# Quantum virology : improved management of viral infections through quantitative measurements

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## Improved management of viral infections through quantitative measurements

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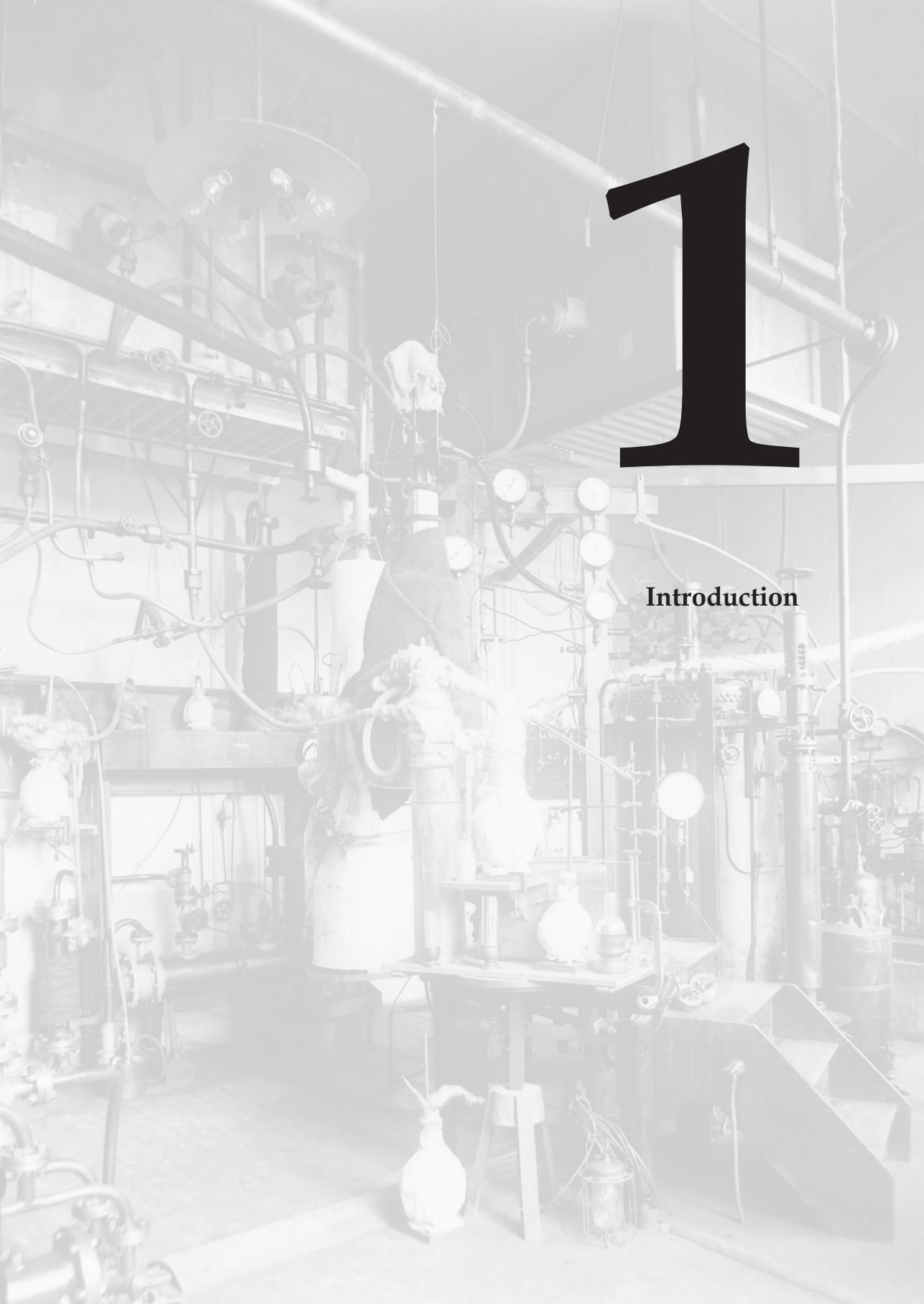
*A lord once asked his physician, a member of healers,*
*which of them was the most skilled in the art.*
*The physician, whose reputation was such*
*that his name became synonymous with medical science replied,*
*"My eldest brother sees the spirit of sickness and removes it before it takes shape,*
*so his name does not get out of the house."*
*"My elder brother cures sickness when it is still extremely minute,*
*so his name does not get out of the neighbourhood."*
*"As for me, I puncture veins, prescribe potions, and massage skin,*
*so from time to time my name gets out and is heard among the lords."*
*Sun Tzu (544-496 BC)*

*Aan Simone Cuypers*

# Contents



# 1

## Introduction

## 1. THE CONCEPT OF THE VIRUS

The concept of the virus as an infectious agent developed slowly in the last decades of the nineteenth century and the beginning of the twentieth century, after the importance of many bacteria and some fungi and protozoa was already firmly established. The principal limitation was the extremely small size of these agents, too small to be observed under the light microscope. Obviously, the fact that viruses are unable to grow in artificial cultures was also relevant. Still, successive elegant and creative experiments by scientists including Adolf Mayer (1843-1942), Dimitry Ivanofsky (1864-1920) and Martinus Beijerinck (1851-1931) were able to define the basic properties of viruses as a new infectious principle, which proved to be extremely widespread in nature.[1,2] Clinical observations and improvements in laboratory techniques throughout the twentieth century enabled the identification and differentiation of many viral illnesses (e.g. smallpox, chickenpox, measles and rubella) and the pathology of many viral diseases could be defined. The work of Louis Pasteur (1822-1895) stimulated systematical studies of the pathogenesis of infectious diseases, including those caused by viruses.[2] It appeared that the outcome of viral infection of a particular host depended on a variety of viral (virulence determinants) and host factors (genetic and physiologic determinants). Viral infections of a susceptible host can result in an infection, resulting in death of host cells and acute illness. However, the interaction of a virus and a host can lead to a variety of other outcomes including the development of persistent infections (chronic or latent infections) and cellular transformation.

### *1.1 Persistent viral infections*

It became apparent that not all viral infections were permanently cleared from the host after acute infection. Several viruses are capable of establishing persistent infections and in general two types of persistence can be defined: chronic infections and latent infections. During chronic viral infections, active virus persists in the host cell and there is a continuous production of virus for a prolonged period of time, for instance in congenital infections with cytomegalovirus (CMV) and rubellavirus and chronic infections with hepatitis B virus (HBV) and hepatitis C virus (HCV). Latent viral infections are characterized by the persistence of inactive virus in the host cell as exclusively the viral genome is maintained in the host cells, in the absence of viral replication. Latent infections are an essential property of herpes viruses. These viruses possess the ability to produce recurrent infections, as they have developed strategies to establish and maintain latency, and to reactivate from the latent state. A prerequisite for viruses to establish persistent infections is a means of evading the host immune response. Indeed, these viruses use several strategies to evade im-

mune-mediated clearance as has been comprehensively reviewed elsewhere.[3-5] The site of persistence within the host is widely variable between different persistent viruses. Several viruses establish persistent infections in the nervous system (e.g. herpes simplex virus [HSV], and varicella-zoster virus [VZV]). Hepatitis B virus (HBV) and hepatitis C virus (HCV) establish persistent infections in the liver, and cytomegalovirus (CMV), Epstein-Barr virus (EBV), human immunodeficiency virus (HIV), and human T-cell leukemia virus (HTLV) establish persistent infections in either lymphocytes or monocytes.
The balance between viral persistence and host immune regulation is of major importance and is well maintained in healthy individuals, but disrupted in many conditions associated with immunosuppression (e.g. transplantation). In such circumstances, viral latency and persistence can lead to reactivation and increased replication, which, if uncontrolled, can cause severe morbidity and mortality.

### *1.2 Virus induced cell transformation*

In addition to acute and persistent infections, interactions between virus and host may lead to alterations of the properties with regard to the regulation of cellular replication (cell transformation), which may eventually lead to cancer formation.[6]
The first virus strongly associated with cancer in humans was discovered in the twentieth century. In 1964, Anthony Epstein observed a virus, which was later named Epstein-Barr virus (EBV), in cultured cells from Burkitt's lymphoma.[7] Since those early days EBV has been associated with several other types of tumors including nasopharyngeal carcinoma and B-cell lymphomas. Several other viruses produce disease by promoting malignant transformation. Hepatitis B virus (HBV) and hepatitis C virus (HCV) are associated with hepatocellular carcinoma. Human papilloma virus (HPV) is associated with cervical cancer and a variety of anogenital and cutaneous neoplasms. Human herpesvirus 8 (HHV 8) is associated with Kaposi's sarcoma and primary effusion lymphoma particularly in persons with HIV infection.
All human tumor viruses require a long latent period to reveal their oncogenic potentials and only a small fraction of the infected hosts eventually develop a virus-induced malignancy. These viruses induce cell transformation and tumor formation through various mechanisms.[6]

## 2. PERSISTENT VIRAL INFECTIONS IN IMMUNOCOMPROMISED HOSTS

Viral infections are a principal cause of morbidity and mortality in immunocompromised patients, and in recent years, the number of immunocompromised patients has grown extensively. The global epidemic of HIV, more intensive and suc-

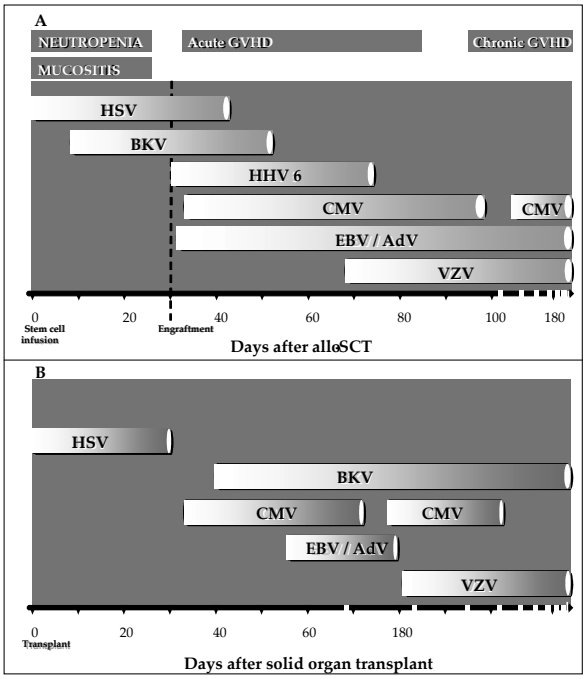
cessful cancer chemotherapy and particularly the availability of more potent immunosuppressive agents for transplant recipients have significantly contributed to this increase.

Transplantations in patients with end-stage organ disease or malignancies provide restoration of function and allow many patients to avert death and also to return to a life without functional limitations.[8,9] This success has been made possible mainly by improved control of rejection, graft versus host disease (GVHD) and infection, the major barriers of successful transplantation. Despite this progress, viral infections are still the most common life-threatening complication of long-term immunosuppressive therapy in transplant recipients. The risk for reactivation of chronic viral infection is closely linked to the nature and intensity of the transplant immunosuppressive program. The most important risk factors are those affecting the net state of immunosuppression: intensity of immunosuppressive therapy (dose, duration and temporal sequence), rejection or GVHD and its treatment, the use of alternate (unrelated, mismatched) donors and graft manipulation (T-cell depletion) in allogeneic stem cell transplantation.[10,11] For both solid organ (SOT) and allogeneic-stem cell transplantation (allo-SCT), protocols for managing immunosuppression have become quite standardized. As a result, similar patterns of reactivating viral infections, linked with the changing immunologic state of the transplant recipient, can be recognized (Figure 1). In general, the timetable of infection for stem cell transplant recipients can be divided into three phases. In the first phase (the neutropenic phase) herpes simplex virus (HSV) infections, mainly due to disruption of the mucosal integrity caused by the preparative regimen, comprise the major concern with respect to reactivating viral infections.[11] Treatment with acyclovir is usually effective. Phase 2, the time between engraftment and day 100, is the peak time period for viral reactivation, especially of cytomegalovirus (CMV) (Figure 1A). Early detection of viral reactivation and treatment, using antiviral drugs or immune modulation, are crucial to prevent severe morbidity and mortality. The third phase is the late transplant phase (>100 days) in which varicella-zostervirus (VZV) infections generally occur frequently, as well as late CMV infection.[11] In solid organ transplant recipients, the time table of infection can also be organized into three segments: the first month, one to six months and more than six months post-transplant, respectively. In general, most significant reactivating viral infections occur in the second phase (Figure 1B), and some patients have chronic or progressive infections with Epstein-Barr virus (EBV), CMV or polyoma virus (BK-virus) in the third phase.[10]

### *2.1 Cytomegalovirus (CMV)*

Like all herpesviruses, cytomegalovirus, a human betaherpesvirus, establishes a lifelong latency in its host after primary infection.[13]

**Figure 1.**

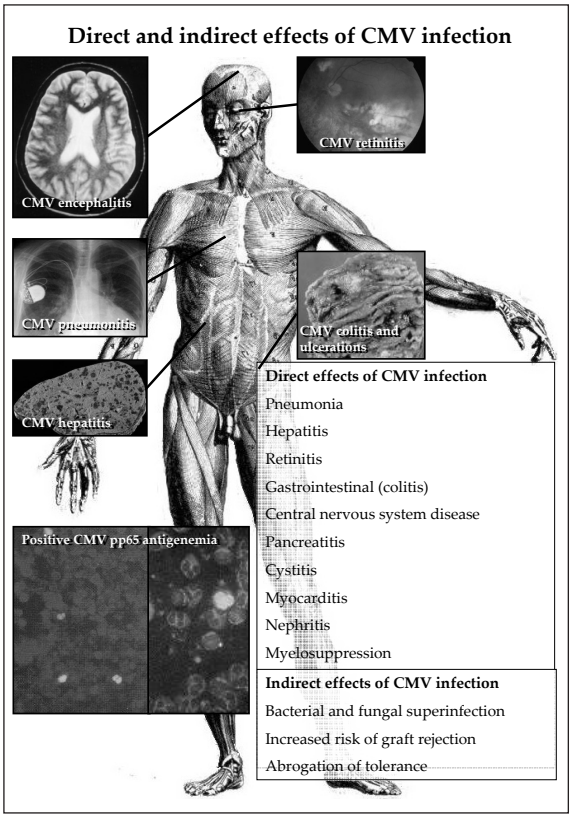



Common sequence of viral infections after (A) allogeneic stem cell transplantation (SCT) and (B) solid organ transplantation. Most common periods for the onset of infections are indicated. Times during which infections can occur are shown in relation to evolving underlying host conditions following allo-SCT (panel A). (AdV, adenovirus; Allo-SCT, allogeneic stem cell transplantation; BKV, BK virus; CMV, cytomegalovirus; EBV, Epstein–Barr virus; GVHD, graft-versus-host disease; HHV6, human herpesvirus-6; HSV, herpes simplexvirus; VZV, Varicella-Zostervirus) [Adapted from references (10-12)]

In stem cell transplant recipients, CMV can cause major complications, such as pneumonitis, fever and gastrointestinal disease and, to a lesser extent, hepatitis, retinitis, myelosuppression and encephalitis[14,15] (Figure 2). In solid organ transplant recipients, CMV disease can present as lung, liver, gastrointestinal, renal or retinal disease and is considered to be the most important infectious complication in these patients.[16,17] In addition to these end-organ diseases, CMV is also associated with "indirect effects"[18], comprising the association with acute and chronic graft rejection as well as bacterial and fungal superinfection (Figure 2).

The most important risk factor for CMV disease, besides factors affecting the net state of immunosuppression, is the pre-transplant serological status of the transplant recipient and the donor,[19,20] which serves to demonstrate the relevance of the previous occurrence of infection as well as the development of immunity before the

**Figure 2.**




Direct and indirect effects of cytomegalovirus infections in transplant recipients

start of immunosuppression. CMV seropositive SCT recipients, regardless of donor serostatus, are at higher risk of CMV disease compared to CMV seronegative recipients. However, in SOT, CMV seronegative recipients from seropositive donors are at the highest risk for CMV disease.[19]

The availability of antiviral agents, ganciclovir, foscarnet, and cidofovir has contributed to the significant reduction of CMV-related mortality and morbidity following transplantation in the past decades. However, the toxicity associated with treatment, mainly hematological and renal, limits the use of these drugs.

Therefore, efforts have been aimed at developing highly sensitive and quantitative virus detection methods to identify patients at risk at the earliest possible stage prior to the onset of disease. As CMV dissemination in blood was shown to be a hallmark of active infection, and viremia was recognized as the major virological risk factor for the progression to clinical disease,[21,22] it was considered likely that

**Table 1. Spectrum of EBV associated disease**

| |
|---|
| **Major EBV Associated diseases** |
| Infectious Mononucleosis (IM) |
| X-linked lymphoproliferative syndrome (X-LPS) |
| B Lymphoproliferative disease (BLPD/PTLD) |
| Burkitt Lymphoma |
| Nasopharyngeal carcinoma |
| EBV-genome positive Hodgkin's disease |
| EBV-genome positive T/NK cell lymphoma |
| EBV-genome positive gastric carcinoma |
| Oral hairy leukoplakia |
| **Possible EBV associated disease** |
| Salivary gland tumors |
| Breast carcinoma |
| Hepatocellular carcinoma |
| Thymoma |

quantification of the systemic CMV load would provide a highly sensitive and specific method to predict the development of CMV disease. The clinical relevance of early assays, such as the traditional plaque assay, the determination of the 50% tissue culture infective dose (TCID50) and other modified tissue culture-based methods, is limited due to time-consuming procedures, poor reproducibility, and a relatively low sensitivity. These assays were almost completely replaced by a direct antigen detection method, aimed at the CMV-pp65 lower matrix antigen.[23,24] However, disadvantages of the pp65 antigenemia assay include its subjective and time consuming nature, the limited reproducibility and a limited reliability in patients with neutropenia, e.g. following stem cell transplantation or sometimes as a consequence of CMV infection.

