoma, adult T cell leukemia/lymphoma, and precursor B- and T-lymphoblastic leukemia/lymphoma (46). Antitumoural treatment, often aiming for cure, constitutes of immunochemotherapy and in selected cases SCT, leading to a subsequent increased risk of serious infectious complications.

2.3.3 Hodgkin lymphoma

HL, accounts for <10% of all lymphomas (17). Classical prognostic factors are age, stage, tumor location, histology, and comorbidities. Primary antitumoural treatment for HL includes combined modality treatment (including radiotherapy and chemotherapy) for localized HL while patients with advanced disease will receive dose-intensive chemotherapy (47). Intensified chemotherapy followed by autoSCT is indicated if residual tumour activity or relapse is seen (48). Antibodies targeting CD30, small molecule inhibitors of cell signaling, and antibodies that inhibit immune checkpoints, have all demonstrated activity in HL (49).

2.4 ETIOLOGY OF BLOODSTREAM INFECTIONS

2.4.1 Definitions and incidence of bloodstream infections

BSI is defined as a positive blood culture in conjunction with symptoms of infection.

Fever is in clinical practice defined as a single oral (or tympanic) temperature of >38.3°C or temperature of ≥38.0°C sustained for 1 hour (50).

Neutropenia is herein defined as an ANC of $<0.5\times10^9$/L, or a count expected to decrease to $<0.5\times10^9$/L over the next 48 hours.

Fever accompanying therapy-induced neutropenia affects approximately 80% of those with hematological malignancy. A BSI is identified in 10-25% of fever episodes after chemotherapy, and in up to 60% following SCT (50-52). During therapy-induced neutropenia, the crude mortality rate of BSI is about 15% (53, 54).
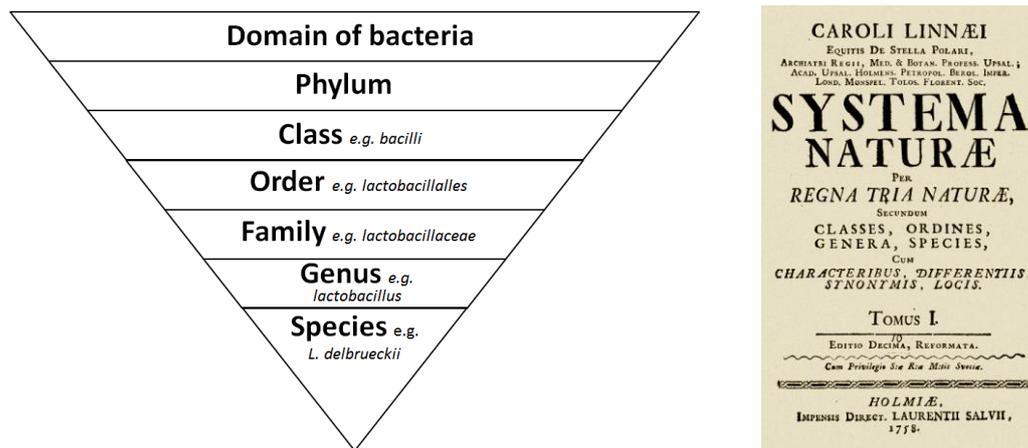
2.4.2 Taxonomy of microorganisms

Figure 3. The taxonomy of bacteria based on the work of Carl von Linné (55).

Based on Carl von Linné's work, organisms are categorized into three domains: eukarya, bacteria and archae. The domain eukarya encompasses several kingdoms, for example fungi and animals. The domain of bacteria consists of 30 phyla which can be further subcategorized into class (for example bacilli), order (for example *Lactobacillalles*), family (*Lactobacillaceae*), genus (Lactobacillus), and species (*L. delbrueckii)* (Figure 3).

2.4.3 Bacteria

The vast majority of all BSI constitutes of bacteria. Historically, *S. aureus* was the most common cause of fatal infection in patients undergoing leukemia treatment. Following the introduction of routine use of β-lactam antibiotics Gram-negative bacteremia, *E. coli*, *Klebsiella* spp., and *P. aeruginosa* dominated in the 1960s and mid 1970s (56). Resistance was low and empirical treatment often constituted of penicillin, or a first generation cephalosporin, with or without an aminoglycoside. During the 1980s and early 1990s Gram-positive bacteria; CoNS, *S. aureus*, enterococci and viridans streptococci dominated (57). During the last two decades our center showed a stable Gram-positive distribution (58), but others in Europe and elsewhere, again, report increasing Gram-negative proportions (59-61).

2.4.4 Fungi

Both yeasts and moulds are associated with an increased morbidity and mortality risk in immunocompromised patients. Invasive fungal infections (IFI) are not infrequently found at autopsy, and only seldomn detected ante mortem (62). Neutropenia (for >10 days), SCT, prolonged treatment (>4 weeks) with corticosteroids, drugs or conditions that lead to chronically impaired cellular immune responses are some of the more major risk factors for IFI. In the hematology setting *Candida* spp., *Aspergillus* spp., Zygomycetes, *Cryptococcus* spp. and *Fusarium* spp. are the most commonly occurring pathogens (63). Conventional histopathological examination and mycological methods for identification (direct microscopy or culture of samples) are often not possible (due to for example thrombocytopenia and need of invasive procedures) or associated with low sensitivity. Nevertheless, a positive culture with susceptibility testing can help to optimize treatment (64). Treatment delays dramatically correlate with increased mortality rates, and therefore molecular methods and algorithms for assessing fungal risk have been developed (65, 66).

1,3-Beta-D-glucan (BDG) is a cell wall component found in a wide variety of fungi that can be analysed from blood; notable exceptions are *Cryptococcus* spp. and Zygomycetes (67, 68). Its favorable negative predictive value could be useful in high risk setting, although, high rates of false positive results of BDG test hampers its utility (69). Galactomannan, another cell wall component that can be sampled from blood, is relatively specific for *Aspergillus* spp. but the test, as BDG, is associated with false positive results (moderate sensitivity) (70).

PCR based methods for identification of fungi are rapid and promising, with high sensitivity and specificity (71, 72). Lack of standardization, difficulty in distinguishing between contamination and true IFI are some of the issues that need to be solved. The ongoing optimization of algorithms for diagnosing IFI will probably use combination of techniques to improve antifungal treatment (73, 74).

2.4.5 Viruses

In 70-90 % of fever episodes accompanying neutropenia no causative microorganism can be identified in blood cultures (75). In a study from our department a viral finding (nasopharynx aspirate or blood) was found in 42% of the fever episodes (76). However, not all viruses give rise to fever and finding a virus with a low titer might have no clinical consequence (77).

Viral infections of importance, developing in the neutropenic time period, include cytomegalovirus infection, herpes virus, varicella zoster virus infection and community acquired respiratory virus infection.

2.4.6 Catheter related infections

Various forms of intravascular devices are essential in the management of patients with hematological malignancies receiving chemotherapy, transfusion therapy or parenteral nutrition. Central venous catheters can be non-tunneled (fixed in place at site on insertion) or more preferred tunneled (passed under skin from insertion). A subcutaneous port has a membranous septum under the skin and is accessed through intact skin. Among the more serious complications are central line-associated bloodstream infections. This is commonly defined (without the removal of the catheter) as growth of the same bacteria in cultures drawn from peripheral and central lines, with the growth of the bacteria in the central line growing two hours earlier than in the peripheral culture, and with no other apparent site of infection other than the catheter (78).

2.5 ANTIBACTERIAL TREATMENT

Classic signs of infection might be missing due to the immunocompromised host's inability to mount an adequate inflammatory response, or concomitant steroids or other anti-inflammatory medication. The aim of the empirical treatment is to cover the most likely and virulent pathogens. Choice of treatment also needs to take into account the patient's history, allergies, signs, symptoms, recent microbiological findings, antibiotic use and antibiotic resistance. International guidelines advocate empirical antibacterial therapy to be initiated in patients with suspected neutropenic sepsis without delay or within 60 minutes from presentation (79-81).

Regular monitoring of local pathogens and antibiotic resistance is pivotal in clinical care of immunocompromised patients.

Internationally, the following different antibacterial approaches have been applied for inpatient treatment: 1) combination of penicillin and an aminoglycoside 2) monotherapy with third or fourth generation cephalosporin 3) combination of a third or fourth generation cephalosporin and an aminoglycoside 4) triple combination therapy containing a combination of a third or fourth generation cephalosporin, aminoglycoside, and glycopeptides (50, 82, 83).

