Epidemiology of most frequent infectious complications in immunocompromised patients:

focusing on bacteraemia, CMV and HHV-6 infections in haematological patients, and following autologous stem cell transplantation

Summary of the Ph.D. Thesis

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LIST OF PUBLICATIONS RELATED TO THE THESIS

I. Piukovics Klára, Terhes G, Bereczki Á, Borbényi Z, Gurbity Pálfi T, Kővári B, Urbán E. Valós idejű polimeráz láncreakció alkalmazása cytomegalovirus-fertőzés és -reaktiváció nyomon követésére malignus hematológiai betegségek kemoterápiás kezelése során és autolog őssejt-transzplantációt követően.

ORVOSI HETILAP **157:**(35) pp. 1403-1409. (2016) **IF: 0,291**

II. Piukovics Klára, Terhes G, Lazar A, Timar F, Borbenyi Z, Urbán E. Evaluation of Bloodstream Infections During Chemotherapy-Induced Febrile Neutropenia in Patients with Malignant Hematological Diseases: Single Center Experience.

EUROPEAN JOURNAL OF MICROBIOLOGY AND IMMUNOLOGY **5:**(3) pp. 199-204. (2015)

III. Piukovics Klára, Borbényi Z, Rajda C, Csomor A, Deák J, Terhes G. Monitoring Human Herpesvirus-6 in Patients with Autologous Stem Cell Transplantation.

IN VIVO **28:**(6) pp. 1113-1117. (2014) **IF: 0,974**

IV. Terhes G, **Piukovics Klára,** Urbán E, Nagy E. Four cases of bacteraemia caused by *Fusobacterium nucleatum* in febrile, neutropenic patients.

JOURNAL OF MEDICAL MICROBIOLOGY **60:**(7) pp. 1046-1049. (2011) **IF: 2,502**

V. Piukovics Klára, Terhes G, Gurbity-Pálfi T, Bereczki Á, Rárosi F, Deák J, Borbényi Z, Urbán E. Cytomegalovirus infection in patients with haematological diseases and after autologous stem cell transplantation as consolidation: a single-centre study.

ANNALS OF HEMATOLOGY 2016 Oct 11. [Epub ahead of print] **IF: 3,022**

VI. Klára Piukovics, Viktória Bertalan, Gabriella Terhes, Ágnes Báthori, Edit Hajdú, Gyula Pokorny, László Kovács, Edit Urbán. Fatal cases of disseminated nocardiosis-challenges to physicians and clinical microbiologist-case report.

ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA **IF: 0,568**
(közlésre elfogadva)

VII. Tóth Eszter, Hajdú Edit, **Piukovics Klára,** Borbényi Zita, Nagy Erzsébet. Véráram fertőzést okozó baktériumok előfordulása acut leukémiás betegeknél.

***BULLETIN OF MEDICAL SCIENCES/ORVOSTUDOMÁNYI ÉRTESÍTŐ* 80:** pp. 32-34. (2007)

VIII. Hajdú Edit, Terhes Gabriella, Bertalan Viktória, **Piukovics Klára**, Nagy Erzsébet. Fatalis kimenetelű nocardiosis immunsupprimált betegeknél.
***INFEKTOLÓGIA ÉS KLINIKAI MIKROBIOLÓGIA* 15:** pp. 46-49. (2009)

1. INTRODUCTION

1.1. INFECTIOUS COMPLICATIONS IN PATIENTS WITH HAEMATOLOGICAL DISEASES

During the past few decades, significant developments have occurred in the treatment of malignant diseases and, because of these, the cancer-related death rate is decreasing. Haematopoietic stem cell transplantation (HSCT) has been incorporated into the consolidating treatment of different types of lymphoproliferative disorders in an autologous setting, or allogeneic stem cell transplantation, as curative intent for acute leukaemias. However, mainly because of the better immunosuppressive effects of these new drugs, neutropenia remains one of the most serious side effects of antineoplastic treatments. Neutropenia may be associated with the malignancy itself or it may be present as a consequence of chemotherapy and this is the major risk factor for developing infectious complications in this patient group. The role of neutropenia in infections was recognised in the 1960's. The majority of patients (about 30-60%) with neutropenia develop infectious complications, and 13-37% of them may develop bloodstream infection (BSI). Most common sites and organs of infections in neutropenic patients are the respiratory tract (35-40%) (paranasal sinuses and lung infiltration), and bacteraemia (15-35%) (central line-associated bloodstream infection, CLABSI). Skin, soft tissues and urinary tract are involved in 5-10% of cases, and also infections originating from the gastro-intestinal tract (neutropenic enterocolitis and perianal inflammation) and oro-pharynx occur in 5-10% of cases. The widespread use of more intensive chemotherapy, and introduction of monoclonal antibodies and biological therapy in the treatment of malignant haematological diseases have produced a persistent and worsening immune-deficient state, and this may also contribute to the development of various infections. During the hospitalisation of these patients, the spectrum of colonising microorganisms on the skin and

mucosal surfaces has radically changed because of therapeutic, surgical and other invasive interventions, hence the colonisation resistance of the normal flora has decreased. In the majority of microbiologically documented infections in patients with cancer, the causative microorganisms are part of the endogenous flora. Only in a few cases might the infection arise from exogenous sources. Because of the above-mentioned factors, bacterial, viral and fungal infections are important causes of morbidity and mortality for patients with malignancies. The spectrum of pathogens causing infections in this patient group is continuously changing, and it is influenced by various factors including local epidemiology, the use of antibiotics, antiviral and antifungal agents, the use of catheters and various medical devices. Viral infections are the most common cause of morbidity in cancer patients especially to pediatric patients. Viruses directly affect the cell-mediated immune system, thus they increase the risk of developing serious and life threatening infections. In the case of viral infections, the most important pathogens are the cytomegalovirus (CMV), herpes simplex virus (HSV), varicella-zoster virus (VZV), respiratory syncytial virus (RSV), parainfluenza and influenza viruses, the human herpesvirus 6 (HHV-6), human poliomavirus 1 (BK virus), adenovirus and the human metapneumovirus. Regarding bacterial infections in patients with haematologic malignancy, the results of various international surveys are available. Earlier, mainly Gram-negative bacteria could be detected from infectious complications, while nowadays, Gram-positive bacteria are frequently associated with sepsis in this patient group and the role of atypical mycobacteria, intracellular pathogens and opportunistic bacteria is becoming evermore important due to developments in microbiological methods. The significance of bacterial infections in patients with haematologic malignancy is well characterised, while the incidence and consequence of viral infections are poorly described in the haematologic patient group.

