From Department of Medicine, Division of Hematology
Karolinska University Hospital and Karolinska Institutet,
Stockholm, Sweden

BLOODSTREAM INFECTIONS IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES

Christian Kjellander

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Bloodstream infections in patients with hematological malignancies

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By

Christian Kjellander

Principal Supervisor:
Assoc. Professor Christian Giske
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Microbiology

Co-supervisors:
Professor Magnus Björkholm
Karolinska Institutet
Department of Medicine, Solna

Professor Sigurður Kristinsson
University of Iceland and Karolinska Institutet
Department of Medicine, Solna

Dr Peter Gyarmati
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Microbiology

Opponent:
Assoc. Professor Dick Stockelberg
Sahlgrenska University Hospital
Department of Internal Medicine
Section for Hematology and Coagulation

Examination Board:
Adjunct Professor Jonas Mattsson
Karolinska Institutet
Department of Oncology-Pathology
Division of Centre for allogeneic stem cell transplantation (CAST)

Assoc. Professor Helene Hallböök
Uppsala University
Department of Medical Sciences
Division of Haematology

Assoc. Professor Robert Schvarcz
Karolinska Institutet
Department of Medicine, Huddinge
Division of Infectious Diseases

“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.

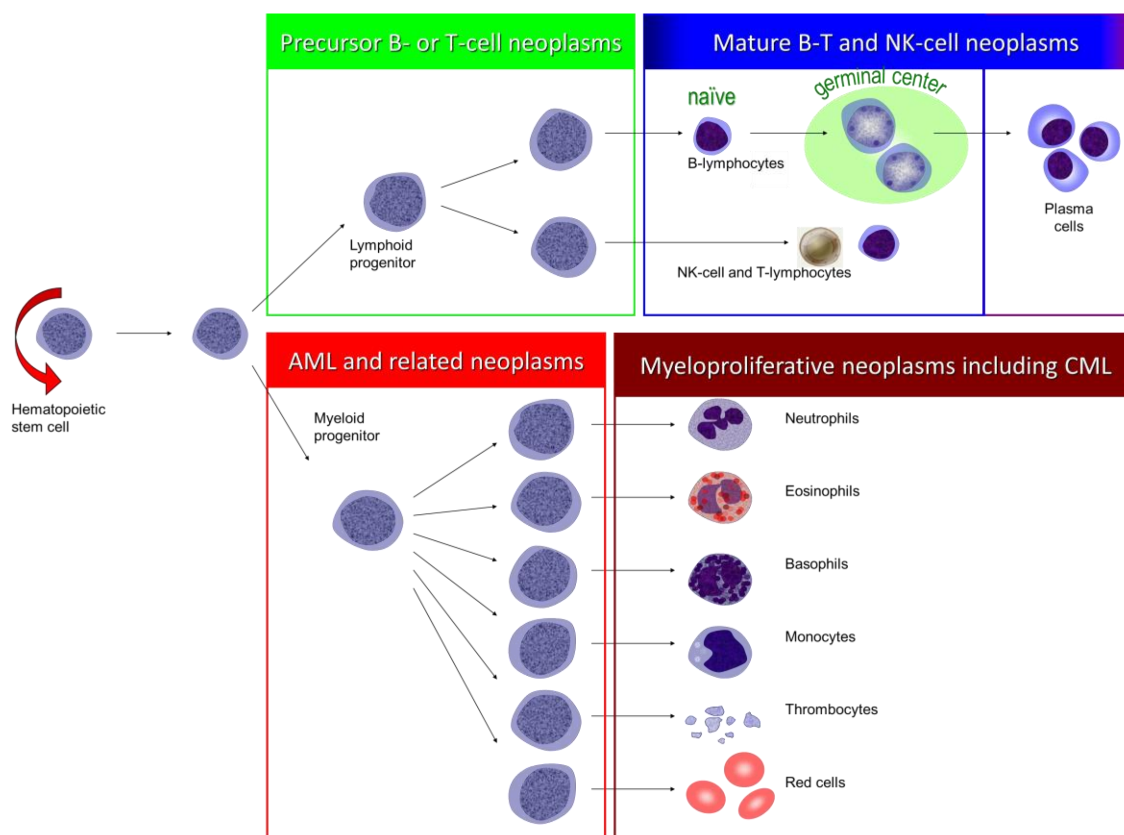

Figure 1. Hematological malignancies, adapted from Dr Dinesh Bhurani (16).

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).

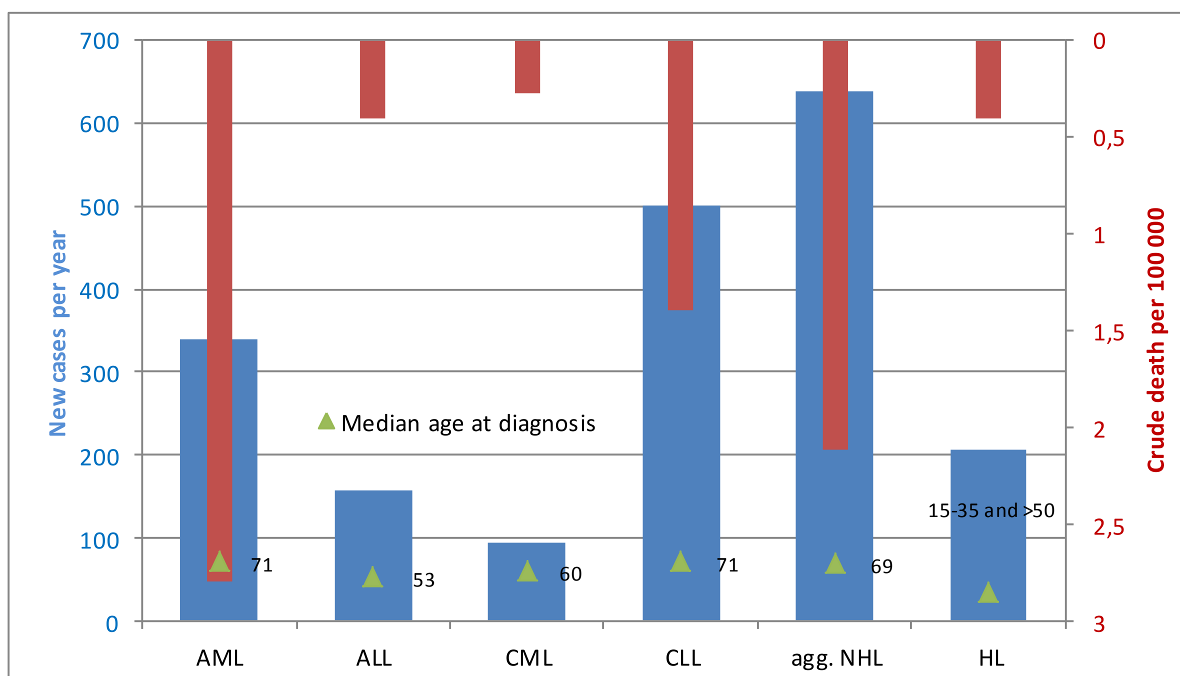

Figure 2. Epidemiological differences in hematological malignancies. Incidence and median age for adults were retrieved from National quality registries for respective diagnosis and estimated deaths per year were extracted from National Cancer Institute's (United States of America) database. Only for 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 $<0.5x10^9$/L, or a count expected to decrease to $<0.5x10^9$/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

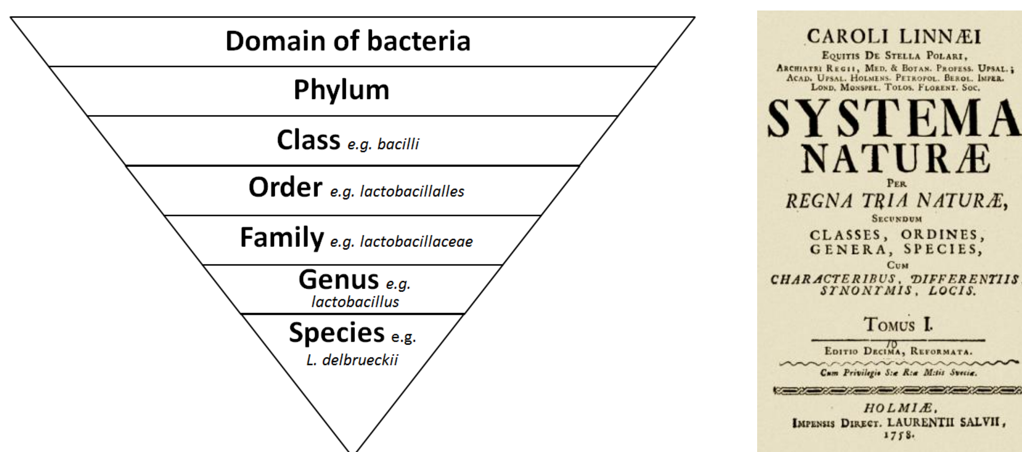

Figure 3. The taxonomy of bacteria based on the work of Carl von Linné (55).

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

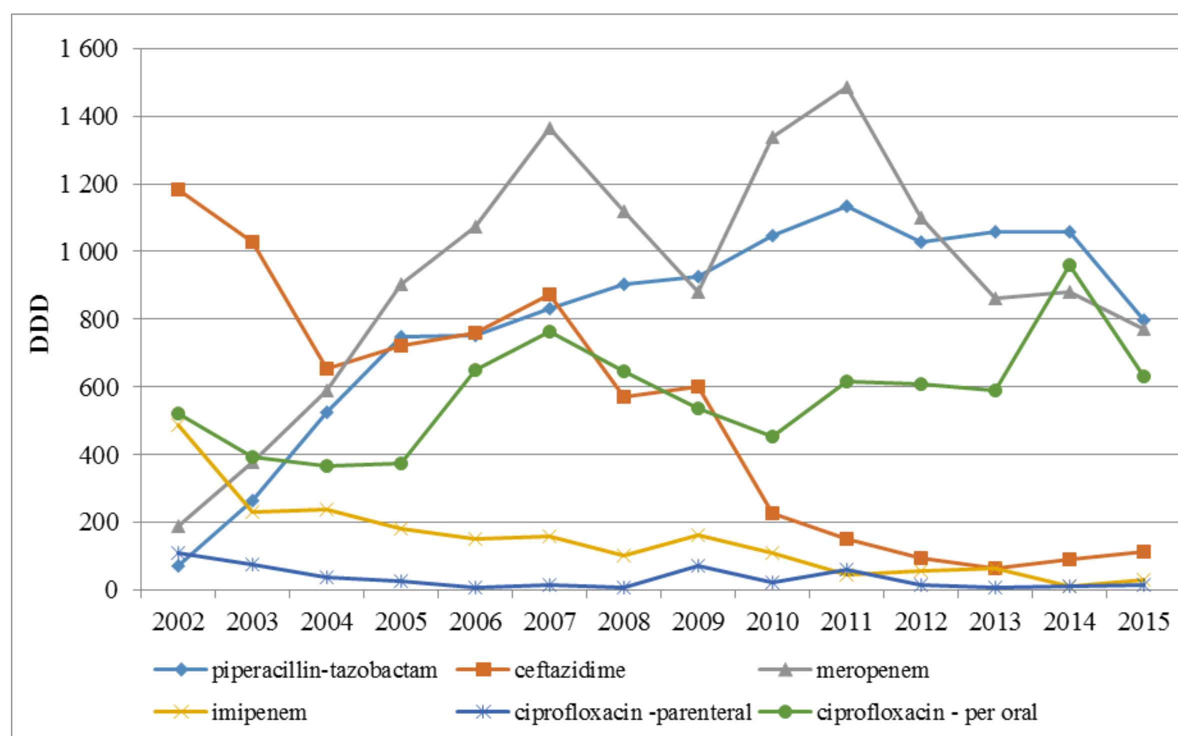

Figure 4. DDD of prescribed antibiotics from the hematology inpatient ward, Karolinska Solna between 2002 and 2015.

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

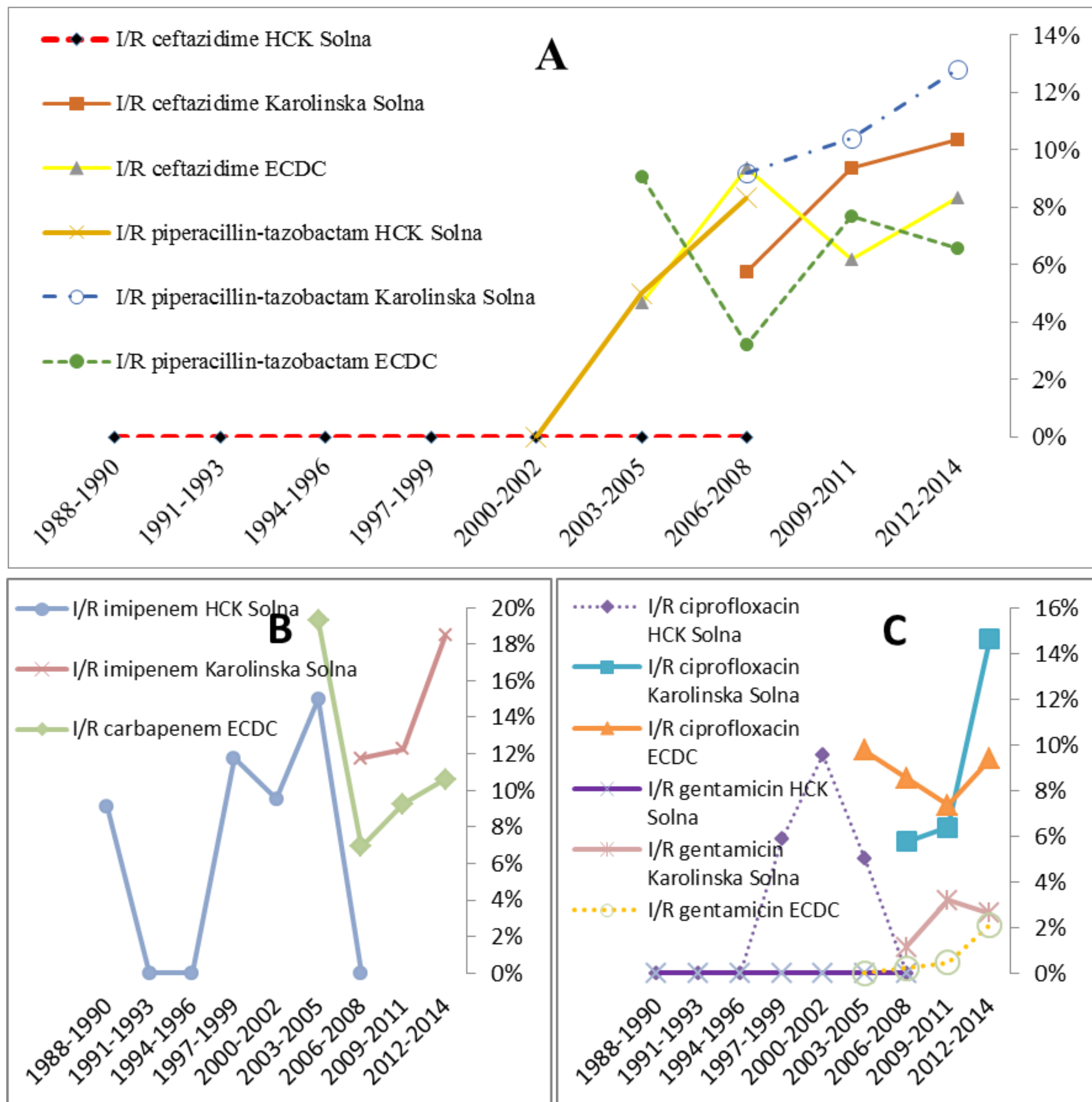

Figure 5. (A,B,C) Pattern of resistance for *P. aeruginosa.*

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).

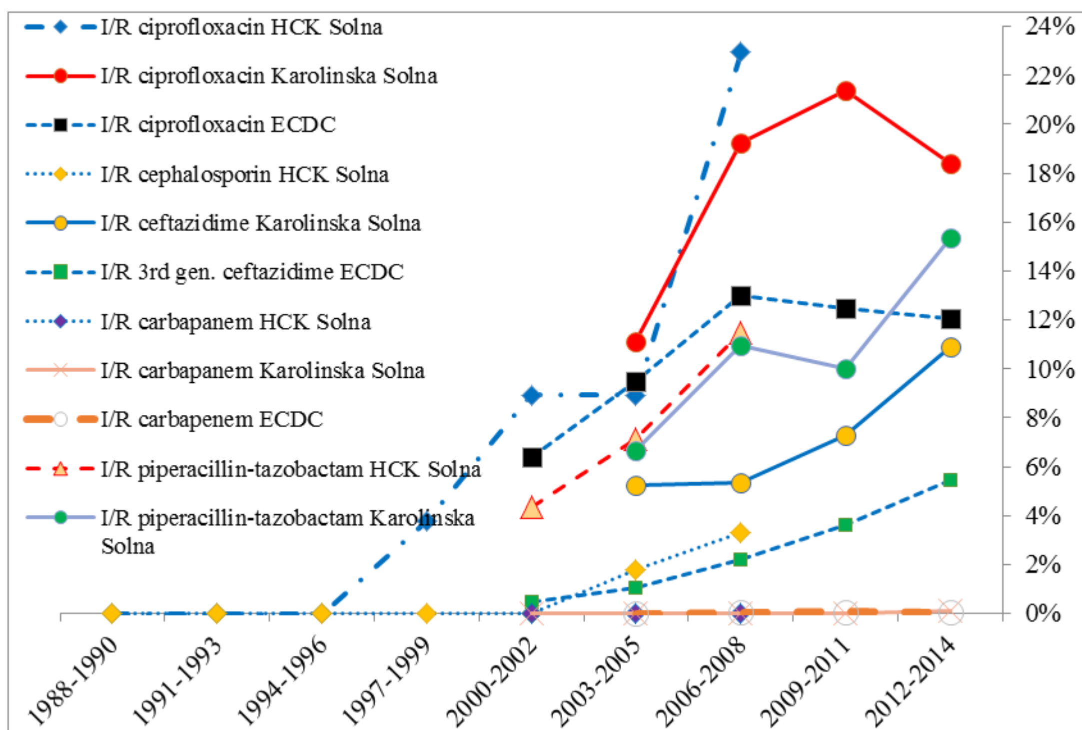

Figure 6. Pattern of resistance for *E. coli*.

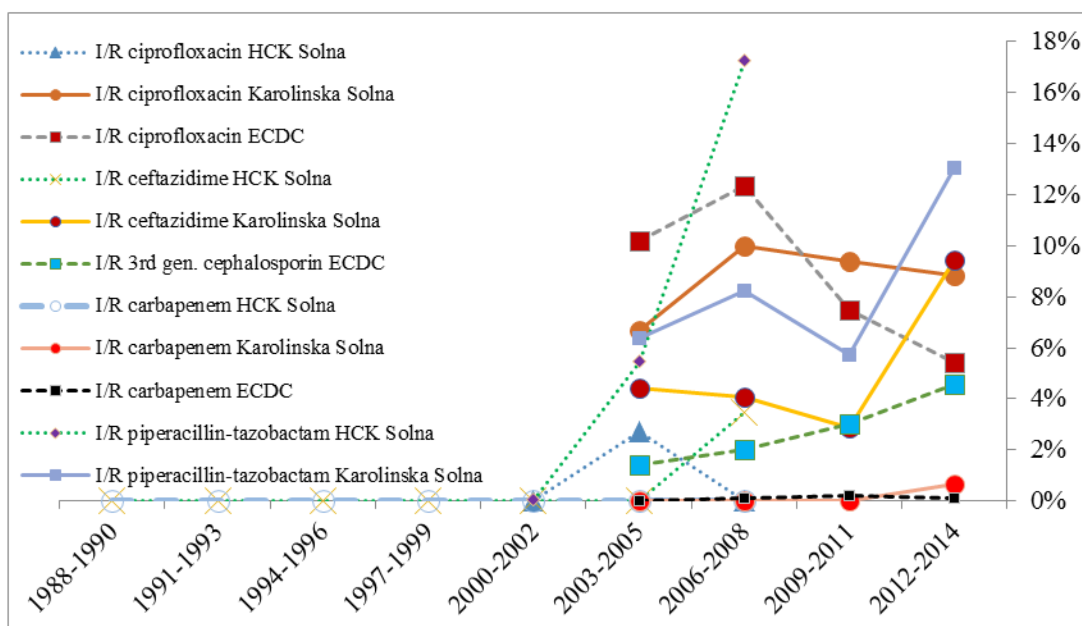

Figure 7. Pattern of resistance for *K. pneumoniae*.

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).

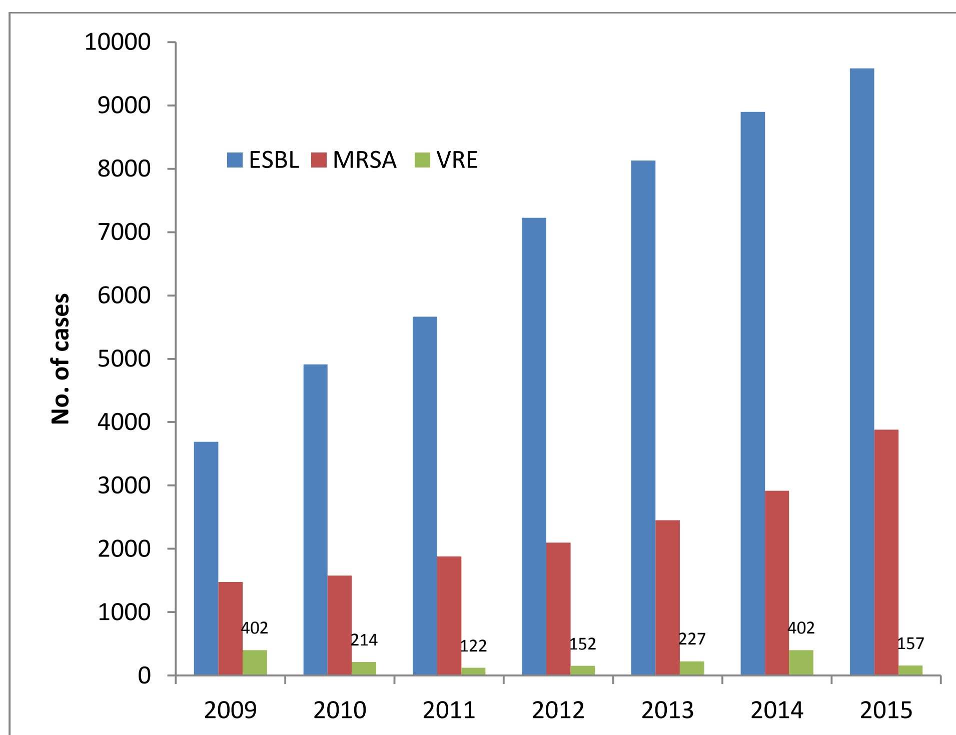

Figure 8. Notifiable resistance in Sweden from 2009 to 2015(Public Health Agency of Sweden (www.folkhalsomyndigheten.se))

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 malignancies 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).

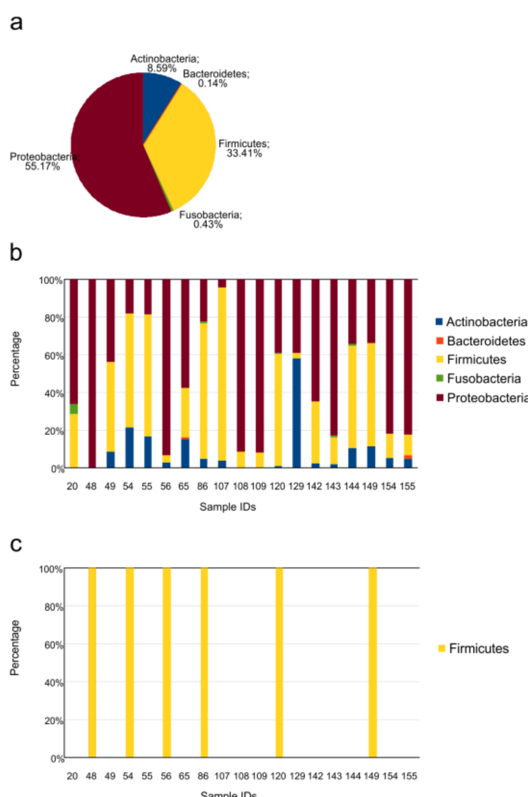

Figure 9. Distribution of phyla from sequences in all samples (A) per sample (B) and from those with positive blood culture (C) (137).

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.

