# Herpesvirus infections in immunocompromised patients : treatment, treatment failure and antiviral resistance

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# Persistence and antiviral resistance of VZV in hematological patients

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## ABSTRACT

*Background:* Varicella-zoster virus (VZV) infections are a relevant cause of morbidity and mortality in hematological patients and especially in hematopoietic stem cell transplant (HSCT) recipients. The present study aimed to investigate the prevalence and clinical significance of viral persistence and antiviral resistance by systematically analyzing all episodes of VZV diagnosed in our laboratory in pediatric and adult hematological patients between 2007 and 2010.

*Methods:* Patient charts were reviewed to document patient and disease characteristics. VZV loads were determined in all available clinical samples from the day of diagnosis and thereafter. Persistent VZV infection was defined as a VZV infection that lasted at least seven days. Analysis of resistance was performed in all patients with persistent VZV infection by sequence analysis of viral thymidine kinase and DNA polymerase genes.

*Results:* In total 89 episodes occurred in 87 patients of whom 65 were recipients of an allogeneic HSCT. Follow up samples were available in 54 episodes. Persistent VZV was demonstrated in 32 of these episodes (59%). Complications occurred in 16 of the persistent episodes (50%) versus 2 of 22 non-persistent episodes (9%). Mutations possibly associated with resistance were found in 27% of patients with persistent VZV, including patients with treatment unresponsive dermatomal zoster that progressed to severe retinal or cerebral infection.

*Conclusions:* In hematological patients, VZV related complications occur frequently, especially in persistent infections. Antiviral resistance is a relevant factor in persistent infections and needs to be investigated in various affected body sites, especially when clinical suspicion of treatment failure arises.

## INTRODUCTION

Varicella-zoster virus (VZV) reactivations can be dermatomal but also disseminated in severely immunodeficient patients.[1] After hematopoietic stem cell transplantation (HSCT) visceral, retinal and neurological VZV infections can occur and result in serious morbidity and mortality.[1-8] Most VZV reactivations respond to treatment with aciclovir (ACV), valaciclovir (vACV) or related antiviral agents,[3;9] but persistent and progressive infections can occur despite treatment.[5;10] This can be due to immunological failure to control viral replication[6;11-13] or due to insufficient drug levels, but resistance of the virus to the antiviral treatment has been described as well.[14-20]

Resistance of VZV to antiviral drugs has not been reported in immune competent patients with primary VZV infections or herpes zoster,[21;22] but it has been demonstrated in AIDS-patients, hemato-oncological patients and HSCT recipients with treatment unresponsive VZV reactivations.[14-20] The prevalence of antiviral drug resistance and its relative contribution to persistent VZV infections in hemato-oncological patients and HSCT recipients is unknown because only case reports and case series have been published thus far. In addition, it is unclear which sample type should be analyzed to determine resistance as compartmentalization of resistant strains has been described.[15] Systematic analysis of the occurrence and localization of resistant VZV in immunocompromised patients with persistent VZV can guide treatment and diagnosis of VZV resistance in this patient group.

The aim of this study was to determine the prevalence and clinical significance of persistent VZV infections and the contribution of antiviral resistance to persistence in hemato-oncological patients, including HSCT recipients.

## PATIENTS AND METHODS

***Patient data***

Patients attending the Leiden University Medical Center, a tertiary care and teaching hospital in the Netherlands, with hematological malignancies and HSCT recipients (adults and children) diagnosed with VZV (laboratory confirmed by PCR and/or culture) between 01-01-2007 and 01-01-2010 were identified from the laboratory information system. Patient charts were reviewed to document patient and disease characteristics. VZV related complications were classified as *recurrence* in case of reappearance

of skin lesions after initial regression and as *dissemination* in case of progression of skin lesions outside the initially affected (and adjacent) dermatomes or spread to visceral organs, the eye and/or the central nervous system (CNS).