During recent years a more risk-adapted approach for choosing antibacterial treatment, venue and route of administration, has gained popularity. An example is the validated Multinational Association for Supportive Care in Cancer risk index (MASCC risk index score) (50, 84). Risk-stratification allows the identification of a sub-population of neutropenic fever patients who can be treated at home with either intravenous monotherapy (often ceftriaxone, dosing once daily) or oral antibiotics (often a combination of fluoroquinolone + amoxicillin-clavulanate, or fluoroquinolone monotherapy).

Moreover, in the general intensive care unit 15-30% of patients with BSI are estimated to receive inappropriate empirical antibiotics (85). In patients with hematological malignancies inappropriate empirical therapy of BSI has been associated with inferior outcome (86).

Interestingly, in our inpatient ward for hematology patients at Karolinska University Hospital in Solna (HCK Solna) daily defined dose (DDD) of antibiotics have changed in the last decade (Figure 4). In the beginning of the period the ward had 24 beds, dropping throughout the period, and in the end of the period approximately 14 beds remained. The proportions hematological diagnoses have though been stable. The DDD of meropenem and piperacillin-tazobactam has increased counterbalancing the decrease of ceftazidime, probably reflecting the notion that ceftazidime has a poor effect against viridans streptococci. Around 2006-2007 an increase of ciprofloxacin, our most commonly prescribed fluoroquinolone, was observed reflecting a change in policy of antibiotic prophylaxis in high risk patients. The peak between 2013 and 2014 in the use of ciprofloxacin could reflect an extreme situation where the HCK Solna and Karolinska Solna were short of beds for
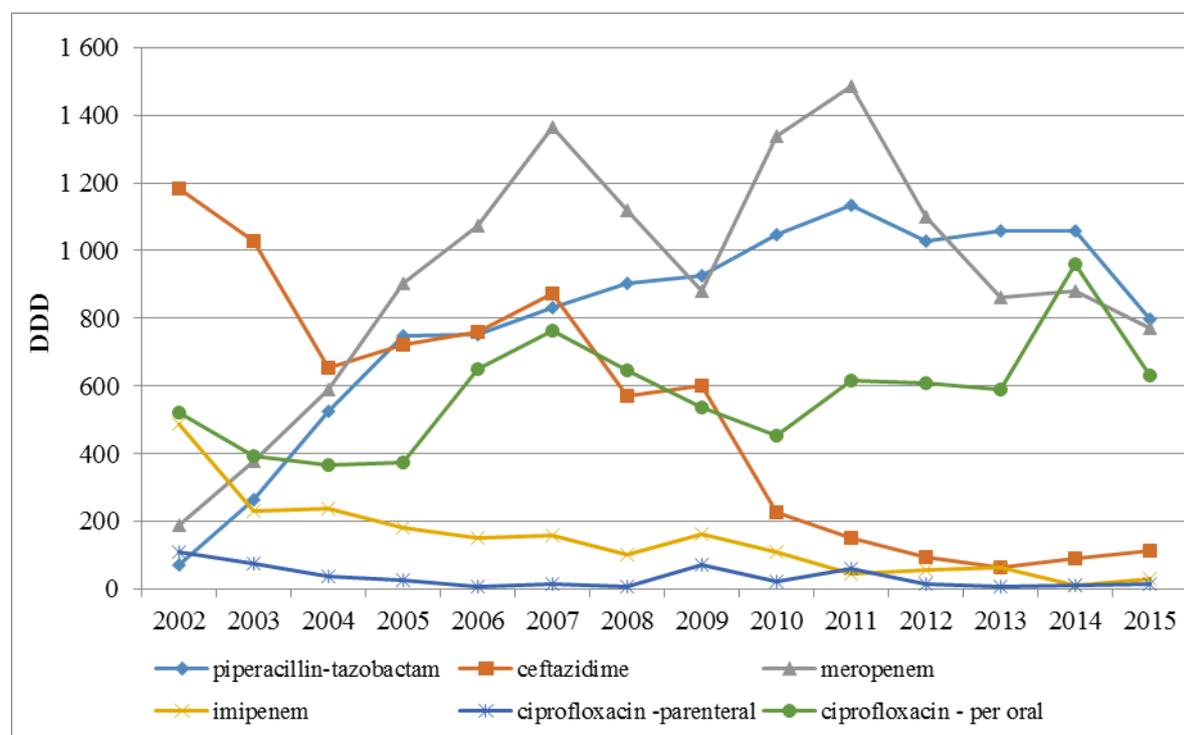

Figure 4. DDD of prescribed antibiotics from the hematology inpatient ward, Karolinska Solna between 2002 and 2015.

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patients with subsequent locally broadened indication of ciprofloxacin to also include less high risk situations.

2.6 ANTIBIOTIC RESISTANCE

Resistance patterns for bacterial BSI in hematological patients follow the resistance patterns of their countries in general. International generalization on outcome of infectious complications is therefore hampered.

2.6.1 Gram-negative bacteria

Among the Gram-negative pathogens, resistance to *P. aeruginosa, E. coli*, *Klebsiella* spp.*, Enterobacter* spp. are of special interest. In our practice the majority of episodes of neutropenic fever episodes are initiated on empirical broadspectrum antibiotics including anti-pseudomonal coverage, often with piperazillin-tazobactam or carbapenem, according to local guidelines. Figures 5,6 and 7 illustrate resistance trends for *P. aeruginosa, E. coli,* and *Klebsiella* spp. based on; blood cultures taken between 2002 and 2008 from HCK Solna, the Karolinska University Hospital Solna (Karolinska Solna) reported data, and nationally reported data to the "Antimicrobial resistance interactive
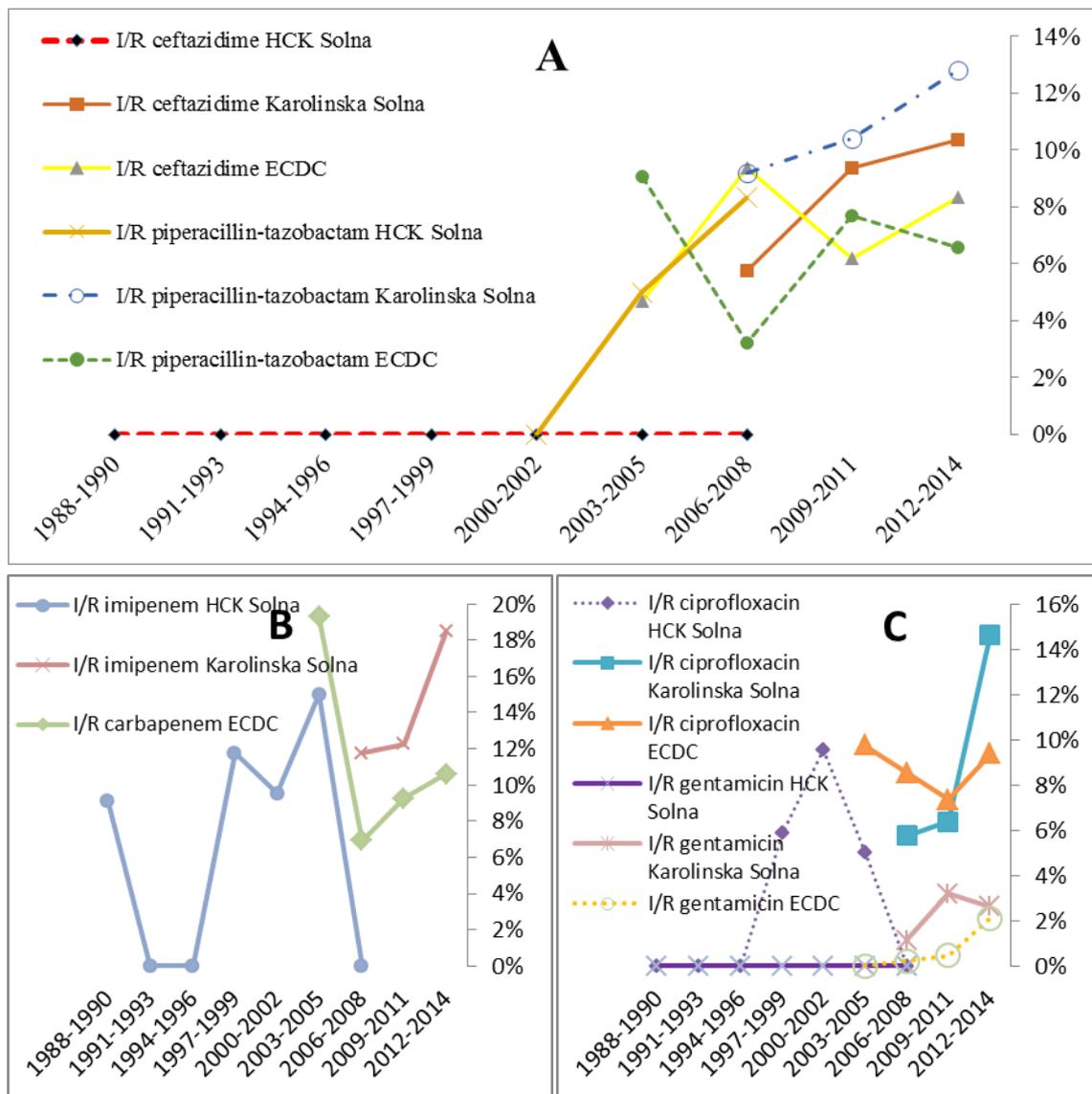

