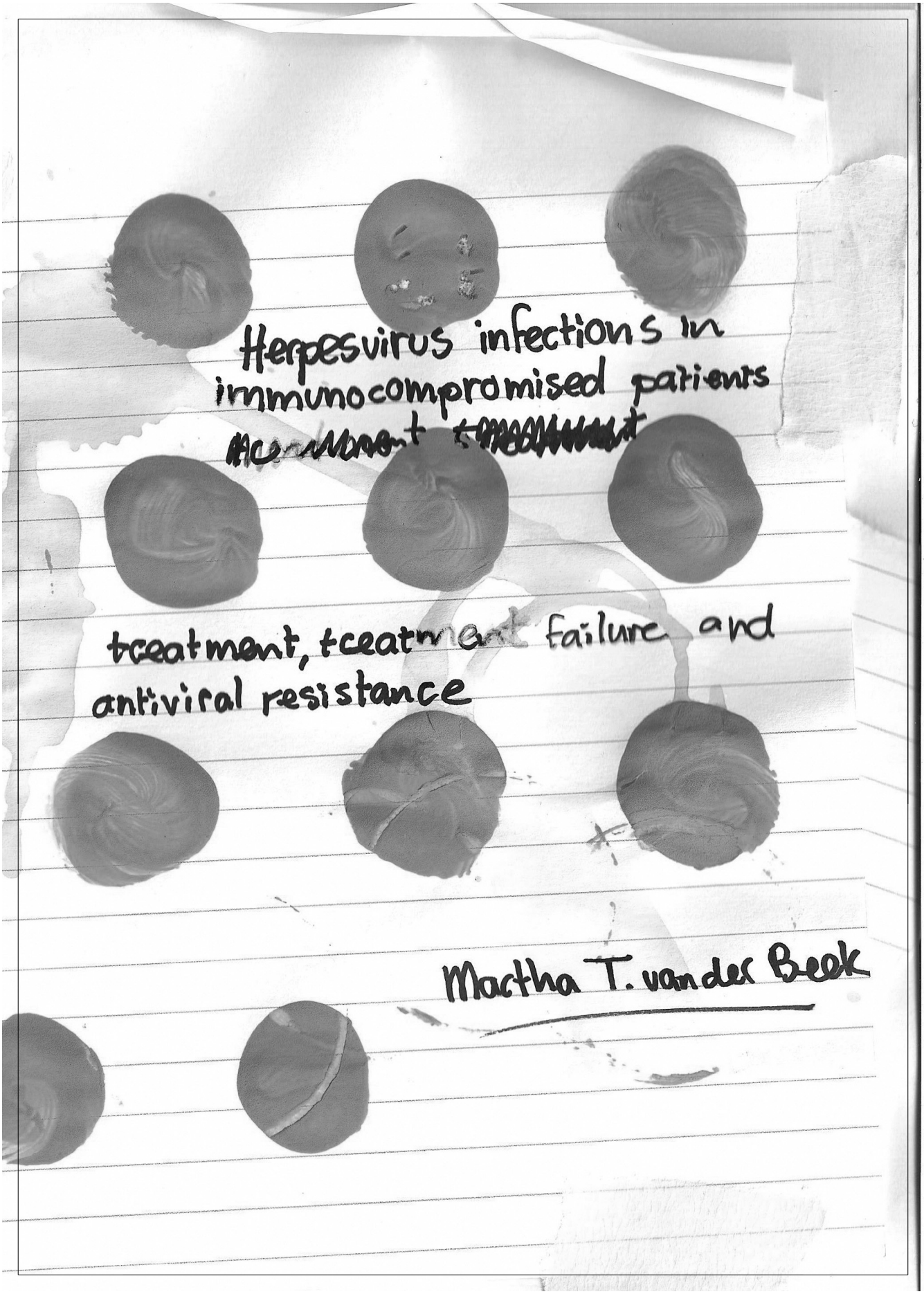

Herpesvirus infections in immunocompromised patients

treatment, treatment failure and antiviral resistance

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Herpesvirus infections in immunocompromised patients: treatment, treatment failure and antiviral resistance
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"Everything you do in life will be insignificant, but it is very important that you do it."

Mohandas Karamchand Gandhi, 1869-1948

Contents

1

Introduction

CONTENTS

HERPESVIRUS INFECTIONS IN IMMUNOCOMPROMISED PATIENTS; GENERAL ASPECTS

Background

The family of Herpesviridae contains hundreds of herpesviruses of which eight naturally infect humans (Table 1).[1] Herpes simplex virus type 1 (HSV-1), varicella-zoster virus (VZV) and cytomegalovirus (CMV) are the human herpesviruses that are investigated in this thesis. All are double-stranded DNA viruses with a lipid envelope.[1] Infections with these herpesviruses are among the most commonly encountered viral infections, occurring in virtually any indivdual.[2]

Table 1. Human herpesviruses.

Virus	Name	Abbreviation
Human herpes virus 1	Herpes simplex virus type 1	HSV-1
Human herpes virus 2	Herpes simplex virus type 2	HSV-2
Human herpes virus 3	Varicella-zoster virus	VZV
Human herpes virus 4	Epstein-Barr virus	EBV
Human herpes virus 5	Cytomegalovirus	CMV
Human herpes virus 6	Human herpes virus 6	HHV-6
Human herpes virus 7	Human herpes virus 7	HHV-7
Human herpes virus 8	Kaposi's sarcoma-associated herpes virus	HHV-8

Herpesviruses are extremely well adapted to their host as they establish widespread lifelong latent infection in humans while causing only low morbidity in healthy individuals. At the first encounter with a herpesvirus, primary infection occurs in which active viral replication leads to death of the infected cells and to the production of infectious progeny virus, so called lytic infection.[1] Primary infection can be either asymptomatic or symptomatic. Examples of common symptomatic primary herpesvirus infections are infectious mononucleosis due to Epstein-Barr virus (EBV) or CMV[3] and chickenpox due to VZV.[4]

After this phase of lytic viral replication, a stage of merely inactive infection occurs which is called latent infection.[1] Latency persists throughout life and is controlled by antiviral immune responses; at times of diminished immunity reactivation towards lytic viral infection can occur.[5,6] Waning antiviral immunity can be seen as an age-dependent phenomenon in otherwise healthy persons and lead to the common, usually mild reactivations of herpesviruses.[1;2;7-11] Because reactivation causes viral shedding at mucosal surfaces it contributes to viral spread between individuals.[2] As such, the latent infection contributes to the high prevalence of herpesvirus infections. Furthermore, viral reac-

tivation may cause symptomatic disease. Common and well known manifestations of herpesvirus reactivation are, for example, herpes labialis due to HSV-1[12] and shingles due to VZV. [4]

Infections in immunocompromised patients

Both primary infections and reactivations from latency are more frequent and more severe in immunocompromised individuals. The more frequent occurrence of primary infections is mostly a feature of infections in transplant recipients and is due to the risk of transmission of herpesviruses by transplantation of cells, tissues or organs latently infected with herpesviruses. This mode of transmission puts transplant recipients at risk of acquisition of a viral infection at a time when they are maximally immunosuppressed.[13]

The more frequent and more severe reactivations in patients with acquired immunodeficiencies occur because immunity against herpesviruses can be severely impaired.[13] For example patients with hematological malignancies receiving chemotherapy and especially hematopoietic stem cell transplant (HSCT) recipients have a temporarily suppressed or eradicated bone marrow function and thus no production of immune cells of any kind.[13] Recipients of a solid organ transplant receive immunosuppressive medication that suppresses mainly T-cell function, although in the induction phase shortly after transplantation and in case of rejection they also receive broader (including B-cell) immunosuppressants.[10;11] Severe herpesvirus reactivations in such patients may cause systemic symptoms, such as fever due to CMV,[14] or may cause organ manifestations, such as VZV retinitis,[4] or malignancies (EBV-related lymphoma).[15]

Asymptomatic herpesvirus reactivation is commonly indicated as herpesvirus infection, whereas a symptomatic reactivation is called herpesvirus disease. Not all immunosuppressed patients develop severe or protracted herpesvirus infection or disease. Various factors may contribute to the successful prevention or control of herpesvirus reactivations, including antiviral immunity[16-27] and antiviral treatment[28-34].

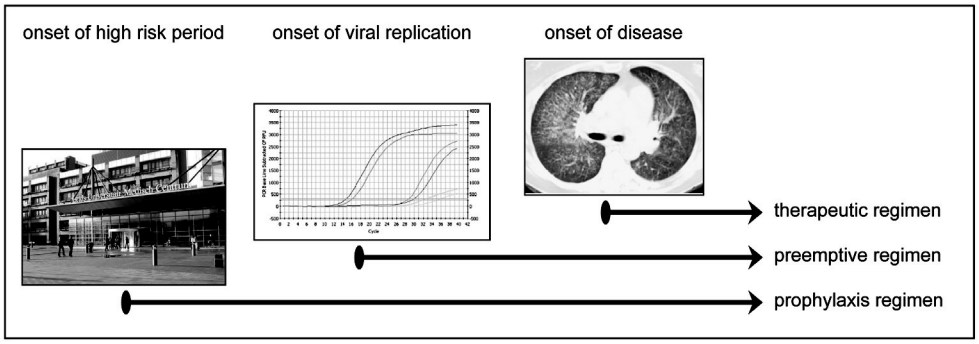
Treatment and treatment failure

Different antivirals can be used to treat infections with HSV-1, VZV and CMV (Table 2). Various strategies for preventing disease due to herpesvirus infections in immunosuppressed patients have been developed. Both prophylaxis, preemptive and symptomatic treatment are applied. In prophylactic regimens antivirals are administered from the time a patient is at risk of infection, whereas in preemptive regimens antivirals are initiated when viral infection is diagnosed before symptomatic infection has occurred (Figure 1). Symptomatic treatment is initiated when viral infection becomes clinically manifest (Figure 1).

Table 2. Antiviral agents used for the treatment of infections with HSV-1, CMV and VZV.

Agent	Abbreviation	Route of administration	Active against
Aciclovir	ACV	Intravenous/ oral	HSV, VZV
Valaciclovir	vACV	Oral	HSV, VZV
Ganciclovir	GCV	Intravenous/ oral	CMV (HSV, VZV)
Valganciclovir	vGCV	Oral	CMV (HSV, VZV)
Foscarnet	FOS	Intravenous	HSV, VZV, CMV
Cidofovir	CDV	Intravenous/ topical	HSV, VZV, CMV

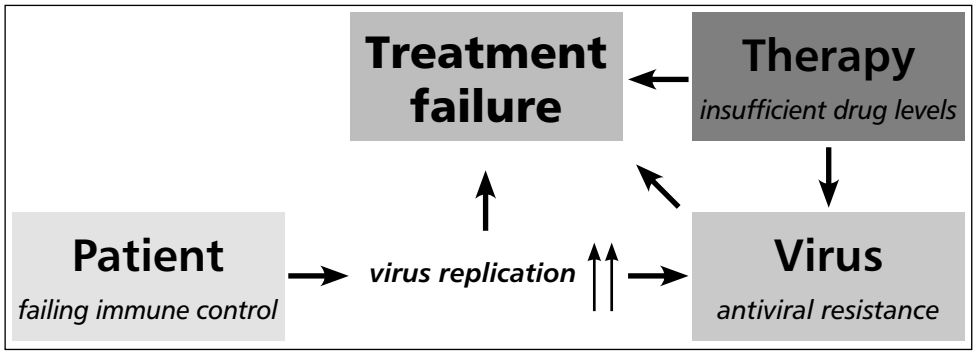
Figure 1. Treatment strategies for herpesvirus infections.



All three strategies can be effective at prevention of morbidity and mortality, but all have their disadvantages as well. Prophylaxis has a high number needed to treat and high costs of medication, while preemptive treatment has the requirement and the costs of regular diagnostic monitoring. Symptomatic treatment does not prevent disease but can only reduce the duration and severity of symptoms and is usually considered inferior and risky. The optimal approach for the various herpesvirus infections in different patient categories at risk has not been established completely.[30;35-37]

Treatment of herpesvirus infections with antiviral medication aims at reduction of viral replication and hence limitation of symptoms of infection. Eventually, only antiviral immunity can cause a return to asymptomatic latent infection. Antivirals merely suppress viral replication awaiting restoration of antiviral immunity. Continuing viral replication despite antiviral medication is considered virological failure of treatment. This may be accompanied by persistent or progressive symptoms, which is considered clinical treatment failure.

Figure 2 Causes of treatment failure of herpesvirus infections.



Treatment failure, either virological or clinical can have various causes (Figure 2). Firstly, it may be due to a profound state of immunodeficiency in which the patient's immune system is unable to control viral replication to any extent. Secondly, inadequate dosing or impaired drug absorption of antivirals can play a role. Lastly, resistance of the virus to antivirals can cause failure of treatment. These factors are interrelated; resistant virus that is less fit may only survive in an immunocompromised host and high levels of viral replication due to immunodeficiency increase the chance of viral resistance (see below).

Antiviral resistance

Resistance of herpesviruses to antivirals is the result of spontaneously occurring mutations during viral replication (Figure 3a). The proportion of spontaneous mutants in a viral population depends on the error rate of the viral DNA polymerase and on the site of the mutations; mutations in viral enzymes that are crucial to viral replication are mostly 'lethal' to the virus and such mutants will disappear from the viral population.[38-41] In the absence of adequate antiviral immunity, viral replication levels are high which increases the chance of a resistance associated mutation occurring. Upon selection pressure due to the administration of an antiviral agent, a resistant mutant subpopulation may become dominant over the wildtype susceptible population (Figure 3b). This is more likely to occur during prolonged therapy and when there is no complete inhibition of viral replication, for example due to low levels of the antiviral drug at the site of replication.[38;42] The latter can be due to incorrect drug dosing, impaired drug absorption or poor penetration of the drug in so called sanctuary sites, such as the cerebrospinal fluid or the eye.

Figure 3a. Illustration of development of resistance in uncomplicated herpesvirus infection.

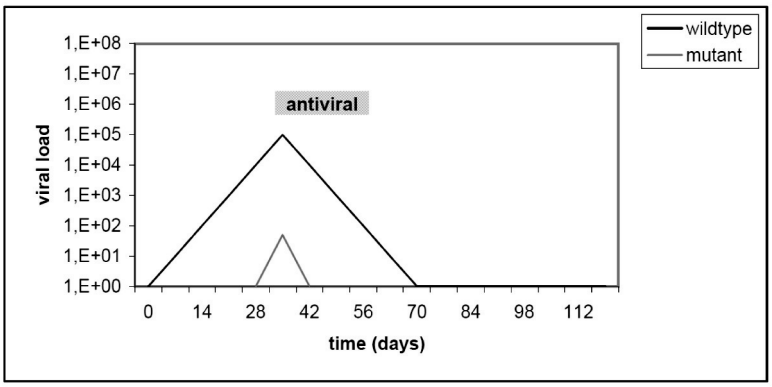
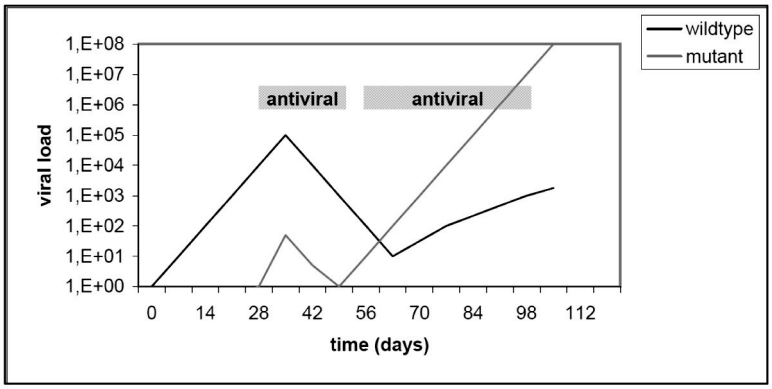


Figure 3b. Illustration of development of resistance in complicated herpesvirus infection.



Spontaneous resistant mutants appear during treatment and disappear after cessation of treatment and after clearance of the infection by the immune system in uncomplicated infections (3a). When immune restoration does not occur and persistent infection with high viral loads necessitates prolonged treatment, the resistant subpopulation may become dominant (3b).

Resistance to antivirals can be detected by culturing a viral isolate in the presence of antivirals and measuring the concentration of antiviral that inhibits viral replication in a so called plaque reduction assay. The success of this phenotypical approach depends on the ability to obtain a viral isolate, which can be difficult for some viruses and body sites (e.g. VZV and CMV in plasma or cerebrospinal fluid). Culture based assays may select for the best replicating viral subpopulation which can lead to false-susceptible results. Furthermore, this type of assay is time consuming and labor intensive. An alternative approach is to detect resistance-associated mutations in the viral genome in a clinical sample by molecular techniques. This approach avoids the need for culture and is fast

and technically easy to perform. However, its applicability depends on the knowledge of the significance of mutations. If this knowledge is not complete or if there is a frequent occurrence of polymorphisms, phenotypical confirmation remains necessary.

HERPES SIMPLEX VIRUS TYPE 1

Background

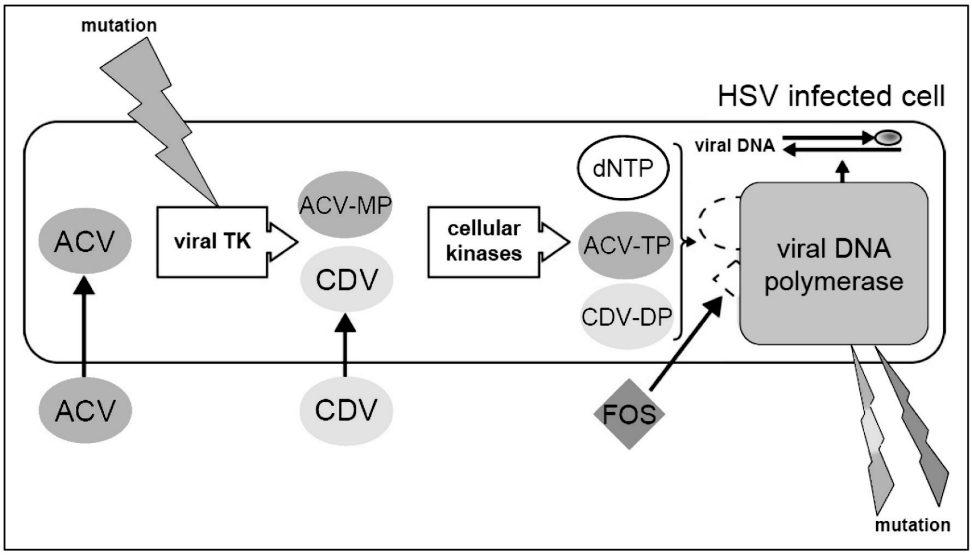
Primary HSV-1 infection occurs by inoculation of susceptible (usually oral) mucosal surfaces or minor skin lesions through direct contact.[43] After viral replication at the inoculation site, the virus traverses the neuroepithelial gap and is transported to a ganglion, most often the trigeminal ganglia in case of HSV-1.[6;12] Latent infection is maintained for life in the infected ganglia. The seroprevalence of HSV-1 in adults depends on geographic area and socioeconomic class and is on average between 50% and 85%.[44-48]

The mechanisms of HSV-1 reactivation are not well understood, but immune control by T-cells appears to play a pivotal role in the prevention of reactivation.[6;12;43;49] The rate of reactivation may be influenced by the site of infection,[50] local (micro)trauma,[51;52] exposure to UV light[53-56] and, possibly, hormonal factors[54;57-59] and psychosocial stressors.[60;61] Upon reactivation, viral replication is reinitiated and HSV-1 travels back along the nerves to the skin or mucosa which leads to local shedding of infectious virus.[43] Primary infection can be asymptomatic, but can also lead to ulcerative stomatitis.[62] Reactivation is most often asymptomatic, but can also lead to painful blistering or ulceration of the affected skin or mucosa.[2;12;43]

Infections in immunocompromised patients

The most common manifestation of HSV-1 reactivation in immunocompromised patients, such as HSCT recipients is (peri)oral ulceration.[13;63] Oral herpetic lesions can cause severe pain and difficulties with eating and drinking.[43] However, chemotherapy with or without total body irradiation can lead to ulcerative oral mucositis as well.[64-68] Chemoraditation usually causes ulcerations of the non-keratinized oral mucosa, whereas HSV-1 infection usually affects the keratinzed oral mucosa.. However, HSV-1 infection may also occur at sites of chemoradiation induced mucositis or aggravate this.[69] Therefore, in clinical practice it can be difficult to distinguish different causes of ulcerations. In addition, a possible role for other Herpesviruses such as CMV[70] and EBV[71] in oral ulceration has been suggested. Knowledge on the relative contribution of chemoradiation and herpesvirus infection to oral ulceration after HSCT is relevant to guide prevention and treatment strategies.

Figure 4. Mechanisms of HSV resistance to antivirals.



Aciclovir (ACV) is activated through phosphorylation by firstly a viral thymidine kinase (TK) and seondly cellular kinases. The other antiviral agents, cidofovir (CDV) and foscarnet (FOS) do not depend on phosphorylation by viral enzymes. After phosphorylation all antivirals inhibit viral replication by the viral DNA polymerase. Resistance associated mutations can occur in the viral TK gene (ACV resistance) or in the viral DNA polyermase gene (ACV, FOS and CDV resistance). Picture adapted from: Gilbert C, Boivin G. Human cytomegalovirus resistance to antiviral drugs. Antimicrob Agents Chemother. 2005, 49(3): 873-83.

Treatment and treatment failure

Oral ulcerations due to HSV-1 in immunocompromised patients are commonly treated with aciclovir (ACV) or its oral prodrug valaciclovir (vACV).[35;72] vACV and ACV have identical working mechanisms. ACV is a deoxyguanosine-analogue which is built into the viral DNA by the viral DNA-polymerase during replication and then inhibits viral replication (Figure 4).[73] Aciclovir only becomes active after phosphorylation by a viral thymidine kinase (TK) and two subsequent phospohrylation steps by cellular kinases (Figure 4).[74] Second line antivirals are TK independent viral DNA polymerase inhibitors (Figure 4), foscarnet[75;76] (FOS) and cidofovir[77-79] (CDV). However, both drugs are nephrotoxic.

Especially in immunocompromised patients debilitating and prolonged HSV reactivations can occur despite antiviral treatment.[13] The relative contribution of antiviral resistance to persistent HSV infections is unknown.

Antiviral resistance

The prevalence of ACV resistance has been shown to be very low in immunocompetent subjects (<1%), whereas in immunocompromised patients with HSV-1 infections, resis-

tance levels up to 27% have been described.[80-82] Investigating antiviral susceptibility of HSV-1 to antivirals can be done using various techniques. Sequence analysis of a viral isolate is the fastest approach. Resistance to ACV in HSV-1 is mostly caused by mutations in the UL23 gene of the viral TK or in the UL30 gene of the viral DNA polymerase (Figure 4).[83-85] Sequencing of these genes may reveal a resistance conferring mutation, but since nucleotide variations are common, mutations of unknown significance are also found frequently.[83-85] In such cases, phenotypical susceptibility testing of HSV-1 is required which is traditionally performed by a plaque reduction assay.[86] This type of assay is labor intensive and time consuming and, hence, results are often not available in a clinically relevant time frame. Real-time pcr based phenotypical susceptibility assays may overcome these limitations and facilitate timely diagnosis of antiviral resistance.[87;88]

VARICELLA-ZOSTER VIRUS

Background

Primary VZV infection occurs by aerosol transmission or through direct contact.[89] After viral replication in the respiratory epithelium, VZV infects T-cells in Waldeyer's ring and then viremia distributes the virus throughout the body via infected T-cells.[90;91] There is uncertainty whether skin involvement occurs only after a second viremia or results directly from the first viremia.[89-93] The seroprevalance of VZV appears to be climate dependent and reaches 95% during childhood in temperate climates but only 50% in tropical areas yet increases thereafter.[94-96]

Lifelong latent infection is established in dorsal root ganglia and cellular immunodefiency predisposes to reactivation.[89] Upon reactivation, viral replication is reinitiated and VZV travels back along the nerve to the skin of the corresponding dermatome.[89] Both primary infection and reactivation are usually symptomatic; primary infection cuases the clinical picture of chickenpox with disseminated itching vesicles, whereas reactivation causes the clinical picture of herpes zoster or shingles with a dermatomal painfull vesicular eruption.[93] In addition, cutaneous VZV reactivations can lead to postherpetic neuralgia with long-lasting and severe morbidity.[89]

Infections in immunocompromised patients

The most common manifestation of VZV reactivation in immunocompromised patients is herpes zoster; this can be dermatomal but is often disseminated in severely immunodeficient patients.[63] VZV infection occurs in 30-40% of HSCT recipients in the first year after transplantation.[34;97] Visceral, retinal en neurological infections can occur in this

patient category and cause serious morbidity and mortality.[23;63;89;93;98-101] Visceral dissemination occurs especially in patients with graft-versus-host disease.[97]

Treatment and treatment failure

To prevent dissemination or other serious manifestations of VZV reactivation, treatment with antiviral agents is given to immunodeficient patients with clinical signs of herpes zoster. Most VZV reactivations respond to treatment with ACV or vACV or related antiviral agents [99;102], but both progressive and persistent infections despite treatment can occur in severely immunocompromised patients [101;103] This can be due to immunological failure and to insufficient drug levels, but resistance of the virus to the antiviral treatment has been described as well.[104-110]

Antiviral resistance

Resistant VZV has not been shown in immunecompetent patients with primary VZV infections or herpes zoster,[111;112] but it has been demonstrated in AIDS-patients with treatment unresponsive VZV reactivations.[104-107] The prevalence of antiviral resistance in hemato-oncological patients and HSCT recipients is unknown, with only some case reports and case series described thus far.[107-110]

Similar to HSV-1 (Figure 4), VZV resistance to (v)ACV is mainly due to mutations in the viral TK gene of VZV, or, in rare cases, in the viral DNA polymerase.[105;108-110;113;114] Resistance can be diagnosed by culture of the virus in the presence of antiviral agents, but culture-based techniques are difficult because VZV is a slowly growing and highly cell-associated virus.[115] Furthermore, VZV often cannot be cultured from clinical samples such a plasma or cerebrospinal fluid. Direct sequence analysis of the target genes in clinical samples is possible in various types of clinical samples and avoids selection by culture. However, it is not completely clear in which sample type to look for resistance as compartmentalization of resistant strains has been described.[108]

CYTOMEGALOVIRUS

Background

Primary CMV infection occurs through direct contact of susceptible mucosal surfaces with infectious body fluids such as saliva and urine, through sexual contact or perinatally, either in utero or from breast milk.[116-118] CMV has a very broad cell tropism including epithelial cells, endothelial cells and polymorphonuclear cells.[5;119] After a phase of viremia, CMV infects many cell types in the body, where subsequently latent infection

is established.[5;13;14;120] The latent presence in various tissues explains the transmission of CMV to recipients of blood transfusions or stem cell and organ transplants.[5;13;14;120]

The seroprevalence in adults varies with ethnicity and socioeconomic status from 40% up to 100% in developing countries.[44;94;121-123] Primary infection is mostly asymptomatic, but a mononucleosis syndrome can occur.[3] Reactivation from latency is known to occur but is asymptomatically in healthy individuals.[1;117;120]

Infections in immunocompromised patients

In immunocompromised individuals both primary infection and reactivation can affect virtually any organ system, with symptoms ranging from fever and malaise to pneumonitis and hepatitis.[14;120] Reactivation occurs more often and is more frequently symptomatic in immunocompromised persons.[1;117;120] Manifestations of CMV infection in immunocompromised patients vary with the underlying disease, the type of the immunodeficiency and the pre-existing CMV immunity. For example, AIDS-patients often suffered from CMV chorioretinitis in the era when effective anti-retroviral treatment was unavailable, whereas this manifestation is rare in transplant recipients.[124] HSCT recipients suffer mostly from CMV pneumonitis or colitis, whereas those organs are less commonly affected in solid organ transplant recipients.[124]

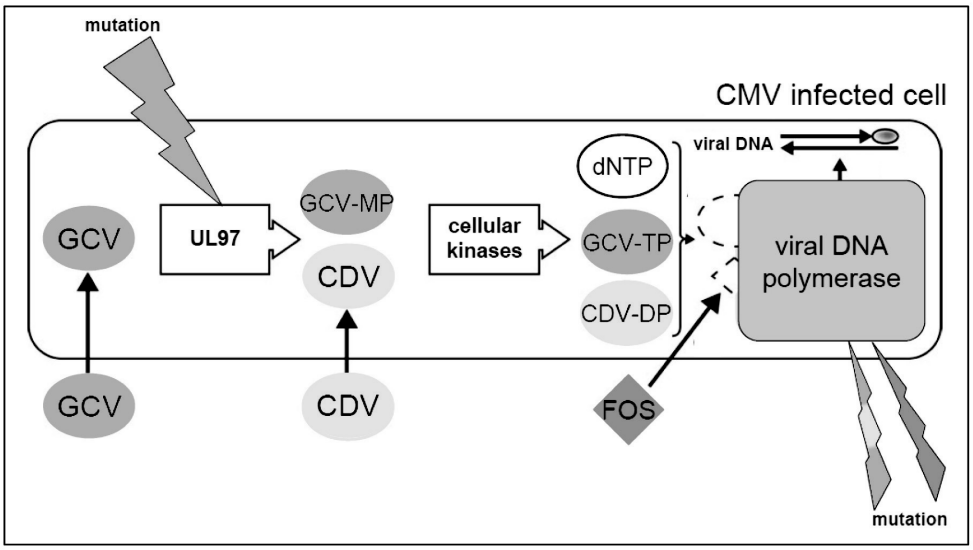
In CMV seronegative individuals, the receipt of a solid organ from a CMV seropositive donor transfers CMV to the recipient. This causes a primary infection at a time when induction immunosuppressive agents are administered and therefore has a high risk of symptomatic and severe disease.[125;126] In contrast, CMV seropositive recipients of a solid organ transplant already have pre-transplant immunity to CMV which decreases the risk of CMV reactivation and of a severe course of CMV infection.[125;126] In the setting of HSCT the situation is reversed. CMV seropositive recipients of an HSCT from a seronegative donor are at a greater risk of severe disease, because of the latently present CMV in the recipient who acquires the CMV-naïve immune system from the seronegative donor.[124;127]

Furthermore, because adaptive immunity is severely suppressed in organ transplant recipients in the early phase after transplantation, the innate immune system probably plays a pivotal role.[128-130] Common single nucleotide polymorphisms in the genes coding for members of e.g. the lectin complement pathway can have potentially important functional implications for the control of CMV infections.[128;131-133]

Treatment and treatment failure

CMV infections can be treated with ganciclovir (GCV)[30;36;37;134] or its oral prodrug valganciclovir (vGCV).[135;136] vGCV and GCV have identical working mechanisms. GCV is

Figure 5. Mechanisms of CMV resistance to antivirals.



Ganciclovir (GCV) is activated through phosphorylation by firstly a viral kinase (UL97) and seondly cellular kinases. The other antiviral agents, cidofovir (CDV) and foscarnet (FOS) do not depend on phosphorylation by viral enzymes. After phosphorylation all antivirals inhibit viral replication by the viral DNA polymerase. Resistance associated mutations can occur in the viral UL97 gene (GCV resistance) or in the viral DNA polyermase gene (GCV, FOS and CDV resistance). Picture adapted from: Gilbert C, Boivin G. Human cytomegalovirus resistance to antiviral drugs. Antimicrob Agents Chemother. 2005, 49(3): 873-83.

a deoxyguanosine-analogue which is built into the viral DNA by the viral DNA-polymerase during replication and then inhibits viral replication (Figure 5).[134;137-140] Ganciclovir only becomes active after phosphorylation by a viral kinase (UL97) and two subsequent phosphorylation steps by cellular kinases (Figure 5).[141;142] FOS[75;76] and CDV[77-79] directly target the viral DNA polymerase of CMV and can be used for treatment as well (Figure 5).

Because symptomatic CMV infection, especially CMV end organ disease, is associated with high morbidity and mortality, most treatment regimens aim at prevention of CMV disease. In high risk solid organ transplant recipients (seropositive donor, D+, seronegative recipient, R-) prophylaxis is often administered and few prospective comparisons with preemptive treatment have been performed.[37;143] For HSCT recipients a preemptive approach is usually preferred to minimize drug toxicity, especially myelosuppression. However, in a preemptive setting, viral DNA can often be detected for days to weeks during and after treatment.[144-147] The significance and optimal management of this finding is unclear.

Antiviral resistance

Only few earlier studies exist in which resistance has been systematically studied in HSCT recipients and none used sensitive CMV monitoring techniques such as real-time pcr.[148;149] Also, the contribution of antiviral resistance to viral persistence despite antiviral treatment is largely unknown. In renal transplant recipients varying rates of resistance have been described; it is unknown which preventive strategy encompasses the lowest risk of development of antiviral resistance. [150-154]

GCV resistance mutations in clinical isolates mainly map to the viral kinase gene UL97 (Figure 5).[154-158] After prolonged treatment, mutations in the viral polymerase gene UL54 can also emerge (Figure 5).[158;159] Sequencing analysis is the fastest method for susceptibility testing of CMV, which often cannot be cultured. Alternative molecular techniques for mutation detection have been studied to reduce hands on time and post-PCR processing,[160-162] but focused on fixed genome positions known to be involved in antiviral drug resistance and, hence, may have missed mutations at other sites. Compared to mutation detection by Sanger based sequencing techniques or by real-time pcr, mass-spectrometry based comparative sequence analysis combines the possibility of detection of all nucleotide variations within a target gene with reduced hands on time due to the automation of post-PCR processing and analysis.[163-165] Application of this technique may facilitate studying and diagnosing antiviral resistance in CMV infections.

SCOPE OF THIS THESIS

The research described in this thesis aims to study determinants of the course and outcome of treatment of herpesvirus infections in immunocompromised patients. Both viral factors, such as antiviral resistance, and patient factors, including immunological parameters, were investigated. Techniques to study antiviral resistance were optimized for use in a clinical diagnostic setting. The aim of this research is to improve and facilitate management of herpesvirus infections in immunocompromised patients.

In **chapter two** the development and validation of a real-time pcr based phenotypical technique to study susceptibility of HSV-1 to antiviral drugs in a routine diagnostic setting is described.

In **chapter three** the role of HSV-1, EBV and CMV in oral ulcerations in HSCT recipients is investigated. Also the course of the oral HSV-1 infections in this setting and the occurrence antiviral resistance are described.

In **chapter four** the course of VZV infections in hematological patients is studied including the role of antiviral resistance in persistent infections. Systematic analysis of the occurrence and localization of resistant VZV is described.

In **chapter five** the application of a novel technique using mass spectrometry-based comparative sequencing to detect ganciclovir resistance in CMV is addressed.

In **chapter six** determinants of the response to antiviral treatment of CMV infections in HSCT recipients are studied, including resistance to antivirals.

In **chapter seven** the response to treatment and the occurrence of antiviral resistance are compared between a preemptive and a sequential prophylactic-preemptive treatment regimen for CMV in D+R- renal transplant recipients.

In **chapter eight** the role of gene polymorphisms influencing components of the innate immunity, mannose-binding lectin and ficolin-2, on CMV infection after orthotopic liver transplantation (OLT) is investigated.

In the discussion, implications for management of herpesvirus infections in immunocompromised patients as well as suggestions for further research are described.

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2

Rapid susceptibility testing for Herpes Simplex Virus type 1 using real-time PCR

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ABSTRACT

Background: Susceptibility testing of herpes simplex virus type 1 (HSV-1) is traditionally performed by a plaque reduction assay (PRA), but this is labor intensive, time consuming and has a manual read out.
Objectives: The goal of this study was to develop an internally controlled real-time PCR-based phenotypical susceptibility test for HSV-1 that is suitable for use in a clinical diagnostic setting.
Study design: A DNA reduction assay (DRA) was developed and validated on a test panel of 26 well-characterized isolates of varying susceptibility to aciclovir or foscarnet, including low-level resistant isolates. The DRA consisted of pre-culture of a clinical sample for 48 hours and subsequent culture in the presence of antivirals for 24 hours. Viral DNA concentration in the culture lysates was measured by an internally controlled quantitative real-time HSV-1 PCR and corrected for cell count and lysis by beta-globin PCR. DRA results were compared to results from PRA and sequence analysis.
Results: DRA results were in accordance with PRA results for both aciclovir and foscarnet susceptibility and appeared to have good discriminative value for low-level resistance due to UL30 gene mutations. Although the direct application of DRA in clinical samples appeared not possible, short pre-culture of 48 hours was sufficient and ensured results within a clinically relevant time frame of 5 days.
Conclusions: DRA is an accurate, rapid and easy to perform phenotypical susceptibility test for HSV-1.