***Antiviral treatment***

In our hospital, immunocompromised patients with a VZV infection are commonly treated with intravenous ACV for at least five days. Prophylaxis with (v)ACV was not routinely given. Individual antiviral treatment data were obtained from the hospital pharmacy database and from patient charts.

***VZV load determination***

Sampling frequencies for follow up had been left to the discretion of the treating physician. The original samples used for VZV diagnosis were retested for confirmation. VZV loads were additionally determined in all available clinical samples from the day of diagnosis and thereafter until two consecutive VZV negative samples were found. Also EDTA plasma samples sent to the laboratory for other diagnostics than VZV were included. Samples included swabs from skin lesions, plasma, serum, cerebrospinal fluid (CSF), aqueous humor and bronchoalveolar lavage (BAL) samples. Persistent VZV infection was defined as a VZV infection lasting at least seven days.

DNA was isolated with the MagNA Pure LC Total Nucleic Acid Isolation Kit-High Performance using a MagNA Pure LC Instrument (Roche Diagnostics, Almere, The Netherlands). A multiplex real-time PCR for VZV and Phocine Herpesvirus (PhHV) as internal control for DNA extraction and PCR inhibition was performed on a CFX96 real-time detection system (Bio-Rad, Veenendaal, The Netherlands) as previously described.[9;23] For quantitation, a standard of VZV (cultured field isolate or ATCC KOS strain) was calibrated using a quantitated DNA control (Advanced Biotechnologies Inc., Columbia, MD, USA).

***Analysis of resistance***

Analysis of resistance was performed in all patients with a persistent VZV infection. The first positive sample of each patient was analyzed as well as subsequent VZV positive samples. Resistance was analyzed in samples from different body sites, when available. Analysis was performed by cycle sequencing after PCR amplification of the entire thymidine kinase (TK) gene. The detection limit of the assay was 2000 copies/ml. In case of possible resistance-associated TK mutations or in patients treated with foscarnet (FOS) or cidofovir (CDV), part of the DNA polymerase (POL) gene containing most previously described resistance conferring mutations was sequenced as well.[24;25]

Amplification and sequencing primers and PCR conditions are shown in Table 1.

*Table 1. Primers and PCR conditions.*

| target | orientation | sequence (5′→3′) | position[a] | amplicon size (nt)[b] | PCR protocol |
|---|---|---|---|---|---|
| TK gene amplification | | CCGTCCCAGAAGATAACC | -43 | 1128 | 15′ 95°C 50x: 30″ 95°C 30″ 60°C 1′ 72°C |
| | | CGCGAGTATGACAATGTGT | +59 | | |
| TK gene sequencing | | CCGTCCCAGAAGATAACC | -43 | | 1′ 96°C 25x: 10″ 96°C 5″ 50°C 4′ 60°C |
| | | CGGCGCTTCCTGGGTTA | 455 | | |
| | | CGCGAGTATGACAATGTGT | +59 | | |
| | | TGACTGGGGAGTGAAAC | 539 | | |
| TK gene codon 220 amplification and sequencing | | GCCGTTTGTTATGGTTCTGA | 588 | 190 | 15′ 95°C 50x: 30″ 95°C 30″ 55°C 1′ 72°C (amplification) 1′ 96°C 25x: 10″ 96°C 5″ 50°C 4′ 60°C (sequencing) |
| | | GGCGAATAACGTGTCTTCAA | 777 | | |
| POL gene amplification | Fragment 1 | AACGGTCTCATATCTCTGGA | 1495 | 533 | 15′ 95°C 50x: 30″ 95°C 30″ 60°C 1′ 72°C (amplification) |
| | | TCGATATAAAATCCCGTATCA | 2027 | | |
| | Fragment 2 | GAGATGGATGAAGACGAGAG | 1911 | 671 | 15′ 95°C 50x: 30″ 95°C 30″ 60°C 1′ 72°C (amplification) |
| | | ACACTAACACCCTTGAATCG | 2591 | | |
| POL gene sequencing | Fragment 1 | AACGGTCTCATATCTCTGGA | 1495 | | 1′ 96°C 25x: 10″ 96°C 5″ 50°C 4′ 60°C |
| | | TGCAAGGCTAGCTAGAATTA | 1746 | | |
| | | TAATTCTAGCTAGCCTTGCA | 1765 | | |
| | | TCGATATAAAATCCCGTATCA | 2027 | | |
| | Fragment 2 | GAGATGGATGAAGACGAGAG | 1911 | | |
| | | AGATGAAGCAGTGTTATTAG | 2283 | | |
| | | CTAATAACACTGCTTCATCT | 2302 | | |
| | | ACACTAACACCCTTGAATCG | 2591 | | |

