Presence of viremia during febrile neutropenic episodes in patients undergoing chemotherapy for malignant neoplasms

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Abstract
The importance of viral infections as a leading cause of morbidity and mortality is well documented in severely immunosuppressed patients undergoing allogeneic stem cell transplantation. By contrast, viral infections generally receive less attention in patients with malignant disorders undergoing chemotherapy, where the onset of neutropenic fever is mostly associated with bacterial or fungal infections, and screening for viral infections is not routinely performed. To address the occurrence of invasive viral infections in a clinical setting commonly associated with less pronounced immunosuppression, we have prospectively screened 237 febrile neutropenic episodes in pediatric (n = 77) and adult (n = 69) patients undergoing intensive chemotherapy, primarily for treatment of acute leukemia. Serial peripheral blood specimens were tested by RQ-PCR assays for the presence and quantity of the clinically relevant viruses CMV, EBV, HHV6 and HAdV, commonly reactivated in highly immunocompromised patients. Viremia was documented in 36 (15%) episodes investigated, including the detection of HHV6 (n = 14), EBV (n = 15), CMV (n = 6), or HAdV (n = 1). While low or intermediate levels of viremia (<10^4 virus copies/mL) were commonly associated with bacterial or fungal co-infection, viremia at higher levels (>10^4 copies/mL) was documented in patients without evidence for other infections, raising the possibility that at least in some instances the onset of fever may have been attributable to the virus detected. The observations suggest that viral infections, potentially resulting from reactivation, might also play a clinically relevant role in patients receiving chemotherapy for treatment of malignant neoplasms, and routine screening for viremia in this clinical setting might be warranted.
1 | INTRODUCTION

Owing to the clear predominance of bacterial and fungal infections in the context of chemotherapy for aggressive hematologic neoplasms, screening for systemic viral infections is rarely part of routine diagnostic surveillance. Nevertheless, viral infections account for or contribute to a proportion of febrile neutropenic episodes in patients with hematological malignancies. A study performed in neutropenic patients with hematologic disorders, including also patients who underwent hematopoietic stem cell transplantation (HSCT), indicated that virus-positive test results were more frequent in the non-transplant patient cohort. We and others have shown that respiratory tract infections in immunocompromised patients with various malignancies, including also recipients of HSCT, display a high virus-associated morbidity. Infections with community respiratory viruses (CRVs) have been recognized as a potential cause of pneumonia and death among patients with hematologic malignancies and HSCT recipients, but reactivation of persistent viral infections might play a more prominent role.

In the past, transmission of viruses by various blood products was a serious problem, particularly in immunocompromised patients, and routine screening for a set of viral pathogens has become part of the standard safety precautions in transfusion medicine. Nevertheless, transmission of viruses via blood products due to contamination with known or yet to be identified human viral pathogens cannot be completely excluded. Protecting the blood supply from emerging infectious threats therefore remains a serious concern in the transfusion medicine community. Moreover, highly immunosuppressive treatment approaches used in hematologic malignancies favor opportunistic infections including viral reactivation. Persistent viral infections that are commonly reactivated during states of impaired immune surveillance include various members of the herpes virus family and different other viruses including particularly human adenovirus (HAdV) as a prominent example. The role of these viruses has been extensively studied in the context of HSCT, and we hypothesized that their contribution to infectious complications in patients undergoing intensive chemotherapy for hematologic neoplasms may be underestimated. To shed more light on the incidence and potential role of viral infection or reactivation in this setting, we have prospectively screened pediatric and adult patients displaying febrile neutropenic episodes during chemotherapy, primarily for malignant hematologic disorders, to assess the occurrence of viremia by targeting select viruses.