### *2.2 Epstein-Barr virus (EBV)*

Like human herpesviruses in general, Epstein-Barr virus (EBV) has co-evolved with humans to become one of the most successful viruses, infecting over 90% of the human population and persisting for the lifetime of the host. Since its discovery in cultured Burkitt's lymphoma cells in 1964,[7] EBV has been implicated in a wide variety of benign as well as malignant diseases, of either lymphoid or epithelial origin (Table 1). A unique set of genes expressed in latency provides EBV with

oncogenic potential and the ability to induce immortalization of B lymphocytes *in vitro*.[25,26] Despite this threatening property, EBV establishes a harmless life-long infection in most infected hosts and rarely causes severe disease unless the host–virus balance is upset.

EBV-associated B cell lymphoproliferative disease emerges as an opportunistic tumor in the setting of intense T cell immune suppression.[27] This is a common life-threatening complication in solid organ and stem cell transplant recipients and its clinical presentations can vary considerably and can mimic graft-versus-host disease, graft rejection, or more conventional infections. Presenting clinical features may resemble mononucleosis to some extent or can include any sign of a lymphoid tumor.[27] As for therapeutic options, reduction of immunosuppressive therapy, if possible, would allow recovery of cytotoxic T lymphocyte (CTL) activity and could lead to tumor regression in early cases, but involves risks of rejection of the transplanted organ or acute graft versus host disease.[27] Furthermore, posttransplant lymphoproliferative disease (PTLD) often recurs, and can become resistant to this conservative treatment. Novel forms of immunotherapy have been tested in PTLD with favorable outcomes.[27] These include humoral approaches, employing humanized mouse monoclonal antibody targeting the CD20 molecule on the surface of all mature B cells (Rituximab) and cell-mediated approaches using infusions of cultured EBV-specific CTL's. However, the varied presentations make the timely clinical diagnosis of PTLD difficult and, consequently, predictive markers have been sought. It appears that high concentrations of EBV DNA in peripheral blood occur in patients with PTLD,[28] which can be applied to the early detection of this disease.

EBV-associated malignancies from epithelial origin include nasopharyngeal carcinoma, which is most prevalent in southern China, in northern Africa, and among Alaskan Inuit, and occurs sporadically in the United States and Western Europe.[29] Nearly 100% of the poorly differentiated nasopharyngeal carcinomas contain EBV genomes and express EBV proteins. Clonal EBV genomes are found in the early pre-invasive carcinoma in situ, indicating that EBV infection precedes the development of malignant invasive tumors.[29] Patients with nasopharyngeal carcinoma often have elevated titers of IgA antibody to EBV structural proteins and measurement of EBV-specific IgA antibodies has been found useful in the screening of patients for early detection of nasopharyngeal carcinoma in southern China. An increase in EBV-specific antibody titers after therapy for nasopharyngeal carcinoma is associated with a poor prognosis, whereas declining or constant levels of antibody reflect a better prognosis.[29] EBV DNA can be detected in peripheral blood in patients with nasopharyngeal carcinoma, and the initially increased number of copies of EBV DNA in the blood during the initial phase of radiotherapy suggests that viral DNA is released after cell death.[30,31]

### *2.3 Varicella-zoster virus (VZV)*

Primary infection by this alphaherpesvirus causes varicella (chickenpox), a common and extremely contagious acute infection that occurs in epidemics among preschool and school-aged children and which is characterized by a generalized vesicular rash.[32] Following primary infection, VZV establishes latency in sensible cranial nerve and dorsal root ganglia, and may reactivate decades later to produce herpes zoster (shingles), a localized cutaneous eruption sometimes accompanied by neuralgic pain.

A primary VZV infection can have a severe course in SCT patients. The risks of cutaneous and visceral dissemination of VZV in severely immunocompromised patients are well recognized.[33] The risk of reactivation leading to herpes zoster is highest between 3 and 6 months after transplantation and commonly reported complications include VZV pneumonia, encephalitis, and hepatitis. Ocular complications such as VZV-associated acute retinal necrosis (ARN) have been described in both immunocompetent and immunocompromised persons.[33] An unusual presentation of herpes zoster in the immunocompromised host is "atypical generalized zoster".[33] These VZV seropositive patients present with diffuse varicella-like skin lesions with no obvious primary dermatomal involvement. Another atypical manifestation of herpes zoster is "abdominal" or "visceral" zoster.[33] These patients present with severe abdominal pain that may precede the appearance of the cutaneous rash by hours to days. The mortality and morbidity associated with disseminated zoster has been substantially reduced by the availability of effective antiviral therapy (aciclovir). However, the diagnosis of herpes zoster is usually not considered until the typical skin vesicles are apparent, which may limit the effects of antiviral therapy. Therefore, appropriate diagnostic assays enabling rapid and accurate diagnosis of clinically relevant VZV infections or reactivation in transplant recipients are essential.

### *2.4 Human herpesvirus 6 (HHV6)*

Primary infection with human herpesvirus 6 (HHV-6), a member of the Roseolovirus genus of the betaherpesvirus subfamily of human herpesviruses, causes acute febrile illness with or without mild skin rash, usually in children between 6 months and 1 year old (roseola infantum or sixth disease). Like other herpesviruses, HHV6 establishes latency after primary infection.[34] Two subtypes of HHV-6 can be distinguished (HHV-6A and HHV-6B), sharing certain biological properties and a high level of sequence homology, but differing significantly in their epidemiology.[34] Human herpes virus 6 reactivates in 40–50% of hematopoietic stem cell transplant (HSCT) recipients and in a similar proportion of solid organ transplantation (SOT) recipients. In both transplant populations, reactivation occurs between 2 and 6 weeks after transplantation and is due mostly to type B virus, with type A accounting for between 2% and

3% of events.[34] In contrast to immunocompetent children where, HHV-6 infections are self-limiting, reactivation of latent virus is associated with serious or even life-threatening complications in immunocompromised individuals. After SOT, HHV-6 has been most frequently associated with encephalitis, other infections including CMV, organ rejection and mortality.[35,36] In SCT recipients, HHV-6 has been most strongly associated with encephalitis, bone marrow suppression and graft versus host disease.[34] No controlled trials of antiviral therapy against HHV-6 have been conducted, and no compounds have been formally approved for the treatment of HHV-6 infections. The drugs clinically used against HHV-6 are the same as those used in CMV therapy, ganciclovir and foscarnet. Isolation by culture of HHV-6 from the blood demonstrates active viral infection. However, culturing HHV-6 is labor-intensive and time-consuming. Detection of viral nucleic acids may indicate active or latent infection depending on the clinical setting and the specimen tested. Detection by PCR of viral DNA in white blood cell fractions can be difficult to interpret since the mononuclear cell is a site of latency. Detection of HHV-6 DNA in plasma or serum correlates well with indicators of active replication and is therefore more directly interpretable.[34,37]

### *2.5 Human herpesvirus 8 (HHV8)*

HHV-8, member of the gammaherpesvirinae subfamily, has been associated with all forms of Kaposi's sarcoma (KS), primary effusion lymphoma, and multicentric Castleman's disease. The rarely occurring post-transplant Kaposi's sarcomas in solid-organ transplant recipients, are particularly seen following renal transplantation, and are caused by two possible mechanisms: HHV-8 transmission from the donor to the recipient or HHV-8 reactivation in patients who were infected before transplantation.[38] Like other herpesviruses, HHV-8 persists in a latent form for life, with $CD19^+$ B cells as the main reservoir.

Similar to other herpesviruses like CMV and EBV, detection of HHV-8 DNA in peripheral blood mononuclear cells (PBMCs) may reflect latent infection, while detection of HHV-8 in serum or plasma reflects active lytic replication. The finding that plasma viremia of HHV-8 is an important event in KS pathogenesis implies that HHV-8 DNA detection in plasma or serum may have a predictive value for disease development and progression.[39-41]

### *2.6 Adenovirus (AdV)*

The human adenoviruses belong to the family of Adenoviridae and consist of at least 51 serotypes, which are grouped into six species (A-F). Different clinical syndromes have been associated with different species. Adenovirus infections are more common in children, with a peak incidence between 6 months and 5 years of age. Fur-

thermore, adenoviruses are increasingly recognized as pathogens causing significant morbidity and mortality, particularly in pediatric allogeneic stem cell transplant recipients.[42-44] In SCT patients, the increased risk of severe adenovirus infection is related to patient age (higher in children than in adults), immunosuppressive regimen (especially the use of T cell-depleted grafts, antithymocyte globulin or anti-CD52 monoclonal antibody), delayed immune recovery post-transplant, presence of graft versus host disease and the degree of genetic discrepancy between the donor and recipient.[43,44] Clinically severe adenoviral disease in these patients presents as fulminant hepatitis, pneumonia or encephalitis. Gastroenteritis and hemorrhagic cystitis may also occur. Species B and C are isolated most often as the cause of severe disease in stem cell transplant recipients.[42,44]

To date, no antiviral treatment of adenovirus infection has unequivocally proven clinically effective. However, ribavirin and cidovofir, agents with *in vitro* activity against adenoviruses, have been used in allo-SCT recipients, but firm conclusions as to the effectiveness of these drugs cannot be drawn.[43,45,46] This uncertainty is related to the importance of the recovery of immunity following SCT, which is considered to be essential for the elimination of AdV infection.[43,47-49]

Laboratory diagnosis of adenoviral infection has traditionally been carried out by viral culture, and conventional monitoring of AdV infection, particularly in pediatric stem cell recipients, is usually performed by culture of feces, urine samples and throat swabs. Even with the rapid detection of adenoviral antigens by immunofluorescence methods, this can take days to weeks to yield results. In the case of this viral infection, it appears that measuring viral nucleic acid load in plasma could provide valuable information with regard to the clinical relevance of the infection. Recently, detection of AdV DNA in plasma by real-time quantitative PCR with high sensitivity and specificity for use on clinical specimens has been described. Studies using this technology have provided new insight into the pathogenesis of AdV reactivation following allo-SCT, including the fact that a relatively high DNA load in serum, rather than the duration of the infectious episode, turned out to be a sensitive and specific marker of a fatal course of the infection.[48,50] Furthermore it was found that in most patients, a time window of several weeks was observed between the first detection of a significant AdV DNA load in plasma and the clinically manifest stages of AdV disease, offering a potential opportunity for intervention.[48,51] Thus, the monitoring of AdV DNA levels in serum, plasma or whole blood by real-time quantitative PCR can be considered to be a sensitive tool for the recognition of pediatric patients at risk of a potentially fatal disseminated AdV infection following allo-SCT and might also be applicable to the adult population, despite the lower incidence of disseminated AdV infections in this population.

### *2.7 BK virus (BKV)*

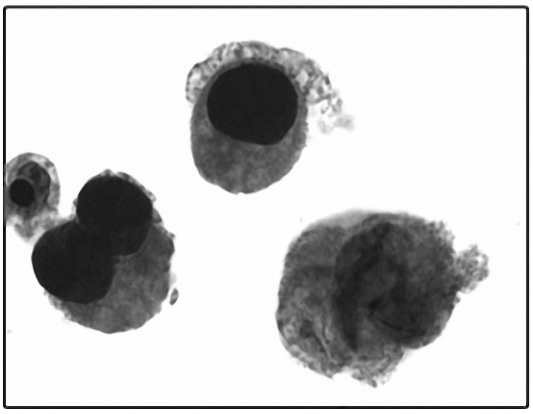
The human polyomavirus type 1, named BK-virus after the initials of the first described patient,[52] belongs to the family Polyomaviridae, which are relatively small double stranded DNA viruses. Primary infection typically occurs during early childhood, after the waning of maternal antibodies and is probably generally asymptomatic. Following primary infection, a state of non-replicative infection (latency) results at multiple sites, including renal tubular epithelial and urothelial cells.[53] In the past decade BKV has emerged as a significant pathogen in kidney transplant recipients, causing polyomavirus-associated nephropathy (PVAN) which is characterized by tubulo-interstitial nephritis progressing to fibrosis and tubular atrophy.[54,55] Increasing prevalence rates of PVAN (1%–10%) have been reported since then, with allograft dysfunction and loss in up to 50% of cases.[56] Immunosuppression is generally accepted as the main factor determining the risk for PVAN. BKV replication is significantly associated with combinations of tacrolimus and mycophenolate mofetil (MMF), and less with other drug combinations.[57-59] The current mainstay of intervention is the reduction of immunosuppressive maintenance therapy, as the specific treatment of PVAN is difficult.[63] The diagnosis of PVAN requires the histological demonstration of BKV replication and resulting organ damage.[56] Although allograft biopsy is highly specific, its sensitivity is limited due to focal involvement, particularly in the early stages, and because the presentation may be mistaken as acute rejection or chronic allograft nephropathy. Early diagnosis of PVAN and timely intervention are associated with a favorable outcome and can be achieved by screening for BKV replication in the urine (cytology and quantitative PCR) and blood (quantitative PCR).[57,60]

Another manifestation of BKV infection is polyomavirus viruria, which is defined by the presence of cytological "decoy cells" (Figure 3) and occassionally is accompanied by hemorrhagic cystitis.[53] Hemorrhagic cystitis is also rare in HIV/AIDS and in solid-organ transplantation but represents a frequent complication in bone marrow transplantation (incidence, 5%–60%). Early-onset hemorrhagic cystitis has been linked to toxic effects of the conditioning procedure, whereas late-onset hemorrhagic cystitis, starting 12 weeks after transplantation, has been associated with BKV viruria.[61]

### *2.8 JC virus (JCV)*

The neurotropic human polyomavirus type 2 or JC virus (JCV), also named after the initials of the first described patient,[62] is the etiologic agent of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system (CNS).[63] Together with BK virus (BKV) and simian virus 40 (SV40), JCV is also a member of the polyomaviruses and is widespread within the

**Figure 3.**



Epithelial cells bearing intranuclear polyoma virus inclusions, so-called "decoy cells", in the urine. Nuclei are enlarged and nuclear chromatin is completely homogenized by the viral cytopathic effect (600x magnification).

human population, as over 80% of adults worldwide exhibit JCV specific antibodies.[56] Subclinical infection with the virus occurs in early childhood and the virus remains in a latent stage throughout life, although on rare occasions the virus becomes reactivated and causes PML when the immune system is impaired. Prior to the AIDS epidemic, PML was considered an extremely rare disorder associated with immunocompromising diseases such as lymphomas. It was also seen in renal transplant and chemotherapy patients as a complication of immunosuppressive therapies. Recently, PML has also been shown to develop during clinical trials of natalizumab, a selective adhesion-molecule blocker, to treat relapsing-remitting multiple sclerosis or Crohn's disease.[64-67]
A brain biopsy is required to demonstrate the typical polyoma virus intranuclear inclusions which can be found in oligodendrial cells. High levels of JCV viral DNA are found in the cerebrospinal fluid of patients with PML[68] and quantitative PCR assays were successfully employed to analyze the correlations between natalizumab treatment and the risk for PML.[64]

As with herpes viruses, diagnosis of the other clinical relevant persisting viruses in immunocompromised patients (e.g. transplant recipients) can be challenging. This is mainly due to the high prevalence and persistence of these viruses and it may limit the effects of appropriate therapy. Therefore, appropriate diagnostic assays enabling rapid and accurate detection of clinically relevant infections with these viruses are

essential. Traditional diagnostic approaches using cell culture and serology based assays are often of limited use, mainly due to time-consuming procedures, poor reproducibility, and a relatively low sensitivity of these assays. Hence, merely demonstrating the presence of these persisting viruses in specimens from immunocompromised patients does not indicate clinical relevant infection or reactivation. An optimal assay for monitoring these reactivating viruses should meet several requirements, including a high sensitivity to enable early detection in patients at high risk for disease. Another prerequisite is the ability to quantify the result, in order to increase the positive predictive value and to enable the monitoring of treatment results. Particularly in situations where the balance between host-immunity and virus replication is disturbed, e.g. in immunocompromised patients, quantification of viral infections would potentially allow the differentiation of clinically relevant viral infection and merely reactivation. Furthermore, a short turnaround time, to allow early interventions and a high degree of reproducibility, is also essential.

## 3. Developments in diagnostic virology

As described in the first paragraph, the virus as an infectious principle was discovered long after bacteria had been established as causes of disease. Also, the development of diagnostic and therapeutic strategies in virology has, for a considerable time, lagged behind substantially compared to bacteriology; at least, until new molecular tools became available in the last decades, revolutionizing the approach to viral infections.[69]

### *3.1 From "filterable agents" to real-time nucleic acid amplification*

The earliest methods used for the characterization of viruses were based on physical properties, rather than the more significant biological features. Hence, the range of methods employed during the first three decades of the twentieth century to characterize viruses included filtration, centrifugation, adsorption, electrophoresis, and optical methods.[70] As it appeared later, a major bottle-neck included the essential role of living cells in virus propagation. Subsequent innovations and improvements in tissue and organ cultures[71] enabled the growth of human pathogenic viruses in tissue cultures, which accounted for a major advancement in diagnostic virology.[72,73] Although expensive and time-consuming, cell cultures formed the basis of diagnostic virology for many years.

More rapid viral diagnosis became possible in the 1980s, with the introduction of fluorescent antibody staining and the development of monoclonal antibodies against a wide variety of viral antigens.[69] The subsequent application of molecular techniques,

particularly the use of polymerase chain reaction led to a rapid evolution of laboratory assays for viral diagnosis which is still ongoing. Presently, major improvements have been made with regard to the application of diagnostic virology in routine medical practice. Besides newly developed technologies, including the polymerase chain reaction (PCR), several other factors have stimulated the expanded role of diagnostic virology. These include the discovery of new pathogenic viruses, among which is HIV, an increased number of patients at risk for opportunistic viral infections and the development of new antiviral agents. Currently, a large number of antiviral agents have become available and their use often depends on laboratory-based diagnosis. However, classical qualitative diagnostic assays, (culture based or even qualitative PCR based assays) are often of limited use when it comes to detecting clinically relevant reactivation of persisting viral infections. By viral culture, CMV can often be detected in throat and urine samples without any clinical relevance while other persisting viruses (EBV, BKV, JCV) are difficult to culture.