Figure 5. (A,B,C) Pattern of resistance for *P. aeruginosa.*

database" of the European Centre for Disease Prevention and Control (ECDC) (58, 87, 88). *Stenotrophomonas maltophilia* is inherently resistant to most antibiotics except trimethoprim-sulfamethoxazole and ticarcillin-clavulanate (89). *Enterobacter* spp. is together with the other *Enterobacteriaceae* important carriers of extended-spectrum β-lactamases (ESBLs). ESBLs can break down penicillins, cephalosporins, monobactams and sometimes carbapenems (90). Growth of ESBL producing bacteria is by law required to be reported to a national registry. ESBL incidence is increasing in Sweden (Figure 8). For *Acinetobacter* spp. resistance mechanisms for merely all antibiotics have been described (91).
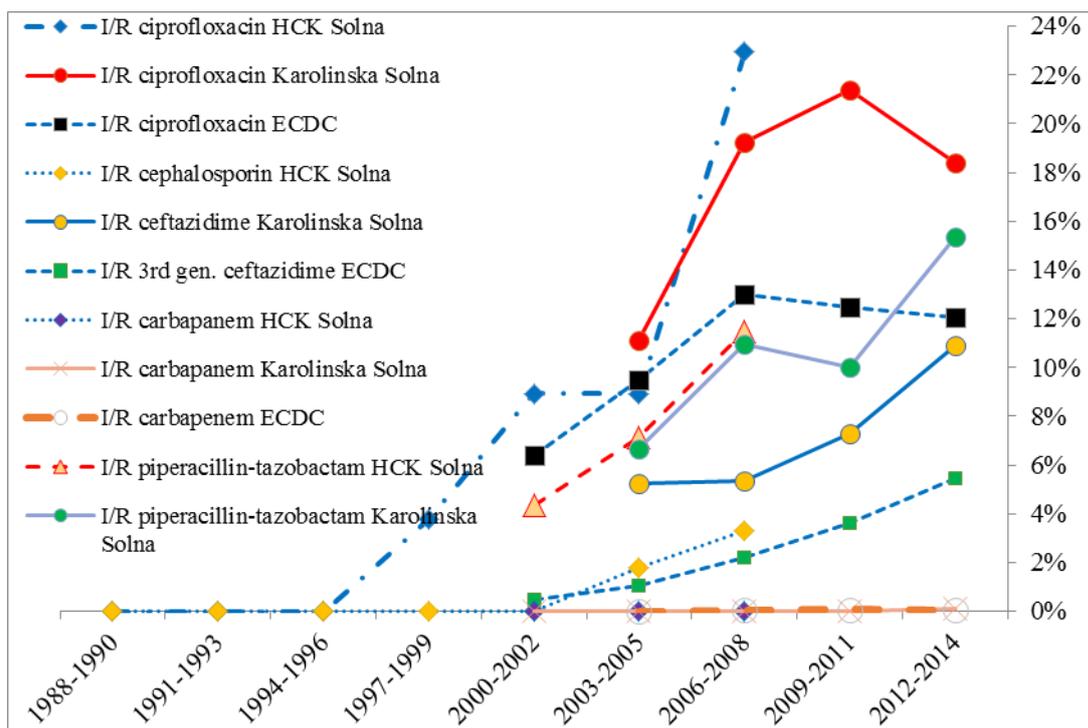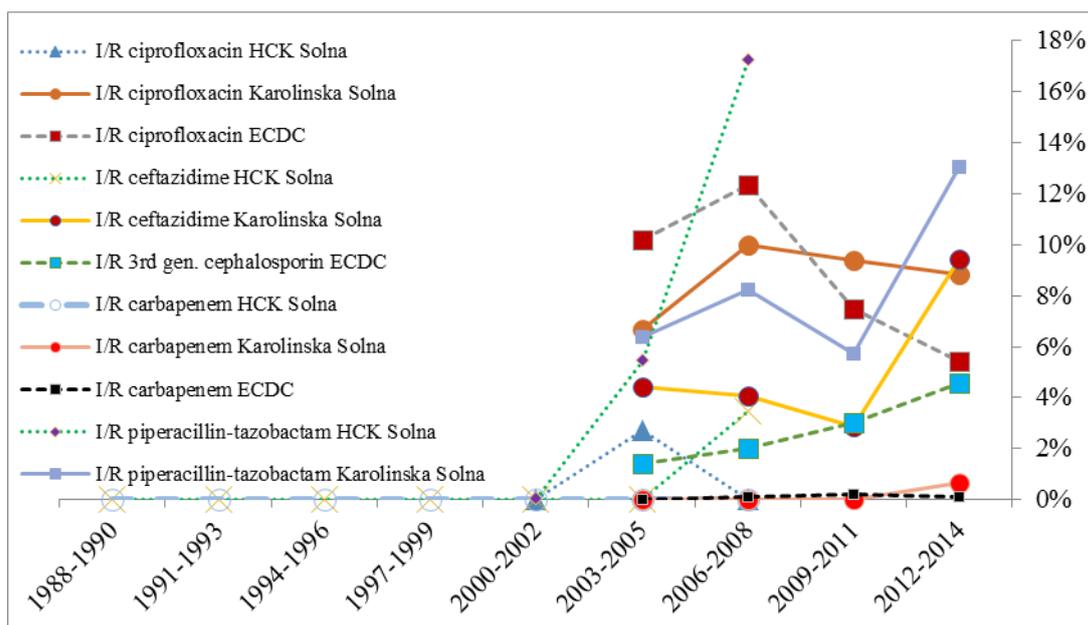

Figure 6. Pattern of resistance for *E. coli.*

Figure 7. Pattern of resistance for *K. pneumoniae.*

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2.6.2 Gram-positive bacteria

Among the Gram-positive pathogens, resistance to staphylococci and enterococci are of special importance. CoNS is among the most frequently identified pathogen in BSI, but is associated with a very low morbidity (92). Nationally reported methicillin-resistant *S. aureus* (MRSA) to ECDC has been constantly low (≤1%) for the last decade. However the incidence is increasing (Figure 8). Enterococcus species, colonizing the intestinal tract, are considered to possess low virulence, but they are significant in terms of multi-drug resistance, predominantly vancomycin-resistant enterococci (VRE) (Figure 8). From 2009 to 2015 VRE in blood culture has been documented on one occasion at Karolinska Solna. Nationally reported incidence of VRE to the ECDC for the last decade is <1% (87, 88).
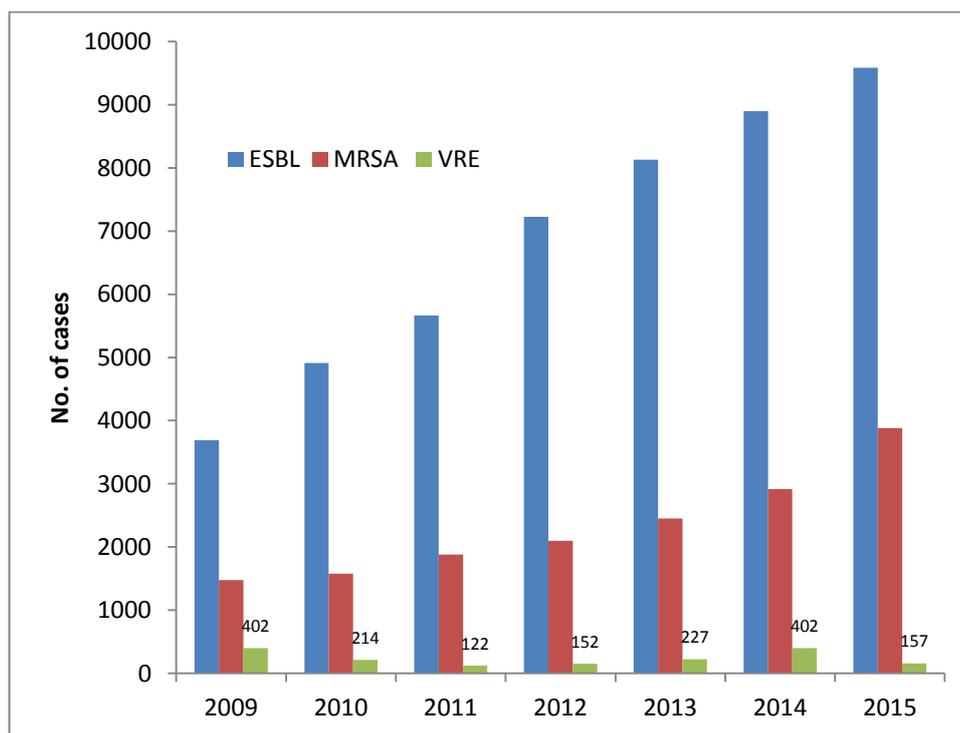