2 | METHODS

2.1 | Patients

From 2015 to 2019, diagnostic surveillance of 237 febrile neutropenic episodes (defined by neutrophil counts < 500/mL blood and body temperature above 38°C) was performed in pediatric (n = 150) and adult (n = 87) settings. The prospective study was performed in patients undergoing intensive chemotherapy for different types of acute leukemia or solid tumors specified below, who carried a high risk for infectious complications. The study was registered at clinicaltrials.gov (identifier: NCT02492594), and the protocol was approved by the respective responsible ethics committees of the Medical University of Vienna (EK 1461/2013), the City of Vienna (EK 14–187–1014), and competent authorities (BASC/AGES, Austrian Federal Office for Safety in Health Care/Austrian Agency for Health and Food Safety). Upon obtaining informed consent, prospective collection of whole peripheral blood (PB) specimens was scheduled as specified below. The study was focused on (a) invasive fungal infections, which are addressed in a separate manuscript, and (b) on the occurrence of invasive virus infections, defined by the presence of viremia, addressed in the present paper. The febrile episodes (n = 237) monitored in 146 patients included a pediatric cohort (77 patients) and an adult cohort (69 patients). The pediatric patients (median age 5 years, range 0–18 years, 48% female) were treated at the St. Anna Children’s Hospital (Vienna, Austria) or the Princess Máxima Center (Utrecht, the Netherlands). The underlying malignancies in the pediatric cohort included acute lymphoblastic leukemia (ALL, 86%), acute myelogenous leukemia (AML, 6%), lymphoma (4%), and rarely other tumor entities including primarily neuroblastoma. The adult patients (median age 58, range 19–81, 38% female) were treated at one of the following centers: Medical University of Vienna, Vienna General Hospital (Vienna, Austria), I.P. Pavlov First Saint Petersburg State Medical University (Saint Petersburg, Russian Federation) or Hanusch Hospital (Vienna, Austria). Neoplasms diagnosed in the adult patients included AML (64%), ALL (20%), lymphoma (10%), and rarely other tumor entities including multiple myeloma (MM) and myelodysplastic syndromes (MDS).

For comparison, a smaller cohort of allogeneic stem cell transplant recipients hospitalized at centers participating in the study was also investigated during febrile neutropenic episodes. The febrile episodes (n = 76) were monitored in 46 patients, including 26 children and 20 adults. The pediatric patients (median age 9 years, range 0–18 years, 42% female) displayed the following underlying malignancies: ALL (62%), neuroblastoma (12%), AML (8%), lymphoma (8%) and MDS (4%). The adult patients (median age 49, range 30–73, 15% female) had the following underlying malignancies: AML (50%), MM (30%), ALL (10%) and lymphoma (10%).

2.2 | Sample collection and microbial pathogen detection

Peripheral blood samples were collected at 0, 24 and 48 hours after onset of neutropenic fever. Bacterial and fungal culture were carried out in external certified laboratories, in line with the routine diagnostic work-up for septic patients. For molecular testing, the specimens were processed immediately or stored as full blood (for RQ-PCR) or plasma (for next generation sequencing - NGS) at −80°C prior to isolation of DNA.
2.3 DNA isolation and RQ-PCR

The Qiagen DNA Minikit (Hilden, Germany) was used for DNA purification according to manufacturer’s instructions. Briefly, 200 µL of PB were spiked with PhHV (phocine herpes virus) DNA as internal control, followed by treatment with SDS-based lysis buffer supplemented with proteinase K. The released DNA was bound on a column, washed and eluted in 240 µL elution buffer, yielding free and cell-bound viral DNA as well as cellular genomic DNA. Detection and quantification of viral DNA was performed using validated real-time quantitative PCR (RQ-PCR) assays for each of the viruses of interest including cytomegalovirus (CMV), Epstein–Barr virus (EBV), human herpesvirus 6 (HHV6), and adenovirus (HAdV) as well as the control virus PhHV spiked into the samples before DNA isolation, according to protocols described previously.19,23,24,31,32 For a subset of virus-positive samples identified by NGS analysis, control testing for BK polyomavirus (BKV) and herpes simplex virus 1 (HSV1) was performed. The RQ-PCR assays for detection of individual viruses were performed in a volume of 25 µL including TaqMan Gene Expression Master mix, primers (7.5–22.8 pmol), TaqMan probe (5 pmol) and DNA template (6 µL of the eluate). Positive controls (ie, plasmids encoding the respective viral sequences) and negative controls (ie, non-template and water controls) were included in each run using a TaqMan 7500 Instrument and the standard 9600 Emulation protocol. Previously determined thresholds and standard curves were employed for the analysis, and the presence of at least two virus-positive blood specimens during a febrile neutropenic episode were required for the diagnosis of viremia. Based on the peak virus copy numbers detected, the level of viremia was regarded as low (<10^3 copies/mL PB), intermediate (10^3–10^4 copies/mL PB) or high (>10^4 copies/mL PB). During the sampling period of 48 hours after onset of neutropenic fever, the virus copy numbers did not change beyond the intrinsic variability of the RQ-PCR assays performed.