A recent development in the evolution of diagnostic virology is the potential for quantitative measurement of viral infections. In contrast to qualitative results, quantitative data provide information with respect to the viral burden in relation to clinical presentation and disease progression. Quantitative measurement of viral infections can be performed on the level of viral culture, in plaque assays and shell vial centrifugation cultures, by detection of viral particles using electron microscopy, by detection of viral antigens using immunological assays, e.g. the CMV pp65 antigenemia assay, or on the level of viral nucleic acids.[74] However, only the quantification of viral nucleic acid, by using techniques like PCR or "nucleic acid sequence-based amplification" (NASBA), provides the high sensitivity and specificity which are essential for such assays to be used in routine clinical settings.[75]

This quantitative approach on the level of viral nucleic acids proved to be highly useful in HIV-infection (76). The management of antiretroviral drug therapy in patients with HIV infection or AIDS is based on assays to measure plasma HIV RNA, and genotypic and phenotypic assays to test HIV drug resistance. Quantitative virology also proved to be of advantage to the management of chronic hepatitis B infections.[77] Driven by this success, the quantitative approach is now increasingly applied to other chronic, persisting or reactivating viral infections, like herpes virus infections. However, earlier assays for nucleic acid based quantification of viral infections, such as quantitative competitive PCR, were time-consuming, lacked adequate sensitivity and were not readily available for routine diagnostic use.[78,79]

Novel developments in PCR technology include the concept of quantification of DNA or RNA products while they accumulate (like "real-time" quantitative PCR). Real-time technology has a number of advantages, such as reduced risk of contamination, high accuracy, the kinetic principle, along with a short turnaround time for

results and ease of performance. These characteristics make it an attractive replacement method for conventional PCR assays as well as traditional culture and antigen based diagnostic methods. With these advantages of real-time PCR, the quantitative approach towards the management of persisting and reactivating viral infections is becoming a reality.

### *3.2 Real-time PCR*

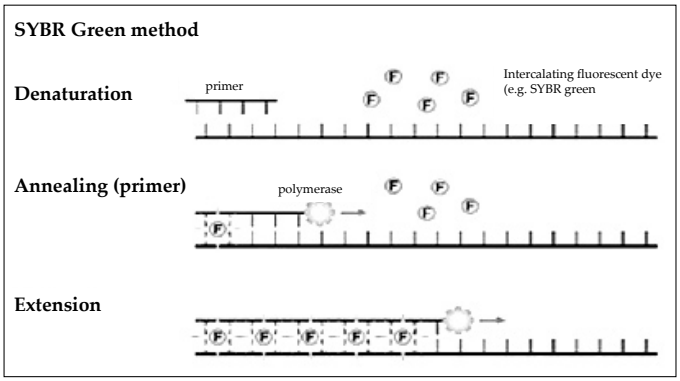
Within the last decade, PCR technology has evolved from a research tool to the widely accepted gold standard in diagnostic virology, providing exquisite sensitivity and specificity for the detection of all viral targets.[80] Besides improvements of technical features, establishing the association of PCR results with the course of viral disease in various categories of patients is essential. Therefore, accurate evaluation to ensure reliability and to establish definitions for clinical workflow is a prerequisite for introducing these assays into clinical diagnostic laboratories.

Real-time PCR combines PCR chemistry with immediate fluorescent probe detection of amplified products in the same reaction vessel.[81] Using this technology, nucleic acid amplification and detection of amplified product are completed considerably faster than by using conventional PCR detection methods. Additionally, these assays provide equivalent sensitivity and specificity to conventional PCR combined with Southern blot analysis. Compared with conventional PCR, the carry-over contamination is negligible, as the nucleic acid amplification and detection steps are performed in the same closed vessel, which reduces the risk of releasing amplified nucleic acids into the environment, and contamination of subsequent analyses. As for ease of use, real-time PCR instrumentation requires considerably less hands-on time than conventional PCR methods and testing is much simpler to perform.[79,82] Additionally, real-time PCR procedures easily allow multiplexing, enabling the simultaneous detection of several targets. Finally, quantification can easily be included, using the emergence of the signal during the course of the reaction, and it boosts an extremely extended dynamic range. The combination of all these advantages (excellent sensitivity and specificity, low contamination risk, ease of performance and speed as well as the possibility of multiplexing and quantification) has supported the broad acceptance of PCR technology as a superior alternative to conventional culture-based or immunoassay-based testing methods used for diagnosing viral diseases.

### *3.3 DNA extraction and detection of nucleic acids*

The first step to real-time PCR, nucleic acid extraction, is generally the most labor-intensive part if performed manually. Besides being a laborious and time-consuming process, manual extraction usually requires multiple manipulations, which in-

**Figure 4.**



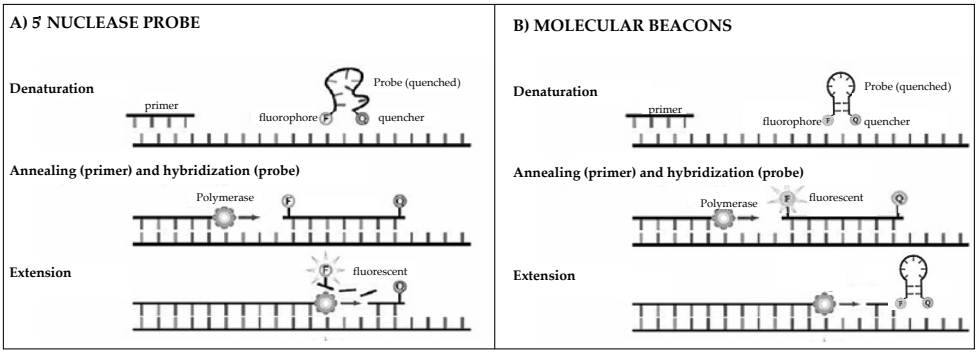

Visualization of nucleic acids using intercalating fluorescent dyes such as SYBR Green, detects accumulation of any double-stranded DNA product.

troduces an increased potential for contamination. Various automated systems for nucleic acid purification, such as the MagnaPure LC (Roche Diagnostics), are now available from commercial companies. The advantages of automated extraction systems over manual methods include consistent and reproducible recovery of nucleic acids combined with a significant reduction of extraction time and hands-on time. Furthermore, as many of the instruments are closed systems and sample manipulation is kept to a minimum, the risk for cross-contamination of samples is reduced.[80] Studies comparing manual and automated extraction have reported automated methods to be equivalent, and in some instances, superior to manual methods.[83-88] These advantages of automated systems enable rapid and efficient nucleic acid extraction and facilitate the efficient use of PCR in diagnostic laboratories.

To detect nucleic acids with real-time PCR, one can use intercalating fluorescent dyes such as SYBR Green, which detects the accumulation of any double-stranded DNA product (Figure 4). SYBR Green provides sensitive detection without any specificity. More sensitive and specific detection of nucleic acids is possible with real-time PCR using novel fluorescent probe technologies such as TaqMan probes and molecular beacons (Figure 5). The mechanisms to achieve a fluorescent signal with various probe technologies are different and all have specific characteristics, making them suitable for a wide range of applications such as quantification, multiplex reactions and single nucleotide polymorphism (SNP) analysis.[80,89]

Various real-time PCR platforms from different manufacturers, such as the LightCycler (Roche), SmartCycler (Cepheid), ICycler (BioRad) and Prism (ABI) are avail-

**Figure 5.**




Real-time probe technologies. (A) 5′ nuclease (TaqMan) probe. (B) Molecular beacon.

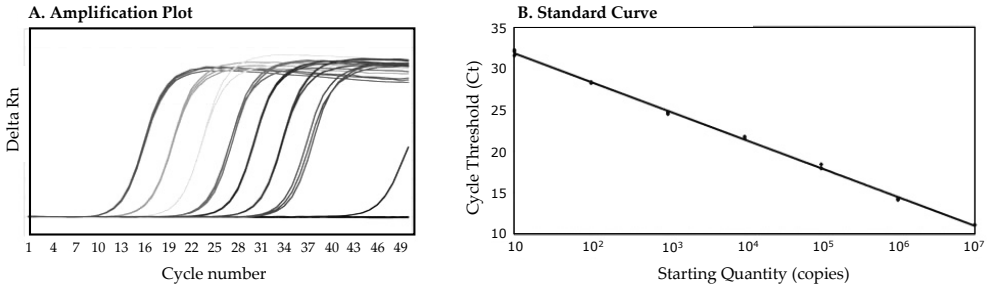
able. These systems differ with respect to supported probe formats, excitation and detection wavelength, maximum number of samples per run, reaction volumes and relative thermocycling times.[80] In general, workload and workflow issues are likely to determine which system will best suit different-sized laboratories and test volumes.

### *3.4 Real-time PCR assay development*

A critical aspect in the application of real-time PCR assays in the laboratory concerns their design. As PCR primers provide the first level of specificity for the real-time PCR assay, accurate primer design is essential, in order to identify a specific organism or organism group with high efficiency and specificity. The most critical variables that must be taken into account when designing PCR primers include the specificity, the primer length, the melting temperature (*T*m), the complementary primer sequences, G/C content and the 3′-end of the primer sequence.[90] Besides these usual PCR requirements for the selection of the assay components, various considerations specific to the real-time format should be taken into account. The size of the PCR product is limited (up to 200 base pairs), the *T*m of the primers should be slightly less than the *T*m of the probe in order to facilitate probe binding prior to primer binding, which is crucial to obtain fluorescence.[80] The use of software (PE/ABI Primer Express [Applied Biosystems] and Beacon Designer [Premier Biosoft]) which has incorporated all the required design parameters, can be of great value when developing real-time PCR assays. Details regarding the design of primer and probes are described extensively in a previous publication.[90]

A prerequisite for real-time PCR assays to be used as a diagnostic tool is that the integrity of the assay is assured through measures concerning quality control and

**Figure 6.**




Relative fluorescence units obtained for a 10-fold dilution series (panel A). Each dilution obtains a Ct value which can be plotted against the starting quantity to obtain a standard curve (panel B). The value obtained for the slope of the line gives a value for the PCR efficiency.

quality assurance.[91,92] In this respect, one particular issue is the inclusion of internal controls to monitor the whole test process from nucleic acids purification to detection. Construction of these internal controls can be performed in various ways.[93] Furthermore, the verification and validation of test performance is of primary importance in order to ensure the consistency of the PCR results. This process includes the establishment of the test accuracy, its reproducibility, and its relevance to clinical practice. Once the evaluation of the PCR assay has been performed, implementation in standard diagnostic laboratory procedures and continued monitoring of its performance and reliability in order to ensure the quality of the assay require careful consideration.

### *3.5 Quantification*

Real-time PCR offers a significant advantage with respect to the quantification of viruses, as real-time based technology can measure viral loads over a wide dynamic range, where other PCR detection systems can only detect the endpoint of an amplification reaction. Quantification can be achieved using internal or external standards, a combination of either method, or competitive amplification.[79] A dilution series of these standards, with known specified or calibrated levels of target nucleic acids, are included in each test run of each quantitative real-time PCR determination. Subsequently, a standard curve can be generated by using the known copy level of the standard reagent to plot fluorescence, a measure of amplified product, against the cycle number in which the nucleic acid target has been detected (Figure 6). The amount of targeted nucleic acids in the specimen can then be determined by comparing the cycle number of the specimen with the standard curve. Quantitative standards (e.g., EBV DNA) from commercial sources

are helpful for developing quantitative tests for viral load levels. Alternatively, nucleic acid targets from viruses cultivated in cell cultures, or target nucleic acids inserted into a plasmid, can also be used to generate standard curves for quantitative assays.

### *3.6 Applications of quantitative real-time PCR in clinical virology*

The clinical relevance of quantitating viral infections is demonstrated by its success in managing HIV infections.[76,94] Quantitation is also relevant with regard to chronic Hepatitis B virus infections, as already recognized in the times of DNA detection by simple hybridization without amplification. Real-time-based TaqMan assays have recently been described to enable detection of HBV DNA for the whole dynamic range of HBV DNA levels in a single assay.[77,95] Quantitative assays have contributed to new insights into the correlation between HBV DNA load and response to treatment,[96] as well as the emergence of drug resistant viruses.[97-99] With respect to Hepatitis C virus infections, it has been clearly established that the two important virological determinants of treatment outcome are the viral genotype and the quantity of the viral presence, or viral load.[100] Subsequently, the application of quantitative real-time PCR methods for the detection and management of various other viral infections is increasingly gaining interest. Particularly the management of nearly all the human herpes virus family members (CMV, EBV, VZV, HHV6 and HHV8) in immunocompromised patients such as transplant recipients, can benefit from this approach. For this reason the clinical relevance and particularly the applicability of this technology in various clinical settings should be established.

Besides the practical relevance of a quantitative approach, there is also interest in the origin of the observed signals. Obviously, detected levels of viral DNA do not necessarily imply the presence of infectious viral particles in the samples. This is clear from the simple comparison of these assays with viral culture, where applicable, which invariably shows a far lower sensitivity of cultures or even a complete lack of positive cultures.[101,102] A more detailed analysis of viral DNA targets that are detected by PCR assays in plasma samples confirms this observation, demonstrating fragmentation.[103] Regardless of its origin, the applicability of the presence and notably the kinetics of viral DNA in plasma as a marker, indicative of either a risk for viral disease (e.g. following organ transplant) or an association with virus induced tumors (e.g. nasopharyngeal carcinoma) should be assessed. For these purposes, the practical relevance and the association with viral disease or tumor activity is of importance, rather than the nature of the DNA, which is either highly fragmented or derived from still infectious virus particles. The clinical evaluation of such markers as well as the standardization of quantitative viral assays constitutes essential challenges for practical applications.

## 4. SCOPE OF THIS THESIS

As discussed in the previous section, real-time monitoring of PCR has strongly supported the increased diagnostic use of nucleic acid detection assays in clinical virology. Particularly the improvements in the ability to quantify target nucleic acid sequences offer new opportunities in the management of viral infections. Real-time PCR is rapidly replacing traditional PCR, and new diagnostic uses will likely emerge. This thesis explores the wide range of potential applications of real-time quantitative PCR technology in clinical virology. This exploration is directed to the design of methods, the application to relevant patient categories, the comparison with established methods where available, and the definition of the clinical relevance of the approach. The focus comprises viral targets where an elaborate balance between viral replication and the host immune system has been established, which brings about viral maintenance without affecting the host, until this balance is disturbed.

**Chapter 2** describes the development, the technical validation and clinical evaluation of a real-time quantitative CMV PCR. The correlation of CMV DNA load in plasma with the CMV pp65 antigen detection assay was assessed in order to enable the definition of criteria for pre-emptive CMV treatment following both solid organ and stem cell transplantation. Using the criteria established in chapter 2, the quantitative real-time CMV PCR was used for routine monitoring of CMV reactivation following solid organ and stem cell transplantation. Subsequently, this assay was employed to evaluate the efficacy of different antiviral agents, the influence of immunosuppressive induction regimens on CMV viremia and the safety of conditioning regimens with respect to CMV infections.

In **chapter 3** the efficacy of pre-emptive CMV treatment with oral valganciclovir or intravenous ganciclovir was evaluated, based on the resulting reduction of CMV DNA load in plasma, both in recipients of solid organ transplants and in recipients of stem cell transplants.

The influence of the duration and intensity of immunosuppressive therapy on CMV infections in transplant recipients is addressed in **chapter 4**. Studying a cohort of simultaneous pancreas-kidney transplant (SPK) recipients, effects on CMV viremia were compared between different immunosuppressive induction therapy regimen containing either ATG or Dacluzimab.

In **chapter 5**, the safety of conditioning regimens prior to allogeneic stem cell transplantation (either reduced-intensity or myeloablative), with respect to CMV infections was assessed in a cohort of 107 adult patients, using the quantitative real-time CMV PCR.

In chapters 6 and 7, the application of monitoring Epstein-Barr virus DNA, using a real-time quantitative PCR was studied. **Chapter 6** describes a cohort of 25 consecu-

tive pediatric stem cell transplant recipients, in which it was assessed whether the identification of patients at risk for EBV-lymphoproliferative disease (LPD) could be improved by the simultaneous analysis of EBV DNA load and EBV-specific T-cell reconstitution.

**Chapter 7** explores the use of monitoring by EBV-DNA PCR in relation to another EBV-associated malignancy, nasopharyngeal carcinoma. The application of real-time quantitative EBV PCR as a tumor marker in a cohort of patients with nasopharyngeal carcinoma living in a low-incidence area is described.

**Chapter 8** addresses the application of quantitative VZV-DNA detection using real-time PCR technology in immunocompromised patients at risk for VZV reactivation; the clinical relevance of quantitative VZV-DNA detection in plasma was assessed in a cohort of 81 adult allo-SCT recipients. Chapter 8 also includes a description of an unusual manifestation of herpes zoster consisting of VZV-related progressive outer retinal necrosis (PORN) and its relationship to detectable VZV DNA load in plasma.

**Chapter 9** concerns a study in a cohort of 107 adult allo-SCT recipients on the occurrence of disseminated infections by adenovirus, as determined using a quantitative real-time adenovirus PCR and compared to a reference cohort of pediatric allo-SCT recipients.

The overall results as well as future issues with respect to the application of quantitative real-time PCR in clinical virology are discussed in **chapter 10**.

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# 2

# Validation of Clinical Application of Cytomegalovirus Plasma DNA Load Measurement and Definition of Treatment Criteria by Analysis of Correlation to Antigen Detection

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## ABSTRACT

Successful preemptive cytomegalovirus (CMV) therapy in transplant patients depends on the availability of sensitive, specific, and timely diagnostic tests for CMV infections. The pp65 antigenemia assay has been used for this purpose with considerable success. Quantification of CMV DNA is currently regarded to be an alternative diagnostic approach. The precise relationship between these two methods has still to be defined, but is essential to compare diagnostic results. This study compared the results of both assays with a large series of transplant recipients in different categories. An internally controlled quantitative real-time CMV DNA PCR was used to test 409 plasma samples from solid organ transplant (SOT) and stem cell transplant (SCT) patients. Levels of CMV DNA in plasma correlated well with classified outcomes of the pp65 antigenemia test. Despite this correlation, the quantitative CMV PCR values in a class of antigen test results were within a wide range, and the definition of an optimal cutoff value for initiating treatment required further analysis by a receiver-operating characteristic curve analysis. This is essential for reactivating infections in particular. For the SCT patients the optimal cutoff value of CMV DNA load defining relevant viral reactivation (in this assay, 10,000 copies/ml) was slightly higher than that for the SOT patients (6,300 copies/ml). Based on a comparison with the established pp65 antigenemia assay, quantification of CMV DNA in plasma appeared to be capable of guiding the clinical management of transplant recipients. This approach may have important advantages, which include a superior reproducibility and sensitivity, allowing the inclusion of kinetic criteria in clinical guidelines.