[a] from startcodon in gene in sense orientation

[b] nt = nucleotide

Additional primers (Table 1) were used to confirm the deletion of codon 220 in patient 5 (Table 4). Amplification was performed in 50 $\mu$l containing 25 $\mu$l HotStart Taq mastermix (Qiagen, Hilden, Germany) and 15 pmol of each primer. All cyclesequencing reactions were performed on bulk amplification product in 20 $\mu$l containing 2 $\mu$l BigDye Terminator v1.1 (Applied Biosystems, Carlsbad, CA, USA), 6 $\mu$l sequencing buffer and 8 pmol primer. Sequence analysis was performed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Sequences were compared to the sequence of the Dumas strain (Genbank Accession Number NC_001348) and to pre-treatment samples of the patient.

## RESULTS

***Patients and episodes***

Characteristics of the 87 included patients are shown in Table 2. Of the 87 patients, 65 (75%) were recipients of an allogeneic HSCT in whom VZV episodes occurred at a median of 153 days after HSCT (range -23 days to +4.8 years). VZV occurred significantly earlier after HSCT in children than in adult HSCT recipients (median of 26.5 versus 44 days, $p<0.001$ Mann-Whitney test).

Characteristics of the 89 VZV episodes are shown in Table 3. One episode of chickenpox occurred in a seronegative patient, whereas all other episodes were herpes zoster in seropositive patients. One patient experienced 3 separate episodes of VZV with months to years between the episodes. Complications were documented in 21 episodes (24%, 95% confidence interval (CI) 15-32%) and consisted of recurrence, dissemination, retinitis, encephalitis, 2 other complications and 3 episodes during which patients died while having VZV in combination with other infectious and/or hematological problems. Antiviral treatment was administered in 61 of the 62 episodes (98%) where treatment was documented (Table 3).

***Virological data***

VZV was detected at the initial presentation in plasma samples in 16 episodes (18%), in both plasma and swabs in 24 episodes (27%), in swabs only in 47 episodes (53%), in plasma and BAL samples in 1 episode (1%) and in CSF in 1 episode (1%). The average viral load in swab samples at diagnosis was $2.6\times10^8$ copies/ml. Plasma samples were available at diagnosis in 49 episodes and were positive in 41 episodes (84%) with an average viral load of $2.8\times10^7$ copies/ml. Of the patients with a dermatomal zoster at presentation 69% had a positive plasma VZV DNA load, whereas all patients with a generalized or visceral zoster as their initial manifestation had VZV DNA positive plasma.