2. AIMS OF THE STUDY

On the basis of findings of various international surveys and the lack of local data on the epidemiology of bloodstream and viral infections in patients with haematologic malignancy, the aims of this study were:

1. To evaluate the occurrence of bacterial species causing bloodstream infections in febrile neutropenic episodes in the Department of Haematology (University of Szeged, Hungary), between 2005 and 2008.

2. To characterise bloodstream infections caused by unusual pathogens including *Fusobacterium* spp., *Nocardia* spp., and *Achromobacter* spp.

3. To present the results of CMV monitoring in patients with haematological diseases, and after autologous stem cell transplantation.

4. To determine the occurrence of HHV-6 infection in stem cell transplant recipients.

3. MATERIALS AND METHODS

3.1. COMMONLY USED DEFINITIONS

Febrile neutropenia. Febrile neutropaenia was defined if a single oral temperature was measured higher than 38.3 °C, or the temperature was 38.0 °C or higher for 1 hour. Neutropenia is defined as when the absolute neutrophil count (ANC) is less than 0.5xG/L or less than 1.0xG/L and it rapidly declines below 0.5XG/L. Three distinct groups of febrile episodes may be distinguished in neutropenic patients. 1. Microbiologically documented infection (MDI) means a positive culture result can be confirmed from different sites (blood or other specimens). 2. Clinically documented infection is diagnosed clinically or by radiographic examinations without positive microbiologic samples. 3. Fever of unknown origin (FUO) means there is no evidence of positive microbiological results or no clinical/radiographic signs of infection could be detected. The incidence of BSI varies between 19-29%, and 15-25% of it is polymicrobial (more than one species of bacteria grew from blood culture (BC) on the same day). About 10-15% of infectious complications of neutropenic patients appear in unusual localisations including central nervous system involvement (meningitis, brain abscess), septic arthritis, and septic involvement of the liver, spleen and kidneys caused by unusual bacteria.

3.2. BLOODSTREAM INFECTIONS IN PATIENTS WITH HAEMATOLOGIC MALIGNANCY

3.2.1. Patients

Between 2005 and 2008, 469 patients with febrile neutropenia (230 females and 239 males, median age 60 years) were examined at our department. The data collected from patient

documentation included the demographics of patients, diagnosis, febrile episodes, source of fever and source of infection, neutrophil count, duration of neutropenia and clinical significance of the isolated organism. A single positive BC was considered significant if the isolated strain was a clinically relevant cause of the infection. Common skin contaminants (CNS (*coagulase-negative staphylococci*) and *propionibacteria*) were considered significant only if they were found in two consecutive BC samples or if there were concurrent skin, soft tissue or catheter–related infections..

3.2.2. Analysis of blood cultures

BCs were collected at the onset of a fever. In patients with central venous catheters, BCs were taken simultaneously from both central and peripheral veins. For the collection of blood culture, blood culture systems (BD Bactec, Beckton Dickinson, USA) including aerobic, anaerobic bottles and bottles for fungi were used. After the collection of blood, the bottles were immediately placed in an incubator, where they were incubated for 5-14 days depending on the type of the putative pathogens. In the case of a positive signal produced by the instrument on the basis of bacterial or fungal growth, microscopic examinations (Gram stained preparations) and culture were performed. For an aerobic culture, Columbia blood agar supplemented with 5% sheep blood (bioMérieux, Marcy l'Etoile, France), chocolate agar supplemented with PolyViteX (bioMérieux, Marcy L'Etoile, France), eosin-methylenblue (Lab M, UK) and Sabouraud Chloramphenicol (Bio-Rad, France) agars was inoculated, while for anaerobic culture Schaedler agar supplemented with 5% sheep blood (bioMérieux, Marcy l'Etoile, France) was inoculated. Plates were incubated at 37 °C for 24 hours in a 5% CO_2 incubator or 37 °C for 24 hours under normal atmospheric pressure or at 37 °C for 48 hours in an anaerobic cabinet (Concept 400; Ruskinn Technology Ltd., Bridgend, UK) under a gas composition of 85% N_2, 10% H_2 and 5% CO_2. From a pure culture, antibiotic susceptibility tests were performed on the basis of recommendations by the Clinical Laboratory Standard Institute.

3.2.3. Identification of bacteria and yeasts

For the precise identification of bacteria and yeasts, traditional biochemical tests or automated identification systems, such as VITEK®2 ID (bioMérieux, France), API 20 A (BioMérieux, France), or RapID™ ANA (bioMérieux, France) were applied on the basis of colony morphology, O_2/CO_2 requirement, and phase contrast microscopy findings. If the identification proved inconclusive using traditional or automated identification systems, the

amplification of 16S rDNA was used. Stated briefly, nucleic acid from the colonies was purified using the QIAamp DNA mini kit (Qiagen) and the 16S rDNA was amplified using universal primers E8F (5'-AGAGTTTGATCCTGGCTCAG-3') and E533R (5'-TTACCGCGGCTGCTGGCA-3'). For amplification, the following cycling conditions were used: starting denaturation at 94ºC for 3 min; 35 cycles: denaturation at 94ºC for 15 s, annealing at 55.5ºC for 30 s, extension at 72ºC for 1 min; final extension at 72ºC for 10 min. The PCR product was purified using the High Pure PCR Cleanup Micro Kit (Roche, Germany) according to manufacturer's instruction and the purified PCR product was sequenced. The sequences obtained were then compared with those stored in GenBank using BLAST alignment software (http://www.ncbi.nlm.nih.gov/blast).