2.4 Isolation of cell-free DNA and unbiased NGS

Plasma was prepared from blood samples by centrifugation for 10 minutes at 292 g and 4°C, snap freezing and storage at −80°C until further processing. Nucleic acids were isolated from thawed plasma after a centrifugation step of 5 minutes at 16000g and 4°C with the QiAsymphony sample preparation (SP) instrument (Qiagen) and the QiAsymphony DSP Circulating DNA Kit according to the manufacturer’s protocol. The cfdNA was quantified with the Qubit dsDNA HS Assay Kit (Life Technologies), and quality was assessed with the HS NGS Fragment Analysis Kit and the Fragment Analyzer instrument (AATI). The levels of cfdNA isolated from patients included in the study were sufficient for library preparation and NGS analysis in a proportion of specimens (n = 99). Library preparation and sequencing were carried out from 1 ng cfdNA using the Nextera XT library preparation kit (Illumina), with a Biomek FXP liquid handling robot (Beckman Coulter). Sequencing of the libraries was performed on a HiSeq2500 (Illumina), resulting, on average, in approximately 30 million 100-bp single end reads per sample. The presence of viral sequences was assessed according to relevance scoring and the SIQ (sepsis indicating quantifier) score established previously.33

2.5 Statistical analysis

Differences between co-infections with viruses, fungi and bacteria were tested using an A/B test (based on N-1 chi-square test) by employing a calculator at MeasuringU.com. Two-tailed P values are indicated (Figure 3). Note, p values <.05 were regarded as significant.

2.6 Role of the funding source

The funding source had no involvement in the study design, in the collection, analysis and interpretation of data, in the writing of the report or in the decision to submit the paper for publication.

3 RESULTS

While the presence of neutropenic fever in various earlier studies was explained by the detection of specific pathogens in less than half of the cases,2–434 the present study failed to reveal microbiological evidence in approximately one third of the 237 febrile neutropenic episodes in the cohorts of 69 adult patients (87 episodes) and 77 pediatric patients (150 episodes) (Figure 1). As expected, bacteria were the most commonly documented infectious agents. While Gram-positive bacteria predominated in the adult cohort (80% of bacterial findings), Gram-negative bacteria were slightly more frequently detected in the pediatric cohort (51% of bacterial findings). Fungal DNAemia was detected in 35% and 22% of adult and pediatric patients, respectively (Figure 1), but proven fungal infections documented by positive culture represented rare observations.

To assess the putative contribution of viruses to febrile episodes in neutropenic patients undergoing intensive chemotherapy for treatment of malignant neoplastic disorders, we focused on select viruses known to commonly reactivate and cause infectious complications in recipients of HSCT. The viruses of primary interest included the herpesviruses CMV, EBV, and HHV6, and the entire spectrum of currently known HAdV types.