*Table 2. Patient characteristics*

| | | Total cohort | | Cohort with follow up samples | |
|---|---|---|---|---|---|
| | | **n** | **(%)** | **n** | **(%)** |
| age | Child (<18 years) | 31 | (36) | 20 | (38) |
| | Adult (≥ 18 years) | 56 | (64) | 33 | (62) |
| sex | male | 51 | (59) | 33 | (62) |
| | female | 36 | (41) | 20 | (38) |
| hematological disease | ALL[a] | 18 | (21) | 12 | (23) |
| | AML[b] | 25 | (29) | 17 | (32) |
| | CLL[c] | 3 | (3) | 2 | (4) |
| | CML[d] | 3 | (3) | 2 | (4) |
| | myelodysplastic syndrome | 4 | (5) | 4 | (8) |
| | Hodgkin lymphoma | 5 | (6) | 0 | (0) |
| | non-Hodgkin lymphoma | 3 | (3) | 1 | (2) |
| | multiple myeloma | 10 | (11) | 5 | (9) |
| | aplastic anemia | 4 | (5) | 1 | (2) |
| | thalassemia | 5 | (6) | 4 | (8) |
| | other | 7 | (8) | 5 | (9) |
| hematological treatment | chemotherapy | 15 | (17) | 4 | (8) |
| | autologous HSCT | 4 | (5) | 2 | (4) |
| | NMA[e] HSCT | 20 | (23) | 14 | (26) |
| | MA[f] HSCT | 45 | (52) | 33 | (62) |
| | other | 3 | (3) | 0 | (0) |
| HSCT donor type | haploidentical | 4 | (6) | 4 | (9) |
| | MSD[g] | 30 | (46) | 19 | (40) |
| | MUD[h] | 30 | (46) | 23 | (49) |
| | cord blood | 1 | (2) | 1 | (2) |
| **Total** | | **87** | | **53** | |

[a] ALL = acute lymphoblastic leukemia, [b] AML = acute myeloid leukemia, [c] CLL = chronic lymphocytic leukemia [d] CML = chronic myeloid leukemia, [e] NMA = non-myeloablative conditioning, [f] MA = myeloablative conditioning, [g] MSD = matched sibling donor, [h] MUD = matched unrelated donor

***Follow up***

On average 4.5 samples were available per episode. Follow up samples were available for at least one week after the diagnosis from 54 episodes in 53 patients. Characteristics of these patients and episodes are shown in Table 2 and 3. Due to the retrospective nature of the study, more samples for analysis and follow up were available from HSCT recipients compared to non-HSCT recipients (mean of 5.4 versus 1.8 samples, $p=0.003$, Student's t-test) and in episodes with complicated VZV compared to uncomplicated VZV (mean of 9.6 versus 2.9, $p<0.001$, Student's t-test).

*Table 3. VZV episodes.*

| | | Total cohort | | Cohort with follow up samples | |
|---|---|---|---|---|---|
| | | **n** | **%** | **n** | **%** |
| presentation | chickenpox | 1 | (1) | 0 | (0) |
| | dermatomal | 53 | (60) | 33 | (61) |
| | generalized cutaneous | 14 | (16) | 7 | (13) |
| | visceral/ eye/ CNS | 10 | (11) | 10 | (19) |
| | unknown | 11 | (12) | 4 | (7) |
| antiviral treatment | none | 1 | (1) | 0 | (0) |
| | vACV | 14 | (16) | 6 | (11) |
| | ACV | 13 | (15) | 9 | (17) |
| | ACV & vACV | 25 | (28) | 19 | (35) |
| | ACV & FOS | 2 | (2) | 2 | (4) |
| | ACV & vACV & FOS | 5 | (6) | 5 | (9) |
| | ACV & FOS & CDV | 2 | (2) | 2 | (4) |
| | unknown | 27 | (30) | 11 | (20) |
| complication | none | 68 | (76) | 36 | (67) |
| | recurrence | 9 | (10) | 9 | (17) |
| | dissemination | 3 | (3) | 3 | (6) |
| | retinitis | 3 | (3) | 3 | (6) |
| | encephalitis | 1 | (1) | 1 | (2) |
| | death | 3 | (3) | 1 | (2) |
| | other | 2 | (2) | 1 | (2) |
| **Total** | | **89** | | **54** | |

***Persistence***

Persistent VZV was demonstrated in 32 of 54 episodes with follow up (59%, CI 46-72%). Plasma samples were positive by PCR in 29 of the 32 persistent episodes and were not available for the remaining 3 episodes where only positive swabs were available. Additional sample types besides plasma were available and positive in 17 persistent episodes. Time since HSCT and conditioning regimen were not associated with occurrence of persistence. Persistence occurred in all VZV episodes in patients with a haploidentical donor (n=4) and in the only patient with a cord blood donor. Antiviral treatment was given in 26 episodes and was unknown in 6 episodes. Persistence occurred after cessation of antiviral treatment in 6 episodes (23%) and during antiviral treatment in 20 episodes (77%). The median duration of VZV positivity was 27.5 days (range 7-179 days) in the 32 persistent episodes. The peak VZV load in plasma was higher in persistent episodes than in non-persistent episodes (median load of $3.2\times10^4$ copies/ml versus $6.4\times10^3$ copies/ml, p=0.039, Mann-Whitney test).