3.2.4. Antibiotic protocol

At the onset of a fever, after the collection of BC samples, broad-spectrum antibiotics were started empirically (piperacillin-tazobactam, cefepime, and imipenem or meropenem). The antibiotic dosage was modified according to the patient's renal function. Patients were examined once a day by their physician to detect any potential source of infection. After 48-72 hours of observation, the patient's condition was re-evaluated. Changes in empiric antibiotic therapy depended on the BC results and clinical response. In afebrile and culture negative patients with a stable clinical state, empiric antibiotic treatment was continued until the ANC reached 0.5xG/L. Vancomycin was used in patients with central venous devices, persistent fever and hypotension. On days 4-5, in patients with a persistent fever that suggested a fungal infection on the basis of clinical signs and CT (computed tomography) scans, amphotericin-B was initiated.

3.3. MONITORING THE PRESENCE OF CMV IN PATIENTS WITH HAEMATOLOGIC DISEASES

3.3.1. Patients and sample collection

Between 2008 and 2014, 1238 plasma samples were collected from 271 patients with haematological diseases in our department (University of Szeged, Hungary). Clinical and demographic data of patients were recruited from the local medical computer-based database. The findings of serological investigations and CMV PCR were analysed retrospectively. Patients were divided into three groups according to the underlying disease, and the first two

categories were further divided into two subgroups: patients who underwent autologous stem cell transplantation (ASCT) or not (non-ASCT). CMV specific antiviral therapy was initiated on the basis of at least two consecutive CMV DNA viral copy numbers if the copy number was >1000 copies/ml in one sample or if the copy number was <1000 copies/ml, but the copy number indicated that there was an increasing tendency in two consecutive specimens.

3.3.2. Determination of the CMV serologic status and monitoring for CMV DNA viral load

Among patients with ASCT, CMV PCR was carried out once a week up to the 30th post-transplant day, and afterwards non-regularly up to the 100th post-transplant day or during hospitalisation and occasionally during the stem cell harvesting period. For the determination of CMV viral load, artus CMV RT PCR (Qiagen, Germany) was applied after nucleic acid isolated from the plasma specimens was tested using the MagNa Pure Compact Nucleic Acid Isolation Kit I (Roche, Swiss). Here, nucleic acid isolation and quantitative real-time PCR were carried out according to the manufacturers' recommendations. A serologic assay using ETI-CYTOK-M reverse Plus CMV IgM EIA (DiaSorin, Italy) and ETI-CYTOK-G Plus CMV IgG EIA (DiaSorin, Italy) was performed in ASCT group before the stem cell harvesting procedure; but in non-ASCT patients these tests were only done occasionally. CMV symptomatic infection was defined if CMV DNAemia was detected in two consecutive specimens along with the clinical suspicion of reactivation. CMV end-organ disease was defined by the presence of symptoms consistent with CMV infection and the simultaneous presence of molecular, histopathological, clinical or imaging findings that suggested CMV infection.

3.4. MONITORING THE PRESENCE OF HHV-6 IN PATIENTS WITH HAEMATOLOGIC DISEASES

3.4.1. Patients

Between 2010 and 2012, 35 consecutive patients that had undergone autologous peripheral stem cell transplantation were observed at our institution for HHV-6 viral reactivation and infection. (They were 19 females and 16 males with a median age of 60 years; range 22-71 years). The underlying diseases were multiple myeloma, Hodgkin lymphoma and non-Hodgkin lymphoma in 29, 2 and 4 cases, respectively. One hundred and twenty-one

anticoagulated (EDTA) blood and 2 cerebrospinal fluid (CSF) samples taken from the 35 patients were collected.

3.4.2. Molecular detection of HHV-6

Plasma specimens and, in the case of neurological manifestations, CSFs were tested for the presence of CMV, HSV-1/2, EBV DNA using real-time PCR (artus CMV LC PCR Kit; Qiagen, Hilden, Germany, artus HSV-1/2 LC PCR Kit; Qiagen and artus EBV LC PCR Kit; Qiagen). HHV-6 nested PCR was performed with the use of Dream Taq Green PCR Master mix (2x) (Thermo Scientific; Waltham, MA, USA) containing 2x DreamTaq Green buffer, 0.4 mM dNTP, 4 mM $MgCl_2$. The primers were derived from the immediate early gene locus of two variants of the HHV-6 strain (U1102) (115). Ten microlitres of 1x DreamTaq Green PCR Master mix, 0.4 µl 25 mM $MgCl_2$, 0.3 µl 25 pmol HHV6-F out primer(5'-TTC TCC AGA TGT GCC AGG GAA ATC C-3'), 0.3 µl 25 pmol HHV6-R out primer (5'- CAT CAT TGT TAT CGC TTT CAC TCT C-3'), 4 µl H_2O, and 5 µl purified DNA template were used for PCR. For the next PCR, 10 µl 1x Dream Taq Green PCR Master mix, 0.4 µl 25 mM $MgCl_2$, 0.3 µl 25 pmol HHV6-F in primer (5'- AGT GAC AGA TCT GGG CGG GCC CTA ATA ACT T-3'), 0.3 µl 25 pmol HHV6-R in primer (5'- AGG TGC TGA GTG ATC AGT TTC ATA ACC AAA-3'), 4 µl H_2O and 5 µl template (from the samples amplified in the course of the previous PCR) were prepared. The amplified PCR product was detected using gel electrophoresis and two variants of HHV-6 were identified on the basis of the size of the amplified products (variant A gave 195 bp of PCR products, while variant B gave 423 bp).