Human cytomegalovirus (CMV) is a ubiquitous member of the human herpesvirus family of viruses. Up to 80% of healthy adults in western countries are seropositive, indicating previous exposure and established latency with the capability of viral reactivation. The mechanism of reactivation is largely unknown but appears to be strongly related to impaired immune control of the virus. For this reason, CMV is one of the most common opportunistic pathogens complicating the care of transplant recipients; potentially it is a major cause of morbidity and mortality. Treatment of CMV disease with specific antiviral drugs such as ganciclovir and foscarnet reduces disease severity and mortality in these patients. Prophylactic and preemptive antiviral strategies have been developed and aim at avoiding aggressive treatment of established end-organ disease. Prophylactic treatment involves the administration of antiviral drugs to all patients at risk for an extended period. Preemptive therapy is specifically directed towards patients identified as having a high risk for CMV disease, thus sparing many from the toxicity of universally applied antiviral prophylaxis. The success of preemptive therapy is dependent upon the availability of appropriate diagnostic tests for early stages of CMV infections. The pp65 antigenemia assay has been used for this purpose with considerable success.[2, 10, 14] However, this assay is labor-intensive and requires samples to be processed within a few hours. In addition, its reading is subjective and therefore requires skilled interpretation. Finally, the assay can be seriously complicated by leukopenia in stem cell transplant (SCT) recipients before engraftment.

Qualitative PCR detection of CMV DNA in leukocytes or plasma appeared to be a sensitive method for detection of CMV in blood but lacked specificity for the diagnosis of CMV disease (2). Quantification of CMV DNA should be able to define more specifically the levels associated with disease.[2]

Real-time PCR-based assays[8] are able to quantify viral DNA accurately over a broad range of input target copies without the necessity for post-PCR handling. As such, these assays provide fast results with less risk of contamination. Recent studies have reported on the application of real-time PCR for the quantification of CMV DNA.[13, 15, 18, 19, 21, 23, 25]

The clinical use of these methods could be evaluated in comparison to the currently widely employed pp65 antigenemia assay, with respect to the diagnosis of CMV infection but also in monitoring of individual transplant recipients during active infection. It is obvious that patients undergoing solid organ transplantation (SOT) or SCT nowadays will always be protected from clinical disease by a monitoring strategy or a preventive regimen, excluding an evaluation solely based on clinical outcome. The establishment of the precise relationship between the two methods is essential to compare diagnostic results, particularly when laboratories consider replacement of the antigen assay.

In this study, an internally controlled quantitative real-time PCR assay has been used to determine the CMV DNA load in plasma. The assay also monitors the efficacy of nucleic acid extraction from the clinical sample and the presence of inhibitors in the PCR. The correlation of this optimized assay to the classical pp65 antigenemia assay has been evaluated with SOT recipients as well as SCT recipients. The aim of this study was to validate the clinical application of real-time measurement of the CMV DNA load in plasma specimens from SOT and SCT recipients and to define criteria for treatment in these groups of patients.

## MATERIALS AND METHODS

### *Patients and samples*

From August 2001 to June 2002, 3,100 EDTA-plasma and whole-blood specimens from SOT (kidney, kidney-pancreas, and liver) and SCT (pediatric and adult) recipients admitted to the Leiden University Medical Center were prospectively collected. From this group, 409 plasma samples from 128 SOT (36 liver and 51 kidney or kidney-pancreas) and SCT (41 adult and pediatric) patients were randomly selected for analysis, irrespective of the CMV serostatus of the donors and recipients. These 409 plasma samples were classified into five groups according to the results of the pp65 antigenemia assay. Group I ($n = 195$) corresponded to CMV antigenemia-negative samples. Group II ($n = 79$) corresponded to samples with low CMV antigenemia values (1 to 3 positive cells), and groups III ($n = 57$), IV ($n = 50$), and V ($n = 28$) corresponded to samples with moderate (4 to 20 positive cells), high (21 to 100 positive cells), and very high (>100 positive cells) CMV antigenemia values, respectively.

Also, 295 corresponding whole-blood samples were selected to address the correlation between CMV DNA loads in plasma and whole blood. During the study period, antigenemia assays were used for patient management, while real-time quantitative CMV PCR was performed retrospectively on the EDTA-plasma and whole blood samples frozen at –80°C.

Additionally, 10 CMV-seronegative kidney or kidney-pancreas transplant patients identified as undergoing a primary CMV infection (donor positive/recipient negative [D+/R–] combinations) were analyzed longitudinally, with a mean follow-up time of 82 days (range, 72 to 180 days) posttransplantation.

### *Viral standards and controls*

A sucrose gradient-purified and electron microscopy-counted HCMV AD169 strain ($5.28 \times 10^{10}$ virus particles/ml; ABI, Columbia, Md.) was used as a standard for quantification. The strain was diluted to a concentration of $10^8$ particles/ml and

subsequently serially diluted to determine a standard curve. The agreement of particle numbers and DNA copies was confirmed by using quantified plasmid DNA (IQ Products, Groningen, The Netherlands) containing the CMV PCR fragment (data not shown). A phocine herpes virus (PhHV) strain, propagated in cell culture, was used as internal control in the real time PCR. For specificity testing, patient samples positive for herpes simplex virus types 1 and 2, varicella-zoster virus, Epstein-Barr virus, human herpesvirus 6, adenovirus, parvovirus B19, and hepatitis B virus were used.

*CMV antigenemia assay*

The CMV antigenemia assay was performed with the CMV Brite Turbo kit (IQ Corporation BV, Groningen, The Netherlands), according to the manufacturer's instructions. Briefly, $2.0 \times 10^6$ leukocytes were applied to a glass slide by cytospin, fixed, and permeabilized to allow subsequent detection of CMV pp65 antigen. The presence of pp65 antigen was detected by the C10/C11 antibody cocktail and visualized by means of a specific secondary fluorescein isothiocyanate-labeled antibody. The number of CMV antigen-positive cells per duplicate stain was counted.

*CMV serology*

CMV-specific immunoglobulin M (IgM) and IgG antibodies in sera from patients were determined by using the Vironostika CMV-IgM assay (BioMerieux/Organon Teknika, Boxtel, The Netherlands) and the AxSYM CMV-IgG assay (Abbott Laboratories, North Chicago, Ill.) according to manufacturers' instructions.

*Extraction of CMV DNA*

Nucleic acids were extracted from 0.2-ml plasma and whole blood samples by using the MagnaPure LC total nucleic acid isolation kit (Roche Molecular Systems, Almere, The Netherlands). During this fully automated purification procedure, lysis-binding buffer is added to the samples, resulting in complete cell lysis and protein denaturation. Subsequently, protein K is added to the samples and cellular proteins are digested. DNA binds to the silica surface of added magnetic glass particles due to the chaotropic salt conditions and the high ionic strength of the lysis-binding buffer. In the next step, wash buffer I removes unbound substances such as proteins, cell membranes, and PCR inhibitors such as heparin and hemoglobin. Wash buffer II further removes impurities and reduces the chaotropic salt concentration. Eventually, purified DNA is eluted in buffer at an elevated temperature.

*Quantitative real-time PCR*

The CMV-specific PCR primers (Table 1) were derived from those previously described[3], and a specific TaqMan probe was developed. The primers (Eurogentec, Se-

**Table 1.** CMV and PhHV primers and probes

| Primer or probe | Sequence |
|---|---|
| Forward CMV primer | 5′-CAAGCGGCCTCTGATAACCA-3′ |
| Reverse CMV primer | 5′-ACTAGGAGAGCAGACTCTCAGAGGAT-3′ |
| TaqMan CMV probe | FAM-TGCATGAAGGTCTTTGCCCAGTACATTCT-TAMRA |
| Forward PhHV primer | 5′-GGG CGA ATC ACA GAT TGA ATC-3′ |
| Reverse PhHV primer | 5′-GCG GTT CCA AAC GTA CCA A-3′ |
| TaqMan PhHV probe | Cy5-TTT TTA TGT GTC CGC CAC CAT CTG GAT C-BHQ2 |

raing, Belgium) amplified a 126-bp fragment from the CMV immediate-early antigen region. The TaqMan probe was labeled at the 5′ end with 6-carboxyfluorescein (FAM) and at the 3′ end with the fluorescent quencher 6-carboxytetramethylrhodamine. The 3′ end was phosphorylated to prevent probe extension during amplification.
The PCR was carried out by using the HotStar *Taq* master mix (Qiagen, Hilden, Germany) in an I-Cycler IQ DNA detection system (Bio-Rad, Veenendaal, The Netherlands). Briefly, 10 µl of either the standard-curve DNA or DNA extracted from the samples was added to 40 µl of PCR mixture containing a 400 µM concentration of each deoxynucleoside triphosphate, a 0.25 µM concentration of each primer, a 0.625 µM concentration of the fluorogenic probe, 4.5 mM MgCl2, and HotStar *Taq* DNA polymerase in HotStar PCR buffer. Template denaturation and activation of HotStar *Taq* DNA polymerase for 15 min at 95°C were followed by 50 cycles of denaturation at 95°C for 20 s, annealing at 63°C for 20 s, and extension at 72°C for 1 min.
To monitor the efficiency of the DNA extraction and PCR inhibition, all clinical samples were spiked with a fixed amount of PhHV virus particles prior to DNA extraction. PhHV DNA was amplified by using a PhHV-specific PCR assay as described previously.[17] Primers used for the PhHV assay amplified a 89-bp fragment of the glycoprotein B gene. The probe was labeled with Cy5 and BHQ2 (Biolegio, Malden, The Netherlands). Primer and probe sequences are shown in Table 1. The CMV and PhHV assays were performed as a duplex PCR in a single tube. The PCR was performed under the same conditions as the CMV assay. During amplification the CMV and PhHV targets generated different reporter fluorescence signals (FAM and indodicarbocyanine, respectively).

***ROC curve analysis***

Receiver-operating characteristic (ROC) curves represent the joined values of the true-positive ratio (sensitivity) and false-positive ratio (1 – specificity) for each value

of the diagnostic variable.[1, 26] In this study, ROC plot analysis was performed to determine a threshold value of the CMV DNA load in plasma for initiating treatment. Current clinical practice is based on the pp65 antigenemia assay, and therefore this assay was chosen to determine the optimal cutoff value for the DNA-based assay. In order to avoid the risk of nonspecific results of the lowest antigen level of one and two positive cells, the outcome for more than three positive cells in the pp65 antigenemia test was taken to be the lower predictive threshold for CMV disease in R+ transplant recipients. The level of three positive cells was deliberately chosen as the lowest convincing positive result. All database entry and statistical analysis were performed with SPSS version 10.0.7.

## RESULTS

### *CMV DNA levels*

With the selected primers and probes, efficient amplification of dilution series of CMV DNA was obtained. When the *Ct* values were plotted, a standard line with a slope of 3.33 could be generated, indicating a PCR efficiency of 99.7%. Based on the dilution series of the CMV AD169 strain, the sensitivity of the assay was found to be approximately 100 to 250 copies/ml, which is 2 to 5 copies of CMV DNA in the reaction.

The specificity was tested with DNAs from a range of other viruses. No amplification was observed with herpes simplex virus types 1 and 2, varicella-zoster virus, Epstein-Barr virus, human herpesvirus 6, adenovirus, parvovirus B19, and hepatitis B virus.

The intra-assay variation was determined by using three CMV standard-curve DNA dilutions with low ($10^3$ copies/ well), medium ($10^5$ copies/well), and high ($10^8$ copies/ well) concentrations. The *Ct* values obtained for the low, medium, and high concentrations of standard CMV DNA in this test of intra-assay variation were 41.9 ± 1.0, 34.5 ± 0.3, and 24.7 ± 0.4, respectively (values are means ± standard deviations).

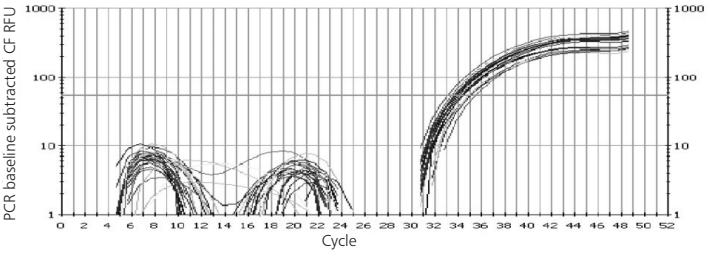
To determine the interassay variation, CMV standard DNA dilutions with low, medium, and high concentrations were subjected to the real-time PCR in 15 distinct experiments. These 15 distinct experiments also included 15 separate DNA extractions. The mean *Ct* values were 39.8 ± 1.4, 33.5 ± 1.1, and 23.5 ± 0.9 (means ± standard deviations), respectively.

### *Monitoring of DNA extraction and detection of PCR inhibition*

Reliable implementation of quantitative assays requires internal controls to avoid false-negative results or underestimation of values. Here an internal control reaction

was used to monitor the nucleic acid extraction procedure and the presence of PCR inhibitors. The amount of internal control spike was arbitrarily set at a concentration which resulted in a *Ct* value of 34 ± 2 (Fig. 1). It was arbitrarily chosen that *Ct* values of the internal control that differed by more than two cycles from the value in the negative control sample were regarded as inhibitory.

**Figure 1**.




Amplification plots obtained with the internal control (PhHV) DNA in a group of plasma samples. No inhibitory samples are detected, as the Ct value of the PhHV control was 34 ± 2.

To analyze possible competition between the CMV and PhHV DNA amplifications, serial dilutions from $10^8$ to $10^3$ of the electron microscopy-counted CMV AD169 strain were spiked with high and low concentrations of PhHV DNA and subjected to the PCR run. In addition, a serial dilution of PhHV DNA was spiked with low ($10^3$) and high ($10^8$) concentrations of CMV AD169 DNA. The results showed no significant difference (data not shown).

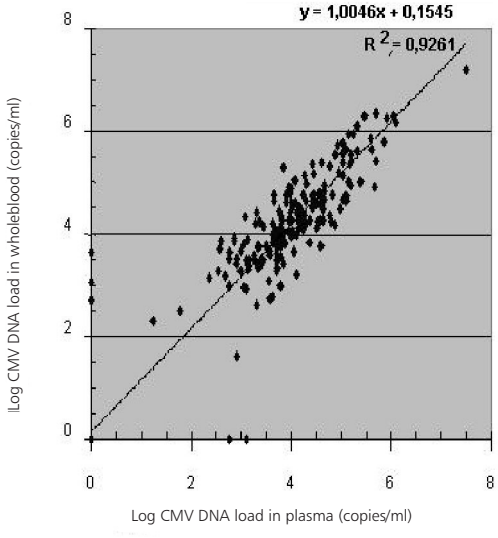
***Correlation between CMV DNA loads in plasma and whole blood***

The CMV DNA loads in corresponding whole blood samples from 295 out of the 409 selected plasma samples were determined. The CMV DNA load in plasma was plotted against the CMV DNA load in whole blood (Fig. 2), and the correlation coefficient (*r*) of 0.962 indicated a high correlation between CMV DNA loads in plasma and whole blood. As can be derived from Fig. 2, the CMV DNA load in whole blood tends to be slightly but not significantly higher than that in plasma. An overall difference of 0.15 log unit (1.4 times) was found (Fig. 2).

***Correlation between pp65 antigenemia and quantitative real-time CMV PCR***

The CMV real-time PCR assay was evaluated with 409 plasma samples from 128 SOT and SCT recipients. The corresponding pp65 antigenemia results were

**Figure 2**.



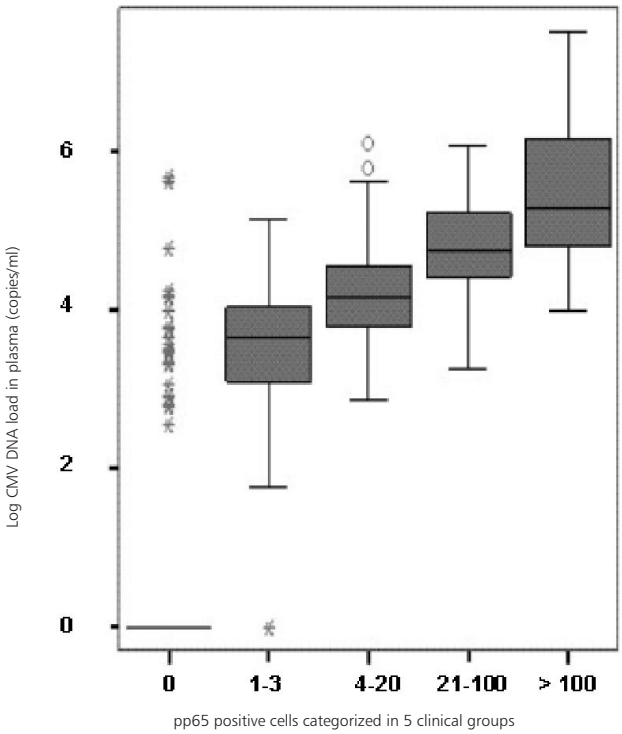

Comparison of the CMV DNA levels in plasma and whole blood from the same patients.

used to group the samples into five clinical categories. The median CMV DNA copy numbers in plasma were 0.00 copies/ml (mean, 2,82 copies/ml) for samples in group I and 4.47 $\times$ $10^3$ copies/ml (mean, 2.14 $\times$ $10^3$ copies/ml), 1.45 $\times$ $10^4$ copies/ml (mean, 2.66 $\times$ $10^4$ copies/ml), 5.50 $\times$ $10^4$ copies/ml (mean, 6.92 $\times$ $10^4$ copies/ml), and 1.91 $\times$ $10^5$ copies/ml (mean, 2.82 $\times$ $10^5$ copies/ml) for samples in groups II, III, IV, and V, respectively. As shown in Fig. 3 the CMV DNA copy numbers in plasma and the CMV antigenemia values seem to correlate well. Despite the correlation, the values for the CMV DNA load within the distinct pp65 groups showed a wide range (Fig. 3).