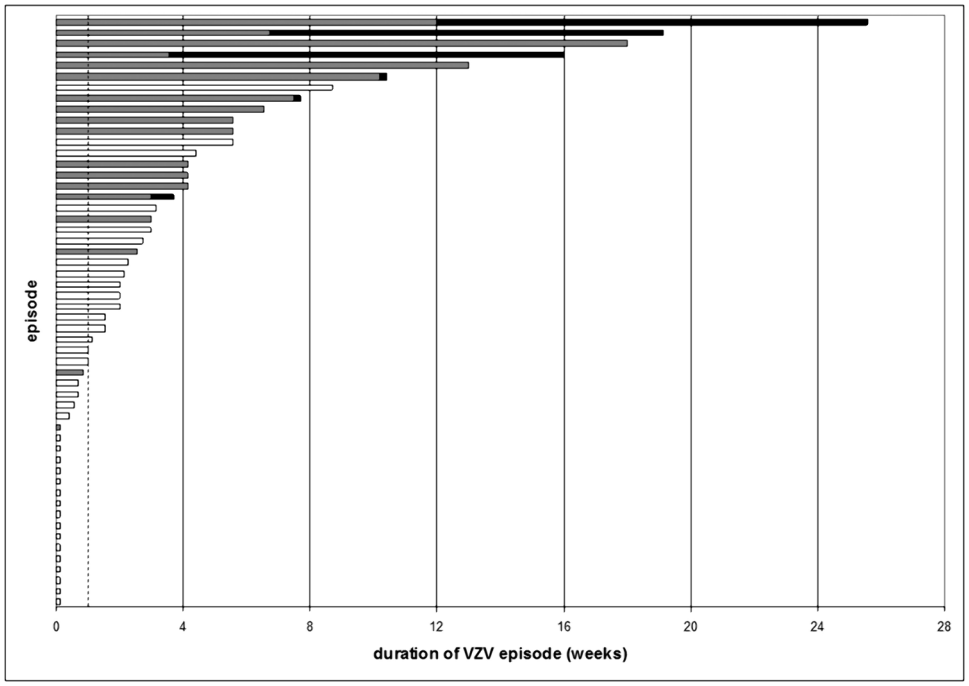
*Table 4. Patients with TK and POL mutations in VZV.*