4. RESULTS

4.1. BLOODSTREAM INFECTIONS IN PATIENTS WITH HAEMATOLOGIC MALIGNANCY

Between 2005 and 2008, 1 361 patients were hospitalised in the haematology department because of haematological diseases, and the average number of cases per year was 340. All together 812 febrile episodes were recorded in 469 (34.5%) patients and blood was collected for microbiological culture. Altogether 3714 blood culture bottles, 6.5 bottles/patient (ranging from 2-12) were sent to the laboratory. In 126 (27%) cases of the 469 patients, only one pair of blood culture bottles was taken following a febrile episode. Clinically documented infections were observed in 430 cases (52.95%) of 812 febrile episodes. The majority of them

were localised to the lung (39.5%). Colitis, and skin and soft tissue infections were the second and third most common types of infection.

Using a microbiological culture, 759 (20.4%) of 3714 blood culture bottles produced positive signals. From the majority of positive blood culture bottles (67.1%), Gram-positive bacteria were detected. Among Gram-positive bacteria, the most frequent isolates were coagulase-negative staphylococci (CNS) (65%), *S. aureus* (10%), *Enterococcus* spp. (6.7%), beta-hemolytic streptococci (3.1%), *S. pneumoniae* (2.8%), alfa-hemolytic streptococci (2.4%) *Clostridium* spp. (1.4%), and others (3%) (including *Listeria monocytogenes*, *Nocardia farcinica*, *Gemella* spp., and Gram-positive non-identified bacteria). Gram-negative bacteria were isolated from 250 (32.9%) blood culture bottles. A high prevalence of *E. coli* (52%) was detected in these specimens, while 14% of samples contained *P. aeruginosa*, 9.6% *Klebsiella* spp., 8% *Enterobacter* spp., and 1.6% *Fusobacterium* spp. Only 6 bottles proved to be positive for fungi during the given period; in 2 cases, *Candida albicans* and also in 2 bottles, *Candida tropicalis* was detected, while 2 other bottles were positive for *Cryptococcus* spp.

Among Gram-positive isolates, CNS was the most commonly identified species. In 50 febrile neutropenic episodes, CNS played a role as a causative agent of fever because of the coexistence of skin, soft tissue and central venous catheter related infections. As for the remaining cases, contamination might have been the source of CNS. *Nocardia farcinica (N. farcinica)* was also detected among rare pathogens in a 37-year-old man diagnosed with large granular lymphocytic leukaemia. He was hospitalised due to several febrile episodes 6 months before present admission, but infectious agents could not be detected. Upon admission, the patient complained of general fatigue, fever, and vomiting. After hospital admission, headache and dizziness started. Neurological examination did not reveal any signs of meningitis. Three days later CT was performed because of central type paresis of facial and hypoglossal nerves. The CT scan revealed multiple lesions (6-13 mm in diameter) with perifocal oedema. A stereotactic core biopsy from lesions could not be carried out because of a deterioration in the patient's clinical status. Blood and urine samples for bacteriological culture were collected, and empiric antibiotic therapy (imipenem-cilastatin 500 mg every 6 hour) was started. Two blood culture bottles gave a positive signal after 52 h and 65 h of incubation; and in the Gram stained preparations, Gram-positive branching filaments could be observed. After 24 h of incubation in an atmosphere with 5% CO_2, small orange colonies had grown. Because of the unsuccessful identification of the isolated strains using a commercial

kit (VITEK 2 GP ID card, BioMérieux), 16S rDNA sequencing was performed. On the basis of sequence analysis of the amplified PCR product, *N. farcinica* was identified as the source of the infection.

Between 2005 and 2008, four cases of bacteremia caused by *Fusobacterium nucleatum* (*F. nucleatum*) were detected in patients with acute leukemia. Another rarely isolated pathogen from blood culture was *Achromobacter xylosoxidans* (*A. xylosoxidans*).

4.2. MONITORING THE PRESENCE OF CMV IN PATIENTS WITH HAEMATOLOGIC DISEASES

Between 2008 and 2014, 271 patients with haematological malignancies were tested for the presence of CMV DNA. A total of 1238 PCRs were performed. A CMV serological assay was carried out for 204 cases, and in 75.5% of these cases, they had CMV specific IgG, while for the remaining 50 (24.5%) patients, the CMV IgG tests proved to be negative.

During the given period, 1238 plasma specimens were tested for the presence of CMV DNA using RT PCR. Out of the total plasma samples examined, 118 (9.5%) were positive. The majority of positive CMV PCR results (45.2%) were observed in the ASCT group between post-transplant 20-40 days, while 5 patients had positive PCR results between post-transplant 10-20 days, and only 3 specimens proved to be positive 100 days after a transplant. The viral load in PCR positive specimens was under 1000 copies/ml in 87 (72%) cases. No significant differences could be observed between the CMV PCR positive group and negative group based on age, sex and an initial diagnosis ($p=0.987$, $p=0.411$, $p=0.416$), respectively. A total of 24 patients received antiviral therapy (intravenous ganciclovir 5 mg/kg twice daily or oral valganciclovir 900 mg daily) until the resolution of clinical signs and after two consecutive negative or decreasing CMV PCR results. For the calculations, the statistical software package applied was IBM SPSS Version 22. Here, we used the Mann-Whitney U-test to compare baseline characteristics among patient groups. The age was compared across CMV PCR positive and CMV PCR negative groups via the Mann-Whitney U-test. The independence of CMV PCR groups and sex, CMV PCR groups and the initial diagnosis was tested by using the chi-square test. A p-value was regarded as statistically significant if $p<0.05$.