Figure 8. Notifiable resistance in Sweden from 2009 to 2015(Public Health Agency of Sweden (www.folkhalsomyndigheten.se))

3 DIAGNOSING BLOODSTREAM INFECTIONS

3.1 BLOOD CULTURE

Blood cultures are routinely drawn from patients with suspected BSI. Among positive blood cultures bacteria are most commonly found but also fungi can be found. Despite the knowledge of the importance of a positive blood culture little has changed in methods of blood culturing, even though incremental progress has been made in pathogen identification by other techniques and susceptibility testing. Continuous blood culture monitoring systems, in Karolinska University Hospital BacT-ALERT (bioMérieux, France), were introduced in the 1990s. Inoculated blood culture vials are loaded on the system, incubated, and monitored for production of CO_2. A change in CO_2 concentration implies biological activity and a positive signal is generated for a predefined threshold. Positive vials are removed from the system and stained with Gram stain to differentiate Gram-positive and Gram-negative organisms. Additional microscopy studies can reveal additional characteristics (morphology and growth pattern) that aid the microbiologist in an early attempt (<12

hours from drawn blood culture) to guide empirical antibiotic therapy. After Gram stain, culture plates can be removed for an attempt of direct identification, based on biochemical characteristics, and susceptibility testing. However, sensitivity is less than using cultured colonies of microorganisms. With conventional methods, a preliminary report on bacterial identification and susceptibility can be made after 18-24 hours and a more definitive usually after 24 hours. Standard incubation in automated monitoring blood culture systems is 5 days.

Guidelines recommend, whenever possible, two to four sets of blood culture bottles to be collected. Viable microbial concentrations in patients with BSI are low and recovered microbial yield is proportional to drawn blood culture volume (93-95). Several key factors may lower contamination rates (goal is <5%): adherence to protocols, sampling from peripheral vein compared to through central venous access, use of sterile gloves, using antiseptics on tops of blood culture bottles and using dedicated phlebotomy teams (96-99).

Recovering fungi from blood cultures can be more troublesome, as the optimal growth temperature and blood culture media varies. Most automated blood culture systems enable growth of yeast, for example *Candida* spp. But if the suspicion is strong for yeast, dimorphic fungi or moulds alternative culture methods should be employed (100). Finally, blood culture growth is impeded in patients on antibiotics and polymicrobial bacteremia (101).)

With above-mentioned limitations for different microorganisms, interpreting a positive blood culture is rarely problematic. In case that a majority of independent drawn blood culture bottles are positive with the same microorganism, the likelihood for a true (i.e. not contamination or unknown significance) BSI is extremely high. Additionally, isolations of certain organisms are also predictive: *S. aureus*, *Enterobacteriaceae*, *S. pneumoniae*, *P. aeruginosa* and *Candida albicans* represent almost always a true BSI. Conversely, from a multicenter study CoNS was isolated in 38% of positive blood cultures but was only considered a pathogen in 10% of cases (102). Similarly, very few of *Corynbacterium* spp., *Bacillus* spp., *Micrococcus* spp., *Lactobacillus* spp. and *Propionbacterium* spp. were considered true pathogens. In a high risk clinical setting, as immunocompromised patients with a hematological malignancy, BSI for skin pathogens mostly requires two positive sets with the same antibiogram or that pathogens are associated with appropriate clinical findings when available (61, 103).

3.2 MUTLIPLEX POLYMERASE CHAIN REACTION

Current methods for diagnosing bloodstream infection are limited in their diagnostic capabilities and timeliness. Molecular methods that target conserved regions of microbial genomes for amplification have been developed. Although they were shown to be useful for detecting blood culture-negative endocarditis (104), no PCR-system has yet, been described to replace blood culture in the setting of BSI due to mainly low and thus unsatisfactory specificity (105, 106).

3.3 MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY

It was proposed already in 1975 that the mass spectrometry could be used in microbiology (107). The last two decades commercial use of Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) was enabled by the discovery of, by the Nobel Prize laureate Kochi Tanaka in 1985, its use on macromolecules. MALDI-TOF MS process begins with

that a microbial colony, or processed clinical specimens, are mixed with a chemical matrix on a MALDI-plate, crystallized and pulsed with a laser (108). The matrix facilitates the ionization process. The laser energy ionizes and ablates the molecules form the sample. In the next phase the time the different molecules travel in an electric field is recorded by the spectrometer. Large molecules travel slower and signal can be converted in a mass (in Dalton) and its intensity correlated with abundance. The mass of the molecule is then checked against a database for phylogenetic characterization. In microbiology MALDI-TOF MS enables quick identification (minutes) of many clinical relevant bacteria and fungi with low false positive results to a low cost. Comparing MADI-TOF MS identification of bacteria to the species and genus level with traditional blood cultures reveals concordance in 80.1 and 87.7%, respectively, for Gram-negative better than for Gram-positive, and for fungi even better (109-111). Some species, already hard do differentiate with biochemical methods, are also hard to distinguish with MALDI-TOF MS due to close relationship, like for example differentiating *Streptococcus mitis* group and *S. pneumoniae*, *Shigella* spp. and *E. coli*, and *Listeria* spp. from each other. Lastly, MALDI-TOF MS has been shown to be less reliable in polymicrobial bloodstream infections (109). Coupling PCR with mass spectrometry has recently been explored for diagnosing bloodstream infections with promising results (112).

3.4 METAGENOMICS

Understanding microbial diversity has been the goal for scientists for decades, but these studies focused only on culturable bacteria. Genomics, with the use of NGS, is a relatively new technology that uses recombinant DNA, DNA sequencing and bioinformatics to analyse the genome. Metagenomics can characterize the genome of microbial communities directly from the environment, as for example the human blood or stool.

The human body contains approximately equal numbers of bacteria and human cells and the human microbiome (bacteria, viruses and fungi) is important in maintaining health (113). Genome characterization started with the bacteriophage by Sanger sequencing in the 1970's (114). In 1986 the first automated apparatus for capillary electrophoresis was introduced which could deliver 1,000bp/day. Until 2005, with developments in sequence terminator chemistry and moving from gel- to capillary electrophoresis with obtainable sequence lengths of ~800bp, Sanger sequencing was the dominant sequencing technique. In 1995 the first whole genome of a strain of *Haemophilus influenzae* was sequenced and in 2001, the human genome (115, 116). Since 2005 methods have developed that massively increase the sequence data, therefore called NGS (117). The platforms share core similarities: 1) no cloning of DNA is required 2) DNA templates are spatially segregated and no physical separation step is needed and 3) DNA is sequenced through synthesis. Platforms differ in how to build template libraries, generating a signal, throughput (amount of sequenced bp pair per run), read lengths and error rates.

The two main approaches for identifying microorganisms remain the amplicon sequencing (mostly restricted to the 16S ribosomal RNA (16sRNA) or 18S ribosomal RNA in bacteria and fungi, respectively) and shotgun metagenomics, which allows untargeted sequencing of DNA in a given sample. Major challenges in metagenomics of human blood samples include 1) low pathogen DNA load versus human DNA 2) inhibitory compounds in whole blood, and 3) contamination and/or bacterial origin from certain reagents (118, 119). Furthermore, most commercial products for identification of microorganism do not immediately report on the viability of the identified microbes. Retrieving viability information requires additional analyses as for example, repetitive

measures (to quantitate bacterial DNA clearance quantitatively) or dyeing the DNA with propidium monoazide (dye only binds to compromised membranes), which inhibits PCR amplification (120).