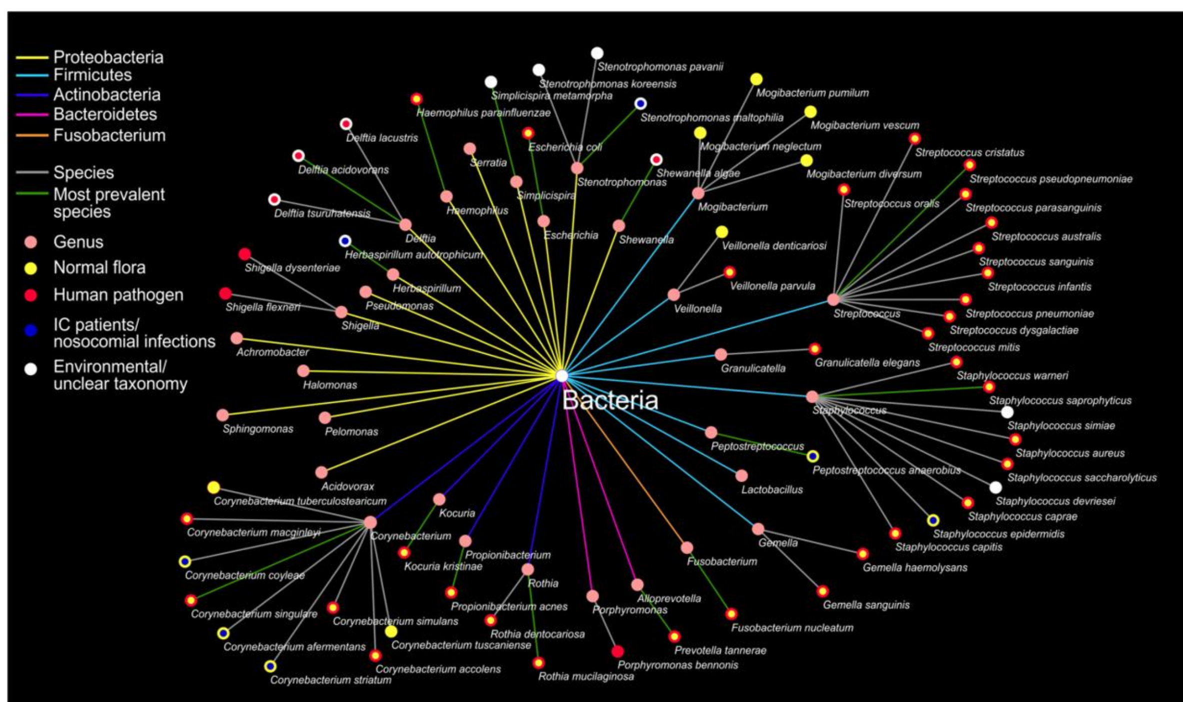

Figure 10. Bacteria on the species level detected with NGS (137).

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.

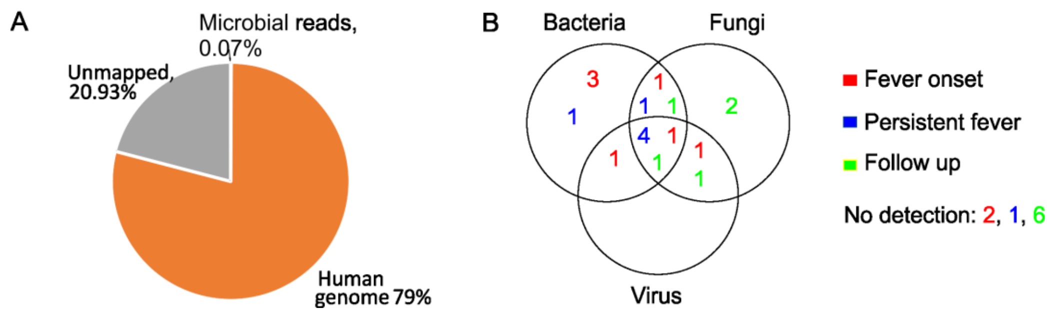

Figure 11. (A) Distribution of sequencing reads. (B) Distribution microbes detected by NGS. Sample category in color and numbers reflecting occurrence (139).

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.

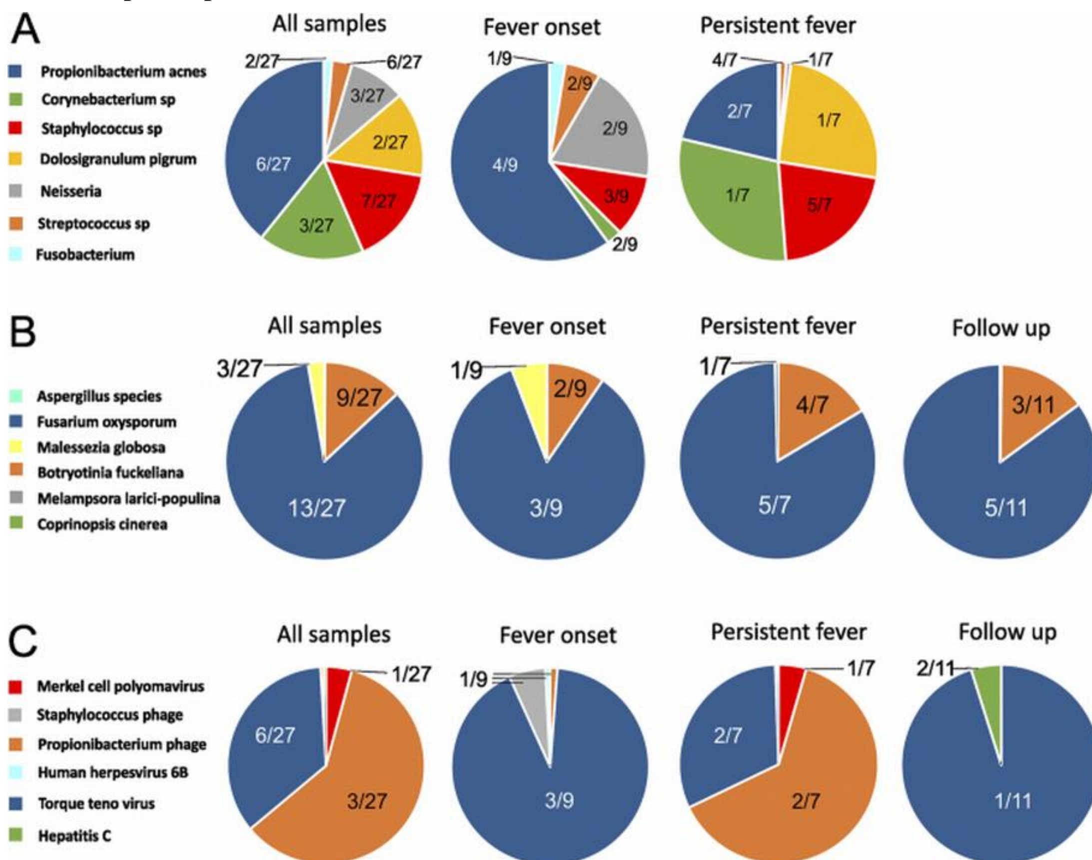

Figure 12. (A) Distributions of bacterial reads at different time points (bacterial taxa with reads of <1% in a sample not shown) (B) fungal reads (C) and viral reads. Numbers within the pie charts indicate the number of samples positive for the given pathogen (139).

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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ORIGINAL ARTICLE

Hematological: Low all-cause mortality and low occurrence of antimicrobial resistance in hematological patients with bacteremia receiving no antibacterial prophylaxis: a single-center study

Christian Kjellander[1], Magnus Björkholm[1], Honar Cherif[2], Mats Kalin[1], Christian G. Giske[3]

[1]Department of Medicine, Karolinska Institutet, Karolinska University Hospital Solna, Stockholm, Sweden; [2]Department of Medical Sciences, Uppsala University Hospital, Uppsala, Sweden; [3]Clinical Microbiology, Karolinska Institutet – MTC, Karolinska University Hospital Solna, Stockholm, Sweden

Abstract

Background: Bacteremia is a major cause of morbidity and mortality in patients with hematological malignancies. *Objectives:* The aim of this study was to define temporal trends in species distribution, antimicrobial susceptibility, and all-cause mortality in bacteremic hospitalized patients receiving no antibacterial prophylaxis during chemotherapy-induced neutropenia. *Methods:* A total of 677 clinical episodes of bacteremia were identified in 463 patients during 2002–2008, and the results were compared with those published from the same institution during 1980–86 and 1988–2001. No major changes in patient selection were introduced during this period. *Results:* Between 2002 and 2008, the dominating pathogens were *Escherichia coli* (18%), coagulase-negative staphylococci (15%), viridans streptococci (14%), *Klebsiella* spp. (10%), and *Enterococcus faecium* (8%). The 7-d crude mortality rate was 5.2%. Polymicrobial bacteremia was seen in 25.7% of the patients who died within 7 d and in 13.1% of the survivors ($P = 0.04$). Acquired resistance was rarely observed, but a statistically significant increase in ciprofloxacin resistance in *E. coli* was observed. Comparing 2002–2008 with historical data from the same institution, the proportion of Gram-positive isolates remained stable at 53–55% from 1988. *Conclusions:* The avoidance of fluoroquinolone prophylaxis may have contributed to a stable proportion of Gram-positive bacteremia. The crude mortality was low in an international perspective. Acquired resistance was uncommon, but ciprofloxacin resistance in *E. coli* increased significantly. We believe that an indiscriminate use of antibacterial prophylaxis could be avoided in neutropenic patients without a negative impact on mortality.

Key words bloodstream infection; hematological malignancy; antimicrobial resistance; hospital epidemiology; polymicrobial bacteremia; temporal trend

Correspondence Christian Kjellander, Department of Hematology, Karolinska University Hospital Solna, SE-171 76 Stockholm, Sweden. Tel: +46 8 517 70000; Fax: +46 8 517 73719; e-mail: christian.kjellander@karolinska.se

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Infectious complications are still a major cause of morbidity and mortality in patients with hematological malignancies, as a consequence of chemotherapy and other immunosuppressive treatment. A prompt and adequate antimicrobial treatment approach may prevent life-threatening complications and avoid delay or limitations of planned antineoplastic treatment (1–3). Bacteria are the most common cause of severe infections, and in about one-third of the episodes of neutropenic fever, bacteremia is diagnosed. Bacterial epidemiology of infections and antibiotic policy should be strictly monitored, particularly in hematological units where prophylactic and widespread use of empirical therapy may modify the ecology of pathogens (4). Moreover, the main mechanism of inducing antibiotic resistance is pressure secondary to antibiotic use (5–7).

The general trend during the last two decades has been a gradual replacement of Gram-negative bacilli by

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Gram-positive cocci as the major cause of bacteremia (8–14). The relative reduction in Gram-negative organisms is probably due to the impact of fluoroquinolone prophylaxis, increased use of indwelling catheters, and intensified chemotherapy (4, 15, 16). In addition, multi-resistant pathogens have become an increasing problem in many healthcare institutions, not the least in hematology units (17–19).

There are limited published data on the bacterial species distribution and antimicrobial resistance in neutropenic hosts who are not exposed to antibacterial prophylaxis. At the Karolinska University Hospital, Solna, culture results have been computerized since 1988, and antibiotic prophylaxis has been used only to a very limited extent. The panorama of hematological disorders and their treatment has not dramatically changed. The exceptions are chronic myelogenous leukemia, where patients are being hospitalized less frequently following the introduction of tyrosine kinase inhibitors and B-cell lymphomas, for which anti-CD20 monoclonal antibodies have increased survival rates. The center offers the full range of hematological treatments apart from allogeneic stem cell transplantation, (which is carried out at another campus of the hospital). We therefore considered a compilation of our microbiological data on bacteremia to be of value. At the same time, we wanted to study trends of emergence of resistance and the impact of bacteremia on mortality.

Patients and methods

Study population

The focus of this study was 10 071 blood cultures from 1855 patients sampled between 2002 and 2008. All patients were treated in the hematology ward of Karolinska University Hospital, Solna, Sweden. Our tertiary urban hospital offers patients receiving neutropenia-inducing chemotherapy fast, direct access to medical services. For temporal trends, a comparison was made with already published data from 1980 to 1986 (20) and from 1988 to 2001 (21). All patients had a hematological disorder with approximately 1/3 being malignant lymphoma, 1/3 being acute leukemia, and 1/3 being multiple myeloma and chronic lymphocytic leukemia. Cancer-specific treatments followed national and international guidelines. All cancer-specific treatments were given at the ward or at the associated day-care unit. We used our laboratory information system (LIS) to search for blood cultures from the hematology department. A small proportion of hematological patients were sampled in the emergency department and therefore were potentially missed in the LIS search. To estimate that proportion of blood cultures, we identified all hematological patients with a positive blood culture, drawn in the emergency department, containing *Klebsiella* spp., *Pseudomonas* spp., and *Enterobacter* spp. Finally, we compared the number of isolates belonging to the aforementioned species in patients sampled in the hematology department with the number of isolates from patients sampled in the emergency room.

Ethical approval for this study was obtained from the Karolinska Institutet Review Board (recordal 2009/1851-31/4), and the study was conducted in accordance with the Helsinki Declaration of 1975.

Antimicrobial treatment, laboratory methods, data extraction, and statistical analysis

Empirical antibiotic treatment during 2002–2008 has been in accordance with national and international guidelines and also guided by local pathogen surveillance programs. As first-line empirical treatment monotherapy with ceftazidime, piperacillin–tazobactam, imipenem or meropenem was used. Antiviral prophylaxis using acyclovir 400 mg orally three times daily and antimycotic prophylaxis using fluconazole 100 mg orally once daily were routinely given to patients expected to obtain absolute neutrophil counts below $0.5 \times 10^9/L$ for more than 7 d. Antibacterial prophylaxis was given only in special cases, as for example, a preceding documented Gram-negative bloodstream infection.

Blood cultures were performed with a minimum of two samples, with at least one retrieved from a peripheral vein. Then, 5–8 mL blood was inoculated into each blood culture bottle, one anaerobic and one aerobic from each site. Until 1993, a regular, manual aerobic and anaerobic blood culture system with agar slants was used. The cultures were incubated for 7 d. On signs of growth, subculture was performed, and the recovered isolate was subjected to species determination following established laboratory procedures. From 1993 onwards, the commercial BacT/Alert 3D system with aerobic and anaerobic bottles was used (bioMérieux, Marcy l'Etoile, France).

Bacteremia was defined as an infection manifested by (1) the presence in at least one blood culture of bacteria other than skin contaminants (i.e., *Corynebacterium* spp., *Bacillus* spp., *Propionibacterium* spp., and coagulase-negative staphylococci [CoNS]) or (2) the presence in at least two blood cultures from the same fever episode of any bacterial species. The date of the first positive blood culture (index culture) was regarded as the date of bacteremia onset. Infections were classified as polymicrobial if two or more different species were recovered from specimens drawn within 24 h, regardless of whether the isolates came from the same or different blood culture sets. Growth of the same microorganism in a new culture

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after a period of ≥7 d from the index culture was considered as a new infectious episode (22).

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

Antimicrobial susceptibility

For all strains isolated, the antibiograms were primarily determined with the disk diffusion method standardized according to the Swedish Reference Group for Antibiotics (SRGA) (23). Isolates were classified either as susceptible (S), intermediate (I), or resistant (R), according to the SRGA breakpoints. Since 1998, the clinical microbiology laboratory at the Karolinska University Hospital has been fully computerized regarding the registration of requests and the reporting of results (ADBakt, Autonik AB, Sköldinge, Sweden). The infection was considered caused by an organism with acquired resistance (I or R) if resistance to defined antibiotics was observed in isolates belonging to the following species: imipenem, piperacillin–tazobactam or metronidazole for anaerobic bacteria.; imipenem, piperacillin–tazobactam, ceftazidime or ciprofloxacin for Enterobacteriaceae and *Pseudomonas* spp.; vancomycin for *Enterococcus faecium;* isoxazolyl-penicillin for *Staphylococcus aureus;* trimethoprim–sulfamethoxazole for *Stenotrophomonas maltophilia*; and benzyl-penicillin for viridans streptococci, β-hemolytic streptococci, and *Streptococci pneumonia*. Owing to the lack of adequate methodology for studying low-grade vancomycin resistance in staphylococci in previous years (disk diffusion can only detect high-grade resistance), a proper comparison of resistance levels could not be conducted (24).

Table 1 Isolated pathogens 2002–2008; 667 episodes in 463 patients

Pathogen[1]	No. (%)
Gram-negative bacilli, aerobes	(46.9)
Escherichia coli	141 (17.8)
Klebsiella spp.	78 (9.8)
Enterobacter spp.	43 (5.4)
Pseudomonas spp.	42 (5.3)
Citrobacter spp.	10 (1.3)
Stenotrophomonas maltophilia	6 (0.8)
Proteus spp.	3 (0.4)
Acinetobacter spp.	1 (0.1)
Haemophilus influenzae	2 (0.3)
Morganella spp.	2 (0.3)
Serratia marcescens	1 (0.1)
Other Gram-negative bacilli	20 (2.5)[1]
Gram-negative anaerobes	23
Bacteroides spp.	14 (1.8)
Fusobacterium spp.	4 (0.5)
Other Gram-negative anaerobes	5 (0.6)[2]
Gram-positive bacteria, aerobes	(53.1)
Coagulase-negative staphylococci	117 (14.7)
Viridans streptococci	111 (14.0)
β-hemolytic streptococci	15 (2.0)[3]
Enterococcus faecium	61 (7.7)
E. faecalis	11 (1.4)
Staphylococcus aureus	55 (6.9)
S. pneumoniae	18 (2.3)
Listeria spp.	2 (0.3)
Bacillus spp.	5 (0.6)
Enterococcus spp.	3 (0.4)[4]
Other Gram-positive bacteria	9 (1.1)[5]
Gram-positive anaerobes	15
Clostridium spp.	14 (1.8)
Other Gram-positive anaerobes not typed	1 (0.1)

[1]Other Gram-negative bacilli include *Moraxella* spp., *Neisseria* spp., *Capnocytophaga* spp., *Aeromonas* spp., *Hafnia alvei*, *Rosemonas gilardii*, *Salmonella* Hadar, *Sphingomonas paucimobilis*, Gram-negative bacillus not typed;
[2]Other Gram-negative anaerobes include *Veillonella* spp., *Leptotrichia* spp., and Gram-negative anaerobes not typed;
[3]β-Hemolytic streptococci include β-hemolytic streptococci group A + B + C + G;
[4]*Enterococcus* spp. excluding *E. faecalis* and *E. faecium;*
[5] Other Gram-positive bacteria include *Lactobacillus* spp., *Corynebacterium* spp. *Rothia mucilaginosa*, Gram-positive not typed, *Stomatococcus mucilaginosus, Gemella spp.*

Results

Characteristics of the period 2002–2008

A total of 10 071 blood cultures were drawn during 2002–2008, out of which 15.3% were positive. Excluding negative results, contaminants, and duplicate results during the same infectious episode, a total of 677 episodes of bacteremia in 463 patients in the hematology ward were observed during the period.

The dominating pathogens were *Escherichia coli* (18%), CoNS (15%), viridans streptococci (14%), and *Klebsiella* spp. (10%). The proportion of Gram-positive isolates was 53% and Gram-negative 47% (Table 1).

Polymicrobial bacteremia within 24 h was seen in 13.7% (Table 2). Common isolates in polymicrobial episodes, with occurrence ≥4 times, were *E. coli* with *Klebsiella* spp., CoNS with viridans streptococci, viridans streptococci with *E. coli*, and *E. coli* with *Pseudomonas* spp. We also looked at the alternative to include isolates within 72 h as constituting one bacteremic episode. However, very few additional isolates were found by increasing the inclusion time from 24 to 72 h, and no statistical difference in species distribution in polymicrobial episodes was seen.

The 7- and 30-d crude mortality rates were 5.2% (35/677) and 13.6% (92/677), respectively. Among the 35 patients who died within 7 d, 12 patients had a diagnosis of acute leukemia, 10 aggressive lymphoma, eight

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Table 2 Isolates included in polymicrobial bacteremic episodes (isolated within <24 h), in the order of occurrence

Pathogen	Occurrence of combination of isolates	No. isolates in polymicrobial episodes	% of all polymicrobial isolates
Escherichia coli	7	32	15.3
Klebsiella spp.		25	12.0
CoNS	7	20	9.6
Viridans streptococci		38	18.2
E. coli	5	32	15.3
Viridans streptococci		38	18.2
E. coli	4	32	15.3
Pseudomonas spp.		13	6.2
Viridans streptococci	3	38	18.2
β-hemolytic streptococci[1]		7	3.3
Klebsiella spp.	3	25	12.0
Enterobacter spp.		8	3.8
Enterococcus faecalis	3	6	2.9
CoNS		20	9.6
Other	≤2	60	
Total		209	

[1] β-Hemolytic streptococci including b-hemolytic streptococci group A + B + C + G; Other includes *Citrobacter* spp., *Stenotrophomonas maltphilia, Proteus* spp., *Morganella* spp., *Moraxella* spp., *Capnocytophaga* spp., *Hafnia alvei*, *Bacteroides* spp., *Fusobacterium* spp., *Veillonella* spp., *Leptotrichia* spp., *Enterococcus* spp, *S. aureus, S. pneumonia, Rothia mucilaginosa,* Gram-positive anaerob bacillus not typed, *Gemella* spp., Gram-positive bacillus not typed and *Gemella* spp.

myeloma, three chronic lymphoctic leukemia, and one patient aplastic anemia and myelodysplastic syndrome, respectively. No bacteremia-associated mortality was recorded in patients undergoing autologous stem cell transplantation. A similar distribution of diagnoses was seen among patients who died within 30 d. Polymicrobial bacteremia was seen in 26% (9/35) of the patients who died within 7 d and in 18% (17/92) of those who died within 30 d. Polymicrobial bacteremia occurred in 25.7% (9/35) of patients with fatal outcome and in 13.1% (84/642) of blood cultures from survivors ($P = 0.04$). Among deceased within 7- and 30-d, 71% (25/35) and 70% (64/92) were men, respectively.

In the group of patients who died within 7 d (35 patients), 49 bacterial isolates were found. Dominating pathogens were *E. coli* (27%, 13 isolates), *E. faecium* (13%, six isolates), and *Pseudomonas* spp. (8%, four isolates). The proportion of Gram-negative bacteremia was higher in patients who died within 7 d (59%) compared with survivors (46%), but the difference was not statistically significant ($P = 0.08$). Among those deceased within 30 d ($n = 92$), 124 isolates were found, dominated by *E. coli* (21%, 25 isolates), *E. faecium* (16%, $n = 19$), CoNS (15%, $n = 18$), and viridans streptococci (10%, $n = 12$). There was no statistically significant difference in the proportion of Gram-negative bacteremias among patients who died within 30 d and survivors (48% vs. 47%, $P = 0.77$).

From those deceased within 7 d, we isolated six strains with acquired resistance (I or R), three isolates of *E. coli* (two ciprofloxacin resistant and one ciprofloxacin plus piperacillin–tazobactam resistant, respectively), one *Klebsiella* spp. (ciprofloxacin), one *Pseudomonas* spp. (imipenem), and one *Enterobacter* spp. (ceftazidime- and piperacillin–tazobactam).

In patients dying within 30 days, 22 resistant isolates were identified (I or R), 9 *E. coli* (six ciprofloxacin resistant, two ciprofloxacin plus piperacillin–tazobactam resistant, and one piperacillin–tazobactam resistant, respectively), four viridans streptococci (benzyl-penicillin), three *Pseudomonas* spp. (two imipenem and one imipenem plus piperacillin–tazobactam resistant), two *Enterobacter* spp. (ceftazidime- and piperacillin–tazobactam), and one each of *Citrobacter* spp. (ceftazidime- and piperacillin–tazobactam), *Klebsiella* spp (ciprofloxacin), *Veillonella* spp. (piperacillin–tazobactam), and *E. faecium* (vancomycin). Resistant *E. coli* was significantly more often isolated from patients who died (36%, 9/25) within 30 d than in those who survived (14.9%, 21/141) the bacteremic episode ($P = 0.02$).

Temporal trends in patient characteristics and mortality during a 28-yr period

Between 1988 and 2008, the median age of the patients increased from 58 to 62 yr, and the male predominance increased from 58% to 62%.

During the four time periods 1980–1986, 1988–1994, 1995–2001, and 2002–2008, a total of 2,457 (263, 605, 795, and 794, respectively) relevant bacterial isolates were isolated from 1520 adult patients (129, 383, 545 and 463). During the last three periods, the proportion of positive blood cultures was stable (16, 17, and 15%, respectively).