4.3. MONITORING THE PRESENCE OF HHV-6 IN PATIENTS WITH HAEMATOLOGIC DISEASES

Four out of 35 (11.4%) patients tested using the PCR method post-transplant had HHV-6 positivity in the peripheral blood or CSF. Two patients had HL, and two patients suffered from MM. The HHV-6 variant A was detected only from MM patients, while the HHV-6 variant B was amplified from HL patients using PCR. Three of the four patients did not display any clinical signs of infection at the time of sample collection, while the fourth patient had limbic encephalitis. A 32- year-old man with stage II/A Hodgkin lymphoma underwent the DHAP regimen because of an early relapse. Autologous stem cell transplantation was performed after he had achieved complete remission. After receiving a second course of DHAP, a peripheral blood stem cell harvest was performed ($5.56x10^6$/kg of body weight; CD34 positive cells). The patient was conditioned according to the BEAM protocol followed by autologous stem cell infusion. On post-transplant day 17, the patient was referred to a neurologist because of mental confusion. After several unsuccessful lumbar punctures, on the basis of findings obtained from an MRI scan of the brain, the possibility of viral encephalitis, most probably herpes-encephalitis caused by HHV-6, in the temporal lobes was presumed. Two days later lumbar puncture was repeated to test HHV-6 nested PCR, and HHV-6 specific nested PCR gave positive results for the HHV-6 B variant from both peripheral blood and CSF samples. Parenteral gancyclovir (500mg twice daily) therapy was given for 21 days and followed by valgancyclovir (450mg twice daily) taken orally for four weeks. Afterwards, his mental state and neurological state gradually improved and he recovered.

DISCUSSION

5.1. BLOODSTREAM INFECTIONS IN PATIENTS WITH HAEMATOLOGIC MALIGNANCY

Although major advances in the care of cancer patients over the past several decades have resulted in improved survival, infectious complications remain a significant cause of morbidity and mortality. To successfully identify, treat, and prevent infections, a comprehensive understanding of risk factors that predispose one to infection and of

commonly encountered pathogens is necessary. In addition, clinicians must keep abreast of the changing epidemiology of infections in this patient population. As therapeutic modalities continue to evolve, as established pathogens become increasingly drug resistant, and as new pathogens are discovered, the successful management of infections will continue to present challenges in the years to come. Febrile neutropenia is the most common complication of chemotherapy in patients with haematologic malignancy. This may have an influence on the chemotherapy applied and dose reduction; moreover, treatment delays can be seen if febrile neutropenia is present. These have unfavourable long-term effects in an otherwise curable malignancy. In patients with haematologic malignancy, bloodstream infections are the most serious bacterial infections. Despite developments in microbiological diagnosis and antimicrobial therapy, these infections are responsible for the large proportion of nosocomial infections worldwide. In the early 1960s, the importance of bloodstream infection in neutropenic patients had been recognised, thus empirical treatment protocols were established for mainly Gram-negative bacteria, because at that time the most common pathogens associated bloodstream infections were Gram-negative bacilli. Later, the spectrum of pathogens associated with BSI shifted from Gram-negative to Gram-positive bacteria due to the increased use of antibiotic prophylaxis and indwelling catheters, which allow colonisation and infections. Nowadays, the most common pathogens isolated from blood are coagulase-negative staphylococci and various antibiotic resistant bacteria including multidrug resistant Gram-negative bacteria and VRE. In the majority of cases, the source of these infections is unknown in spite of efforts to find them. The recognition of changes in the epidemiology of BSIs is needed to modify the antibiotic policy because on the basis of these findings we can reduce the infection-related morbidity and mortality. In our retrospective survey, during the 4-year study period, the average incidence of bacteraemia was 20.4%. Similar findings were reported in the literature; Klastersky *et al*. discovered that the incidence of bacteraemia was 23% in cancer patients, while Viscoli *et al*. found that bacteraemia occurred in 29% of patients with febrile neutropenia. Our results are consistent with the above-mentioned literature data, because from the majority of blood culture bottles (13.7%), Gram-positive bacteria were isolated. 65% of Gram-positive bacteria belonged to coagulase-negative staphylococci. However, in a study by Winston *et al.* in North America, Gram-negative bacteria (55.6%) were responsible for the majority of bacteraemia in febrile neutropenic patients. At the same time, other researchers from Italy and France demonstrated that the most important isolates in neutropenic patients are Gram-positive bacteria, including coagulase-negative staphylococci or streptococci, while Gram-negative organisms including

E. coli or *Klebsiella* spp., *P. aeruginosa* constituted a smaller portion of the isolates. In our case, the most frequently used empiric treatment in this patient group is piperacillin/tazobactam, or if the patient has colitis or the possibility of abdominal infection has arisen, imipenem or meropenem are frequently used antibiotics. Thus the increased incidence of Gram-positive bacteria can be explained by the empiric antibiotic treatment being applied, while the presence of coagulase-negative staphylococci may be attributed to the frequent use of central venous catheters. The incidence of bacterial species in blood cultures may be influenced by the chemotherapy applied. In our case, 30% of patients with acute leukaemia received high dose Ara-C chemotherapy, and 15% and 12% of patients were treated with fludarabin and Ara-C plus idarubicin, respectively. On the basis of literature data, an increasing prevalence of Gram-positive cocci in febrile neutropenic patients could be observed after high-dose cytarabine chemotherapy, and this was confirmed by our findings. Cordonnier *et al*. showed that the prevalence of staphylococci is higher than the prevalence of streptococci and enterococci in febrile neutropenic patients. Similarly, our results were in general agreement with this, because among Gram-positive bacteria the majority of the isolated strains were coagulase-negative staphylococci, 6.7% and only 2.4% of Gram-positive bacteria belonged to *Enterococcus* spp. and beta-hemolytic streptococci, respectively. 331 blood culture samples proved to be positive for coagulase-negative staphylococci, and these had been collected from 161 febrile episodes of 149 patients. In 50 febrile neutropenic episodes, coagulase-negative staphylococci were identified as the cause of fever, and these were confirmed with the coexistence of skin, soft tissue and central venous catheter-related infection. Based on the clinical data and examination of the manifestations, the remaining 111 cases were thought to be due to contamination.