3.5 AMPLICON SEQUENCING: 16S RIBOSOMAL RNA

The 16sRNA gene, around 1,550bp long, is present in all bacteria and is composed of interspersed variable regions flanked by relatively conserved regions. Probes can be designed to hybridize the conservative regions of the extracted DNA for PCR amplification, and subsequent sequencing of the variable regions allows identification of different bacteria. For most clinical isolates, the initial 500bp is enough for bacterial differentiation (121). For the Illumina technology, used in our laboratory, the next step is a simultaneous tag- and fragmentation. The modified molecule bounds to the complementary oligonucleotide on a flow cell. Next step is a parallel synthesis of a library that can work as a template for the fluorescently tagged nucleotides during sequencing. Only one complementary fluorescent nucleotide can bind per cycle, the rest is washed away. At the end of a cycle a camera takes a shot of the chip and a computer can, based on the wavelength and intensity emitted, determine which base and where it was produced.

Bioinformatic analysis begins with quality checking and preprocessing of reads. Reads with similar base calls are aligned and those who are similar are clustered, forming operational taxonomic units (OTUs). OTUs are defined by how the sequences under a particular percentage diverge from each other. OTUs are annotated by comparison with well-known database libraries via analytic software tools or the World Wide Web, for example from the Ribosomal Database project (122).

3.6 SHOTGUN METAGENOMICS

With shotgun metagenomics the entire DNA content of a sample, instead of a targeted locus, is fragmented, and then the fragments are sequenced in parallel. The way reads are produced depends on which NGS-method is used. In metagenomics the reads usually vary between 100 and 400bp, depending on the NGS-method used. Reads with similar base calls are assembled, forming contigs, Taxonomic information is ascribed to each contig by sequence comparison with well-known database libraries via the World Wide Web, for example GenBank (http://www.ncbi.nlm.nih.gov/) for identification and variant analysis. Potentially, shotgun metagenomics can not only describe taxonomic diversity, but also functionality, since entire microbial genes can be assembled (123).

4 STUDY AIMS

With the goal of improving management of infectious complication in patients with hematological malignancies studies with the following objectives were conducted

Retrospective epidemiological studies

- Defining trends in bacterial BSI: pathogen distribution, antibiotic resistance and mortality in patients with aggressive hematological malignancies in general (I)
- Defining bacterial distribution and mortality in BSI in patients with CLL (II)

Prospective comparative cross-sectional and longitudinal studies

- Revealing bacterial DNA in BSI in hematological malignancies during chemotherapy-induced neutropenia by the use of NGS and conventional methods (blood cultures) (III)
- Investigating microbial DNA in BSI at different time points during chemotherapy-induced neutropenia in hematological malignancies (IV)

5 RETROSPECTIVE COHORT STUDIES (I, II)

5.1 MATERIAL AND METHODS

5.1.1 Study I

In this study we investigated 10,071 blood cultures from 1,855 patients sampled between 2002 and 2008 from HCK Solna. Out tertiary urban hospital offers a variety of antitumoural treatments for adults according to national guidelines, excluding alloSCT. For temporal trends patients were compared with earlier published data (1980-86 and 1988-2001) from the same institution. Roughly, 1/3 of patients were diagnosed with malignant lymphoma, 1/3 with leukemia and 1/3 with myeloma and CLL. Sampling indication and procedures have been unchanged during observed periods.

Study patients were identified through the laboratory information system. Antimicrobial prophylaxis, most importantly fluoroquinolone-prophylaxis, has been low and unchanged during investigated and compared study periods. Laboratory procedures for detecting positive blood cultures were automated in 1993.

A positive blood culture was defined by the presence of bacteria (1) other than typical skin contaminants in at least one blood culture or (2) in two blood cultures from the same fever episode (same day). A polymicrobial bacteremia episode was defined by growth of more than one bacterial species within 24 hours. Growth of the same bacteria <7 days was not considered a new positive blood culture.

Isolated strains had antibiogram determined; either as susceptible (S), intermediate (I) or resistant (R), according to the Swedish Reference Group for Antibiotics. Isolated bacteria was considered resistant (I or R) if reduced susceptibility was observed in isolates belonging to the following species: imipenem, piperacillin-tazobactam or metronidazole for anaerobic bacteria; piperacillin-tazobactam, ceftazidime or ciprofloxacin for *Enterobacteriaceae* and *Pseudomonas* spp.; vancomycin for *Enterococcus faecium*; isoxazolyl-penicillin for *S. aureus*; trimethoprim-sulfamethoxazole for *S. maltophilia*; and benzyl-penicillin for viridans streptococci, β-hemolytic streptococci and S. *pneumoniae*. Due to lack of adequate methodology for studying low-grade vancomycin resistance in staphylococci in previous years (disc diffusion can only detect high-grade resistance) a proper comparison of resistance levels could not be conducted.

For statistical analysis comparing categorical variables we used the two-tailed Fisher's exact test. P-values <0.05 were considered statistically significant

5.1.2 Study II

Individuals who had a blood culture analysed between 1988 and 2006 at the Karolinska University Hospital's Clinical Microbiology Laboratory were linked to national Swedish Cancer Registry (ICD 7 204.1). 275 patients (1,092 blood cultures) had a preceding diagnosis of CLL.

A bloodstream infection was defined as growth of any microorganism excluding common skin contaminants. Growth of the same microorganism within seven days was considered as the same BSI episode. Identified individuals were linked to nationwide Cause of Death Registry to retrieve information on death and the Swedish Patient Registry to retrieve discharge diagnosis. Depending on

year of diagnosis individuals were grouped into three separate time periods 1988-1993, 1994-1999, and 2000-2006.

Survival analysis with BSI as exposure were analysed both as a dichotomous and time-varying variable. Cox proportional regression models were used to calculate risk hazards in the three different timer periods. A sensitivity analysis, including patients diagnosed within a year of their bloodstream infection was made to evaluate potential selection bias. For patients undergoing splenectomy survival was evaluated in a separate model.

Differences in proportion of BSI between time periods were tested using chi-square test or Fisher's exact test. Differences in mean time to blood culture were tested using a t-test.

5.2 RESULTS AND DISCUSSION

5.2.1 Study I

When this study was initiated there was no consensus on antibacterial prophylaxis for dose-intensive chemotherapies in hematological malignancies (124), except for those at risk of *Pneumocystis jirovecii* pneumonia. Our study group had demonstrated, in our center, a low prevalence of bacterial resistance, stable distribution of pathogens and a stable Gram-positive to Gram-negative distribution. A Cochrane meta-analysis by Gafter-Gvili *et al*., first published in 2005 (and later updated in 2007 and 2012) presented a reduced risk by 48% (95% CI 33% to 65%) in infectious related death in the fluoroquinolone-prophylaxis group compared to placebo; but also significantly with respect to overall mortality, fever and clinically documented infections (125). Meanwhile reports internationally implied an increase in Gram-positive bacteria among hematological patients receiving quinolone-prophylaxis (126), and an increase in fluoroquinolone-resistance in the community (127).

For above reasons, we therefore performed study I. Between 2002 and 2008 we found 794 relevant isolates in 463 patients, making 667 bacteremia episodes (Table 1). Compared with earlier published results from the same institution 1980-1986 and 1988-2001 the proportion of positive blood cultures and ratio Gram-positive to Gram-negative were stable. Polymicrobial bacteremia was also common (13.7%).

The 7- and 30 day crude mortality rates were 5.2% (35/677) and 13.6% (92/677) and polymicrobial bacteremia was found in 26% and 18% of those who died within 7 and 30 days, respectively. Internationally the death rates were comparable with the literature (128), and the associated increase of polymicrobial bacteremia among serious outcomes in neutropenic fever, well known (129). In our center practice has been not to give fluoroquinolone-prophylaxis 2002-2008. Nevertheless, we saw a significant increment of resistance among *E. faecium*, *Enterobacter* spp. and *E. coli.*; and found more resistant *E. coli* among patient who died within 30-days of bacteremia.

Fluoroquinolone-prophylaxis use in hematological treatment has remained an issue of discussion. A new meta-analysis presented by Imran *et al* of only placebo controlled trials showed a non-statistically reduced mortality risk with fluoroquinolone-prophylaxis in patients with neutropenia (130).