Screening for these viruses in serial peripheral blood samples by RQ-PCR assays revealed the presence of viremia in 35 cases, including 27 pediatric (18%) and eight adult (9%) patients. This represents 15% of the febrile neutropenic episodes and 18% of the patients screened, because two or three febrile neutropenic episodes with viremia were observed in four and two patients, respectively. In adult patients, EBV was detected most frequently, while in pediatric patients HHV6 showed the highest prevalence, followed by EBV and CMV (Figure 2). Most patients displaying viral DNAemia had low (<10^3 copies/mL PB) or intermediate (10^3–10^4 copies/mL PB) virus copy numbers, involving EBV (n = 15), CMV (n = 5), HHV6 (n = 11),
and HAdV (n = 1). High copy numbers (>10^4 copies/mL PB) were observed in a smaller proportion (11%) of viremic patients, involving infections or reactivations of HHV6 (n = 3) or CMV (n = 1) (Figure 2). In patients with low virus copy numbers, co-infections with bacterial or fungal pathogens detected by culture or molecular screening were observed in 64% of the cases (Figure 3). The rate of bacterial or fungal co-infections was considerably lower (41%) in the presence of intermediate-level viremia, and no co-infections by other pathogens were detected in patients with high-level viremia (Figure 3). To further analyze the association of viral infection with clinical symptoms during febrile neutropenic episodes, patients displaying viremia in absence of other detectable pathogens were evaluated in more detail (Figure 4). The detectability of viremia already at first onset of fever correlated with the virus copy number level (Figure 4(A)). Moreover, the assessment of body temperature during the indicated days of sample collection revealed that fever persisting for more than 24 hours correlated with increasing virus copy numbers (Figure 4(B)).

To facilitate direct comparison between patients receiving intensive chemotherapy and patients undergoing allogeneic HSCT, where the important role of viral infections or reactivations is well established, we have prospectively screened for the indicated viruses in a smaller cohort of transplant recipients hospitalized at centers participating in the study, who displayed febrile neutropenic episodes during the study period. The allogeneic HSCT cases included 76 episodes monitored in 46 patients (20 adult and 26 pediatric), and viremia was detected in nine episodes (12%), including CMV (n = 2), EBV (n = 2), and HHV6 (n = 5). Similar to the patient cohorts undergoing chemotherapy, co-infections with bacterial or fungal pathogens were common in the presence of low or intermediate virus copy numbers (67% and 60%), and in the only patient displaying high-level viremia no co-infection was detected by routine diagnostic screening (Figure S1, Additional file 1). However, NGS analysis indicated the presence of *Legionella* (see below).

**FIGURE 1** Frequency of microbiological pathogens detected during febrile neutropenic episodes. Microbiological findings obtained by culture (bacteria, fungi) and/or molecular screening (fungi, viruses) in the pediatric, adult and combined (All) cohorts are indicated in percent, highlighting the predominance of bacterial pathogens in this setting. However, a large proportion of febrile neutropenic episodes remained unexplained by routinely performed microbiological assays. n = number of evaluated episodes.

**FIGURE 2** Virus detection in A, adult and B, pediatric cohorts. Peripheral blood (PB) samples from patients after the onset of neutropenic fever were analyzed by RQ-PCR. The number of febrile episodes in which CMV, EBV, HHV6 or HAdV were detected are depicted, and the presence of low (<10^3 copies/mL PB), intermediate (10^3–10^4 copies/mL PB), and high (>10^4 copies/mL PB) virus copy numbers is indicated. CMV: cytomegalovirus, EBV: Epstein–Barr virus, HHV6: Human herpesvirus 6, HAdV: human adenovirus.

**FIGURE 3** Occurrence of co-infections with bacteria or fungi in patients with viremia. The frequency of co-infections in low, intermediate and high copy number virus-positive samples is indicated in percent, revealing decreasing frequencies of co-infection with rising virus copy numbers. n = number of virus-positive episodes, p = p value.
Since the RQ-PCR-based screening was focused on select viruses, we have employed unbiased NGS (next generation sequencing) analysis in a subset of PB samples, including 99 specimens from 40 febrile episodes, to validate the findings by an independent method and to assess the possible presence of other DNA viruses in the patients investigated. The specimens available for NGS analysis included samples from two patients with positive findings by RQ-PCR (HHV6, n = 2), and the data obtained by RQ-PCR screening were confirmed by NGS. Unbiased NGS analysis indicated the presence of viruses in five additional patients, including two cases of BK polyomavirus (BKV), one case of Herpes simplex virus 1 (HSV1), one case of Alphapapillomavirus (α-HPV), as well as two CMV cases not detected by RQ-PCR screening. The specimens were re-analyzed by virus-specific assays validated for clinical diagnostics (with the exception of α-HPV, for which no diagnostic RQ-PCR test was available), and the presence of BKV, but not that of HSV1 and CMV, could be confirmed. Interestingly, no other viruses have been identified by unbiased NGS analysis in the samples investigated.