| | patient | day since diagnosis | clinical & treatment details at time of sampling | mutations in TK gene | mutations in POL gene |
|---|---|---|---|---|---|
| 1 | Female, 52 years, CML, MA HSCT +403 days, MSD | 0 | pneumonia and generalized cutaneous VZV, ACV iv 15 days | none (plasma, swab, broncho alveolar lavage) | none |
| | | 25 | recurrent skin lesions after treatment cessation, ACV iv with good response | **A176G → E59G** (plasma, swab) | none |
| | | 51 | second recurrence under ACV iv treatment, FOS iv, recovery | N.D.[a] | N.D. |
| 2 | Female, 16 years, AML, MA HSCT +106 days, haploidentical donor | 0 | zoster of left arm, hand and shoulder, ACV iv and vACV | N.D. | N.D. |
| | | 18 | persistent zoster of the arm, vACV | none (plasma) | N.D. |
| | | 54 | progressive outer retinal necrosis, FOS iv, loss of vision in affected eye, recovery | **addC493 → stop 194** (aqueous humor)[b]<br>plasma N.D. | N.D. |
| 3 | Male, 9 years, erythropoietic protoporphyria, OLTx[c], MA HSCT +34 days, haploidentical donor | 0 | facial zoster, vACV | N.D. | N.D. |
| | | 84 | persistent zoster oticus, ACV iv | **delA76 → stop 38** (plasma)[d] | N.D. |
| | | 121 | zoster improved, hepatitis, follow up treatment with vACV | **delC493 → stop 171** (plasma day 121),<br>*C919T → stop 307* (plasma day 147),<br>*C205T → stop 69* (plasma day 154) | none,<br>none,<br>none |
| | | 161 | persistent facial zoster, progressive headache and loss of vision, ACV iv and FOS iv | **delA76 → stop 38** (plasma, CSF),<br>none (swab) | none |
| | | 179 | loss of vision in both eyes, death due to organ failure | **delA76 → stop 38** (CSF) | none |
| 4 | Male, 17 years, ALL, MA HSCT +28 days, cord blood donor | 0 | zoster n. ophtalmicus, ACV and vACV | none (swab) | none |
| | | 26 | persistent skin lesions, keratitis | none (swab) | none |
| | | 47 | progressive skin lesions, convulsion, encephalitis, ACV and FOS | *delAAC658 → del codon 220* (plasma day 47)<br>*delAAC658 → del codon 220* (CSF day 57)<br>none (plasma day 57) | N.D.,<br>none |
| | | 115 | convulsions, ACV and FOS, recovery | N.D. | N.D. |
| 5 | Female, 64 years, AML, NMA HSCT +262 days, MUD | 0 | abdominal pain, pneumonia, convulsion and generalized cutaneous VZV, ACV iv 10 days | none (plasma, swab) | none |
| | | 12 | recurrent skin lesions after treatment, ACV iv 10 days with rapid response | none (plasma day 12-17),<br>*T209C → T70I* (mixture, plasma day 21) | none,<br>none |
| | | 24 | follow up treatment with vACV, recovery | *C118T → P40S* (plasma day 24)<br>none (plasma day 26) | none |
| 6 | Female, 2 years thalassemia, MA HSCT -1 days, MSD | 0 | facial zoster, ACV iv | N.D. | N.D. |
| | | 15 | persistent facial zoster and dissemination to pubic region, ACV iv and FOS iv and CDV iv (concomitant adenovirus reactivation) | none (swab) | none |
| | | 73 | recurrent skin lesions, FOS iv and CDV topical, recovery | none (swab)<br>plasma N.D. | *G2066A → S689N*<br>plasma N.D. |

Resistance conferring mutations are depicted in bold, novel mutations in italics.

[a] N.D. = not determined, because of too low load for resistance analysis or no sample available , [b] add = addition, [c] OLTx = ortothopic liver transplantation, [d] del = deletion

*Figure 1. Occurrence of persistence, complications and resistance.*




Episodes with follow up samples at least 1 week after diagnosis are depicted (n= 54). The dotted line separates non-persistent (n = 22) from persistent VZV episodes (duration at least 1 week, n = 32). Complicated episodes (n = 18) are depicted by gray bars and episodes with resistant virus (n = 6) are shown in black.

Complications occurred in 16 of 32 persistent episodes (50%, CI 32-68%) versus 2 of 22 non-persistent episodes (9%, CI 0-21%, p=0.002, Chi-Square, Figure 1). In pediatric patients, complicated episodes occurred earlier after HSCT than uncomplicated episodes (median of 5 versus 33 days, p=0.025 Mann-Whitney test). Time after HSCT was not associated with the occurrence of complications in adult patients. Conditioning regimen was not associated with complications. Three of the four patients with a haploidentical donor and the only recipient of a cord blood transplant suffered from complications.