A nonsignificant decrease in 7- and 30-d mortality was observed in 2002–2008 period compared with the preceding 7-yr period, when crude mortality rates were 6.1% and 15.1%, respectively.(21) Mortality seemed even higher in 1980–86, when 26% of the patients with bacteremia were considered to have died of infection, but crude 30-d mortality was not presented and could not be calculated from the published data.

From 1995–2001 to 2002–2008, we observed an increase in *E. coli* bacteremia in patients who died both within 7 d (4.7–9.2%, $P = 0.24$) and within 30 d (12–18%, $P = 0.31$). Moreover, a numerical decrease in CoNS (7.3–2.6% $P = 0.14$ and 14–15% $P = 0.86$) and viridans streptococci (7.6–2.7% $P = 0.14$ and14–11% $P = 0.54$) was observed for the 7- and 30-d crude mortality rate, respectively (Table 3).

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Table 3 Common pathogen distribution over time in deceased patients

	7-d mortality				30-d mortality			
	1995–2001		2002–2008		1995–2001		2002–2008	
Species	No.	%	No.	%	No.	%	No.	%
Escherichia coli	6	12.2	13	26.5	15	12.5	25	20.2
Coagulase-negative staphylococci	9	18.4	3	6.1	17	14.2	18	14.5
Viridians streptococci	8	16.3	3	6.1	15	12.5	12	9.7
Enterococcus faecium	7	14.3	6	12.2	14	11.7	19	15.3
Klebsiella spp.	4	8.2	3	6.1	14	11.7	7	5.6
Pseudomonas aeruginosa	4	8.2	4	8.2	6	5	8	6.5
Stenotrophomonas maltphilia	1	14.3	3	6.1	8	6.7	3	2.4
Other	5	10.2	14	28.6	31	25.8	32	25.8
Total	49	100	49	100	120	100	124	100

P-values for difference, not significant.

Table 4 Percentage of different isolated species among episodes during four time periods

Pathogen	1980–1986 no. 253	1988–1994 no. 605	1995–2001 no. 795	2002–2008 no. 794
Gram-negative bacteria	52.6	45.8	45.0	46.9
Escherichia coli	27.3	16.7	16.0	17.8
Klebsiella spp.	11.8	9.0	9.9	9.8
Enterobacter spp.	2.8	3.3	3.1	5.4[1]
Pseudomonas spp.	6.7	6.3	4.9	5.3
Other Gram-negative bacilli	2	8.4	7.3	5.7
Gram-negative anaerobes	2.0	1.3	1.9	2.9
Gram-positive bacteria	47.4	54.2	55.2	53.2
CoNS	13.0	21.5	15.6	14.7
Viridans streptococci	13.0	12.7	13.2	14.0
Enterococcus faecium	–[2]	2.8	7.2	7.7
Other enterococcal spp	2.4[3]	2.2[3]	2.8	1.8[4]
Staphylococcus aureus	9.5	7.1	10.3	6.9[5]
Other Gram-positive bacteria	5.6	6.5	4.0	6.2
Gram-positive anaerobes	3.9	2.7	2.6	1.9

[1]Compared to 1995–2001, when 25 out of 795 were *Enterobacter* spp., there has been a significant increase ($P = 0.0352$).
[2]Not specified 1980-86, mostly *E. faecalis* 1988-2008.
[3]*Enterococcus spp.* were only identified to the genus level.
[4]*Enterococcus spp.* includes *E. faecalis, E. durans, E. gallinarum*.
[5]Compared to 1995–2001, when 82 out of 795 were *S. aureus,* there has been a significant decrease ($P = 0.0025$).

Temporal trends in species distribution and antimicrobial susceptibility during a 28-yr period

A significant increase in *Enterobacter* spp. (from 3.1% to 5.4%) and decrease in *S. aureus* (from 10.3% to 6.9%) was seen in the last 7-yr period compared with 1995–2001. *E. faecium* increased significantly between the second (1988–1994) and third (1995–2001) period and has remained frequent and stable at 8%. (21) The proportion of Gram-positive isolates has remained stable at a level of 53–55% from 1998, increasing nonsignificantly from 1980 to 1986 (47.4%, $P = 0.07$). (Table 4).

No general increase in resistance among Gram-negative bacteria was seen when comparing the last 7-yr period with the previously reported 21-yr time period. Only the increase in ciprofloxacin resistance in *E. coli* during the last 7-yr period was statistically significant. In general, the prevalence of acquired antimicrobial resistance according to the predefined definitions was low (Tables 5 and 6).

Blood cultures from hematological patients drawn in the emergency department vs. blood cultures drawn in the hematology department

To investigate the potential bias of excluding hematological patients with blood cultures drawn from the emergency department, we searched for blood cultures drawn from hematological patients admitted through the emergency department between 2002 and 2008. We limited the search to positive cultures with findings of *Klebsiella* spp., *Pseudomonas* spp., and *Enterobacter* spp. A total of

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Table 5 Resistance of gram-positive bacteria 2002–2008

	ISO no. (%)	CLI no. (%)	GEN no. (%)	PCG no. (%)	VAN no. (%)
Species					
CoNS	83 (71)	46 (39)	47 (40)	–	–
Staphylococcus aureus	2 (3.7)	1 (1.8)	0	–	0
Viridans streptococci	–	10 (9.0)	–	22 (20)	–
Enterococcus faecium	–	–	–	–	1 (1.6)
E. faecalis	–	–	–	–	0

ISO, isoxazolyl-penicillin; CLI, clindamycin; GEN, gentamicin; PCG, benzyl-penicillin; VAN, vancomycin.

Table 6 Resistance of gram-negative bacteria 2002–2008

	TZP no. (%)	CAZ no. (%)	IPM no (%)	CIP no (%)	GEN no (%)
Species					
Escherichia coli	13 (9.2)	3 (2.1)	0	22 (16)[1]	2 (1.4)
Klebsiella spp.	7 (9.0)	1 (1.3)	0	1 (1.3)	0
Enterobacter spp.	11 (26)	11 (26)	1 (1)	2 (4.7)	0
Acinetobacter spp.	–	–	0	0	–
P. aeruginosa	2 (4.8)	0	5 (12)	3 (7.1)	0

TZP, piperacillin–tazobactam; CAZ, ceftazidime; IPM, imipenem; CIP, ciprofloxacin; GEN, gentamicin.
[1]Significant increase from 2 to 16% ($P = 0.0005$) compared with 1995–2001.

30 new episodes were identified; 13 of *Klebsiella* spp., 14 of *Pseudomonas* spp., and three of *Enterobacter* spp. When comparing patients sampled in the emergency room and in the hematology department, we saw no statistically significant differences in median age, sex, or fatal outcome.

Discussion

This is the largest Scandinavian epidemiological description of bacteremia in patients with chemotherapy-induced neutropenia. Comparing the study period with the preceding 14 yr, there is a trend toward an increase in median age, atleast in part attributable to an aging population. In spite of this, there was a trend, however, not statistically significant, toward decreased mortality. In accordance with other studies, males seem to be over-represented in bacteremia, have a higher incidence of hematological malignancies, and increased mortality (25–29).

During the entire time period, comprising almost 30 yr, there has been a negligible use of fluoroquinolone prophylaxis at our institution and also in Sweden in general, contrary to the situation in most high-income countries outside of Scandinavia. The mortality rates in this study were lower than in international reports in general, despite the almost non-existent use of antibacterial prophylaxis and a relatively high prevalence of Gram-negative bacteremia and polymicrobial bacteremia (30–32). In the Cochrane meta-analysis by Gafter-Gvili *et al.*, first published in 2005 and later updated 2009, prophylaxis with fluoroquinolones in neutropenic patients resulted in a significant decrease in the risk of infection-related death, RR 0.58 (95% CI 0.45–0.74), and in the frequency of bacteremia (33). Without a head-to-head comparison, conclusions regarding the bacteremia incidence between published studies are hard to draw. However, in light of the already low mortality in our material such a reduction in crude mortality as noted in the Cochrane analysis would seem a remarkable achievement in the case general fluoroquinolone prophylaxis would be instituted in our institution.

The incidence of the main pathogens in our institution have remained essentially unchanged, with *E. coli,* CoNS, viridans streptococci, *Klebsiella* spp., *E. faecium,* and *S. aureus* dominating. However, there has been a significant increase in the incidence of bacteremia with more resistant bacteria, like *E. faecium* and *Enterobacter* spp. and also with fluoroquinolone-resistant *E. coli* during the last 21 yr. Interestingly, antibiotic-resistant *E. coli* was also observed more frequently in patients who died within 30 d. β-Lactam resistance in viridans streptococci was 20%, and strains with high MIC values to benzyl-penicillin are fairly often seen, although historical comparison could not be made owing to the lack of data. In general, however, the rates of antibiotic resistance were low and essentially stable over a monitored period of 28 yr compared with many other countries in the world (34). Comparing 2002–2008 with the previous 14-yr period, there was no shift in the distribution between Gram-negative and Gram-positive bacteria, probably as a result of the negligible use of fluoroquinolone prophylaxis.

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Among Scandinavian countries, the levels of antimicrobial resistance are similar, but prophylactic antibiotic use seems to differ (35, 36). Mortality rates in Norwegian centers are slightly higher than those found in this study. According to a recent Norwegian publication, Gram-negative resistance did not contribute to this mortality, although the absolute number of bacteremia episodes ($n = 95$) was small (37). The prevalence of fluoroquinolone resistance in *E. coli* was higher than that reported in the annual European blood culture statistics for the entire Sweden (38), but this could be due to methodological factors, as the definition of fluoroquinolone resistance has recently changed in Sweden following the European harmonization of S-I-R categorization. In a historic material such as this one, isolates with low-level resistance to fluoroquinolones, which are considered susceptible in Europe (24), are here considered non-susceptible. Nevertheless, an increase in fluoroquinolone resistance has been observed at Karolinska University Hospital, which probably reflects the increasing resistance to fluoroquinolones in the community. This could be due to factors such as misuse of fluoroquinolones and could also be attributable to, for example, veterinary use of antimicrobials and exposure through food (39). The impact of fluoroquinolone prophylaxis on the total burden of resistance is probably quite small, but it is still possible to observe that resistance levels in our study were in general lower than in centers using fluoroquinolone prophylaxis (30, 40).

Polymicobial bacteremia was common in this study and associated with a significantly higher mortality than monomicrobial bacteremia. One reason for this is that polymicrobial infections are common in breakthrough infection, where mortality is higher than in monomicrobial infections (41, 42). This is in accordance with a population-based Danish study where polymicrobial infection was associated with increased mortality (43). Extending the accepted time to 72 h resulted in very few additional cases of polymicrobial bacteremia or change in distribution of species. This supports the proposed definition of definite polymicrobial bacteremia where isolation of more than one bacterial species from blood cultures obtained within 24 h is sufficient for the diagnosis (44).

In our study, the 7- and 30 d mortality rates, 5.2% and 13.6%, respectively, decreased slightly compared with previous periods. Among deceased patients, the distribution of pathogens did not significantly differ from the distribution in surviving patients apart from when *Stenotrophomonas maltophilia* was the causative pathogen ($P = 0.01$). The trend for *E. coli* was almost significant ($P = 0.14$) among those who died <7 d from a positive culture. This could be due to the common occurrence of *E. coli* in polymicrobial bacteremia. Moreover, resistant *E. coli* was significantly more often isolated in patients who died within 30-d compared with survivors ($P = 0.02$).

Most hematological patients with fever in our hospital are admitted directly to the hematology ward, but some patients first seek medical attention via the emergency department. A limitation to the present study is that blood cultures drawn from hematological patients in the emergency department were not included in the analysis. Thus, there could be a selection bias and external validity problems. We therefore investigated whether the distribution of species, age, sex, or crude mortality differed between hematological patients who had blood cultures drawn in the hematology ward and in the emergency department, but no significant differences were observed. Hence, we believe that the additional cases would not have had a significant impact on the conclusions.

Our low and slowly decreasing mortality rate could be regarded to support the correctness of our antibiotic policy. The prevalence of different pathogens in bacteremia is essentially stable, but with a significant trend toward more antimicrobial resistance. Moreover, polymicrobial bacteremia was common and in accordance with other studies associated with a higher mortality rate. It is therefore important to have a high suspicion of antibiotic resistance in patients who do not respond to the initially selected antibiotic drug also in centers as ours with limited resistance problems. At the same time, it is evident that antibiotic therapy can be shortened and simplified in a majority of patients who do not have bacteremia or a serious course, as we have shown previously (45, 46). A majority of patients can thereby return to their home with continued outpatient/day-care observations after an initial inpatient management. This will lead to increased patient satisfaction and decreased risks of hospital-acquired infections and emergence of antibiotic resistance. With this policy, fluoroquinolones will be used for early shift from intravenous broad-spectrum antibiotics to oral outpatient antibiotics instead of for prophylaxis.

In conclusion, the avoidance of fluoroquinolone prophylaxis may have contributed to a stable proportion of Gram-positive bacteremia. The crude mortality was low in an international perspective. Acquired resistance was uncommon, but ciprofloxacin resistance in *E. coli* increased significantly. We believe that an indiscriminate use of antibacterial prophylaxis could be avoided in neutropenic patients without a negative impact on mortality.

Authorship and disclosures

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ORIGINAL ARTICLE

Bloodstream infections in patients with chronic lymphocytic leukemia: a longitudinal single-center study

Christian Kjellander [1,2] · **Magnus Björkholm** [1] · **Owe Källman** [3] · **Christian G. Giske** [3] · **Caroline E. Weibull** [4] · **Thorvardur J. Löve** [5,7] · **Ola Landgren** [6] · **Sigurdur Y. Kristinsson** [1,7]

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Abstract Infectious complications in chronic lymphocytic leukemia (CLL) represent a major cause of morbidity and mortality. The aim of the study was to investigate temporal trends in bloodstream infections (BSIs) among patients with CLL. Individuals with blood cultures were linked to Swedish Cancer Registry and divided into three time periods (1988–1993, 1994–1999, and 2000–2006) according to year of CLL diagnosis. CLL patients ($n = 275$) with 1092 blood culture episodes were identified and linked to the nationwide Cause of Death Registry and Swedish Patient Registry (to retrieve information on splenectomies). The most common causes of BSI among CLL patients were *Escherichia coli* (11/43, 15/78, and 9/33), *Streptococcus pneumoniae* (7/43, 13/78, and 6/33), *Pseudomonas aeruginosa* (2/43, 8/78, and 3/33), *Staphylococcus aureus* (1/43, 6/78, and 6/33), and *Viridans streptococci* (5/43, 6/78, and 2/33). Coagulase-negative staphylococci was the most frequent microorganism found in blood cultures (22/70, 23/106, and 5/41, respectively) but is a frequent contaminant. Based on the largest study to date on BSI in CLL patients, we found a stable proportion of Gram-positive to Gram-negative bacteria and no temporal change of distribution was observed for BSIs 1988–2006.

Keywords Chronic lymphocytic leukemia · Infections · Prognosis · Bloodstream infections · Splenectomy

✉ Christian Kjellander
Christian.Kjellander@karolinska.se

[1] Division of Hematology, Department of Medicine, Karolinska University Hospital Solna and Karolinska Institutet, Stockholm, Sweden

[2] Hematologiskt Centrum, Karolinska Universitetssjukhuset Solna, 17176 Stockholm, Sweden

[3] Clinical Microbiology, Karolinska University Hospital and Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden

[4] Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, Sweden

[5] Centre for Rheumatology Research, Landspitali University Hospital and University of Iceland, Reykjavik, Iceland

[6] Myeloma Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

[7] Faculty of Medicine, University of Iceland and Landspitali University Hospital, Reykjavik, Iceland

Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults in the Western world with an incidence of approximately 5 per 100,000 person-years in Sweden [1]. It affects men approximately twice as frequently as women and is a disease of older people with a median age at diagnosis of approximately 71 years. Clinical management of CLL patients has improved considerably over recent years, to which an increased use of prognostic markers, introduction of new therapeutic agents and procedures, and improved supportive care measures all have contributed [2, 3].

Infectious complications have since long been recognized as a major cause for morbidity and mortality in patients with CLL [4, 5]. Based on studies from 1970s to 1990s, the overall mortality from infections ranges from 30 to 50 %; however, large studies on CLL patients diagnosed in more recent years are lacking [6]. The pathogenesis of infections in CLL patients is multifactorial and is related to inherent immune defects and therapy-related immunosuppression [7]. The inherent immune dysfunction is characterized by defects in both humoral and cell-mediated immunity. Cytotoxic chemotherapy also targets

normal immune cells, thus exacerbating this immunosuppression. Splenectomy or hyposplenism (secondary to irradiation to spleen) performed for complications of CLL including autoimmune hemolytic anemia or thrombocytopenia, hypersplenism, and symptomatic splenomegaly is associated with complex innate and adaptive immune responses [8]. Overwhelming post-splenectomy infection is a rare medical emergency [9, 10]. Use of alemtuzumab is associated with neutropenia and reductions in B, T, and NK cells and rituximab with transient reduction of B cell counts; both antibodies are associated with increased risk of infections [11].

Apart from data based on clinical trials and smaller case studies, there is a lack of detailed knowledge on bloodstream infections (BSIs) in CLL patients, their impact on survival, and the effect of the anti-tumoral treatments on the distribution of causative pathogens over time. We thus performed a study, based on blood cultures sampled from CLL patients which were analyzed 1988–2006 at the Clinical Microbiology Laboratory, Karolinska University Hospital Solna, Sweden. The aims were to identify all culture-verified blood cultures, to identify potential changes of causative agents over time, and to record changes in patient survival.

Patient and methods

Data sources and study population

In Sweden, more than 96 % of all new cancers are reported to the national Swedish Cancer Registry, which was established in 1958 and has a high coverage for hematological malignancies [1, 12, 13]. Every physician and pathologist/cytologist is obliged by law to report each occurrence of cancer to the registry.

All blood cultures that were analyzed at the Karolinska University Hospital's Clinical Microbiology Laboratory were identified, and individuals who had at least one blood culture analyzed between 1988 and 2006 were registered (Fig. 1). Individuals with a drawn blood culture were linked to the nationwide Swedish Cancer Registry to identify all persons diagnosed with CLL. Only CLL patients with a blood culture drawn at the same date or after CLL diagnosis were included in the analyses. A positive blood culture included growth of any microorganism in blood culture. A BSI was defined as growth of bacteria in blood culture excluding commonly found contaminants [14]. Growth of the same microorganism within 7 days was considered as the same BSI episode.

Information from the nationwide Cause of Death Registry (with data from 1954) was used to retrieve dates of death, and the Swedish Patient Registry (with data from 1964) was used to retrieve information on individual patient-based discharge diagnoses from inpatient and outpatient care [15].

Approval for this study was obtained from the Regional Ethical Review Board in Stockholm (record 2007/1307-31/5).

Statistical methods

Patient characteristics as well as bacterial findings were analyzed for three separate time periods of CLL diagnosis (1988–1993, 1994–1999, and 2000–2006; June 30). To evaluate survival after diagnosis of CLL, by blood culture pattern, data was analyzed using survival analysis methods with the event of interest being all-cause mortality during follow-up. Risk time was accumulated from date of first blood culture following diagnosis until death or December 31, 2009, whichever occurred first. Time since CLL diagnosis was used as the underlying timescale in all analyses. Crude mortality rates (and 95 % confidence intervals [CIs]) were calculated as number of events per 1000 person-years. The exposure of interest was a BSI following CLL diagnosis and was analyzed both as a dichotomous variable ("ever" vs "never") and as time-varying ("after" vs "before" or "never"). The effect of having had a splenectomy on survival was evaluated in a separate model. To evaluate possible associations, Cox proportional hazard models were used, yielding hazard ratios (HRs) with 95 % CIs. The proportional hazard assumption was formally assessed in all models, and no evidence suggested that this assumption was violated. Analyses were adjusted for time since diagnosis, sex, and age at diagnosis. The effect of the exposure was estimated separately for the three time periods of diagnosis by including an interaction term in the models. As a sensitivity analysis, to obtain similar time to first culture between different calendar periods, the cohort was restricted to patients diagnosed before June 30, 2005 who had their first culture within 1 year following diagnosis. Difference in proportion of BSI between time periods was tested using a chi-squared test or Fisher's exact test. Difference in mean time to culture was tested using a *t* test.

Stata v.12 software was used for the statistical analyses (StataCorp, College Station, TX, USA).

Results

A total of 275 CLL patients, diagnosed between January 1, 1988 and June 30, 2006, were included in the study. Among these CLL patients, a total of 1092 blood culture episodes were identified. The mean time between CLL diagnosis and first drawn blood culture was significantly shorter in the most recent time period (6.2, 4.7, and 2.4 years for the time periods 1988–1993, 1994–1999, and 2000–2006, respectively (p value <0.001); Table 1).

In the last time period, patients had a significant lower percentage of positive blood cultures (51.5, 45.9, and 28.2 %, respectively (p value 0.007)). Similarly, the percentage of patients with at least one BSI decreased significantly (38.2, 38.5, and 22.4 %, respectively (p value 0.033)). In a sensitivity analysis including only patients diagnosed until 2005 with their

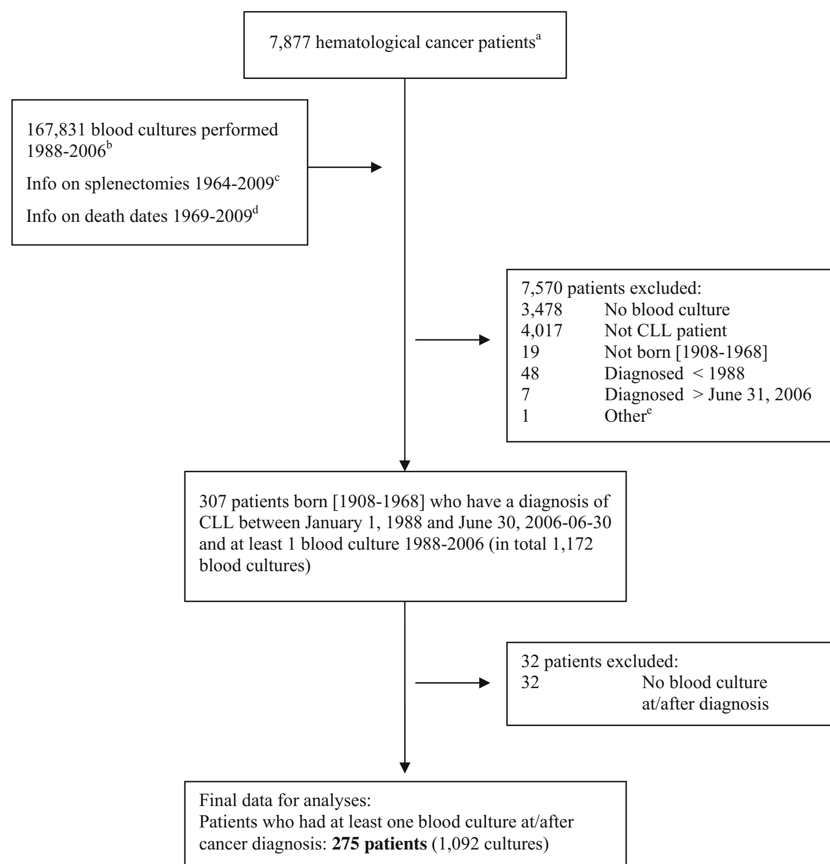

Fig. 1 Flowchart describing the study cohort. [a]Using the national registration number unique to all Swedish citizens, individuals with a drawn blood culture were linked to the nationwide Swedish Cancer Registry to identify all persons diagnosed with CLL (ICD-7 204.1 with morphology codes 98223, 98231, 98233, 982335, 982336, or missing morphology code (not 80003)) between January 1, 1988 and June 30, 2006. [b]One negative blood culture is defined as a negative culture with no previous cultures on the same date. One positive blood culture is defined as a positive culture with no previous positive cultures with the same finding within the same week. [c]The centralized Swedish Patient Registry contains information on individual patient-based discharge diagnoses from inpatient (since 1964, with nationwide coverage since 1987) and outpatient (since 2000) care. [d]Information from the nationwide Cause of Death Registry was used to retrieve dates of death. [e]Error in dataset (patient registered dead before blood culture date)

positive blood culture within a year of diagnosis, the decline remained statistically significant both for BSI (71.4, 36.4, and 13.8 %; *p* value 0.008) and for all positive blood cultures (71.4, 50.0, and 24.1 %; *p* value 0.033; Fig. 2).