Table 1 Bacterial isolates (n=794) and distribution of Gram-positive to gram-negative (53.1% vs 49.9%) between 2002 and 2008 (58)

Gram-negative bacilli, aerobes	No.(%[1])	Gram-positive bacteria, aerobes	No.(%[1])
E. coli	141 (17.8)	Coagulase-negative staphylococci	117 (14.7)
Klebsiella spp.	78 (9.8)	Viridans streptococci	111 (14.0)
Enterobacter spp.	43 (5.4)	β-hemolytic streptococci[4]	15 (2.0)
Pseudomonas spp.	42 (5.3)	*E. faecium*	61 (7.7)
Citrobacter spp.	10 (1.3)	*E. faecalis*	11 (1.4)
Stenotrophomonas maltophilia	6 (0.8)	*S. aureus*	55 (6.9)
Proteus spp.	3 (0.4)	*S. pneumonia*	18 (2.3)
Acinetobacter spp.	1 (0.1)	*Listeria* spp.	2 (0.3)
Haemophilus influenzae	2 (0.3)	*Bacillus* spp.	5 (0.6)
Morganella spp.	2 (0.3)	*Enterococcus* spp[5]	3 (0.4)
Serratia marcescens	1 (0.1)	Other gram-positive bacteria[6]	9 (1.1)
Other Gram-negative bacilli[2]	20 (2.5)		

Gram-negative anaerobes		Gram-positive anaerobes	
Bacteroides spp.	14 (1.8)	*Clostridium* spp.	14 (1.8)
Fusobacterium spp.	4 (0.5)	Other gram-positive anaerobes not typed	1 (0.1)
Other gram-negative anaerobes[3]	5 (0.6)		

[1]Due to rounding, not all percentages add up to 100%
[2]Other Gram-negative bacilli includes *Moraxella* spp., *Neisseria* spp., *Capnocytophaga* spp., *Aeromonas* spp., *Hafnia alvei*, *Rosemonas gilardii*, *Salmonella* Hadar, *Sphingomonas paucimobilis*, Gram-negative bacillus not typed;
[3]Other Gram-negative anaerobes includes *Veillonella* spp., *Leptotrichia* spp., and Gram-negative anaerobes not typed;
[4]β-hemolytic streptococci includes β-hemolytic streptococci group A+B+D+G;
[5]*Enterococcus* spp excluding *E. faecalis* and *E. faecium;*
[6]Other gram-positive bacteria including *Lactobacillus* spp., *Corynebacterium* spp. *Rothia mucilaginosa*, Gram-positive not typed, *Stomatococcus mucilaginosus, Gemella spp.*

There is a concern of fluoroquinolone prophylaxis leading to more bacterial resistance and more use of carbapenems (131). Even so, growing resistance must take the whole community in account when evaluating changing patterns (132). Increased resistance can lead to increased mortality and lack off prophylactic effect (133). In centers with high resistance discontinuation of prophylaxis has led to mixed outcomes regarding infectious complications, but reduced resistance (134, 135). Upon reinstitution of prophylaxis, fever episodes and resistance levels reassumed to pre-discontinuation levels (136) .

5.2.2 Study II

Treatment for CLL has evolved during the last two decades, even more in the last years. Standard treatment now constitute not only of chemotherapy, but also of immunotherapy and small molecules interfering cell-signaling.

In study II, the biggest study based on high quality registries on BSI in CLL, we found a decrease in positive blood cultures in patients between 1998-2008. We speculate that the more effective treatment in recent years, with deeper responses and longer time to next treatment, is behind the reduction of positive blood cultures. The distribution of bacterial species was stable, as was the proportion of Gram-positive to Gram-negative bacteria which is reassuring. Here again, there has been reports of increasing Gram-negative bacteremia as a consequence, most likely, of decrease use of indwelling accesses and antibiotic prophylaxis. Dominating BSI pathogens were *E. coli*, *S. pneumoniae*, *P. aeruginosa*, *S. aureus* and viridans streptococci. CoNS was the most frequent detected microorganism

in blood cultures, but is a frequent contaminant; and analysis were made with or without common contaminants of BSI.

As intuitively foreseen, we demonstrated that BSI was associated with worse prognosis, especially during the last time period. Splenectomies did not affect prognosis for patients with CLL and BSI, but power to detect this was hampered by low numbers. We speculate if the introduction of immunotherapy could negatively affect the adaptive immune response. By doing that, a BSI prevailing the non-specific immune response has a more dismal prognosis. The study is also important for future comparisons of infectious complications with the new therapies for CLL that are in late phases of clinical trials.

6 PROSPECTIVE COHORT STUDIES (III, IV)

6.1 MATERIAL AND METHODS

6.1.1 Study III

Patients with hematological malignances fit for dose-intensive chemotherapy between 2013 and 2014 at HCK Solna were eligible for enrollment. Patients could be included at any time point during their treatment phase, but predominantly and especially for AML, inclusion was made before commencement of chemotherapy. In all 33 patients were included in the study; 19 patients with AML and 14 with other aggressive hematological malignancies.

Included patients were sampled with 2 EDTA tubes at different time points; 1) at diagnosis 2) at neutropenic fever before initiation of antibiotics 3) follow-up samples to fever-onset sample (only for AML), and 4) persisting fever during broad spectrum antibiotic treatment. Patient data was extracted retrospectively.

Bacterial DNA extraction was done within 1-24 hours with MolYsis Complete 5 kit (Molzym Life Science, Bremen, Germany). All samples were checked for 16S rRNA gene positivity. Positive samples were then subject for library preparation with primer pair covering the V1-V3 regions of the 16S rRNA, and processed to a 2x300bp paired end (PE) sequencing on an Illumina MiSeq instrument. Reads below Q20 and 246bp, and unmerged PE and chimeras were removed. Subsequent overlapping PE reads were merged and phylogenetically analysed. Abundance was calculated from reads within the different clusters. Presentation of bacteria in the study required sample to contain at least 0.5% of the total operational taxonomic unit (OTU)-assigned reads in each sample.

6.1.2 Study IV

From the same cohort as in study III, 8 out of 19 patients with AML that had full availability of results from inclusion, fever onset, and follow up samples were selected for shotgun metagenomics.

Bacterial content was extracted with MolYsis and 1 µg eluted DNA was enriched with NebNext (New England Biolabs, Ipwich, MA, USA). 10 ng DNA was then subject for multiplex displacement amplification and 2 µg for library preparation with Nextera XT Kit and subsequently sequenced on HiSeq 2500 instrument. Received data reads were then bioinformatically processed using the Fastx toolkit; for disregarding shorter than 30bp reads and low quality reads; Flash software merged PEs and, RTG Core 3.4 was used to map sequencing reads against microbial databases and to filter against the human genome. The ARG-ANNOT database was used to detect antibiotic resistance genes and the

gene ontology analysis was completed using the Blast2Go v3 software. Mann-Whitney U-test was performed to detect statistical significance.

6.2 RESULTS AND DISCUSSION

6.2.1 Study III

Standard diagnostics for BSI, mainly blood culture and PCR-techniques has several limitations as already stated. NGS is a quickly growing field, allowing billions of reads in a few days' time. At the time of publication, the bacterial landscape of bloodstream infections in patients with hematological malignancies and neutropenia was unexplored.

130 samples were analysed; 27 from AML patients at diagnosis, 38 at fever onset, 41 during follow up and 24 due to persistent fever (Figure 8). 16SrRNA positivity was 23.7% (9/38) for fever onset, 7.3% (3/41) for follow up and 29.2% (7/24) for persisting fever and in none of the inclusion samples. Blood cultures were positive in 15.4% (10/65); 21.1% (8/38) at fever onset and 8.3% (2/24) in persisting fever and none in follow up samples.

Sequencing yielded 2,764,592 reads assigned to bacterial OTUs. Five bacterial phyla: 55.2% *Proteobacteria* (Gram-negative bacteria like *Pseudomonas* and *Escherichia*), 33.4% *Firmicutes* (mostly Gram-positive normal flora like *Staphylococus* and *Streptococcus*, 8.6% *Actinobacteria* (Gram-positive bacteria found in the environment, potentially opportunistic pathogens like *Corynbacterium*), *Fusobacterium* 0.4% (anaerob Gram-negative potentially opportunistic pathogens normally found in the oral flora, like *Leptotrichia* and *Fusobacterium*) and 0.1% *Bacteroidetes* (Gram-negative bacteria in soil, gut and oral flora; potentially opportunistic pathogens like *Alloprevatella* and *Bacteroides*) (Figure 9).
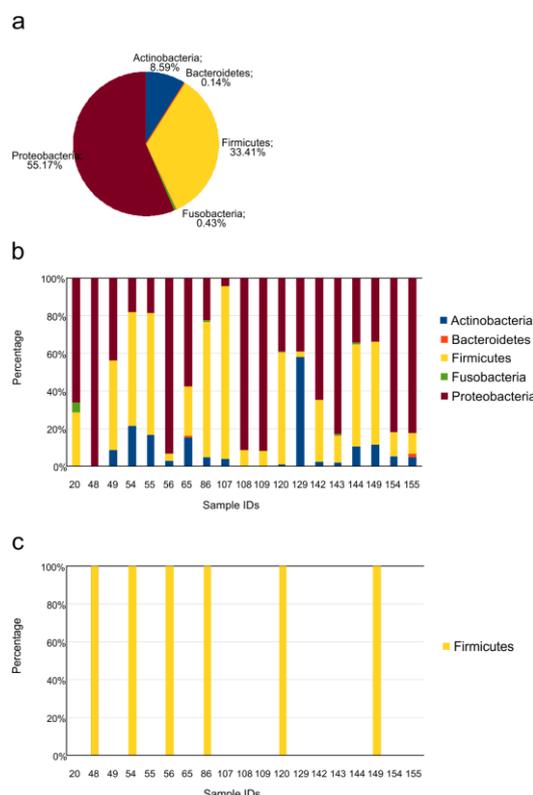

Figure 9. Distribution of phyla from sequences in all samples (A) per sample (B) and from those with positive blood culture (C) (137).