In the case of blood cultures that had positive results, 32.9% of them had Gram-negative bacteria, and the majority of these contained *E. coli* (52%). The second most common isolate was *P. aeruginosa* (14%), while the third was *Klebsiella* spp (9.6%). Similar findings were obtained by Ramphal. In this review, the results of four articles were analysed, and among Gram-negative organisms, the most important pathogens were also *E. coli, Klebsiella* spp. and *P. aeruginosa*.

Among rarely isolated bacteria, *A. xylosoxidans*, *B. cepacia* are usually associated with catheter-related sepsis, while *S. maltophilia* usually causes nosocomial bacteraemia. However, a possible source of *H. influenzae*, *Neisseria* spp., *Gemella* spp. is the damaged oral mucosa. The identification of the isolated strain from bloodstream infection using traditional

biochemical methods or automatic identification systems is sometimes unsuccessful because of the low biochemical activity of the isolated strain; or if the isolated species cannot be found in the library of the automatic identification system, the result of identification will be misleading. In such cases, molecular methods like universal bacterial PCRs and sequencing or the use of MALDI-TOF provide the opportunity to obtain the correct species name as we saw in the case of bacteraemia caused by *Nocardia* sp., and *A. xylosoxidans*. Because of the fastidious or slow-growing nature of some rarely isolated bacteria, special nutriments and culture conditions are required for a successful culture test. Thus in the case of the possible presence of these opportunistic pathogens, the past medical history of the patients and communication between clinicians and microbiologists are essential to help confirm the presence of these bacteria. This situation can be observed in the case of nocardiosis in patients with cancer. Nocardiosis is most frequently described as an opportunistic infection in immunocompromised patients, especially in patients with depressed cell-mediated immunity. Pulmonary nocardiosis is the most common clinical presentation of this infection, while among extrapulmonary forms, central nervous system (CNS) involvement is quite common. Nocardia bacteraemia is a rarely described clinical entity and in the majority of patients (64%) concurrent pulmonary, cutaneous (28%) and CNS involvements (19%) may be observed. In the case of disseminated nocardiosis, two or more organs are infected, and in the majority of cases, due to airway transmission the lung is also affected. Several publications described case reports about the presence of *N. farcinica* in various infections, but according to Christidou *et al*. up to 2004, only 11 English publications were found where *N. farcinica* bacteraemia was described. The majority of patients were male and had one or more predisposing factors, and the most common primary site of infection were the lungs. The mortality rate was 41.7%, and a similarly poor outcome associated with *N. farcinica* was reported earlier by Torres *et al*. General treatment recommendations for nocardiosis are difficult to provide owing to the lack of controlled trials and variable *in vitro* antibiotic susceptibility patterns. What is more, it is difficult to perform antimicrobial susceptibility testing because of the slow-growing nature of several *Nocardia* sp., inoculum consistency and interpretation of the cut-off. The treatment of *N. farcinica* infections is also problematic, because the majority of clinical isolates are multi-resistant - hence in our case, the isolated strain was susceptible only to imipenem and amikacin.

Because of the possible presence of unusual pathogens, such as anaerobic bacteria in bloodstream infections, the use of various blood culture bottles including anaerobic bottles

was considered. These confirmed those cases where a microbiological investigation revealed the presence of *F. nucleatum* in the blood cultures of four patients. In three cases, only one anaerobic bottle gave a positive signal. All the patients had clinical symptoms of sepsis; however, since the isolated *F. nucleatum* is not a member of the skin flora, its role as a possible contaminant was excluded. The mean number of days required for the blood culture bottles to become positive was 2.6 days (range 35 to 87 h). All four *F. nucleatum* strains proved to be susceptible to all tested anti-anaerobic antibiotics (penicillin, amoxicillin/clavulanic acid, clindamycin, cefoxitin, imipenem and metronidazole). In all cases, the underlying disease was haematological malignancy, acute myeloid or lymphoblastic leukaemia. Two patients (cases 1 and 3) received chemotherapy before the development of a febrile period, one of them had confirmed oropharyngeal mucositis, while with the other there were no clinical symptoms of this, but the therapy used is known to make patients susceptible to the development of oral mucositis. In case 4, the patient had severe oropharyngeal mucositis without previous chemotherapy, while in case 2, the peritonsillar region and the soft palate were oedematous. Fanourgiakis et al. reported fifteen cases of bacteraemia caused by *Fusobacterium* spp. over a period of 6.5 years. Among these, thirteen patients were neutropenic, their mean age was 53 years, and the majority of them had underlying haematological malignancies and oral postchemotherapy mucositis. As oral mucositis could be observed in almost all the patients, and this was the most likely source of the bacteraemia. This study also demonstrated that a relatively long incubation period (about 5 days) was sometimes necessary to get positive signals in the case of anaerobic bottles. On the basis of literature data, in the 1960s and 1970s, the incidence of anaerobic bacteraemia increased due to improvements in anaerobic laboratory culture methods; and later this was followed by a decrease. Then between 1993 and 2004, the mean incidence of anaerobic bacteraemias once again grew.

5.2. MONITORING THE PRESENCE OF CMV IN PATIENTS WITH HAEMATOLOGIC DISEASES

Because of the increasing number of drugs associated with severe and prolonged cell-mediated immunodeficiency, CMV infection is a major opportunistic infection among patients with malignant haematological diseases. The vast majority of publications have presented data about the occurrence of CMV reactivation and disease in haematological