4 | DISCUSSION

The ongoing SARS-CoV-2 (severe acute respiratory syndrome-related coronavirus 2) pandemic has increased the interest in viral infections in many clinical settings. The current study was conducted before the onset of the pandemic, but our findings nevertheless highlight the role of viruses as important and underdiagnosed pathogens in patients with malignant neoplasms. In contrast to recipients of allogeneic HSCT, studies on the role of viral infections in cancer patients receiving intensive chemotherapy are less common. In the present study, we demonstrate the presence of viremia upon onset of febrile neutropenia in 18% of patients undergoing intensive chemotherapy for different malignant disorders, including particularly acute leukemias. In the presence of low-level viremia (<10^3 copies/mL PB), concomitant detection of bacterial or fungal microorganisms was common (Figure 3), suggesting that the febrile episodes were likely attributable to non-viral pathogens. Due to the fact that the herpes viruses included in the PCR-based screening often reveal persistence in individual leukocyte fractions, detection of low virus copy numbers in PB specimens may reflect the presence of persistently infected blood cells rather than circulating viral particles indicative of an active infection. This notion is further supported by the frequent absence of detectable viremia at first onset of neutropenic fever in these instances, rendering a causative role of the virus less likely (Figure 4(A)). Definitions of virus reactivation differ between studies owing to the assessment of different parameters (antigen or DNA) and due to the application of different thresholds. While some studies define reactivation as detection of DNA at any level, others set the threshold at thousand copies per mL blood. In patients displaying intermediate-level viremia (10^2–10^4 copies/mL PB), co-infection with other pathogens was less frequent (Figure 3), but high-level viremia (>10^4 copies/mL) was not associated with the presence of any other pathogens screened for by routine diagnostic testing, indicating that the onset of fever might have been attributable to the virus detected. Indeed, high-level viremia was more frequently associated with virus detection already at the onset of fever (Figure 4(A)) and more common persistence of fever (Figure 4(B)). Because of the high prevalence of persistent infections involving the viruses analyzed, reactivation rather than new infection may be a more probable cause of the infectious process, as described in recipients of allogeneic HSCT. In contrast to patients undergoing HSCT, where concomitant reactivation of multiple viruses reflects the state of severe immunosuppression, viral co-infection in the febrile neutropenic patients on chemotherapy was found in a single case only. This observation could be attributable to the lower severity of immunosuppression in the chemotherapy setting, although the degree of immunosuppression conveyed by chemotherapeutics can be considerable. In contrast to earlier analyses in HSCT recipients performed at our center, no cases of adenoviremia have been detected in the transplant recipients in the present study (Figure S1B, Additional file 1). In the cohort of patients undergoing chemotherapy, one case of adenoviremia was detected (Figure 2(B)), and screening of
other clinical specimens such as nasal or respiratory tract aspirates would have likely revealed the presence of adenoviruses more commonly, as shown by our earlier study in a similar set of patients treated in the St. Anna Children’s Hospital. Some of the viruses detected have been previously linked to individual tumor entities (eg, EBV and Burkitt lymphoma) and other links are suspected. However, the patients displaying viremia in the present study did not reveal malignant neoplasms previously associated with specific viruses, as outlined in Table S2.

The virus-associated morbidity and mortality is well documented in allogeneic HSCT recipients, but the role and clinical impact of viral infections or reactivations in patients receiving intensive chemotherapy for treatment of malignant neoplasms needs to be firmly established. Although asymptomatic viral infections have been reported in such patients, it is conceivable that particularly high-level and possibly also intermediate-level viremia might be associated with febrile reactions in this setting. However, it is necessary to consider that the onset of fever may be attributable to other causes, possibly unrelated to infectious complications, and the reactivation of viral infections might occur as a secondary event. Assessment of the potential correlation of viremia with morbidity therefore requires systematic screening in appropriately designed large-scale studies.