Positive blood cultures were observed in 115 CLL patients, with 215 positive blood cultures for bacteria and two episodes of candidemia. Dominating bacterial species were coagulase-negative staphylococci (CoNS) (22 (31 %), 23 (22 %), and 5 (12 %), for the three time periods, *Escherichia coli* (11 (16 %), 15 (14 %), and 9 (22 %)), *Streptococcus pneumoniae* (7 (10 %), 13 (12 %), and 6 (15 %)), *Pseudomonas aeruginosa* (2 (3 %), 8 (8 %), and 3 (7 %)), *Staphylococcus aureus* (1 (1 %), 6 (6 %), and 6 (15 %)), and *Viridans streptococci* (5 (7 %), 6 (6 %), and 2 (5 %)); Table 2). Excluding common contaminants (CoNS, *Micrococcus* spp., *Corynbacterium* spp., *Bacillus* spp., *Proprionbacterium acnes*, and Gram-positive bacillus (not further specified), 152 bacterial BSI (hereafter BSI) episodes (42, 77, and 33 in respective time period) and two episodes of candidemia were found. Excluding strictly anaerobes and fungi, the percentage of Gram-positive vs Gram-negative bacteria remained stable in the three time periods, respectively (61.4 vs 31.4 %, 54.7 % vs 34.0 %, and 65.9 vs 31.7 %; Table 2).

Mortality rates and relative risks of death among CLL patients with at least one positive blood culture or BSI at or following diagnosis, compared to patients with only negative blood culture episodes, or negative blood culture episodes including cultures with finding of contaminants, are presented in Table 3. In the last time period, mortality among patients with a BSI was significantly higher compared to patients with either a contaminant or a negative blood culture (HR = 2.52, 95 % CI 1.44–4.41), as well as compared to patients with negative blood cultures alone (HR = 2.44, 95 % CI 1.38–4.30), when adjusting for time since diagnosis, age at diagnosis, and sex. Being splenectomized did not significantly affect mortality during any of the three time periods (HR = 0.97, 95 % CI 0.38–2.48; HR = 0.45, 95 % CI 0.19–1.06; and HR = 0.64, 95 % CI 0.25–1.60; Table 3).

Table 1 Demographic and infection characteristics among CLL patients having at least one blood culture episode at/following diagnosis, by time period of diagnosis

	Year of CLL diagnosis. Number of patients (%)[a]			
	1988–1993	1994–1999	2000–2006[b]	Total
Total	68 (100)	122 (100)	85 (100)	275
Sex				
Male	39 (57.4)	77 (63.1)	66 (77.7)	182
Age at diagnosis (years)				
30–39	1 (1.5)	2 (1.6)	0 (0.0)	3
40–49	2 (2.9)	1 (0.8)	3 (3.5)	6
50–59	14 (20.6)	17 (13.9)	7 (8.2)	38
60–69	20 (29.4)	36 (29.5)	27 (31.8)	83
70–79	28 (41.2)	44 (36.1)	26 (30.6)	98
80–	3 (4.4)	22 (18.0)	22 (25.9)	47
Splenectomy				
Yes	6 (8.8)	10 (8.2)	8 (9.4)	24
Distribution of patients with one or more blood cultures drawn				
1	26 (38.2)	44 (36.1)	33 (38.8)	103
2	11 (16.2)	15 (12.3)	16 (18.8)	42
3+	31 (45.6)	63 (51.6)	36 (42.4)	130
Distribution of patients with positive blood cultures	35 (51.5)	56 (45.9)	24 (28.2)	115
Distribution of patients with BSI	26 (38.2)	47 (38.5)	19 (22.4)	92
Median age at first culture, irrespective of result (years)	73.5 (IQR 66.5–79.5)	74.0 (IQR 67.0–81.0)	75.0 (IQR 64.0–80.0)	74.0 (IQR 66.0–80.0)
Mean time to first culture in years (SD)	6.18 (3.38)	4.67 (3.06)	2.44 (1.76)	

[a] Due to rounding, not all percentages add up to 100

[b] Until June 30, 2006

In a sensitivity analysis, including only patients diagnosed until 2005 with their first culture within a year of diagnosis, the mortality rate was higher during 1994–1999 among patients with a BSI, as compared to patients with a contaminant or a negative blood culture (HR = 2.94, 95 % CI 1.04–8.33), as well as compared to patients with a negative blood culture alone (HR = 5.72, 95 % CI 1.74–18.7). During the same time period, the mortality rate was also higher comparing all positive blood cultures to negative blood cultures (HR = 3.55, 95 % CI 1.34–9.44; Table 3, lower panel).

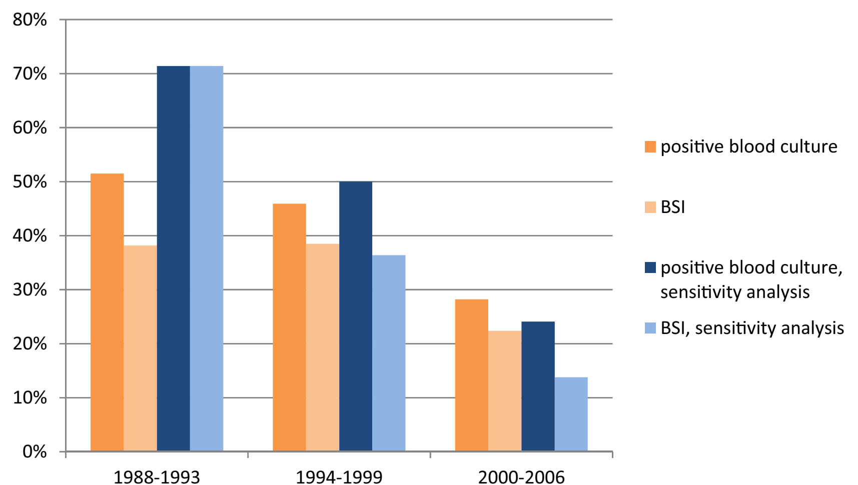

Fig. 2 Proportion positive blood cultures (BSI and all positive blood cultures) for the whole study population (diagnosed 1988–2006) as well as for the sensitivity analysis (diagnosed 1988–2005 with a blood culture within a year), at/following diagnosis by time period of diagnosis

Table 2 Distribution of positive blood cultures ($n = 217$ among 115 patients), and BSIs (in brackets), by time period of diagnosis

Type of finding, text	1988–1993		1994–1999		2000–2006[a]	
	Frequency	Percent	Frequency	Percent	Frequency	Percent
Total Gram-negative, aerobes	22 (22)	31.4	36 (36)	34.0	13 (13)	31.7
E. coli	11 (11)		15 (15)		9 (9)	
Klebsiella spp.	3 (3)		5 (5)		1 (1)	
Enterobacter cloacae	3 (3)		3 (3)		0	
Pseudomonas aeruginosa	2 (2)		8 (8)		3 (3)	
Haemophilus influenzae	0		1 (1)		0	
Other Gram-negative aerobes[b]	3 (3)		4 (4)		0	
Total Gram-positive, aerobes	43 (18)	61.4	58 (32)	54.7	27 (19)	65.9
Coagulase-negative staphylococci	22 (0)		23 (0)		5 (0)	
E. faecium	1 (1)		4 (4)		1 (1)	
E. faecalis	0		1 (1)		1 (1)	
Viridans streptococci[c]	5 (5)		6 (6)		2 (2)	
S. aureus	1 (1)		6 (6)		6 (6)	
β-hemolytic streptococci[d]	3 (3)		1 (1)		0	
S. pneumonia	7 (7)		13 (13)		6 (6)	
Other Gram-positive aerobes[e]	4 (1)		4 (1)		6 (3)	
Total strictly anaerobes	4 (2)	5.7	11 (9)	10.4	1 (1)	2.4
Bacteroides spp.	0		5 (5)		0	
Clostridium septicum	0		0		1 (1)	
Other, not specified anaerobes[f]	4 (2)		6 (4)		0	
Candida spp.[g]	1 (1)	1.4	1 (1)	1.4	0	
Total	70 (43)	100.0	106 (78)	100.0	41 (33)	100.0

[a] Until June 30, 2006

[b] Other Gram-negative aerobes includes *Chryseobacterium meningosepticum*, *Rhizobium* spp., *Capnocytophaga* spp., *Aeromonas* spp., *Moraxella* spp., and *Salmonella typhimurium*

[c] Includes *Streptococcus salivarius*, *Streptococcus mitis*, *Viridans streptococci* (not further specified), *Streptococcus milleri* group, *Streptococcus bovis*, and *Streptococcus acidominimus*

[d] Includes *β-hemolytic streptococci* group A and G

[e] Other Gram-positive aerobes includes *Bacillus* spp., *B. cereus*, *Corynebacterium* spp., Gram-positive bacillus (not further specified), *Micrococcus* spp., and *Listeria monocytogenes*

[f] Other, not specified anaerobes, includes *Veilonella* spp., anaerobic Gram-negative coccus (not further specified), anaerobic Gram-positive bacillus (not further specified), *Propionibacterium acnes*, *Fusobacterium* spp., *Leptotrichia* spp., and *Peptostreptococcus* spp

[g] *Candida* spp., includes *Candida lusitaniae* and *Candida albicans*

When analyzing positive blood culture as a time-varying exposure, mortality was significantly higher among patients with a BSI both during 1994–1999 (HR = 2.59, 95 % CI 1.74–3.85) and 2000–2006 (HR = 3.21, 95 % CI 1.83–5.62; Table 4). In a sensitivity analysis, the mortality among patients with BSI was significantly higher (HR = 5.07, 95 % CI 1.33–19.3) in the middle time period (data not shown).

Discussion

In this large study based on 1092 blood culture episodes from 275 CLL patients analyzed between 1998 and 2006 at Karolinska University Hospital Solna, we found a stable proportion of Gram-positive to Gram-negative bacteria and no changes in distribution of bacterial species were observed, with the dominating BSI pathogens being *E. coli*, *S. pneumoniae*, *P. aeruginosa*, *S. aureus*, and *V. streptococci*. CoNS was the most frequently detected microorganism in blood cultures but is a frequent contaminant. Our study is of importance given concerns of infectious complications using new treatment options and highlights real-life data in a large cohort of CLL patients.

We found a significant decrease in proportion of positive blood cultures and BSI between 1994 and 1999 and between 2000 and 2006. This is in contrast to earlier experiences from blood cultures collected from the hematology ward 1988–2008 in the same institution, where we observed stable

Table 3 Frequencies, mortality rates, and hazard ratios (HRs) with 95 % confidence interval (CI) among patients with at least one positive blood culture at/following diagnosis, by time period of diagnosis

–	1988–1993			1994–1999			2000–2006[a]		
	Number (deaths)	Mortality rate[b] (95 % CI)	HR[c] (95 % CI)	Number (deaths)	Mortality rate[b] (95 % CI)	HR[c] (95 % CI)	Number (deaths)	Mortality rate[b] (95 % CI)	HR[c] (95 % CI)
Total	68 (60)	250.4 (194.4–322.5)	–	122 (107)	290.7 (240.5–351.3)	–	85 (64)	303.7 (237.7–388.0)	–
Culture result									
BSI[d]	26 (24)	229.8 (154.0–342.9)	0.70 (0.41–1.21)	47 (45)	327.0 (244.2–438.0)	1.37 (0.92–2.03)	19 (18)	550.5 (346.8–873.7)	2.52 (1.44–4.41)
Contaminant or negative blood culture episode	42 (36)	266.3 (192.1–369.1)	1.00	75 (62)	269.0 (209.7–345.0)	1.00	66 (46)	258.3 (193.5–344.9)	1.00
BSI[d]	26 (24)	229.8 (154.0–342.9)	0.75 (0.42–1.35)	47 (45)	327.0 (244.2–438.0)	1.44 (0.96–2.18)	19 (18)	550.5 (346.8–873.7)	2.44 (1.38–4.30)
Negative blood culture episode	33 (27)	248.1 (170.2–361.8)	1.00	66 (53)	252.5 (192.9–330.6)	1.00	61 (42)	256.6 (189.6–347.2)	1.00
Positive blood culture (all)	35 (33)	252.3 (179.3–354.8)	0.80 (0.47–1.37)	56 (54)	341.3 (261.4–445.6)	1.43 (0.97–2.10)	24 (22)	467.4 (307.8–709.9)	1.63 (0.96–2.75)
Negative blood culture episode	33 (27)	248.1 (170.2–361.8)	1.00	66 (53)	252.5 (192.9–330.6)	1.00	61 (42)	256.6 (189.6–347.2)	1.00
Splenectomy									
Yes	6 (5)	253.3 (105.4–608.5)	0.97 (0.38–2.48)	10 (6)	95.3 (42.8–212.2)	0.45 (0.19–1.06)	8 (5)	177.5 (73.9–426.6)	0.64 (0.25–1.60)
No	62 (55)	250.1 (192.0–325.8)	1.00	112 (101)	331.0 (272.3–402.3)	1.00	77 (59)	323.1 (250.3–417.0)	1.00
Culture result of sensitivity analysis[e]	7 (6)	173.0 (77.7–385.0)	–	22 (21)	298.9 (194.9–458.4)	–	29 (24)	323.8 (217.0–483.1)	–
BSI[d]	5 (5)	370.3 (154.1–889.6)	0.58 (0.06–5.70)	8 (8)	511.1 (255.6–1022)	2.94 (1.04–8.33)	4 (4)	578.8 (217.2–1542)	2.33 (0.74–7.32)
Contaminant or negative blood culture episode	2 (1)	47.2 (6.65–335.1)	1.00	14 (13)	238.1 (138.2–410.0)	1.00	25 (20)	297.6 (192.0–461.2)	1.00
BSI[d]	5 (5)	370.3 (154.1–889.6)	0.60 (0.06–5.98)	8 (8)	511.1 (255.6–1022)	5.72 (1.74–18.7)	4 (4)	578.8 (217.2–1542)	2.07 (0.64–6.64)
Negative blood culture episode	2 (1)	47.2 (6.65–335.1)	1.00	11 (10)	202.1 (108.8–375.6)	1.00	22 (17)	288.4 (179.3–463.9)	1.00
Positive blood culture episode (all)	5 (5)	370.3 (154.1–889.6)	0.56 (0.06–5.42)	11 (11)	529.3 (293.1–955.7)	3.55 (1.34–9.44)	7 (7)	461.3 (219.9–967.7)	1.25 (0.47–3.38)
Negative blood culture episode	2 (1)	47.2 (6.65–335.1)	1.00	11 (10)	202.1 (108.8–375.6)	1.00	22 (17)	288.4 (179.3–463.9)	1.00

[a] Until June 30, 2006

[b] Per 1000 person-years

[c] Estimated from Cox regression adjusted for time since diagnosis, age at diagnosis, sex, and with interaction between exposure and time period of diagnosis. Patients start being at risk at date of first culture following diagnosis

[d] BSI excludes probable contaminants (CoNS, *Micrococcus* spp., *Corynbacterium* spp., *Bacillus* spp., *Propionibacterium acnes*, Gram-positive bacillus (not further specified), and anaerobic Gram-positive bacillus (not further specified))

[e] Patients diagnosed until June 30, 2005 who had their first blood culture within 1 year following diagnosis

Table 4 Frequencies, mortality rates, and hazard ratios (HRs) with 95 % confidence interval (CI) comparing patients before or without BSI to after a BSI, by time period of diagnosis

	1988–1993		1994–1999		2000–2006[a]	
	Mortality rate[b] (95 % CI)	HR[c] (95 % CI)	Mortality rate[b] (95 % CI)	HR[c] (95 % CI)	Mortality rate[b] (95 % CI)	HR[c] (95 % CI)
All patients						
After BSI[d]	304.0 (203.8–453.5)	1.27 (0.75–2.17)	513.9 (383.7–688.3)	2.59 (1.74–3.85)	666.1 (419.7–1057)	3.21 (1.83–5.62)
Before/without BSI[d]	224.0 (161.6–310.6)	1.00	221.0 (172.3–283.5)	1.00	250.4 (187.5–334.2)	1.00

[a] Until June 30, 2006

[b] Per 1000 person-years

[c] Estimated from Cox regression adjusted for time since diagnosis (timescale), age at diagnosis, sex, and with interaction between exposure and time period of diagnosis

[d] BSI excludes possible contaminants

percentage of positive blood cultures over time [16]. A possible explanation could be the introduction of combination treatments including monoclonal antibodies [17–19]. We speculate that more effective therapy given in recent years, which leads to deeper responses and longer time to next progression, results in the observed reduction of positive blood cultures and BSI. Our results are supported by a study from the UK where a trend to lower rates of BSI over three 12-month periods was observed [20]. On the other hand, in more aggressive hematological diseases, BSI seems to have increased in another UK center between 2004 and 2010 [21]. However, anti-bacterial prophylaxis, non-comparable use of anti-cancer therapy, and different overall survival make comparisons difficult. Moreover, stated limitations preclude us from interpreting any trends from our data in proportion of positive blood cultures or BSI.

The stable distribution between Gram-positive and Gram-negative bacteria observed in this study is similar to an observation by Francis et al. [22]. Nevertheless, in two reviews of BSI in hematology and cancer patients and a questionnaire on the etiology of BSI in conjunction with the Fourth European Conference on Infections in Leukemia (2011), a recent trend in increased number of infections caused by Gram-negative bacilli was found [23, 24]. The proposed explanation is decreased use and shorter duration of anti-bacterial prophylaxis and, to a lesser extent, decreased use of indwelling catheters and cytotoxic chemotherapy. Trimethoprim/sulfamethoxazole effectively reduces risk of *Pneumocystis jirovecii* pneumonia and also reduces the incidence of BSI [25–27]. Considering the scarce use of fluoroquinolone prophylaxis and indwelling catheters in CLL, comparisons of trends in BSI with other malignancies must be done with caution.

During the study period, the anti-tumoral armamentarium has expanded and treatment has remained reserved for patients with systemic symptoms or progressive disease [28]. The monoclonal anti-CD-20 antibody rituximab in combination with FC was shown in 2005 to further improve response rates [18, 19]. For rituximab and other anti-tumoral agents, only scarce information on BSI in CLL exists [6, 11, 18, 19, 29–32]. Furthermore, major infectious complications increase with previously received anti-tumoral therapies [33, 34]. Our results on the most common pathogens in BSI might affect anti-biotic choice when empirical therapy in CLL patients with signs and symptoms of BSI is considered.

We found BSI to be associated with worse prognosis especially during the latter time periods, but limitations of our study may bias our results. The effect was seen both when treating blood cultures as a dichotomous exposure and as time-varying. This has to our knowledge not been shown previously in CLL patients. To evaluate this further, we did sensitivity analysis on a homogenous group of more high-risk patients, by only including patients diagnosed 1988 to 2005 who had a culture within 1 year of diagnosis. The point estimates during 1999–2005 remained of similar magnitude, although slightly smaller as compared to those from the full cohort analysis, and they were no longer statistically significant. However, fewer deaths in this analysis made power to detect potential differences small, which is reflected in the wide confidence intervals. During the middle period, where event counts were larger, a significantly higher mortality was observed among patients with BSI. This, together with the non-significant risk increase in the last time period, support the hypothesis that among more high-risk patients, a positive blood culture results in higher mortality. In a retrospective study of BSI 1988–2008, from the same institution, antibiotic resistance was stable and should therefore not influence our results on temporal trends in mortality rate [16]. We speculate if CLL patients in later time periods, due to introduction of immunotherapy, had developed therapy-related immunodeficiencies in their adaptive immune response, with less favorable outcomes of BSI. Our study includes a stable number of splenectomies among CLL patients during the study period. No statistically significant differences in mortality were seen when comparing patients with splenectomy to those without.

However, the numbers of splenectomies were small, and thus, the results are limited by power.

To our knowledge, this is the first study to examine the risk for and distribution of BSI in patients with CLL. Previous reports have presented BSI in all hematological diseases as a group [16, 35], presented mortality rates for CLL with BSIs or major infections without displaying CLL-specific pathogen distribution [36, 37], or only presented very few BSIs among CLL patients [17]. The main strengths are the large sample size from a single institution, the use of high-quality registries, and the uniform treatment according to national guidelines. Our study has some important limitations. Generalizability is impeded by the retrospective data and that data consists of CLL patients with drawn blood culture, rather than all CLL patients. Information on important clinical descriptors were not available as, for example, anti-tumoral treatment, anti-microbial prophylaxis, anti-microbial resistance, and site of drawn blood culture. For clinical purpose, we therefore excluded pathogens known to be possible contaminants even though delineation of clinical significance can be difficult in the immunocompromised host. For the latter time period, due to administrative censoring, time window for having a blood culture drawn was shorter as compared to that for patients diagnosed during the earlier time periods. Low counts of exposure and events further hampered our possibilities to draw strong conclusions on temporal differences in the sensitivity analyses of BSI and mortality. Lastly, the design of the study, organizing 1.8 million microbial analyzes and then cross-matching data against three national registries, rendered delays in presentation of results.

In summary, based on the largest study to date on BSI in CLL patients, we found a stable proportion of Gram-positive to Gram-negative bacteria for BSIs 1988–2006. The most common pathogens of BSI were *E. coli*, *S. pneumoniae*, *P. aeruginosa*, *S. aureus*, and *V. streptococci*, and no temporal change of distribution was observed. Surveying treatment-related infections and optimizing prophylaxis will be important objectives when improving anti-tumoral treatments for CLL.

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Contribution C Kjellander, SY Kristinsson, O Källman, and M Björkholm designed the study. C Kjellander and SY Kristinsson drafted the manuscript. O Källman, TJ Löve, O Landgren, and SY Kristinsson set up the database. CE Weibull and C Kjellander performed the statistical analyses. All authors contributed to the writing of the final manuscript. All the authors were involved in the interpretation of the results; read, gave comments, and approved the final version of the manuscript; had full access to the data in the study; and take responsibility for the accuracy of the data analysis.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethic approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study, formal consent is not required.

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RESEARCH ARTICLE

Bacterial Landscape of Bloodstream Infections in Neutropenic Patients via High Throughput Sequencing

Peter Gyarmati[1,2]*, Christian Kjellander[3☯], Carl Aust[4☯], Mats Kalin[5], Lars Öhrmalm[4], Christian G. Giske[1,2]

1 Karolinska Institutet, Department of Microbiology, Tumor and Cell Biology, Nobels väg 16, Stockholm, Sweden, **2** Karolinska University Hospital, Department of Clinical Microbiology L2:02, Stockholm, Sweden, **3** Karolinska Institutet, Department of Medicine, Division of Hematology, Stockholm, Sweden, **4** Karolinska Institutet, Department of Medicine, Solna, Infectious Diseases Unit, Center for Molecular Medicine, Karolinska University Hospital, Stockholm, Sweden, **5** Karolinska Institutet, Department of Infectious Diseases, Stockholm, Sweden

☯ These authors contributed equally to this work.
* peter.gyarmati@ki.se

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Abstract

Background

Bloodstream infection (BSI) is a common and potentially life-threatening complication in patients with hematological malignancies and therapy-induced neutropenia. Administration of broad spectrum antibiotics has substantially decreased the mortality rate in febrile neutropenia, but bacterial infection is documented in only one-third or fewer of the cases. BSI is typically diagnosed by blood culture; however, this method can detect only culturable pathogens.

Methods

In the present study, a total of 130 blood samples from hematological patients receiving dose-intensive antitumoural treatment were subjected to 16S rRNA PCR and 62 of them were cultured. PCR positive samples were processed to high throughput sequencing by amplifying the V1-V3 regions of the 16S rRNA gene to obtain a full spectrum of bacteria present in BSI.

Results

Five phyla and 30 genera were identified with sequencing compared to 2 phyla and 4 genera with culture. The largest proportion of bacteria detected by sequencing belonged to Proteobacteria (55.2%), Firmicutes (33.4%) and Actinobacteria (8.6%), while Fusobacteria (0.4%) and Bacteroidetes (0.1%) were also detected. Ninety-eight percent of the bacteria identified by sequencing were opportunistic human pathogens and 65% belonged to the normal human microbiota.

Competing Interests: The authors have declared that no competing interests exist.

Conclusions

The present study indicates that BSIs in neutropenic hosts contain a much broader diversity of bacteria, likely with host origin, than previously realized. The elevated ratio of Proteobacteria in BSI corroborates the results found in other systemic inflammatory diseases, such as inflammatory bowel disease or mucosal infections. This knowledge may become of value for tailoring antimicrobial drug administration.

Introduction

Infection during neutropenia is one of the most common causes of mortality in patients receiving chemotherapy. Mortality rates vary between 5–11% depending on the co-existing conditions and can rise even higher if bacteremia is present [1]. The standard microbiological diagnostic method in febrile episodes in neutropenic patients is blood culture. Its positivity rate is dependent on whether the patient has received antibiotic prophylaxis or not, but typically bacteremia may be identified in around 7–17% of the patients on antibiotics and in 14–31% of those who are not on antibiotic treatment. Around 50–70% of the identified bacteria are Gram-positive organisms [1–3], the high number is probably at least partly related to the use of prophylactic antibiotics in many clinical settings. Febrile neutropenia is treated with empirically chosen broad-spectrum antibiotics and a more comprehensive identification of the incriminated microorganisms would have the potential to reduce antibiotic overuse by targeting only specific bacteria, a strategy which could reduce the generation of resistant strains.