BACKGROUND

Severe and persistent infections with herpes simplex virus type 1 (HSV-1) are common in immunocompromised patients, especially patients receiving chemotherapy and hematopoietic stem cell transplants, and are frequently associated with antiviral resistance.[1] The fastest approach to HSV-1 susceptibility testing is sequence analysis of the UL23 gene of the HSV-1 thymidine kinase that catalyzes a necessary phosphorylation step of aciclovir or of the UL30 gene of the HSV-1 DNA polymerase. Sequencing of these genes may reveal a resistance conferring mutation, but since nucleotide variations are common, mutations of unknown significance are also found frequently.[1-4] In such cases, phenotypical susceptibility testing of HSV-1 is still required which is traditionally performed by a plaque reduction assay (PRA).[5] PRA requires viral titration and prolonged incubation until viral cytopathogenic effect (CPE) is visible and is labor intensive, subjective and time consuming.

Faster phenotypical assays using more sensitive and objective endpoints are preferable. Real-time PCR has previously been applied successfully to measure viral concentrations in HSV-1 phenotypical susceptibility tests.[6;7] Stranska et al[6] measured inhibition of viral DNA replication by antivirals in culture supernatant, which may be less indicative of intracellular viral replication. The protocol described by Thi et al[7] measured viral DNA in cells but used crude cell lysate in an uncontrolled PCR.

OBJECTIVES

The goal of this study was to design, optimize and validate a rapid internally controlled real-time PCR-based phenotypical susceptibility test for HSV-1 for routine use in a clinical diagnostic setting.

STUDY DESIGN

Viral isolates and clinical samples

For DNA reduction assay (DRA) validation using viral isolates (Table 1), susceptible reference strain HSV F (ATCC number VR-733 Manassas, VA, USA), and 25 previously characterized viral isolates from 14 patients that were clinically suspected of having a resistant virus and that had been sent for susceptibility testing to an external laboratory

Table 1. Features of previously characterized HSV-1 isolates used for DNA reduction assay validation.

patient/ source	isolate	clinical & treatment details	UL23 mutation	UL30 mutation
ATCC	HSV-F	susceptible reference strain	none	none
A	#1	SCT, mucositis, pre-treatment	none	none
	#11	mucositis, ACV iv 13 days	delG180 → fs61	none
	#12	ulcerative esophagitis, ACV iv 13 days & vACV 4 days	delG180 → fs61	none
	#13	ulcerative esophagitis, ACV iv 22 days & vACV 9 days	delG180 → fs61	none
B	#2	SCT, lip lesion, pre-treatment	none	none
	#21	severe facial an days oral lesions, ACV iv 52 days & vACV 32 days	G488A → subR163H	none
	#24	lip lesion, ACV iv 44 days	none	G2171A → subS724N
C	#3	SCT, mucositis, pre-treatment	none	none
	#22	persistent mucositis, ACV iv 13 days	C566T → subA189V	none
D	#4	SCT, mucositis, pre-treatment	none	none
	#14	persistent mucositis, ACV iv 5 days & GCV iv 12 days	insG430 → fs146	none
E	#5	SCT, mucositis, pre-treatment	none	none
	#23	persistent mucositis, vACV 8 days & ACV iv 7 days	insG430 → fs146	none
F	#10	SCT, encephalitis, ACV iv 15 days	T1033C → subS345P	G1684A → subA562T
G	#20	SCT, persistent mucositis, ACV iv 26 days	A314C → subH105P	none
H	#7	SCT, stomatitis, pre-treatment	none	none
	#8	persistent stomatitis, ACV iv 5 days	none	none
	#15	persistent stomatitis, ACV iv 12 days	insG430 → fs146	none
I	#16	SCT, treatment unknown	delG430 → fs146	none
J	#18	SCT, treatment unknown	insC548 → fs185	none
K	#19	SCT, treatment unknown	C310T → stop104	none
L	#25	SCT, treatment unknown	none	C2156T → subA719V
M	#6	chemotherapy, mucositis, vACV 5 days	none	none
	#17	persistent mucositis, vACV 12 days & ACV iv 10 days	delC460 → fs155	none
N	#9	immunocompetent, recurrent genital HSV-1 despite vACV prophylaxis	none	G1947T → subE649D

All samples were isolated from swab samples from mucous membranes or skin, except for sample 10 which was isolated from cerebrospinal fluid. Patient B[10], patients I-L[8] and patients A, C, D and H[9] were described in previous studies.

ACV = aciclovir; d = days; del = deletion; fs = frameshift; GCV = ganciclovir; ins = insertion; SCT = stem cell transplantation; sub = substitution; vACV = valaciclovir.

or that were described in previous studies were used.[8-10] The test panel included pre-treatment viral isolates if available. Aliquots of viral isolates were stored at -80°C. For DRA validation directly on clinical samples (swabs), the clinical samples from which isolates #5 and #17 had been cultured and 7 randomly selected HSV-1 positive clinical samples were used.

UL23 and UL 30 gene sequence analysis

Genotypical resistance analysis was performed by cycle sequencing after PCR amplification on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Amplification and sequencing primers and PCR conditions are shown in Table 2. UL23 amplification was performed in 50 μl containing 25 μl HotStart Taq mastermix (Qiagen, Hilden, Germany) and 15 pmol of each primer. For UL30 amplification, nested PCR was necessary for clinical samples, but not for viral isolates. PCR and nested PCR were performed in 50 μl containing 1 μl Advantage®-GC 2 Polymerase mix (Clontech, Westburg, Leusden, The Netherlands), 10 μl Advantage®-GC 2 PCR Buffer, 25 μmol GC-melt, 0.2 mM dNTP mix and 15 pmol of each primer. All cycle sequencing reactions were performed in 20 μl containing 2 μl Big Dye Terminator v1.1 (Applied Biosystems, Carlsbad, CA, USA), 6 μl sequencing buffer and 8 pmol primer. The detection limit of the assay was around 1000 copies/ml for the UL23 gene and around 5000 copies/ml for the UL30 gene. Sequences were compared to pre-treatment isolates if available and to the sequence of HSV F.

Plaque Reduction Assay

The protocol M33-A *Antiviral Susceptibility Testing: Herpes Simplex Virus by Plaque Reduction Assay* of the Clinical and Laboratory Standards Institute[5] using Vero cells was modified by including the use of microcrystalline cellulose (Avicel® RC/CL, FMC BioPolymer, Philadelphia, USA) as overlay.[11] For viral titration, the overlay consisted of 1.5 ml of 0.6% Avicel® RC/CL in sterile water mixed 1:1 with 2x EMEM without phenol red (Gibco) with 4% FCS and 4 mM glutamine. After incubation, cells were fixed with formalin and, after aspiration of the overlay, stained with crystal violet. For PRA, the overlay consisted of twofold serial dilutions of aciclovir (acycloguanosine, Sigma-Aldrich, Schnelldorf, Germany) or foscarnet (sodium phosphonoformate tribasic hexahydrate, Sigma-Aldrich) in 2x EMEM without phenol red with 4% FCS and 4 mM glutamine mixed 1:1 with 0.6% Avicel® RC/CL in sterile water. The aciclovir concentration range was 0,12 to 16 mg/L and for foscarnet the concentration range was 16,7 to 400 mg/L. Isolates having an IC_{50} value $\geq$ 2 mg/L for aciclovir or $\geq$ 100 mg/L for foscarnet were considered resistant.[5]

Table 2. Primers, probes and PCR conditions used for UL23 and UL30 gene sequence analysis and DNA reduction assay.

target	orientation	sequence (5′→3′)	position[a]	amplicon size (bp)	pcr protocol
UL23 gene HSV-1 amplification	sense	TCCACTTCGCATATTAAGGT	-146	1335	15′ 95°C 40x: 1′ 95°C 1′ 50°C 1′ 72°C 1x: 10′ 72°C
	antisense	CTGTCTTTTTATTGCCGTCA	+38		
UL23 gene HSV-1 sequencing	sense	GCTTAACAGCGTCAACAGC	-84		1′ 96°C 25x: 10″ 96°C 5″ 50°C 4′ 60°C
	sense	AGACAATCGCGAACATCTAC	284		
	antisense	CCGATATCTCACCCTGGTC	321		
	sense	ATACGGTGCGGTATCTGC	731		
	antisense	AAAGCTGTCCCCAATCCT	770		
	antisense	TGTCTTTTTATTGCCGTCAT	+37		
UL30 gene HSV-1 amplification	sense	GAGGACGAGCTGGCCTTTCCG	1126	1810	3′ 94°C 40x: 30″ 94°C 30″ 60°C 2′ 68°C 1x: 3′ 68°C
	antisense	AAAACAGCAGGTCGACCAGGGC	2914		
UL30 gene HSV-1 nested amplification	sense	GACCTCCCCGAATCCCA	1252	1652	3′ 94°C 30x: 30″ 94°C 30″ 60°C 2′ 68°C 1x: 3′ 68°C
	antisense	CGGTTGATAAACGCGCAGTTG	2883		
UL30 gene HSV-1 sequencing	sense	GAGGACGAGCTGGCCTTT	1126		1′ 96°C 25x: 10″ 96°C 5″ 50°C 4′ 60°C
	sense	GAGTACTGCATACAGGATTC	1726		
	antisense	TGGCCGTCGTAGATGGTG	1833		
	antisense	TGCTGCACTCCCGTGAAC	2457		
	antisense	AAAACAGCAGGTCGACCAG	3708		
	sense[b]	GACCTCCCCGAATCCCA	1252		
	antisense[b]	CGGTTGATAAACGCGCAGTTG	2883		
Glycoprotein B gene HSV-1 amplification	sense	TCAAGACCACCTCCTCCATC	1484-1503	130	15′ 94°C 50 x: 30″ 94°C 30″ 55°C 30″ 72°C
	antisense	AGGGTCAGCTCGTGATTCTG	1594-1613		
	probe	FAM AACATATCGTTGACATGG BHQ-1	1545-1562		
Glycoprotein B gene PhHV-1 amplification	sense	GGGCGAATCACAGATTGAATC	159-179	89	15′ 94°C 50 x: 30″ 94°C 30″ 55°C 30″ 72°C
	antisense	GCGGTTCCAAACGTACCAA	229-247		
	probe	Cy5 TTTTTATGTGTCCGCCACCATCTGGATC BHQ-2	197-224		
ß hemoglobin gene	sense	AAGTGCTCGGTGCCTTTAGTG	200-220	97	15′ 94°C 50 x: 30″ 94°C 30″ 55°C 30″ 72°C
	antisense	ACGTGCAGCTTGTCACAGTG	222-245		
	probe	YAK TGGCCTGGCTCACCTGGACAACCT BHQ-1	277-296		

[a] from startcodon in gene in sense orientation, [b] in case of nested amplification

Real-time PCR based phenotypical susceptibility test

DRA was performed on supernatants from either HSV-1 culture isolates (Table 1) or directly on positive clinical samples inoculated onto A549 cells and incubated for 48 hours. Supernatants from pre-cultured clinical samples were diluted 1:100 in EMEM regardless of viral titers whereas stored viral isolates were diluted to obtain viral loads between 10^4 and 10^7 copies/ml. A confluent monolayer of Vero cells was cultured in 24 wells plates. Subsequently 300 μl of the diluted sample was added to each well after removal of culture medium. After one hour of incubation at 37°C in a humidified atmosphere of 5% CO_2 the inoculum was removed and replaced by 1 ml of aciclovir or foscarnet in EMEM with 5% FCS. The concentration range of the serial dilutions (6 dilutions per isolate) of aciclovir was 0.12 to 16 mg/L and for foscarnet 8.35 to 100 mg/L. All samples were assayed in quadruplicate and incubated for 24 hours.

Then, after removal of the culture medium, 200 μl of AL Lysis buffer (QIAamp® DNA Mini and Bloodkit, Qiagen) spiked with Phocid Herpes Virus (PhHV, kindly provided by Dr. M Schutten, Erasmus Medical Center, Rotterdam, The Netherlands) was added to the monolayer and incubated for 15 minutes at room temperature. Lysate was harvested and all wells were rinsed with 200 μl PBS to include any remaining viral DNA which was then added to the lysate. After addition of 20 μl Proteinase K (Qiagen), samples were incubated 10 minutes at 56°C and 10 minutes at 95°C. Samples were mixed with 200 μl ethanol 100% and transferred to a Qiamp® mini spin column (Qiagen) and centrifuged for 1 minute at 6000 x g. Washing steps were omitted to limit hands on time. DNA was eluted in 200 μl AE buffer (Qiagen) by centrifugation for 1 minute at 6000 x g. A real-time PCR for beta-globin and a multiplex real-time PCR for PhHV and HSV-1 were performed on a CFX96 real-time detection system (Bio-Rad, Veenendaal, The Netherlands). Primers, probes and PCR conditions are shown in Table 2. PCRs were carried out in 50 μl volume containing 25 μl HotStart Taq mastermix (Qiagen) and 4 mM $MgCl_2$ (5 mM for beta-globin).

For quantitation, a standard of HSV-1 (cultured field isolate or ATCC KOS strain VR-1493D) was calibrated using a quantitated DNA control of the HSV-1 MacIntyre strain (Advanced Biotechnologies Inc., Columbia, MD). PhHV served as an internal control for DNA extraction and PCR inhibition[12] and those wells that were outliers in the PhHV PCR (more than 3 CT-values different from assay average) were considered inhibited and were excluded from the analysis. The beta-globin PCR was performed as a control for cell concentration and cell lysis for which the same exclusion criteria were used. The concentration of antiviral agents to inhibit viral DNA replication by 50% (IC_{50}) was calculated after plotting the viral concentration versus the concentration of antiviral agent. Provisional resistance breakpoints were adapted from Thi at al.[7] and set at ≥0.23 mg/L for aciclovir and at ≥ 13 mg/L for foscarnet.

RESULTS

Viral isolates

PRA and sequence analysis of the UL23 and UL30 genes were repeated on aliquots of the viral isolates to be used for DRA. PRA results for aciclovir were concordant with genotypic analysis in 25 isolates including 11 with no mutations in either the UL23 or UL30 genes, 13 with a mutation in UL23, and 1 with a mutation in UL30. One isolate (#25) had a UL30 mutation but was susceptible to aciclovir by PRA but with an elevated IC50 of 1.03 mg/L (Table 3). PRA results for foscarnet were concordant in 23 isolates, including 10 with no mutations and 13 with a mutation in UL23, failed in one isolate (#2) and were discordant with genotypical analysis in the two isolates with UL30 mutations (#24 and #25, Table 3). Both isolates #24 and #25 showed diminished susceptibility to foscarnet although not meeting the proposed resistance criteria according to CLSI.

DRA was validated for aciclovir on all isolates and for foscarnet in UL30 mutants and in a subset of UL23 mutants. DRA failed in one isolate (#9). DRA results (Table 3) for aciclovir were in concordance with genotyping of all 25 isolates using the provisional breakpoint of 0.23 mg/L, but not in concordance with PRA for 1 isolate (#25). By DRA, IC50 values of all 15 UL23 and UL30 mutants, including isolate #25, were at least at least 2.8-fold the tentative breakpoint and 6.4-fold the IC50 value of the susceptible reference isolate. DRA results (Table 3) for foscarnet were concordant with genotypical results in the 8 tested isolates, but were not concordant with PRA in two isolates (#24 and #24) . These UL30 mutants had IC50-values that were at least 1.7 fold the tentative breakpoint and at least 2.5 fold the IC50 value of the susceptible reference isolate.

Clinical samples

DRA was applied to 9 clinical samples (Table 4) and demonstrated aciclovir resistance in two UL23 mutants (Table 4) and failed in one of the clinical samples that had a relatively low viral load after 48 hours of pre-culture (7.9×10^4 copies/ml).

DISCUSSION

This study optimized and validated a protocol for phenotypical susceptibility testing of HSV-1. A fast and easily applicable protocol was developed that compared very well to results obtained by genotypical tests and by PRA. Although only tentative breakpoints

Table 3. UL23 and UL30 sequence analysis, plaque reduction assay and DNA reduction assay results on HSV-1 isolates.

isolate	resistance-associated mutation	PRA			DRA		
		IC50 ACV mg/L	**IC50 FOS** mg/L	**conclusion**	**IC50 ACV** mg/L	**IC50 FOS** mg/L	**conclusion**
HSV-F	none	0.08	21.9	ACV S FOS S	0.10	8.75	ACV S FOS S
#1	none	0.10	21.3	ACV S FOS S	0.08	n.d.	ACV S FOS n.d.
#2	none	0.45	failed	ACV S FOS n.d.	0.06	n.d.	ACV S FOS n.d.
#3	none	0.16	18.2	ACV S FOS S	0.12	n.d.	ACV S FOS n.d.
#4	none	0.09	26.0	ACV S FOS S	0.07	n.d.	ACV S FOS n.d.
#5	none	0.15	19.4	ACV S FOS S	0.07	n.d.	ACV S FOS n.d.
#6	none	0.23	16.7	ACV S FOS S	0.12	n.d.	ACV S FOS n.d.
#7	none	0.28	11.4	ACV S FOS S	0.08	n.d.	ACV S FOS n.d.
#8	none	0.28	17.8	ACV S FOS S	0.18	n.d.	ACV S FOS n.d.
#9	none	0.06	10.5	ACV S FOS S	failed	failed	failed
#10	none	0.11	35.7	ACV S FOS S	0.08	12.81	ACV S FOS S
#11	UL23	**16.21**	11.3	ACV **R** FOS S	**2.65**	n.d.	ACV **R** FOS n.d.
#12	UL23	**15.16**	16.3	ACV **R** FOS S	**3.67**	n.d.	ACV **R** FOS n.d.
#13	UL23	**11.20**	10.1	ACV **R** FOS S	**0.76**	n.d.	ACV **R** FOS n.d.
#14	UL23	**11.82**	12.5	ACV **R** FOS S	**6.17**	n.d.	ACV **R** FOS n.d.
#15	UL23	**15.56**	9.1	ACV **R** FOS S	**2.40**	n.d.	ACV **R** FOS n.d.
#16	UL23	**13.92**	25.0	ACV **R** FOS S	**3.18**	5.98	ACV **R** FOS S
#17	UL23	**16.70**	17.4	ACV **R** FOS S	**3.06**	n.d.	ACV **R** FOS n.d.
#18	UL23	**37.95**	25.0	ACV **R** FOS S	**4.62**	4.81	ACV **R** FOS S
#19	UL23	**6.88**	25.0	ACV **R** FOS S	**2.42**	9.11	ACV **R** FOS S
#20	UL23	**10.55**	15.5	ACV **R** FOS S	**1.63**	n.d.	ACV **R** FOS n.d.
#21	UL23	**6.15**	21.6	ACV **R** FOS S	**4.69**	n.d.	ACV **R** FOS n.d.
#22	UL23	**6.33**	5.8	ACV **R** FOS S	**0.67**	n.d.	ACV **R** FOS n.d.
#23	UL23	**14.25**	11.7	ACV **R** FOS S	**0.95**	5.63	ACV **R** FOS S
#24	UL30	**5.81**	88.7	ACV **R** FOS S	**0.64**	**22.13**	ACV **R** FOS **R**
#25	UL30	1.03	43.4	ACV S FOS S	**0.99**	**29.73**	ACV **R** FOS **R**

IC50 values greater than or equal to resistance breakpoint (see text) in bold. ACV = aciclovir; DRA = DNA reduction assay; FOS = foscarnet; n.d. = not determined; PRA = plaque reduction assay; R = resistant; S = susceptible.

exist for susceptibility testing of HSV-1 using a real-time-PCR based approach, classification of the DRA results according to the tentative breakpoints adopted from Thi et al.[7] resulted in very high concordance. DRA failed in only one isolate.

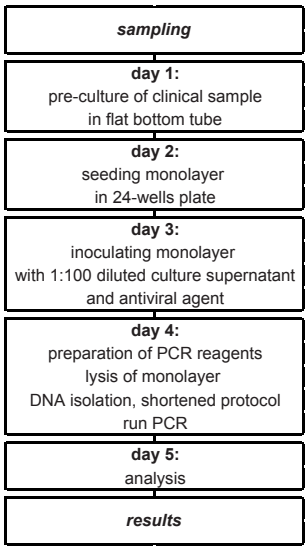
DRA provided results within five days after sampling (Figure 1). No visible viral CPE is required when viral replication is measured by sensitive methods such as real-time

Table 4. UL23 sequence analysis and DNA reduction assay results performed directly on HSV-1 positive clinical samples.

patient/ source	**clinical & treatment details**	**viral load (^{10} log copies/ml)**				**resistance associated UL23 gene mutations**		**IC50 ACV in DRA (mg/L)**
		in sample	*after pre-culture*	*at input in DRA*	*at read-out of DRA*	*in sample*	*after pre-culture*	*after pre-culture*
E**	SCT, mucositis, pretreatment	7.4	8.4	4.9	7.2	none	none	0.07
M*	chemotherapy, persistent mucositis, vACV 12 days & ACV iv 10 days	5.3	7.3	5.4	6.6	delC460 → fs155	delC460 → fs155	**3.06**
O	SCT, HSV mouth, no antivirals	4.1	5.9	3.9	5.9	none	none	0.06
P	chemotherapy, HSV lip, no antivirals	5.3	7.1	5.1	6.1	none	none	0.16
Q	infant, eczema herpeticum, treatment unknown	5.4	4.9	2.9	3.9	none	none	Failed***
R	SCT, HSV mouth, no antivirals	6.4	7.1	5.1	7.5	none	none	0.06
S	unknown	6.9	6.5	4.5	6.1	none	none	0.06
T	SCT, HSV mouth, ACV iv 17 days	6.9	6.6	4.6	6.5	insG430 → fs146	insG430 → fs146	**5.74**
U	infant, eczema herpeticum, treatment unknown	8.3	6.2	4.2	6.6	none	none	0.06

IC50 values greater than or equal to resistance breakpoint (see text) in bold. * see Table 1, isolate #17, ** see Table 1 isolate #5, *** IC50 value 0.06 mg/L at 1:10 dilution; ACV = aciclovir; d = days; del = deletion; DRA = DNA reduction assay; fs = frameshift; ins = insertion; SCT = stem cell transplantation; vACV = valaciclovir.

Figure 1



Time frame (workings days) of susceptibility testing by DNA reduction assay versus plaque reduction assay.

PCR and hence incubation time can be reduced compared to PRA. Pilot experiments using both susceptible and resistant isolates showed comparable IC50 values over broad ranges of viral inoculum, given sufficient viral input (10.000 copies/ml), hence allowing a fixed dilution of pre-cultured clinical samples without the need for viral titration. Hands on time could be reduced by the use of a simplified DNA isolation procedure. DRA was applicable to all clinical samples with a viral load of at least 10.000 copies/ml. In the final format of quadruplicate testing the coefficient of variation of the log-transformed viral concentrations was 5% on average. On average 0.3 wells were excluded per 24-wells assay because of PCR inhibition and 1.2 wells because of insufficient beta-globin concentration.

As shown in previous studies,[1-3] mutations of unknown significance are commonly found in HSV-1 clinical isolates and a pre-treatment sample for comparison of mutations is often not available. Three isolates contained UL30 mutation Asp-672→Asn which has recently been described both as a natural polymorphism[2] and as an aciclovir and foscarnet resistance-associated mutation.[13] Phenotypical susceptibility tests showed aciclovir and foscarnet susceptibility in an isolate with no other resistance-associated mutations making Asp-672→Asn unlikely as a resistance-associated mutation. This nicely demonstrates the usefulness of phenotypical susceptibility testing.

Low level resistance to aciclovir and foscarnet due to mutations in the UL30 gene is possibly more accurately detected by DRA than by PRA. Both our PRA and PRA performed initially (by others) showed a level of resistance around or below the specified breakpoint, as previously described for UL30 mutations A719V and S724N.[8;10;14;15] This may be due to the specific UL30 gene mutations or because low-level resistance or intermediate susceptibility may not be accurately defined by the CLSI breakpoints.[5] Nevertheless, DRA consistently showed increased IC50 values above the tentative breakpoints in these isolates. Because knowledge on the clinical significance of such mutations is lacking, it would be interesting to study how infections with such isolates should be treated.

Some limitations apply to the DRA. Firstly, without a short pre-culture, no viral replication was observed in DRA, but our protocol was suitable for clinical samples after a fixed short term pre-culture. This may be due to the low inoculum present in clinical samples, although pre-culture did not increase viral loads very much. It can also be related to the short duration of incubation of the DRA, although prolonging the assay to 48 hours did not solve the problem. Possibly, adaptation of virus to cell culture is necessary for a successful DRA. To study the possible selection pressure during pre-culture of a clinical sample, nine clinical samples were cultured for 48 hours. The nucleotide sequence of the entire UL23 gene was compared between the clinical samples and the viral isolates after 48 hours of pre-culture (Table 4) and no differences were found.

Secondly, the detection limit of DRA precluded its use for weakly positive samples. This limitation may be partly overcome by adaptation of the dilution in samples with a lower viral load, which was applied successfully in one of our clinical samples in which a 1:10 diluted culture supernatant was used instead of 1:100 (Table 4). Finally, the assay was validated for HSV-1 only.

In clinical diagnostics, a genotypical approach has the advantages of speed and technical ease. A two-step approach may therefore be practical in this setting, starting with UL23 and UL30 gene sequencing of, preferably, a pre- and on-treatment sample and subsequent phenotypical confirmation of resistance if mutations of unclear significance are encountered. A multicenter trial evaluating assay applicability and reproducibility and the clinical outcome in relation to the tentative breakpoints, should confirm the role of DRA in phenotypical susceptibility testing of HSV-1.

In conclusion, DRA is an accurate, rapid and easy to perform phenotypical susceptibility test for HSV-1.

AUTHOR CONTRIBUTIONS

MB, EC, AK and AV conceived and designed the experiments. MB, LR and CB performed the experiments. MB, EC, FM and AV analyzed the data. MB wrote the paper. All authors read and approved the final manuscript.

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3

Viral loads and antiviral resistance of Herpesviruses and oral ulcerations in hematopoietic stem cell transplant recipients

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ABSTRACT

Background: Ulcerative oral mucositis and infection are frequent complications in HSCT recipients. The aim of this study was to investigate the relationship between oral ulcerations and HSV-1, EBV and CMV excretion and the presence of aciclovir resistant HSV-1 strains in HSCT recipients.

Methods: This prospective observational study included 49 adult patients that underwent allogeneic HSCT. Twenty-six patients received myeloablative and 23 received non-myeloablative conditioning. Ulcerations on non-keratinized and keratinized oral mucosa were scored and oral rinsing samples were taken twice weekly. Viral loads were determined by real-time PCR. Samples from patients remaining HSV-1 positive despite antiviral treatment were studied for resistance to antivirals.

Results: Having an HSV-1 or EBV DNA positive sample was a significant predictor for ulceration of keratinized mucosa. HSV-1 was a significant predictor for ulcerations on non-keratinized mucosa as well. Persistent HSV-1 infection occurred in 12 of 28 patients treated with antiviral medication and aciclovir resistant HSV-1 was found in 5 persistent infections.

Conclusions: In conclusion, HSV-1 is a predictor of ulcerations on non-keratinized as well as keratinized oral mucosa following HSCT. The role of EBV deserves further study. Persistent HSV-1 replication despite antiviral treatment is common and is due to resistance in 18% of treated patients.

INTRODUCTION

Ulcerative oral mucositis is a frequent and serious complication in patients receiving a hematopoietic stem cell transplant (HSCT). Previous studies reported that between 76% and 89% of patients undergoing myeloablative HSCT suffered from oral mucositis;[1-5] reduced-intensity regimens may decrease the prevalence as well as the severity of oral mucositis.[2] Patients reported that oral mucositis was the single most debilitating side effect of HSCT conditioning.[6] Furthermore, it is associated with lower survival.[7]

Following chemotherapy or HSCT, reactivation of latent HSV-1 occurs frequently.[8-10] In such patients, HSV-1-induced ulcers may develop at the typical non-keratinized predilection sites for oral mucositis induced by chemoradiation (e.g., floor of mouth, buccal and labial mucosa, lateral side and tip of the tongue, and soft palate) and may aggravate mucositis, or be confused with this condition.[8] In addition, HSV-1- induced ulcerations may develop at keratinized oral mucosa and peri-orally, where mucositis as a result of chemoradiation is very uncommon. These sites include the hard palate, the dorsum of the tongue, the gingiva and the vermillion lip. However, HSV-1 reactivation may also result in asymptomatic shedding.[8;11] It is unknown whether the viral load in an oral sample can distinguish between asymptomatic and symptomatic HSV-1 reactivation. Also, it is unknown whether prolonged HSV-1 excretion in HSCT recipients despite antiviral treatment is caused by the profound immunosuppressed status of HSCT recipients, or due to resistance of the virus to the antiviral medication. The prevalence of antiviral resistance has been shown to be very low in immunocompetent subjects, whereas in immunocompromised patients with HSV-1 infections, resistance levels up to 27% have been described.[12-14]

A possible role for other Herpesviruses such as CMV[15] and EBV[16] in oral ulceration has been suggested but their role has at present only been supported by a very limited number of studies.[9] Shedding of EBV in the saliva of healthy carriers occurs frequently[17] whereas oral shedding of CMV, apart from congenital or primary infection, is seen mostly in immunocompromised patients.[18]

To our knowledge, no studies have been performed that prospectively sampled HSCT patients, regardless of the presence of oral ulcers, and quantitatively analyzed the presence of HSV-1, EBV and CMV DNA. Also, no data are available on the differential role of herpesvirus excretion on ulcerations at oral sites that may be affected by chemoradiation-induced oral mucositis (non-keratinized mucosa) and at sites atypical for chemoradiation-induced mucositis (keratinized mucosa). Therefore, the aim of this study in adult HSCT recipients was to prospectively explore the relationship between

the presence and localization of oral ulcerations and the duration and amount of HSV-1, EBV and CMV DNA shedding in the oral cavity. Furthermore, persistence of HSV-1 despite antiviral treatment and the occurrence of antiviral resistance in HSV-1 were studied.

PATIENTS AND METHODS

Patients

Data were collected from 49 adult patients that underwent HSCT for hematological malignancies at the Leiden University Medical Center between November 2006 and June 2009. The medical ethical committee of the Leiden University Medical Center approved of this study. All patients gave their informed consent. Oral assessment data were noted on standardized forms, whereas other data, including HSV, CMV and EBV serostatus were retrieved from patient charts and from the laboratory information system.

Transplantation protocol

T cell-depleted transplantation was performed either according to a reduced intensity conditioning (RIC) protocol or a myeloablative conditioning (MAC) regimen as described previously.[19-21] Prophylaxis for GVHD was only administered to recipients of grafts from matched unrelated donors in the MAC regimen (cyclosporine 3 mg/kg intravenously starting on day -1). During granulocytopenia, all patients received oral selective digestive tract decontamination, antifungal prophylaxis and, in case of myeloablative conditioning, systemic streptococcal prophylaxis. All patients received a basic oral care regimen aimed at preventing accumulation of dental plaque and keeping oral tissues moist and free of debris. Antiseptic washings were not part of basic oral care. Patients did not receive antiviral prophylaxis. In case of an oral lesion suspect of HSV-1 infection, sampling was performed using a sterile cotton swab and patients were treated with intravenous aciclovir or oral valaciclovir at the discretion of the treating physician.

Oral assessment

Oral assessment was performed twice weekly starting before or shortly following HSCT conditioning until hospital discharge by one trained investigator. Any type of oral ulceration was recorded in all patients. Mucositis was scored according to the WHO criteria.[22] For the WHO score ulcerations were evaluated at non-keratinized oral mucosal sites only. Ulcerations on keratinized oral mucosa and the vermillion lips were assessed and noted separately. .

Sampling

Oral rinsing samples were taken at each oral assessment. Patients were asked to rinse their mouth for 30 seconds with 10 ml of a 0.9% saline solution. Samples were frozen in -20°C within 3 hours after collection and stored at -80°C until analysis.

Viral load determination

DNA was isolated from a 200 ml aliquot of the samples with the MagNA Pure LC Total Nucleic Acid Isolation Kit using a MagNA Pure LC Instrument (Roche Diagnostics, Almere, The Netherlands). Viral loads were determined by real-time PCR on a CFX96™ optical reaction module (Bio-Rad, Veenendaal, The Netherlands). Real-time PCRs for beta-globin, CMV, EBV, Phocine Herpesvirus (PhHV) and HSV-1 were performed as previously described.[23-25] PhHV served as an internal control for DNA extraction and PCR inhibition and the beta-globin PCR was performed as a control for cell concentration in the samples.

Resistance analysis

Samples from patients whose samples remained positive despite antiviral treatment for at least 5 days were studied for development of resistance by comparing pre-treatment samples with subsequent samples during treatment as long as samples remained positive. In addition to the oral rinsing samples, samples routinely submitted to the clinical microbiology laboratory for diagnosis of HSV-1 were included in the resistance analysis. Resistance was determined by sequencing the viral thymidine kinase (TK) gene, and in case of TK mutations, by additional sequencing of the viral DNA-polymerase gene.[25] Phenotypical susceptibility testing was performed on viral isolates containing mutations of unknown significance, as described elsewhere.[25]

Statistical analysis

Possible predictors of ulcerations at non-keratinized and keratinized mucosa were analyzed separately in all patients. Positive samples with viral loads below 250 copies/ml were set at 125 copies/ml to correct for imprecise quantification at very low loads. To adjust for skewness of the data, ^{10}log transformed viral and beta-globin loads were used in the analysis. To correct for cell count in an oral sample, the beta-globin load was included as a predictor in the model. WHO mucositis scores were recoded into a binary variable: mucositis WHO grade 0-1 was scored as no ulceration present and mucositis WHO grade 2-4 was scored as ulcerative mucositis present. In view of the repeated measurements of ulcerative mucositis and ulcerations on keratinized mucosa within patients, the outcome was modeled as a repeated measures logistic regression. Param-

eters were estimated using the Generalized Estimating Equations procedure in SPSS with first-order autoregressive correlation structure and a robust estimation procedure. Univariable analysis of potential predictors was performed with p-values <0.20 as a criterion for possible inclusion in a multivariable model.

RESULTS

Patient characteristics and oral assessment outcomes

The characteristics of the 49 patients participating in this study are summarized in Table 1. The mean duration of hospitalization was 27 days. During the assessment phase, 33 patients (67%) developed ulcerative mucositis and maximum WHO grade 2, 3 and 4 occurred in 23, 7 and 3 patients respectively. During the assessment phase 23 out of 48 (due to missing data on 1 patient) patients (48%) developed an ulceration on the keratinized intra-oral mucosa or the vermillion lip. There was a weak, but significant, association between the peak mucositis score and the length of stay in the hospital (Spearman's rho=0.31, p=0.036).

Oral ulcerations and viral loads

HSV-1 could be analyzed in 191 samples and was detected in 23 patients (47%) starting at a median of 2 days before HSCT (range -8 days to + 6 days). Most HSV-1 positive samples occurred between days 4 to 6 after HSCT. The median HSV-1 DNA load in the 55 positive samples was $10^{5,3}$ copies/ml (range $10^{2,1}$-$10^{7,4}$ copies/ml). HSV-1 DNA was detectable in 24 of 61 samples (39%) from patients with ulcerations on non-keratinized mucosa ("ulcerative mucositis") and in 30 of 129 samples (23%) from patients without ulcerative mucositis (Figure 1). In 31 of 45 samples (69%) from patients with ulcerations on the keratinized areas of the mouth HSV-1 DNA was detected, whereas it was detectable in 24 of 140 samples (17%) from patients without ulcerations on keratinized mucosa (Figure 1). Of the 55 HSV-1 positive samples 7 (13%) were from patients with ulcerative mucositis, 14 (25%) from patients with ulcerations on keratinized mucosa, 17 (31%) from patients with ulcerations at both locations and 17 (31%) from patients without any ulceration at the time of sampling. In the case of 15 of the 17 samples (88%) from patients without ulcerations, ulcerations occurred directly preceding or following sampling within days. There was no difference in the presence or the load of HSV-1 between patients in the MAC and RIC conditioning regimens (data not shown).