patients following stem cell transplantation - predominantly in an allogeneic setting-, hence little information is available about patients treated for haematological diseases without stem cell support. In our study, we retrospectively analysed epidemiological data and the incidence of CMV infection in patients with malignant haematological diseases over a 6-year period. In the ASCT group, a higher rate of CMV infection (33.3%) was observed than that in non-ASCT group (16.6%). Similarly, a higher rate of CMV infection (26-39%) was observed in autologous transplantation recipients in other international studies, mainly in patients who had received $CD34^+$ selected autograft. Holmberg *et al.* also reported higher reactivation rates (22.6%) in patients who had received a $CD34^+$ selected autograft than those in unselected cases (4.2%) during the first 100 post-transplant days. However a low risk of CMV infection was observed earlier in patients undergoing autologous stem cell transplantation. In patients who had received pre-emptive anti-CMV treatment, CMV disease was not observed in association with ASCT. CMV pneumonia was identified in 2% of patients with ASCT and these were associated with CMV seropositivity before transplantation. Marchesi *et al.* also examined the rate of CMV reactivation after ASCT and he found that 11% of patients had required antiviral treatment because of CMV infection or end-organ diseases. The majority of CMV reactivation occurred in patients with lymphoma (16%), while CMV PCR positivity was detected in 8% of myeloma patients. Al-Rawi *et al.* (2015) examined CMV reactivation with a CMV pp65 antigenemia test, and observed CMV reactivation in 37 out of 210 patients (17.6%), and 94.6% of patients with a positive antigenemia assay were treated with anti-CMV therapy. Due to the anti-CMV therapy, symptomatic CMV infection or end-organ disease was not observed, and the mortality was 29.7% in this group. In our case, the majority of ASCT patients had asymptomatic reactivation. Eight patients (19%) suffered from symptomatic reactivation; while only one patient had CMV disease with 6225 copies/ml, and in this case, interstitial pneumonia, acute respiratory distress syndrome were demonstrated by a chest radiographic examination. The patient's condition rapidly declined and the subject died two days later. The postmortem histological examination confirmed the earlier diagnosis. As data published previously showed, the incidence of CMV pneumonia was 2-9% in ASCT recipients, with an extremely high mortality rate. Marchesi and colleagues found a significantly higher transplant related mortality rate in CMV reactivated patients (8.4%+/-4.7% versus 1.7%+/-0.8%; p=0.047), while sex, diagnosis (NHL versus MM) and types of conditioning regimen did not display any significant differences. Likewise in our observations, for ASCT and non-ASCT patients no observable differences were found between CMV PCR positive and negative groups according to age, sex (p=0.671, p=0.411),

and mortality rate (p=0.429). On the basis of a statistical analysis, in the ASCT group, regarding the types of malignancies (MM, NHL, HL) no difference could be detected in the rate of CMV reactivation at a significance level of 5%, while differences were confirmed at a significance level of 10% (p=0.076 versus p=0.038). NHL patients with B-cell phenotype who had had a transplant were treated with rituximab as part of a pretransplant induction therapy, while only thirteen patients were treated with rituximab as part of a conditioning therapy. In a comparison of two types of conditioning treatment (BEAM+R-BEAM versus melphalan), significant differences were found to exist across the prevalence of CMV reactivations (23.6% versus 42.3%; p=0.029). For R-BEAM, only one patient with NHL had a CMV positive PCR result. Because of the low number of cases, we did not look for a correlation between R-BEAM versus BEAM and CMV reactivation. Jain *et al.* (2016) analysed the results of CMV PCR in ASCT patients. In that study, 24% of patients were treated with rituximab as part of pretransplant induction therapy or conditioning treatment. The rate of CMV PCR positivity was 2.9% in the case of the 239 patients who were examined. CMV viraemia was detected in seven patients, and three of them were being treated with rituximab; therefore this treatment was not a significant risk factor for developing symptomatic CMV reactivation (p=0.34). Although the number of patients with NHL was lower in our study, we obtained similar findings. In the case of patients with MM, no significant differences were seen between the type of pretransplant induction treatment (bortezomib versus thalidomide) and a CMV positive/negative group (45% vs 46.7%; p=0.912). According to Marchesi et al, with MM patients treated with tandem ASCT, CMV reactivation after the first ASCT was more common when the induction treatment contained novel agents (odds ratio [OR]: 9.897; 95% confidence interval [CI]); p=0.021). It seems that a high dose of dexamethasone applied in cases of patients undergoing this type of induction treatment has a detrimental effect on cell-mediated immunity. Still, proteasome inhibitors and immunomodulators may have an adverse effect on the T cell (NK, CD4+ and CD8+) count and function. In our study, the majority of patients with lymphoid malignancies had CMV reactivation in the non-ASCT group, while the number of patients with myeloid malignant disease who suffered from CMV infection was low (just 1 case). In non-ASCT group, fourteen patients had symptomatic CMV infections, and among these, CMV disease was observed in two cases (colitis and hepatitis). Similarly, the authors of several publications said that the risk of reactivation was highest among patients with lymphoproliferative diseases and those who were being treated for decreasing T-cell function with a drug such as alemtuzumab. Han et al obtained similar findings when they examined CMV reactivation using a CMV pp65

antigenemia assay in patients with malignant haematological diseases. They demonstrated that a significantly higher rate of reactivation could be detected in lymphoid malignancies than in myeloid ones. Another centre also (Peter MacCallum Cancer Center in Melbourne, Australia) examined the presence of CMV DNA taken from whole blood in patients undergoing different chemotherapeutic regimens [hyper-CVAD (cyclophosphamide, vincristine, doxorubicin, dexamethasone, methotrexate and cytarabine), fludarabine-containing regimens, alemtuzumab] and ASCT. The rate of CMV reactivation was 4.6, 4.2, 9.7, 2.6 and 50% in patients that had a fludarabine-based therapy, ASCT, hyperCVAD, rituximab treatment and alemtuzumab treatment, respectively. Likewise, in our non-ASCT patient's group with CMV reactivation, five patients were treated with alemtuzumab, two with fludarabine-based therapy, and those who had the hyper-CVAD therapy did not have reactivation. Our findings are consistent with those found in observations published earlier.