***Resistance***

Resistance analysis could be performed in 22 of the 32 persistent episodes, because in 10 episodes the VZV load was either insufficient to enable sequence analysis or samples were unavailable. Mutations developed during persistence in 6 of the 22 (27%, CI 8-46%) investigated episodes (Table 4, Figure 1). Two patients (patients 1 and 2) had mutations in VZV TK that had previously been associated with ACV resistance. In one

of the patients (patient 3) we found both previously characterized (premature stop codon 38 and 171) and novel mutations (premature stop codon 69 and 307, GenBank accession numbers JQ745671 and JQ745672). These mutations were alternately present in subsequent plasma and CSF samples. Two patients (patients 4 and 5) were found to have mutations that have not been described in association with resistance before. Patient 4 had a deletion of codon 220 in the VZV DNA present in plasma (GenBank accession number JQ745673) and in a subsequent CSF sample. Patient 5 had two different previously uncharacterized mutations (T70I and P40S, GenBank accession numbers JQ745669 and JQ745670) in two different samples, both of which are outside conserved or functional domains of VZV TK making their role as resistance associated mutation unclear. A DNA polymerase mutation of unknown significance (S689N, GenBank accession number JQ745674) was found in patient 6 after treatment with ACV, FOS and CDV. This mutation is outside functional domains of the viral DNA polymerase, but adjacent mutations at codon 684 [26] and 692 [27] have been associated with FOS resistance. No resistance associated mutations were found in the DNA polymerase gene in samples from the 5 patients with TK mutations.

Clinical details on the patients with mutations in VZV TK and POL are shown in Table 4. Two adult HSCT patients (patient 1 and 5) presented with recurrent skin lesions that responded well to retreatment with aciclovir. One pediatric patient (patient 6) presented with persistent cutaneous zoster that eventually recovered. In contrast, two pediatric patients (patient 2 and 3) with resistant VZV presented with treatment unresponsive dermatomal zoster that progressed to severe retinal infection with unfavorable outcome. One pediatric patient (patient 4) presented with persistent skin lesions and keratitis that progressed into encephalitis.

All 6 patients with mutations in VZV developed VZV related complications, versus 9 of 16 (56%, CI 31-81%) patients with persistent VZV without mutations ($p=0.12$, Fisher's Exact test). The median duration of VZV was 92.5 days (range 26-179 days) in patients with persistent VZV with mutations versus 29 days (range 7-126 days) in patients with persistent VZV without mutations ($p=0.015$, Mann-Whitney test).

## DISCUSSION

VZV infections are a relevant cause of morbidity and mortality in hemato-oncological patients.[3;28] Case reports on the sometimes severe and protracted course of infection in such patients have previously been published.[15;16;19;29] Our present study is among the first to report a systematical investigation of the prevalence and clinical significance of

viral persistence and antiviral resistance by analyzing all episodes of VZV diagnosed in our laboratory in hematological patients between 2007 and 2010. It was demonstrated that 24% of all episodes of VZV in this patient group were associated with complications such as recurrence, dissemination or severe organ manifestations. In patients from whom follow up samples were available, VZV was shown to be persistent in 59% of the episodes, despite the use of antiviral treatment in the majority of cases. Persistent episodes were associated with complications in 50% of the cases and possible resistant virus was identified in 27% of these cases.

Persistence of VZV DNA in whole blood or blood mononuclear cells after dermatomal zoster has been described as a common phenomenon lasting several months in immunocompetent individuals.[30;31] Persistence in our study was defined as the presence of a positive PCR of any relevant sample available at least 7 days after the diagnosis. Firstly, this was chosen in accordance with common antiviral treatment policies[10] that advocate a treatment course of about 7 days at the end of which the treating clinician has to decide whether or not to continue or change treatment if the patient has either clinical or virological signs of infection. Secondly, 7 days was chosen in order to include as many patients as possible in the resistance analysis. Persistence was found to occur in various body sites simultaneously in many episodes. As expected, the peak VZV load was associated with persistence, since the time to clear viral infection is most likely related to the viral load both of which are related to the immunosuppressive state of the patient.