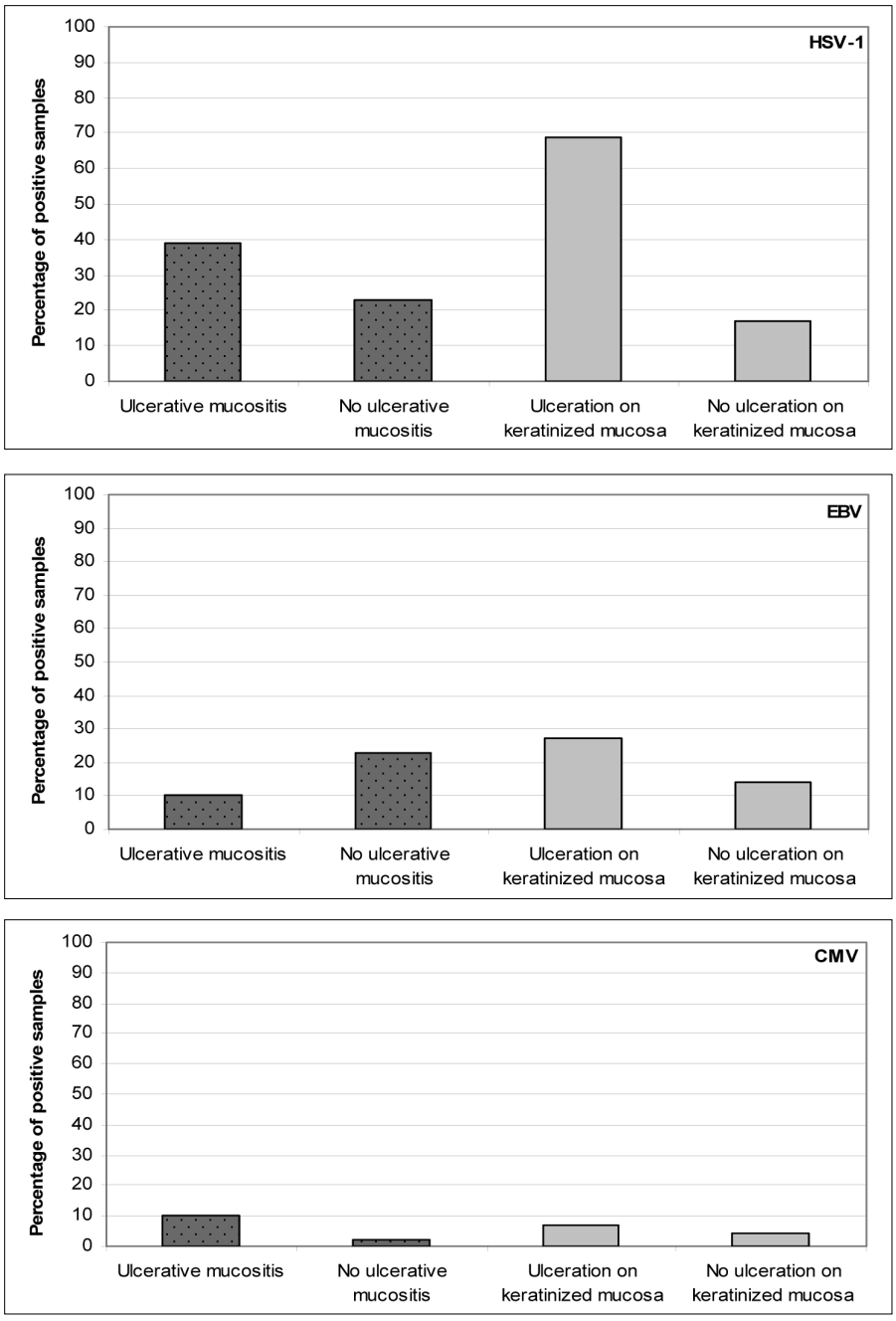
From 18 patients at 24 sampling moments both oral rinsing samples and swabs from oral lesions were available; 20 samples (83%) showed concordant results (12 were HSV-

Table 1. Patient characteristics.

number of patients	49
sex	
Male	27 (55%)
Female	22 (44%)
age (mean; SD)	48.8; 13.6 years
diagnosis	
Acute myeloid leukemia	19 (39%)
Multiple myeloma	10 (20%)
Acute lymphoblastic leukemia	5 (10%)
Non-Hodgkin lymphoma	5 (10%)
Myelodysplastic syndrome	3 (6%)
Chronic myeloid leukemia	2 (4%
Hodgkin lymphoma	2 (4%)
Chronic lymphocytic leukemia	2 (4%)
Others	1 (2%)
donor type	
Matched Sibling	17 (35%)
Matched Unrelated	28 (57%)
Other[1]	4 (8%)
conditioning regimen	
Myeloablative	25 (51%)
Reduced intensity	24 (49%)
length of stay in the hospital (mean; SD)	27; 8.9 days
HSV serostatus (IgG pre-HSCT)	
Positive	39 (80%)
Negative	9 (18%)
Unknown	1 (2%)
EBV serostatus (IgG pre-HSCT)	
Positive	48 (98%)
Negative	1 (2%)
Unknown	0
CMV serostatus (IgG pre-HSCT)	
Positive	24 (49%)
Negative	21 (43%)
Unknown	4 (8%)

[1] cord blood donor for two patients, mismatched related donor for one patient, autologous transplant in one patient

Figure 1. Detection of herpesviruses and presence of oral ulcerations.



Percentage of HSV-1 (top), EBV (middle) and CMV (bottom) DNA positive samples from patients with or without oral ulcerations at the time of sampling.

1-positive and 8 were HSV-1-negative in both samples types), whereas 4 showed discordant results (all were negative oral rinsing samples with a positive swab). In two of the discrepant cases, swabs were weakly positive (Ct-value above 34) and in the other two cases swabs were from ulcerations on the lip.

EBV and CMV could be analyzed in 186 samples. EBV was detected in 12 patients (24%) with a median load of $10^{3,8}$ copies/ml (range $10^{2,1}$-$10^{5,7}$ copies/ml) in the 35 positive samples. CMV was detected in 6 patients (12%) with a median load in the 9 positive samples of $10^{2,5}$ copies/ml (range $10^{2,1}$-$10^{4,1}$ copies/ml). EBV was detected in 6 of 59 samples (10%) from patients with ulcerative mucositis, in 29 of 126 samples (23%) from patients without mucositis, in 12 of 44 samples (27%) from patients with ulcerations on keratinized mucosa and in 19 of 136 samples (14%) from patients without ulcerations on keratinized mucosa (Figure 1). For CMV, these percentages were 10,2%, 2,4%, 6,8% and 4,4% respectively (Figure 1).

Predictors of oral ulcerations

In uni- and multivariable analyses, the presence of HSV-1 DNA in a sample (Table 2) was a significant positive predictor of having ulcerative mucositis (multivariable OR=2.62, p=0.049) and of having ulcerations on the keratinized mucosa (multivariable OR=4.37, p=0.003). The HSV-1 DNA load was a significant predictor of both ulcerative mucositis and of ulcerations on keratinized mucosa in the univariable analysis (OR=1.17, p=0.023 and OR=1.41, p<0.001, respectively) In multivariable analysis, HSV-1 DNA load was a positive predictor of ulcerations on keratinized mucosa (OR=1.35, p=0.0005), but it was no predictor of ulcerative mucositis (OR=1.18, p=0.08). No threshold of the HSV-1 DNA load could be established above which all samples were derived from patients with oral ulcerations (Table 3).

The presence of EBV DNA, but not the EBV DNA load, was a positive predictor of ulcerations on keratinized mucosa (multivariable OR=3.82, p=0.02, Table 2). Neither the presence nor the load of EBV DNA were significant predictors of ulcerative mucositis in the multivariable analysis.

CMV was not a significant predictor of ulcerative mucositis or of ulcerations on keratinized mucosa. Interestingly conditioning regimen was no significant predictor of ulcerative mucositis or ulcerations on the keratinized mucosa. Sex, age, body mass index, donor type and time after HSCT were no significant predictors of oral ulcerations either.

Persistent HSV-1 and viral resistance

Twenty-nine patients had at least one HSV-1-positive oral rinsing sample or oral swab of whom 28 received antiviral treatment for 5 days and 12 (43%) still had detectable levels

Table 2. Uni- and multivariable GEE analyses of predictors of ulcerative mucositis and of ulcerations on non-keratinized mucosa.

	ulcerations on non-keratinized mucosa (ulcerative mucositis)						**ulcerations on keratinized mucosa**					
	univariable analysis			multivariable analysis			univariable analysis			multivariable analysis		
	OR	95% CI	p-value	OR	95% CI	p-value	OR	95% CI	p-value	OR	95% CI	p-value
sex												
male	1,16	0,62-2,19	0,64				0,99	0,41-2,39	0,98			
female	reference category						reference category					
age	0,98	0,95-1,00	0,048*	0,97	0,93-1,01	0,13	1,02	0,98-1,06	0,39			
(years, mean)												
body mass index	0,94	0,86-1,04	0,24				1,03	0,92-1,15	0,65			
(kg/m^2, mean)												
underlying disease[2]												
conditioning regimen												
myeloablative	1,77	0,97-3,22	0,06	1,26	0,44-3,58	0,67	0,76	0,32-1,79	0,52			
non-myeloablative	reference category			reference category			reference category					
donor type												
matched sibling	0,64	0,16-2,56	0,53				1,74	0,34-8,90	0,51			
matched unrelated	1,46	0,40-5,35	0,57				2,17	0,45-10,4	0,33			
other	reference category						reference category					
time of sampling	1,08	1,02-1,14	0,008*	1,06	0,99-1,12	0,08	1,05	0,99-1,10	0,09	1,04	0,99-1,10	0,13
(day after HSCT, median)												
HSV present in sample												
yes	2,54	1,24-5,18	0,01*	2,62	1,00-6,86	0,049*	6,19	2,62-14,6	<0.001*	4,37	1,67-11,4	0,003*
no	reference category			reference category			reference category			reference category		
CMV present in sample												
yes	3,05	0,96-9,71	0,06	1,80	0,55-5,92	0,33	2,31	1,00-5,37	0,051	1,22	0,46-3,23	0,69
no	reference category			reference category			reference category			reference category		
EBV present in sample												
yes	0,31	0,11-0,92	0,04*	0,38	0,13-1,10	0,08	3,21	1,21-8,56	0,02*	3,82	1,27-11,5	0,02*
no	reference category			reference category			reference category			reference category		
total												
clinical data available	232						226					
samples available	185						180					

[1] In multivariable analysis either virus detectability or virus load (results shown in text) were included as potential predictors. Beta-globin load (^{10}log transformed) was included in the multivariable analysis to correct for cell count in the sample (ulcerative mucositis: OR=0.93, p=0.78; ulcerations on keratinized mucosa: OR=1.56, p=0.12). [2] not determined because of insufficient power for analysis of various small strata * p-value < 0.05

Table 3. The number and percentage of samples coming from patients with oral ulcerations at the time of sampling given for various HSV-1 load thresholds.

Viral load in oral washing sample	total	ulcerative mucositis		ulcerations on keratinized mucosa		ulceration anywhere in the mouth	
	n	**n**	%	**n**	%	**n**	%
< 1000 copies/ml	136	34	25,2	17	13,1	47	34,6
≥ 1000 copies/ml	49	22	44,9	28	57,1	34	69,4
≥ 10.000 copies/ml	39	17	43,6	23	59,0	28	71,8
≥ 100.000 copies/ml	32	13	40,6	19	59,4	22	68,8
≥ 1.000.000 copies/ml	13	7	53,8	8	61,5	10	76,9
≥ 10.000.000 copies/ml	6	4	66,7	4	66,7	5	83,3
total	185	56		45		81	

of HSV-1 after 5 days of antiviral treatment. Clinical data were available in 11 of the 12 patients and all patients suffered from oral ulcerations. No mutations were found in pre-treatment samples, but resistance-associated mutations in the TK-gene were found in on-treatment isolates from 5 patients with treatment failure (Table 4); a frameshift mutation (insG@436) known to confer ACV resistance [26] was found in 4 patients and a substitution (G59W) in one patient (GenBank Accession Number JN191624). The G59W substitution has not been described before in a clinical isolate. Phenotypical susceptibility testing showed ACV-resistance in the isolates with mutation G59W (IC50-value 20-fold of pre-therapy isolates without mutation). No resistance-associated mutations were found in the viral DNA-polymerase gene of these samples.

DISCUSSION

The aim of our study was to prospectively investigate the relationship between Herpesvirus excretion and the presence of oral ulcerations. In our study the presence of HSV-1 was a significant positive predictor for ulcerations on non-keratinized and keratinized mucosa. Primary HSV-1 infection may cause ulcerative stomatitis resembling the typical presentation of HSV-1 reactivation in immunocompromised patients; therefore a causative role for HSV-1 in oral ulcerations in these patients is very plausible. Our findings support the use of antiviral prophylaxis in HSV-seropositive HSCT recipients.[27] A previous study found an association between HSV-1 and stomatitis in autologous HSCT recipients as well,[28] whereas another study failed to establish a relationship between the presence of HSV-1 and mucositis in HSCT recipients.[4] The use of antiviral prophylaxis

Table 4. Details on samples from patients with ACV resistant isolates.

patient	day after SCT	clinical & treatment details at time of sampling	mutations in thymidine kinase gene	mutations in DNA polymerase gene	ACV IC50[1], mg/L
39	1	Pre-treatment	S23N, E36K, Q89R, G240E, R281Q, G371E, V372M	failed	n.d.
	15	Grade 3 ulcerative mucositis and ulceration on keratinized mucosa, ACV i.v. 9 days	S23N, E36K, Q89R, **insG@436**, G240E, R281Q, G371E, V372M	S920P	n.d.
44	4	Pre-treatment	G371E, V372M	V905M, S920P	n.d.
	76	Ulceration on hard palate, ACV i.v. 52 days	**insG@436**, G371E, V372M	V905M, S920P	n.d.
50	-2	Pre-treatment	S23N, E36K, Q89R, G240E, Q261K, R281Q, G371E, V372M	S920P	n.d.
	12	Grade 2 ulcerative mucositis and ulceration on keratinized mucosa, vACV 6 days, ACV i.v. 4 days	S23N, E36K, Q89R, **insG@436**, G240E, Q261K, R281Q, G371E, V372M	S920P	n.d.
54	6	Pre-treatment	C6G, R41H, Q89R, A192V, G251C, V267L, P268T, D286E, Y305T, G371E, V372M, N376H	V905M, S920P	n.d.
	22	Ulceration on keratinized mucosa, ACV i.v. 10 days	C6G, R41H, Q89R, **insG@436**, A192V, G251C, V267L, P268T, D286E, Y305T, G371E, V372M, N376H	V905M, S920P	n.d.
60	1	Pre-treatment	C6G, R41H, Q89R, A192V, G251C, V267L, P268T, D286E, Y305T, G371E, V372M, N376H	V905M, S920P	0.07
	25	Ulceration on keratinized mucosa, vACV 5 days	C6G, R41H, **G59W**, Q89R, A192V, G251C, V267L, P268T, D286E, Y305T, G371E, V372M, N376H	V905M, S920P	1.4 [2]

Resistance-associated mutations in bold. [1] ACV IC50 as determined by DNA reduction assay.[25] [2] average from 2 independent experiments on two viral isolates with G59W mutation.ins = insertion, n.d. = not determined

probably limited the role of HSV in this latter study.[4] No other studies performed standardized and prospective quantitative sampling in all patients, with and without oral ulcers or used sensitive real-time PCR.

Although the HSV-1 load was a predictor of ulcerations on the non-keratinized mucosa, a clear quantitative relationship was difficult to establish. No cut-off value of the HSV-1 load could be established above which all samples were derived from patients with oral ulcerations, irrespective of correction for the betaglobin load. Various factors may contribute to this phenomenon, such as the presence of non cell-associated virus in the sample, the unknown interval between virus detection and the development of ulcerations and the unknown significance of viral dynamics. Possibly the increase in load is more predictive than the actual load. However, truly asymptomatic shedding without preceding or subsequent oral ulceration was found in only 2 samples. Therefore, in the absence of antiviral prophylaxis, prompt initiation of antiviral treatment upon detection of HSV-1 DNA in an oral sample may be warranted, regardless of the virus quantity.

Persistent HSV-1 shedding despite treatment occurred in 43% of the treated patients and resistance-associated mutations were found in 18% of the treated patients. Of course, sensitive detection of HSV-1 DNA by real-time PCR after ulcerations have healed may account for part of the persistence. Nevertheless, resistance to antivirals appears to play a role in persistent infections in a relevant proportion of patients. The prevalence of resistant HSV-1 in our study as assessed by sequence analysis is comparable to previous studies in HSCT recipients using different methodologies to detect resistance. In a setting of antiviral prophylaxis, HSV-1 resistance was demonstrated by viral culture in 27% of patients with HSV.[13] In other studies, a prevalence of resistance of 14% was found using a colorimetric yield reduction assay[14] and of 18% using another colorimetric assay.[12] The retrospective analysis of resistance in our study hampers addressing the clinical significance of the infections with resistant HSV-1. We can only speculate that faster healing of oral ulcerations would have occurred if treatment had been switched early and in all patients with resistant isolates, but a role for viral susceptibility testing in case of persistent symptomatic HSV-1 infection seems clear.

Mutation G59W has not been described in a clinical isolate before. The mutation is located in the ATP-binding site of the TK protein and it has been selected *in vitro* under selection pressure with brivudin[29] and ACV[30] and an ACV-resistant HSV-2 isolate with mutation G59P has been found in an AIDS-patient.[31] This makes the G59W mutation the likely cause of the ACV-resistant phenotype of the isolate in our study.

The presence of EBV in a sample was an independent predictor of oral ulcerations on the keratinized mucosa, but not of ulcerative mucositis. The rate of EBV shedding of 25% was comparable to a previous study using DNA hybridization which detected EBV in mouth washes from 19% of HSCT patients.[32] EBV has clearly been associated with oral hairy leukoplakia in various categories of immunocompromised patients.[33] Its role in oral ulcerations is not well-known. Recently, EBV positive oral ulcers with Hodgkin-like features that responded well to reduction of immunosuppression have been described in immunosuppressed patients, including one HSCT recipient.[34] Also, EBV-associated oral lesions resembling oral hairy leukoplakia with extensive mucosal ulceration have been described in an HSCT recipient.[16] In our patient group oral EBV shedding was not a predictor of systemic EBV complications, as there was no difference in the occurrence of EBV plasma DNAemia or the occurrence of post-transplant lymphoproliferative disorder between patients with or without EBV in the oral samples (data not shown). The possible role for EBV in oral lesions in HSCT recipients is currently very unclear but certainly merits further study.

No relationship between the presence of CMV in the oral cavity and oral ulcerations was found. Furthermore, the occurrence of oral CMV shedding and the viral loads were low, despite the fact that half of the patients were seropositive for the virus prior to HSCT.

In our patient group 67% of the patients developed ulcerative mucositis during hospitalization, which is a lower percentage than reported previously.[1-5] Although Takahashi et al.[2] reported a profound decrease in the presence of ulcerative mucositis when patients underwent RIC conditioning instead of a MAC regimen, the conditioning regimen was not a significant predictor of oral ulcerations in our study. The important role of HSV-1 in the development of oral ulcerations in the absence of antiviral prophylaxis in our study possibly masks the effects of the conditioning regimen. In addition, the fact that RIC and GvHD prophylaxis regimens vary between our hospital and that of Takahashi et al.[2] may account for some differences as well.

In order to obtain a standardized and quantitative oral sample, oral rinsing samples were used. Results from oral rinsing samples were concordant with results from swabs from intraoral ulcerations, with discrepancies only in the case of lesions on the lips or in the case of weakly positive swabs. Since taking a rinsing sample is less painful than swabbing an ulceration, oral washings might be a suitable alternative for viral diagnostics of intraoral ulcerations.

A possible limitation of our study is the co-occurrence of various types or oral ulcerations with a different etiology simultaneously, which is expected to occur in this severely immunocompromised patient category. In patients in whom both types of ulcerations occur, risk factors for oral each type of ulceration, including viral shedding, may be interrelated.

In conclusion, reactivation of HSV-1 is an important factor in ulcerations involving the non-keratinized and keratinized oral mucosa after HSCT in the absence of antiviral prophylaxis. Persistent HSV-1 replication despite antiviral treatment is common and is due to resistance in a relevant proportion of patients. Oral shedding of EBV may play a role in ulcerations on the keratinized mucosa, but the mechanism of action remains to be elucidated.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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4

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 μl containing 25 μl HotStart Taq mastermix (Qiagen, Hilden, Germany) and 15 pmol of each primer. All cyclesequencing reactions were performed on bulk amplification product in 20 μl containing 2 μl BigDye Terminator v1.1 (Applied Biosystems, Carlsbad, CA, USA), 6 μl 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 2.6×10^8 copies/ml. Plasma samples were available at diagnosis in 49 episodes and were positive in 41 episodes (84%) with an average viral load of 2.8×10^7 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 3.2×10^4 copies/ml versus 6.4×10^3 copies/ml, p=0.039, Mann-Whitney test).

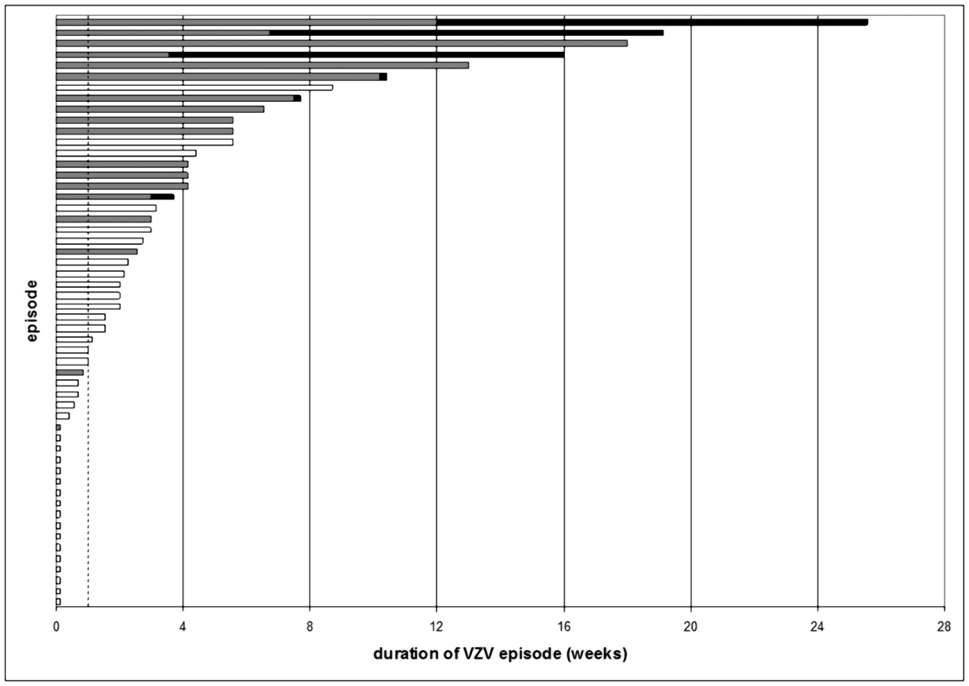
Table 4. Patients with TK and POL mutations in VZV.

	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] 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), *C919T → stop 307* (plasma day 147), *C205T → stop 69* (plasma day 154)	none, none, none
		161	persistent facial zoster, progressive headache and loss of vision, ACV iv and FOS iv	**delA76 → stop 38** (plasma, CSF), 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) *delAAC658 → del codon 220* (CSF day 57) none (plasma day 57)	N.D., 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), *T209C → T70I* (mixture, plasma day 21)	none, none
		24	follow up treatment with vACV, recovery	*C118T → P40S* (plasma day 24) 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) plasma N.D.	*G2066A → S689N* 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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5

Mass spectrometry-based comparative sequencing to detect ganciclovir resistance in the UL97 gene of human cytomegalovirus

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ABSTRACT

Background: Persistent infections with herpesviruses such as human cytomegalovirus (HCMV) frequently occur after solid organ or stem cell transplantation, and are due to either failure of the host to immunologically control the virus or emerging resistance of the virus to the antiviral drug(s) used. Antiviral therapy can be guided by viral drug susceptibility testing based on screening for known resistance-inducing mutations in the viral genome. Mass spectrometry-based comparative sequence analysis (MSCSA) might be advantageous for this purpose because of its suitability for semi-automation.
Objectives: The applicability of MSCSA to detect sequence polymorphisms and drug resistance-inducing mutations in the HCMV genome was investigated.
Study design: We analyzed the 3′ part of the HCMV UL97 gene, which encodes the kinase that is activated by the commonly used anti-HCMV drug ganciclovir. Sequences obtained by MSCSA of material from HCMV-infected patients (43 samples) and the HCMV type strain were compared to conventional cycle sequencing results.
Results: In 94.1% of all samples the results obtained by MSCSA of the UL97 gene were identical to those from conventional cycle sequencing. The threshold to detect mutant sequences in a mixture with wildtype material was 20% using either technique. Furthermore, MSCSA was successfully applied to study the development of drug resistance in a patient who developed encephalitis due to ganciclovir-resistant HCMV.
Conclusions: MSCSA was found to be equally accurate compared to conventional cycle sequencing in the analysis of UL97 of HCMV.

BACKGROUND

Human cytomegalovirus (HCMV) is a beta-herpesvirus with an approximately 235-kbp double-stranded (ds) DNA genome that encodes at least 165 gene products.[1] Primary infection in adult, immunocompetent individuals is mostly mild or asymptomatic.[2] However, as with other herpesviruses, infections with HCMV can cause major problems after solid organ or stem cell transplantation (SCT). When the host fails to immunologically control the virus infection, HCMV can persist for weeks and cause severe morbidity and mortality. Antiviral drug resistance is relatively rare,[3-5] but can occur in immunocompromised patients after prolonged antiviral therapy.[6-11] HCMV infections are commonly treated with ganciclovir (GCV) or its orally administered prodrug valganciclovir (vGCV).[12;13] Activation of this deoxyguanisone analogue requires three successive phosphorylation steps carried out by the kinase encoded by the viral UL97 gene and two cellular kinases.[14] This yields the active triphosphate form of the drug that selectively inhibits the viral DNA polymerase UL54 by disrupting viral DNA synthesis.[15] GCV resistance mutations in clinical isolates mainly map to the viral kinase gene UL97,[16-19] but after prolonged treatment mutations in the viral polymerase gene UL54 can also emerge.[19;20] In the case of a persistent infection, drug susceptibility testing of the virus can support antiviral therapy management.

OBJECTIVES

We have evaluated the application of a recently developed method for mass spectrometry-based comparative sequence analysis (MSCSA) to the identification of drug resistance-associated mutations in HCMV. The results were systematically compared to those obtained using a conventional cycle sequencing method. Furthermore, we studied the development of antiviral drug resistance in a SCT patient who developed encephalitis caused by a GCV resistant HCMV mutant.

STUDY DESIGN

Virus strains and patient samples

Forty-three CMV DNA positive clinical samples (EDTA-plasma) from forty-three different (immunocompromised) patients with HCMV infection were selected for comparison of single nucleotide polymorphism (SNP) detection. Clinical and antiviral treatment

Table 1. List of amplicons and primers used for MSCSA and cycle sequencing analysis of UL97 of HCMV.

Application	Forward primer[a]	HCMV-specific sequence	Reverse primer[b]	HCMV-specific sequence	Amplified region[c]
MSCSA Amplicon 97AB	CMV97A F	gtgctcacggtctggatgtcg	CMV97B SP6R	ggtaacattcgcgcagacggtg	1090–1558
MSCSA Amplicon 97CD	CMV97C F	gcgcgccgcatccccaactg	CMV97D SP6R	catggtctgcgagcattcgtg	1513–1980
Cycle sequencing analysis	1125CMVRs	gtgctcacggtctggatgt	1135CMVRas	cggtgggtttgtaccttctc	1090 ± 65

[a] Forward primers for MSCSA PCR were tagged with T7 promoter: 5′-cagtaatacgactcactatagggagaaggct-3′.
[b] Reverse primers for MSCSA PCR were tagged with SP6 promoter: 5′-cgatttaggtgacactatagaagagaggct-3′.
[c] Numbers indicate most 5′ or 3′ nucleotide in UL97 of the AD169 HCMV type strain (GenBank accession number X17403) that anneals with the forward or reverse primer, respectively.

Table 2. Comparison of the performance of MSCSA and cycle sequencing analysis of UL97 of HCMV.

	Total	Sequence match[a]	SNP in MSCSA[b]	SNP in cycle sequencing[c]	Amplicons in duplicate (MSCSA)	Identical duplicate MSCSA sequences
Samples analyzed	44	n.a.	n.a.	n.a.	n.a.	n.a.
MCSCA reactions (97AB + 97CD)	136	128 (94.1%)	1 (0.7%)	7 (5.1%)	58	56 (96.6%)[d]
SNPs detected	289	281 (97.2%)	1 (0.3%)	7 (2.4%)	n.a.	n.a.
Nucleotides sequenced	58,015	58,007 (99.99%)	n.a.	n.a.	n.a.	n.a.

n.a. not applicable.
[a] Identical between MSCSA and cycle sequencing results.
[b] SNPs (mutations compared to the AD169 reference sequence) found by MSCSA, but not by cycle sequencing.
[c] SNPs found by cycle sequencing, but not by MSCSA.
[d] The non-matching duplicate MSCSA sequences were identical to the cycle sequence results of the individual PCR products that were used for MSCSA analysis.

data were derived from patient charts (see Table S1).[5] Additionally, nine plasma-EDTA and four cerebrospinal fluid (CSF) samples from a case patient with HCMV encephalitis (see below) were included for analysis of GCV resistance. DNA was isolated from all samples with the MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics) using a MagNA Pure LC Instrument (Roche Diagnostics). HCMV strain AD169 was obtained from American Type Culture Collection, Manassas, VA, USA. HCMV DNA loads were determined by real-time PCR.[21]

Mass spectrometry-based comparative sequence analysis (MSCSA) and cycle sequencing
The MassARRAY® /iSEQTM – comparative sequence analysis technique (Sequenom, San Diego, USA;[22]) uses PCR amplification of a target and its subsequent in vitro transcription to produce RNA strands that are cleaved to produce a sequence-specific set of fragments for analysis by mass spectrometry. The obtained spectra are then compared to theoretical spectra derived from a database with reference sequences.

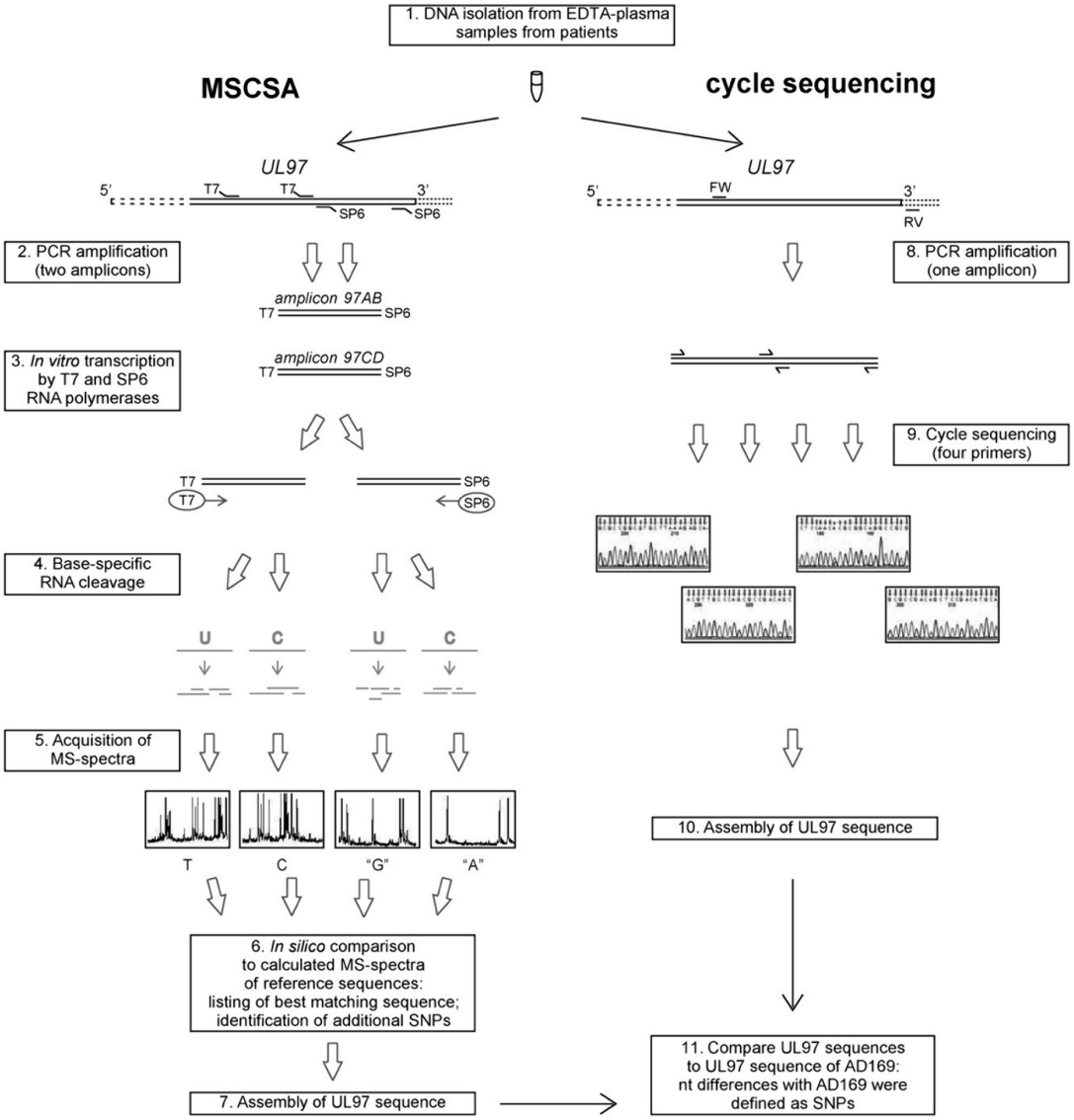
A schematic representation of the MSCSA and cycle sequencing approaches for HCMV UL97 analysis is provided in Figure 1. The 3′ region of the HCMV UL97 gene was PCR amplified in two amplicons, UL97AB and UL97CD, of 469 and 468 bp, respectively, by use of T7 promoter-tagged forward primers and SP6-tagged reverse primers (see Table 1). PCR reactions were performed in a 10-ml volume in 384-well microtiter plates, as previously described.[5]

The sample was further processed by shrimp alkaline phosphatase treatment, in vitro transcription, and Cor U-specific RNaseA cleavage, according to the manufacturer's instructions and using a MassARRAY® Liquid Handler (Matrix + FusioTM Chip Module; Sequenom, San Diego). The fragments resulting from RNA cleavage were diluted in double-distilled water and desalted with clean resin (Sequenom), transferred to a SpectroCHIP array (Sequenom), and analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MassARRAY Compact Analyzer, Sequenom). The acquired spectra were analyzed using iSEQTM Software Version 1.0.0.2.

A set of reference sequences was created for both the 97AB and the 97CD amplicon, based on previously published UL97 sequences from HCMV clinical isolates that were GCV-sensitive in phenotypic assays.[23] The set was later supplemented with three and nine sequences for amplicons 97AB and 97CD, respectively, derived from clinical isolates from Leiden University Medical Center (GenBank accession numbers GU992367–GU992375).

Cycle sequencing reactions were performed on an ABI Prism
3100 Genetic Analyzer (Applied Biosystems) after PCR amplification of the 3′ region of the UL97 gene from all samples, as previously described (see Figure 1).[5] Assembled

Figure. 1. Schematic overview of HCMV UL97 genotyping by MSCSA and cycle sequencing.



Step 1. After isolation of CMV DNA from patient samples, each sample was analyzed by either method. **Step 2**. (MSCSA) Amplification of the 3′ part of the HCMV UL97 gene in two amplicons (UL97AB and UL97CD) by use of T7 promoter-tagged forward primers and SP6-tagged reverse primers. **Step 3**. In vitro transcription of both amplicons by T7 and SP6 RNA polymerase (two reactions with each enzyme). **Step 4.** C- or U-specific RNaseA cleavage of plus and minus sense RNA transcripts. **Step 5**. Acquisition of MS-spectra of the resulting RNA cleavage products. Using these four base-specific cleavage reactions per sample each nucleotide in the sequence is specifically probed. **Step 6**. Comparison of the acquired MS spectra using iSEQTM Software Version 1.0.0.2, which lists the best-matching sequence from a database of reference sequences and any additional sequence variations for each target region. **Step 7**. Assembly of the amplicon 97AB and 97CD sequences. **Step 8**. (cycle sequencing) Amplification of the 3′ part of the HCMV UL97 gene in one amplicon. **Step 9**. Four cycle sequencing reactions were performed per PCR product. **Step 10**. Assembly of the four obtained sequences. **Step 11.** Comparison of MSCSA and cycle sequencing results. Nucleotide differences compared to the UL97 sequence of AD169 were defined as SNPs.

UL97 sequences were aligned to the sequence of the AD169 strain (GenBank accession number X17403) and SNPs were defined as nucleotide variations compared to this reference sequence. Data from both methods were compared (Figure 1, Step 11).

Analysis of mixed wild-type and mutant sequences

HCMV gene UL97 and a region of gene UL96 were cloned by inserting a 2.9 kb BglII-XhoI restriction fragment from construct pHB5[24] (kindly provided by Dr. Albert Zimmerman and Dr. Hartmut Hengel, Düsseldorf, Germany) into a pBluescript KS-derived (Stratagene) shuttle vector with the appropriate restriction sites. A 434-base pair fragment from this plasmid (corresponding to position 1587–2021 in UL97) was replaced by a PCR-amplified UL97 gene product from an HCMV isolate of the encephalitis patient described below, introducing a C1781T mutation (encoding a A594V amino acid substitution) and three translationally silent mutations. Wild-type and mutant constructs were mixed in various ratios to give a final plasmid DNA concentration of 1 ng/ml.