A substantial proportion of bacteria cannot be cultivated [4–7]. Diagnostic methods used to diagnose bloodstream infections (BSI) are mostly limited to blood culture, which can detect only culturable pathogens, or to real-time PCR, which detects microorganisms pre-defined by primers [8]. By blood culture only a restricted range of pathogens may be identified, it might take several days before a positive result is indicated and large volumes of blood are needed to obtain optimal sensitivity, typically 20–40 ml/fever episode. Molecular methods, using 16S rRNA amplicon sequencing, have the potential to reveal pathogens present in BSIs, which may be undetected by culture-dependent methods. It requires $\leq$ 1 ml of blood and because it uses the variable regions of the 16S rRNA gene, identification of bacteria to genus or species level is possible [4, 6, 9].

High-throughput sequencing is a quickly growing field, and has helped to characterize microorganisms in several different habitats. Its expansion is powered by the development of high throughput sequencing techniques, allowing sequencing billions of reads in a few days' time. Sequencing of the 16S rRNA gene is commonly used for culture-independent analysis, as this gene is universally present in bacteria, it is amplifiable by targeting conserved regions but also allows characterization of microbes through its variable regions. Although massively parallel sequencing makes species identification and estimating species abundance possible by its high coverage, targeting multiple regions of the 16S rRNA gene allows a more accurate identification of microorganisms [9]. In the present study, the variable V1-V3 regions were sequenced in blood samples from neutropenic patients with fever and suspected BSI.

This study aimed to characterize the bacterial content in blood samples of immunocompromised hematological patients in BSIs using high-throughput sequencing. Sequencing data were then compared with results from blood culture, the current gold standard for the diagnosis of BSIs.

Materials and Methods

Study population and sampling

Patients with hematological malignancies fit for dose intensive antitumoural treatment at the Hematology Center, Karolinska University Hospital in Stockholm, Sweden, were eligible for enrollment. Patients with acute myeloblastic leukaemia (AML) were included upon diagnosis whereas patients with other diagnoses could be asked to participate at any time points of the antitumoural treatment. Included patients were then sampled with two 4.5 mL EDTA tubes at different time point; 1) at diagnosis (only patients with AML), 2) at fever onset during neutropenia before intravenous broad spectrum antibiotic treatment was initiated, 3) follow-up samples to the fever-onset sample (only patients with AML), and 4) persisting fever during intravenous broad spectrum antibiotic treatment.

Samples were taken over a 1-year period (2013 March-2014 March). Data on white blood cell count (WBC), absolute neutrophil count (ANC), C-reactive protein (CRP) levels as well as age, gender and hematological diagnosis were extracted retrospectively from the patients' medical records. Samples were handled anonymously.

Ethics statement

Written consents were obtained from all patients. All adult subjects provided written, informed consent, and a parent or guardian of any child participant provided written, informed consent on their behalf. The study (recordal 2012/1929-31/1) was approved by The Regional Ethical Review Board in Stockholm.

Definitions

Fever was defined as a single oral temperature of $\geq$38.5°C or a temperature of $>$38.0°C persisting for $>$1 hour. Neutropenia was defined as a neutrophil count of $\leq 0.5 \times 10^9$ cells/L, or a higher count with a predicted decrease to $\leq 0.5 \times 10^9$ cells/L within 24 hours.

Culture

Commercial BacT-Alert 3D system with 2–2 aerobic and anaerobic bottles was used (bioMérieux, Marcy l`Etoile, France). BSI was defined as an infection manifested by the presence of bacteria in at least one culture bottle, or at least two blood culture bottles with the same microorganism growing in the case of common skin contaminants.

Sample preparation and sequencing

Blood samples for sequencing were drawn into sterile 4.5 ml Vacutainer (Becton Dickinson, Franklin Lakes, NJ USA) tubes, were kept at 4°C and processed to DNA extraction within 1–24 hrs. MolYsis Complete5 kit (Molzym Life Science, Bremen, Germany) was used to extract bacterial DNA following the manufacturer's instructions with the following exceptions: 5 minutes were used for the final elution instead of 1, and samples were dissolved in 50 ul water instead of 100 ul. Positivity for the 16S rRNA gene was controlled by the 520F (AYTGGGYD TAAAGNG)-802R (TACNVGGGTATCTAATCC) primer pair [10] with 1x Phusion High Fidelity master mix (New England Biolabs, Ipswich, MA, USA) and 200 nM primer concentration. Reactions were incubated at 98C for 2 min, then 98C for 30 sec, 40C for 30 sec, 72 for 1min 30sec, cycled 35 times and incubated at 72C for 5 min. Amplicon sizes were controlled on a 2% agarose gel. No template controls (NTCs) were run with each set of samples and all DNA extraction reagents were tested for 16S rRNA PCR as well in order to investigate the possible contamination from the reagents used [11], but no detectable amplification was noted.

Since longer 16S rRNA fragments result in more accurate identification [12], PCR positive samples were subjected to library preparation with the 27F (AGAGTTTGATCCTGGCTCA G)– 534R (ATTACCGCGGCTGCTGG) primer pair covering the V1-V3 regions of the 16S rRNA [10], and were processed to 2x300 bp paired end (PE) sequencing on an Illumina MiSeq instrument at GATC Biotech (Konstanz, Germany) as recommended by the manufacturer. In order to examine possible contamination originated from the human blood and/or the environment, a blood sample from a healthy donor and NTC samples were overamplified with 45 PCR cycles and were processed to Sanger sequencing. The resulting reads did not show significant (≥95%) similarity to any known bacteria when compared to the NCBI *nr/nt* database. Sequencing reads generated in this study were deposited to Sequencing Read Archives under experiment SRA:SRX668701, while background controls were deposited to NCBI GenBank under accession number KR152337-KR152338.

Data analysis

Reads below Q20 and 246 bp, and PE reads that could not be merged (FLASH, [13]) were removed. Cd-hit [14] was used for clustering with 99% similarity. Chimeras were removed using UCHIME [15]. BLASTn was used for similarity search [16] with $e \leq 10^{-6}$ and minimum similarity set to 97%, with references from the Ribosomal Database project (RDP 11, [17]). Taxonomic classification was based on NCBI Taxonomy [18]. Numbers of reads within each cluster were used to calculate relative abundances. Identified genera and species were included in the study if they contained at least 0.5% of the total number of operational taxonomic unit (OTU)-assigned reads in each sample. The Qiime package [19–22] was used for phylogenetic analysis with FastTree 2.1.3 [23] using the Silva_111 reference database [24] and was visualized with FigTree v1.4.2. The *exclude_seqs_by_blast.py* was used to check human DNA contamination as part of the Qiime package.

Results

Clinical characteristics

A total of 33 patients were included in the study; 19 with AML and 14 with other highly malignant hematological diagnoses. In total 130 blood samples were collected; 27 from AML patients at diagnosis, 38 at fever onset, and 41 follow-up samples. A total of 24 samples were collected from patients with persisting fever during broad spectrum antibiotic treatment (S1 Table).

Ninety-two samples were from patients with AML as the underlying diagnosis (70.8%), acute lymphoblastic leukaemia for 21 samples (16.2%), acute promyelocytic leukaemia for 8 samples (6.2%), mantle cell lymphoma for 4 samples (3.1%), Burkitt lymphoma for 3 samples (2.3%), and diffuse large B-cell lymphoma for 2 samples (1.5%).

The average age of the total study population was 52.2 years ± 16.3 (mean ± SD, n = 130) with 40% females, WBC = 0.8 ± 2.1 (n = 87), ANC = 0.3 ± 0.9 (n = 59), CRP level = 75 ± 53 (n = 78).

In fever onset samples, the age of patients was 51 ± 17.9 (n = 38), 39.5% females, WBC = 1.2 ± 3.1 (n = 33), ANC = 0.4 ± 1.2 (n = 29), CRP = 51.6 ± 37.5 (n = 30).

In persisting fever samples, the age was 53.1 ± 15.5 (n = 24), 33.3% females, WBC = 0.3 ± 0.7 (n = 20), ANC = 0.1 ± 0.4 (n = 10), CRP = 117.8 ± 49.5 (n = 16).

In follow up samples, the average age was 50.3 ± 15.8 years (n = 41), 41.5% females, WBC = 0.8 ± 1.1 (n = 34), ANC = 0.2 ± 0.4 (n = 20), CRP = 75.6 ± 54.6 (n = 32).

Positivity rates

A total of 130 blood samples were investigated with 16S rRNA PCR in this study and 65 of them with blood culture. Nineteen samples were positive by PCR out of 130 (14.6%) and 10 by blood culture out of 65 (15.4%), with 6 samples positive by both methods (S1 and S2 Tables). Positivity rate in fever onset samples (n = 38) was 23.7% (9/38) with PCR and 21.1% (8/38) with culture; in persisting fever samples (n = 24) the corresponding rates were 29.2% (7/24) and 8.3% (2/24); in follow up samples (n = 41) 7.3% (3/41) were positive with PCR and none with culture. In the 27 inclusion none was found to be positive with PCR. Accordingly, a total of 19 samples were positive by PCR and thereby processed to sequencing.

Sequencing

PCR positive samples were processed to sequencing. A total of 2,764,592 reads were assigned to bacterial OTUs (S3 Table, average per sample: 145,504). In the entire dataset, sequencing detected members of five bacterial phyla; most reads were assigned to Proteobacteria (55.2%) and Firmicutes (33.4%). Apart from these, Actinobacteria (8.6%), Fusobacteria (0.4%) and Bacteroidetes (0.1%) were also detected (Fig 1). All samples except ID_48 contained bacteria from more than one phylum. Of the total number of reads, 55.7% belonged to Gram-negative bacteria.

Within the 5 phyla, 30 genera were identified, where *Streptococcus* (detected in 18 cases out of 19), *Pseudomonas* (17/19), *Shewanella* and *Staphylococcus* (16/19), *Pelomonas* and *Propionibacterium* (14/19) were the most prevalent. Sixteen genera occurred in only 1 case (Fig 2A) and 20 genera had <1% of all assigned reads (Fig 2B). Although *Streptococcus*, *Propionibacterium* and *Pelomonas* were amongst the most commonly occurring genera (detected in 18/14/14 cases, respectively; Fig 2A), the total read percentages show that they contain a relatively small proportion of all assigned reads (*Streptococcus*: 8.8%, *Propionibacterium*: 5.3%, *Pelomonas*: 1.2%; Fig 2B). On the other hand, *Delftia* and *Halomonas* genera occurred only in 3 and 2 cases, respectively, but contained 2.7% and 3% of all assigned reads.

Over 98% of the identified reads belonged to reported human pathogens, and 65% of them belonged to the normal human microbiota (Fig 2C). Most of the identified genera (80.5% of the total reads) belonged to anaerobic or facultative anaerobic bacteria. Altogether 58 species were identified; 16 genera contained multiple species, typically with one dominant (Fig 3). Even though species were identified with ≥97% similarity, the 16S rRNA gene can have ≤1% diversity in between some species [9]. Eight genera could not be classified to the species level due to the high inter-species similarity. Sequencing of the V1-V3 regions also enabled phylogenetic analysis (S1 Fig).

The diversity of the samples illustrated with rarefaction curves (S2 Fig) indicate that in some cases >10,000x coverage might be necessary to identify all pathogens present with high-throughput sequencing.

Culture

Viridans group streptococci (7), coagulase-negative staphylococci (CoNS, 2), *E. coli* (1) and *Enterococcus faecalis* (1) were identified in blood cultures, with one polymicrobial infection (CoNS + *E. faecalis*, in sample ID_86). All of the bacteria detected by both culture and sequencing belonged to the Firmicutes phylum (Fig 1C, S2 Table).

Effect of antibiotic treatment on bacterial composition

In three pairs of samples (before-after antibiotic treatment: samples 48–49, 54–55, 120–129) we found PCR positive samples despite of antibiotic treatment. In two cases (48–49 and 120–129)

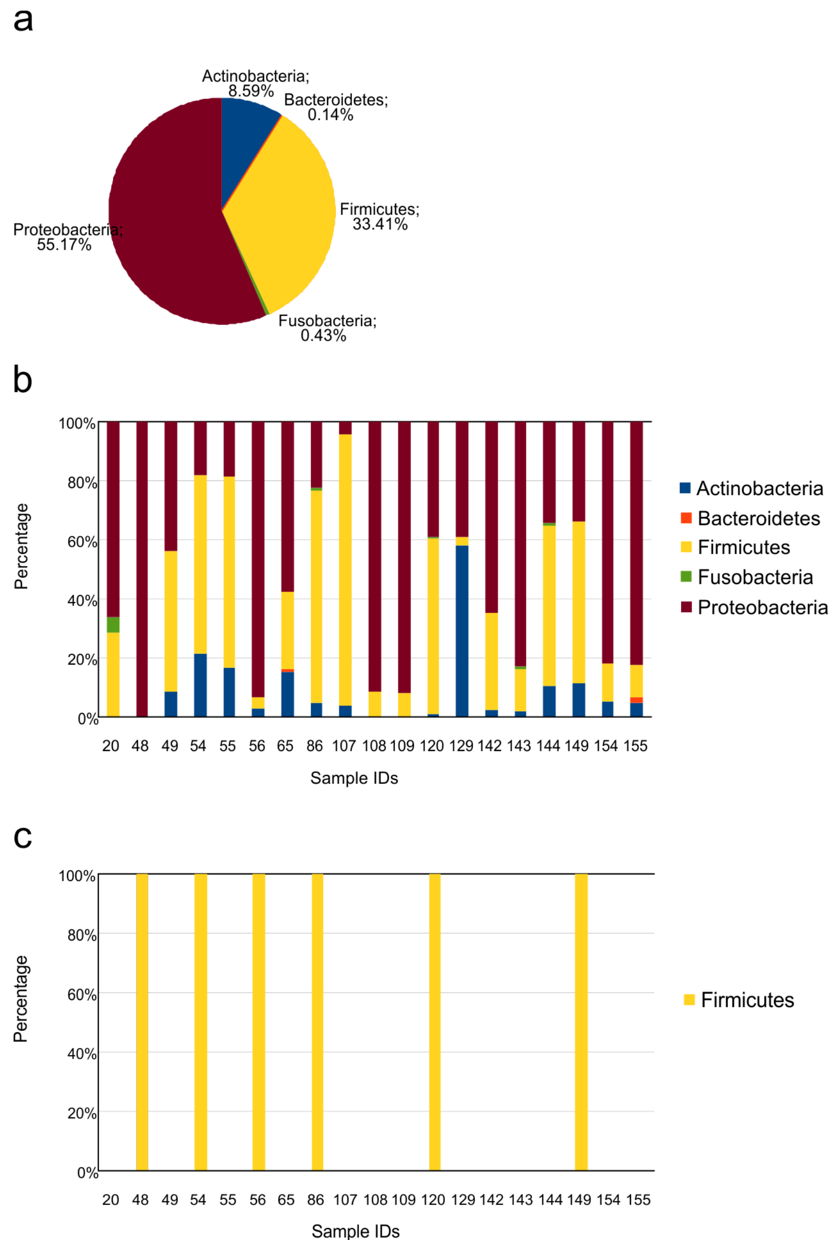

Fig 1. Representation of the distribution of phyla with sequencing in all samples (a) and in individual samples (b). Detected phyla per sample with blood culture (c).

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the bacterial composition underwent a major change after the antibiotic treatment, but in one case (54–55) the bacterial composition remained identical (Fig 4).

Discussion

Febrile neutropenia is a severe medical condition in immunocompromised patients and in those undergoing chemotherapy; and is a common cause of death when coupled with bacteremia [25].

Routine diagnosis of BSIs is based on the identification of pathogens by use of blood culture bottles. However, blood cultures have several limitations: the growth in the bottle can be slow,

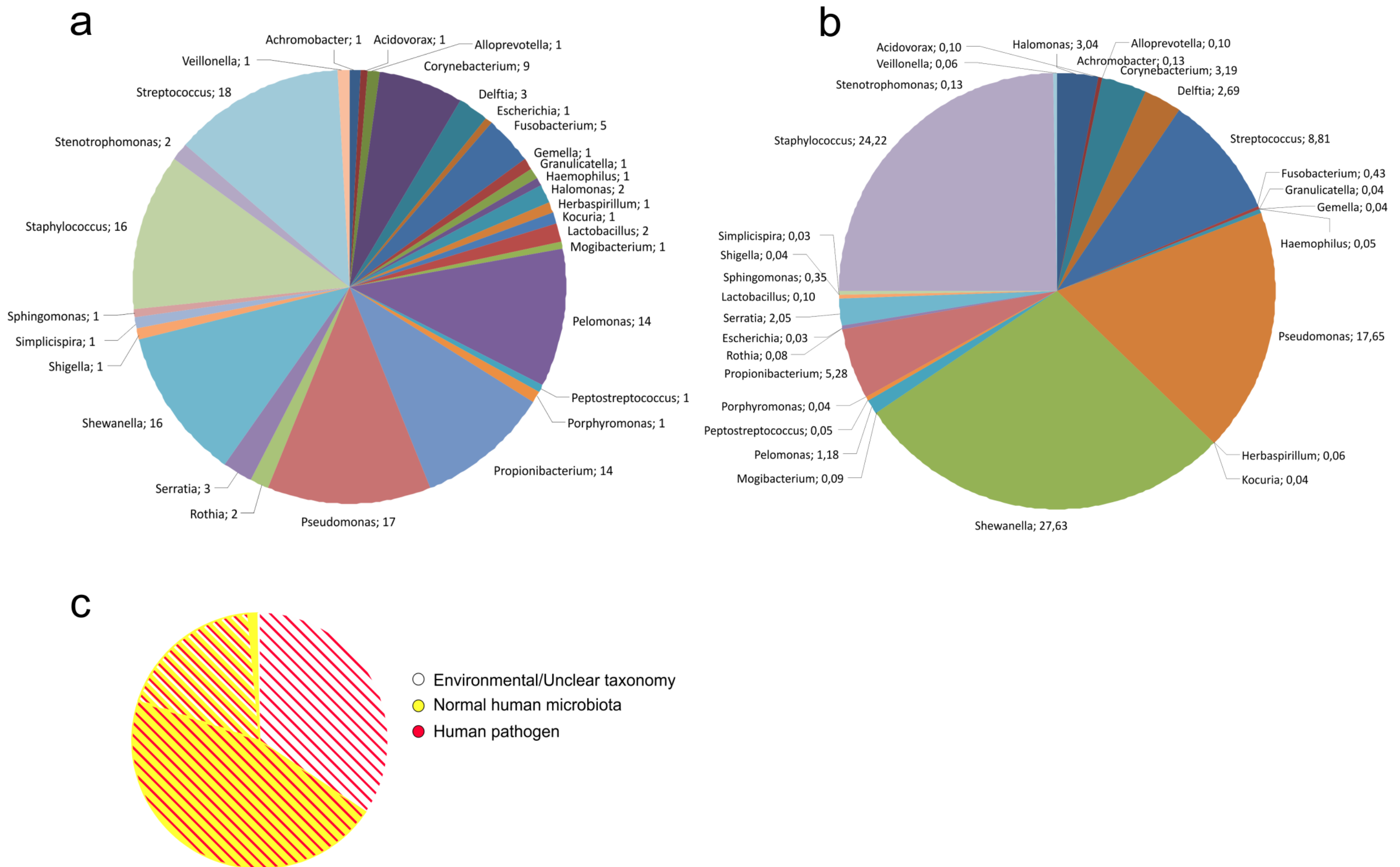

Fig 2. Occurrence of genera in 19 samples identified by sequencing (a). A genus was included if it reached or exceeded 0.5% of the total number of filtered reads in at least one sample. Distribution of OTU-assigned reads per genera in all samples in percentage (b). Diagram shows the pathogenicity and natural habitat of the detected genera based on read percentages (c). Over 96% of the identified reads belonged to opportunistic human pathogens (black stripes), while 64% belonged to the normal human microbiota (grey background).

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and several days may be required before growth can be noted; a large volume of blood is required to optimize sensitivity and only culturable pathogens can be detected. In one study, high-throughput sequencing was proven to detect more bacterial pathogens and was shown to be more sensitive than culture or Sanger sequencing in CSF samples [26]. In the presented work, blood culture was shown to detect fewer microorganisms in fewer cases compared to high-throughput sequencing. Thus, results obtained with blood culture may not reveal optimal data for management and might lead to inadequate treatment. The Firmicutes phylum were dominant with blood culture (Fig 1C), indicating a narrow range of detectable pathogens, possibly due to a competition in the growth of culturable pathogens in blood cultures. Blood culture typically detects only one pathogen per sample, while the bacterial composition of BSIs in neutropenic febrile hosts seems to be much wider according to the results from high-throughput sequencing (Figs 1–3). It has to be noted that the efficacy of both classical and molecular diagnostics methods depends on several factors regarding the detection of pathogens in blood, including sampling, bacterial load, bacterial interference, etc.

Despite its numerous advantages, high-throughput sequencing raises considerable challenges as well: although sequencing costs continue to decrease, the cost of an instrument and the reagent costs remain high. Sequencing runs can take a few days to complete and the large

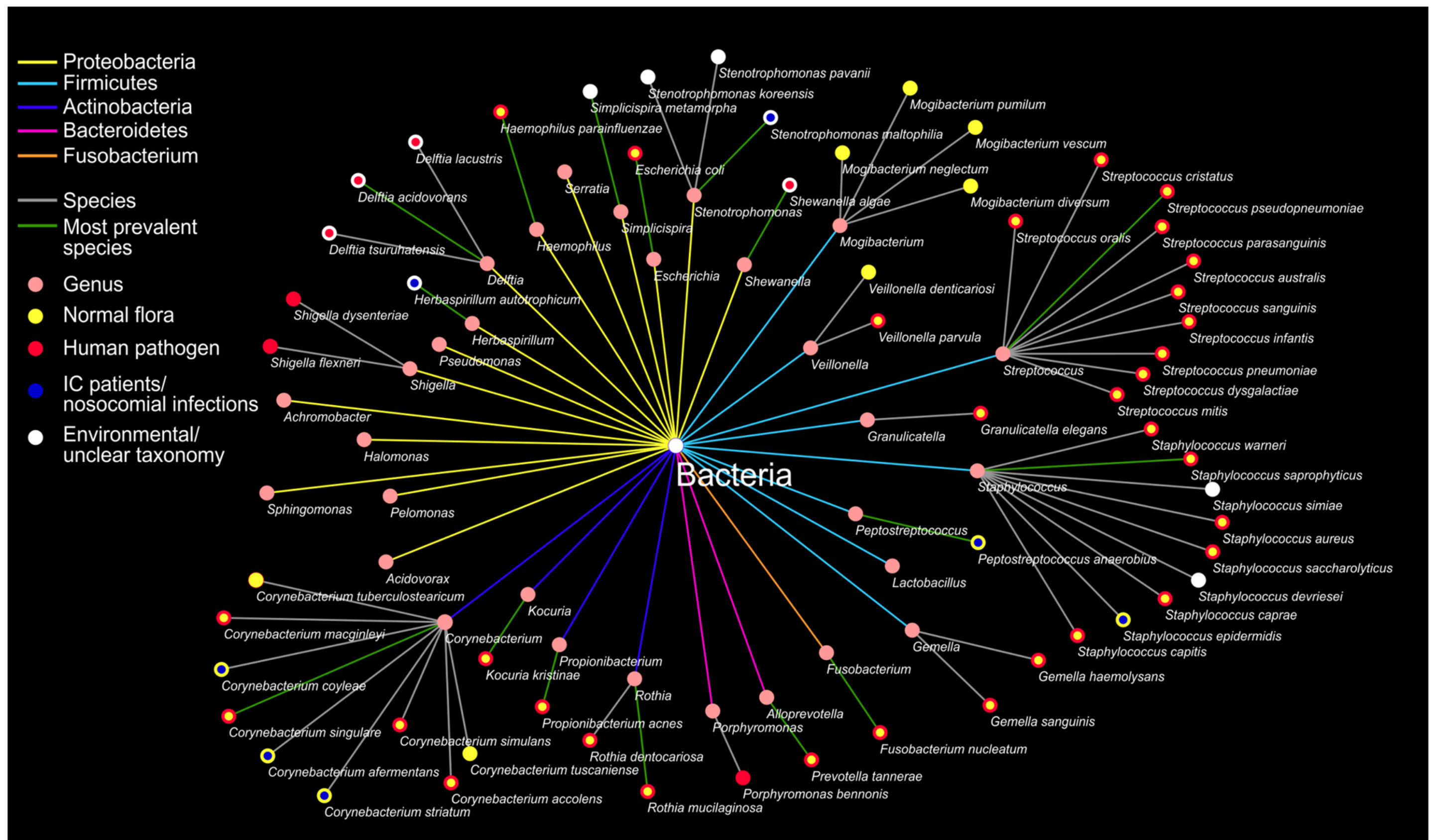

Fig 3. Schematic representation of microorganisms detected by sequencing on the species level. Pink nodes represent the given genera connected to the species. Green lines indicate the most prevalent species. Color lines from Bacteria to genera indicate phyla (Proteobacteria-yellow, Firmicutes-light blue, Actinobacteria-dark blue, Bacteroidetes-purple, Fusobacterium-orange). Species nodes indicate infectious properties: yellow-normal microbiota, red-human pathogen, blue-typically occurs in immunocompromised patients and/or nosocomial infections, white: taxonomy unclear/recently changed or environmental bacteria.