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From the 5 phyla, 30 genera were identified and all previously isolated from BSI, except *Pelomonas*, which, however recently was found in the endometrial bacterial community (138) (Figure 10). The notion that identified bacteria belonged to the normal human microbiota supports the idea of translocation of bacteria playing a pivotal role in inflammation associated with sepsis. Another notion is that the *Shewanella* genus (previously *Pseudomonas*) was detected in 80% of the samples. *Shewanella* bacteremia, although well described, is not routinely diagnosed with standard methods and could constitute one of more underdiagnosed microorganisms in BSI found in our material.

NGS was also able to estimate efficacy of antibiotics, calculated as change in reads, but also identify bacterial DNA content in patients with ongoing antibiotics, as shown in the persisting fever group when comparing NGS with blood cultures. This is most probably due to non-cleared non-functional bacterial DNA in the blood. This type of translocation of bacterial content is most likely to be involved in the host's response to bacteria, and might thereby help our understanding of sepsis.
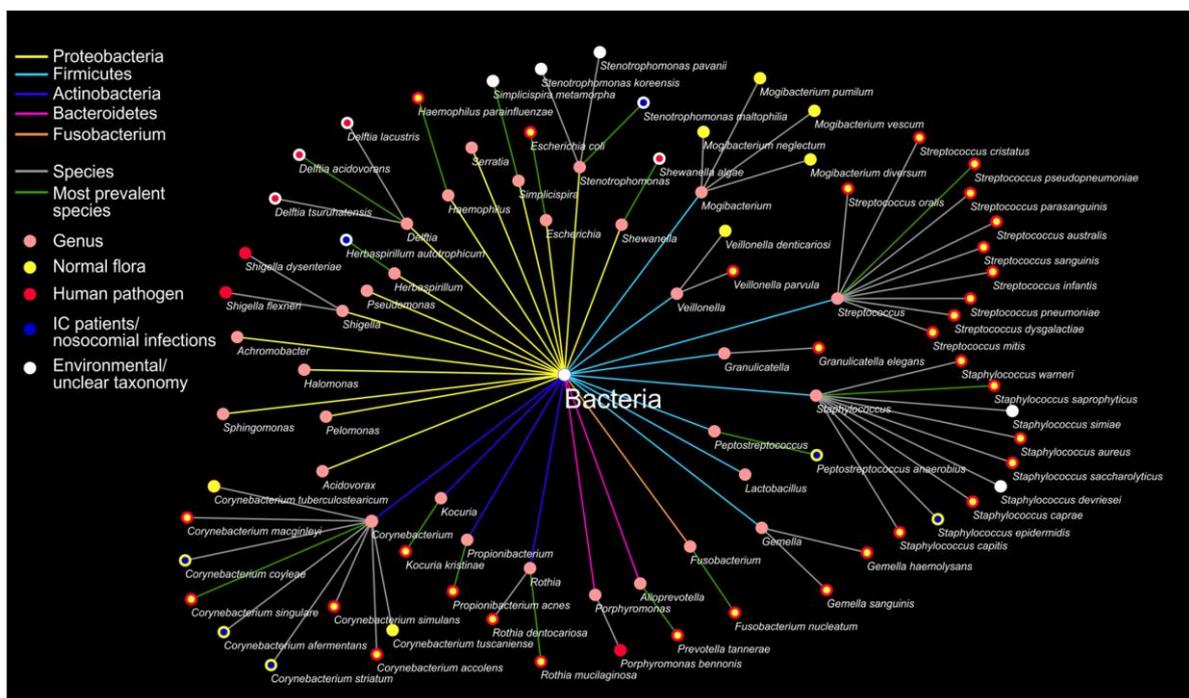

Figure 10. Bacteria on the species level detected with NGS (137).

6.2.2 Study IV

As expected with shotgun metagenomics, analysis yielded more reads, compared to the 16sRNA study. Out of the 27 samples; 1 sample was from inclusion, 9 samples were from fever onset (day 0, before antibiotic treatment), 7 samples were from the day after antibiotic commenced, and 11 samples were from day 1-5. In 2 patients no microbial content was found (Figure 11). No microorganism was detected in 2 of the fever onset samples, in 1 of the persistent fever samples and in 6 of the follow up samples.

Antibacterial treatment only caused, as foreseen, a significant effect on Shannon's diversity index for bacteria. In follow up samples bacterial reads were very low.
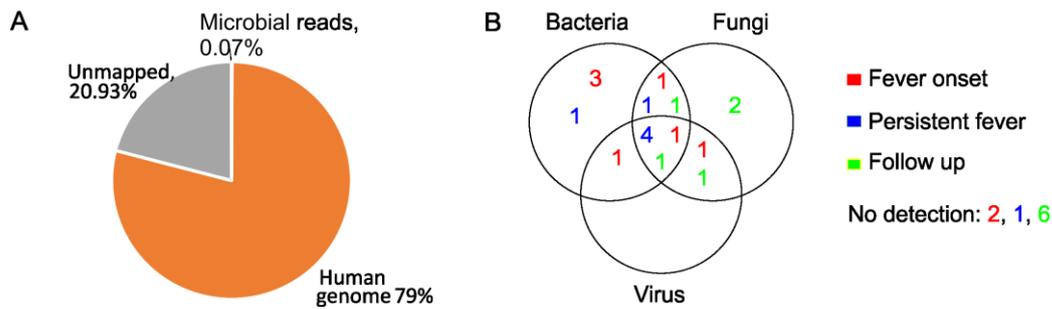
Figure 11. (A) Distribution of sequencing reads. (B) Distribution microbes detected by NGS. Sample category in color and numbers reflecting occurrence (139).

Distribution of reads for bacteria, fungi and viruses are depicted in Figure 12. *Propionbacterium acnes* dominated in fever samples, but was not found at all in follow up samples. Persistent fever samples were dominated by *Corynbacterium* spp., *Dolosigranum pigrum* and *Staphylococcus* spp.. All species should have been susceptible to given antibiotics, and a contamination can therefore not be ruled out. The most reoccurring bacteria in persistent fever samples was *Streptococcus* spp., also covered by given antibiotics, could mean a reinfection as the reads were repeatedly low in these samples. *Acinetobacter*, known for acquiring resistance mechanisms was found in 5 samples from 2 patients, and most reads were found among samples obtained at persistent fever. *Fusarium oxysporum* reads dominated and was the most frequent fungi at all time-points for sampling. Also, *Aspergillus* spp. was detected in one sample with persistent fever. All patients received posaconazole, known to be effective against *Aspergillus* spp. but resistance can occur against *Fusarium* spp.. Phages, most probably resulting from dead bacteria, were more common in persistent fever than fever onset. No certain neutropenia specific virus was detected.
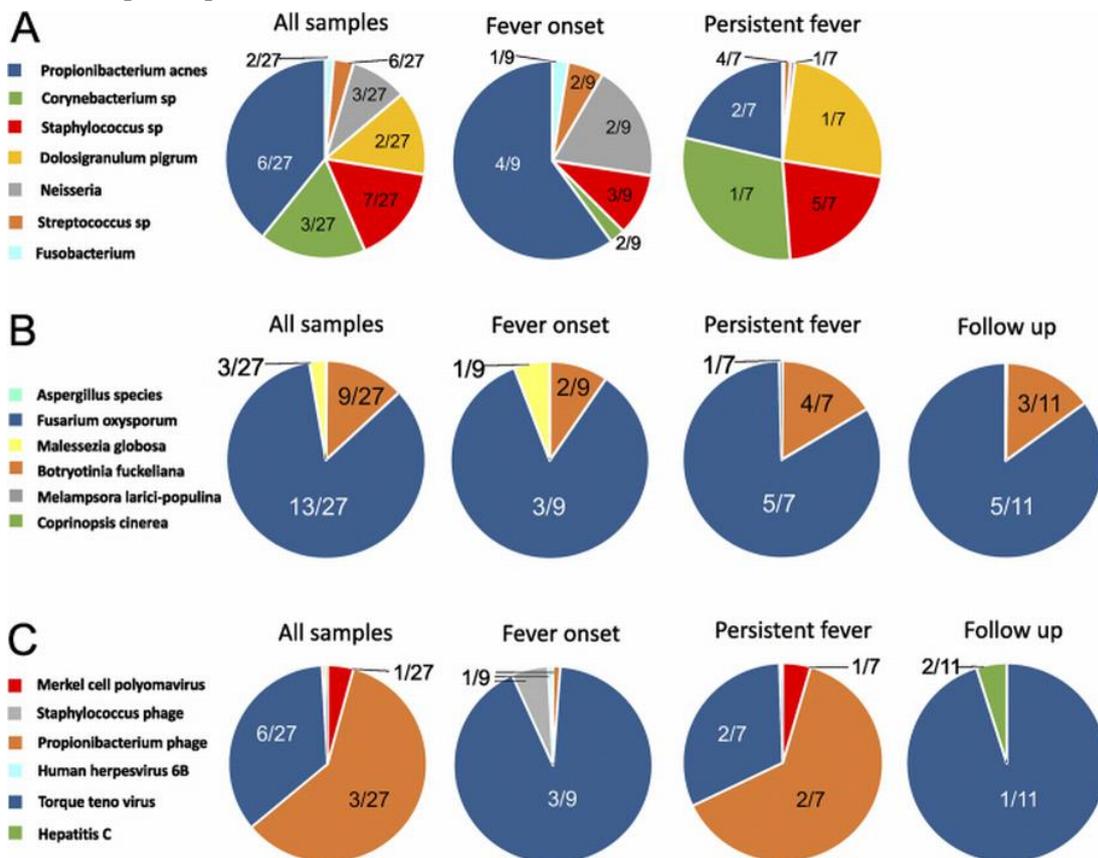