RESULTS

Comparing the accuracy of MSCSA and cycle sequencing

To assess the accuracy of MSCSA, we analyzed amplicons 97AB and 97CD derived from 43 clinical samples and from the HCMV type strain AD169 in duplicate. This set included plasma samples from 43 immunocompromised patients containing GCV-sensitive and GCV-resistant HCMV. The sequences obtained were verified by conventional cycle sequencing and every mismatch with the AD169 sequence was defined as a SNP. The 97AB or 97CD PCR amplification needed for MSCSA analysis failed for some of these samples (in 40 of 176 reactions). As a result, amplicon 97AB could be analyzed in 35 samples and amplicon 97CD in 43 samples, and duplicate reactions were available for 22 and 36 samples, respectively. The detection limit for clinical samples was comparable for both techniques (10 log3.6 copies/ml), although especially the amplification of the 97AB amplicon regularly failed for samples with lower viral loads (see Table S2).

After optimization of the reference database, 128 MSCSA sequences (94.1%) were identical to the cycle sequencing data of the same sample. Discrepancies were found in 8 samples, with one sequence containing a SNP recognized by MCSCA and seven by cycle sequencing only (Table 2). Accordingly, 97.2% of the SNPs were recognized by both methods. Overall more than 58 kb of UL97 sequence were analyzed by both methods, with as few as eight (0.01%) nucleotides differing between the MSCSA and cycle sequencing-derived sequences.

Fifty-eight reactions were performed in duplicate, which revealed that the variability between independent MSCSA experiments was low: for 56 samples (96.6%) identical MSCSA results were obtained. Discrepancies between duplicate MSCSA sequences or between MSCSA and cycle sequencing results were confirmed in two thirds of samples by cycle sequencing of the MSCSA PCR product (see Table S2).

Seven of the analyzed patient samples contained GCV resistance associated mutations.[16;25-28] Of these, four samples containing A594V; C592G, A591V and L595F substitutions in UL97 were identified by cycle sequencing and subsequently confirmed by MSCSA analysis. Three other resistance-associated SNPs could not be verified by MSCSA as PCR reactions failed due to low viral loads (in two samples; M460I and M460V) or probably due to the presence of mixed viral populations resulting in discrepancies between MSCSA and cycle sequencing results (C603W; see above and Table S2).

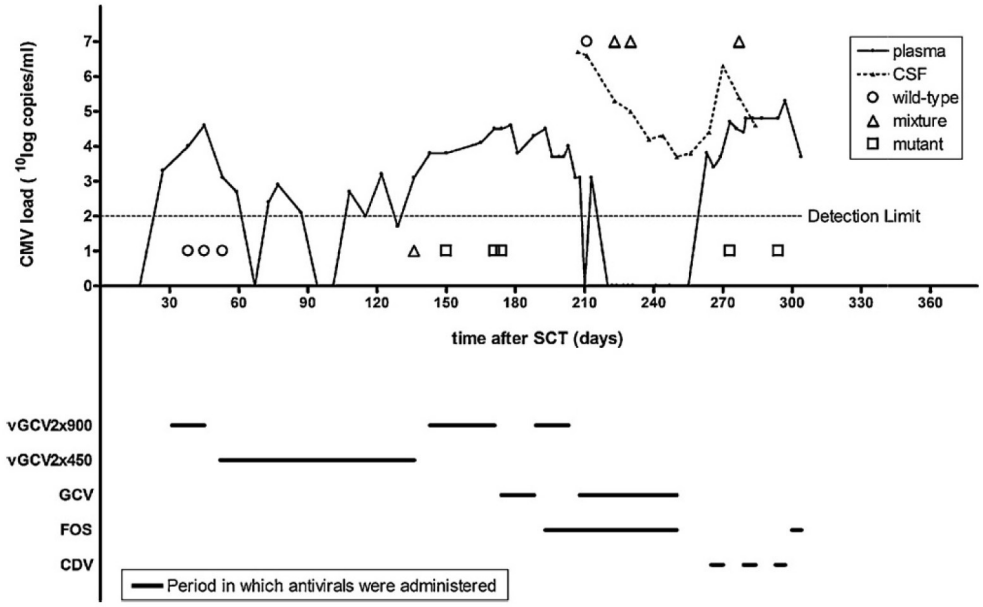
To mimic the clinical situation of a patient in which a drug resistant viral subpopulation begins to emerge due to the selection pressure of antiviral treatment, samples containing a mixture of wild type and mutant (C1781T) UL97 plasmids were analyzed. Using either method, the mutation and three translationally silent SNPs could be detected in mixtures containing 20% or more of the mutant sequence.

Application of MSCSA to a case of HCMV encephalitis after SCT

A 52-year old female with relapsed acute myeloid leukemia, secondary to myelodysplastic syndrome, received a haplo-identical T-cell depleted peripheral blood SCT after a myeloablative conditioning regimen. The patient was seropositive for HCMV, whereas the donor was seronegative. The patient was treated according to a preemptive HCMV treatment protocol, which was guided by HCMV DNA loads in plasma.[21] The patient's plasma became positive for HCMV DNA at 27 days after transplantation and she was treated with oral vGCV, 900 mg twice daily for two weeks. Because of persistent low levels of HCMV DNA and a lack of T cell recovery, treatment with a reduced vGCV dose was started at day 45 after transplantation (450 mg twice daily) and continued for 4 months. However, during the last month of treatment, HCMV DNA loads steadily increased and resistance to GCV was suspected. This was treated with intravenous GCV (5 mg/kg twice daily) for two weeks and, subsequently, with valganciclovir (900 mg twice daily) and foscarnet (60 mg/kg three times daily) for two weeks.[29]

Because the patient developed cognitive and neurological impairments, CSF was analyzed, which revealed a HCMV DNA load of 10 log 6.7 copies/ml. Other causes of encephalitis were excluded and MRI of the brain showed diffuse non-specific white matter abnormalities, supporting the diagnosis of HCMV encephalitis. Combination treatment with foscarnet (60 mg/kg three times daily) and GCV (5 mg/kg twice daily)

Figure 2. Case report.



In a patient with a persistent HCMV DNA load, despite antiviral treatment with valganciclovir (vGCV) and ganciclovir (GCV), encephalitis developed and high levels of HCMV DNA were found in the cerebrospinal fluid (CSF). Sequence analysis of the HCMV UL97 gene, derived from nine plasma and four CSF samples, was performed in duplicate using cycle sequencing and MSCSA and revealed consistently wild-type isolates (circles), mixed sequences (wild-type UL97 and the mutant encoding the A594V substitution; triangles) or exclusively mutant virus (squares). The results between both methods matched for all samples tested. vGCV2 × 900: twice a day 900 mg vGCV; vGCV2 × 450: twice a day 450 mg vGCV.

was started, followed after two months by cidofovir (5 mg/kg once weekly for three weeks). Unfortunately, the patient died from urosepsis.

Using MSCSA and cycle sequencing, plasma samples taken between days 38 and 53 showed no mutations, but later a mixture of wild-type and C1781T mutant UL97 sequences was detected (Figure 2). This mutation, specifying a A594V mutation in the UL97encoded kinase is known to be associated with resistance to GCV.[25;30] It remained present in subsequent DNA samples isolated from plasma. The HCMV DNA extracted from CSF samples also contained mixed sequences with this mutation.

DISCUSSION

In immunocompromised patients, information on the presence or absence of drug resistance-associated mutations may help to guide treatment of persistent or recurrent

HCMV infections. In such situations, virus characterization needs to be sensitive, accurate, and rapid in order to be of clinical relevance. We investigated the applicability of an MSCSA method for automated high-throughput DNA sequence analysis for the detection of sequence polymorphisms and drug resistance-inducing mutations in the 3′ region of the HCMV UL97 gene, and compared the results to cycle sequencing analysis of the same genome region. This method was previously developed by Sequenom and validated for genotyping of Neisseria meningitidis. The method was presented as a highly reproducible alternative to sequence analysis methods relying on chain termination by dideoxynucleotide incorporation.[22] Recently the first reports on the use of this technique for viral genotyping were published.[31;32]

We conclude that Sequenom's MassARRAY protocol, in combination with the iSEQ software, can be equally accurate compared to conventional sequencing techniques (see Table 2). The presence of mixed virus populations in patient samples may have contributed to the differences between duplicate MSCSA experiments and between MSCSA and cycle sequencing experiments, as the presence or absence of the discrepant SNPs in the MSCSA sequences were confirmed in many of the samples by cycle sequencing of the MSCSA PCR product. The use of two amplicons to cover the UL97 region of interest resulted in a clinically relevant detection limit of 10 log3.6 HCMV copies per ml. The sensitivity of mutation detection in the mixed plasmid DNA samples was comparable to cycle sequencing as well. We would like to stress that the accuracy of SNP detection by MSCSA is largely dependent on the quality (and quantity) of the sequences in the reference database, as performance improved considerably when the databases were supplemented with new sequences (data not shown).

Various genotypic screening methods have been developed for mutation detection in viral genomes, including that of HCMV, each with its own advantages and limitations.[33-35] Some methods allow rapid screening, but only of fixed genome positions known to be involved in antiviral drug resistance. MSCSA combines the possibility of detection of all nucleotide variations within a designated region of a viral genome with reduced hands on time due to the automation of post-PCR processing and analysis. Recently developed deep-sequencing methods allow for detection of minor variants of HCMV in patients[36], but are laborious and may be less suitable in a clinical setting. MSCSA is performed in a 96-well format and the experimental processing of one plate (48 samples, as the UL97 gene was amplified in two amplicons) is comparable to the workload of conventional cycle sequencing of about 32 samples: results are obtained in about twelve hours, of which four hours are hands-on time. The costs for the analysis

of one sample by MSCSA (two amplicons) are estimated to be twice as high as compared to cycle sequencing (one amplicon): the needed chemicals and disposables were estimated to cost around twelve to fourteen euro (excl VAT). Optimization of the PCR step in MSCSA (e.g., PCR amplification of the 3′ end of UL97 using a single amplicon, instead of the current two) would further reduce the average processing time and cost needed for MSCSA.

The MSCSA method evaluated here was successfully applied to a case of HCMV encephalitis, a rare complication after SCT that is attributed to antiviral drug resistance.[37-40] In agreement with previous cases, the prolonged use of GCV in combination with failing T-cell immune recovery likely contributed to the progression of infection in this patient. The association in this case between the treatment with a low dose of vGCV, motivated by possible myelotoxicity of the drug, and the subsequent development of resistance and viral encephalitis is compelling although not completely proven. Based on the results presented in this study, we conclude that MS-based comparative sequence analysis constitutes a reliable medium to high-throughput screening method that can be applied to detect resistance markers and other point mutations in viral genomes.

COMPETING INTERESTS

None declared.

FUNDING

None.

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Table S1. Details about the patient samples that were analyzed by MSCSA and cycle sequencing.

Patient	underlying disease[1)]	antiviral treatment (days)	CMV load (^{10}log copies/ml)	polymorphisms/ mutations[2)]
AD169	ATCC strain	N.A.	N.A.	none
1	RT	GCV 8d	5,2	none
2	myc fun	none	6,3	none
3	SCT	vGCV 2d FOS 6d	3,6	none
4	NHL	GCV 4d	4,3	none
5	SCT	GCV 6d	4,3	**C592G**
6	RT	none	5,9	D605E
7	RT	vGCV 14d	4,2	R686Q
8	RT	none	4,8	none
9	RT	none	3,5	H469Y
10	RT	none	4,8	none
11	RT	vGCV 15d	3,8	N510S, D441N
12	RT	vGCV 50d	4,9	N510S; **A594V**
13	RT	vGCV 70d	4,0	A478T; N510S; **A594V**
14	SCT	vGCV 3d	4,5	none
15	RT	none	5,0	none
16	RT	none	3,6	N467S
17	RT	none	5,3	none
18	RT	none	5,6	none
19	RT	vGCV 60d GCV 13d	3,3	H469Y; **C603W**
20	RT	vGCV 28d	3,5	E575K
21	SCT	none	4,0	none
22	SCT	vGCV 14d	4,5	H469Y
23	SCT	vGCV 48d GCV 22d FOS 14d	4,6	none
24	SCT	none	3,4	none
25	SCT	vGCV 14d	4,5	none
26	RT	GCV 13d	4,8	none
27	SCT	vGCV 4d GCV 2d	4,5	none
28	post partum hepatitis	unknown	4,0	none
29	renal disease	unknown	5,8	none
30	unknown	unknown	4,9	H469Y
31	RT	GCV 2d	3,9	none
32	RT	none	4,9	none
33	SCT	none	4,3	none
34	SCT	vGCV 66d GCV 25d CDV 3 doses	3,9	**A591V** or **L595F**
35	OLT	GCV 18d	3,4	none
36	HIV	none	4,6	none
37	SCT	vGCV 3d	4,3	none
38	SCT	none	4,9	none
39	RT	vGCV 65d GCV 39d	3,1	**M460V**
40	SCT	vGCV 26d	6,5	none
41	RT	vGCV 45d GCV 39d	2,3	**M460I**
42	SCT	none	5,1	none
43	OLT	GCV 25d	4,5	none

[1)] CDV = cidofovir; d = days; FOS = foscarnet; GCV = ganciclovir; MSCSA = mass spectrometry-based comparative sequence analysis; myc fun = mycosis fungoides; N.A. = not applicable; NHL = non-Hodgkin lymphoma; OLT = orthotopic liver transplantation; RT = renal transplantation; SCT = stem cell transplantation; vGCV = valganciclovir.
[2)] resistance associated mutations in bold.[16;25-28] Polymorphisms/mutations were identified by cycle sequencing of the samples.

Table S2. Detailed comparison of results obtained by MSCSA and cycle sequencing.

Patient	results cycle sequencing[1)]		results MSCSA[2)]			concordance MSCSA and cycle sequencing	comment
	nt changes in amplicon (1110-2124)	Polymorphisms/ Mutations	nt changes in amplicon 97AB (1090-1558)	nt changes in amplicon 97CD (1513-1980)	Polymorphisms/ Mutations		
AD169	none	none	no SNPs	no SNPs	none	yes	
1	c/T@1287; t/C@1509; t/C@1794	none	c/T@1287; t/C@1509	t/C@1794*	none	yes	
2	t/C@1509; t/C@1671; t/C@1794; c/A@1854; c/T@2064	none	t/C@1509	t/C@1671; t/C@1794; C/A@1854	none	yes	
3	c/T@1368; t/C@1509; c/T@1657; c/T@1737; t/C@1794; g/A@1902	none	c/T@1368; t/C@1509*	c/T@1657; c/T@1737; t/C@1794; g/A@1902	none	yes	
4	c/T@1368; t/C@1509; c/T@1657; c/T@1737; t/C@1794; g/A@1902; c/A@2076; c/T@2106	none	c/T@1368; t/C@1509	c/T@1657; c/T@1737; t/C@1794; g/A@1902	none	yes	
5	c/T@1122; c/T@1203; t/C@1509; t/C@1671; c/A@1773; **t/G@1774**; t/C@1794	**C592G**	c/T@1122; c/T@1203; t/C@1509	t/C@1671; c/A@1773; **t/G@1774**; t/C@1794	**C592G**	yes	
6	t/C@1509; c/T@1575; t/C@1794; c/G@1815	D605E	t/C@1509	c/T@1575; t/C@1794; c/G@1815	D605E	yes	

Table S2. Continued.

Patient	results cycle sequencing[1)]		results MSCSA[2)]			concordance MSCSA and cycle sequencing	comment
	nt changes in amplicon (1110-2124)	**Polymorphisms/ Mutations**	**nt changes in amplicon 97AB (1090-1558)**	**nt changes in amplicon 97CD (1513-1980)**	**Polymorphisms/ Mutations**		
7	g/A@2057	R686Q	no SNPs*	no SNPs	R686Q	yes	
8	c/T@1368; t/C@1509; t/C@1794; c/A@1869	none	c/T@1368; t/C@1509	t/C@1794; c/A@1869	none	yes	
9	c/T@1287; c/T@1405; t/C@1509; t/C@1794; c/T@2106	H469Y	failed	t/C@1794*	H469Y	yes	
10	c/T@1242; c/T@1368; g/A@1467; t/C@1509; c/T@1657; c/T@1737; t/C@1794; g/A@1902	none	c/T@1242; c/T@1368; g/A@1467; t/C@1509	c/T@1657; c/T@1737; t/C@1794; g/A@1902	none	yes	
11	g/A@1321; c/T@1368; t/C@1509; a/G@1529; c/T@1657; c/T@1737; t/C@1794; g/A@1902	N510S, D441N	c/T@1368; t/C@1509; a/G@1529*	c/T@1657; c/T@1737; t/C@1794; g/A@1902*	N510S	no; see comment	g/A@1321 discrepancy with original cycle sequencing result was confirmed by sequencing of MSCSA PCR products
12	c/T@1368; t/C@1509; a/G@1529; c/T@1657; c/T@1737; **c/T@1781**; t/C@1794; g/A@1902	N510S; **A594V**	c/T@1368; t/C@1509; a/G@1529	c/T@1657; c/T@1737; **(c/T@1781)**; t/C@1794; g/A@1902	N510S; **(A594V)**	yes; see comment	c/T@1781 discrepancy between duplicates were confirmed by cycle sequencing of MSCSA PCR products

Patient	results cycle sequencing[1)]		results MSCSA[2)]			concordance MSCSA and cycle sequencing	comment
	nt changes in amplicon (1110-2124)	Polymorphisms/ Mutations	nt changes in amplicon 97AB (1090-1558)	nt changes in amplicon 97CD (1513-1980)	Polymorphisms/ Mutations		
13	c/T@1368; g/A@1432; t/C@1509; a/G@1529; c/T@1657; c/T@1737; **c/T@1781**; t/C@1794; g/A@1902; c/T@2106	A478T; N510S; **A594V**	failed	c/T@1657; c/T@1737; **c/T@1781**; t/C@1794; g/A@1902	A478T; N510S; **A594V**	yes	
14	c/T@1368; t/C@1509; c/T@1657; c/T@1737; t/C@1794; g/A@1902	none	c/T@1368; t/C@1509	c/T@1657; c/T@1737; t/C@1794; g/A@1902	none	yes	
15	none	none	no SNPs	no SNPs	none	yes	
16	c/T@1242; a/G@1400; g/A@1467	N467S	failed	no SNPs	none	yes	polymorphism was not identified because MSCSA of amplicon 97AB failed
17	c/T@1368; t/C@1509; c/T@1657; c/T@1737; t/C@1794; g/A@1902	none	c/T@1368; t/C@1509	c/T@1657; c/T@1737; t/C@1794; g/A@1902	none	yes	
18	none	none	no SNPs	no SNPs	none	yes	
19	c/T@1287; c/T@1405; t/C@1509; t/C@1794; **c/T@1809**	H469Y; **C603W**	c/T@1287; c/T@1405; t/C@1509*	t/C@1794*	H469Y	no; see comment	c/T@1809 discrepancy with original cycle sequencing result was confirmed by sequencing of MSCSA PCR products

Table S2. Continued.

Patient	results cycle sequencing[1)]		results MSCSA[2)]			concordance MSCSA and cycle sequencing	comment
	nt changes in amplicon (1110-2124)	Polymorphisms/ Mutations	nt changes in amplicon 97AB (1090-1558)	nt changes in amplicon 97CD (1513-1980)	Polymorphisms/ Mutations		
20	t/C@1509; g/A@1723; t/C@1794	E575K	failed	t/C@1794	none	no; see comment	g/A@1723 discrepancy with original cycle sequencing result was confirmed by sequencing of MSCSA PCR products
21	t/C@1509; t/C@1794	none	t/C@1509*	t/C@1794*	none	yes	
22	c/T@1287; c/T@1405; t/C@1509; t/C@1794	H469Y	c/T@1287; c/T@1405; t/C@1509*	t/C@1794	H469Y	yes	
23	t/C@1509; t/C@1794	none	t/C@1509*	t/C@1794	none	yes	
24	none	none	failed	no SNPs	none	yes	
25	g/A@1188; c/T@1287; c/T@1368; t/C@1509; a/G@1529; c/T@1657; c/T@1737; t/C@1794; g/A@1902; c/T@1959	none	a/G@1188; c/T@1287; c/T@1368; t/C@1509; a/G@1529	c/T@1657; c/T@1737; t/C@1794; g/A@1902; c/T@1959	none	yes	
26	c/T@1368; t/C@1509; c/T@1657; c/T@1737; t/C@1794; g/A@1902	none	c/T@1368; t/C@1509	c/T@1657; c/T@1737; t/C@1794; g/A@1902	none	yes	
27	c/T@1119; t/C@1509; t/C@1671; t/C@1794	none	c/T@1119; t/C@1509	t/C@1671; t/C@1794	none	yes	
28	none	none	no SNPs	no SNPs	none	yes	
29	c/T@1245; t/C@1509; t/C@1794	none	t/C@1509	t/C@1794	none	no	c/T@1245 only found by cycle sequencing

Patient	results cycle sequencing[1)]		results MSCSA[2)]			concordance MSCSA and cycle sequencing	comment
	nt changes in amplicon (1110-2124)	Polymorphisms/ Mutations	nt changes in amplicon 97AB (1090-1558)	nt changes in amplicon 97CD (1513-1980)	Polymorphisms/ Mutations		
30	c/T@1287; c/T@1405; t/C@1509; t/C@1794	H469Y	c/T@1287; c/T@1405; t/C@1509	t/C@1794	H469Y	yes	
31	c/T@1368; t/C@1509; c/T@1657; c/T@1737; t/C@1794; g/A@1902	none	c/T@1368; t/C@1509*	c/T@1657; c/T@1737; t/C@1794; g/A@1902	none	yes	
32	t/C@1509; t/C@1671; t/C@1794	none	c/T@1287; t/C@1509*	t/C@1671; t/C@1794	none	no	c/T@1287 only found by MSCSA
33	g/A@1227; c/T@1368; t/C@1509; c/T@1657; c/T@1737; t/C@1794; g/A@1902	none	c/T@1368; t/C@1509*	c/T@1657; c/T@1737; t/C@1794; g/A@1902	none	no	g/A@1227 only found by cycle sequencing
34	t/C@1509; **c/T@1772**; t/C@1794 or t/C@1509; **g/T@1785**; t/C@1794	**A591V** or **L595F**	t/C@1509*	(**c/T@1772**); (**g/T@1785**); t/C@1794	**(A591V); (L595F)**	yes; see comment	c/T@1772 and g/T@1785 discrepancies between duplicates were confirmed by cycle sequencing of MSCSA PCR products
35	t/C@1671; t/C@1794	none	failed	t/C@1671; t/C@1794	none	yes	
36	c/T@1368; t/C@1509; c/T@1657; g/A@1719; c/T@1737; t/C@1794; g/A@1902	none	c/T@1368; t/C@1509*	c/T@1657; g/A@1719; c/T@1737; t/C@1794; g/A@1902	none	yes	

Table S2. Continued.

Patient	results cycle sequencing[1)]		results MSCSA[2)]			concordance MSCSA and cycle sequencing	comment
	nt changes in amplicon (1110-2124)	Polymorphisms/ Mutations	nt changes in amplicon 97AB (1090-1558)	nt changes in amplicon 97CD (1513-1980)	Polymorphisms/ Mutations		
37	c/T@1657; c/T@1737; c/T@1752; t/C@1794; g/A@1902	none	failed	c/T@1657; c/T@1737; c/T@1752; t/C@1794; g/A@1902*	none	yes	
38	c/T@1368; t/C@1509; c/T@1657; c/T@1737; t/C@1794; g/A@1902	none	c/T@1368; t/C@1509*	c/T@1657; c/T@1737; t/C@1794; g/A@1902	none	yes	
39	c/T@1368; **a/G@1378;** t/C@1509; c/T@1657; c/T@1737; t/C@1794; g/A@1902; c/T@2106	**M460V**	failed	c/T@1657; c/T@1737; t/C@1794; g/A@1902*	none	yes	Mutation was not identified because MSCSA of amplicon 97AB failed
40	g/A@1467	none	g/A@1467	no SNPs	none	yes	
41	c/T@1368; **g/T@1380**; t/C@1509; c/T@1657; c/T@1737	**M460I**	failed	failed	none	n.a.	Mutation was not identified because MSCSA failed
42	none	none	no SNPs	no SNPs	none	yes	
43	c/T@1287; c/T@1405; t/C@1509; t/C@1794	none	c/T@1287; c/T@1405; t/C@1509	t/C@1794	none	yes	

[1)] SNPs in bold mark resistance associated mutations; SNPs outside MSCSA amplicon underlined. [2)] SNPs between brackets were found in one of two duplicate experiments. * unable to amplify duplicate product. n.a. not applicable

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Failure of preemptive treatment of cytomegalovirus infections and antiviral resistance in stem cell transplant recipients

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ABSTRACT

Background: Treatment of cytomegalovirus (CMV) infections after stem cell transplantation (SCT) does not always lead to a rapid viral response. The causes of treatment failure may be either viral resistance or immunological failure to control viral replication. This study investigated the response to preemptive treatment in CMV infections in order to define risk factors for treatment failure, including the role of antiviral resistance.
Methods: Adult recipients of allogeneic T-cell depleted SCT were studied retrospectively (n = 92). CMV infections were treated with (val)ganciclovir according to a CMV DNA load based preemptive strategy. Treatment failure was defined as a CMV DNA load of 1000 copies/ml or more after at least 2 weeks of treatment. Resistance was analyzed by nucleotide sequence analysis of the UL97 and UL54 genes in the first CMV DNA positive sample and in samples during treatment failure.
Results: Treatment failure occurred in 26 of the 47 preemptively treated patients (55%) and in 39 of 86 (45%) treatment episodes. The risk of treatment failure was increased during first treatment episodes ($p = 0.01$) and during the use of immunosuppressive medication ($p = 0.02$). Antiviral resistance was found in only 1 patient (4%) with treatment failure.
Conclusions: A slow response to preemptive antiviral treatment occurred frequently in CMV infections in SCT recipients. Antiviral resistance was observed but played a minor role in treatment failure.

INTRODUCTION

Preemptive therapy of cytomegalovirus (CMV) infections based on virological monitoring has been proven effective and has become common practice after hematopoietic stem cell transplantation (SCT) in many centres. Quantitative viral load measurements can additionally be used for monitoring treatment responses. In this way, it has been shown that preemptive treatment of CMV infections after SCT does not always lead to a rapid, complete and sustained viral response. By sensitive assays such as real-time PCR, viral DNA can be detected for days to weeks after treatment,[1;2] frequently considerably longer as compared to detection of pp65 antigenemia. The clinical significance of prolonged CMV DNAemia is not always clear. It may predict recurrence of CMV DNAemia or development of CMV disease, but persistent infections may also be cleared spontaneously or respond to a repeated treatment course without complications.[1;3;4] The causes of viral DNA persistence despite antiviral treatment may be viral resistance on the one hand or immunological failure to control viral replication on the other. Risk factors associated with treatment failure and in particular the role of resistant CMV in a preemptive treatment setting after SCT are largely unknown.

In guiding treatment of persistent CMV infections, it is important to know the contribution of the various causes of treatment failure. This study investigated the occurrence and risk factors of failure of preemptive treatment of CMV infections in SCT patients, focusing on the possible role of antiviral resistance.

METHODS

Patient data

Consecutive adult recipients of an allogeneic T-cell depleted stem cell transplant between 2005 and 2008 at risk for CMV (donor and/or recipient CMV seropositive) were included (n = 92). Follow-up was one year after transplantation. T cell-depleted transplantation was performed either according to a reduced intensity conditioning protocol or a myeloablative conditioning regimen as described previously [5-7]. In the absence of graft versus host disease (GvHD) or graft failure, patients received donor lymphocyte infusion for treatment of mixed chimerism or relapsed disease, at least 3 months (median of 187 days) after transplantation.

CMV treatment

CMV DNA loads in EDTA plasma were determined prospectively by quantitative real-time PCR as previously described.[8] A preemptive treatment protocol[8] with valganciclovir (vGCV, 900 mg twice daily for 14 days) was applied based on CMV DNA load. Treatment was initiated if the load exceeded 10^4 copies/ml or was above 10^3 copies/ml combined with an at least tenfold increase in comparison to the preceding week. Subsequent DNAemia episodes were treated if the load was above 10^5 copies/ml or above 10^4 copies/ml and increasing at least tenfold in one week. Treatment was continued as long as the CMV DNA load was above these thresholds. Symptomatic CMV infection was treated with intravenous ganciclovir (GCV, 5 mg/ kg twice daily for 14 days). Oral ganciclovir was not applied.

Data collection

Patient and treatment data were collected retrospectively from patient charts. The recorded baseline data are shown in Table 1. The follow-up data included the use of antiviral medication, the use of immunosuppressive medication (therapeutic, i.e. non-prophylactic, use of systemic corticosteroids, cyclosporine, rituximab) and the total lymphocyte count, signs and symptoms of CMV end-organ disease[9] and patient survival. Renal function was recorded to check for adequate dosing of antiviral medication.

Analysis of resistance

Analysis of resistance was performed by nucleotide sequence analysis of CMV DNA from plasma samples as previously described.[10] The amplified region ranged from codon 370 to 708 of the *UL97* gene, covering all previously described mutations.[11] Sequence analysis of codon 262 to 1169 of the *UL54* gene was performed on DNA isolates with *UL97* mutations or from patients using foscarnet or cidofovir. Sequences were compared to the sequence of the GCV susceptible AD169 strain and to pre-treatment samples from the patient. Baseline resistance was determined in the first sample from each patient containing at least 1000 copies/ ml of CMV DNA. Resistance during treatment was determined by analysis of subsequent plasma samples containing at least 1000 copies/ ml of CMV DNA after at least two weeks of treatment of CMV infection. Genotypic resistance was defined as the presence of resistance-associated mutations that have been published previously (as proven by marker transfer).

Statistical analysis

CMV DNAemia was defined as any detectable CMV DNA load in plasma. A treatment episode was defined as a period of antiviral treatment of 14 days. Treatment failure was

Table 1. Baseline characteristics of the 92 patients.

	Patients	
	n	(%)
Sex		
Male	54	(59)
Age		
Years (mean, range)	48	(21-70)
Underlying disease		
ALL	9	(10)
AML	29	(32)
CLL	3	(3)
CML	9	(10)
MDS	5	(5)
MM	14	(15)
Other	23	(25)
Transplant type		
Peripheral blood stem cells	91	(99)
Bone marrow	1	(1)
Conditioning regimen		
Myeloablative	48	(52)
Non-myeloablative	44	(48)
Donor type		
Matched sibling donor	51	(55)
Matched unrelated donor	39	(42)
Other[1]	2	(2)
Serostatus donor		
CMV-seropositive	56	(61)
Serostatus recipient		
CMV-seropositive	75	(82)
Serostatus combination		
Donor -/ recipient +	36	(39)
Donor +/ recipient -	17	(19)
Donor +/ recipient +	39	(42)
Total	**92**	(100)

Abbreviations: ALL = acute lymphoblastic leukaemia, AML = acute myelogenous leukaemia, CLL = chronic lymphocytic leukaemia, CML = chronic myelogenous leukaemia, MDS = myelodysplastic syndrome, MM = multiple myeloma

[1] haplo-identical related donor (n = 1), mismatched related donor (n = 1)

defined as the presence of at least 1000 copies/ ml of CMV DNA in plasma at the end of a treatment episode. In view of the repeated measurement of treatment failure as a binary variable (failure yes/no) within patients, the outcome was modelled as a repeated mea-

sures logistic regression. Parameters were estimated using the Generalized Estimating Equations procedure in SPSS 16 (SPSS Inc., Chicago, IL, USA) with first-order autoregressive correlation structure and a robust estimation procedure. Univariable analyses of potential predictors was performed with p-values <0.20 as a criterion for possible inclusion in a multivariable model.

RESULTS

CMV infections

Plasma samples and treatment data were available from all 92 transplanted patients at risk for CMV (donor and/or recipient CMV seropositive). Baseline characteristics of the patients are shown in Table 1. CMV DNAemia was detected in 67 of all 92 patients (73%). Pre-emptive treatment for CMV infections was administered in 47 of 67 patients with CMV DNAemia; in total 96 treatment episodes occurred of which 86 were evaluable with respect to treatment response. CMV treatment was initiated after a median of 33 days after transplantation. CMV disease occurred in five patients; three patients with pneumonitis, one patient with encephalitis and one patient with retinitis. During the first year after transplantation 30 patients (33%) died, including the five patients with CMV disease.

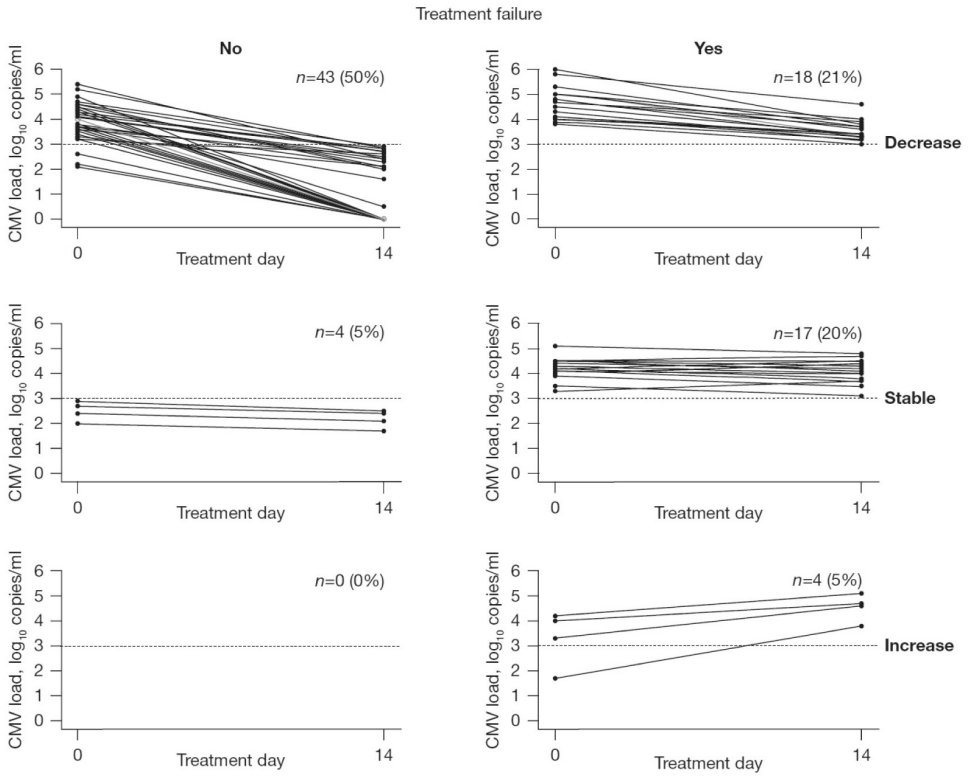
Treatment failure

Treatment failure occurred in 55% of the treated patients (26 of 47), corresponding to failure in 39 of 86 (45%) treatment episodes. In 4 of the failing episodes (4.7%) the viral load increased during treatment, in 17 episodes (19.7%) the viral load remained stable above 1000 copies/ml despite treatment and in 18 episodes (20.9%) the viral load decreased but remained above 1000 copies/ml (Figure 1). Nineteen patients (73%) experienced a single episode of treatment failure, three patients (12%) had persistent treatment failure lasting multiple episodes of antiviral treatment and four patients (15%) had recurrent episodes of treatment failure.

In 25 of the 26 patients (96%) treatment failure occurred during the first treatment episode. The cumulative duration of all treatment episodes in the first year after transplantation was 15 and 29 days (median) in the patients without and with treatment failure respectively. CMV disease was present in 4 of 26 treated patients with (recurrent or persistent) treatment failure (15%) and in 1 of 21 patients (4.8%) without treatment failure (odds ratio 3.63, $p = 0.27$).

In univariable analysis (Table 2), the risk of treatment failure was increased during first treatment episodes, during the therapeutic use of immunosuppressive medication and in patients with a higher CMV load at the start of treatment. Conditioning regimen, donor

Figure 1. Cytomegalovirus (CMV) treatment episodes categorized according to viral kinetics and treatment failure.



Treatment course and failure could be evaluated in 86 of 96 treatment episodes (14 days of antiviral treatment). Treatment failure was defined as the presence of at least 1000 copies/ ml of cytomegalovirus DNA in plasma after at least two weeks of treatment. A change in viral load of ≥ 0,5 ^{10}log copies/ml was considered an increase or decrease.

type, CMV serostatus, lymphocyte counts and the timing after SCT were not significant predictors of treatment failure. Underdosage of antiviral medication (defined as a dosage lower than valganciclovir 900 mg twice daily or ganciclovir 5 mg/kg twice daily excluding cases of dose adjustments for impaired renal function) was associated with a decreased occurrence of treatment failure (Table 2). In multivariable analysis (Table 2), the risk of treatment failure was increased during first treatment episodes and during the use of immunosuppressive medication.