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amount of data generated from a sequencing run requires bioinformatics solutions. Due to sequence similarities of the 16S rRNA genes between microorganisms, identification of lower taxonomic categories (eg., species level) can be less certain [27] and antibiotic resistance patterns cannot be identified with this method. Also, while blood culture detects only viable microorganisms, pathogens identified by 16S rRNA sequencing might not necessarily be functional as shown in this study: positivity rate was the highest in fever onset samples both with PCR and culture (26% and 21%, respectively), indicating the presence of a high load of viable bacteria. However, in persisting fever samples, as the antibiotic treatment started, positivity rate of culture decreased to 4% while PCR maintained 24%, implying the presence of non-viable bacteria. PCR therefore offers an extended time for detecting BSIs during and after antibiotic treatment (S1 Table).

High-throughput sequencing

Five phyla and thirty genera were identified with this method. All genera found in these samples have been previously reported in bacteremia except the *Pelomonas* genus, which, however, has been isolated from haemodialysis water [28]. The majority of sequencing reads belonged to bacteria which form the normal human microbiota (Fig 2C), supporting the notion that translocation of the human microbiota plays a decisive role in bacteremia [29]. In addition, mostly

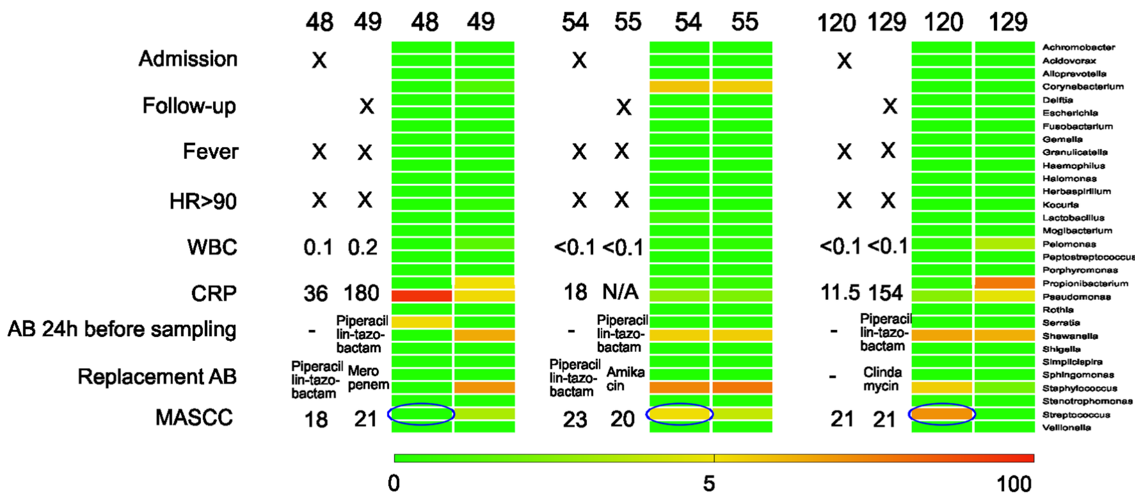

Fig 4. Heat map showing 3 pairs of samples before and after antibiotic treatment with the corresponding clinical characteristics (follow up: 1 day for samples 48/49 and 54/55, 5 days for samples 120/129). Bacteria were detected in all cases, but in 2 pairs (48–49, 120–129), the composition of bacteria changed, while in one patient (54–55), the composition of bacteria remained the same despite of antibiotic treatment. Blue ellipses indicate culture results. N/A: not available.

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anaerobic bacteria were detected in the presented samples and similarly, the human microbiota largely consists of anaerobic bacteria [7].

The *Shewanella* genus (formerly classified as *Pseudomonas*) was detected in over 80% of the samples (Fig 3). Although *Shewanella* bacteremia is a well-reported phenomenon [30–33], our results suggest that its relevance may be underestimated as it is not routinely diagnosed. Studies suggest that especially immunocompromised patients might be commonly infected with this pathogen, although its clinical significance is not fully known [33]. Additionally, because of their different clinical characteristics and susceptibilities to antimicrobial agents, it is important to differentiate *S. algae* from *S. putrefaciens* and as shown, sequencing of the 16S rRNA gene can identify *Shewanella* on the species level.

Similarly to another report [34], *Staphylococcus* and *Pseudomonas* were amongst the most commonly identified pathogens in patients with neutropenia, although *Escherichia* was identified in only one case in the present report in contrast to the findings reported by Ortega *et al.* [34]. *Pseudomonas* commonly occurs in hospital-acquired infections in immunocompromised patients [35]; it is one of the most genetically divergent genera and it was one of the most prevalent genera in the examined samples. However, the similarity of the 16S rRNA gene between *Pseudomonas* strains can be >99% [36]; therefore sequencing of the 16S rRNA gene does not discriminate appropriately between species for the *Pseudomonas* genus [37, 38].

The microbiota composition of BSI shows highest similarity to that of inflammatory bowel disease

The composition of microbiota can change in pathophysiological conditions associated with systemic inflammation, such as allergy or autoimmune diseases, due to the microbiota's ability to participate in the regulation of the host's immune system [39–43]. In order to investigate bacterial composition in BSIs, we compared the distribution of the four major phyla found in the reported samples with other studies characterizing the microbiota in different parts of the body (Fig 5).

In comparison to the microbiota in BSIs, Firmicutes and Bacteroidetes are the main phyla in the gut, while Proteobacteria can be found in very low percentages ([44, 45], Fig 5).

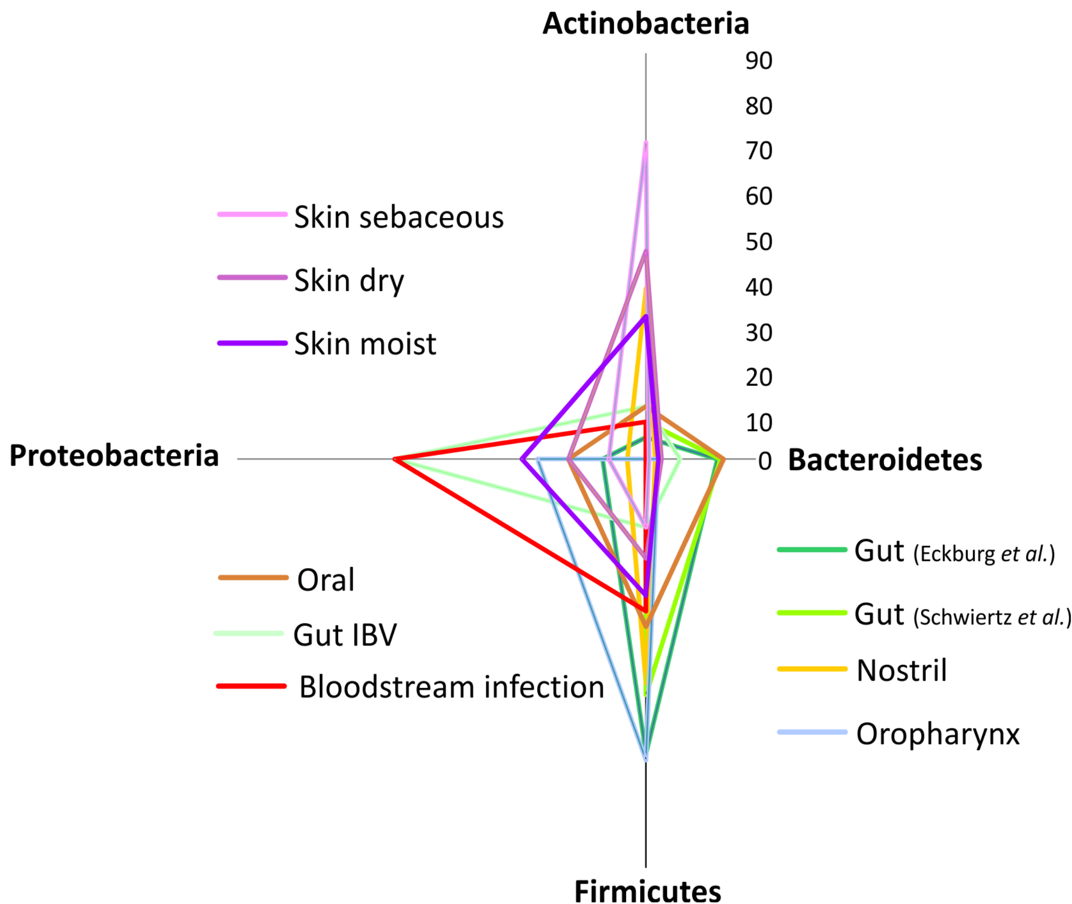

Fig 5. Composition of microbiota from different parts of the body classified by four major bacterial phyla. The graph was reconstructed based on the data from [44, 45, 51, 53, 55]. Axes show percentages.

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However, gut microbiota from inflammatory bowel disease [44] showed the largest overlap with our samples from all compared microbiota, indicating that the formation and composition of microbes play an important role in systemic inflammation [39], represented by an increased proportion of Proteobacteria as demonstrated in the present study and in other cases [46–49].

On the skin, Firmicutes, Proteobacteria and Actinobacteria are also commonly found [50], although that depends on various factors, such as dryness of the skin and sampling sites [51]. Typically, the skin is dominated by Actinobacteria [52], and the moist skin sites had the largest overlap with our samples amongst different skin microbiota, possibly due to sampling or translocation [40]. Grice et al. reported a larger proportion of Proteobacteria when the skin was sampled from the inner elbow [52].

Lemon and colleagues reported [53] an inverse correlation between Actinobacteria and Firmicutes in the microbiota of the nostril. This effect has been detected in the presented samples as well, e.g., in samples ID_129 (with *Propionibacterium* detected) and 120 (with *Streptococcus* detected), where sample 120 represent the situation before antibiotic treatment.

Our results show partial similarity with the lung microbiota [40, 54], where, based on multiple studies, Proteobacteria and Firmicutes are consistently the most commonly identified phyla and *Pseudomonas*, *Streptococcus* and *Prevotella* are the most common genera. It has to be noted however, that methodologies for characterizing microbiota vary widely which might

influence any comparison. Apart from the methodological aspects, microbiota carry-over might also be considered in clinical samples [40].

Bacteria identified in BSIs are dominated by Proteobacteria (Figs 1 and 5)–a phylum, which has been identified in local inflammations and has been recently associated with systemic inflammation [46–49]. The elevated ratio of Proteobacteria might be caused by the special metabolism of this phylum to utilize nitric metabolites abundant on inflammatory sites [39]. A large proportion of the identified bacteria belonged to the normal human microbiota (Figs 2 and 3), implying its role in the formation of systemic inflammatory response.

High-throughput sequencing as a potential tool to assess the efficacy of antibiotic treatments

Characterization of the microbiota in BSIs would not only help in choosing antibiotic treatment options, but it would also enable to estimate the efficacy of antimicrobial treatment (Fig 4, S3 Fig). Interestingly, we could detect different effects of the antibiotic treatments in different samples of the same patient. In one case the content of bacteria did not change while in another case drastic changes could be observed. The former indicates that treatment did not eliminate the invading microorganisms, while in the latter case, elimination of the bacteria led to re-population or co-infection by different strains. One patient had *Pseudomonas* and *Serratia* detected before sampling (Fig 4, sample 48), and after piperacillin-tazobactam treatment, the proportion of these genera decreased, indicating the effect of antibiotic treatment consistent with these genera often being susceptible to this compound in our clinical setting. However instead *Propionibacterium* and *Staphylococcus* were present in the follow-up sample (sample 49), which could also be related to contamination from the skin microbiota. In samples 54 and 55, *Staphylococcus*, *Shewanella* and *Corynebacterium* were detected both before and after piperacillin-tazobactam treatment, possibly due to resistance to this drug. In samples 120 and 129, *Shewanella* prevailed, while *Staphylococcus* and *Streptococcus* disappeared after drug administration, which could be consistent with the administered combination treatment of piperacillin-tazobactam and clindamycin treatment.

Although these preliminary findings are based on a very limited number of samples, the data indicate that high-throughput sequencing may have the potential to become a promising tool in evaluating the efficacy of antibiotic therapy.

Conclusion

Promoting rational antimicrobial use is essential to restrict the development of antibiotic resistance. As shown, high-throughput sequencing is able to identify a wide range of pathogens undetected by classical methods. By knowing the relative abundance of pathogens, a more customized treatment could be administered. Additionally, revealing the composition of microbiota in BSIs might help to understand its role in the pathomechanisms behind sepsis and provide information on the factors relevant in systemic inflammatory responses.

Supporting Information

S1 Fig. Phylogenetic representation of sample ID_149 showing distribution of the identified genera and phyla based on the assigned OTUs.
(TIF)

S2 Fig. Rarefaction curves show species count in relation to number of reads in OTU-assigned unique clusters.
(TIF)

S3 Fig. Heat map with a correlation scale shows the distribution of genera per sample. Blue ellipses indicate culture results.
(TIF)

S1 Table. Representation of samples used in this study, cells indicating PCR / culture results, respectively. Brown = PCR positive, yellow = culture positive, green = positive with both methods, na = no culture taken, NA = no sample taken, sum = sum of positive samples. Empty cells = no sample available.
(DOCX)

S2 Table. Comparison of bateria detected by sequencing and blood culture. [1] In patients 9 and 25, sequencing identified *S. mitis*, *S. pneumoniae* and *S. pseudopneumoniae*, while, apart from these three species, in patient 27 *S. infantis*, *S. oralis* and *S. australis* were also detected, confirming the presence of viridans streptococci. [2]In patient 10, *S. dysgalactiae* (β-hemolytic streptococci) was detected by sequencing. [3]In patient 17, in agreement with the culture result, sequencing detected a coagulase-negative Staphylococcus (*S. Saphrophyticus*). CoNS: Coagulase-negative staphylococci.
(DOCX)

S3 Table. Sequencing statistics of assigned reads per sample.
(DOCX)

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Author Contributions

Conceived and designed the experiments: PG CGG LÖ. Performed the experiments: PG CK CA. Analyzed the data: PG CK CA. Contributed reagents/materials/analysis tools: PG CK CGG LÖ MK. Wrote the paper: PG CK CGG LÖ MK.

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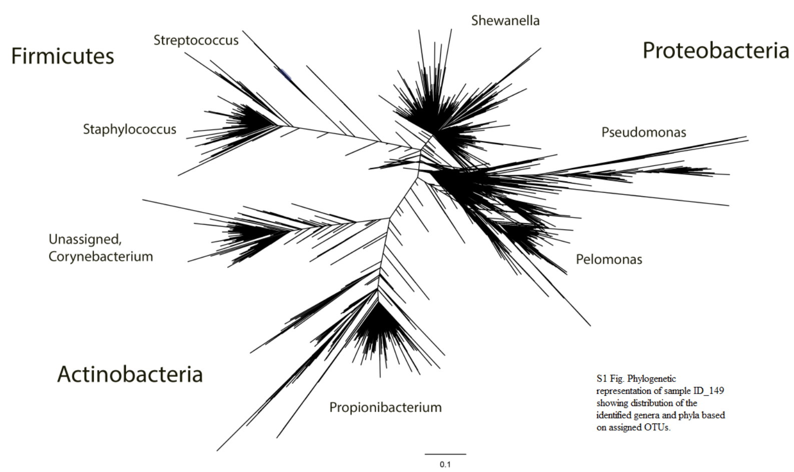
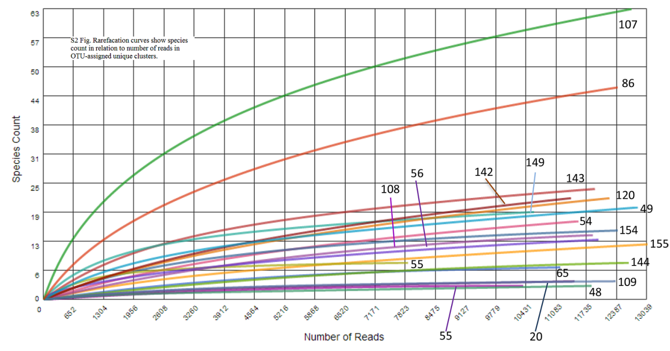


S1 Fig. Phylogenetic representation of sample ID_149 showing distribution of the identified genera and phyla based on assigned OTUs.


S2 Fig. Rarefaction curves show species count in relation to number of reads in OTU-assigned unique clusters.
Species Count
63
57
50
44
38
31
25
19
13
6
0
Number of Reads
0
652
1304
1956
2608
3260
3912
4564
5216
5868
6520
7171
7823
8475
9127
9779
10431
11083
11735
12387
13039
107
86
149
142
56
143
108
120
49
54
154
155
55
144
65
109
48
55
20

S3 Fig. Heat map with correlation scale shows the distribution of genera per sample.

Sample ID 20 48 49 54 55 56 65 86 107 108 109 120 129 142 143 144 149 154 155

Achromobacter
Acidovorax
Alloprevotella
Corynebacterium
Delftia
Escherichia
Fusobacterium
Gemella
Granulicatella
Haemophilus
Halomonas
Herbaspirillum
Kocuria
Lactobacillus
Mogibacterium
Pelomonas
Peptostreptococcus
Porphyromonas
Propionibacterium
Pseudomonas
Rothia
Serratia
Shewanella
Shigella
Simplicispira
Sphingomonas
Staphylococcus
Stenotrophomonas
Streptococcus
Veillonella

0 5 100

S1 Table. Representation of samples used in this study, cells indicating PCR / culture results, respectively.

	Fever	Persisting fever	Follow up 1	Follow up 2	Follow up 3
Patient 1	- / -		- /na	- / -	NA
Patient 2		+ / -	NA	NA	NA
Patient 3-1	- / -		- /na	NA	NA
Patient 3-2	- / -		- /na	- /na	NA
Patient 4-1	- / -		- /na	- /na	NA
Patient 4-2		- / -	NA	NA	NA
Patient 4-3	- / -		- /na	- /na	NA
Patient 5-1	- / -		- /na	NA	NA
Patient 5-2		- / -	NA	NA	NA
Patient 6		- / -	NA	NA	NA
Patient 7	+ / +		+ / na	- / -	NA
Patient 8		- / -	NA	NA	NA
Patient 9	+ / +	+ / -	- /na	- /na	NA
Patient 10	+ / +		NA	NA	NA
Patient 11-1		- / -	NA	NA	NA
Patient 11-2		- / -	NA	NA	NA
Patient 11-3	- / -		NA	NA	NA
Patient 12-1	- / -	- / -	+ /na	- /na	NA
Patient 12-2	- / -		NA	NA	NA
Patient 13-1	- / -	- / -	- /na	NA	NA
Patient 13-2		- / -	NA	NA	NA
Patient 13-3	- / -		- /na	- /na	- /na
Patient 14	- / -		NA	NA	NA
Patient 15-1	- / -		- /na	- /na	- /na
Patient 15-2	- / -	- / -	- /na	- /na	NA
Patient 15-3	- / -	+ / -	NA	NA	NA
Patient 16		- / -	NA	NA	NA
Patient 17		+ / +	NA	NA	NA
Patient 18	- / +		NA	NA	NA
Patient 19-1	- / -	- / -	- /na	NA	NA
Patient 19-2	- / -	- / -	- /na	NA	NA
Patient 20	- / -		NA	NA	NA
Patient 21-1	+ / -		NA	NA	NA
Patient 21-2	- / -		- /na	NA	NA
Patient 22-1	- / -		NA	NA	NA
Patient 22-2	- / -		NA	NA	NA
Patient 23	+ / -		NA	NA	NA
Patient 24	- / -		- /na	NA	NA
Patient 25-1	+ / +	- / +	- /na	NA	NA
Patient 25-2		+ / -	NA	NA	NA
Patient 26-1	- / -		- /na	- /na	- / -
Patient 26-2	- / -		- /na	- /na	+ / na
Patient 27-1		- / -	NA	NA	NA
Patient 27-2	+ / +	+ / -	- /na	NA	NA
Patient 28-1	- / -		NA	NA	NA
Patient 28-2		- / -	NA	NA	NA
Patient 28-3		- / -	NA	NA	NA
Patient 29	- / +		- /na	- /na	NA
Patient 30	+ / -		NA	NA	NA
Patient 31	+ / -		NA	NA	NA
Patient 32	- / -	+ / -	- /na	NA	NA
Patient 33	- / +		- /na	NA	NA
sum	**17 (9/8)**	**9 (7/2)**	**2 (2/0)**	**0**	**1 (1/0)**

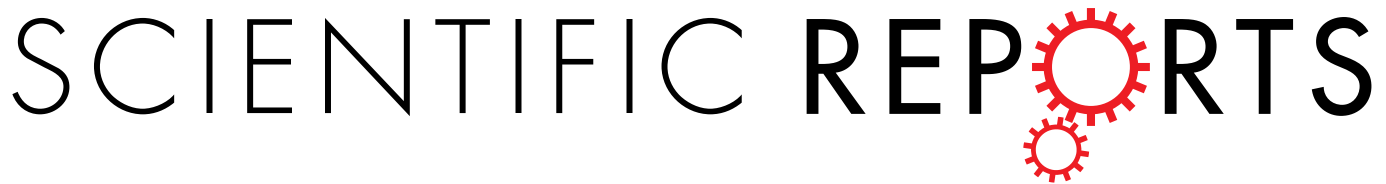
S2 Table. Comparison of bacteria detected by sequencing and blood culture.

	Sample type	Sequencing	Culture
Patient 2	Persisting fever	Shewanella Staphylococcus Fusobacterium Acidovorax Pelomonas	-
Patient 7	Fever onset	Pseudomonas Serratia Delftia	Viridans streptococci
Patient 7	Follow up	Staphylococcus Shewanella Pseudomonas Propionibacterium Streptococcus	-
Patient 9	Fever onset	Staphylococcus Corynebacterium Shewanella Streptococcus[1] Pseudomonas	Viridans streptococci
Patient 9	Persisting fever	Staphylococcus Corynebacterium Shewanella Streptococcus Pseudomonas	-
Patient 10	Fever onset	Pseudomonas Sphingomonas Corynebacterium Delftia Streptococcus[2]	Viridans streptococci
Patient 12	Follow up	Shewanella Streptococcus Corynebacterium Staphylococcus Pseudomonas	NA
Patient 15	Persisting fever	Staphylococcus Streptococcus Corynebacterium Shewanella Haemophilus	-
Patient 17	Persisting fever	Staphylococcus[3] Streptococcus Shewanella Corynebacterium Pelomonas	CoNS Enterococcus faecalis
Patient 18	Fever onset	-	Escherichia coli
Patient 21	Fever onset	Delftia Pseudomonas Streptococcus Propionibacterium	-
Patient 23	Fever onset	Pseudomonas	-

		Serratia Staphylococcus Shewanella Halomonas	
Patient 25	Fever onset	Streptococcus[1] Shewanella Staphylococcus Pseudomonas Propionibacterium	Viridans streptococci
Patient 25	Persisting fever	-	Viridans streptococci
Patient 25	Persisting fever	Propionibacterium Shewanella Pseudomonas Pelomonas Staphylococcus	-
Patient 26	Follow up	Halomonas Shewanella Staphylococcus Pseudomonas Streptococcus	NA
Patient 27	Fever onset	Staphylococcus Shewanella Propionibacterium Streptococcus[1] Pseudomonas	Viridans streptococci
Patient 27	Persisting fever	Shewanella Pseudomonas Staphylococcus Propionibacterium Streptococcus	-
Patient 29	Fever onset	-	Viridans streptococci
Patient 30	Fever onset	Shewanella Staphylococcus Propionibacterium Pseudomonas Streptococcus	-
Patient 31	Fever onset	Staphylococcus Shewanella Streptococcus Propionibacterium Pseudomonas	-
Patient 32	Persisting fever	Shewanella Pseudomonas Streptococcus Propionibacterium Pelomonas	-
Patient 33	Fever onset	-	Staphylococcus epidermis

S3 Table. Sequencing statistics of assigned reads per sample.