*Discrepancy analysis*

The quantitative real-time CMV PCR viral load values generally correlated well with the groups of antigenemia values (Fig. 3). However, some discrepancies between the pp65 antigenemia test and the CMV viral load were observed. Of the 195 antigenemia-negative samples in group I, 24 samples (from 16 patients) had a detectable CMV DNA load (mean CMV DNA load, 4.37 $\times$ $10^3$ copies/ml; median, 3.09 $\times$ $10^3$ copies/ml). In the CMV serostatus analysis, 15 of these 16 patients were either CMV IgG positive or had seroconverted (IgM positive), indicating that a positive CMV

**Figure 3**.




Comparison of CMV DNA loads in plasma specimens from SOT and SCT recipients by the pp65 antigenemia assay. The CMV DNA load was plotted to five pp65 antigenemia groups. For each group, the median load, the interquartile 50% range, and the range of values are represented. Open circles indicate the outliers (values between 1.5 and 3 box lengths from the upper or lower edge of the box). Asterisks represent the extreme values (values more than three box lengths).

DNA load can be expected. For one patient, no serostatus or other follow-up data were available.

In nine patients the CMV DNA load was positive at the moment that CMV IgG was negative. Follow-up of these nine patients revealed that all of them seroconverted subsequently, indicating that a persisting seronegative status with a detectable load did not occur.

Finally, one pp65-positive sample (three cells) showed an undetectable CMV DNA load. The negative CMV DNA PCR was caused by either PCR inhibition or inefficient DNA isolation, since the PhHV internal control PCR remained negative during 50 amplification cycles.

**Table 2.** Sensitivities and specificities of different threshold levels of CMV DNA load in plasma[a]

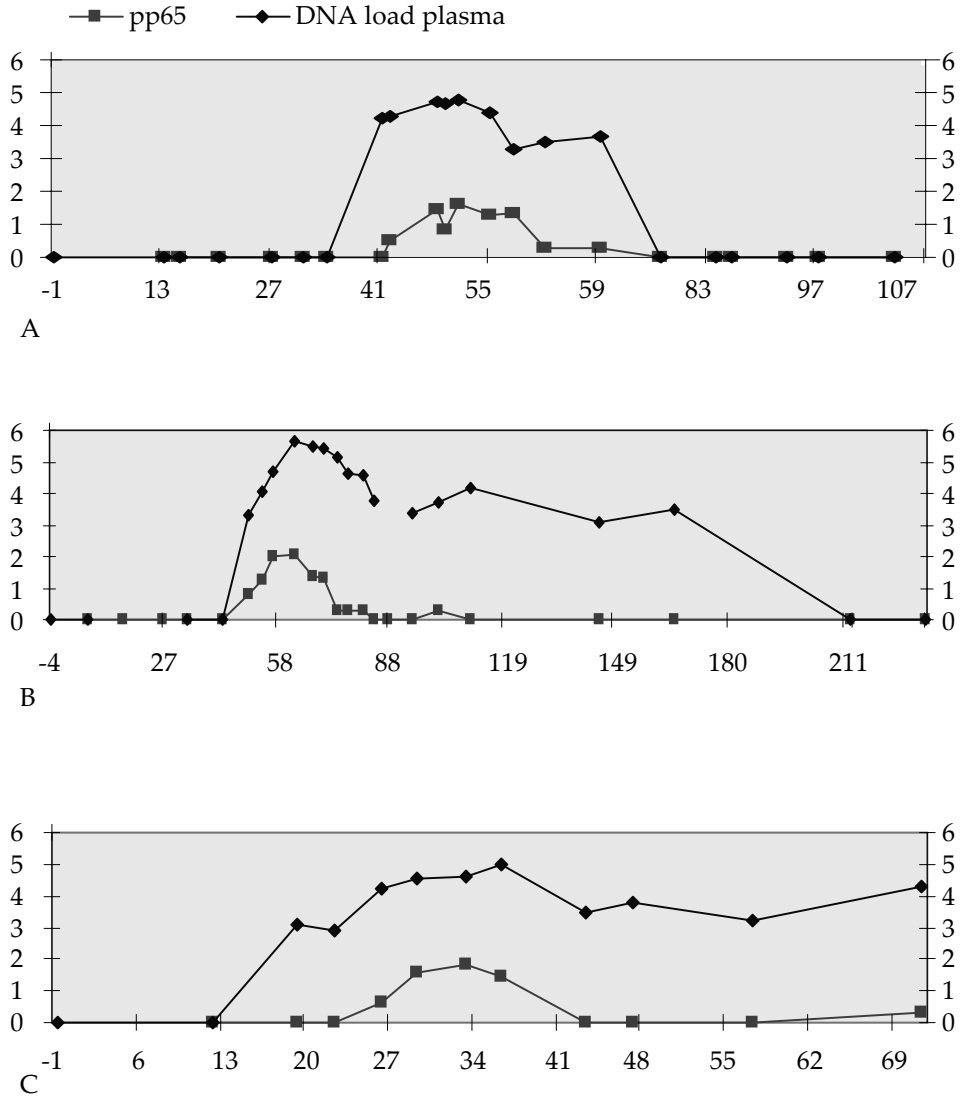
| Group | Threshold CMV DNA level (copies/ml) in plasma as reference | Sensitivity (%) | Specificity (%) | Optimal CMV DNA level[b] (% sensitivity, % specificity) |
|---|---|---|---|---|
| SCT recipients | $10^2$ | 98 | 48 | $1.00 \times 10^4$ (83, 82) |
| | $10^3$ | 98 | 56 | |
| | $10^4$ | 83 | 82 | |
| SOT recipients | $10^2$ | 99 | 72 | $5.37 \times 10^3$ (87, 90) |
| | $10^3$ | 98 | 78 | |
| | $10^4$ | 80 | 93 | |
| SCT and SOT recipients | $10^2$ | 99 | 66 | $1.00 \times 10^4$ (81, 90) |
| | $10^3$ | 98 | 72 | |
| | $10^4$ | 81 | 90 | |

[a] ROC analysis was performed by using the outcome of more than three positive cells in the pp65 antigenemia test as the value indicating which category should be considered positive. SOT and SCT recipients were considered as one group as well as separately.
[b] Values are numbers of copies per milliliter.

*Determination of CMV DNA load threshold values*

As the pp65 assay has been used for guiding CMV therapy in current clinical practice, it is important to establish the corresponding threshold values for the CMV DNA assay. In order to define the optimal cutoff value of CMV DNA load for initiating treatment in transplant patients at risk for CMV disease, an ROC curve analysis was performed, using existing treatment criteria based on pp65 test results. The outcome of more than three positive cells in the pp65 antigenemia test was taken to be the lower predictive threshold for CMV disease in R+ transplant patients. If subsequently a CMV DNA level of 100 copies/ml was used as a threshold for predicting CMV disease, the sensitivity and specificity were 99 and 66%, respectively (Table 2). The sensitivity decreased to 81% and the specificity increased to 90% when the CMV DNA level was increased to 10,000 copies/ml. When the ROC curve analysis was performed for SOT and SCT patients separately, the sensitivity and specificity for the above-mentioned CMV DNA levels were essentially the same (Table 2). For the SCT patients the optimal cutoff value of CMV DNA load was $10^4$ copies/ml (sensitivity and specificity of 83 and 82%, respectively), and for the SOT recipients the optimal cutoff value was $5.37 \times 10^3$

**Figure 4.**




Three observed patterns in pp65 antigenemia and CMV DNA load follow-up in 7 of the 10 D+/R– kidney or kidney-pancreas transplant recipients studied. Three patients remained negative in both assays during follow-up. (A) The assays demonstrated that the patient responded well to antiviral treatment. This pattern was seen in four patients. (B). After antiviral treatment, the CMV DNA load persisted for some weeks at a level below the cutoff value of $10^4$ copies/ml before it decreased to an undetectable level (one patient). (C) The pp65 assay and CMV DNA load measurement showed two episodes of CMV activation. Between these episodes, pp65 are negative, whereas the CMV DNA load persisted at levels below the defined cutoff value (two patients).

copies/ml (sensitivity and specificity of 87 and 90%, respectively). The results are shown in Table 2.

To assess the relationship between the CMV DNA level and the number of pp65-positive cells in transplant patients who are at risk for a primary CMV infection (D+/R–), 10 kidney or kidney-pancreas transplant recipients with a mean follow-up time of 82 days (range, 72 to 180 days) posttransplantation were longitudinally analyzed. In three (30%) of the patients, the CMV pp65 assay remained negative during the follow-up period. In the other seven patients (70%), positive pp65 results were observed (representative cases are shown in Fig. 4). The mean (median) numbers of days to the first positive test were 75 (41) and 78 (42) for CMV DNA load and antigenemia, respectively. In two patients from whom samples were obtained more frequently, a positive DNA load was detected prior to antigenemia positivity (18 versus 25 days in one patient and 19 versus 26 days in the other patient). CMV DNA never became positive later than pp65.

## DISCUSSION

This study describes the application of an internally controlled real-time quantitative CMV PCR to plasma and whole-blood samples of SOT and SCT recipients. When these quantitative PCR results were compared with results of the pp65 antigenemia assay, the median CMV DNA copy numbers in plasma increased proportionally with the CMV antigenemia values, confirming results obtained in earlier studies.[5, 6, 11, 12, 13, 16, 20, 23, 24] Nevertheless, some discrepancies were observed in this analysis of 409 plasma samples. These discrepancies could largely be explained by the increased sensitivity of the PCR compared to the pp65 test.[7, 9] CMV DNA in plasma thus can be detected earlier than pp65 antigen in leukocytes. In addition, it should be noted that CMV DNA in plasma tended to persist longer during or after therapy than pp65 antigens; the rates of decline in the values of the two assays may well be different. Therefore, it is likely that these factors contribute to the finding that CMV DNA can be detected in antigenemia-negative samples. Since anti-CMV IgG antibodies were detectable in all of these pp65 and CMV DNA discrepant samples, lack of specificity of the CMV PCR is unlikely to be an issue. It was also shown that persisting seronegative status with a detectable DNA load did not occur.

The analytical results of the two assays correlated well, and subsequently the clinical application of the results was assessed. This implies the development of criteria to initiate treatment based on CMV DNA values. For this purpose, patients at risk for a primary CMV infection and patients at risk for a CMV reactivation must be considered separately. Since patients at risk for a primary infection have never encountered

**Figure 5.**

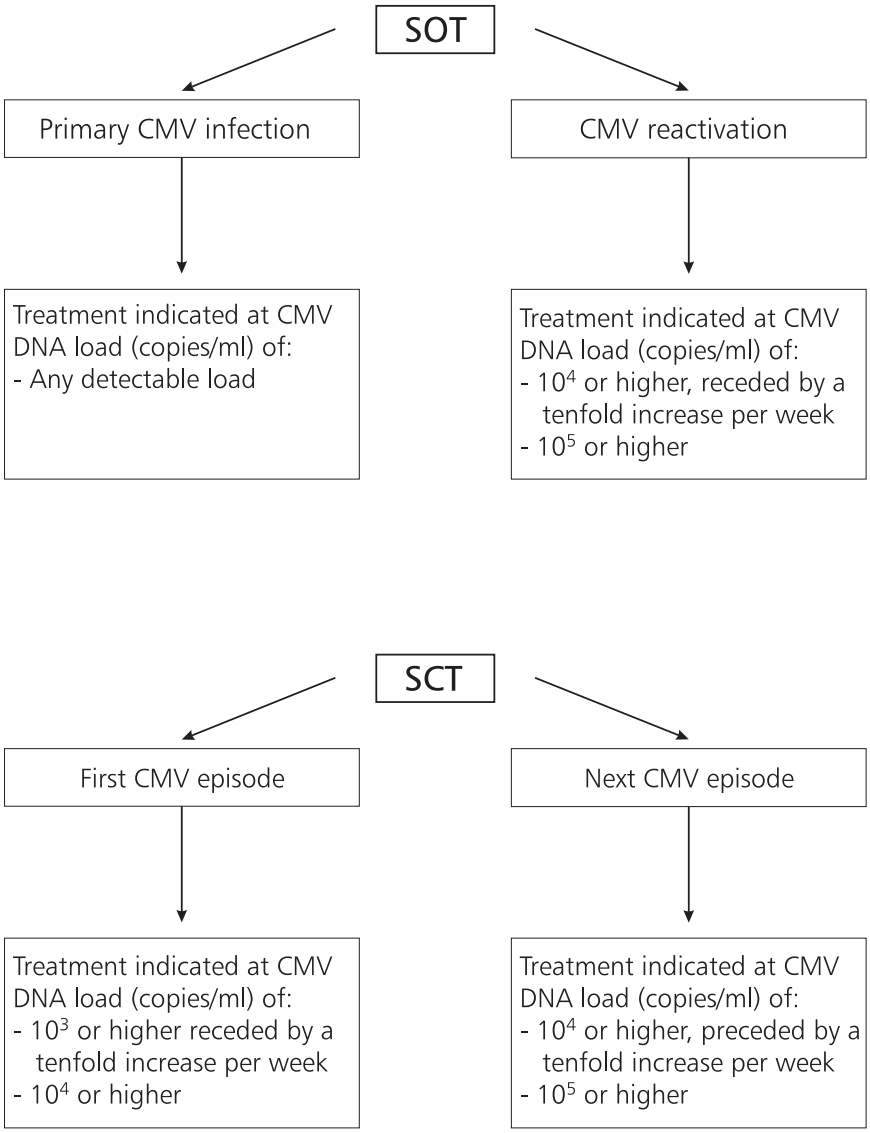



Illustration of possible guidelines for the interpretation of plasma CMV DNA values after transplantation, based on the observations described in this study. To formulate these guidelines, the threshold values as derived from the ROC analysis were rounded off to the nearest decimal. In these guidelines, some practical approaches are different for SOT and SCT recipients. In the case of SOT recipients, thresholds are defined only for recipients at risk for CMV reactivation. SOT recipients at risk for primary CMV infection (D+/R–) have never encountered CMV, and therefore any level of CMV DNA is considered to be evidence of imminent clinically relevant infection. Primary infections are more difficult to define in the case of SCT recipients, as the level of grafted donor immunity is highly variable with regard to CMV. Therefore, a distinction is made between the first CMV episode and any subsequent episodes after transplantation. During the first episode, the threshold is set more stringently than for the subsequent episodes.

CMV, any level of CMV DNA load will be predictive for the development of CMV infection. Treatment can be initiated on the basis of any positive result. However, patients at risk for a CMV reactivation may have a background CMV DNA level in plasma which is not necessarily correlated with disease. Determination of an optimal cutoff value for the CMV DNA level in plasma that is predictive for CMV disease is essential for the management of these patients. ROC analysis indicated optimal cutoff values of 10,000 copies of CMV DNA/ml in SCT patients and 5,370 copies/ml in SOT patients, with a sensitivity and specificity of more then 80% each. If, during monitoring, viral DNA loads exceed these threshold levels, antiviral therapy could be initiated to prevent disease. However, it has been shown previously that in addition to the viral DNA load, the kinetics of the DNA load should be taken into account for treatment decisions.[4, 22] The present study results might enable the replacement of pp65 antigenemia tests by quantitative real-time CMV PCR. Figure 5 provides an illustration of possible practical guidelines which can be formulated for monitoring and management of CMV-related problems in transplant recipients based on the CMV DNA load in plasma. For further validation, the CMV DNA load measurement and pp65 antigenemia assays were performed simultaneously for all samples from SOT and SCT recipients during 2 months. A comparison of clinical decisions based on the guidelines as depicted in Fig. 5 with the clinical decisions based on the simultaneously obtained pp65 values demonstrated that the number of treatment periods was identical (results not shown). However, most of the CMV episodes were detected earlier with the CMV DNA load measurement.

It was shown that the CMV DNA loads in plasma and whole blood correlated very well. This finding demonstrated that both blood compartments are likely to be adequate for use in the diagnosis and monitoring of CMV disease in transplant recipients.

In summary, the quantitative CMV real-time PCR is a useful tool for monitoring the risk of development of CMV disease in transplant recipients. If the results obtained by this assay are compared with those obtained by the pp65 antigenemia assay, it is possible to define cutoff values for the CMV DNA load in plasma to be used in the management of transplant patients at risk for CMV reactivation.

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# 3

## Efficacy of pre-emptive cytomegalovirus treatment using intravenous ganciclovir or oral valganciclovir in solid organ and stem cell transplant recipients

# 3a

## Similar reduction of cytomegalovirus DNA load by oral valganciclovir and intravenous ganciclovir on pre-emptive therapy after renal and renal–pancreas transplantation

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## ABSTRACT

***Background:*** Pre-emptive treatment of CMV infection in transplant recipients aims at prevention of clinical disease by early detection. However, current treatment requires the intravenous (iv) administration of ganciclovir for 2 weeks, which is a considerable burden for the patient. In this observational study, the efficacy of the new oral prodrug valganciclovir was compared with iv ganciclovir.

***Methods:*** To facilitate the introduction of valganciclovir, a therapeutic guideline was developed to use this drug under controlled conditions with regard to safety in renal/renal–pancreas transplant recipients requiring CMV therapy. Subsequently, a group of 57 consecutive transplant recipients was evaluated. Onset and treatment of CMV infections were followed by frequent monitoring of CMV DNA in plasma by quantitative real-time PCR. Details of antiviral therapy were documented.

***Results:*** In 15 out of 57 transplant recipients, a total of 27 anti-CMV treatment episodes were recorded: 18 with valganciclovir (900 mg twice daily) and nine with iv ganciclovir (5 mg/kg twice daily) as initial treatment. Median CMV DNA load reduction during treatment was 0.12 $\log_{10}$/day in the valganciclovir group and 0.09 $\log_{10}$/day in the ganciclovir group. There were no haematological side effects in any group and no patient developed signs of clinical CMV disease.

***Conclusion:*** Similar reduction of CMV DNA load was observed during pre-emptive treatment with oral valganciclovir and iv ganciclovir in transplant recipients. Oral valganciclovir would provide an attractive and safe alternative for pre-emptive CMV treatment in renal/ renal–pancreas transplant patients, however, confirmation in larger randomized studies would be desirable.