Antiviral resistance

Resistance development could be monitored by analyzing baseline samples from 58 of 67 patients with CMV DNAemia and follow-up samples from 23 of 26 patients with

Table 2. Treatment failure.

	Treatment failure		Univariable analysis			Multivariable analysis		
	No	Yes						
	(% of episodes)	(% of episodes)	Odds-ratio	95% confidence interval	*p*-value	Odds-ratio	95% confidence interval	*p*-value
Sex								
Male	51,3	48,7	1,26	(0,53-2,96)	0,60			
Female	57,4	42,6		reference category				
Age, years, median	53,0	51,0	0,97	(0,94-1,01)	0,15	0,98	(0,94-1,03)	0,49
Underlying disease								
ALL	46,2	53,8	[b]			[b]		
AML	63,3	36,7	[b]			[b]		
CLL	50,0	50,0	[b]			[b]		
CML	71,4	28,6	[b]			[b]		
MDS	66,7	33,3	[b]			[b]		
Other	45,2	54,8	[b]			[b]		
Conditioning regimen								
Myeloablative	50,0	50,0	1,61	(0,67-3,86)	0,29			
Non-myeloablative	61,1	38,9		reference category				
Donor type								
Matched sibling donor	54,5	45,5	1,19	(0,61-2,34)	0,61			
Matched unrelated donor	53,5	46,5	1,25	(0,65-2,40)	0,51			
Other	60,0	40,0		reference category				
CMV serostatus donor (D)/ recipient (R)								
D -/ R +	53,1	46,9	1,20	(0,50-2,87)	0,69			
D +/ R -	50,0	50,0	1,40	(0,43-4,50)	0,58			
D +/ R +	58,1	41,9		reference category				
Treatment onset	58,0	44,0	1,00	(0,99-1,00)	0,47			
day after SCT, median								

Table 2. Continued.

	Treatment failure		Univariable analysis			Multivariable analysis		
	No	Yes						
	(% of episodes)	(% of episodes)	Odds-ratio	95% confidence interval	*p*-value	Odds-ratio	95% confidence interval	*p*-value
Ranking of treatment								
First episode	44,4	55,6	4,05	(1,60-10,28)	0,003*	4,44	(1,39-14,16)	0,01*
Subsequent episode	65,9	34,1		reference category			reference category	
Underdosage of antiviral medication								
No	51,3	48,7		reference category			reference category	
Yes	87,5	12,5	0,15	(0,06-0,35)	0,000*	1,01	(0,33-3,04)	0,99
CMV load at start of treatment	3,8	4,2	2,31	(1,25-4,30)	0,01*	2,16	(0,68-6,87)	0,19
^{10}log copies/ml, median								
Immunosuppressive medication [a]								
No	62,7	37,3		reference category			reference category	
Yes	37,0	63,0	2,82	(1,14-6,98)	0,03*	4,10	(1,23-13,66)	0,02*
Lymphocyte count	0,27	0,15	0,70	(0,43-1,15)	0,16	0,58	(0,25-1,33)	0,20
$\cdot 10^9$ cells/litre, median								
	55	45						
Total								
	(n = 47)	(n = 39)						

Predictors of treatment failure were analyzed by repeated measures logistic regression using the Generalized Estimating Equations procedure in SPSS 16 with first-order autoregressive correlation structure and a robust estimation procedure. For multivariable analysis potential predictors were included with p-values <0.20 in univariable analysis.
Abbreviations: ALL = acute lymphoblastic leukaemia, AML = acute myelogenous leukaemia, CLL = chronic lymphocytic leukaemia, CML = chronic myelogenous leukaemia, MDS = myelodysplastic syndrome, MM = multiple myeloma
[a] Therapeutic use of (combinations of) corticosteroids (22 episodes), cyclosporine (21 episodes) or rituximab (11 episodes), mainly for graft-versus-host disease or post-transplant lymphoproliferative disorder; [b] not determined because of insufficient power for analysis of various small strata; *significant at a 0.05 level.

treatment failure. Each patient with treatment failure was investigated for antiviral resistance in samples every two weeks until the CMV DNA load fell below 1000 copies/ml. The median number of samples for each patient was 2 (range 1-7). Most follow-up samples (75%) were retrieved after two weeks of treatment (range 2-13 weeks of treatment). No resistance associated mutations were found in the *UL97* gene in the 58 pre-treatment samples. Resistance at treatment failure was found in 1 of 23 patients (4%). In this previously described patient[12] with recurrent treatment failure, CMV DNAemia first occurred at day 27 after transplantation, which was treated with vGCV (900 mg twice daily). Subsequently, the patient was treated with vGCV prophylaxis (450 mg twice daily) because of persistent low levels of CMV DNA. However, from day 136 onward, CMV DNA loads steadily increased and eventually progression to CMV encephalitis occurred. Viral DNA with the resistance associated A594V mutation in the *UL97* gene was found in plasma and cerebrospinal fluid samples from day 136 onwards, after 109 days of antiviral treatment. In the other 22 patients with treatment failure, no resistance associated mutations were found in the *UL97* gene (30 samples) or the *UL54* gene (7 samples).

DISCUSSION

In this study, the response to preemptive antiviral treatment of CMV infections after SCT and the role of antiviral resistance in treatment failure were studied. Based on regular monitoring of CMV DNA in plasma, CMV DNA levels during treatment were analyzed. Approximately half of the patients still had CMV DNAemia of at least 1000 copies/ml after a standard course of antiviral treatment of two weeks. Antiviral resistance played only a minor role in such persistent CMV DNAemia, with resistance-associated mutations found in only one patient with treatment failure and in none of the baseline samples.

The definition of treatment failure as a CMV DNA load of at least 1000 copies/ ml after at least two weeks of treatment was chosen to include those patients in whom prolongation of treatment is commonly considered. It appeared that patients with treatment failure according to our definition indeed had a longer duration of treatment for CMV than patients without treatment failure. Treatment failure can also be defined using viral dynamics and, for example, treatment failure defined as less then 2log decrease in viral load after 14 days of treatment would have resulted in a comparable prevalence of failure in 49% of the episodes (MTvdB *et al.*, data not shown). Although the persistence of viral DNA in plasma is no direct proof of ongoing viral replication, it is likely that the

half-life of viral DNA is short and, therefore, that plasma DNAemia is a correlate for recent viral replication.[13] This view is supported by the fact that viral loads decreased rapidly in many patients in our study.

The rate of treatment failure of 45% of all episodes was somewhat higher than observed in an earlier study in our hospital (Leiden University Medical Center, Leiden, The Netherlands), which found failure in 20-25% of episodes, despite comparable patient and episode characteristics.[3] This may be due to a longer duration of follow up in the current study. Our results are comparable to two other studies where rising antigenemia under treatment occurred in 45% of patients[4] and PCR positivity was found in 39% of patients after treatment.[14] A recent prospective cohort study showed persistent CMV infection after 21 days of antiviral treatment in only 4 of 59 (7%) SCT patients.[15] The different definition of treatment failure (21 versus 14 days and inclusion of DNAemia as well as antigenemia, viral culture and histopathology) probably contributed to this variation. Differences in antiviral treatment regimen, which was not further specified, may also play a role. A lack of association between treatment failure and CMV disease in our study may be explained by the small number of patients with CMV disease due to the effective preemptive treatment strategy.

In our study, all patients treated for CMV DNAemia were tested for resistance, regardless of symptoms or clinical suspicion of resistance. Most previous publications on resistance comprised case reports or small case series of patients with CMV disease and did not assess the overall prevalence of resistant CMV. Only a few earlier studies are available in which resistance has been systematically studied in persistent CMV infections in SCT patients undergoing preemptive treatment.[4;15;16] A prospective study found resistance in 1 of 4 SCT patients with persistent viral replication.[15] Low-level resistance (as determined by phenotypic methods) was found in 1 of 15 patients with rising antigenemia levels under treatment[4] and in none of 10 patients with positive PCR results after two weeks of antiviral treatment.[16] In the setting of recurrent CMV after preemptive treatment, one study observed resistance in 1 of 13 patients.[17] These results are in accordance with the low prevalence of resistance found in our study, demonstrating that this does not provide a likely explanation for the majority of cases with treatment failure. There is a small chance that *UL54* mutations have been missed due to our strategy of only analyzing samples from patients harbouring *UL97* mutants or from patients who had been treated with the antivirals (foscarnet and cidofovir) that directly target the viral DNA polymerase *UL54.*[18;19] However, previous studies have shown that the majority of resistance-associated mutations are found the *UL97* gene.[15;20]

Treatment failure was found most often during first treatment episodes. Probably, the development of CMV-specific immunity during CMV infection facilitates treatment responses in subsequent episodes. Furthermore, the use of immunosuppressive medication, mainly for GvHD or post-transplant lymphoproliferative disorder treatment, during antiviral treatment increased the risk of treatment failure. Likewise, in previous studies, the use of high-dose corticosteroids was a significant risk factor for persistent or increasing pp65 antigenemia.[4;21] Obviously, the time needed to clear DNAemia or antigenemia is related to the amount of virus at start of treatment[22] and, indeed, in univariable analysis, a high viral load at start of treatment was associated with a higher risk of treatment failure. In multivariable analysis this effect was insignificant, however, suggesting a more complex relationship between viral load, treatment failure, failure of immunological recovery and, likely, viral resistance. A sustained lack of (CMV-specific) T-cells after transplantation, for example due to the use of immunosuppressive medication, probably allows for high viral loads and, hence, persistent or recurrent CMV infection.[23] This necessitates repeated and prolonged antiviral treatment and may also lead to the development of antiviral resistance.

Indeed, the prolonged treatment with a low dose of vGCV in the only patient with a *UL97* mutant, may have led to the development of resistance due to the erroneous use of secondary prophylaxis during an ongoing viral reactivation.[12] Surprisingly, suboptimal dosages of antiviral medication were not associated with treatment failure, but were administered more often during successful treatment episodes. This appears to be the confusing result of the off-protocol use of secondary prophylaxis with vGCV 900 mg once daily in certain patients. Those patients had a low viral load after a standard treatment course and then received secondary prophylaxis with low dose valganciclovir. Either spontaneously or due to the antiviral treatment, CMV DNAemia was rapidly cleared. This unexpected association was insignificant in multivariable analysis.

In conclusion, in SCT patients, CMV infections with a slow response to antiviral treatment occurred frequently. Antiviral resistance was observed but played a minor role in treatment failure.

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MB designed and performed the study research, analysed the data and wrote the paper, EM designed the study, AV analysed the data, CB performed the research, RW analysed the data, CH contributed clinical data, EC analysed the data, AK designed the study.

DISCLOSURE STATEMENT

The authors declare no competing interest.

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7

Preemptive versus sequential prophylactic-preemptive treatment regimens for CMV in renal transplantation: comparison of treatment failure and antiviral resistance.

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ABSTRACT

Background: Cytomegalovirus (CMV) infections after transplantation are commonly treated using a prophylactic or preemptive regimen with (val)ganciclovir. It remains unclear which approach is most effective in preventing CMV disease in D+R- patients. The aim of this retrospective study was to compare treatment response and antiviral resistance in CMV infections between two treatment regimens in D+R- renal transplant recipients.

Methods: Before 2006, a preemptive treatment regimen with valganciclovir was applied (42 patients). From 2006 onwards, patients first received prophylaxis with valganciclovir for 90 days, followed by a preemptive regimen (29 patients). CMV infections were monitored by regular determination of the CMV-DNA load in plasma. Patient charts were reviewed for antiviral treatment data and resistance was analyzed by nucleotide sequence analysis of the UL97 and UL54 genes in CMV-DNA positive samples.

Results: Treatment failure, defined as a CMV DNA load ≥ 1000 copies/ml after at least 2 weeks of treatment, occurred less frequently in the prophylaxis cohort than in the preemptive cohort (14% versus 71%, $p < 0.001$). No CMV end-organ disease occurred in either cohort. Resistant viral isolates were found during treatment in one patient in the prophylaxis cohort versus in three patients in the preemptive group. All CMV infections with resistant virus were cleared without switch of (val)ganciclovir treatment.

Conclusions: Treatment failure of CMV infections occurred less frequently in D+R- renal transplant patients on a sequential prophylaxis-preemptive regimen, than in patients on a purely preemptive regimen. Antiviral resistance was observed infrequently and apparently played a minor role in treatment failure.

INTRODUCTION

Prophylactic and preemptive regimens are widely applied in renal transplant recipients to reduce morbidity and mortality associated with cytomegalovirus (CMV) disease. Patients at high risk for CMV disease after transplantation are transplant recipients who are CMV-seronegative prior to transplantation and receive an organ from a CMV-seropositive donor (D+R-). Without preventive measures, CMV disease occurs in about 70% of D+R- patients.[1;2]

It remains unclear whether a prophylactic or a preemptive approach is most effective in preventing CMV disease in D+R- patients.[3] In a direct comparison of prophylaxis versus preemptive treatment in renal transplant recipients using valganciclovir (vGCV), prophylaxis reduced the incidence of CMV infection in all transplant recipients, except for D+R- patients.[4] Furthermore, no reduction in symptomatic episodes was observed.[4] In a trial using oral ganciclovir (GCV) for prophylaxis versus intravenous GCV for preemptive treatment, prophylaxis also reduced the incidence of CMV infection and increased long-term graft survival in all recipients[5]. No separate data were available for D+R- patients.[5] After prophylaxis, late onset CMV disease still occurs in about 30% of D+R- transplant patients, necessitating follow up after prophylaxis.[6-9]

Treatment of CMV infections in D+R- patients does not always lead to a rapid and sustained viral response.[4;10] Such treatment failure may be related to the type of treatment regimen, to immunological factors or it may be caused by viral resistance. Resistance of CMV to antiviral agents can occur after prolonged exposure to antiviral drugs,[11] both in prophylactic and preemptive treatment regimens. Yet, it is unknown if either type of CMV treatment regimen predisposes to the development of antiviral resistance.

Our hospital has recently chosen to change its strategy for the prevention of CMV disease in D+R- renal transplant recipients. The purely preemptive strategy has been replaced by a three-month prophylaxis regimen followed by preemptive treatment. In this retrospective study, we compared the incidence and course of CMV infections, the frequency of treatment failure of CMV infections and the role of antiviral resistance between the purely preemptive versus the prophylaxis followed by preemptive treatment regimen.

PATIENTS AND METHODS

Patients

Consecutive D+R- kidney and kidney-pancreas transplant recipients transplanted in the Leiden University Medical Center between 2004 and 2008 were included (n = 78).

Table 1. Baseline characteristics of the included patients.

		regimen		
		preemptive	**prophylaxis**	
		n = 42	n = 29	
		n (% of patients)	n (% of patients)	
age at Tx	age (years, mean)	46	49	n.s.
gender	male	24 (57)	18 (62)	n.s.
underlying disease	PKD	7 (17)	3 (10)	n.s.
	diabetes mellitus	9 (21)	8 (28)	
	hypertension	4 (10)	3 (10)	
	other	22 (52)	15 (52)	
type of Tx	kidney	35 (83)	25 (86)	n.s.
	kidney-pancreas	7 (17)	4 (14)	
type of donor	living related	11 (26)	8 (28)	n.s.
	living unrelated	6 (14)	7 (24)	
	post mortal HB	12 (29)	10 (35)	
	post mortal non-HB	13 (31)	4 (14)	
immunosuppressive regimen directly after Tx	prednisone	42 (100)	29 (100)	n.s.
	cyclosporine	32 (76)	27 (93)	
	tacrolimus	10 (24)	2 (7)	n.s.
	mycophenolate	42 (100)	29 (100)	

Tx, transplantation; HB, heart beating; PKD, polycystic kidney disease; n.s., not significant ($p > 0.05$).

Follow-up was one year after transplantation. The initial immunosuppressive regimen consisted of prophylaxis with either antibodies against the IL-2 receptor alpha-chain (basiliximab/daclizumab) or anti-thymocyte globulin (ATG, Fresenius Kabi, Utrecht, The Netherlands) for kidney and kidney-pancreas transplant recipients, respectively. Maintenance immunosuppression consisted of three immunosuppressive drugs including prednisolone, either tacrolimus or cyclosporine and either mycophenolate mofetil or mycophenolic acid (see Table 1). Exposition to tacrolimus and cyclosporine was monitored by area-under-the-curve (AUC) measurements. First acute rejection episodes were treated with methylprednisolone and second rejection episodes were treated with ATG (Merieux, Genzyme, Naarden, The Netherlands). The immunosuppressive regimen, target AUC ranges and rejection treatment were unchanged throughout the study period.

CMV treatment

During the study period, the protocols for CMV prevention and treatment were changed from a purely preemptive strategy to initial prophylaxis followed by a preemptive regimen. In all regimens, (v)GCV dosing was adapted according to renal function (dose reduction if creatinin clearance was below 60 ml/min). Before 2006, a preemptive treatment regimen with valganciclovir (900 mg twice daily for 14 days) was applied which was guided by the CMV DNA load. Treatment was initiated at the first detectable CMV DNA load after transplantation. Subsequent DNAemia episodes were treated if the load was above 100.000 copies/ml or above 10.000 copies/ml and increasing at least tenfold in one week. From 2006 onwards, patients received prophylaxis with vGCV (900 mg once daily) for 90 days, followed by a preemptive regimen. CMV DNAemia occurring after prophylaxis was treated (900 mg twice daily for 10-14 days) if the load was above 100.000 copies/ml or above 10.000 copies/ml and increasing at least tenfold in one week. In both regimens, symptomatic CMV infection was treated with intravenous GCV (5 mg/kg twice daily for 14 days) and, if possible with regard to the risk of rejection, the use of mycophenolate mofetil or mycophenolic acid was reduced or temporarily discontinued.

Data collection

Patient and treatment data were collected retrospectively from patient charts. Baseline data that were registered included age, sex, underlying renal disease, type of donor and transplantation, CMV serostatus of donor and recipient prior to transplantation and immunosuppressive medication directly after transplantation. Follow-up data that were collected were immunosuppressive medication at onset of each CMV DNAemia episode, rejection treatment episodes, signs and symptoms of CMV end-organ disease,[12] renal function at six and twelve months after transplantation, patient survival and graft survival. Start and end date, and dosage of antiviral medication were documented. Creatinin clearance based on 24-hour urine collection and creatinin serum levels was registered at start of antiviral medication and subsequently monthly during antiviral medication to check for adequate dosing of antiviral medication in relation to renal function.

CMV monitoring

CMV DNA loads in EDTA plasma had been determined prospectively by quantitative real-time PCR.[13] CMV IgG (Vidas 30, Biomérieux, Zaltbommel, The Netherlands) was determined retrospectively in EDTA plasma samples.

Antiviral resistance analysis

Analysis of resistance was performed by nucleotide sequence analysis on CMV DNA from plasma samples. Cycle sequencing reactions were performed after PCR and nucleotide sequence analysis was performed on an ABI Prism 3100 Genetic Analyser (Applied Biosystems, Warrington, United Kingdom). For amplification and cycle sequencing forward primer GTGCTCACGGTCTGGATGT and reverse primer CGGTGGGTTTGTACCTTCTC were used. Additional cycle sequencing primers were forward primer GATTACAGCCTCAGCGAG and reverse primer CGTCTCCTGAAAGACGG. The detection limit of the assay was 1000 copies/ml of CMV DNA and the amplified region ranged from codon 370 to 708 of the UL97 gene, covering all previously described mutations.[14;14] Sequences were compared to the sequence of the wild type AD169 strain and to pre-treatment samples from the patient.

Baseline resistance was determined in the first sample from each patient containing at least 1000 copies/ml of CMV DNA. Resistance during treatment was determined by analysis of subsequent plasma samples containing at least 1000 copies/ml of CMV DNA after at least two weeks treatment of CMV infection. Sequence analysis of codon 262 to 1169 of the UL54 gene was performed on DNA from samples from patients using foscarnet or cidofovir.

End points

The two treatment regimens were compared for timing, incidence and course of CMV DNAemia and the occurrence of treatment failure and resistance. The course of CMV DNAemia was expressed as the AUC of the CMV loads of each patient. The AUC is a reflection of the total exposure of the patient to CMV and was calculated using the trapezoidal model as previously described.[15] Since quantification of low plasma loads in the range below 250 copies/ml is less precise, the AUC above this threshold was calculated. Treatment failure was defined as the presence of at least 1000 copies/ml of CMV DNA in plasma after at least two weeks of treatment of CMV infection. Resistance was defined as the presence of resistance-associated mutations in the UL97 or UL54 gene.[14;14]

Statistical analysis

For statistical analysis of dichotomous variables the Pearson Chi-square test or Fisher's exact test were used. Because for most continuous variables a normal distribution was not assumed, the Mann-Whitney-U-test was used.

RESULTS

Patients

Plasma samples and treatment data were available from 78 transplanted D+R- patients. Baseline characteristics of the patients from the two regimens are shown in Table 1. Of the 71 included patients, 42 had been treated according to a purely preemptive regimen and 29 primarily with prophylaxis. Seven patients were excluded from the analysis for the following reasons; two patients died within one month after transplantation, two patients received prolonged courses of cidofovir for BK-virus and three patients were treated with delayed prophylaxis after rejection treatment. No significant differences were present in baseline characteristics between the two treatment regimens.

Viral Monitoring

The number of plasma samples submitted for CMV DNA load determination during the first three months after transplantation was higher in the preemptive cohort than during prophylaxis (median of 10 versus 6 samples, $p < 0.01$), but was similar during the remainder of the year (median of 11 versus 10 samples, p 0.39).

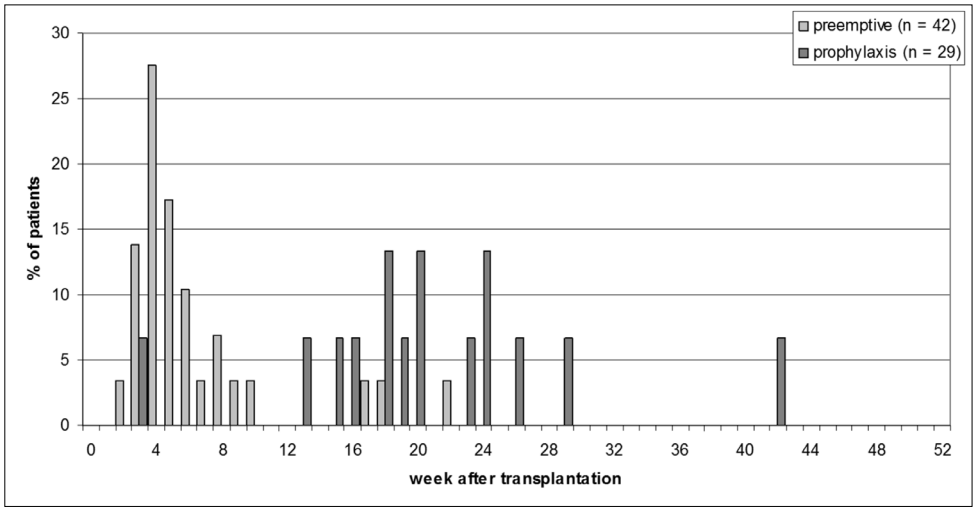
CMV DNAemia

Timing of CMV DNAemia differed significantly between the two treatment regimens; the first CMV DNA load was detected after a median of 33 days after transplantation in the preemptive cohort and after 138 days in the prophylaxis cohort ($p < 0.01$, Figure 1). Breakthrough CMV DNAemia occurred in one patient in the prophylaxis cohort, who already had a CMV infection at the start of prophylaxis (Figure 1).

The incidence of CMV DNAemia was similar in both cohorts; 29 patients (69%) in the preemptive cohort and 15 patients (52%) in the prophylaxis cohort had CMV DNAemia in the first year after transplantation (p 0.14, Figure 2). The percentage of patients reaching a CMV DNAemia of at least 1000 copies/ml was significantly higher in the preemptive cohort than in the prophylaxis cohort; 69% of the patients in the preemptive cohort versus 45% of the patients in the prophylaxis cohort (p 0.04, Figure 2). The threshold of 10.000 copies/ml was reached by 55% and 24% of patients in each cohort respectively (p 0.01, Figure 2).

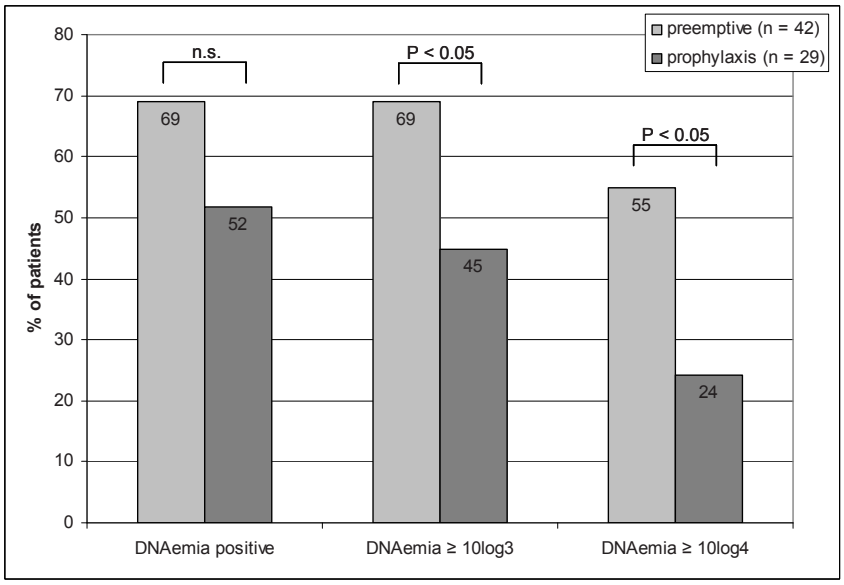
The course of CMV DNAemia was significantly different between the two regimens; the AUC above 250 copies/ml was significantly higher in the preemptive cohort than in the prophylaxis cohort (mean of $2.1 \cdot 10^6$ versus $0.9 \cdot 10^6$ copies·day/ml, Mann-Whitney U-test p 0.03). In the patients with CMV DNAemia, the period in which CMV DNA was present after transplantation was longer in the preemptive cohort than in the prophylaxis cohort (median of 203 versus 79 days, $p < 0.01$).

Figure 1. Time to first CMV DNAemia.



The median time to first CMV DNAemia was 33 versus 138 days in the preemptive versus the prophylaxis cohort ($p < 0.01$).

Figure 2. Incidence of CMV DNAemia.



The percentage of patients having DNAemia, reaching 1000 copies/ml CMV DNA in plasma and reaching 10.000 copies/ml CMV DNA in plasma in the first year after transplantation. n.s., not significant.

No CMV end-organ-disease occurred in the study cohort. One patient died and one graft was lost, but both events were unrelated to CMV infection. Seroconversion for CMV IgG occurred in the first three months after transplantation in 57% of the patients in the preemptive cohort and in 3% of the patients in the prophylaxis cohort ($p < 0.01$). The two treatment regimens did not show a significant difference in the number of rejection episodes or the renal function at six and twelve months after transplantation. Rejection treatment was not associated with the occurrence of CMV DNAemia (data not shown).

Treatment failure

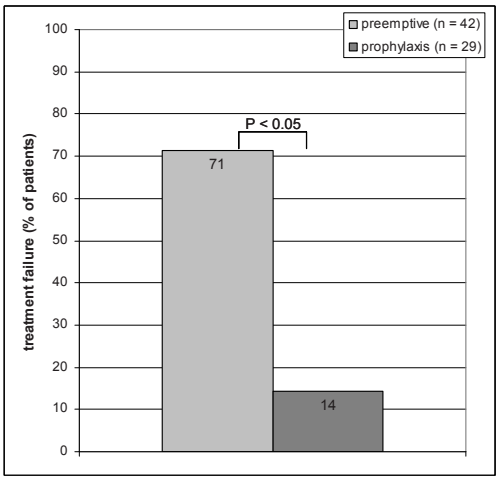
The percentage of patients receiving preemptive treatment was not significantly different between the two regimens; 67% of the patients (n = 28) in the preemptive cohort versus 48% of the patients (n = 14) in the prophylaxis cohort (p 0.12). Treatment failure was present in a significantly higher proportion of the treated patients in the preemptive cohort than in the prophylaxis cohort; 71% (n = 20) versus 14% (n = 2) ($p < 0.01$, Figure 3a). The median duration of preemptive treatment was longer in the preemptive cohort than in the prophylaxis cohort (45 versus 29 days, p 0.02, Figure 3b).

The maximum CMV DNA load was significantly higher in the patients with treatment failure compared to the patients without treatment failure (median of ^{10}log 4.8 versus ^{10}log 4.0, $p < 0.01$). No significant differences were found regarding transplantation characteristics, rejection episodes and immunosuppressive medication directly after transplantation or at the onset of the first antiviral treatment episode between patients with and without treatment failure. Suboptimal dosages of antiviral medication during infection, based on renal function, were administered to 30% of the patients with treatment failure and to 42% of the patients without treatment failure (p 0.43). CMV-specific IgG was present in 9% of the patients with treatment failure and in 35% of the patients without treatment failure at the start of the first treatment episode (p 0.06).

Antiviral resistance

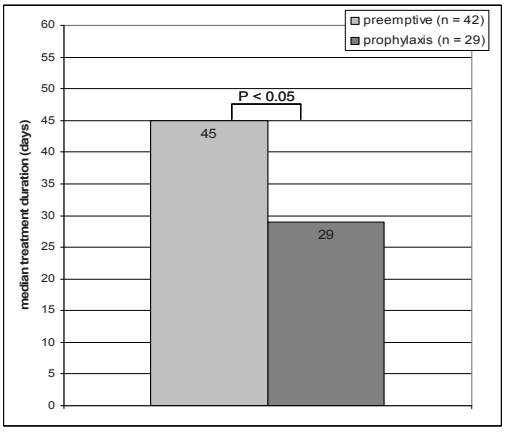
No antiviral resistance was found in 33 baseline samples from the 42 patients who had a CMV DNAemia of at least 1000 copies/ml. In nine of these 42 patients baseline resistance could not be determined. Resistance during treatment could be analyzed in 21 of 22 patients with treatment failure. Antiviral resistance was found in four of the 21 patients (19%). The four patients with resistant virus were recipients of kidney transplants and had various UL97 mutations (A594V, M460I/V, C603W). In one patient, resistance occurred during prophylaxis that had been initiated at the moment when CMV DNAemia was already present at a very low level (Figure 4a). The three other patients belonged

Figure 3a. Treatment failure.



Treatment failure was defined as a CMV DNA load of at least 1000 copies/ml in plasma after at least two weeks of treatment.

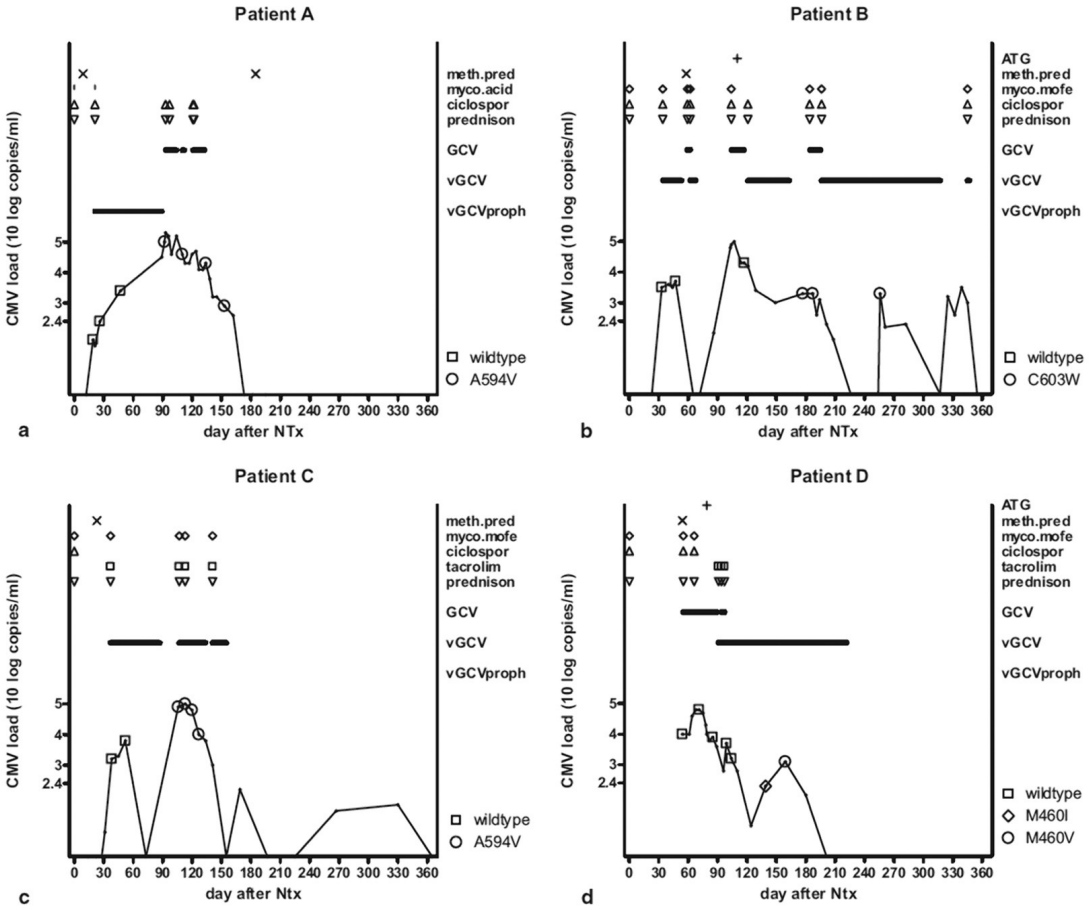
Figure 3b. Duration of treatment.



Antiviral treatment was defined as either preemptive or symptomatic treatment for CMV. Prophylaxis was not included in the calculation of the treatment duration.

to the preemptive cohort and developed resistance during preemptive treatment (Figure 4b-d). All these patients experienced episodes of rejection and bacterial urinary tract infections at the time of the occurrence of the resistant virus. This hindered a retrospective conclusion regarding the contribution of their CMV infection to symptoms, such as fever or deterioration of renal function. In all four patients the CMV infection eventually disappeared without switching the (val)ganciclovir treatment (Figure 4a-d).

Figure 4a-d. Patients with resistant virus.



In patient A resistant virus was found during prophylaxis, whereas in patients B, C and D resistant virus was found during preemptive treatment. Meth.pred = methylprenisolone, ATG = anti-thymocyte globulin, myco.acid = mycophenolic acid, myco.mofe = mycophenolate mofetil, ciclospor = ciclosporine, tacrolim = tacrolimus, prednison = prednisolone, GCV = ganciclovir, vGCV = valganciclovir, vGCVprohp = valganciclovir prophylaxis. A594V, C603W, M460I, M460V: resistance associated mutations.

DISCUSSION

In this retrospective study the two most frequently applied regimens for the prevention of CMV disease in D+R- renal transplant recipients were systematically compared for the course of CMV DNAemia, treatment failure, and antiviral resistance. Due to a recent change in protocols, patients treated in a purely preemptive strategy could be compared with patients who were treated initially with three months

of prophylaxis followed by a preemptive regimen. CMV infections were effectively postponed by prophylaxis with valganciclovir, with only one CMV infection in the prophylaxis cohort occurring already before the start of prophylaxis. No CMV end-organ disease occurred in either cohort. Prophylaxis followed by preemptive treatment reduced the percentage of patients reaching high CMV loads, as well as the AUC of CMV DNAemia and the duration of subsequent preemptive treatment episodes. Therefore, the sequential prophylaxis-preemptive approach proved very practical in our outpatient setting. Furthermore, CMV infections with a slow response to preemptive antiviral treatment occurred less frequently in patients who had received prophylactic treatment than in patients on a strictly preemptive regimen.

The relatively mild course of CMV DNAemia during a preemptive regimen after initial prophylaxis in our study and in studies by others[7] demonstrates the effectiveness of regular CMV monitoring after prophylaxis to prevent CMV disease. The severe outcomes of late-onset CMV disease reported in previous studies may be explained by the fact that prophylaxis was not combined with a subsequent preemptive regimen.[6;7] Apart from the early detection of the primary CMV-infection due to preemptive monitoring, the decreased level of immune suppression at three months after transplantation probably facilitates the response to antiviral treatment as well. Firstly, the immunosuppressive effect of induction therapy lasts for six to twelve weeks after transplantation. In addition, the dosage of maintenance immunosuppressive medication is often reduced from six to twelve weeks after transplantation. Furthermore, reducing or temporarily stopping mycophenolate mofetil or mycophenolic acid at the occurrence of CMV infection harbors less risk of rejection at three months after transplantation. In accordance with previous findings, seroconversion for CMV during prophylaxis was nearly absent in our patients.[16;17] This renders preceding seroconversion an unlikely explanation for the milder course of CMV after prophylaxis.