Persistence was associated with complications in half of the cases. It appears that combined clinical and virological persistence may predict complications. Our findings are in accordance with the previous finding that the clinical course of VZV is correlated to the plasma load.[9] In pediatric patients, VZV infection early after HSCT was associated with a higher frequency of complications, which can be explained by the severe immunosuppressive state of the patient at that time. In adult patients, this relation was not found, possibly due to differences in transplantation protocols or in the occurrence of GvHD. Possibly, donor type is of importance as well with persistence and complications occurring frequently in recipients of a haploidentical or cord blood transplant, all of whom were children.

In 27% of the episodes of persistent VZV, possible resistance associated mutations were detected suggesting potential antiviral resistance. Resistance to antivirals is the result of spontaneously occurring mutations during viral replication, especially when replication levels are high due to the absence of adequate antiviral immunity. Upon selection

pressure by the administration of an antiviral agent, particularly during prolonged therapy and when there is no complete inhibition of viral replication, as in sites with poor penetration of the drug, such as the cerebrospinal fluid or the eye, a resistant mutant subpopulation may become dominant. In our study, resistance was found at various times after diagnosis and also in various sites including CNS and eye.[15] Interestingly, patient 3 even had four different mutations that were never detected simultaneously under continuous treatment with vACV and ACV. Possibly, a mixture of mutant viruses was present of which the relative amounts varied over time. Additional sequencing and real-time PCR with specific probes failed to identify various mutant viruses in a single sample, probably because of the relatively low load of the variants in the samples (data not shown).

Although antiviral resistance mutations occurred at a rather high frequency, clinical treatment failure cannot always be explained by antiviral resistance, as lack of antiviral immunity or insufficient dosing of antivirals may play a role as well.[18;19] These factors emphasize the need for timely and comprehensive diagnostics in complicated and persistent cases of VZV in immunocompromised patients. As the majority of VZV was detected in samples from which VZV cannot be cultured,[35] nucleotide sequence analysis of the genes most involved in antiviral resistance[15;16;18;19;36;37] was chosen to diagnose resistance. Several new mutations were found, that might be clinically relevant because they appeared under antiviral therapy. However, due to the lack of viral isolates, we could not confirm their actual contribution to resistance, which remains to be established. Comparing sequences between pre-treatment and on-treatment samples from a patient can partly overcome this problem. Marker transfer studies may be another approach, but are not routinely available in most laboratories.[37]

Some limitations apply to our study. Firstly, due to the retrospective nature of the study more samples were probably available from patients with clinically persistent or complicated infection in whom the treating clinician found an indication for diagnostics and for follow up. Therefore, both attrition and selection bias towards more persistence and more complications in the part of our cohort that had sufficient follow up samples is likely, despite the fact that about 45% of the included samples consisted of plasma samples submitted for other diagnostic requests. For accurate estimation of the risks and interrelations of complications, persistence and resistance, a prospective study including all VZV episodes in hematological patients is required. Secondly, due to limitations in the sensitivity of sequence analysis in comparison to the diagnostic assay, resistance could not be determined in patients with low VZV loads and thus the potential role in

low-level persistence could not be established. Also, the VZV DNA extracted from CSF and eye samples was often difficult to amplify. This may be related to low viral loads in some of these samples, but may also be due to the presence of fragmented viral DNA.[38]

Previous studies have demonstrated the efficacy of long term (v)ACV prophylaxis in preventing VZV after HSCT.[32-34] Many of the patients in our cohort were HSCT recipients and VZV was found to occur at a broad range of time points after HSCT. It is likely that prophylaxis can prevent VZV related complications in periods of maximum immunodeficiency after HSCT. However, the optimal timing and dosage remain to be established.

In conclusion, in hematological patients, VZV related complications occur frequently, especially in persistent infections. Antiviral resistance is a relevant factor in persistence and needs to be investigated timely and in samples from various body sites as soon as clinical or virological suspicion of persistence or other complications arises.

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## POTENTIAL CONFLICTS OF INTEREST

All authors: No reported conflicts.
All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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