## Introduction

Cytomegalovirus (CMV) is the most common opportunistic pathogen complicating the care of solid organ transplant (SOT) recipients. Potentially, it is a major cause of morbidity and mortality, frequently necessitating treatment with specific antiviral drugs such as ganciclovir and foscarnet. Current strategies for the prevention of CMV disease aim at avoiding aggressive treatment of established end-organ disease and include ganciclovir or valganciclovir prophylaxis[1,2] or ganciclovir pre-emptive therapy, initiated upon early detection of CMV infection by antigenaemia or CMV DNA.[2,3] The relative merits of both regimens have been debated extensively in the literature.[4,5] Prophylactic treatment involves the administration of oral ganciclovir to all patients at risk for an extended period. Universally applied antiviral prophylaxis below therapeutic dosages for extended periods is considered a risk factor in the development of antiviral drug resistance.[6] Pre-emptive therapy is of short duration and specifically directed towards patients identified as having a high risk for CMV disease, thus sparing many from the toxicity related to long-term use of antiviral prophylaxis. Monitoring is essential in this instance and antiviral therapy is initiated at the moment when relevant CMV activity is detected. The efficacy of preemptive therapy is supported by the results of randomized controlled trials.[7,8] The major drawback limiting the use of ganciclovir concerns its poor bio-availability, which precludes therapeutic use by oral administration.[3] This has now changed with the recent introduction of valganciclovir, which is an orally administered prodrug of ganciclovir. Previous pharmacokinetic studies showed similar drug exposure to ganciclovir after a single dose of 900 mg valganciclovir orally as compared with 5 mg/kg intravenous (iv) infusion of ganciclovir.[9–11]

The clinical efficacy of valganciclovir was first confirmed in studies on the treatment and prevention of CMV retinitis in AIDS patients.[12] Also, ganciclovir resistance was not observed more frequently with the use of valganciclovir compared with iv ganciclovir in these patients.[13]

Recently, the efficacy of prophylactic oral ganciclovir versus valganciclovir was studied in a high-risk group [CMV positive donors and negative recipients (D+/R–)] of SOT recipients.[14] This double-blind randomized trial was designed to demonstrate the equivalence of the two prophylactic regimens with CMV disease as the specified primary endpoint. It was concluded that valganciclovir 900 mg once daily was at least as effective as oral ganciclovir 1000 mg three-times daily in the prevention of CMV disease.

Up to now, no data are available on the efficacy of 900 mg valganciclovir twice daily as compared with iv 5 mg/kg ganciclovir twice daily in pre-emptive therapy of CMV infections. In this observational study, the efficacy and safety of CMV DNA load-

guided pre-emptive valganciclovir therapy compared with iv ganciclovir in renal and renal–pancreas allograft recipients were evaluated by providing a treatment guideline to the physicians enabling them to use valganciclovir under controlled conditions with regards to safety. This would allow the comparison of the treatment effects of both drugs, using CMV DNA load reduction as the therapeutic endpoint, used either individually or administered consecutively.

## METHODS

In this study, all consecutive patients undergoing renal or renal–pancreas transplantation at the Leiden University Medical Centre between 1 January 2003 and 15 August 2003 were included. All patients were routinely monitored by CMV DNA load in plasma at weekly intervals starting on the day of transplantation, continuing until 180 days after transplantation or beyond day 180 until CMV DNA became undetectable. The real-time quantitative PCR for detection of CMV DNA in plasma was performed according to the method previously described.[15] The primary endpoint for this study was CMV DNA load reduction after treatment with oral valganciclovir or iv ganciclovir.
Data were available on demographic characteristics, cause of end-stage renal disease, donor and recipient CMV status, immunosuppressive therapy, initiation, cessation, dose and form of administration of antiviral therapy, CMV DNA load measurements, rejection therapy and general laboratory parameters. The initial immunosuppressive regimen consisted of prophylaxis with either antibodies against the IL-2 receptor alpha-chain (basiliximab/daclizumab) or anti-thymocyte globulin (Fresenius) for kidney and kidney/pancreas transplant recipients, respectively. Maintenance immunosuppression included prednisolone, tacrolimus and mycophenolate mofetil. First rejection episodes were treated with solumedrol for 3 days. Second rejection episodes were treated with ATG for 10–14 days. A third rejection episode was treated similarly to the first episode. Upon CMV reactivation post-transplantation (positive CMV DNA load in plasma), immunosuppression dosages were reduced at the discretion of the treating physician.
CMV DNA load-guided pre-emptive therapy was initiated according to a guideline derived from findings in a previous study.[15] In short, any symptomatic CMV infection would be treated with iv 5 mg/kg ganciclovir twice daily. In the case of a primary infection or a significant viraemia (CMV DNA load $>10^5$ copies/ml or CMV load $>10^4$ copies/ml and more than one $\log_{10}$ increase as compared with previous measurement) without clinical symptoms of CMV disease, 900 mg valganciclovir twice daily or iv 5 mg/kg ganciclovir twice daily was administered for 2 weeks. The choice of initial treatment with valganciclovir or ganciclovir was at the discretion of the

**Table 1.** Cytomegalovirus infections and treatment episodes

| | Renal and renal/pancreas transplant recipients (*n*=57) | | | |
|---|---|---|---|---|
| | D+/R– | D+/R+ | D–/R+ | D–/R– |
| Follow-up days after transplantation, median (IQR) | 165 (142–255) | 164 (116–239) | 134 (111–186) | 209 (115–275) |
| Cumulative incidence of positive CMV DNA load, *n* (%) | 8/9 (89) | 15/18 (83) | 5/12 (42) | 0/18 (0) |
| Cumulative incidence of anti-CMV treatment, *n* (%) | 8/9 (89) | 5/18 (28) | 2/12 (17) | 0/18 (0) |
| Median anti-CMV treatment duration, days (range, IQR) | 14 (6–36, 14–27) | 14 (8–17, 10–14) | 15 (13–16, 13–16) | 0 (0) |
| Anti-CMV treatment episodes, *n* (%) | 17/9 (188) | 8/18 (44) | 2/12 (16) | 0 (0) |

D, donor; R, recipient; +, positive; –, negative.

treating physician. With regard to treatment with valganciclovir, safety precautions were taken by a frequent follow-up of therapeutic responses by means of CMV DNA load measurements during therapy. A switch to iv ganciclovir would be made when more than one $\log_{10}$ CMV DNA load increase was observed during valganciclovir treatment in the first week.

Ganciclovir and valganciclovir doses were adjusted to renal function as previously described.[14] Serum creatinin levels and haematological parameters (that is, haemoglobin, leucocytes and thrombocytes) were monitored throughout treatment episodes.

All database entries and statistical analysis were performed with SPSS v10.0.7 (SPSS, Inc., Chicago, IL, USA).

## RESULTS

A total of 57 patients were included with a median age of 51 years (IQR 39–59), 47 receiving a kidney and 10 a combined kidney–pancreas transplant. With regard to donor and recipient CMV serostatus, 18 D+/R+ (31.6%), nine D+/R– (15.8%), 12 D–/R+ (21.1%) and 18 D–/R– (31.6%) combinations were recognized.

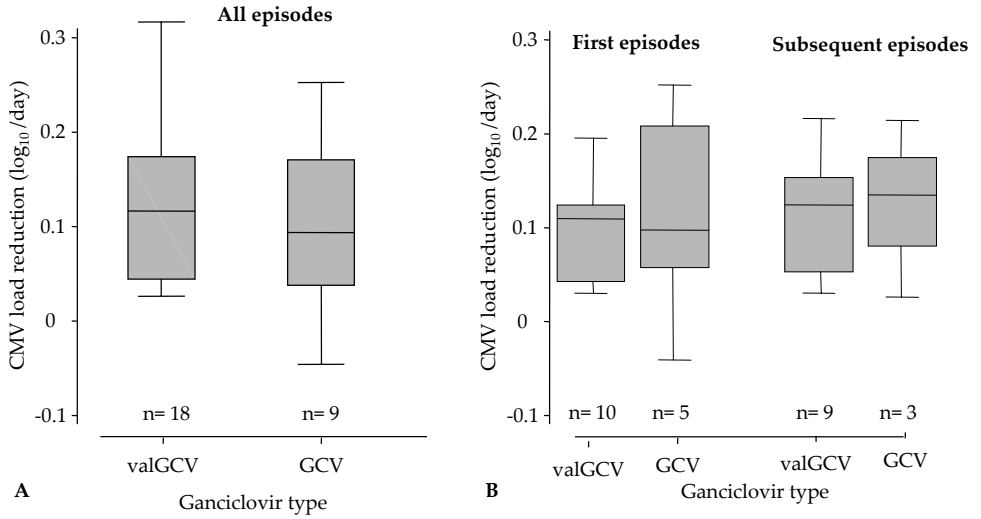
The follow-up period after transplantation was 164 days (IQR 116–255) in D+/R+ patients, 165 days (IQR 142–255) in D+/R– patients and 134 days (IQR 111–186) in D–/R+ patients (Table 1). During the follow-up period, 27 anti-CMV treatment episodes

**Table 2.** Characteristics of the study population in both treatment groups

| | ValGCV (*n*=12) | GCV (*n*=7) |
|---|---|---|
| Age, years (median, range) | 54 (24–67) | 46 (24–55) |
| Gender (male), % | 66.7 | 42.9 |
| Underlying disease, % | | |
| Glomerulonephritis | 33.3 | 28.6 |
| Hypertension | 25.0 | 14.3 |
| Cystic disease | 16.7 | 28.6 |
| Unknown | 16.7 | 28.6 |
| Diabetic | 8.3 | 0 |
| Type of Tx, % | | |
| Postmortal kidney | 66.7 | 42.9 |
| Kidney/pancreas | 16.7 | 42.9 |
| Living family kidney | 16.7 | 14.3 |
| CMV serostatus, % | | |
| D+/R– | 66.7 | 71.4 |
| D+/R+ | 33.3 | 14.3 |
| D–/R+ | 0 | 14.3 |

with either ganciclovir or valganciclovir were recorded in 15 patients. Most of these episodes, [17 (63%)], were in D+/R– patients (Table 1). Progression to CMV disease was observed in none of the patients and, as a consequence, iv ganciclovir was never administered for CMV disease. The characteristics of the study population in both treatment groups are depicted in Table 2. When the first treatment episode as well as all subsequent episodes per patient were considered, median CMV DNA load at start of therapy was 4.6 $\log_{10}$ copies/ml (range 3.0–6.2 $\log_{10}$ copies/ml) in the valganciclovir group compared with 3.7 $\log_{10}$ copies/ml (range 3.2–5.4 $\log_{10}$ copies/ml) in the ganciclovir group. When only the first episode for each patient was considered, median CMV DNA loads at start of therapy were 3.6 $\log_{10}$ copies/ml and 3.5 $\log_{10}$ copies/ml for the valganciclovir and ganciclovir groups, respectively. Initial treatment with valganciclovir was administered in 18 of the 27 episodes resulting in a CMV DNA load reduction below the level of 3.0 $\log_{10}$ within 14 days. However, in one of the episodes during valganciclovir treatment (in a D+/R– patient), CMV DNA load was not significantly reduced after 14 days of treatment. Therefore, a switch was made to iv ganciclovir after which the CMV DNA load decreased to undetectable levels within 14 days. However, a relapse occurred within 2 weeks that was initially treated with iv

**Figure 1.** CMV DNA load reduction per day during treatment with valganciclovir (ValGCV) and iv ganciclovir (GCV)




For both treatment groups the median load, the interquartile 50% range (box) and the range of values (whiskers) are represented. **(A)** CMV DNA load reduction per day when treatment of first episodes and subsequent episodes are combined. **(B)** Load reduction per day was analysed for first episodes and subsequent episodes separately.

ganciclovir. After 10 days of ganciclovir treatment, CMV DNA load was significantly reduced and the patient was discharged with oral valganciclovir for an additional 8 days after which CMV DNA load reduced below the detection level.

In nine out of 27 episodes, iv ganciclovir was used as the initial treatment resulting in a load reduction below the level of 3.0 $\log_{10}$ in eight of these episodes. In one episode, again in a D+/R– patient, no load reduction was observed after initial treatment with ganciclovir for 7 days. After a switch was made to valganciclovir, CMV DNA load decreased to undetectable levels within 14 days. However, 17 days later a relapse occurred, which was successfully treated with oral ganciclovir (CMV DNA load reduction below detection level within 14 days).

The effect of anti-CMV treatment with either valganciclovir or ganciclovir on CMV DNA load was also assessed by comparing the CMV DNA load at the start and at the completion of the treatment episodes. When all episodes for each patient according to treatment assignment were considered, the median CMV DNA load reduction after treatment with valganciclovir was 0.12 $\log_{10}$ copies/ml/day (IQR 0.03–0.39 $\log_{10}$ copies/ml) (Figure 1A). In episodes treated with iv ganciclovir, the CMV DNA load reduction was 0.09 $\log_{10}$ copies/ml/day (IQR –0.04–0.25 $\log_{10}$ copies/ml) (Figure 1A).

When only the first episodes for each patient according to treatment assignment were compared, the median CMV DNA load reduction was 0.11 $\log_{10}$ copies/ ml/day and 0.09 $\log_{10}$ copies/ml/day with valganciclovir and ganciclovir, respectively (Figure 1B). The median haemoglobin concentration at the start of iv ganciclovir and valganciclovir treatment was 6.9 mmol/ml (IQR 5.8–7.5 mmol/ml) and 7.4 mmol/ml (IQR 5.9–9.8 mmol/ml), respectively. At the start of therapy, the median leucocyte counts were $6.2\times10^9$ (IQR $1.7\times10^9$–$14.5\times10^9$) in the ganciclovir group and $6.1\times10^9$ (IQR $3.2\times10^9$–$15.9\times10^9$) in the valganciclovir group, whereas median thrombocyte counts were $216\times10^9$ (IQR $128\times10^9$–$366\times10^9$) and $209\times10^9$ (IQR $99\times10^9$–$488\times10^9$) in the ganciclovir and the valganciclovir groups, respectively. Using a mixed model analysis with repeated measurements in time, no significant changes of haemoglobin concentration, leucocyte and thrombocyte counts were observed throughout treatment episodes in both groups.

## DISCUSSION

Pre-emptive treatment of post-transplant CMV infections guided by the frequent quantitative measurement of CMV DNA load in plasma is a successfully applied strategy to prevent CMV disease. Intravenously administered ganciclovir at a dose of 5 mg/kg twice daily for 2 or 3 weeks is the most commonly used dosing regimen. Consequently, the early detection of a relevant CMV infection in otherwise asymptomatic patients usually requires hospitalization for iv drug administration. There is an urgent need for an effective oral formulation for pre-emptive CMV therapy, which would enable prevention and treatment of CMV in an outpatient setting and would consequently reduce health care costs significantly.

Recently, Paya *et al.* presented the results of a double-blind randomized trial, demonstrating the equivalent efficacy of prophylaxis with oral ganciclovir versus oral valganciclovir in a high risk (D+/R–) transplantation population.[14] This study did not employ a pre-emptive treatment strategy and did not include a comparison with the iv administration of ganciclovir, as commonly used in this approach.

In the current study we found a similar CMV DNA load reduction with orally administered valganciclovir as compared with intravenously administered ganciclovir, as part of a pre-emptive strategy to prevent CMV disease in renal and renal/pancreas transplant recipients. Treatment of CMV viraemia episodes according to predefined criteria with either valganciclovir or ganciclovir led to a similar median CMV DNA load reduction in plasma of approximately 0.1 $\log_{10}$ copies/ml per day, corresponding to a virus load half-life of 2.3 days for both treatment options (half-life of the virus in plasma was calculated using the equation $t_{1/2} = -\ln 2/\text{slope}$ of CMV DNA load decline

after initiation of therapy). These latter observations are in agreement with those by Emery *et al.* who showed a half-life of CMV DNA load decline in blood of between 1.1 days and 2.9 days after treatment with iv 5 mg/kg ganciclovir twice daily.[16] Others also found a half-life of CMV DNA load in blood of 2.56 ±0.36 days after treatment with iv ganciclovir in transplant recipients with a first episode of CMV retinitis.[17]
In all cases, CMV DNA load declined to a level below 3 $\log_{10}$ copies/ml after anti-CMV treatment with oral valganciclovir, except for one episode where a switch to iv ganciclovir was needed to reach sufficient CMV DNA load reduction. Treatment failure in this respect was also observed in one patient initially treated with iv ganciclovir. Here, a switch to oral valganciclovir resulted in a CMV DNA load reduction beyond the detection level. Reasons for these failures are not clear. In the current study, the pre-emptive strategy was effective as no disease related to CMV infection was reported in any treated patient and also safe, as no adverse effects were observed. This study demonstrated that pre-emptive treatment with oral valganciclovir and iv ganciclovir were equally effective in reducing CMV DNA load in renal and renal–pancreas allograft recipients. Therefore, we conclude that in this pre-emptive setting, iv ganciclovir can safely be replaced by valganciclovir, leading to a reduction in hospitalization rates and associated costs, both financial and in terms of patients' quality of life. Based on rational precautions, it remains advisable that patients with symptomatic CMV infections are treated with the intravenously administered drug, as the course of CMV disease can be serious and rapidly progressive. In conclusion, the large majority of patients who are without disease when the first laboratory signs of CMV infection are detected may benefit from treatment with an oral drug with maintenance of adequate anti-CMV therapy and without the need and burden of hospitalization. However, larger randomized studies are desirable to confirm the results of this observational study.