In this study, patients with a higher CMV DNA load more often had a slower treatment response. Obviously, the time needed to clear DNAemia is related to the amount of virus. Apart from the preemptive treatment regimen (i.e. without prior prophylaxis) and the height of the CMV load, no other risk factors were found to be associated with treatment failure. The type of immunosuppressive medication or preceding rejection treatment could not be identified as risk factors. Under-dosage of antiviral medication was systematically recorded and occurred frequently, probably due to the rapidly changing renal function in these patients. However, this did not predict treatment failure. Patients without CMV-specific IgG at the start of treatment

only showed a trend towards treatment failure. Based on our findings, the predictive value of IgG in the management of CMV DNAemia remains unclear, as found earlier.[16]

Our definition of treatment failure as a CMV DNA load of at least 1000 copies/ml after at least two weeks of treatment corresponds to those patients for whom extension of treatment is commonly considered. In previous studies, preemptive treatment resulted in a median time to clear DNAemia of 13 tot 20 days.[4] In the setting of treatment of symptomatic CMV infections, the median time to DNAemia eradication was reported to be 21 days.[10]

In this study, antiviral resistance was observed but apparently played a minor role in treatment failure. Baseline resistance was absent and resistance during treatment was found in a minority of patients (19%) with treatment failure. Furthermore, CMV infections with resistant virus were eventually cleared without switching antiviral therapy. Due to the retrospective nature of this study, it is unknown whether a switch of treatment to UL97-independent antiviral medication, such as cidofovir or foscarnet, would have lead to a more rapid clearance of DNAemia.

Our findings are in contrast to those from studies in the setting of symptomatic CMV in which resistant CMV caused CMV disease often with an unfavorable outcome.[18;19] Explanations for this discrepancy may lie in the fact that CMV disease was effectively prevented in our cohort and that resistance was systematically studied, regardless of symptoms. The sensitivity of our PCR allowed us to study plasma samples from all patients who had a CMV load of at least 1000 copies/ml. Therefore, no selection bias was present towards patients with an extreme course of CMV. Previous studies addressing resistance after prophylaxis without selection for patients with symptomatic disease also reported resistance mutations in patients without CMV disease that were cleared spontaneously.[20;21]

There was no significant relation between the treatment regimen and the occurrence of resistance, but numbers were small in our study. However, since baseline resistance was absent, resistance is mainly expected to occur during treatment failure. Treatment failure occurred less frequently after prophylaxis and, therefore, resistance is not expected to occur more often after prophylaxis. Indeed, in a previous trial with valganciclovir for prophylaxis in D+R- patients, no resistance was found at the end of prophylaxis nor during infections after prophylaxis.[21] Based on our findings, the role of antiviral resistance testing in guiding preemptive therapy in case of treatment failure seems of limited significance, with the possible exception of patients with CMV disease and patients with CMV infections during prophylaxis.

The cost-effectiveness of both regimens is a relevant aspect in decision making. Costs may vary locally, but depend among other things on the number of samples for CMV DNA load measurement, the amount of antiviral medication administered and the number of admissions for CMV infections. In our study, the total number of samples for CMV DNA load measurement in the first year after transplantation decreased by 27% in the sequential prophylaxis-preemptive cohort compared to the purely preemptive cohort. Because breakthrough CMV infections were very rare in our study, monitoring for CMV during prophylaxis may be very limited. In the study cohort, this would have decreased the number of samples for CMV monitoring further, up to 50%. The total number of antiviral treatment days including prophylaxis increased by 2,5 fold in the sequential prophylaxis-preemptive cohort. However, the number of hospital days for intravenous antiviral treatment decreased by 55%. Furthermore, based on pharmacokinetic studies, a lower dose of valganciclovir may be effective for prophylaxis.[22;23] This may further reduce costs of prophylaxis and is an interesting topic for further study.

Some limitations to the present study apply. Firstly, it was small and due to its retrospective nature it was neither blinded nor randomized. Nevertheless, both cohorts were comparable with regard to baseline characteristics. Secondly, no clinical end points could be studied in view of the effective treatment strategies applied. Since both the CMV DNA load and the AUC have already been shown to predict the risk of CMV disease these parameters were used in our analysis.[15;24]

In conclusion, in D+R- renal transplant recipients, prophylaxis with low dose valganciclovir for three months followed by a preemptive regimen not only effectively postponed CMV infections, but also made subsequent infections more manageable without increasing the occurrence of antiviral resistance.

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8

Mannose-binding lectin and ficolin-2 gene polymorphisms predispose to cytomegalovirus (re)infection after orthotopic liver transplantation

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ABSTRACT

Background & Aims: The lectin pathway of complement activation is a crucial effector cascade of the innate immune response to pathogens. Cytomegalovirus (CMV) infection occurs frequently in immunocompromised patients after orthotopic liver transplantation (OLT). Single-nucleotide polymorphisms (SNPs) in the lectin pathway genes determine their, liver-derived, protein level and functional activity. We examined the association between these SNPs and the risk for CMV infection in OLT.

Methods: OLT patients (n=295) were genotyped for recipient and donor SNPs in mannose-binding lectin (MBL2), Ficolin-2 (FCN2) and MBL-associated serine protease (MASP2) genes.

Results: Combined analysis of independently associated variant MBL2 [HR 1.65, P<0.02] and wild-type FCN2 [1.85; P<0.02] SNPs in the donor liver showed an increased risk of CMV infection for either and both risk genotypes [HR 2.02 and HR 3.26, respectively, P=0.004], especially in CMV Donor-/Recipient+ patients [HR 4.7 and HR 10.0, respectively, P=0.01]. A genetic donor-recipient mismatch for MBL2 and for FCN2 increased the CMV risk independently, also combined [HR 5.35; P<0.001], particularly in CMV D-/R+ patients [HR 16.6; P=0.009]. Multivariate Cox analysis showed a consistent stepwise increase in CMV infection risk with the gene profile of the donor [up to HR 2.77; P<0.005] and the combined MBL2 and FCN2 donor-recipient mismatch profile [up to HR 4.57; P<0.001], independent from donor-recipient CMV serostatus, also at higher CMV (re)infection cut-off values.

Conclusions: MBL2 and FCN2 risk alleles of donor liver and recipient constitute independent risk factors for CMV infection after OLT. Patients with these risk genes probably need intensified CMV monitoring and anti-viral therapy.

INTRODUCTION

Cytomegalovirus (CMV) is an important cause of opportunistic infection in immunocompromised individuals and can have severe manifestations.[1] The administration of immunosuppressive drugs after orthotopic liver transplantation (OLT) has a broad range of inhibitory effects on the adaptive immune system which prevent graft rejection but increase the susceptibility to opportunistic infection, including CMV infection. After OLT the patient thus becomes critically dependent on the innate immunity and the complement system is a pivotal component of this innate defense against pathogens. The structurally similar pattern-recognition receptors mannose-binding lectin (MBL) and ficolin-2 bind carbohydrate ligands on pathogens and initiate the lectin pathway of complement activation via MBL-associated serine proteases (MASPs), predominantly through MASP-2. Common single nucleotide polymorphisms (SNPs) in the genes coding for these three members of the lectin complement pathway result in diverse protein variants and altered protein levels with potentially important functional implications, as recently shown by us for bacterial infections after OLT.[2]

Structural variations in the exon 1 region of the MBL gene (*MBL2*) interfere with the oligomerization of the protein and polymorphisms in the promoter regions alter the rate of synthesis of the protein, leading to changes in avidity and protein level of the lectin, respectively.[3;4] Ficolin-2 (*FCN2*) gene polymorphisms within the carbohydrate-recognition domain encoding region of the *FNC2* gene are associated with a decreased (*FCN2*-B) or increased (*FCN2*-C) ligand binding of Ficolin-2 compared to wild-type Ficolin-2 (*FCN2*-A).[5] The relation between MBL and CMV in solid organ transplantation has been studied before, mainly in kidney transplantation[6-8] and only in a small number of patients after liver transplantation.[9] Data concerning the relation between development of CMV infection and other components of the lectin pathway of complement, i.e., the ficolins and the MASPs, have not been reported before. The combination of an immunocompromised patient and a new donor liver, as the major site where these proteins are synthesized, makes OLT a unique *in vivo* patient model to study the MBL-Ficolin-MASP gene profile in relation to CMV infection.

METHODS

Study population

Data were retrieved from the records of all patients undergoing orthotopic liver transplantation (OLT) at the Leiden University Medical Center (LUMC, from 1992 to 2006)

and the University Medical Center Groningen (from 2000 to 2006) in The Netherlands. Follow-up data were collected until the end of December 2006. The study was performed on prospectively collected data and samples obtained with informed consent from the patients according to the guidelines of both Medical Ethics Committees, in compliance with the Helsinki Declaration.

For the present study, 426 patients were identified who underwent OLT from whom we were able to unselectively include 310 patients of whom DNA was available from both donor and recipient, and who had at least 7 days of follow-up after transplantation, thus excluding patients with perioperative morbidity and mortality. From 248 cases blood CMV pp65 antigen follow-up within the first year after OLT was available. CMV-DNA quantification data were available in 47 cases because of transition from pp65 in the LUMC from June 2002 onwards. Thus, a final cohort of 295 patients undergoing an OLT was used for the present study.

Covariates and definitions

Data on covariates of interest were indentified from the general patient database and from transplantation databases and included: age of recipient and donor at the time of OLT, gender, primary liver disease, donor and recipient CMV IgG antibody status, acute cellular rejection and date of death or last follow-up. Allograft rejection was diagnosed by liver allograft biopsy using the Banff classification scheme,[10] and these patients received additional immunosuppression (corticosteroids and other) which is known to increase the risk of CMV reactivation. Donor (D) and recipient (R) CMV serology was coded as a 4-level covariate (D-/R-, D-/R+, D+/R-, D+/R+).

Assessment of CMV infection and disease

CMV pp65 lower matrix protein had been detected routinely in leukocytes from ethylenediaminetetraacetic acid (EDTA) blood samples. Laboratory evidence for CMV infection was defined as a result of one or more cells positive for CMV pp65 antigen per 50.000 leukocytes. CMV-DNA loads had been determined prospectively in EDTA plasma by a validated quantitative real-time polymerase chain reaction (PCR) using the I-Cycler IQ DNA detection system (Bio-Rad, Veenendaal, The Netherlands), as previously described[11] with a detection limit of 0.90 log(10).

Both CMV screenings were performed twice a week during the first 21 days post-transplantation. Thereafter, the test was performed at each outpatient visit, at least every other week until postoperative day 90. Subsequently screening was performed at least once every month until postoperative day 365. Since all patients had an undetectable CMV load at the time of transplantation, any subsequent detection of pp65 or

CMV-DNA was considered CMV (re)infection in our study. Time to first positive CMV test result was calculated from the date of transplantation until the date of the first positive test. Those with persistently negative CMV assays were censored at day 365 and patients who died within 365 days after OLT were censored on the day of death.

Primary CMV infection was defined as the detection of CMV infection in an individual previously found to be CMV seronegative. Recurrent infection was defined as new detection of CMV infection resulting from either reactivation of latent virus (endogenous) or reinfection (exogenous) in a patient who has had previously a documented infection.[12] CMV disease was defined as the presence of clinical signs and symptoms compatible with the diagnosis, i.e., presence of fever (temperature, >38°C), neutropenia (<1.5 x 10^9/L) or thrombocytopenia (<100,000/mm^3), accompanied by the isolation of CMV from blood.[12] Tissue invasive CMV disease was defined when tissue samples were found to be positively stained for CMV.

Immunosuppression and antibiotic prophylaxis

Patients received routine immunosuppressive therapy consisting of corticosteroids, a calcineurin inhibitor (cyclosporine or tacrolimus) and either azathioprine or mycophenolate mofetil and/or basiliximab. With respect to the immunosuppressive therapy, azathioprine was used until 2001, and thereafter mycophenolate mofetil was given in case of impaired renal function. From 2001 on basiliximab was used on days 0 and 4. In addition, patients received 24 hours of prophylactic antibiotics intravenously (gentamycin, cefuroxim, penicillin G and metronidazol) and 3 weeks of selective digestive tract decontamination (polymyxin/neomycin, norfloxacin and amfotericin B) after OLT (131 patients) or amoxicillin-clavulanate and ciprofloxacin without selective digestive tract decontamination (164 patients).

CMV prophylaxis

All patients transplanted before 2002 received anti-CMV prophylaxis irrespective of their CMV status: D+/R- received 3 weeks of intravenous ganciclovir (induction), followed by oral acyclovir up to 3 months after OLT (maintenance). Patients with other serostatus combinations received oral acyclovir for 3 months. From 2002 onwards CMV prophylaxis was restricted to patients with a D+/R- serostatus combination: these received prophylaxis with valganciclovir for 3 months, while the other D/R combinations did not receive prophylaxis. In both periods preemptive therapy was used, meaning that a primary infection was treated upon first positivity of CMVpp65 test or PCR, while reactivations were treated if a relevant increase in CMVpp65 or CMV-DNA occurred after first positivity. The use of acyclovir, ganciclovir and valganciclovir as prophylaxis

was included as one variable to consider the combined effects of these medications in the analyses.

Genotyping and MBL-deficiency single-nucleotide polymorphisms

Genomic DNA was from peripheral blood and/or tissue samples. SNPs in the *MBL2*, *FCN2* and *MASP2* gene were determined with the use of high resolution DNA melting assays (HRMA) with the oligonucleotide primers as indicated in Table S1.[2;13;14] Briefly, HRMA of PCR products amplified in the presence of a saturating double-stranded DNA dye (LCGreenPlus, Idaho Technology) and a 3'-blocked probe, identifies both heterozygous and homozygous sequence variants by a change in melting temperature curves, verified by DNA sequenced controls.

Genotypic MBL studies have shown that different SNPs in the promoter and exon 1 of the MBL gene (B, C and D collectively called O, whereas the wild-type allele for each position is called A) are in strong linkage disequilibrium. The association between MBL genotype and phenotype is very strong: sufficient functional MBL concentration is associated with YA/YA, YA/XA, XA/XA and YA/O genotypes, whereas relatively deficient functional MBL concentration is associated with XA/O and O/O genotypes.[15;16]

Statistical analysis

In the univariate analysis, pretransplantation and post-transplantation covariates were analyzed for their association with CMV infection. Cox proportional-hazards survival analysis was used for both discrete categorical variables and continuous variables. For survival analysis, cases were censored at the date of the last follow-up, death or liver retransplantation. The multivariate Cox proportional hazards regression analysis was used to evaluate the independence of covariates with a P value <0.15 in the univariate analyses. Results were considered statistically significant when P values were <0.05. Bonferroni correction for multiple comparison tests was not performed because SNPs were selected on the basis of a deducible hypothesis, i.e., the reported associations with bacterial infections[2] and preliminary studies on CMV in OLT[9] consequence of the SNP on the function of the respective proteins and the liver as their production organ. All analyses were performed with SPSS Statistical Software Package (version 16.02, SPSS Inc., Chicago, IL).

RESULTS

We evaluated 295 orthotopic liver transplantation recipient-donor pairs. Pre- and post-transplantation clinicopathological features of the patients are shown in Table 1. In 120

Table 1. Characteristics of Orthotopic Liver Transplant Recipients and Donors (n=295).

Variable	n (%)
Age (median, range)	
Recipient	51 (16-70)
Donor	46 (13-72)
Gender	
Male recipient	181 (61.4)
Male donor	146 (49.5)
Primary liver disease	
Viral	57 (19.3)
Alcoholic	44 (14.9)
Cholestatic	78 (26.4)
Other disease§	116 (39.3)
Immunosuppressive regimen	
Prednisone / CNI (+ azathioprine)	94 (31.9)
Prednisone / CNI / basiliximab (+ MMF)	201 (68.1)
Antiviral prophylaxis	
None	126 (42.7)
Acyclovir	127 (43.1)
Ganciclovir+ Acyclovir	8 (2.7)
Valganciclovir	34 (11.5)
Acute cellular rejection	103 (34.9)
Child-Turcotte-Pugh class	
A	65 (22.0)
B	134 (45.4)
C	96 (32.5)
Lab MELD score (median, SD)	14+9
CMV serostatus	
D+/R+	90 (30.5)
D+/R-	42 (14.2)
D-/R+	114 (38.6)
D-/R-	49 (16.6)

§ Other diseases included predominately autoimmune hepatitis, cryptogenic cirrhosis and metabolic disorders.
CNI denotes calcineurin inhibitors, MMF mycophenolate mofetil, MELD Model for End-Stage Liver Disease, CMV cytomegalovirus, D donor, R recipient.

out of the 295 patients (35%) there was laboratory evidence of CMV infection within the first year after OLT, of which 90 (75%) were recurrent infections and 30 (25%) were primary infections. The median time to infection was 36 days (range 1-348 days) after transplantation. Out of these 120 patients 37 (31%) [13% (37/295) of all patients] had

CMV disease. All 295 recipients and their donor liver were tested for 9 functional polymorphisms in the *MBL2*, *FCN2* and *MASP2* genes (Table S2).

MBL2 and FCN2 polymorphisms of the donor are associated with CMV infection.
The *MBL2*, *FCN2* and *MASP2* genotype distributions of the donor livers were analyzed in relation to the cumulative incidence of CMV infection within the first year post-OLT. In a univariate Cox proportional hazards model, patients receiving a liver from an MBL deficient donor (XA/O or O/O) were found to have a significantly increased risk of CMV infection compared to those receiving a wild-type liver [54% (27/50) versus 38% (93/245) HR 1.65; CI, 1.07-2.54 P=0.02] (Fig.1A). In addition, there was a significant association with increased CMV infection in patients receiving a donor liver with an absence of the minor T-allele of *FCN2* SNP rs7851696 (*FCN2*-A) compared to a donor liver with at least one copy of the minor T-allele (*FCN2*-C) [44% (103/232) versus 27% (17/63) [HR 1.85; CI, 1.11-3.13 P<0.02] (Fig.1B). Particularly the recurrent CMV infection in seropositive recipients was increased with an MBL deficient compared to a wild type donor liver [75% (24/37) versus 40% (66/167), respectively, P=0.005], and it was again found to be increased when receiving a *FCN2*-A donor liver compared to *FCN2*-C donor liver [49% (78/159) versus 29% (12/42), P=0.02].

The *FCN2*-B (rs17549193) and *MASP2* donor gene polymorphisms showed no significant association with CMV infection. Moreover, none of the gene polymorphisms related with CMV infection in the donor showed a direct association in the recipients (Table S2). However, the genotype of the recipient in relation to the donor genotype was found to be a major determinant regarding the occurrence of CMV infection after OLT. Recipients with a sufficient (wild type) MBL genotype (A/A and YA/O) receiving a donor liver with a deficient MBL genotype (XA/O and O/O) developed significantly more frequently an CMV infection than the other patients [58% (26/45) versus 38% (94/250), respectively, P=0.006]. Conversely, CMV infection was less frequent in low-binding *FCN2*-A recipients who received a high-binding *FCN2*-C donor liver compared to the other genotype combinations [22% (10/46) versus 44% (110/249) P=0.009].

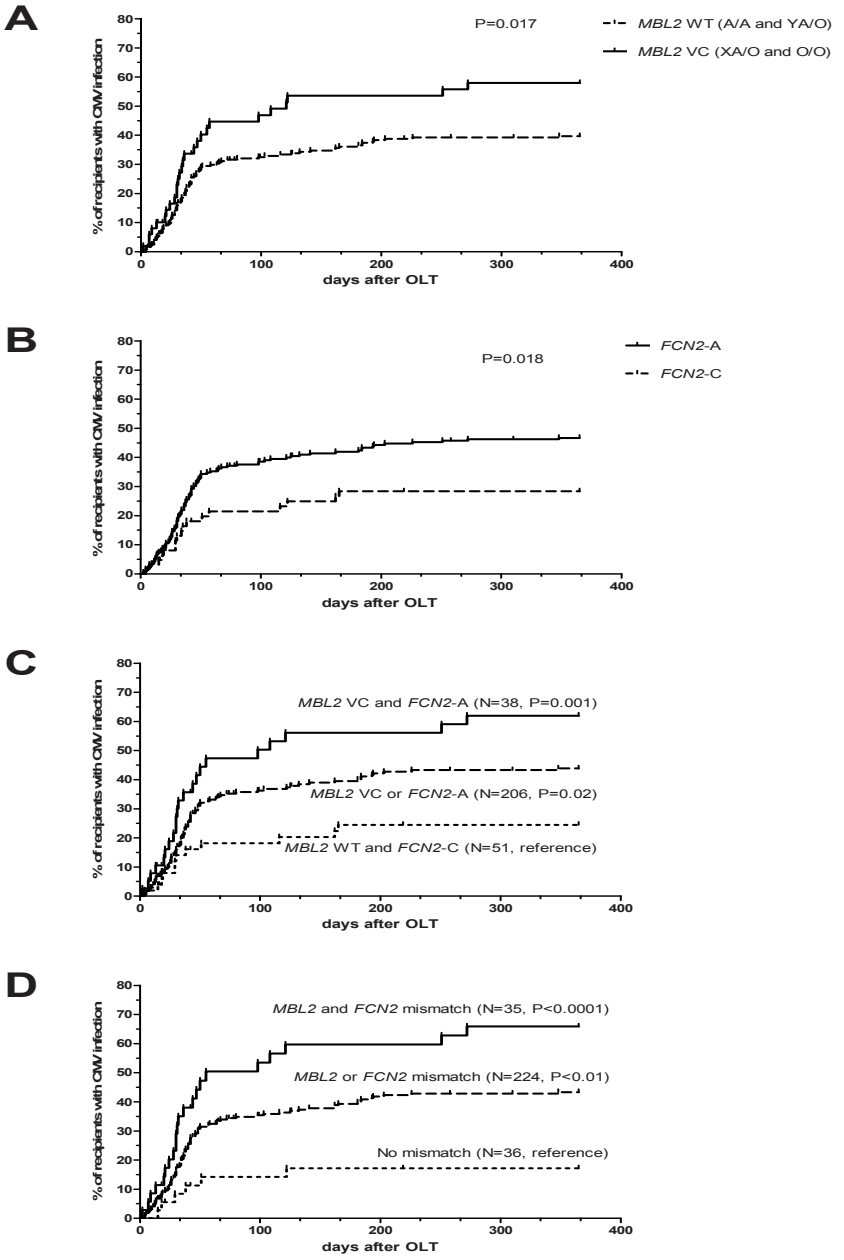
Combined analysis of MBL2 and FCN2-C SNP in the risk of CMV infection
The combination of MBL deficiency genotypes (XA/O or O/O) and presence of *FCN2*-A in the donor liver revealed a significant relation with CMV infection (Table 2). To extend the findings of the univariate model, the combined impact of donor *MBL2* and *FCN2*-A genotype on the cumulative probability of CMV infection was assessed as shown in Figure 1C.

Table 2. Univariate and Multivariate Analysis of the Association of Risk Factors of CMV Infection after OLT According to Combined Donor MBL2 and FCN2 Genotypes (n=295).

		CMV infection	Univariate Model		Adjusted Multivariate Model	
Variable		% (n)	HR (95% CI)	P Value	HR (95% CI)	P Value
Donor genotypes*	*MBL2* XA/O and O/O and *FCN2*-A	58 (22/38)	3.26 (1.61-6.60)	0.001	2.77 (1.35-5.67)	0.005
	MBL2 XA/O and O/O or *FCN2*-A	42 (86/206)	2.02 (1.10-3.70)	0.02	2.03 (1.10-3.75)	0.02
	MBL2 A/A and YA/O and *FCN2*-C	24 (12/51)	1 [Reference]		1 [Reference]	
CMV serostatus	D+/R+	60 (54/90)	14.49 (4.53-46.40)	<0.001	16.64 (4.90-56.50)	<0.001
	D+/R-	64 (27/42)	13.88 (4.21-45.82)	<0.001	13.01 (4.02-42.10)	<0.001
	D-/R+	32 (36/114)	6.31 (1.94-20.48)	0.002	6.48 (1.98-21.23)	0.002
	D-/R-	6 (3/49)	1 [Reference]		1 [Reference]	
Age recipient	Continuous	41 (120/295)	1.00 (0.98-1.01)	0.59		
Age donor	Continuous	41 (120/295)	1.00 (0.99-1.01)	0.88		
Gender recipient	Male	45 (81/181)	1.48 (1.01-2.18)	0.04	1.39 (0.93-2.09)	0.11
	Female	34 (39/114)	1 [Reference]		1 [Reference]	
Gender donor	Male	40 (59/146)	1.06 (0.74-1.51)	0.77		
	Female	41 (61/149)	1 [Reference]			
Immunosuppressive regimen	P/CNI/BAS (+MMF)	42 (85/201)	1.21 (0.82-1.80)	0.34		
	P/CNI (+AZA)	37 (35/94)	1 [Reference]			
Acute rejection	Yes	36 (37/103)	0.75 (0.51-1.11)	0.15	0.94 (0.63-1.40)	0.75
	No	43 (83/192)	1 [Reference]		1 [Reference]	
Antiviral treatment	Yes	41 (69/169)	0.90 (0.63-1.30)	0.59	0.62 (0.41-0.93)	0.02
	No	40 (51/126)	1 [Reference]		1 [Reference]	
Primary liver disease	Viral	49 (28/57)	1.38 (0.86-2.21)	0.19	1.06 (0.64-1.75)	0.82
	Alcoholic	55 (24/44)	1.63 (0.99-2.68)	0.06	1.11 (0.67-1.85)	0.69
	Cholestatic	31 (24/78)	0.78 (0.47-1.28)	0.32	0.73 (0.44-1.21)	0.23
	other	38 (44/116)	1 [Reference]		1 [Reference]	

OLT denotes orthotopic liver transplantation; CMV cytomegalovirus; D donor, R recipient, HR hazard ratio, CI confidence interval, P prednisone, CNI calcineurin inhibitors, BAS basiliximab, MMF mycophenolate mofetil, AZA azathioprine; *Four single nucleotide polymorphisms in MBL2 were genotyped: MBL2 -221 (X/Y), rs7096206; MBL2 codon 52 (D), rs5030737; MBL2 codon 54 (B), rs1800450 and MBL2 codon 57 (C), rs1800451. Structural variants D, B and C are collectively called O; where A is considered wild type; Secretor haplotypes were constructed: A/A and YA/O versus XA/O and O/O.

Figure 1. Kaplan-Meier estimation of cytomegalovirus (CMV) infection in liver transplant recipients with functional lectin pathway polymorphisms.



In the donor liver: A MBL2 variant carriers (VC) compared to MBL2 wild types (WT). B minor T-allele of FCN2-C SNP compared to absence of the minor T-allele (FCN2-A). C combination of donor MBL2 (XA/O or O/O) and FCN2 (FCN2-A or FCN2-C). In donor-recipient: D combined MBL2 and FCN2 donor-recipient mismatch profile. P values were calculated with the use of the log-rank test.

In order to determine the risk of true CMV reactivation we additionally analyzed the rates of CMV infection in the subgroup of seropositive recipients who received a liver from a seronegative donor, i.e., the D-/R+ subgroup. In this particular group cumulative CMV infection occurred significantly more often in recipients who received a liver from an MBL deficient (XA/O or O/O) and *FCN2*-A (54%, HR 10.0, P=0.004) donor or in those with either one of these genotypes in the donor liver (35%, HR 4.7, P=0.04) compared to those with the *MBL2* wild type and the presence of *FCN2*-C (9%, reference) (Figure S1).

Furthermore, in these D-/R+ patients an *MBL2* mismatch, i.e., a sufficient recipient with a deficient donor liver, conferred a significantly increased risk for developing CMV reactivation compared to the other *MBL2* combinations [59% (10/17) versus 27% (26/97) respectively, P=0.003]. The *FCN2* mismatch, i.e., a *FCN2*-A recipient and a *FCN2*-C donor liver showed a trend towards less frequent CMV reactivation when compared to the other combinations [17% (4/23) versus 35% (32/91) respectively, P=0.09].

The combined *MBL2* and *FCN2* mismatch profile showed a notably higher risk for developing CMV infection when compared to the combined donor SNPs alone (5.35 versus 3.26, see Table 3 and Table 2, respectively). Particularly the rates of reactivation in the CMV D-/R+ subgroup increased with the number of mismatches compared to no mismatches (mismatch in both *MBL2* and *FCN2* 58%, HR 16.6, P=0.009; mismatch in *MBL2* or *FCN2* 33%, HR 7.3, P=0.05).

CMV disease in all OLT patients showed no significant relation with the number of variants present in the donor liver [10% (5/51) with no genetic variant; 13% (27/206) in those with one and 13% (5/38) in those with two variants] or the combined mismatch profile [8% (3/36) with no mismatch; 13% (29/224) in those with one and 14% (5/35) in those with two mismatches]. Occurrence of CMV disease in the D-/R+ subgroup showed a trend to increase with the number of variants present in the donor liver but this did not reach statistical significance [9% (2/23) with no genetic variant; 12% (9/78) in those with one and 23% (3/13) in those with two variants]. The mismatch profile showed an increase of CMV disease in case of an *MBL2* mismatch [29% (5/17) versus 9% (9/97) respectively, P=0.02] but no relation with *FCN2* mismatches (data not shown). The combined mismatch profile also showed a trend to increase with the number of mismatches but this did not reach statistical significance [6% (1/18) with no mismatch; 12% (10/84) in those with one and 25% (3/12) in those with two mismatches].

Covariates

Analysis of other factors associated with development of CMV infection using the univariate Cox proportional hazards model is shown in Table 2. In this model the four strata

Table 3. Univariate and Multivariate Analysis for the Association of Risk Factors of CMV infections in all OLT Patients Stratified According Donor-Recipient Genotypes (N=295).

Variable			**CMV infection** % (n)	**Univariate Model**		**Multivariate Model†**		**Final Multivariate Model†**	
				HR (95% CI)	P Value	HR (95% CI)	P Value	HR (95% CI)	P Value
Donor-Recipient Genotypic mismatch	*MBL2**	XA/O and O/O donor and A/A and YA/O recipient	58 (26/45)	1.84 (1.19-2.84)	0.006	1.73 (1.10-2.71)	0.02		
		Other combinations	38 (94/250)	1 [Reference]		1 [Reference]			
	FCN2#	*Other combinations*	44 (110/249)	1.33 (1.07-1.65)	0.009	1.29 (1.04-1.61)	0.02		
		FCN2-C donor And *FCN2*-A recipient	22 (10/46)	1 [Reference]		1 [Reference]			
Combined *MBL2* and *FCN2* mismatch profile‡		*MBL2* and *FCN2* mismatch	63 (22/35)	5.35 (2.17-13.20)	<0.001			4.57 (1.83-11.40)	<0.001
		MBL2 or *FCN2* mismatch	41 (92/224)	2.97 (1.30-6.78)	<0.01			3.09 (1.34-7.11)	<0.01
		No mismatch	17 (6/36)	1 [Reference]				1 [Reference]	

OLT denotes orthotopic liver transplantation, CMV cytomegalovirus, HR hazard ratio, CI confidence interval. *The presence of a deficient donor MBL2 secretor haplotypes in a sufficient recipient haplotype conferred the highest risk compared to other donor-recipient combinations. #The presence of a FCN2-A (low binding) recipient receiving a FCN2-C (high binding) donor liver showed a clear trend to less CMV infections as opposed to the other genotypes. ‡The presence of donor-recipient mismatch associated with CMV within the individual were clustered as mismatch profile groups. †Adjusted for CMV serostatus (4 categories), gender recipient, acute rejection (yes/no), antiviral prophylaxis (yes/no), underlying disease (4 categories), as in table 2.

based on the CMV serostatus of donor and recipient showed a strong association with infection. Patients who were CMV D+/R- had the highest cumulative incidence of CMV infection (64%), followed by CMV D+/R+ (60%), CMV D-/R+ (32%) and CMV D-/R- (6%). CMV disease was most frequently observed in the CMV D+/R+ patients with a cumulative incidence of 17% (15/90), and 17% (7/42, 1 with suspected CMV hepatitis) in CMV D+/R-, followed by 12% (14/114, 1 with CMV gastritis) in CMV D-/R+, and 2% (1/49) in CMV D-/R- patients. Male gender of the recipient was significantly associated with more frequent CMV infection. Furthermore, a (non-significant) trend was observed concerning the type of primary liver disease in relation to the risk of CMV infection.

Multivariate analysis

In the multivariate Cox proportional hazards model, adjusting for risk factors associated with CMV infection ($P<0.15$), both donor *MBL2* (XA/O or O/O) genotypes [HR 1.56 (CI 1.01-2.42), $P<0.05$] and *FCN2*-A [HR 1.69 (CI 1.01-2.86), $P<0.05$] showed an independent relation with CMV infection. In the multivariate Cox proportional hazards model, adjusting for risk factors associated with CMV infection, the gene-dose related combinations of genetic variations in *MBL2* and *FCN2* in the donor liver [either gene HR 2.03, $P<0.02$; both genes HR 2.77, $P<0.005$], donor-recipient CMV serostatus pairs [HR $\geq$ 6.47, $P<0.002$] and antiviral treatment [HR 0.62, $P<0.02$] remained the only significant independent predictors of an CMV infection after OLT (Table 2). All other clinicopathological factors lost their predictive impact on the incidence of CMV. Despite the fact that antiviral prophylaxis was not associated with CMV infection in the univariate analysis ($P=0.59$) it was included in the multivariate analysis as a possible confounder. Since the multivariate model showed that the individual mismatches in *MBL2* and *FCN2* were independently associated with an increased CMV infection risk (Table 3) both donor-recipient mismatch genotypes were included in the final multivariate model. An even higher CMV infection risk profile, compared to the donor gene profile alone (Table 2), was found for one or two mismatches as compared to no mismatch, with adjusted hazard ratios of 3.09 [CI 1.34-7.11] and 4.57 [CI 1.83-11.40], respectively. To substantiate these observations we performed similar analyses at increasing cut-off values of pp65 and CMV-DNA for CMV positivity. The univariate and multivariate analysis results, broken down by extent of CMV positivity showed a consistent association between *MBL2* and *FCN2* genotype combinations, particularly the donor-recipient mismatches, and CMV infection.

Table 4. Univariate and Multivariate Analysis for the Association of Risk Factors by extent of CMV positivity according to MBL2 and FCN2 genotype combinations in all OLT Patients (N=295).

		Pp65≥3 or CMV-DNA log≥2*					Pp65≥10 or CMV-DNA log≥3*				
		CMV infection	Univariate Model		Multivariate Model†		CMV infection	Univariate Model		Multivariate Model†	
Variable		% (n)	HR (95% CI)	P Value	HR (95% CI)	P Value	% (n)	HR (95% CI)	P Value	HR (95% CI)	P Value
Donor genotypes	*MBL2* XA/O and O/O and *FCN2*-A	45 (17/38)	3.05 (1.39-6.66)	0.005	2.55 (1.15-5.65)	0.02	34 (13/38)	2.60 (1.11-6.10)	0.03	1.99 (0.83-4.73)	0.12
	MBL2 XA/O and O/O or *FCN2*-A	30 (62/206)	1.76 (0.90-3.42)	0.10	1. 80 (0.91-3.53)	0.09	24 (49/206)	1.54 (0.76-3.15)	0.23	1.48 (0.72-3.03)	0.29
	MBL2 A/A and YA/O and *FCN2*-C	20 (10/51)	1 [Reference]		1 [Reference]		18 (9/51)	1 [Reference]		1 [Reference]	
Combined *MBL2* and *FCN2* mismatch profile	*MBL2* And *FCN2* mismatch	49 (17/35)	6.26 (2.10-18.62)	<0.001	5.18 (1.72-15.62)	0.003	37 (13/35)	6.43 (1.83-22.58)	0.004	4.64 (1.30-16.54)	0.02
	MBL2 Or *FCN2* mismatch	30 (68/224)	3.31 (1.21-9.07)	0.02	3.43 (1.24-9.49)	0.02	25 (55/224)	3.58 (1.12-11.44)	0.03	3.27 (1.01-10.54)	0.05
	No mismatch	11 (4/36)	1 [Reference]		1 [Reference]		8 (3/36)	1 [Reference]		1 [Reference]	

OLT denotes orthotopic liver transplantation, CMV cytomegalovirus, HR hazard ratio, CI confidence interval.

*Pp65 or CMV-DNA log increase in pre-OLT CMV positive recipients and any positive pp65 or CMV-DNA in pre-OLT CMV negative recipients.

† Adjusted for CMV serostatus (4 categories), gender recipient, acute rejection (yes/no), antiviral prophylaxis (yes/no), underlying disease (4 categories), as in table 2.