Figure 12. (A) Distributions of bacterial reads at different time points (bacterial taxa with reads of <1% in a sample not shown) (B) fungal reads (C) and viral reads. Numbers within the pie charts indicate the number of samples positive for the given pathogen (139).

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7 CONCLUDING REMARKS

From the retrospective studies, first on a diverse cohort of hematological malignancies and later on a CLL-specific cohort, we have found stable distribution of pathogens and mortality over time; corroborating other international reports. The reported increase in fluoroquinolone-resistant *E. coli* in blood cultures is in parallel with the trend in our hospital, and nationally reported data. It is my personal opinion that the more liberal use in the last decade of fluoroquinolone-prophylaxis in hematological malignancies lacks good scientific support. In awaiting future prospective comparative trials fluoroquinolone-prophylaxis should only be used for the very high risk patients. For optimal use of antibiotics; more should be done for endorsing adherence to existing guidelines and development of technical tools aiding clinical work.

Next generation sequencing, as opposed to traditional infectious disease diagnostics, requires little or no prior knowledge of possible findings. Our prospective studies have given us novel insight into unculturable bacteria as well as culturable microorganisms; and dynamics of infectious episodes. Clinically, many microbiological diagnostic methods can be replaced by NGS. However, NGS for microbial detection need to improve in: timeliness, cost, sensitivity, discriminative power, and complexity of data analysis and interpretation; before being clinical useful. Nonetheless, it is my belief that NGS in the future will learn us much more about the important microbiome-host interaction, the panorama of pathogenic microorganisms and their management.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Blodcancer är den fjärde vanligaste cancerformen och en ledande orsak till cancerrelaterad död i Sverige. Patienter med blodcancer tvingas ofta till tuffa behandlingar, som i princip alltid upprepas under lång tid. Grundsjukdomen och dess behandlingar; ofta kombinationer av cellgifter, strålterapi, målstyrda antikroppar och stamcellstransplantation, leder till sänkt immunförsvar. Nedsatt immunförsvar är förenat med infektioner som i sin tur kan leda till exempelvis blodförgiftning, sänkt livskvalité och ökad risk för ohälsa och död. Att drabbas av en blodförgiftning med virus, svamp eller bakterie tillhör den absolut allvarligaste infektionskomplikationen som patienter med hematologisk malignitet kan drabbas av. Utan korrekt antimikrobiell behandling ökar dödligheten markant för varje timme. Dagens diagnostiska metoder för blodförgiftning vilka är relativt oförändrade under de senaste decennierna, är långsamma och fångar upp bara en bråkdel av attackerande mikroorganismer. Även om förebyggande antimikrobiell behandling med till exempel antibiotikaprofylax minskar risken för infektiösa komplikationer är risken ändå inte försumbar. I händelse av misstänkt infektion tvingas därför ofta behandlande läkare att inleda kraftfull antimikrobiell behandling innan svar på eventuell mikrobiologisk diagnostik finns tillgängligt. Hög antibiotikaförbrukning leder till ökad antibiotikaresistens, som i sin tur leder till minskad effekt av antibiotikabehandling och därmed ökad dödlighet. För att förbättra vården vid blodförgiftning är det viktigt att ha god kunskap om vilka mikrobiella organismer som drabbar patienterna, att känna till antibiotikaresistensläget bland vanliga bakteriefynd och att utveckla bättre diagnostiska verktyg.

År 2003 publicerades mikrobiologiska fynd vid blododlingar tagna 1988-2001 på hematologens slutenvårdavdelning vid Karolinska Universitetssjukhuset i Solna. Studien påvisade i huvudsak stabil distribution av bakteriella arter i blododlingar och oförändrad låg förekomst av antibiotikaresistens. Nya studier 2003-2008 beskrev ökad resistensproblematik såväl nationellt som internationellt, och vår restriktiva strategi avseende antibiotikaprofylax kunde ifrågasättas.

Studie I beskriver blododlingsfynd hos patienter med hematologisk malignitet som vårdats på hematologens slutenvårdavdelning vid Karolinska Universitetssjukhuset i Solna 2002-2008. I Studie I jämförs också nämnda resultat med historiska data (1980-2001) från samma institution. Återigen noteras stabil distribution av bakteriella arter, en viss ökning av antibiotikaresistens hos tarmbakterier såsom *E. coli, Enterobacter* spp. och *Enterococcus faecium.* Studien visar att flera olika bakterier i blodet vid blodförgiftning är vanligt förekommande och förenat med ökad dödlighet. Dödligheten 7 respektive 30 dagar efter en blodförgiftning är låg, även vid en internationell jämförelse. Dödligheten är ökad hos patienter med resistent *E. coli* i blodet jämfört med patienter med *E. coli* utan resistensproblematik. Studien bekräftar också att det inte fanns några tecken på att vi under aktuell period borde ha övergivit vår kliniks restriktiva antibiotikaprofylaxpolicy.

Kronisk lymfatisk leukemi (KLL) är den vanligaste kroniska leukemin i västvärlden. Cancerbehandlingen mot KLL har under åren förändrats. Inte minst på senare år har flera nya behandlingsalternativ tillkommit. **Studie II** utgår från patienter som påvisats ha en bakterie i blodet vid analys på Mikrobiologen vid Karolinska Universitetssjukhuset, 1988-2003. Studie II visar att *E. coli, S. pneumoniae, Pseudomonas aeruginosa, S. aureus,* och viridans streptococci är de vanligast förekommande bakterierna vid blodförgiftning vid KLL. Resultaten från studien kommer att fungera som viktig referens i framtiden, då nya cancerbehandlingars komplikationer i form av blodförgiftningar ska analyseras.

I **studie III** tillämpas en ny teknologi för att identifiera arvsanlag från bakterier i blodet. Prov från patienter med hematologisk malignitet och nedsatt immunförsvar togs i samband med feber. Traditionell diagnostik av blodförgiftningar, d.v.s. blododlingar, begränsas av att alla bakterier inte lika lätt tillåter sig att odlas fram och att det är svårt med blododling att påvisa mer än en bakterie. Med den nya tekniken, som vi är först i världen med att applicera på hematologiska patienter med infektion, kunde fler bakterier identifieras, även under pågående antibiotikabehandling. Studien ger en fingervisning om mekanismer som kan vara aktuella för de patienter som har kvarstående feber trots tillsynes korrekt antibiotikabehandling.

I **Studie IV** använde vi snarlik teknologi som i studie III, men här letade vi förutom efter arvsanlag från bakterier i blodet även efter arvsanlag från svampar och virus. Studien beskriver bland annat dynamiken vid feberepisoder, d.v.s. hur förekomst av infektionsalstrande mikroorganismers arvsanlag stiger och sjunker i blodet som svar på antimikrobiell behandling. Vidare ges spekulativa förklaringar till några av de episoder av oförklarlig feber trots kraftfull antimikrobiell behandling som vi som kliniker ser hos denna patientgrupp.

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