Similarly to other earlier publications, approximately one third of the febrile neutropenic episodes did not reveal any potentially causative pathogen, raising the possibility that a microorganism not covered by the diagnostic screening strategy may have caused or contributed to the clinical symptoms. Febrile neutropenic episodes without any microbiological evidence for an infectious pathogen were less frequently associated with profound or prolonged neutropenia, elevated C-reactive protein (CRP) levels or lung damage (Table S1, Additional file 2), and detection of bacteria or fungi was generally associated with these clinical findings more frequently than viremia (Table S1, Additional file 2). We had the opportunity to employ unbiased NGS in a proportion of specimens, and the analyses revealed only a few additional viruses, some of which could not be confirmed by subsequently performed specific RQ-PCR tests. Since the sensitivity of well-established targeted PCR assays is not likely to be inferior to NGS analysis, the discrepant findings might be attributable to different technical approaches to nucleic acid isolation prior to the indicated molecular assays, which may have resulted in preferential detection of individual sequences. Moreover, unbiased NGS-based sequencing analysis may have permitted virus identification from any free circulating viral fragment DNA (cfDNA), which escaped detection by RQ-PCR tests relying on the amplifiability of a single defined viral fragment. It was somewhat surprising that the employment of unbiased NGS analysis did not permit identification of a greater number and variety of viruses that could have been related to febrile episodes in the patients investigated. It is conceivable, however, that the ongoing infectious process is not reflected by blood stream infections in many instances. Moreover, the NGS analyses performed were restricted to sequencing of DNA specimens, and the inclusion of RNA analysis might - at least occasionally - have identified viral pathogens representing causative agents of the clinical symptoms observed.

Patients with febrile neutropenia not responding to different antibiotic treatment approaches for 72–96 hours commonly receive systemic antimycotic therapy without any evidence for the presence of fungal infection. This empirical treatment strategy is associated with considerable toxicity and costs, and it is desirable therefore to greatly reduce or eliminate overtreatment by antifungal agents. Results of the present study indicate the possibility that viral infection or reactivation may be responsible for a proportion of febrile episodes in this clinical setting. Screening for viral infections might therefore be warranted to elucidate their potential role in patients undergoing intensive chemotherapy for malignant disorders. If future studies support the notion presented, detection of high-level viremia in the absence of evidence for other microbial infections might serve as a basis for more judicious administration of antimicrobial treatment.

AUTHORS’ CONTRIBUTIONS

K.O., S.G., M.R., I.K. and L.G. performed sample analysis; K.O., S.G., M.R., S.C., I.K. and L.G. performed data acquisition and analysis; K.O., S.G., K.V.G., W.R.S., G.E., A.A., H.P., A.L., P.V., K.S. and T.L. contributed to data interpretation; S.H., K.V.G., W.R.S., T.E.-M., C.P., G.E., M.D., A.A., M.v.G., M.M.v.d.H.-E., I.S.M., Y.R., L.Z., N.Z., H.P., A.L., E.K., F.K. and P.V. contributed to data acquisition and analysis; K.O., M.R. and S.C. supervised experimental aspects of the study; N.A., K.S. and T.L. designed and coordinated the study; S.H., K.V.G., W.R.S., T.E.-M., C.P., G.E., M.D., A.A., M.v.G., M.M.v.d.H.-E., I.S.M., Y.R., L.Z., N.Z., H.P., A.L., E.K., F.K. and P.V. provided clinical samples and clinical data; G.E. provided expert consultation of clinical data; T.F. provided essential regulatory approvals, contributed to G.C.P. compliance; maintained ClinicalTrialsGov data; N.A. provided essential support in raising the funding; T.L. led the study and raised the funding; K.O. and T.L. wrote the manuscript; S.G., K.V.G., W.R.S., A.A., H.P., A.L., P.V. and K.S. provided feedback and input to the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

The study protocol was approved by the respective responsible ethics committees of the Medical University of Vienna (EK 1461/2013), the City of Vienna (EK 14–187-1014), and competent authorities (BASG/AGES, Austrian Federal Office for Safety in Health Care/Austrian Agency for Health and Food Safety).
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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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