DISCUSSION

The present study revealed the association of polymorphisms in liver-derived members of the lectin pathway of complement activation with the occurrence of CMV infection after liver transplantation. In particular, liver transplant patients with single-nucleotide *MBL2* polymorphisms (XA/O or O/O) in the donor liver had significantly increased rates of CMV infection compared to recipients of *MBL2* wild type donor livers. Furthermore, the major allele of the *FCN2* SNP rs7851696 (*FCN2*-A) of the donor liver increased the chance of CMV infection compared to the minor allele (*FCN2*-C). The joint genetic effect of these *MBL2* and *FCN2* genotypes in the donor liver and donor-recipient mismatches were even stronger. The involvement of mannose-binding lectin in the immune response against CMV after solid organ transplantation was suggested earlier.[6-9] For example, the study from Cervera et al.[7] reported that low-MBL genotypes of the pretransplant CMV positive recipient were associated with a higher proportion of CMV disease in renal transplantation, Despite the small number of patients (13%) who developed CMV disease in our OLT group we also found a trend between genotypically variant donor livers or donor-recipient mismatches and the development of CMV disease in pretransplant CMV positive recipients.

Our results indicate there is a pathogenetic link between several components of the lectin pathway of complement activation and the initial immune response against CMV after OLT, as we recently also reported for bacterial infections.[2] Both lectins i.e., MBL and Ficolin-2 are known to exert their effects via binding of specific carbohydrates presented on the surface of pathogens leading to activation of the complement cascade and enhanced phagocytosis in the host. CMV glycoproteins consist of proteins which are covalently linked to carbohydrates, like mannose and N-acetylglucosamine. The single nucleotide polymorphisms in the *MBL2*-gene are associated with changes in avidity and production level of this lectin.[4] The *FCN2*-C genotype was previously reported to be associated with stronger N-acetylglucosamine binding capacity compared to the wild type (*FCN2*-A).[5] Therefore, gene polymorphisms of both lectins of the donor liver may very well contribute to their functional activity in the innate immune response against CMV after OLT.

The lectin complement pathway genes of the OLT recipients were not found to be intrinsically related to the risk of CMV infection but a major indirect contribution related to the genetic constitution of the donor liver was observed. Particularly, MBL sufficient recipients receiving an MBL deficient donor liver were found to be at high risk for CMV

infections, especially for CMV reactivation in the D-/R+ subgroup. In addition, a *FCN2*-C donor liver reduced the chance for CMV infection in a *FCN2*-A recipient as opposed to the other *FCN2* genotype combinations. These finding are to some extent similar to the observations we made previously with bacterial infections[2], i.e., an association with the variant *MBL2* genotype and the donor recipient mismatch. In contrast, however, with bacterial infections an association with *FCN2*-B and with *MASP2* was found which was not observed with the CMV infection. These observations illustrate intrinsic differences in the involvement of the lectin complement pathway in bacterial versus viral (CMV) infections after OLT.

Viruses have developed different strategies to evade complement-mediated destruction. Modulation of host immune responses is a common strategy for promoting virus persistence and avoiding clearance. CMV is known to encode numerous immunomodulatory genes and is capable of altering several innate immune responses in favor of its own survival, for instance cytotoxicity, inhibition of NF-kB, etc.[17-19] Complement activation is potentially harmful to the host by inducing tissue damage and is therefore carefully kept in place by regulators of complement activation. Inhibition of complement activation is important for CMV replication. So far no virus-encoded complement inhibitors have been identified for CMV, but several studies show that CMV upregulates the expression of host-encoded (surface) complement inhibitors[20] and counteracts complement activation by incorporation of host cell-derived complement regulatory proteins CD55 and CD59.[21] Previous research also showed that the Toll-like receptor 2 (TLR2) Arg753Gln gene polymorphism seems to be associated with CMV replication after liver transplantation.[22] Interestingly, MBL is able to interact with TLR 2 in the phagosome to initiate proinflammatory signaling[23] and, although speculative, this might also play a role in CMV infection after OLT.

In addition, there may be a subsequent mechanism by which MBL could be involved in CMV replication. MBL naturally occurs in two forms, S-MBL and I-MBL, which are synthesized mainly in the liver and translated from a single form of mRNA. S-MBL activates complement via the lectin pathway, whereas I-MBL acts as a putative intracellular cargo lectin for glycoprotein transport between the endoplasmic reticulum and Golgi apparatus.[24] The intracellular interaction of I-MBL with HMCV glycoproteins may thereby disrupt CMV virion assembly or formation, restricting CMV replication and transmission.

Liver transplant recipients who were initially seronegative for CMV, but received a graft from a CMV positive donor are at greatest risk for CMV infection, similar to the observations with other organ transplants.[25] These D+/R- OLT patients always receive

CMV prophylaxis and constitute only a small percentage (14% 42/295) of our patients. Probably, therefore no association with the lectin and pathway genes was found in this group. Of the CMV seropositive recipients only some will develop CMV reactivation and the optimal strategy for prevention of CMV infection in this subgroup has not yet been established. In our study, in CMV seropositive recipients who received a liver from a CMV negative donor, reactivation of CMV occurred significantly more often when the donor liver was MBL-deficient. The presence of the minor allele of the *FCN2*-C SNP of the donor liver limited CMV infection compared to the presence of the major A-allele.

The impact of the lectin pathway genes in the CMV (re)infection process was reinforced by our observation that their contribution remained impressive even at higher cut-off values in the CMV seropositive recipients. In the CMV seronegative recipients any CMV detection is already indicative for infection and necessitates treatment. Although the associations we found are observational findings, it is tempting to speculate that in CMV negative donors, due to the primary exposure of the liver to CMV from the recipient, the mentioned CMV-induced immune evasion responses have not yet been modulated by the virus, and therefore functional MBL and FCN2-C from the donor might still exert their initial protective effect.

At present genetic risk factors for CMV infections are insufficiently understood, but a possible clinical application of our findings would be to screen for presence of *MBL2*-*FCN2* risk alleles in the donors for the CMV serostatus D-/R+ patients and subsequently intensify viral load or antigenemia monitoring in high risk patients.

There are some theoretical limitations to our study. Firstly, it is a retrospective study; nevertheless, frequencies of the studied SNPs associated with MBL deficiency and of the *FCN2*-C SNP in both donors and recipients were comparable to the frequency reported by others in healthy Caucasian populations.[26-29] Furthermore, the two independent cohorts showed a consistent association and were comparable with regard to the genetic profile and the occurrence of CMV infection/reactivation (Figure S2) but separately did not have enough power, i.e., numbers to be conclusive. Secondly, despite a relatively large combined patient cohort, only a small number of patients (13%) developed CMV disease or tissue invasive disease (2 patients). These numbers were too small to perform robust statistical analyses on CMV disease in the different subgroups. Therefore we limit our interpretation to the observation that donor and recipient *MBL2* and *FCN2* polymorphisms contribute to the CMV infection and /or reactivation in OLT recipients.

In conclusion, the present study strongly suggests an association between the occurrence of CMV infection and donor/recipient *MBL2* and *FCN2* gene polymorphisms in

liver transplant patients, independent from other risk factors. These observations may have potential clinical implications, as recently discussed regarding this component of the innate immune system and infections after liver transplantation in general.[30] Therefore, further studies on these genetic risk factors in liver transplantation, including longer follow-up, more patient cohorts, and patients with invasive CMV disease, could contribute to novel CMV infection prevention strategies in these patients.

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COMPETING INTEREST

None to declare

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Table S1. Oligonucleotide Primers for Polymerase Chain Reaction - High Resolution DNA Melting Assay.

Gene	SNP		dbSNP ID	sequence forward primer	reverse primer	3′-blocked probe (C6 spacer)
MBL2						
promoter	H/L	-550	rs11003125	CCAGGGCCAACGTAGTAAGA	CCAGCCCAGAATTAACTGGA	CAGGCAAGCCTGTCTAAAACACCAAGG
	Y/X	-221	rs7096206	TTCCCTAAGCTAACAGGCATAA	GCACTATGATGAGCAGTGGG	CCACGGAAAGCATGTTTATAGTCTT
exon 1*						
	D (codon 52)	+223	rs5030737			
	B (codon 54)	+230	rs1800450	CTGCAGTGATTGCCTGTAGC	GCCCAACACGTACCTGGTTC	GGCAAAGATGGGCGTGATGGCACCAAGGGA
	C (codon 57)	+239	rs1800451			
FCN2						
exon 8	B	+6359	rs17549193	GATGTTACTGCCTGTAACGATGC	GGTGGAGAAGGACTGGTTGTT	GAGATTCCCTGACGTTCCACAACAAC
	C	+6424	rs7851696	CCAGGACAATGATCTTAACACC	CACATGGCAGTTTTTGTACCAC	GGAAATTGTGCTGTGATGTTTCAGGG
MASP2						
exon 1		+105	rs72550870	TGCATAGAAGGCCTCGAACC	GGCAAGGACACTTTCTACTCGC	CTCGTTGGAGTAGCCGGAGCGGAAGG
exon 9		+371	rs12711521	AGCTTTGTAGGTGGTCACTCC	CCCTCCCATGCTTCTTTCTT	CCACTGGGTAGATCATCAGGAGGGCCA

*Ref. 14

Table S2. Frequencies of Lectin Pathway Gene Polymorphisms in Orthotopic Liver Transplant Recipients and Donors.

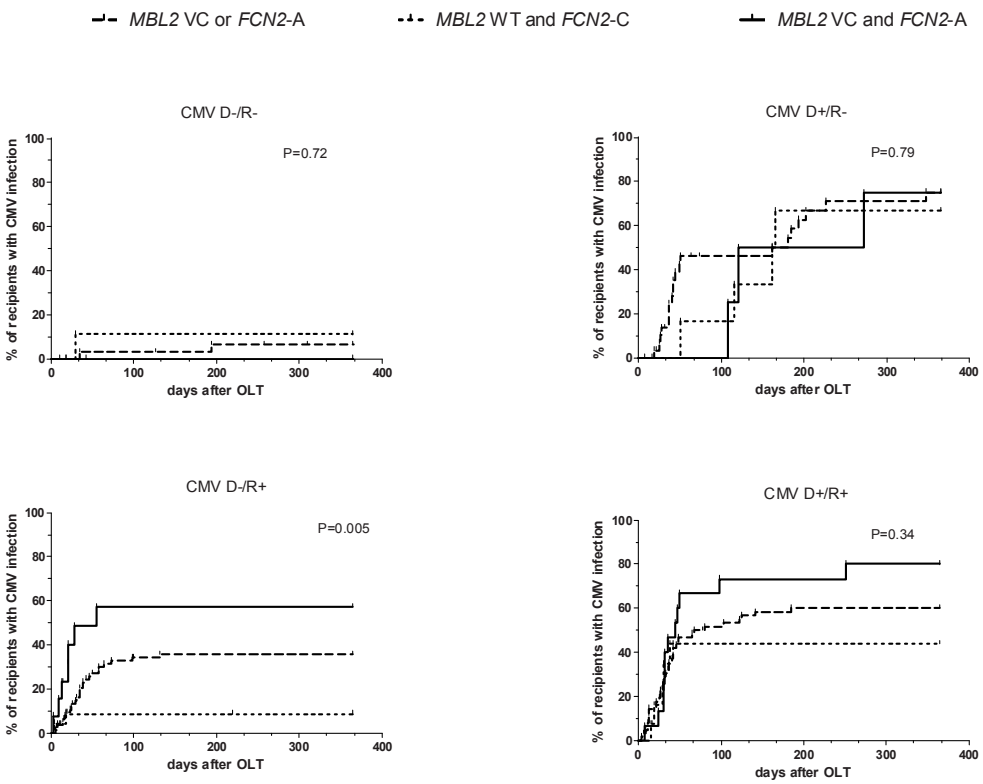
dbSNP ID	SNP			Genotype	Donor		Recipient	
					CMV %(n=120)	No CMV %(n=175)	CMV %(n=120)	No CMV %(n=175)
MBL2								
secretor haplotype [a, b]				A/A and YA/O	38 (93)*	62 (152)	43 (107)	57 (144)
				XA/O and O/O	54 (27)*	46 (23)	30 (13)	70 (31)
FCN2								
rs17549193	+6359 (B)	C→T	T236M	CC	40 (55)	60 (84)		
				CT	41 (52)	59 (75)		
				TT	45 (13)	55 (16)		
rs7851696 [b]	+6424 (C)	G→T	A258S	GG	44 (103)*	56 (129)	41 (96)	59 (136)
				GT	28 (17)*	72 (43)	38 (23)	62 (37)
				TT	0 (0)*	100 (3)	33 (1)	67 (2)
MASP2								
rs72550870	+105	A→G	D105G	AA	41 (114)	59 (164)		
				AG	35 (6)	65 (11)		
				GG	0 (0)	0 (0)		
rs12711521	+371	G→T	D371Y	AA	39 (74)	61 (117)		
				AC	44 (41)	56 (53)		
				CC	50 (5)	50 (5)		

CMV denotes cytomegalovirus, *P Values <0.05, calculated with the use of the univariate Cox proportional-hazards survival analysis

[a] Five single nucleotide polymorphisms in MBL2 were genotyped: MBL2 -550 (H/L), rs11003125; MBL2 -221 (X/Y), rs7096206; MBL2 codon 52 (D), rs5030737; MBL2 codon 54 (B), rs1800450 and MBL2 codon 57 (C), rs1800451. Structural variants D, B and C are collectively called O; where A is considered wild type; Secretor haplotypes were constructed: A/A and YA/O versus XA/O and O/O.

[b] SNP distribution (%) of the donors and recipients were comparable to the frequency reported by others in healthy Caucasian populations.

Figure S1. Kaplan-Meier estimation of cytomegalovirus (CMV) infection in liver transplant recipients with functional lectin pathway polymorphisms in the donor liver according to the four strata based on the CMV serostatus of donor (D) and recipient (R).



MBL2 wild types (WT): A/A or YA/O; MBL2 variant carriers (VC): XA/O or O/O; FCN2-A: absence of the minor T-allele; FCN2-C: presence of minor T-allele. P values were calculated with the use of the log-rank test.

Figure S2. Kaplan-Meier estimation of cytomegalovirus (CMV) infection in liver transplant recipients with functional lectin pathway polymorphisms in the donor liver for both independent centers.

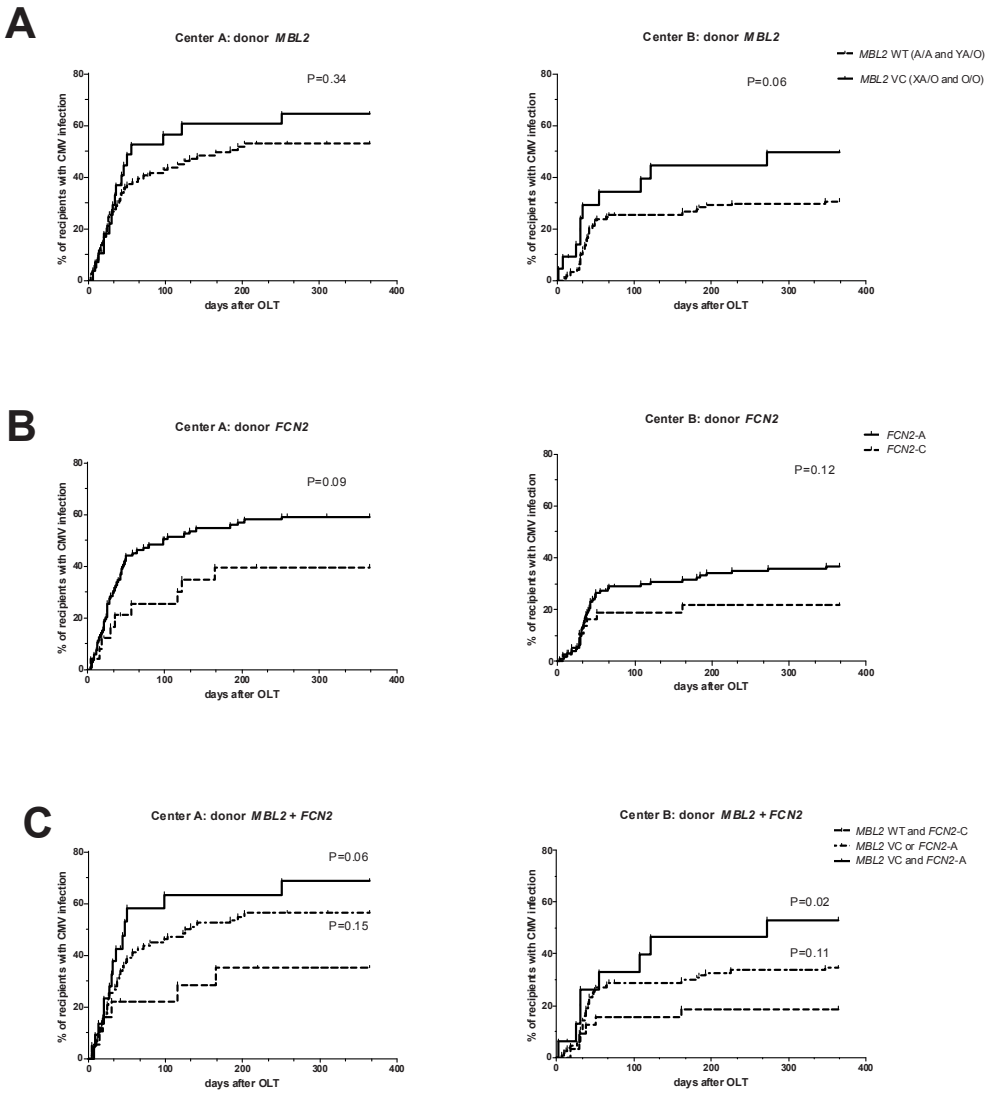
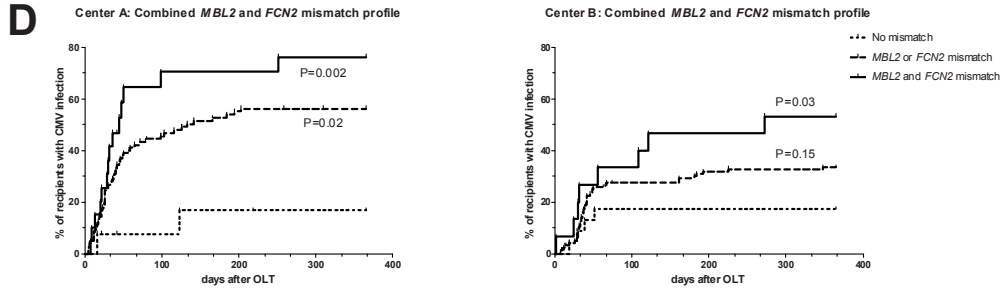


Figure S2. Continued



P values were calculated with the use of the log-rank test.
A MBL2 variant carriers (VC) compared to MBL2 wild types (WT).
B minor T-allele of FCN2-C SNP compared to absence of the minor T-allele (FCN2-A).
C combination of donor MBL2 (XA/O or O/O) and FCN2 (FCN2-A or FCN2-C).
D combined MBL2 and FCN2 mismatch profile.

9

General discussion

CONTENTS

HERPES SIMPLEX VIRUS TYPE-1

Role in oral ulcerations

Based on our knowledge of the pathogenesis of HSV-1 infections a causative role for HSV-1 in oral ulcerations in hematopoietic stem cell transplant (HSCT) recipients is very plausible. However, the contribution of herpesviruses to oral ulcerations in this setting has never been studied systematically, by sampling all cases regardless of the presence of ulcerations. The multifactorial etiology of oral ulcerations during and after HSCT and the fact that asymptomatic oral shedding of serveral viruses has been described during immunesuppression warrant this type of study, as described in chapter three.

Hence, the relative contribution of chemoradiation and different herpesvirus infections to oral ulceration after HSCT was studied. In the study in chapter three the presence of HSV-1 was a significant positive predictor for both ulcerative mucositis and ulcerations of the keratinized mucosa. Truly asymptomatic oral HSV-1 shedding occurred rarely. Since conditioning regimen and donortype were no predictors of oral ulcerations and since the rate of HSV-1 shedding was high, the relative contribution of HSV-1 to oral ulcerations after HSCT should be considered large. These findings support the use of antiviral prophylaxis with (val)aciclovir ((v)ACV) in this patient group.[1-6] Without prophylaxis, prompt administration of antivirals upon the development of oral ulcerations awaiting viral diagnostics should certainly be considered.

Interestingly, also the presence of EBV in oral washing samples was an independent predictor of oral ulcerations on the keratinized mucosa, but not of ulcerative mucositis. The pathogenetic role of EBV in oral ulcerations is not well-known. EBV has clearly been associated with oral hairy leukoplakia in various categories of immunocompromised patients.[7] EBV-associated oral ulcerations have also sporadically been described.[8,9] However, a causative role of EBV in oral ulcerations shortly after HSCT has not been proven by our study nor by others. Its presence may rather be the consequence of increased shedding in the presence of (HSV-1-induced) ulcers rather than being the cause of ulceration. Prophylaxis of HSV-1 with (v)ACV may decrease oral shedding of EBV, because EBV is susceptible to ACV during lytic infection.[10-15] Still, it is unclear whether EBV in oral ulcerations represents lytic infection, comparable to mononucleosis and oral hairy leukoplakia, or expansion of latently EBV infected cells, comparable to EBV related lymphoproliferative disorders, and the response to antiviral prophylaxis is therefore uncertain.[16-21] Certainly, the possible role of EBV in oral lesions in HSCT recipients merits further study.

Antiviral resistance

In clinical diagnostics, viral sequence analysis to detect resistance-associated mutations has the advantages of speed and technical ease. Nevertheless, as shown previously[22;23] and in chapter two and three, mutations of unknown significance are commonly found in HSV-1 clinical isolates. This demonstrates the need for a phenotypical susceptibility test to establish the significance of such mutations. The classical plaque reduction assay (PRA) can be used for this purpose, but because of its long assay time, a faster assay is needed.

In the study described in chapter two a faster and more easily applied protocol for phenotypical susceptibility testing of HSV-1 was developed. Results of the DNA reduction assay (DRA) compared very well to results obtained by genotypic tests and by PRA. Moreover, low level resistance to ACV and FOS was more accurately detected by DRA than by PRA. However, low-level resistance or intermediate susceptibility is not defined in the CLSI protocol[24] or by breakpoints suggested previously[25] and it remains to be investigated if infections with such isolates should be treated differently from high-level resistant isolates.

A two-step approach is likely to be most practical in the clinical setting, starting with target gene sequencing of, preferably, a pre- and on-treatment sample and subsequent phenotypical confirmation of resistance if mutations of unclear significance are encountered. As described in chapter two, DRA was successfully applied to confirm susceptibility to ACV in an isolate with a previously not well characterized mutation.[22;26] Also, in chapter three DRA demonstrated ACV resistance in an isolate with a novel mutation. This demonstrates the utility of a two-step approach for HSV-1 resistance analysis.

Treatment failure and antiviral resistance

In chapter three, HSCT recipients were systematically monitored for persistent oral replication of HSV-1. Oral shedding after a standard course of antiviral therapy for five days occurred in 43% of the patients and was due to resistance in 18% of treated patients. Of course, sensitive detection of HSV-1 DNA by real-time PCR after ulcerations have (almost) healed may account for part of the persistence. However, given the very short half-life of DNA,[27-31] such detection must represent at least recent viral replication rather than being a remnant of past replication.

The retrospective analysis of resistance in our study hampers establishing the clinical significance of the infections with resistant HSV-1. Probably, resistant isolates have reduced fitness and virulence[32;33] or are rapidly cleared as soon as immunological recov-

ery occurs. Nevertheless, a protracted course with severe ulceration occurred in several patients in our study and as patients have reported that oral mucositis was the single most debilitating side effect of HSCT conditioning,[34] its possible clinical relevance should not be ignored. Therefore, persistent oral ulcerations despite antiviral treatment demand viral diagnostics and antiviral resistance testing, to optimize treatment, both for patients with and without resistant HSV-1.

VARICELLA-ZOSTER VIRUS

Antiviral resistance

Because VZV is a rather slow growing and highly cell-associated virus and is often present in samples from which it cannot be cultured (plasma, CSF), resistance analysis is usually performed by sequence analysis of the genes involved in antiviral resistance.[35-40] As described previously[41] compartmentalization of antiviral resistance in sanctuary sites such as CSF and eye was found in a relevant proportion of the patients with resistant virus in our study in chapter four. In addition, amplification of full length viral genes from CSF and eye samples was often problematic necessitating us to adapt the protocol for such samples (using smaller amplicons). This may be related to the viral loads in the samples, but may also be due to the presence of fragmented viral DNA.[42]

Using sequence analysis as a resistance assay, the significance of mutations that have not been described before, which were found in half of the patients with resistant virus in chapter four, cannot be determined with certainty. Comparing sequences between pre-treatment and on-treatment samples from a patient can partly overcome this limitation. Nevertheless, phenotypical confirmation assays[43;44] that can be performed without the need for a viral isolate will be a useful addition to the diagnostics of antiviral resistance of VZV.

Treatment failure and antiviral resistance

The study in chapter four aimed to investigate the occurrence and significance of persistence and antiviral resistance systematically by analyzing all episodes of VZV in hematological patients between 2007 and 2010. VZV episodes with a duration of at least 7 days were demonstrated in 59% of the episodes and were associated with complications in 50% of the episodes. Persistence was accompanied by antiviral resistance in 27% of the cases and some cases of resistant VZV concerned very complicated cases with unfavorable outcome.

Due to the retrospective and biased nature of the study, the significance of virological persistence without clinical persistence is unclear. However, it was shown that combined clinical and virological persistence may predict complications. Therefore, routine follow up after a VZV episode by PCR on blood samples has no proven additional predictive value, also because the consequence of asymptomatic persistence for antiviral treatment is unclear. Since antiviral resistance explained a relevant part of the persistent episodes, antiviral susceptibility testing should be performed timely and comprehensively (i.e. studying all affected body sites), to optimize patient management.

CYTOMEGALOVIRUS

Predictors of infection

Immunological determinants of the occurrence and outcome of CMV infection in transplant recipients have been studied widely.[45-67] Improved prediction on the basis of these immunological determinants of patients at risk for (complicated) CMV infections after transplantation could individualize prevention strategies.

As described in chapter eight, orthotopic liver transplant recipients were shown to have significantly increased rates of CMV infection if single-nucleotide polymorphisms (SNPs) were present in the gene for mannose-binding lectin 2 (MBL2) in the donor liver that are associated with decreased synthesis of MBL2. Furthermore, the risk of CMV infection was decreased in the presence of the minor allele of the Ficolin-2 (FCN2) gene in the donor liver that is associated with improved ligand binding. The joint genetic effect of these MBL2 and FCN2 genotypes in the donor liver was even stronger.

Interestingly, especially patients with the favorable genotype combination who received a liver with the unfavorable genotype combination had an increased risk for developing CMV reactivation compared to all other recipient-donor combinations. In contrast, recipients with an unfavorable genotype were not at increased risk of CMV. This suggests that some adaptation to or compensation for the potentially unfavorable genotypes of MBL2 and FCN2 occurs normally that is not transferred with the donor liver. The absence of this compensation in recipients with a favorable genotype combination, increases the risk of infection when receiving a liver from a donor with the unfavorable genotype combination.

The effects of MBL2 and FCN2 SNPs were most clear in CMV seropositive recipients of a liver from a CMV seronegative donor, who constitute a relevant proportion of liver transplant recipients. For this group, the optimal CMV prevention strategy has not been

defined and identifying immunological correlates of protection against severe CMV may aid in choosing the optimal strategy. However, as shown in our study the effect of SNPs in innate immunity genes is complex and their predictive potential should be studied in clinical trials before their use can be implemented in routine clinical practice.

Treatment

The optimal prevention strategy for CMV disease in renal transplant recipients has not been established yet.[68-71] In chapter seven the two most frequently applied regimens for the prevention of CMV disease in D+R- renal transplant recipients were systematically compared retrospectively. Patients treated in a purely preemptive strategy were compared with patients who were treated initially with three months of valganciclovir (vGCV) prophylaxis followed by a preemptive regimen. Prophylaxis effectively postponed CMV infections and reduced the percentage of patients reaching high CMV loads, as well as the AUC of CMV DNAemia, the duration of subsequent preemptive treatment episodes and the occurrence of treatment failure. No CMV end-organ disease occurred in either cohort.

The relatively mild course of CMV DNAemia during a preemptive regimen after initial prophylaxis in our study and in studies by others[72] demonstrated the effectiveness of regular CMV monitoring to prevent CMV disease. The severe outcomes of late-onset CMV disease reported in previous studies in which prophylaxis was not combined with a subsequent preemptive regimen emphasize the importance of regular monitoring after the end of prophylaxis in preventing late-onset CMV disease.[72;73] The optimal frequency and duration for monitoring remain to be studied.[74] Reluctance to include CMV monitoring is probably explained by the presumed costs of continued CMV monitoring after transplantation.[68-71] However, timely and thus more effective CMV treatment reduces the number of expensive hospital days for intravenous antiviral treatment and reduces long-term morbidity, including graft loss.[75-78]

Antiviral resistance

In case of treatment failure a rapid diagnosis of resistance is valuable. GCV resistance associated mutations in clinical isolates mainly map to the viral kinase gene UL97.[79-83] Sequence analysis is the fastest method for susceptibility testing of CMV. Novel techniques such as mass-spectrometry based comparative sequence analysis (MSCSA) combine the possibility of detection of all nucleotide variations within a target gene with reduced hands on time due to the automation of post-PCR processing and analysis.[84-86]

In chapter five, we investigated the applicability of an MSCSA method for automated high-throughput DNA sequence analysis for the detection of mutations in the UL97 gene. MSCSA was found to be equally accurate compared to conventional sequencing techniques and the sensitivity of mutation detection in a mixture was comparable as well. The accuracy of SNP detection by MSCSA was largely dependent on the quality (and quantity) of the sequences in the reference database, as performance improved considerably when the databases were supplemented with new sequences. Since MSCSA did not improve mutation (mixture) detection, its benefit lies mainly in its suitability for high-throughput analysis. With the relatively rare occurrence of GCV resistant CMV there is no such requirement. However, its ability to accurately detect resistance associated mutations in CMV is a proof of principle for its applicability in settings where larger amounts of samples can be expected, such as detection of resistance mutations in human immunodeficiency virus or influenza.

Currently, CMV resistance testing can be rapidly and easily performed by routine sequencing techniques. As shown in chapter six and seven, phenotypical confirmation of mutations is seldom required, since the number of mutations is rather limited and knowledge on the significance of these mutations is sufficiently available.

Treatment failure and antiviral resistance

The occurrence and possible causes of persistent CMV infection despite antiviral treatment were studied in two different cohorts of transplant recipients in chapter six and seven. In both studies, treatment failure was defined as the presence of at least 1000 copies/ ml of CMV DNA in plasma after a standard course of two weeks of treatment of CMV infection. Because the half-life of viral DNA in blood is probably very short,[27-31] plasma DNAemia is a correlate for recent viral replication.[87] This view is supported by the fact that viral loads decreased rapidly, with or without antiviral treatment, in many patients in our studies.

In D+R- renal transplant recipients treatment failure occurred in 52% of the treated patients (chapter seven). Undergoing a preemptive treatment regimen (i.e. without prior prophylaxis) and a high peak CMV load were found to be associated with treatment failure. In recipients of an allogeneic T-cell depleted HSCT who were at risk for CMV (donor and/or recipient CMV seropositive) treatment failure occurred in 55% of the treated patients (chapter six). The risk of treatment failure was increased during first treatment episodes and during the use of immunosuppressive medication. A high CMV load at the start of treatment was a predictor in univariable analysis only.

In both patient groups a comparable incidence of treatment failure of approximately 50% was found. This number is in accordance with previous studies in renal transplant recipients in which preemptive treatment resulted in a median time to clear DNAemia of 14-15 days and between 13 to 20 days, respectively.[88;89] For HSCT recipients varying rates of treatment failure have been reported from 7% up to 45%.[90-93] Different definitions of failure as well as differences in antiviral treatment regimens probably play a role in these variations. The similarity in the rates of treatment failure in the two very different patient groups studied in this thesis at least demonstrates that CMV replication often persist during antiviral treatment in transplant recipients.[69-71]

Predictors of treatment failure were different between the two types of transplant recipients. Apart from differences of patient characteristics and types of immune deficiencies between the two groups, variations in the analysis may account for some of the differences. In chapter seven, predictors were analyzed on a patient level, whereas in chapter six predictors were studied for each CMV episode. The latter method takes into account the effects of repeated measures per patients and increases statistical power and, in retrospect, would have been the preferred method in the study described in chapter seven. Nevertheless, as expected,[89;94;95] the height of the viral load at the beginning of antiviral treatment was a predictor (albeit univariable) in both studies. The relation with immunosuppressive medication and first episodes in HSCT recipients is logical, since lack of antiviral immunity allows for higher levels of viral replication hence increasing the time to clear CMV DNAemia.

The contribution of antiviral resistance to persistence was studied in both cohorts as well. Resistance was found in only one of 47 HSCT patients (2%) with CMV treatment. In D+R- renal transplant recipients resistance was found in four of 42 patients (9.5%) with CMV treatment. Hence, in both patient groups low although different rates of GCV resistance were found and four of five CMV infections with resistant virus were eventually cleared without switching antiviral therapy.

This implicated that in the majority of the cases persistent infection despite treatment is not due to antiviral resistance and that switching antiviral treatment to more toxic second line agents was often not necessary. Also, it demonstrated that antiviral resistance does not appear to be a negative consequence of a sequential prophylaxis-preemptive treatment regimen.[96] Our findings are in accordance with previous studies systematically addressing resistance,[91;92;96-98] but in contrast to studies on symptomatic CMV, in

which resistant CMV was found more frequently and more often caused CMV disease with an unfavorable outcome.[99;100] Explanations for this discrepancy may lie in the fact that in our studies resistance was studied regardless of symptoms and that CMV disease was effectively prevented in our patients. This emphasizes, again, the need for CMV monitoring to prevent disease. The results from both studies show that the role of antiviral resistance testing lies merely in reassuring clinicians to continue first line treatment awaiting viral clearance by the immune system. In addition, cessation of treatment in some cases of persistent infection may be safe as well and should be further studied.

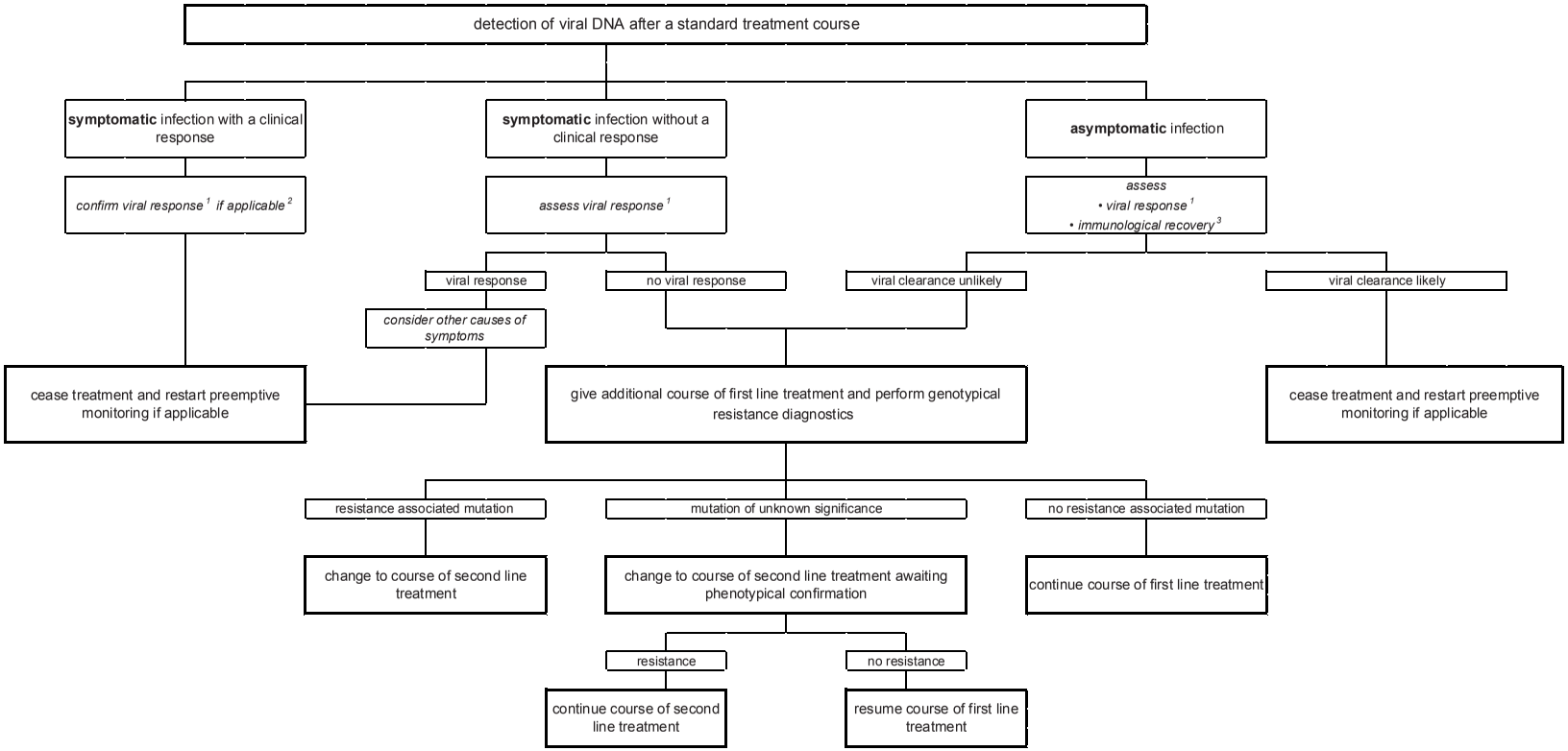
FUTURE DIRECTIONS

The aim of this research was to develop and improve diagnostic tools in order to rapidly diagnose HSV-1, VZV and CMV resistance to antiviral agents. Subsequently, the contribution of antiviral resistance and other predictors to persistent infections with HSV-1, VZV and CMV were studied.