5.3. MONITORING THE PRESENCE OF HHV-6 IN PATIENTS WITH HAEMATOLOGIC DISEASES

Autologous peripheral stem cell transplantation is a widely used procedure in the treatment of multiple myeloma, Hodgkin's and non-Hodgkin's lymphoma. In this patient group, viral infection is one of the most important causes of post-transplant morbidity and mortality. In this setting, HHV-6 encephalitis represents a very rare type of viral infection and it is a severe life-threatening complication in ASCT recipients. Pagter *et al*. summarised several studies in which they analysed HHV-6 reactivation during haematopoietic stem cell transplantation. Eighteen studies were examined and, in the case of twelve of these, one month after haematopoietic stem cell transplantation, HHV-6 reactivation occurred. The incidence of HHV-6 reactivation ranged from 28% to 78% in allogeneic and autologous patients. Delayed platelet engraftment displayed a significant association with the presence of HHV-6 DNA. Imbert-Marcille *et al*. studied 846 peripheral blood samples obtained from 92 consecutive patients (526 from autologous and 320 from allogeneic transplant recipients) for HHV-6 DNA; 18.3% of these samples gave positive results. Similar to other studies, the incidence of active HHV-6 infection was 42.5 % after autologous transplantation (27/64) and also allogeneic transplantation (12/28). Ljungman *et al*. also confirmed that a high HHV-6 viral load was associated with the development of HHV-6 disease, myelosuppression and prolonged engraftment, mainly in megakaryocytic and erythroid cell lineages. In addition to

this, only patient 4 displayed clinical signs of infection owing to HHV-6 reactivation. In our study, the number of HHV-6 positive samples was lower than that in previous studies; however, the number of patients analysed was also lower. Delayed engraftment was not detected in our study. In immunocompromised patients with signs of encephalopathy, a change in mental status and loss of short-term memory may help us to differentiate several causes - not only intra-cerebral bleeding from thrombocytopaenia, (the?) direct toxicity of drugs, electrolite and other metabolic disturbances, but also infectious complications. Bommer *et al*. described encephalitis and pneumonitis caused by HHV-6 in a young patient with relapsed Hodgkin's lymphoma who had undergone autologous stem cell transplantation. In this case, mental disturbances and convulsion developed without abnormality and signs were noted in the first MRI image of the brain on the twelfth post-transplant day, while a CT scan of the chest showed diffuse interstitial pneumonia on both sides of the lung. Bronchoalveolar lavage and CSF were positive for HHV-6 DNA. The patient was successfully treated with foscarnet followed by oral valgancyclovir for another six weeks. We also had a patient with encephalitis due to HHV-6 reactivation shortly after transplantation. Multiple EDTA blood and two CSF specimens taken from this patient gave positive HHV-6 PCR results, while no viral or bacterial pathogens were detected as a cause of encephalitis. Because of confirmed HHV-6 encephalitis, parenteral gancyclovir was started followed by oral valgancyclovir. During this course of treatment, the neurological signs were successfully resolved with minimal short-term memory dysfunction; and his haematological disease is now in complete remission. Similar to our findings, Imbert-Marcille *et al*. confirmed that active HHV-6 infection frequently occurred three or four weeks after transplantation. However, without a randomised clinical trial, treatment with gancyclovir, foscarnet or cidofovir is recommended for patients with HHV-6 encephalitis. In our case, gancyclovir treatment resolved the neurological signs with minimal sequelae.

CONCLUSIONS

In this study our key aim was to evaluate the local epidemiology of most frequent infectious complications in immunocompromised patients, especially bacteraemia, CMV and HHV-6 infections in haematological patients, and following autologous stem cell transplantation.

1. We evaluated the local occurrence of bacterial species that caused bloodstream infections in febrile neutropenic episodes between 2005 and 2008. In this study period among patients with febrile neutropenic episodes using microbiological culture, 20.4% of BC samples were positive, and in 67.1 % of positive BC samples Gram-positive bacteria were detected. The most frequent Gram-positive isolates were CNS (65%), *S. aureus* (10%) and the *Enterecoccus* species (6.7%). Here, CNS was identified in 331 cases and it was collected from 161 febrile neutropenic episodes of 149 patients. In 50 FN episodes, CNS was relevant as a causative agent of fever, because of the coexistence of skin, soft tissue and central venous catheter-related infections. The remaining 111 cases that had a positive BC result for CNS were presumed to be contamination, hence it is necessary to stress importance of a well-timed and precise sampling of a BC to avoid contamination. Gram-negative bacteria were isolated in 32.9 % of the positive BC results, and a high prevalence of *E. coli* (52%) was detected. This can be explained by the widespread use of indwelling catheters and more toxic high-dose chemotherapeutic regimens that can cause severe mucosal damage.

2. In this study we characterised bloodstream infections caused by “unusual” pathogens and described some instructive cases that were caused by these interesting, rarely isolated pathogens. The identification of rarely isolated pathogens (*A. xylosoxidans*, *B. cepacia*, *F. nucleatum* and *N. farcinica*) not only required the use of traditional biochemical methods and automatic identification systems, but also special conditions for a successful culture; and in these cases bacterial PCRs and sequencing or MALDI-TOF led us to determine the exact microbial species.

3. Out of the 271 patients with haematological malignancies all were tested using the CMV DNA method, and in 66 cases (24.4%) positive results were detected. Among CMV PCR positive patients, 42 underwent ASCT. In the non-ASCT patient-group the risk of reactivation was highest among patients with lymphoproliferative disorders, and those that had received treatment associated with decreasing T-cell function (purine analogues, alemtuzumab). In the ASCT group according to the type of underlying disease (MM versus NHL patients), borderline significant differences were detected in the rate of CMV reactivation, and by comparing the type of conditioning regimen, it was found that significant differences exist across BEAM+R-BEAM versus Melphalan ($p=0.029$) regimens.

4. After ASCT, 11.4% of the patients examined were found to be positive for HHV-6 after taking a sample of PB or CSF and applying the PCR method. Only one patient had been diagnosed with limbic encephalitis caused by HHV-6, and he was treated successfully with GCV. However without a randomised clinical trial, GCV, Foscarnet or CDV is recommended for patients with HHV-6 encephalitis.

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