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# 3b

# Oral valganciclovir as pre-emptive therapy has similar efficacy on cytomegalovirus DNA load reduction as intravenous ganciclovir in allogeneic stem cell transplantation recipients

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## ABSTRACT

The efficacy and safety of oral valganciclovir was compared to ganciclovir i.v. in pre-emptive treatment of cytomegalovirus (CMV) in T-cell-depleted allogeneic stem cell transplant (allo-SCT) recipients. A therapeutic guideline was developed to allow the safe application of valganciclovir in allo-SCT recipients requiring CMV therapy. In total, 107 consecutive transplant recipients were evaluated. Cytomegalovirus DNA load in plasma was monitored longitudinally; details on antiviral therapy and treatment responses were analyzed retrospectively. Fifty-seven CMV treatment episodes were recorded in 34 patients: 20 with valganciclovir (900 mg twice-daily) and 37 with ganciclovir (5 mg/kg twice-daily). Median CMV DNA load reduction was 0.079 and 0.069 $\log_{10}$ copies/ml/day in the ganciclovir and valganciclovir group, respectively. Good response on CMV DNA load(reduction below 3.0 $\log_{10}$ copies/ml) was observed in 75.7% of ganciclovir and 80.0% of valganciclovir treatment episodes. Severe adverse effects were not observed and CMV-related disease did not occur. However, the percentage of patients receiving erythrocyte transfusion was higher in the group of patients receiving ganciclovir as compared to valganciclovir (41 versus 20%, $P = 0.116$). In conclusion, pre-emptive treatment with valganciclovir and ganciclovir, led to similar reduction of CMV DNA load. Oral valganciclovir is an attractive and safe alternative for pre-emptive CMV treatment in T-cell-depleted allo-SCT recipients.

## INTRODUCTION

In myeloablative (MA) allogeneic stem cell transplant (allo-SCT) recipients, cytomegalovirus (CMV) infection contributes significantly to morbidity and mortality.[1] Primary infection results in a lifelong persistence of the virus with reactivation and potentially fatal disease when immunity fails. Cytomegalovirus seropositivity in a patient before transplantation is associated with the highest risk of CMV disease.[2] Furthermore, graft-versus-host disease (GVHD) and T-cell depletion (TCD) of the transplant are important contributing factors.[3] Current strategies for the prevention of CMV disease aim at preventing end-organ disease by using ganciclovir or valganciclovir prophylaxis[4,5] or ganciclovir pre-emptive therapy, initiated upon early detection of CMV infection by antigenemia or CMV DNA in plasma.[5,6] The relative merits of both strategies have been debated extensively in the literature.[7,8] The major drawback limiting the use of oral ganciclovir is its poor bioavailability, which precludes therapeutic use by oral administration.[6] This has now changed with the introduction of valganciclovir, which is an orally administered prodrug of ganciclovir with good bioavailability. Previous pharmacokinetic studies showed similar drug exposure to ganciclovir after a single oral dose of 900 mg valganciclovir as compared to an intravenous dose of 5 mg/kg ganciclovir.[9–11] Recently, oral valganciclovir and intravenous ganciclovir were shown to have similar efficacy in pre-emptive CMV treatment in solid organ transplant recipients.[12–14] As a consequence, the prevention of CMV disease in high-risk renal, renal–pancreas and heart transplant patients was added as another indication to the original approval of valganciclovir for the treatment of CMV retinitis in AIDS patients. So far, no data are available on the efficacy of 900 mg valganciclovir twice daily as compared to intravenous 5 mg/kg ganciclovir twice daily in the pre-emptive therapy of CMV infection in stem cell transplant recipients and therefore valganciclovir is not licensed for use in allogeneic stem cell transplantation patients. A comparison with intravenous ganciclovir in allo-SCT patients is warranted, as haematological toxicity is a common side effect of ganciclovir and of particular significance in this population. In this observational prospective study, we compared the efficacy and safety of CMV DNA load-guided pre-emptive therapy with valganciclovir to ganciclovir intravenously in allo-SCT recipients.

## PATIENTS AND METHODS

### *Patients*

All consecutive patients undergoing MA and reduced-intensity allogeneic stem cell transplantation at the Leiden University Medical Center between January 2001 and

December 2004 were included in this analysis. All patients at risk for CMV infection (i.e. CMV sero-positivity in either the recipient (R+), the donor (D+) or both (D+/R+)) were routinely monitored by CMV DNA load detection in plasma. Data were available on demographic characteristics, underlying diseases, donor and recipient CMV serostatus, occurrence of GVHD and treatment (i.e. initiation, duration, type and dosage of drugs used) and the ganciclovir formulation (i.e. valganciclovir or ganciclovir), CMV DNA load measurements and general laboratory parameters.

***Transplantation***

T-cell-depleted transplantation was performed either according to a reduced-intensity conditioning (RIC) protocol or a conventional MA regimen as described previously.[15,16] The RIC regimen consisted of fludarabine (30 mg/m$^2$, intravenously, days –10 to –6), busulphan (3.2 mg/kg, intravenously, days –6 and –5) and ATG (10 mg/kg/day intravenously, days –4 to –1), for both sibling and matched unrelated donor (MUD) grafts. The MA conditioning regimen consisted of cyclophosphamide (60 mg/kg/day intravenously for 2 consecutive days) followed by single dose of total body irradiation (TBI, 9 Gy, day –1) in patients receiving sibling donor grafts. Recipients of MUD grafts, in the MA regimen, received additional Campath1G or -1H (days –8 and –4) and cyclosporine (3 mg/kg intravenously, starting on day –1) and TBI (6 Gy, days –8 and –7). The stem cell product was infused on day 0. In all conditioning regimens, TCD of the graft was performed by *in vitro* incubation of the graft with Campath-1H (20 mg). Assessment of acute and chronic GVHD was performed using the Glucksberg and Shulman criteria.[17,18] In the absence of GVHD or graft failure, patients received donor lymphocyte infusion (DLI) after RIC transplantation or in mixed chimerism or relapsed disease after MA transplantation. Donor lymphocyte infusion was administered at least 6 months following transplantation. Donor lymphocyte infusion was not used as a therapeutic modality for CMV infection.

***Cytomegalovirus monitoring and treatment***

CMV DNA load was measured at weekly intervals for at least 180 days following transplantation, until death occurred or beyond day 180 until CMV DNA became undetectable. The real-time quantitative PCR for detection of CMV DNA in plasma was performed according to the method described previously.[19] Cytomegalovirus DNA load-guided pre-emptive therapy was initiated according to a guideline as described previously.[13] In short, any symptomatic CMV infection would be treated with intravenous 5 mg/kg ganciclovir twice daily. In case of a first reactivation or a significant viraemia (CMV DNA load $>10^4$ copies/ml, or CMV load $>10^3$ copies/ml and more than 1.0 $\log_{10}$ increase as compared to preceding measurement) without clinical symptoms of CMV disease, either 900 mg valganciclovir twice daily

or intravenous 5 mg/kg ganciclovir twice daily was administered for 2 weeks. Until 2003 intravenous ganciclovir was used as primary preemptive treatment. From 2003 onwards, as soon as it became available for clinical use, valganciclovir was used as preferred primary treatment of outpatients, only limited to approval by the patients' medical insurance. When such approval was not granted, or if hospital admission was indicated for other reasons, intravenous ganciclovir was administered. Ganciclovir and valganciclovir dosages were adjusted to renal function as described previously.[20] During (val)ganciclovir treatment, CMV DNA load and haematological parameters were monitored at least weekly; G-CSF prophylaxis was not routinely used. Donor lymphocyte infusion was not used as a therapeutic modality for CMV infection.

***End points and statistical analysis***

The effect of CMV treatment on CMV DNA load in plasma, following a full course of either ganciclovir or valganciclovir, was defined as good response (CMV DNA load reduction of more than 0.5 $\log_{10}$ and to a level below 3.0 $\log_{10}$ copies/ml), moderate response (reduction of CMV DNA load of more than 0.5 $\log_{10}$, but not to a level below 3.0 $\log_{10}$ copies/ml) and no response (equal DNA load (i.e. reduction of less than 0.5 $\log_{10}$) or an increase). The levels of 3.0 $\log_{10}$ and 0.5 $\log_{10}$ were chosen as reference values based on a previous report on pre-emptive CMV treatment in SCT recipients.[19] In addition, absolute reduction in number of CMV DNA copies/ml was calculated to compensate for differences in baseline CMV load before treatment. To avoid bias owing to possible differences in CMV reduction rate in first episodes as compared to subsequent episodes, the effect of antiviral medication in first and subsequent episodes was analyzed separately. Cytomegalovirus load reduction per day was calculated by dividing the difference in pre-and post-treatment CMV DNA load by the number of treatment days.

Haematological toxicity was assessed by comparing the number of erythrocyte and thrombocyte transfusion units administered during and following antiviral treatment and by comparing leucocyte ratios (calculated by dividing the leucocyte count before treatment by the count at the end of treatment). Criteria for erythrocyte and thrombocyte transfusion were haemoglobin concentration below 6.0 mmol/l and platelet count below $10 \times 10^{10}$/l, respectively. Definitions of CMV infection, CMV disease and CMV detection in blood were consistent with internationally accepted criteria.[21]

All statistical analyses were performed using SPSS version 12.0.1. Differences in the distribution of categorical data were tested using $\chi^2$ test. For comparison of the antiviral effect between the two treatments (i.e. ganciclovir or valganciclovir) and comparison of baseline non-categorical data we used Mann–Whitney *U*-test. Paired ob-

**Table 1.** Characteristics of the study population in both treatment groups

| Parameter | ValGCV | GCV |
|---|---|---|
| Treatment episodes, *n* | 20 | 37 |
| Number of patients, *n* | 14 | 26 |
| Median age in years (range) | 51 (41–62) | 50 (24-62) |
| Male gender, *n (%)* | 9 (64) | 17 (65) |
| Type of conditioning, *n (%)* | | |
| Reduced intensity | 6 (40) | 14 (54) |
| Myeloablative | 8 (60) | 12 (46) |
| Type of donor, *n (%)* | | |
| Related | 11 (80) | 20 (76) |
| Unrelated | 3 (20) | 6 (24) |
| Underlying disease, *n (%)* | | |
| Acute leukaemia | 5 (38) | 9 (35) |
| CML | 2 (14) | 3 (12) |
| CLL | 1 (7) | 1 (4) |
| MM | 1 (7) | 6 (23) |
| NHL | 4 (29) | 1 (4) |
| Other | 1 (7) | 6 (23) |
| GVHD, *n (%)* | | |
| No GVHD | 10 (70) | 19 (73) |
| Grade I/II | 4 (25) | 6 (24) |
| Grade III/IV | 1 (5) | 1 (3) |
| Treatment | 3 (20) | 5 (19) |
| CMV serostatus, *n (%)* | | |
| D+/R– | 0 (0) | 1 (3) |
| D+/R+ | 7 (50) | 13 (51) |
| D–/R+ | 7 (50) | 12 (46 |
| Median duration of treatment in days (range) | 14 (7–36) | 14 (7-28) |
| Haematological parameters at start of treatment (Median values (range)) | | |
| Haemoglobin (mmol/l) | 7.3 (5.1–8.3) | 6.9 (4.5–10.6) |
| Leucocyte count ( $\times 10^9$/l) | 5.0 (1.9–8.0) | 3.1 (0.7–11.5) |
| Thrombocyte count ( $\times 10^9$/l) | 88.0 (62.0–264.0) | 100 (12.0–206.0) |

Abbreviations: CLL = chronic lymphocytic leukaemia; CML = chronic myelogenous leukaemia; CMV = cytomealovirus; GVHD = graft-versushost disease; MM = multiple myeloma; NHL = non-Hodgkin's lymphoma. In total, 57 CMV treatment episodes were observed in 34 patients. No statistically significant differences were observed between the two treatment groups. Systemic treatment of GVHD consisted of oral prednisone, intravenous methylprednisolone and/or oral cyclosporine.

servations (e.g., pre-treatment versus post treatment measurements) were analyzed non-parametrically using the Wilcoxon signed ranks test for paired observations.

## RESULTS

A total of 107 patients were included in this study. The demographic and disease characteristics for both CMV treatment groups are shown in Table 1. Distribution of the characteristics across the two groups was similar. Briefly, 48 patients received a transplantation following an RIC protocol, whereas 59 patients received their transplants following an MA conditioning regimen. With regard to donor and recipient CMV serostatus, 40 D+/R+ (37.4%), eight D+/R– (7.5%), 30 D–/R+ (28.0%) and 29 D–/R– (27.1%) combinations were observed. The D–/R– patients were excluded from further analysis, as they are not considered to be at risk for CMV infection. The median follow-up period following transplantation was 200 days (range: 30–611). During the follow-up period, CMV DNA load became detectable in 42 out of 78 (54%) patients at risk for CMV infection, resulting in 57 CMV treatment episodes with either ganciclovir or valganciclovir in 34 patients. The incidence of GVHD and the percentage of patients treated for GVHD were similar in the two CMV treatment groups. In none of the patients DLI was administered during treatment episodes.

The CMV treatment results are shown in Table 2. Intravenous ganciclovir was used in 37 episodes. A good response was observed in 28 episodes (76%). A moderate response was observed in five episodes (14%) occurring in four separate patients. One of these patients died as a result of extensive GVHD without signs of CMV disease. The remaining three patients reached a good response following a second course of intravenous ganciclovir. In four ganciclovir treatment episodes (11%), occurring in four individual patients, no response on CMV load was observed. In three of these four non-responding patients, CMV DNA load decreased below undetectable levels within 2 weeks after cessation of ganciclovir. In the remaining patient, CMV DNA load increased from 3.5 to 4.8 $\log_{10}$ copies/ml, despite 4 weeks of ganciclovir treatment, and subsequently foscarnet was administered, resulting in a CMV DNA load below detectable levels within 14 days of treatment. Treatment with valganciclovir was administered in 20 of the 57 episodes, resulting in a good response in 16 out of these 20 episodes (80%). Moderate response was observed in three out of these 20 episodes (15%) occurring in three individual patients. One of these patients died as a result of extensive GVHD without signs of CMV disease, and the remaining two patients showed a good response following a second course of valganciclovir. In one out of the 20 valganciclovir treatment episodes (5%), no response on CMV DNA

**Table 2.** Characteristics of 57 CMV treatment episodes in 34 patients and response on CMV DNA load according to treatment group

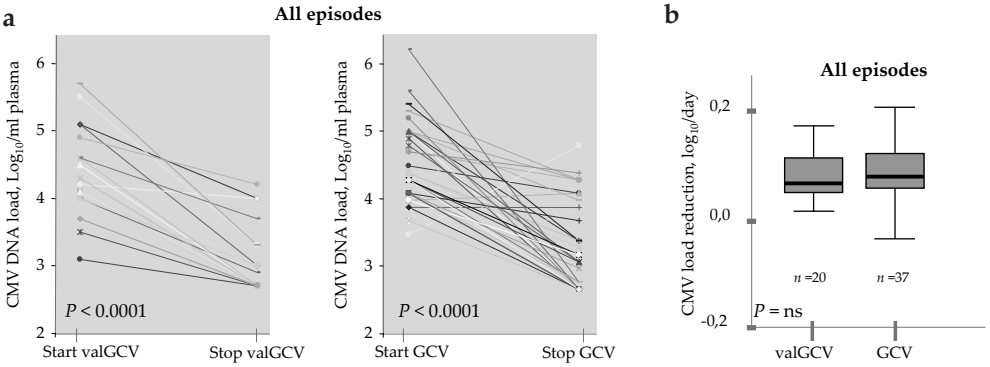
| Parameter | valGCV (n = 20) | GCV (n = 37) |
|---|---|---|
| First treatment episodes, *n (%)* | 8 (40) | 26 (70) |
| Subsequent treatment episodes, *n (%)* | 12 (60) | 11 (30) |
| Response on CMV DNA load | | |
| Good response, *n (%)* | 16 (80) | 28 (76) |
| Moderate response, *n (%)* | 3 (15) | 5 (14) |
| No response, *n (%)* | 1 (5) | 4 (11) |
| Erythrocyte transfusion, *n (%)* | 4 (20) | 15 (41) |
| Thrombocyte transfusion, *n (%)* | 3 (15) | 5 (14) |
| Leucocyte ratio[a] (median (range)) | 1.6 (0.6–27.1) | 1.2 (0.2–11.0) |
| Leucocyte count $\times 10^9$/l (median, (range)) | | |
| Pre-treatment | 5.0 (1.9–8.0) | 3.1 (0.7–11.5) |
| Post-treatment | 3.6 (0.1–9.7) | 3.0 (0.4–8.6) |

Abbreviations: CMV = cytomegalovirus; GCV = ganciclovir; valGCV = valganciclovir. No statistically significant differences were observed between the two treatment groups.
[a]Calculated by dividing leucocyte count before treatment by the count at the end of treatment.

load was observed; this patient showed a good response upon a second course of valganciclovir.