Treatment failure

As shown in various chapters, persistence of herpesvirus DNA after a standard course of treatment is detected in about 50% of immunocompromised patients. If persistence is accompanied by clinical disease it is evident that additional antiviral treatment is required. However, the treatment of virological persistence without signs or symptoms of infections is a matter of debate. Of course, one should treat the patient and not the laboratory results, but in a preemptive setting this distinction is absent by definition. In addition, prolonged treatment of ongoing viral replication harbors the risk of selecting resistant virus. This emphasizes the limitations of our knowledge of the required treatment duration for herpesvirus infections after transplantation. In our studies the treating physicians usually have chosen to prolong treatment in patients with treatment failure. However, the duration of treatment of herpesvirus infections after transplantation is not based on controlled clinical trials and it is unknown whether treatment should be continued if viral replication persists after a course of treatment. Possibly, immune monitoring and viral dynamics may aid not only in deciding in whom to start antiviral treatment, but also in whom to safely end treatment.[94;101] At least one clinical trial is currently being performed on this topic.[101] Such studies may tailor the duration of antiviral treatment. For a flow chart on the suggested treatment of persistent herpesvirus infections, see Figure 1.

Figure 1. Flow chart for diagnostics and treatment in case of failure of antiviral therapy of DNA virus infections in immunocompromised patients.



Global approach to diagnostics and treatment in case of persistent DNA virus infections in immunosuppressed patients. [1]Assessment of viral response may include the viral load after treatment and the decrease in viral load in response to the given treatment. [2]In case of severe symptomatic disease. [3]Assessment of immunological recovery may include total and virus-specific T-cell counts and the possibility to decrease the use of immunosuppressive medication. Details may differ per virus.

It appears from the presented observations that antiviral immunity is the main determinant of treatment response. Interesting and promising in this regard are the current trials (http://clinicaltrials.gov/) on the effectiveness of pre-transplantation CMV and VZV vaccination strategies in preventing the occurrence or changing the course of these herpesvirus infections after transplantation.[102]

Antiviral resistance

Also shown in various chapters is the fact that persistent herpesvirus infection is explained by resistance in only a minority of the cases. Often, resistance was associated with a severe clinical course of the infection, but sometimes resistant viral isolates cleared spontaneously without appreciable clinical problems. These findings emphasize the need for rapid and adequate diagnostics of antiviral resistance in cases of persistent clinical and virological infection. Susceptibility testing will rule out resistance in most cases thus avoiding the need for second line agents with their associated toxicity and need for intravenous administration. In addition, it will optimize treatment in patients with resistant virus who not seldomly have serious organ manifestations.

Diagnosing antiviral resistance was found to be most straightforward in CMV where simple sequencing analysis suffices in most instances. Web based software tools, such as ReCall (RECall beta v2.6, http://pssm.cfenet.ubc.ca/home/index) can be applied for herpesvirus sequence analysis and may facilitate sequence analysis in routine diagnostics. For HSV and VZV, a two step approach is required, starting with sequence analysis and followed by phenotypical confirmation if mutations of unclear significance are found. An additional difficulty for VZV is the fact that usually viral isolates cannot be obtained. The latter point deserves further assay development. Possible compartmentalization demands investigation of virus in all affected body sites. For a flow chart on suggested diagnostics in case of persistent herpesvirus infections, see Figure 1.

There is a relevant need for less toxic and oral alternatives for antiviral treatment in case of antiviral resistance. For HSV several trials exploring novel antiviral drugs have been done (e.g. helicase-primase inhibitors) and are ongoing (e.g. NB-001, BTL TML HSV, http://clinicaltrials.gov/) and pre-clinical studies have been performed for other new drugs.[103;104] For CMV novel agents for treatment including CMX001 are being investigated.[105-107] For VZV an as yet unpublished clinical trial on the effectiveness of FV-100 has been performed (http://clinicaltrials.gov/). This increases the number of options for antiviral treatment in case of resistance. As a consequence, susceptibility testing needs to be adapted continuously to include novel antivirals.

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Summary

The research described in this thesis aimed to study determinants of the course and outcome of treatment of herpesvirus infections in immunocompromised patients. Both viral factors, such as antiviral resistance, but also patient factors, including immunological parameters, were investigated. Techniques to study antiviral resistance were optimized for use in a clinical diagnostic setting. The aim of this research was to improve and facilitate management of herpesvirus infections in immunocompromised patients.

In **chapter two** the development and validation of a real-time pcr based phenotypical technique to study susceptibility of HSV-1 to antiviral drugs in a routine diagnostic setting is described. This assay was designed to be faster and less labor intensive than classical culture based phenotypical susceptibility tests for HSV-1, such as the plaque reduction assay.

The results from our DNA reduction assay (DRA) were in accordance with plaque reduction assay results and with sequence analysis. DRA appeared to have a better discriminative value for low-level resistance. Although the direct application of DRA in clinical samples appeared not possible, short pre-culture of 48 hours was sufficient and ensured results within a clinically relevant time frame of 5 days.

Oral ulcerations are frequent and debilitating complications after hematopoietic stem cell transplantation (HSCT). In **chapter three** the role of HSV-1, EBV and CMV in oral ulcerations in HSCT recipients was investigated. Insight on the precise role of herpesviruses in this setting may improve management.

In a prospective observational cohort study in 49 adult patients that underwent allogeneic HSCT, the occurrence and localization of oral ulcerations and the presence and quantity of HSV-1, EBV and CMV in oral washing samples were systematically documented. Persistent HSV-1 infection was defined as an infection that lasted at least 5 days despite antiviral treatment. Antiviral resistance was studied in all persistent HSV-1 infections by viral sequence analysis.

Having an HSV-1 or EBV DNA positive sample was found to be a significant predictor for ulceration of keratinized mucosa. HSV-1 was a significant predictor for ulcerations on non-keratinized mucosa as well. Furthermore, persistent HSV-1 infection occurred in 12 of 28 patients treated with antiviral medication and aciclovir resistant HSV-1 was found in 5 persistent infections. In conclusion, given the important role of HSV in oral ulcerations after HSCT, prophylaxis or rapid treatment of oral HSV-1 infection and timely resistance diagnosis are warranted after HSCT.

VZV infections are a relevant cause of morbidity and mortality in hematological patients and especially in HSCT recipients. However, little is known on the course and on the occurrence of antiviral resistance of VZV in this setting. In **chapter four** the course of VZV infections in hematological patients was studied including the role of antiviral resistance in persistent infections.

In a retrospective study including all 87 pediatric and adult hematological patients diagnosed with VZV in our laboratory between 2007 and 2010, the clinical and virological course of VZV infections was studied. Persistent infection was defined as an infection that lasted at least 7 days. Antiviral resistance was studied in all persistent infections by viral sequence analysis.

Persistent VZV was demonstrated in 59% of the 54 episodes with follow-up available. Complications occurred in 50% of the persistent episodes and possible resistance associated mutations were found in 27% of the patients with persistent VZV, including patients with treatment unresponsive dermatomal zoster that progressed to severe retinal or cerebral infection. Therefore, antiviral resistance of VZV needs to be investigated timely and in all affected body sites in persistent infections.

In **chapter five** the application of a novel technique using mass spectrometry-based comparative sequencing to detect ganciclovir resistance in CMV is addressed. Mass spectrometry-based comparative sequence analysis (MSCSA) might be advantageous for this purpose because of its suitability for semi-automation.

Comparison of results from MSCSA with conventional cycle sequencing showed 94.1% concordance. The threshold to detect mutant sequences in a mixture with wild-type material was 20% using either technique. Thus, MSCSA was found to be equally accurate compared to conventional cycle sequencing in the analysis of the UL97 gene of HCMV

Pre-emptive treatment of CMV infections does not always lead to a rapid viral response. The causes of this type of treatment failure can be diverse and include antiviral resistance, pharmacological aspects and immunological factors. In **chapter six** various determinants of the response to antiviral treatment of CMV infections in HSCT recipients were studied, including resistance to antivirals.

Consecutive adult recipients of allogeneic T-cell depleted SCT were studied retrospectively (n=92). Treatment failure was defined as a CMV DNA load of 1000 copies/ml or more after at least 2 weeks of treatment. Resistance was analyzed in all failure episodes by viral sequence analysis.

Treatment failure occurred in 45% of pre-emptive treatment episodes and occurred

more often during first treatment episodes and during the use of immunosuppressive medication. Antiviral resistance was found in only 1 patient with treatment failure. Hence this study showed that a slow response to pre-emptive antiviral treatment occurred frequently in this setting and that antiviral resistance played a minor role.

In **chapter seven** the response to treatment and the occurrence of antiviral resistance are compared between a preemptive and a sequential prophylactic-preemptive treatment regimen for CMV in D+R- renal transplant recipients.

Consecutive adult D+R- recipients of a renal transplant were studied retrospectively. Before 2006, a preemptive treatment regimen with valganciclovir was applied (42 patients). From 2006 onwards, patients first received prophylaxis with valganciclovir for 90 days, followed by a preemptive regimen (29 patients). Treatment failure was defined as a CMV DNA load of 1000 copies/ml or more after at least 2 weeks of treatment. Resistance was analyzed in all failure episodes by viral sequence analysis.

Treatment failure occurred less frequently in the prophylaxis cohort than in the preemptive cohort (14% vs. 71%). Resistant viral isolates were found during treatment in one patient in the prophylaxis cohort versus in three patients in the preemptive group. All CMV infections with resistant virus were cleared without switch of (val)ganciclovir treatment. In conclusion, treatment failure occurred not frequently in the sequential prophylaxis-preemptive cohort and antiviral resistance played a minor role in treatment failure.

The lectin pathway of complement activation, component of the innate immunity, is a crucial effector cascade of the innate immune response to pathogens. Because many effector proteins from this pathway are synthesized in the liver, after liver transplantation a unique situation exists in which a recipient acquires the donor derived genotype en phenotype. In **chapter eight** the role of gene polymorphisms influencing mannose-binding lectin (MBL2), ficolin-2 (FCN2) and MBL-associated serine protease (MASP2) on CMV infection after orthotopic liver transplantation was investigated.

Transplant recipients (n=295) and donors were genotyped for polymorphisms in MBL2, FCN2 and MASP2 genes. Combined analysis of independently associated variant MBL2 and wild-type FCN2 SNPs in the donor liver showed an increased risk of CMV infection for either and both risk genotypes, especially in D-R+ patients. A genetic donor–recipient mismatch for MBL2 and FCN2 increased the CMV risk independently, also combined, particularly in CMV D-/R+ patients. In conclusion, MBL2 and FCN2 risk alleles of donor liver and recipient constitute independent risk factors for CMV infection after OLT.

In the discussion, implications for management of herpesvirus infections in immunocompromised patients as well as suggestions for further research are described.

Nederlandse samenvatting

NEDERLANDSE SAMENVATTING VOOR NIET (BIO)MEDISCH GESCHOOLDE LEZERS

Inleiding

Het onderzoek zoals beschreven in dit proefschrift betreft het beloop en de gevolgen van infecties met herpesvirussen bij immuungestoorde patiënten. Herpesvirussen komen veel voor en leiden na besmetting tot een levenslange, zogenoemd latente, infectie. Doorgaans verlopen deze latente infecties onopgemerkt, maar wanneer sprake is van een verminderde afweer kunnen zij opvlammen, reactiveren genoemd, en tot ernstige ziektebeelden leiden. De herpesvirussen die behandeld worden in dit proefschrift staan weergegeven in Tabel 1.

Tabel 1. Humane herpesvirussen behandeld in dit proefschrift.

virus	**ziektebeeld bij eerste besmetting**	**ziektebeeld bij reactivatie**
Herpes simplex virus type 1	blaasjes in de mond	koortslip, zweren in mond/keel
Varicella-zoster virus	waterpokken	gordelroos
Epstein-Barr virus	ziekte van Pfeiffer	tumoren, slijmvliesafwijking mond
Cytomegalovirus	ziekte van Pfeiffer	koorts, ontsteking van inwendige organen, ogen, hersenen

Zo kan een infectie met het herpes simplex virus type 1 (HSV-1) bij reactivatie leiden tot een koortslip en bij ernstig gestoorde afweer tot uitgebreide pijnlijke afwijkingen in en om de mond. Wanneer een latente infectie met varicella zoster virus (VZV) opvlamt kan dit leiden tot een lokale ophoping van pijnlijke blaasjes, beter bekend als gordelroos, maar uitgebreidere huidafwijkingen en aantasting van interne organen, ogen en hersenen kan ook voorkomen. Tenslotte kan reactivatie van een latente infectie met cytomegalovirus (CMV) koorts veroorzaken, evenals infectie van bijna alle interne organen, ogen en hersenen.

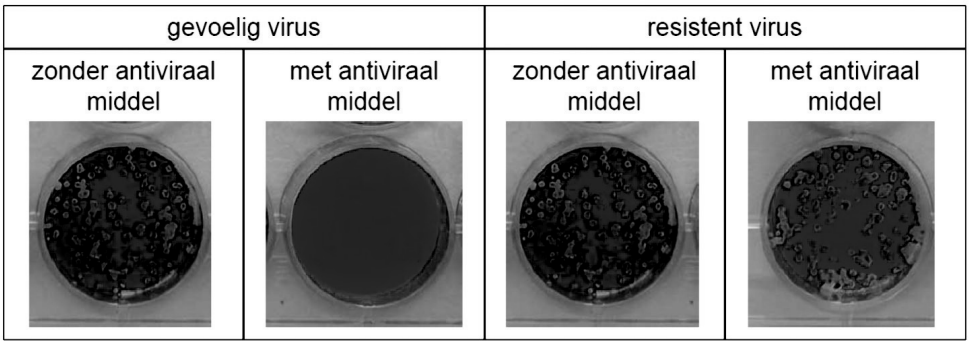
Bij immuungestoorde patiënten wordt daarom behandeling met antivirale middelen toegepast om symptomen te voorkomen of verminderen. In dit proefschrift is bij patiënten met een verminderde afweer onderzocht hoe deze drie herpesvirussen reageren op behandeling met antivirale middelen en of er sprake is van resistentie tegen antivirale middelen, wanneer de infectie niet vlot herstelt. Daarnaast zijn technieken opgezet om resistentie te kunnen aantonen bij de verschillende virussen.

Hoofdstuk 2

In **hoofdstuk 2** wordt de opzet beschreven van een nieuwe techniek om de gevoeligheid van HSV-1 voor antivirale middelen te bepalen. Bij de zogenaamde fenotypische

gevoeligheidstesten wordt bepaald in welke mate antivirale middelen de vermenigvuldiging van een virus remmen (zie figuur 1). Door de hoeveelheid virus na kweek in aan- en afwezigheid van antiviraal middel te vergelijken kan nagegaan worden in hoeverre er remming is door het antivirale middel. Afwezigheid van remming duidt op resistentie tegen het betreffende middel.

Figuur 1. Fenotypische gevoeligheidsbepaling HSV-1.



HSV-1 wordt gekweekt in een laag cellen in een kweekplaat. Daar waar het virus zich vermenigvuldigt worden de cellen vernietigd door het virus en dat zorgt voor de ophelderingen (plaques) in de blauwe cellaag (linker foto voor beide virussen). Elke plaque staat voor 1 virusdeeltje dat zich vermenigvuldigt. Wanneer antiviraal middel de vermenigvuldiging van een virus remt, ontstaan er geen plaques (rechter foto gevoelig virus). Wanneer virus resistent is tegen het antivirale middel, zal er ondanks de aanwezigheid van antiviraal middel vermenigvuldiging optreden en ontstaan er plaques (rechter foto resistent virus).

Eerder ontworpen methodes zijn afhankelijk van zichtbare en voldoende groei van het virus, waardoor deze testen lang duren en veel handwerk vergen. De nieuwe methode die is opgezet ondervangt deze nadelen door gebruik te maken van snelle en gevoelige detectie van DNA van HSV-1. Vergeleken met de traditionele testen, voldeed de methode goed in een testpanel van opgekweekt HSV-1 en in bepaalde gevallen (bij een geringe mate van resistentie) zelfs beter. Met het nieuwe protocol werd bovendien vlot een resultaat verkregen.

Bij patiënten die een stamceltransplantatie ondergaan worden de eigen bloed- en afweervormende cellen vernietigd om ziektes die hierin aanwezig zijn te genezen. Vervolgens krijgen deze patiënten stamcellen uit het beenmerg of bloed van een donor die weer kunnen uitgroeien tot effectieve bloed- en afweercellen. In de fase waarin de donorcellen nog niet volledig zijn uitgegroeid zijn deze zogenoemde stamcel transplantatie (SCT) ontvangers zeer ernstig immuungestoord. Een frequente en vervelende bijwerking van SCT is het ontstaan van pijnlijke zweren in en om de mond. Deze kunnen veroorzaakt worden door de chemotherapie die gegeven wordt

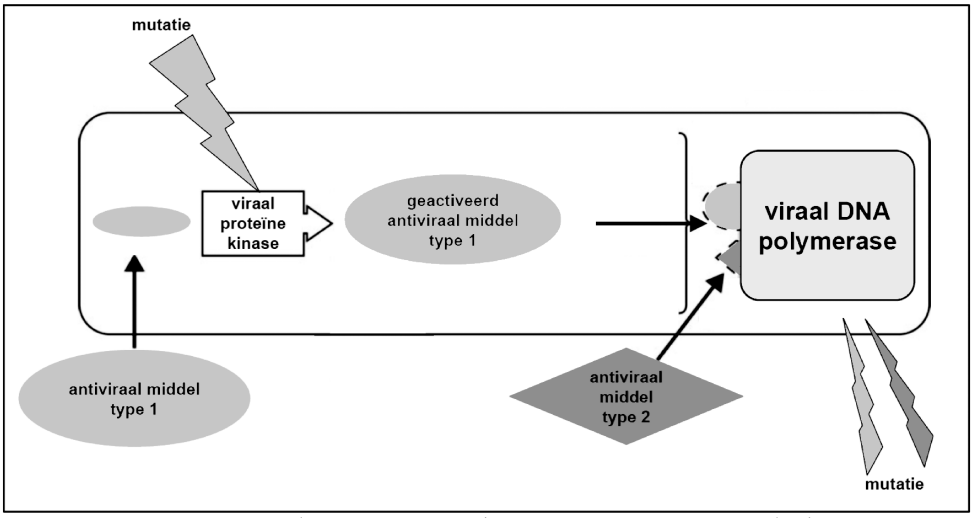
om de eigen cellen van de patiënt te vernietigen, maar het reactiveren van HSV-1 of andere herpesvirussen zoals Epstein-Barr virus (EBV) kan hierbij ook een rol spelen.

Hoofdstuk 3

In **hoofdstuk 3** is een groep patiënten tijdens het SCT traject gevolgd. Geregistreerd werd of zij last kregen van afwijkingen in de mond en of er HSV-1, CMV of EBV in hun mond aanwezig was. Daarnaast werd bij de patiënten die HSV-1 bleken te hebben onderzocht hoe vaak er sprake was van een blijvende infectie in de mond ondanks behandeling. Bij de patiënten met een blijvende infectie werd tenslotte bekeken of er sprake was van een resistent virus.

De aanwezigheid van HSV-1 bleek verband te houden met zweren in de gehele mond. EBV leek verband te houden met het optreden van zweren in specifieke gedeelten van de mond. Een blijvende HSV-1 infectie ondanks behandeling trad op bij 43% van de behandelde patiënten en ging gepaard met een resistent virus bij 42% van de blijvende infecties. Dit betekent dat preventie, diagnose en behandeling van HSV-1 infecties na SCT van belang zijn evenals resistentie onderzoek, wanneer de infectie niet reageert op behandeling. De rol van EBV verdient nog nader onderzoek.

Figuur 2. Werkings- en resistentiemechanismen antivirale middelen tegen herpesvirussen.



Vereenvoudigde weergave van het werkingsmechanisme van anti-herpesvirus middelen in een virus geïnfecteerde cel. Deze middelen kunnen in 2 typen verdeeld worden op grond van hun aangrijpingspunt in het virus. Middelen van type 1 dienen voordat zij werkzaam zijn geactiveerd te worden door het virale enzym proteïne kinase. Daarna blokkeren zij de werking van het virale enzym DNA polymerase waardoor het virus zich niet meer kan vermenigvuldigen. Middelen van type 2 zijn direct actief tegen het virale DNA polymerase. Hieruit volgt dat mutaties in het virale proteïne kinase de werking van type 1 middelen teniet doen. Mutaties in het virale DNA polymerase kunnen de werken van beide typen antivirale middelen blokkeren.

Hoofdstuk 4

In **hoofdstuk 4** zijn het beloop, het effect van antivirale behandeling en het vóórkomen van resistent virus onderzocht bij hematologische patiënten met een VZV infectie. Hoewel VZV veel voorkomt in deze patiëntengroep, is over het voorkomen van langdurige VZV infecties en de rol van resistent virus hierbij weinig bekend. Daarom is dit terugkijkend onderzocht bij een groep volwassenen en kinderen die ofwel chemotherapie voor bloed- of lymfeklierkanker ofwel een SCT hadden ondergaan in het recente verleden en daarna een VZV infectie ontwikkelden. Van deze patiënten is nagezocht hoe lang zij symptomen hadden en wat voor soort symptomen dat waren. Ook is bij hen achteraf bepaald in opgeslagen monsters hoeveel en hoe lang VZV aantoonbaar was. Tenslotte is bij langdurige infecties gekeken of er sprake was van resistent virus.

Van de patiënten bij wie dit achteraf onderzocht kon worden had 59% een langdurige infectie en dit ging bij de helft van de patiënten gepaard met complicaties. Resistent virus werd gevonden in 27% van de patiënten en ging in een aantal gevallen gepaard met zeer ernstige infecties met resistent virus in ogen of hersenen. Dit betekent dat bij langdurige VZV infectie tijdig en uitgebreid resistentie onderzoek van virus uit alle mogelijk aangedane organen noodzakelijk is.

Hoofdstuk 5

Antivirale middelen die CMV en andere herpesvirussen remmen hebben twee aangrijpingspunten in het virus (zie figuur 2). De eerste is het virale enzym proteïne kinase dat door het antivirale middel benut wordt om zich te laten omzetten tot actieve remmende stof. De tweede is het enzym DNA polymerase waarvan de werking door de antivirale middelen geblokkeerd wordt, waardoor het virus zich niet meer kan vermenigvuldigen.

In het DNA van CMV liggen de twee genen die, wanneer zij afgelezen en vertaald worden, leiden tot de productie van deze twee enzymen. Resistentie ontstaat in een virus zoals CMV bij toeval, omdat tijdens de vermenigvuldiging van een virus het DNA ervan wordt gekopieerd voor het nieuwe virusdeeltje en hierbij wel eens fouten, mutaties genoemd, ontstaan. Wanneer zo'n mutatie gelokaliseerd is in een stuk viraal DNA waarin zich de genen voor proteïne kinase of DNA polymerase bevinden is het mogelijk dat deze enzymen niet meer of anders gaan werken. Daardoor kan het virus het aangrijpingspunt van een antiviraal middel verliezen. Het virus is dan resistent geworden tegen het middel.

Resistentie kan bij een virus daarom gedetecteerd worden door op zoek te gaan naar mutaties in de genen van de enzymen proteïne kinase en DNA polymerase. De gebrui-

kelijke techniek hiervoor bestaat uit het volledig aflezen van de betreffende genen. Dit behelst vrij veel handwerk en veel analysetijd. Massa spectrometrie gebaseerde vergelijkende sequentie analyse (mass spectrometry-based comparative sequence analysis, MSCSA) is een nieuwe techniek waarbij het niet nodig is een heel gen af te lezen. Met massa spectrometrie worden het gewicht en de lading van een molecuul bepaald. Dit kan gebruikt worden voor het analyseren van DNA door een gen in ontelbare kleine fragmentjes te knippen en daarvan de lading en massa te bepalen. Software kan de gegevens van alle kleine fragmentjes vergelijken met gegevens over hoe het gen samengesteld zou moeten zijn en zo herleiden op welke volgorde de fragmenten zich in het oorspronkelijke gen bevonden. Als het gewicht van een fragment veranderd is betekent dit dat er iets veranderd is in het gen en zo kan bepaald worden of er mutaties in aanwezig zijn. De bewerking van het virus monster en de analyse kunnen beide halfautomatisch verricht worden en dat bespaart dus tijd.

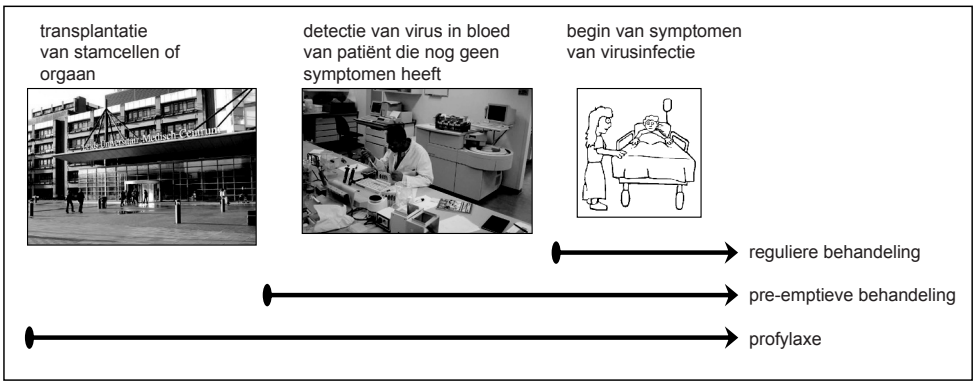
In **hoofdstuk 5** is deze nieuwe techniek om resistentie veroorzakende mutaties op te sporen onderzocht voor het proteïne kinase gen van CMV. In een testpanel van gevoelige en resistente CMV monsters werkte de nieuwe techniek net zo goed als de gebruikelijke voor het opsporen van mutaties in het proteïne kinase gen. Wanneer er een groot aanbod is van monsters voor resistentie analyse zou deze techniek dus ingezet kunnen worden.

Hoofdstuk 6

CMV infecties kunnen bij SCT patiënten ernstig verlopen in de fase waarin hun donor stamcellen nog onvoldoende zijn uitgegroeid. Om dit te voorkomen worden deze infecties al behandeld voordat de patiënt er symptomen van heeft door regelmatig in het bloed van de patiënten te kijken of er CMV aantoonbaar is. Het monitoren van het ontstaan van een infectie en het starten met behandelen voordat deze klinisch merkbaar is heet pre-emptieve therapie (zie Figuur 3). Soms is na een pre-emptieve behandeling toch nog virus meetbaar in het bloed van patiënten. Het is onduidelijk hoe dit komt.

In **hoofdstuk 6** is bij SCT patiënten terugkijkend onderzocht hoe vaak er ondanks pre-emptieve behandeling van CMV sprake was van een aanhoudende infectie in het bloed. De risicofactoren hiervoor en de rol van resistent virus zijn bestudeerd. Bij 45% van de ziekte episodes bleek sprake van een aanhoudende infectie ondanks behandeling. Dit kwam vooral voor tijdens de eerste CMV infectie sinds de SCT en bij patiënten die veel afweer onderdrukkende medicijnen gebruiken. Resistent virus kwam slechts bij één patient voor. Dit betekent dat een langdurige CMV infectie na SCT vooral verband houdt met een slechte afweer en dat het meestal niet nodig is een ander antiviraal middel te geven.

Figuur 3 Preventie- en behandelingsstrategieën van herpesvirus infecties na transplantatie.



Om een ernstig verloop van infecties met herpevirussen bij transplantatiepatiënten te voorkómen kunnen verschillende strategieën worden toegepast. Profylaxe bestaat uit het geven van antivirale middelen vanaf het moment van transplantatie. Pre-emptieve behandeling omvat het regelmatig monitoren van virus in het bloed om therapie te kunnen starten zodra virus aantoonbaar is nog voordat de patiënt hier verschijnselen van heeft. Tenslotte kunnen infecties ook pas behandeld worden wanneer de patiënt er symptomen van heeft, maar dan is de kans op een ernstig beloop groter.

Na een orgaan transplantatie komen CMV infecties ook veelvuldig voor. Dit komt enerzijds doordat patiënten sterke afweer onderdrukkende medicatie gebruiken na transplantatie om afstoting van het donor orgaan te voorkomen. Anderzijds komt dit doordat ontvangers van een orgaan geïnfecteerd kunnen worden doordat CMV zich in het donor orgaan bevindt. Vooral wanneer de ontvanger zelf nog nooit CMV heeft opgelopen en hiertegen dus geen afweer heeft opgebouwd is dit gevaarlijk, omdat de patiënt dan een eerste CMV infectie oploopt op een moment waarop er sprake is van een sterk onderdrukte afweer. Om ernstige ziekteverschijnselen te voorkomen krijgen patiënten zonder afweer tegen CMV daarom soms preventief antivirale middelen toegediend na transplantatie van een orgaan dat CMV bevat (profylaxe, zie Figuur 3). Een pre-emptieve strategie, zoals in de vorige paragraaf beschreven kan ook worden toegepast. Het is onbekend welke van deze twee strategieën het meest effectief is en het laagste risico op resistentie geeft.

Hoofdstuk 7

In het LUMC is men recent van een pre-emptieve strategie overgegaan op een strategie van 3 maanden profylaxe gevolgd door een pre-emptieve strategie ter voorkoming van CMV infecties bij niertransplantatie patiënten uit de genoemde hoog risico categorie. In **hoofdstuk 7** is terugkijkend een vergelijking gemaakt van het vóórkomen van langdurige infecties en het vóórkomen van resistent virus tussen beide behandelingsstrategieën. Blijvende CMV infecties ondanks behandeling kwam voor bij 71% van de patiënten uit

het pre-emptieve cohort en slechts 14% van de patiënten in het profylaxe gevolgd door pre-emptieve behandeling cohort. Resistentie kwam voor bij respectievelijk 3 en 1 patient uit de beide groepen en deze infecties verdwenen zonder behandeling. Dit betekent dat profylaxe leidt tot minder langdurige infecties en dat, wanneer profylaxe gevolgd wordt door pre-emptieve therapie, er geen grotere kans op resistentie is en misschien zelfs een kleinere kans.

Hoofdstuk 8

Omdat bepaalde delen van de afweer na orgaantransplantatie sterk onderdrukt worden met medicatie, spelen de minder onderdrukte componenten zoals de aangeboren afweer waarschijnlijk een belangrijke rol in de afweer tegen infecties. De zogenoemde aangeboren afweer bestaat onder andere uit verschillende eiwitten die ziekteverwekkers zoals CMV kunnen herkennen en onschadelijk kunnen maken. Voorbeelden van deze eiwitten zijn mannose-bindend lectine (MBL), ficoline-2 (FCN2) en MBL-geassocieerd serine protease (MASP2). Door verschillen in de genen die voor deze eiwitten coderen, zijn er verschillen tussen mensen wat betreft de hoeveelheid en de werking van deze eiwitten. Mogelijk leidt dit ook tot verschillen in de effectiviteit van de aangeboren afweer tegen infecties zoals CMV. De eiwitten MBL, FCN2 en MASP2 worden geproduceerd door de lever. Na een levertransplantatie erft de ontvanger daarmee de genen van de leverdonor en gaat voortaan eiwitten produceren in de hoeveelheid en werking zoals voorgeschreven door die genen.

In **hoofdstuk 8** is onderzocht of dit gevolgen heeft voor de kans op CMV infecties na levertransplantatie. Terugkijkend is van ontvangers van een levertransplantaat gekeken naar hun MBL, FCN2 en MASP2 genen en die van de leverdonor. Van alle patiënten is vervolgens terugkijkend geregistreerd of zij een CMV infectie opliepen na transplantatie.

Ontvangers van een levertransplantaat met genen die zorgden voor verminderde productie van MBL2 of een minder effectief FCN2 hadden inderdaad een grotere kans op CMV infecties, vooral wanneer zij beide ongunstige genvarianten hadden. Dit risico was het grootst bij patiënten die zelf vóór transplantatie gunstige genvarianten hadden. Bij de patiënten die zelf al ongunstige genvarianten hadden was dit risico minder groot. Misschien komt dit door het feit dat deze patiënten al 'gewend' waren aan minder effectieve onderdelen van de aangeboren afweer en ter compensatie andere onderdelen van de afweer extra hadden benut. Een patiënt die van nature geen ongunstige genvarianten had en dus geen compensatiemechanismen had ontwikkeld en een lever met een verminderde productie van effectief MBL2 en FCN2 ontvangt heeft daardoor mis-

schien op een moment van sterk onderdrukte afweer ook nog eens een minder optimale aangeboren afweer. In de toekomst kan het in detail bepalen van onderdelen van de afweer bij een patiënt vóór transplantatie mogelijk leiden tot betere risico inschattingen en preventie van CMV infecties na transplantatie.

Conclusie

Concluderend kan gesteld worden dat langdurige infecties met HSV, VZV en CMV frequent voorkomen bij immuungestoorde patiënten. Resistent virus is bij een minderheid van de patiënten een oorzaak van een blijvende infectie. Echter, infecties met resistent virus kunnen ernstig verlopen. Daarom is bij blijvende infecties tijdig onderzoek naar resistentie noodzakelijk. Hiervoor kunnen onder andere de beschreven nieuwe technieken gebruikt worden.

Om in de toekomst infecties met herpesvirussen bij immuungestoorde patiënten beter te kunnen voorkómen en behandelen is meer kennis nodig over het voorspellen en versterken van de afweer tegen herpesvirussen, over aanvullende technieken voor resistentiebepaling en over nieuwe middelen voor behandeling van infecties met resistente virussen. Ook is meer inzicht nodig in de optimale behandelduur van deze infecties om het beleid bij een langdurige infectie beter te kunnen vaststellen.

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Curriculum Vitae

Martha van der Beek werd geboren in 1977 te Zoetermeer. Zij doorliep de middelbare school van 1989 tot en met 1995 aan het Christelijk Gymnasium Sorghvliet te Den Haag. In 1995 begon zij aan de studie Biomedische Wetenschappen aan de Universiteit Leiden en in 1998 begon zij daarnaast aan de studie Geneeskunde aan deze universiteit. Wetenschappelijke stages werden achtereenvolgens in het Leids Universitair Medisch Centrum doorgebracht bij de afdelingen Endocrinologie bij dr. M.M. Deckers en prof. dr. C.W. Löwik en bij de afdeling Experimentele Hematologie, bij dr. M. Vogt en prof. dr. J.H.F. Falkenburg. De afstudeerstage naar determinanten van de ontstekingsrespons op gele koorts vaccinatie werd verricht bij de Divisie Vaat- en Bindweefselonderzoek van TNO Preventie en Gezondheid in samenwerking met de afdeling Infectieziekten van het Leids Universitair Medisch Centrum onder begeleiding van dr. M.P.M. de Maat, dr. L.G. Visser, prof. dr. A. van der Laarse. In 2001 behaalde zij voor beide studies cum laude het doctoraal examen en in 2003 behaalde zij het artsexamen cum laude.

In 2003 en 2004 werkte zij als assistent-geneeskundige niet in opleiding bij de afdeling Interne Geneeskunde van het Diaconessenhuis te Leiden. In 2004 werd begonnen aan de opleiding tot arts-microbioloog bij prof. dr. A.C.M. Kroes bij de afdeling Medische Microbiologie van het Leids Universitair Medisch Centrum. Alhier werd in 2007 een aanvang gemaakt met het promotieonderzoek onder begeleiding van dr. A.C.T.M. Vossen en prof. dr. A.C.M. Kroes. In 2009 werd zij geregistreerd als arts-microbioloog en sindsdien is zij werkzaam als zodanig in het Leids Universitair Medisch Centrum.

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