Sample ID	Total nr of reads	Nr reads after filtering and merging	Chimeric	OTU assigned
20	466,652	273,794	20,444	132,667
48	474,277	273,969	36,726	121,854
49	795,628	433,277	26,279	166,931
54	523,375	257,355	26,182	128,237
55	364,535	149,576	21,246	76,419
56	475,885	286,183	30,297	85,901
65	383,000	186,300	13,523	74,459
86	584,211	315,811	46,292	132,412
107	629,698	319,846	42,250	143,208
108	717,971	396,013	53,756	227,974
109	594,559	319,827	26,067	141,713
120	799,542	439,307	57,150	213,283
129	574,904	288,058	7,697	92,281
142	681,706	379,007	37,799	214,555
143	659,540	344,781	36,145	172,019
144	632,071	333,763	28,610	136,684
149	607,915	314,855	40,279	173,335
154	687,741	366,034	44,607	184,111
155	737,094	407,992	33,912	146,549
Total	11,390,304	6,085,748	629,261	2,764,592
Average per sample	599,489	320,302	33,119	145,504



OPEN

Metagenomic analysis of bloodstream infections in patients with acute leukemia and therapy-induced neutropenia

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P. Gyarmati[1,2], C. Kjellander[3], C. Aust[4], Y. Song[5], L. Öhrmalm[4] & C. G. Giske[1,2]

Leukemic patients are often immunocompromised due to underlying conditions, comorbidities and the effects of chemotherapy, and thus at risk for developing systemic infections. Bloodstream infection (BSI) is a severe complication in neutropenic patients, and is associated with increased mortality. BSI is routinely diagnosed with blood culture, which only detects culturable pathogens. We analyzed 27 blood samples from 9 patients with acute leukemia and suspected BSI at different time points of their antimicrobial treatment using shotgun metagenomics sequencing in order to detect unculturable and non-bacterial pathogens. Our findings confirm the presence of bacterial, fungal and viral pathogens alongside antimicrobial resistance genes. Decreased white blood cell (WBC) counts were associated with the presence of microbial DNA, and was inversely proportional to the number of sequencing reads. This study could indicate the use of high-throughput sequencing for personalized antimicrobial treatments in BSIs.

Systemic infections, such as BSIs can develop in hematological malignancies due to inherent immune defects and therapy-related immunosuppression, and are associated with increased mortality[1]. BSIs are routinely diagnosed by blood culture and treated by empirical broad-spectrum antimicrobials. Antimicrobial treatment might be inappropriate due to the lack of coverage of the underlying pathogen(s), or antimicrobial resistance of the causative pathogens[2]. Blood culture requires relatively large sample volumes, only detects culturable pathogens and represents a narrow spectrum of the microbes present in a sample[3]. Furthermore, only 10–30% of blood cultures from febrile neutropenia, and 50% of blood cultures from septic shock are positive[1,4,5]. Although bacteria are the most commonly detected pathogens, fungal and viral infections also represent major complications in hematological malignancies[4,6,7]. *Candida* and *Aspergillus* species are the most commonly occurring fungal pathogens, but others, including *Fusarium* and *Trichosporon*, can also be detected[8].

With the recent advantages of high-throughput sequencing technologies, it has become possible to reconstruct the taxonomic diversity of uncultured microbial communities, even in complex clinical samples. The two main approaches remain the amplicon sequencing (mostly restricted to the 16S rDNA universal gene in bacteria) and shotgun metagenomics, which allows untargeted sequencing of DNA in a given sample. Compared to the 16S rDNA sequencing approach, shotgun metagenomics not only allows the analysis of bacterial diversity in clinical samples, but can also provide information on the presence of parasitic, fungal and viral pathogens. An important clinical aspect is microbial resistance: sequencing of the 16S rDNA gene does not provide information regarding resistance patterns, while shotgun sequencing can identify resistance genes, their point mutations and also chromosomal resistance mechanisms. The latter approach also permits functional annotation of the sequencing data by assigning molecular functions to sequencing reads.

[1]Karolinska Institutet, Department of Laboratory Medicine, Alfred Nobels Allé 8, Stockholm, 17177 Sweden. [2]Karolinska University Hospital, Department of Clinical Microbiology L2:02, Stockholm, 17176 Sweden. [3]Karolinska Institutet, Department of Medicine, Division of Hematology, Stockholm, 17176 Sweden. [4]Karolinska Institutet, Department of Medicine, Solna, Infectious Diseases Unit, Center for Molecular Medicine, Karolinska University Hospital, Stockholm, 17176 Sweden. [5]Royal Institute of Technology, Science for Life Laboratory, Stockholm, 17176 Sweden. Correspondence and requests for materials should be addressed to P.G. (email: peter.gyarmati@ki.se)

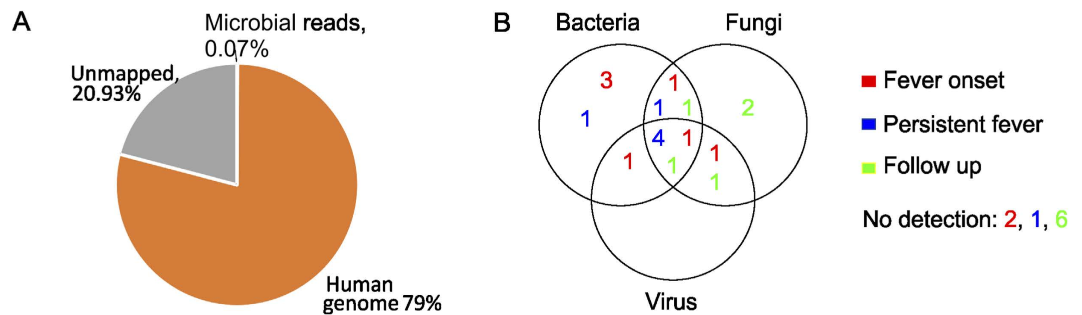

Figure 1. (**A**) Distribution of sequencing reads between human and microbial origins. (**B**) Distribution of the detected microbes. Colored numbers indicate the number of occurrences per sampling category (red numbers: fever onset, blue: persistent fever, green: follow up). Two fever samples did not contain microorganisms, and there were no detections of pathogen DNA in 1 persistent fever sample and 6 follow up samples.

The aim of this study was to characterize the microbial content of blood samples from neutropenic patients with newly diagnosed acute leukemia. Sampling was performed at different time points before and after the initiation of antimicrobial treatments, and sequence analysis was combined with functional annotation.

Results

Sequencing reads. In total, 1,005,260,502 reads were generated (excluding spike in samples), with 33.5 million reads being sequenced per sample on average. Seventy-nine percent of the reads were mapped to the human genome, while 20.93% of the reads were unmapped. Microbial reads consisted 0.07% of the total reads (Fig. 1). As shown in previous studies, human DNA exceeds microbial DNA approximately 10^7–10^8 –fold in sepsis[9,10]. The applied background suppression treatments reduced human DNA approximately 10,000-fold as sequencing reads aligned to the human genome outweighed microbial reads by 10^4 on average.

Microbial content of blood samples per sample category. Samples from nine patients were included in this study; in two patients, no microbial detection occurred. Samples (n = 27) were analyzed at different time points during antimicrobial treatments: 1) at inclusion (n = 1), 2) at fever onset (before antibiotic treatment started, n = 9), 3) 1 day after antibiotics were given (n = 7), and 4) follow up (1–5 days after the start of antibiotic treatment started, n = 11).

Three out of nine fever onset samples only contained bacteria, while four out of seven persistent fever samples contained bacteria, fungi and viruses (Fig. 1B).

Bacterial reads were detected mostly at fever onset and in persistent fever (69% of all reads in both categories), but in less than 1% in follow up samples. The diversity of bacterial taxa did not change during fever, but showed a significant decrease in follow up samples, likely due to antibiotic treatment (Fig. 2A). Viral reads were dominantly detected during persisting fever (Fig. 2), and mostly consisted of bacteriophages. The diversity of fungal and viral taxa did not show significant changes between disease states.

Distribution of bacterial taxa in different time points of sampling. The bacterial content of the samples was dominated by *Propionibacterium acnes*, *Corynebacterium spp* and *Staphylococcus spp* in agreement with a 16S rDNA study on BSIs[3]. *Dolosigranulum pigrum* (in follow up samples) and *Neisseria spp* (during persistent fever) were also frequently detected (Fig. 3A).

The distribution of bacterial taxa per sample type revealed a difference between sampling time points: *P. acnes* was the dominant taxa only in fever onset samples, and was not detected in follow up samples at all. In samples with persisting fever, *Corynebacterium spp*, *D. pigrum* and *Staphylococcus spp* were dominant, while *S. spiritivorum* was the most prominent taxa in follow up samples.

Distribution of fungal taxa in different time points of sampling. *Fusarium oxysporum*, a known fungal pathogen in immunocompromised patients, was detected most frequently in every sampling category (in 13 samples out of 27, Fig. 3B). Other detected fungal taxa also represent opportunistic infections (e.g., *Aspergillus spp* and *Malessezia globosa*) characteristic for immunocompromised patients.

Distribution of viral taxa in different time points of sampling. Overall, phage related to *Propionibacterium* were the most dominant viral taxon (Fig. 3C), along with *Torque Teno Virus* (TTV). TTV was detected in 6 samples from 3 patients, while *Propionibacterium* phages were detected in 3 samples, from 3 patients. Amongst the known oncoviruses, *Merkel cell polyomavirus* and *Hepatitis C* were detected. Human endogenous retroviruses, as part of the human genome were removed from the analysis.

Changes in microbial content in individual samples throughout the disease. As shown, antibiotic treatments were typically efficient against bacterial infections (e.g., *P. acnes* or *Neisseria spp.* in patients 1, 4, 5, 7), but less effective against species such as Pseudomonas (patient 2). Viruses and fungi were also often detected and were not affected by the antiviral/-fungal prophylaxis. When the infection was dominantly fungal or viral (patients 3, 5, 6), persistent fever samples contained the most reads, while in bacterial infections, fever onset samples had the highest number of reads.

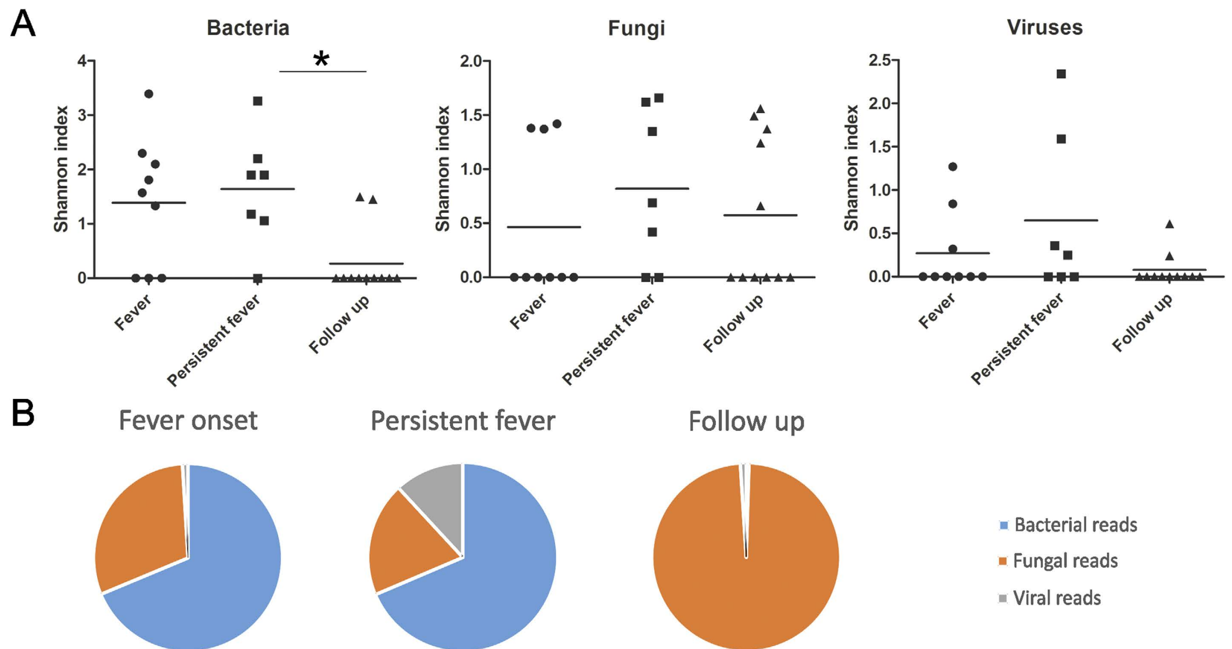

Figure 2. (**A**) Shannon's diversity index is shown for bacteria, fungi and viruses at different time points of neutropenic fever. Significant decrease can be seen in bacterial diversity in follow up samples, due to antibiotic treatment. *denotes $p \leq 0.05$. (**B**) Distribution of microbial reads per time points.

In patients 3 and 6, fungal and viral taxa were dominant, and the number of reads increased with fever (Fig. 4).

Laboratory data. WBC and ANC were compared between patients in different disease stages (Fig. 5A), and with and without detected microbial DNA. WBC was significantly lower in patients when microbial DNA was identified ($p \leq 0.05$, Fig. 5B).

Samples with the highest microbial reads (>10,000 reads, $n = 6$) had significantly lower WBC ($p \leq 0.05$) and lower ANC ($p \geq 0.05$) than samples with less microbial reads found (Fig. 5C).

Bacterial resistance. Tetracycline resistance genes were found in 2 samples (patient 4, fever sample, and patient 7, persistent fever sample 1). MLS (Macrolide Lincosamide Streptogramin) resistance genes were found in 4 samples (patient 3 (persistent fever), patient 4 (fever and persistent fever samples), and patient 7 (persistent fever sample 1)).

Gene ontology (GO) analysis. In bacterial reads, significant differences were found in molecular processes (binding processes and hydrolase activity) between persistent fever and follow up samples ($p \leq 0.05$, Fig. S1a). Metabolic processes also showed a difference between these two groups ($p \leq 0.05$, Fig. S1b).

In fungal reads, molecular functions and biological processes did not show changes throughout the disease, assuming the lack of active antifungal drugs (Fig. S2).

The molecular functions and biological processes did not show a significant change in viral reads, although the hydrolase activity was elevated in persistent fever samples ($p \geq 0.05$, Fig. S3).

Discussion

Although the blood is considered sterile in physiological state, it may harbor dormant microbes, or microbes can enter the bloodstream due to translocation or pathological conditions[11]. BSI is treated with empirical broad-spectrum antimicrobials, which are often not efficient against the invading microbes due to the lack of specificity or resistance mechanisms[2]. Even though detection of microbial DNA in the blood does not necessarily imply the presence of viable pathogens, it is associated with worse clinical outcomes[5,12,13]; Fig. 5 in present study).

In this study, blood samples from patients with acute leukemia and suspected bloodstream infection were subjected to characterizing their microbiota using shotgun metagenomics. To our knowledge, this is the first investigation of uncultured microbial content and their resistance genes in blood samples from BSIs. Our results corroborate previous findings that the routinely used blood culture only detects a proportion of pathogens[3], and our findings confirm the presence of viral and fungal pathogens in immunocompromised patients[7,8].

The risk of infection is much greater in leukemic patients due to comorbidities and side effects of chemotherapy. BSI is often caused by bacteria, but can also be caused by fungi and viruses[7,8]. While bacterial infections are diagnosed with blood culture and treated with antibiotics, fungal and viral infections often go undetected in BSI cases, although they are associated with higher mortality rates in cancer patients[8,14]. As expected, the employed antibiotic treatment was effective against the majority of bacteria, but dominantly viral (including bacteriophages) and fungal pathogens were detected in 4 patients (Fig. 4). Fungal infections are an important cause of mortality in neutropenic cancer patients[8,15], with A. fumigatus and C. albicans being mainly responsible for the infections,

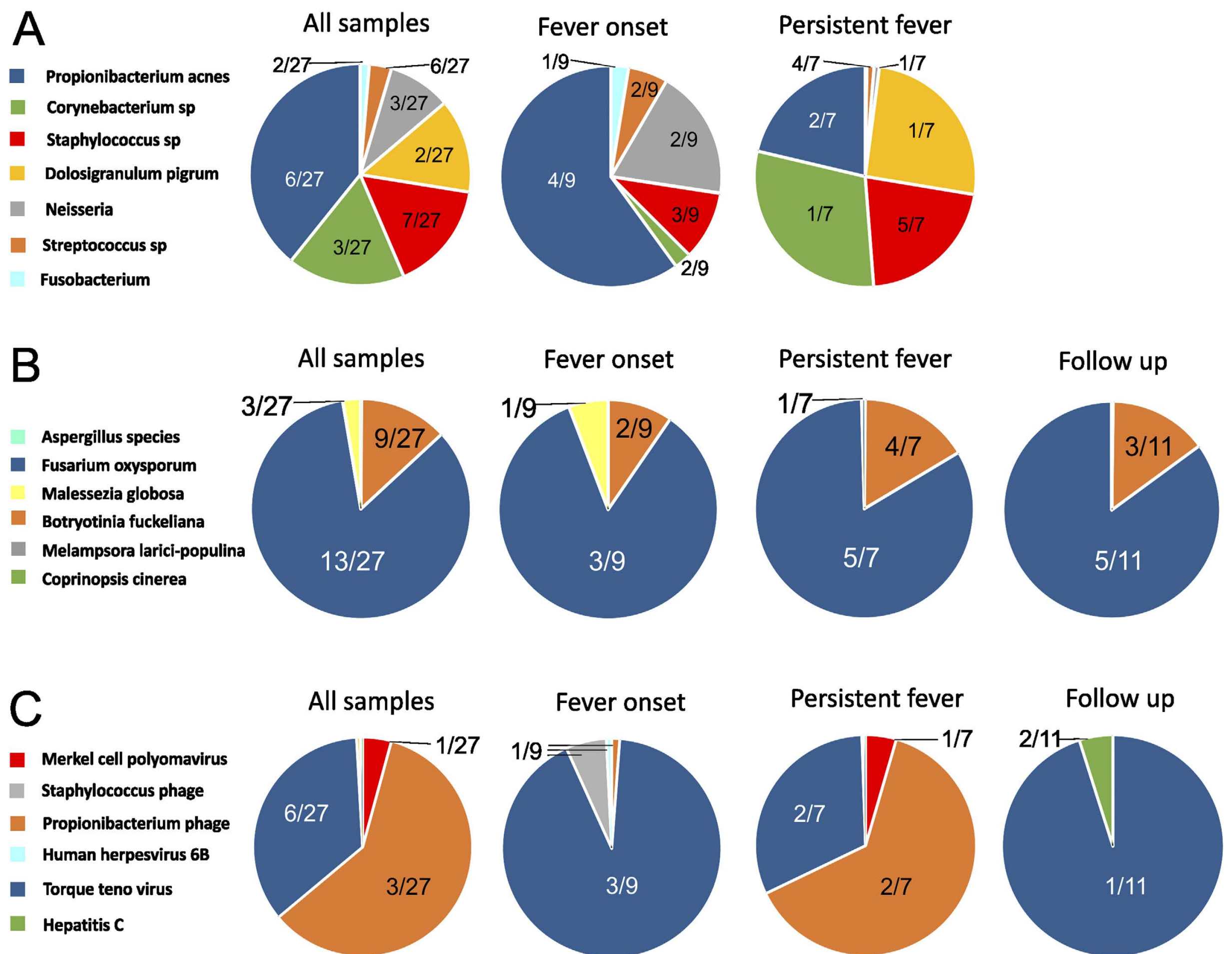

Figure 3. (**A**) Distributions of bacterial reads are shown in different diseases stages, with *Propionibacterium* dominating during the initial fever episode, while *Corynebacterium, Staphylococcus and Dolosigranulum* were also detected in persisting fevers. Bacterial taxa which consisted of < 1% of all reads in a sample are not shown. (**B**) Distribution of fungal taxa detected in the samples used in this study indicate the presence of *Fusarium* in different disease stages, possibly implying subclinical fungal infections. (**C**) Distribution of reads belonging viral taxa at different sampling time points. Numbers within the pie charts indicate the number of samples positive for the given pathogen.

along with Fusarium and Trichosporon species. Posaconazole, which was used as fungal prophylactic is effective against most fungal pathogens but resistance can occur against this drug in Fusarium species[15–18]. Fusarium oxysporum was the most commonly detected fungal species in the current study (patients 5–7, Fig. 3B) with fungal load changing with fever (Fig. 4), which might indicate low level of persistent, possibly subclinical fungemia.

Viruses commonly occur in leukemic patients with febrile neutropenia[6,7]. For the patients included in this study, acyclovir was used as a prophylactic drug to prevent Herpesvirus-related infections. Herpesviruses are commonly detected in leukemic patients with neutropenia[6,7], and were detected in 2 samples from 1 patient in our study as well, while TTV was the most commonly detected viral species (3 patients, 6 samples). TTV has been detected in leukemic patients but its causative role has not been proven; it was hypothesized however that TTV can be a co-virus in participating in the disease etiology in leukemia[19]. Bacteriophages are considered as part of the normal microbiota[20]; in this study, phages have been detected dominantly during persisting fever directly after antibiotic treatment, indicating that the origin of phages were bacteria degraded by antibiotics (Figs 2 and 3C). Additionally, antibiotic treatment is a strong inducer of the release of bacteriophages[21,22], which can explain their elevated hydrolase activity (Fig. S3). In case of detection of bacteriophages, their corresponding host bacteria were also detected as phages exert high level of host specificity[23].

Bacteria mostly occurred in fever and persistent fever samples, but were almost non-existent in follow up samples, due to the antibiotic treatment (Fig. 2). *P. acnes, Staphylococcus and Corynebacterium spp* were detected commonly in samples included in this study (Fig. 3A). *Dolosigranulum pigrum* and *Neisseria spp* bloodstream infections were also frequently detected, as they can be opportunistic pathogens in patients with immunosuppression[24–27]. Many bacteria have been detected and/or have been implied as causative in cancers[28]. Acinetobacter has been detected in acute leukemia and was postulated to play a role in bacteria-human cell lateral gene transfer[29]; this genus was detected in 5 samples from 2 patients in this study. Also, mucosal injury or damage in the lumen

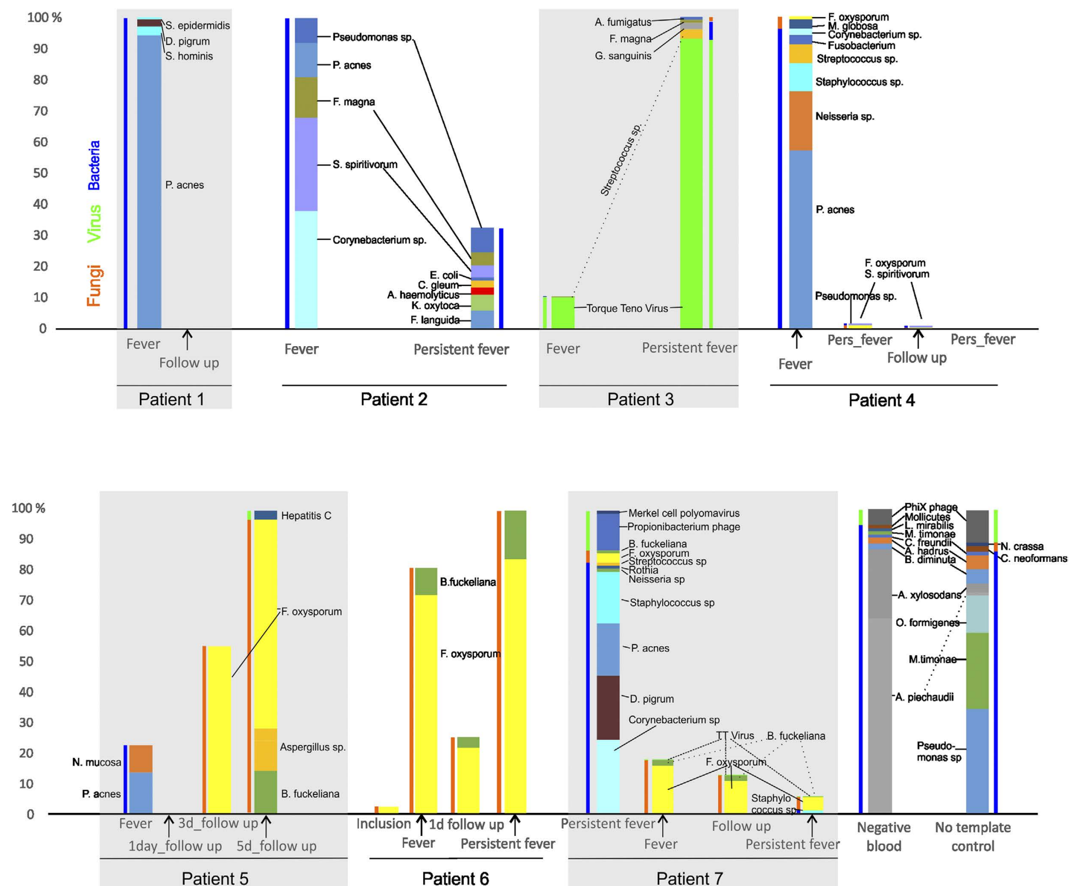

Figure 4. Relative abundances of bacterial, fungal and viral taxa are shown in individual samples. The highest bar for each patient represents the highest number of reads; the heights of other bars of the same patient are proportional to the highest. Wider bars represent taxa on the species level, slim bars show domains. Only taxa with ≥1% relative abundance are shown.

can also occur as a side effect of chemotherapy and opportunistic bacteria can enter the bloodstream in hematological malignancies[3,4,28].

GO analysis of bacteria showed significant changes with antibiotic administration (Fig. S1), implying the effectiveness of antimicrobial drugs and adaptation of bacteria. GO analysis also confers the notion of resistance of the F. oxysporum strains as there were no changes in molecular functions and biological processes throughout the treatment (Fig. S2) despite of antifungal prophylaxis. The elevated ratio of transport processes in fungal reads might indicate resistance to drugs as the rate of transport of antimicrobial agents can be insufficient compared to the efflux pumps, resulting in inefficient doses[30]. The elevation of hydrolase activity in viral reads (Fig. S3) is most likely due to the release of phages with the degradation of bacteria in persistent fever samples (1 day after AB administration), as no changes were observed between fever onset and follow up samples.

In summary, this work confirms the presence of viral and fungal pathogens alongside bacteria and antibiotic resistance genes in leukemic patients with neutropenic fever. The presence of microbial DNA and the number of sequencing reads were correlated with lower WBC counts in blood samples included in this study. Altogether, our results imply the possible utility of this technology in personalized medicine in the antimicrobial treatment of patients with acute leukemia.

Materials and Methods

Study population and sampling. Eight patients with acute myeloid leukemia and one patient with acute lymphocytic leukemia considered suitable for dose intensive antitumoural treatment at the Hematology Center, Karolinska University Hospital in Stockholm, Sweden, were enrolled for this study upon diagnosis. Included patients were sampled with two 4.5 mL EDTA tubes for venous blood at different time points: 1) at hematological diagnosis, 2) at fever onset during neutropenia before intravenous broad-spectrum antibiotic treatment was initiated, 3) persisting fever during intravenous broad-spectrum antibiotic treatment, and 4) follow-up samples 1–5 days after the initiation of antibiotic treatment.

Data on white blood cell count (WBC), absolute neutrophil count (ANC), C-reactive protein (CRP) levels, Multinational Association for Supportive Care in Cancer (MASCC) Risk Scores, and hematological diagnoses were extracted retrospectively from the patients' medical records. Samples were handled anonymously.

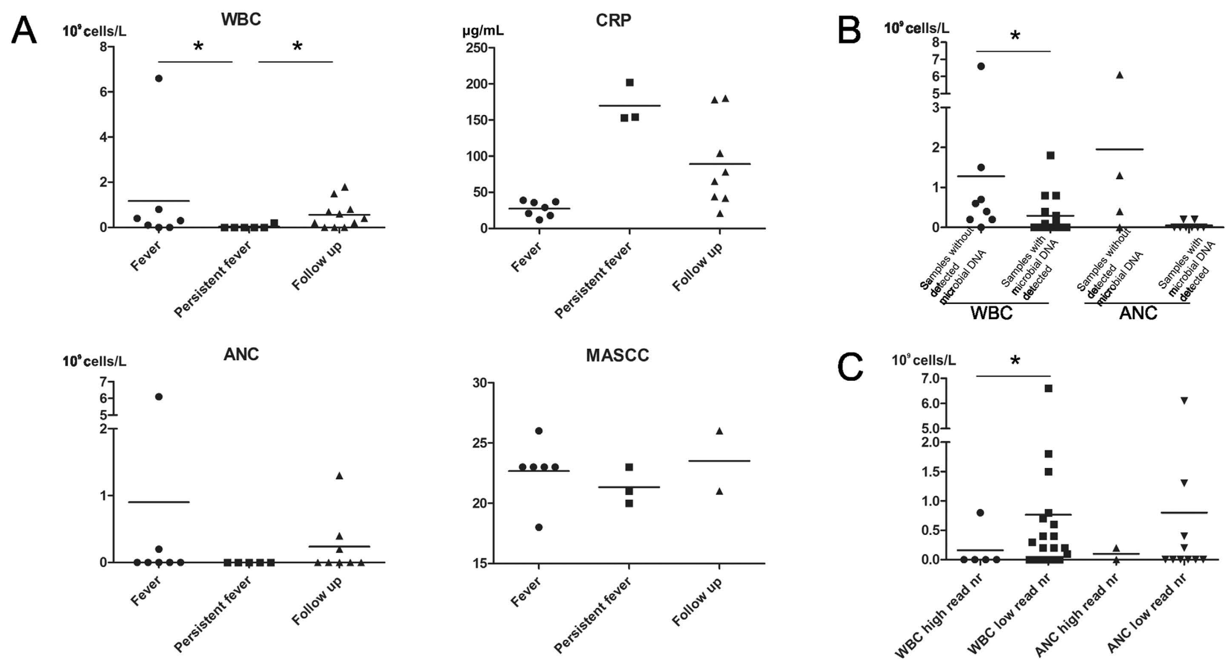

Figure 5. (**A**) Clinical characteristics of the samples used in this study, measured at the time of sampling. (**B**) Comparison of white blood cell count and absolute neutrophil count between samples with and without the presence of microbial DNA. (**C**) Comparison of WBC and ANC between samples with the highest microbial reads (>10,000) versus samples with microbial reads lower than 10,000. *denotes $p \leq 0.05$. WBC: white blood cell count, ANC: absolute neutrophil count, CRP: C-reactive protein, MASCC: Multinational Association for Supportive Care in Cancer risk score.

Antimicrobial treatments. All patients were treated with empirical broad-spectrum antibiotics at fever onset, according to international IDSA guidelines. Five of the patients received ciprofloxacin prophylaxis. Acyclovir and posaconazole were used as antiviral and antifungal prophylactics for all patients, in accordance with the local hospital guidelines (Table S1).

Ethics statement. All subjects provided written, informed consent. The study, and all experimental protocols used in this study was approved by The Regional Ethical Review Board, Stockholm (Regionala Etikprövningsnämnden Stockholm, recordal 2012/1929-31/1), and were carried out in accordance with the approved guidelines.

Definitions. Fever was defined as a single oral temperature of ≥38.5 °C or a temperature of >38.0 °C persisting for >1 hour. Neutropenia was defined as a neutrophil count of $\leq 0.5 \times 10^9$ cells/L, or a higher count with an expected decrease to $\leq 0.5 \times 10^9$ cells/L within 24 hours.

Sample preparation and sequencing. Blood samples for sequencing were drawn into sterile 4.5 ml Vacutainer (Becton Dickinson, Franklin Lakes, NJ USA) tubes, were kept at 4 °C and processed to DNA extraction within 1–24 hrs. MolYsis Complete5 kit (Molzym Life Science, Bremen, Germany) was used to extract bacterial DNA following the manufacturer's instructions with the following exceptions: 5 minutes were used for the final elution instead of 1, and samples were dissolved in 50 μl water instead of 100 μl. One μg of the eluted DNA was then processed to NebNext microbiome enrichment (New England Biolabs, Ipswich, MA, USA) following the manufacturer's instructions. Ten ng DNA was subjected to multiplex displacement amplification, using the GenomiPhi V2 DNA amplification kit (GE Healthcare, Little Chalfont, United Kingdom), with 90 minutes amplification resulting in 2–4 μg DNA. Two μg DNA was used for library preparation with the Nextera XT kit, and libraries were processed to a 2*100 base pair PE sequencing on a HiSeq 2500 instrument. The data generated in this study was uploaded to the NCBI Sequencing Read Archives under experiment SRX1381258.

Validation of the assay. *Escherichia coli* NCTC 9001 genomic DNA was spiked in to microfiltered human blood (Sigma-Aldrich) in 10-fold dilutions from 1 million CFU to 0. No template controls did not generate sufficient background amplification with the standard protocol for sequencing, therefore the MDA reaction was performed for 3 hours and processed as described above. *E. coli* DNA spiked into sterile blood was detected down to 10 CFU. Control samples (microfiltered human blood, "Negative blood") and a sample without added DNA ("No template control", Fig. 4) were dominated by bacterial species, *Achromobacter piechaudii* and *xylosodans*, *Pseudomonas* species and *Massilia timonae*, with several of them have already been reported as reagent contaminants[31].

Data analysis. Reads shorter than 30 bp and with Phred quality scores below 30 were discarded using the Fastx toolkit. Paired end reads were merged using the Flash software[32], while unpaired reads were discarded. RTG Core 3.4[33] was used to filter reads against the human genome (build Hg_19), and to map the unmapped reads to fungal, bacterial and viral whole genome sequences (downloaded from the NCBI Microbial Genomes Resources (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html)), with minimum *e* value set to 10^{-6}. Duplicate reads were removed from the analysis using RTG Core 3.4. Taxa detected in the no template control sample, or in the spike in sample with 0 copies of *E. coli*, or with ≤ 10 reads/taxon were not included. The ARG-ANNOT database was used to detect antibiotic resistance genes. GO analysis was performed using the Blast2GO v3 software, with $e = 10^{-6}$. Statistical analysis was performed using the Mann-Whitney U-test, with significance set to 0.05.

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Author Contributions

Conceived and designed the experiments: P.G. and C.G. Performed the experiments: P.G., C.K., C.A. and Y.S. Analyzed the data: P.G., C.K. and L.Ö. Contributed reagents/materials: P.G., C.K., L.Ö. and C.G. Contributed to the writing of the manuscript: P.G., C.K., C.A., Y.S., L.Ö. and C.G.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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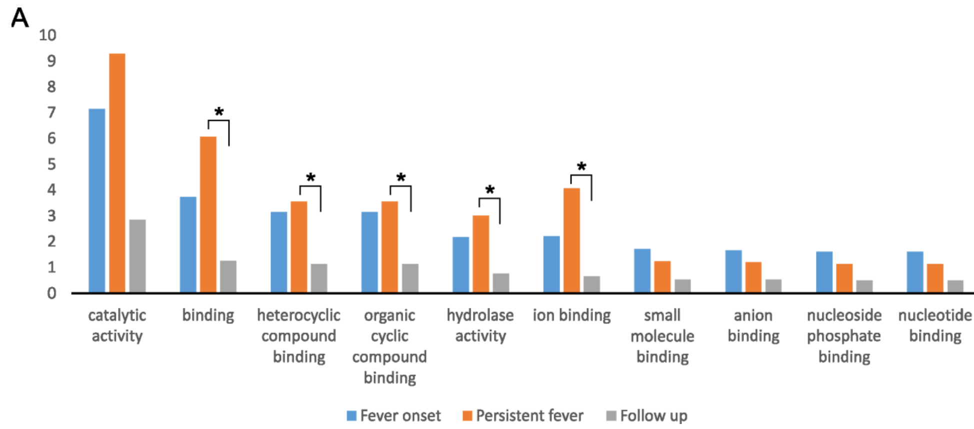


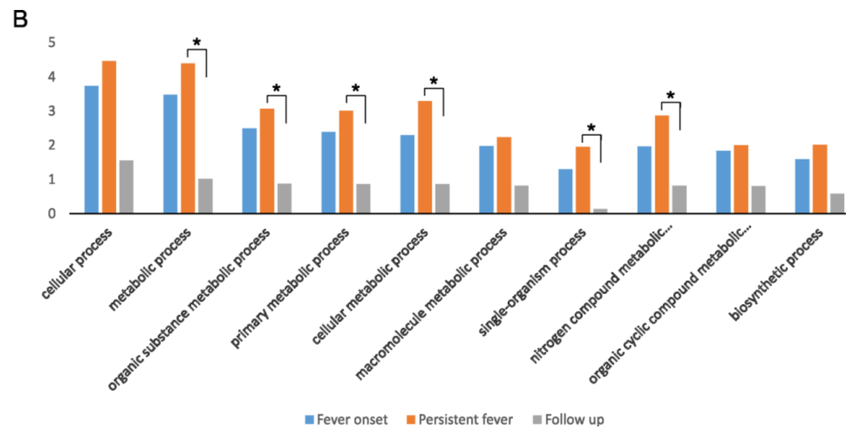
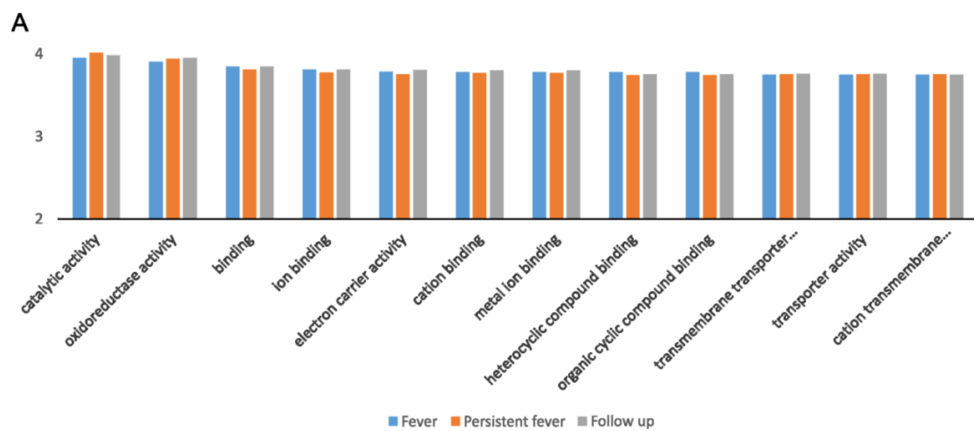


Fig. S1 (A) Relative abundance of molecular functions in bacterial reads based on the gene ontology analysis. Ten processes with the highest abundances in fever onset samples are shown. (B) Relative abundance of biological processes in bacterial reads based on the gene ontology analysis. Ten processes with the highest abundances in fever onset samples are shown.



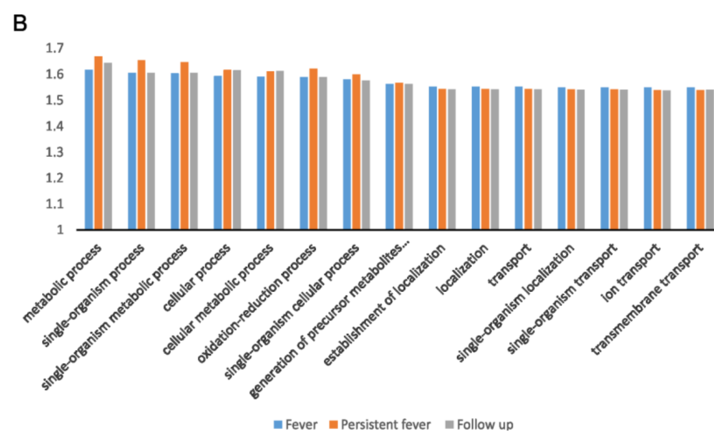


Fig. S2 (A) Relative abundance of molecular functions in fungal reads based on the gene ontology analysis. Ten processes with the highest abundances in fever onset samples are shown. (B) Relative abundance of biological processes in fungal reads based on the gene ontology analysis. Ten processes with the highest abundances in fever onset samples are shown.

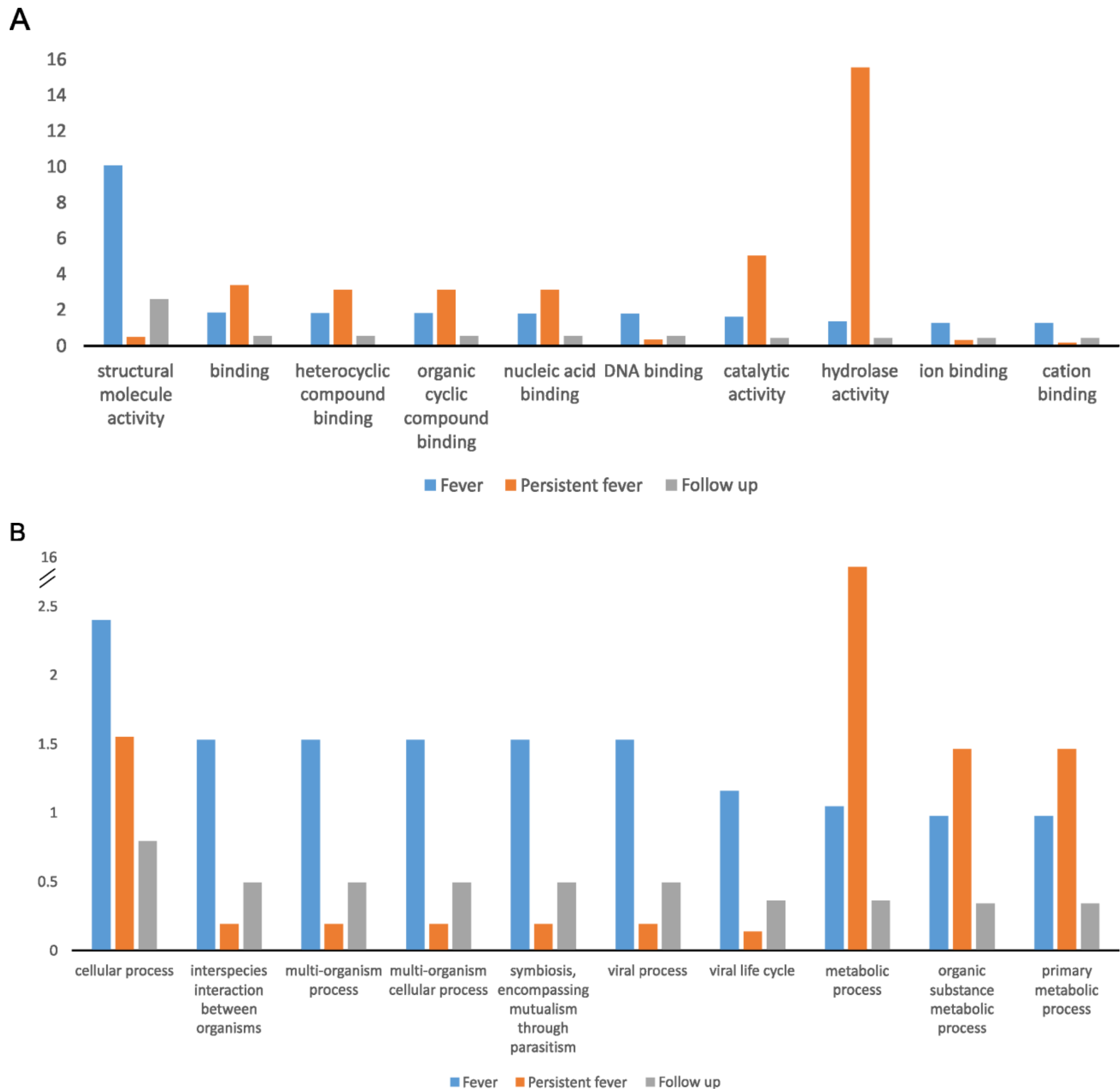


Fig. S3 (A) Relative abundance of molecular functions in viral reads based on the gene ontology analysis. Ten processes with the highest abundances in fever onset samples are shown. (B) Relative abundance of biological processes in viral reads based on the gene ontology analysis. Ten processes with the highest abundances in fever onset samples are shown.

	Age	Gender	Chemotherapy		Fever	Blood culture result	WBC	ANC	CRP	Broad-spectrum antibiotics	Antibiotic prophylaxis	Antiviral prophylaxis	Antifungal prophylaxis	MASCC	Hematological diagnosis
Patient 1	50	M	cytarabine + daunorubicin	Sample 1	x	Alpha Streptococcus	0.1	0	36	piperacillin/tazobactam	-	valacyclovir	posaconazole	18	acute myeloblastic leukaemia
				Sample 2	-	ND	0.2	ND	180	meropenem	-	valacyclovir	posaconazole	21	acute myeloblastic leukaemia
Patient 2	54	M	cytarabine + daunorubicin	Sample 1	x	Alpha Streptococcus	0	0	18	piperacillin/tazobactam	ciprofloxacin	valacyclovir	posaconazole	23	acute lymphoblastic leukaemia
				Sample 2	x	negative	0	0	ND	amikacin	-	valacyclovir	posaconazole	20	acute lymphoblastic leukaemia
Patient 3	65	F	cytarabine + daunorubicin	Sample 1	x	negative	0.3	0	29	meropenem	ciprofloxacin	valacyclovir	posaconazole	ND	acute myeloblastic leukaemia
				Sample 2	x	negative	0.2	ND	ND	meropenem	ciprofloxacin	valacyclovir	posaconazole	ND	acute myeloblastic leukaemia
Patient 4	25	M	cytarabine + daunorubicin	Sample 1	x	Alpha Streptococcus	0	0	12	piperacillin/tazobactam	ciprofloxacin	valacyclovir	posaconazole	ND	acute myeloblastic leukaemia
				Sample 2	x	Alpha Streptococcus	0	ND	ND	piperacillin/tazobactam	-	valacyclovir	posaconazole	ND	acute myeloblastic leukaemia
				Sample 3	-	negative	0	ND	178	piperacillin/tazobactam	-	valacyclovir	posaconazole	ND	acute myeloblastic leukaemia
				Sample 4	x	ND	0	ND	154	clindamycin	-	valacyclovir	posaconazole	21	acute myeloblastic leukaemia
Patient 5	51	M	cytarabine + daunorubicin	Sample 1	x	negative	0.4	ND	37	piperacillin/tazobactam	ciprofloxacin	valacyclovir	posaconazole	26	acute myeloblastic leukaemia
				Sample 2	-	ND	0.4	ND	44	piperacillin/tazobactam	-	valacyclovir	posaconazole	ND	acute myeloblastic leukaemia
				Sample 3	-	ND	0.8	0	ND	piperacillin/tazobactam	-	valacyclovir	posaconazole	ND	acute myeloblastic leukaemia
				Sample 4	-	ND	1.8	0.2	ND	piperacillin/tazobactam	-	valacyclovir	posaconazole	26	acute myeloblastic leukaemia
Patient 6	50	F	cytarabine + daunorubicin	Sample 1	-	ND	ND	ND	ND	piperacillin/tazobactam	-	valacyclovir	posaconazole	ND	acute myeloblastic leukaemia
				Sample 2	x	negative	0.8	0.2	ND	piperacillin/tazobactam	-	valacyclovir	posaconazole	23	acute myeloblastic leukaemia
				Sample 3	x	negative	ND	ND	ND	piperacillin/tazobactam	-	valacyclovir	posaconazole	ND	acute myeloblastic leukaemia
				Sample 4	-	ND	ND	ND	65	piperacillin/tazobactam	-	valacyclovir	posaconazole	ND	acute myeloblastic leukaemia
Patient 7	48	M	cytarabine + daunorubicin	Sample 1	x	negative	0	ND	153	piperacillin/tazobactam	-	valacyclovir	posaconazole	23	acute myeloblastic leukaemia
				Sample 2	x	Alpha Streptococcus	ND	ND	39	piperacillin/tazobactam	ciprofloxacin	valacyclovir	posaconazole	23	acute myeloblastic leukaemia
				Sample 3	-	ND	0	ND	78	piperacillin/tazobactam	-	valacyclovir	posaconazole	ND	acute myeloblastic leukaemia
				Sample 4	x	negative	0	ND	202	piperacillin/tazobactam	-	valacyclovir	posaconazole	ND	acute myeloblastic leukaemia

Table S1. Patients characteristics showing age, gender, chemotherapeutic treatment, blood culture findings, the applied broad-spectrum antibiotics, hematological diagnoses, antimicrobial prophylaxis and laboratory data. ND=no data; WBC=white blood cell count, 10^9 cells/L; ANC=absolute neutrophil count, 10^9 cells/L; CRP=C reactive protein, µg/ml; MASCC=Multinational Association for Supportive Care in Cancer risk score.