The effect of anti-CMV treatment with ganciclovir and valganciclovir was further assessed by comparing the CMV DNA load at the start and at the completion of the treatment episode. When first treatment episodes as well as all subsequent episodes were evaluated, CMV DNA load at start of therapy in the ganciclovir and the valganciclovir group was similar (median 4.3 (range: 3.3–6.2) and 4.2 $\log_{10}$ copies/ml (range: 3.1–5.7), $P > 0.4$, respectively, Figure 1b). The kinetics of CMV DNA following treatment with ganciclovir and valganciclovir for individual patients are shown in Figure 1a. A median reduction of 1.20 and 1.10 $\log_{10}$ DNA copies/ml was reached in the ganciclovir-($n = 37$) and the valganciclovir- ($n = 20$) treated patients, respectively ($P < 0.0001$ for both groups). No difference in the magnitude of CMV DNA load reduction/treatment day was observed between the ganciclovir and valganciclovir groups (median 0.0786 (range: –0.0464–0.767) and 0.0690 $\log_{10}$ copies/ml/day (range: 0.0182–0.171), $P > 0.8$, respectively; Figure 1b). Cytomegalovirus treatment episodes were further subdivided into 34 first episodes (26 ganciclovir,

**Figure 1.**



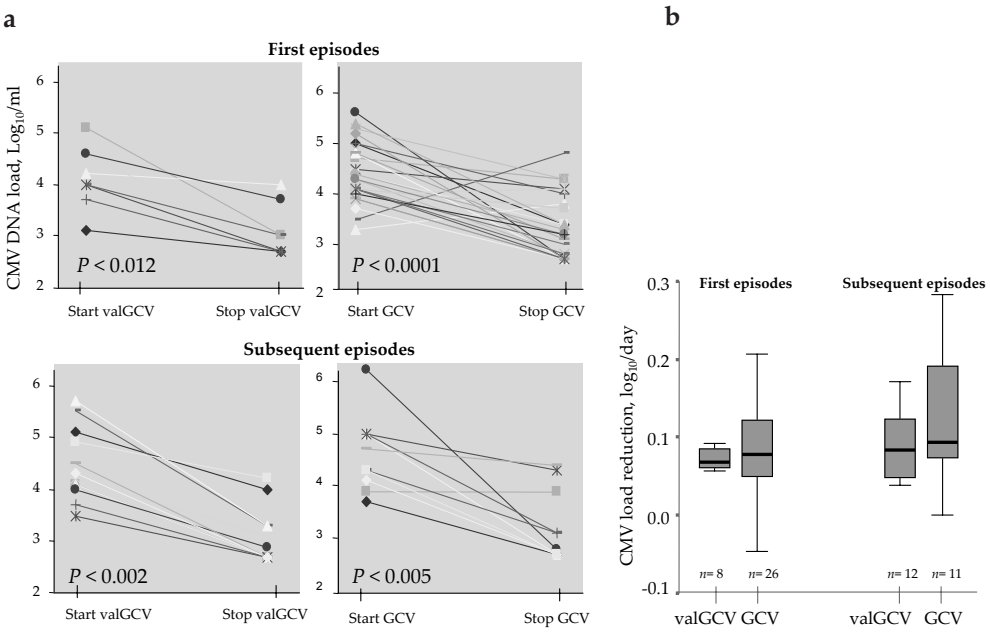

Course of cytomegalovirus (CMV) DNA load before and after treatment with valganciclovir or ganciclovir for individual patients (all treatment episodes). (b) CMV DNA load reduction per treatment day with valganciclovir and intravenous ganciclovir (all treatment episodes). The box plots display the median, the 25th and 75th percentiles (box), and the smallest and largest values (whiskers).

eight valganciclovir) and 23 subsequent episodes (11 ganciclovir, 12 valganciclovir) (Figure 2a). Cytomegalovirus DNA load at start of therapy, according to treatment episode, was similar in the ganciclovir and valganciclovir groups (median 4.4 (range: 3.3–5.6) versus 4.1 $\log_{10}$ copies/ml (range: 3.1–5.1) in first episodes, $P >$ 0.3, respectively and 4.3 (range: 3.5–5.7) versus 4.3 $\log_{10}$ copies/ml (range: 3.5–5.7) in subsequent episodes, $P > 0.7$, respectively). The magnitude of CMV load reduction/treatment day in first treatment episodes was similar for the ganciclovir and valganciclovir group (median 0.0941 (range: 0.000–0.767) and 0.0833 $\log_{10}$ copies/ml/day (range: 0.0381–0.171), $P >$ 0.6, respectively, Figure 2b). For subsequent episodes, the same result was obtained (median 0.0786 (range: 0.0464–0.260) and 0.0685 $\log_{10}$ copies/ml/ day (range: 0.0182–0.150), $P > 0.4$, for ganciclovir and valganciclovir, respectively; Figure 2b).

Erythrocyte transfusions were administered in 15 out of the 37 (41%) ganciclovir treatment episodes (median number of units: 2, range 2–6 units) as compared to four out of the 20 (20%) (median number of units: 2, range 2–6 units) of the valganciclovir treatment episodes ($P = 0.116$). The percentage of patients receiving thrombocyte transfusions was similar in the ganciclovir-and valganciclovirtreated groups (15.0 and 13.5%, $P > 0.8$, respectively). Furthermore, the leucocyte ratio was not significantly different between ganciclovir and valganciclovir treatment episodes (median 1.16 and 1.55, $P > 0.1$, respectively).

No signs of CMV disease and no severe adverse reaction (NCI grade 3–4) of (val)ganciclovir treatment were observed.

**Figure 2.**




(a) Courses of cytomegalovirus (CMV) DNA load before and after treatment with valganciclovir or ganciclovir for individual patients. First (upper panel) and subsequent (lower panel) treatment episodes are plotted separately. (b) The cytomegalovirus DNA load reduction per day during treatment with valganciclovir and intravenous ganciclovir. First (left box plots) and subsequent (right box plots) episodes are shown separately. The box plots display the median, the 25th and 75th percentiles (box), and the smallest and largest values (whiskers). No significant differences are present.

## DISCUSSION

This study demonstrates that pre-emptive treatments with oral valganciclovir and intravenous ganciclovir are equally effective in reducing CMV DNA load in allogeneic stem cell recipients. Pre-emptive treatment of CMV viraemia episodes in allogeneic stem cell recipients with either valganciclovir or ganciclovir led to a similar median CMV DNA load reduction in plasma of approximately 0.1 $\log_{10}$ copies/ml/day, which is in accordance with our previous report on renal and renal/pancreas transplant recipients.[13]

Although initially no response was seen upon treatment with intravenous ganciclovir in four patients, CMV DNA load spontaneously declined in three of these whereas in only one patient a switch to foscarnet was made. Furthermore, in four other patients (five treatment episodes), treatment with intravenous ganciclovir for

14 days did not reduce the CMV DNA load below the level of 3.0 $\log_{10}$ copies/ml and a subsequent course was needed to further reduce CMV DNA load. Similarly, in four patients treated with valganciclovir, either a subsequent course or a switch to foscarnet was needed to reduce CMV DNA load beyond detectable levels. Reasons for these failures are not clear and this study was not designed to identify factors associated with antiviral treatment failure. Therefore, further investigation with regard to these treatment failures is warranted.

As soon as valganciclovir became available in our institution in 2003, it was used as preferred primary treatment of asymptomatic patients, only limited to approval by the patient's medical insurance. In case such an approval was not granted or in case of co-morbidity leading to hospitalization, intravenous ganciclovir was administered. Patient selection might therefore have occurred, as co-morbidity was more likely to be present in admitted patients treated with ganciclovir. However, we do not expect that this possible bias has influenced our results to such an extent that the conclusions drawn might be incorrect. The baseline CMV loads in the ganciclovir and valganciclovir-treated groups were similar, indicating similar CMV activity. Furthermore, the magnitude of CMV decline in all analysed subgroups was similar, substantiating our conclusion on the equal efficacy of both drugs in CMV infection.

In our study, the haematological toxicity of oral valganciclovir in allo-SCT patients was similar as compared to ganciclovir intravenously. The slightly higher, although not statistically significant, percentage of patients receiving erythrocyte transfusions in the intravenous ganciclovir group might be the result of co-morbidity in the admitted patients treated with ganciclovir intravenously. Mainly owing to the retrospective nature of this study, differences in non-haematological toxicity, such as gastrointestinal and neurological complications, between the two treatment groups could not be assessed adequately and further evaluation in a prospective study is warranted.

So far, no other studies have been reported on the use of valganciclovir compared to intravenous ganciclovir in stem cell recipients. In conclusion, based on our findings, oral valganciclovir (900 mg, twice daily) is equally effective and safe as intravenous ganciclovir (5 mg/kg, twice daily) in the pre-emptive treatment of CMV disease following allo-SCT. There is an urgent need for an effective oral treatment for pre-emptive CMV therapy, which would enable prevention and treatment of CMV in an outpatient setting leading to reduced patient burden and health-care cost. The finding of the therapeutic equivalence of oral valganciclovir and intravenous ganciclovir is a confirmation of previous reports with respect to pre-emptive[12–14,22] and prophylactic treatment[20] in solid organ transplant recipients.

The large majority of allo-SCT recipients, without any signs and symptoms of CMV

disease when the first laboratory signs of CMV infection are detected, can benefit from treatment with an oral drug, without the need of hospitalization. Based on rational precautions, intravenously administered ganciclovir remains the first choice drug for patients with suspected symptomatic CMV infections, as the course of CMV disease can be serious, rapidly progressive and ultimately fatal.

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# 4

# Choice of antibody immunotherapy influences cytomegalovirus viremia in simultaneous pancreas-kidney transplant recipients

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## ABSTRACT

***Objective:*** Simultaneous pancreas-kidney (SPK) transplantation in type 1 diabetic patients requires immunotherapy against allo- and autoreactive T-cells. Cytomegalovirus (CMV) infection is a major cause for morbidity after transplantation and is possibly related to recurrent autoimmunity. In this study, we assessed the pattern of CMV viremia in SPK transplant recipients receiving either antithymocyte globulin (ATG) or anti-CD25 (daclizumab) immunosuppressive induction therapy.

***Research design and methods***: We evaluated 36 SPK transplant recipients from a randomized cohort that received either ATG or daclizumab as induction therapy. Patients at risk for CMV infection received oral prophylactic ganciclovir therapy. The CMV DNA level in plasma was measured for at least 180 days using a quantitative real-time PCR. Recipient peripheral blood mononuclear cells were cross-sectionally HLA tetramer-stained for CMV-specific $CD8^+$ T-cells.

***Results:*** Positive CMV serostatus in donors was correlated with a higher incidence of CMV viremia than negative serostatus. In patients at risk, daclizumab induction therapy significantly prolonged CMV-free survival. CMV viremia occurred earlier and was more severe in patients with rejection episodes than in patients without rejection episodes. CMV-specific $CD8^+$ T-cell counts were significantly lower in patients developing CMV viremia than in those who did not.

***Conclusions:*** Despite their comparable immunosuppressive potential, daclizumab is safer than ATG regarding CMV infection risk in SPK transplantation. ATG-treated rejection episodes are associated with earlier and more severe infection. Furthermore, high CMV-specific tetramer counts reflect antiviral immunity rather than concurrent viremia because they imply low viremic activity. These findings may prove valuable in the discussion on both safety of induction therapy and recurrent autoimmunity in SPK and islet transplantation.

Type 1 diabetes is an autoimmune disease characterized by T-cell–mediated destruction of insulinproducing β-cells.[1] Simultaneous pancreas-kidney (SPK) transplantation is a well-established treatment option for type 1 diabetic patients with (or approaching) end-stage renal failure.[2–5] The foremost challenge in SPK transplantation is to prevent alloreactivity as well as recurrence of autoimmunity against β-cells.

Recurrent autoimmunity and alloreactivity can be effectively reduced by immunosuppressive induction therapy,[6,7] in combination with maintenance immune suppression.[8] Polyclonal rabbit antithymocyte globulin (ATG) has been widely accepted as an effective form of induction therapy in pancreatic and islet transplantation.[9] It depletes different subsets of the T-cell repertoire[10] and is also commonly used as rejection therapy for steroid-resistant rejection episodes.[11] Unfortunately, it can cause a number of unwanted side effects, the most important being prolonged immunodeficiency and a subsequent increased risk of infections.[12] In our institute, ATG Fresenius ($ATG_F$) (derived from a rabbit anti-Jurkat cell line)[13] is used for induction therapy, whereas ATG Merieux ($ATG_M$) (derived from a rabbit antihuman thymocyte line)[10] is used as rejection therapy in SPK transplantation.

More recently, monoclonal antibodies directed against specific T-cell surface molecules have been developed for clinical use for immunosuppression. One of these is anti-CD25 (daclizumab), a humanized IgG1 monoclonal antibody directed against the low-affinity interleukin-2 receptor α-chain.[14] This antibody is supposed to solely affect activated T-cells.[15] Its use in a clinical setting has increased in recent years.[16–19] Similar immunosuppressive properties for both ATG and daclizumab in terms of preventing alloreactivity have been reported.[14]

The most common opportunistic pathogen complicating the care of immunosuppressed solid organ transplant recipients is cytomegalovirus (CMV). It causes both direct effects, including tissue injury and clinical disease, and a variety of indirect effects, such as allograft rejection.[20] Because protection from CMV infection is mainly dependent on cellular-mediated immunity,[21] CMV-related problems are typically encountered primarily between 1 and 6 months after transplantation as a consequence of the intensity of immunosuppressive therapy in that period.[20,22]

In pancreas and islet transplant recipients, the possible role of CMV in the pathogenesis of type 1 diabetes is of additional interest. This mechanism is proposed to be mediated by an autoimmune reaction provoked by molecular mimicry between CMV and autoantigen GAD65[23] and/or by impaired insulin release.[24] As a consequence, adequate prevention and treatment of CMV infection can have additional value for the prevention of recurrent autoimmunity in recipients of SPK transplants as well as islet allografts.

The severity of an episode of CMV viremia is determined not only by its level but also

by its duration.[25,26] Both quantities can be combined by calculation of the area under the curve of viral load over time,[25] a universal means of assessing the interrelationship among peak viral load, initial viral load, and rate of increase of viral load, parameters that have been described as independent risk factors for CMV disease.[26] In this retrospective study, (re)activation of CMV, as measured by DNA load in plasma, was used as a safety parameter to evaluate the efficacy of ATG versus daclizumab in SPK transplant recipients. Additionally, CMV-specific tetramer staining was used as a marker for antiviral immunity to further assess its role in CMV (re)activation in this patient group.

## RESEARCH DESIGN AND METHODS

Thirty-nine consecutive patients received SPK transplants at the Leiden University Medical Center between October 1999 and May 2002. In all patients duodenocystostomy was used for exocrine drainage of the pancreatic graft. Patients were randomly assigned to receive either a single dose of $ATG_F$ (9 mg/ kg) intraoperatively or five consecutive doses of daclizumab (1 mg/kg) administered in 2-week intervals, starting before transplantation. Relevant patient characteristics were comparable between groups. No differences in clinical outcome were observed between either induction protocols or occurrence of CMV viremia with regard to transplant survival, insulin independence, and cumulative numbers of rejection episodes (Table 1). From 36 patients, sufficient plasma samples could be collected for the CMV DNA quantification used in this study. Two patients lost their pancreas graft at an early stage (3 and 4 days after transplantation, respectively) due to technical complications (venous graft thrombosis), and one patient died with functioning grafts 70 days after transplantation.

CMV serostatus of both donor and recipient was determined before transplantation. Patients at risk for CMV infection (based on donor [D]/receptor [R] serostatus: D+/R–, D+/R+, or D–/R+) received antiviral prophylaxis (1,000 mg ganciclovir orally three times daily for 3–4 months) starting within 14 days after transplantation. Maintenance immunosuppression in all patients consisted of cyclosporin A microemulsion (Neoral) with dose adjustments based on trough level monitoring, mycophenolate mofetil 1,000 mg twice per day, and prednisolone, which was gradually tapered to 10 mg/day by 3 months. Clinical rejection episodes were treated with high-dose intravenous steroids (Solu-Medrol 1,000 mg/day for 3 consecutive days). Recurrent or steroid-resistant rejection episodes were treated with a 10-day course of $ATG_M$ (starting at 4 mg/kg), with subsequent dosing guided by absolute lymphocyte counts in peripheral blood.

**Table 1.** Characteristics of the study population according to type of induction therapy

| Characteristic | Induction therapy | | *P* value |
|---|---|---|---|
| | ATG | Daclizumab | |
| *n* | 19 | 20 | |
| Recipient age (years) | 44.1 ± 8.3 | 40.3 ± 7.4 | 0.14 |
| Recipient sex (male/female) | 10/9 | 14/6 | 0.33 |
| Diabetes duration (years) | 29.2 ± 8.3 | 26.9 ± 6.5 | 0.35 |
| Diabetic retinopathy (%) | 100 | 100 | 1.00 |
| Diabetic neuropathy (%) | 88.9 | 70.0 | 0.24 |
| Maintenance dialysis (%) | 68.4 | 75.0 | 0.73 |
| Time on dialysis (years) | 2.2 ± 1.3 | 1.3 ± 0.7 | 0.03 |
| HLA-A mismatch | 1.4 ± 0.6 | 1.4 ± 0.6 | 0.88 |
| HLA-B mismatch | 1.4 ± 0.6 | 1.7 ± 0.5 | 0.12 |
| HLA-DR mismatch | 1.4 ± 0.6 | 1.2 ± 0.8 | 0.33 |
| Donor age (years) | 39.3 ± 8.4 | 32.2 ± 12.6 | 0.04 |
| Donor sex (male/female) | 10/9 | 11/9 | 1.00 |
| Cold ischemic time pancreas (h) | 12.0 ± 3.4 | 13.3 ± 3.4 | 0.23 |
| Cold ischemic time kidney (h) | 12.2 ± 4.1 | 13.6 ± 3.4 | 0.28 |
| CMV IgG serostatus (%) | | | |
| D+/R+ | 16 | 20 | 1.00 |
| D+/R– | 26 | 20 | 0.72 |
| D–/R+ | 11 | 20 | 0.66 |
| D–/R– | 47 | 40 | 0.89 |
| Ganciclovir prophylaxis (days) | 92 ± 18.6 | 107 ± 19.4 | 0.55 |
| Acute rejection at 6 months (%) | 36.8 | 45.0 | 0.85 |
| Patient survival at 6/12/36 months (%) | 100/100/100 | 95/95/90 | 0.11 |
| Kidney graft survival at 6/12/36 months (%) | 100/94.7/94.7 | 100/100/94.7 | 0.98 |
| Pancreas graft survival at 6/12/36 months (%) | 89.5/84.2/84.2 | 100/100/94.7 | 0.27 |

Data are means ±SD unless otherwise indicated.

***Sample collection, quantification of CMV DNA load in plasma, and determination of area under the viremia curve***

EDTA plasma samples were collected at a frequency of about once a week for at least 180 days after transplantation and stored at –80°C until further processing. Nucleic acids were extracted from 0.2-ml plasma samples with the automated purification

procedure of the MagNA Pure LC system (Roche Molecular Systems, Almere, the Netherlands) using the total nucleic acid isolation kit. Subsequently, CMV DNA quantification was performed using an internally controlled real-time quantitative CMV PCR. Sensitivity, specificity, and reproducibility of this assay were described in more detail previously.[27] The course of CMV DNA load in plasma was documented longitudinally for each patient within 180 days of follow-up. Individual areas under the CMV viremia curves between 0 and 180 days after transplantation were calculated using the trapezoidal rule as described previously.[25,28]

***CMV tetramer staining***

HLA-A2–restricted, CMV-specific phytoerythrin-labeled tetramers have been shown to be a valuable tool both for the detection of cytotoxic lymphocytes directed against CMV and potentially for diagnostic use.[29] Blood from 16 HLA-A2–positive SPK transplant recipients was drawn and heparinized cross-sectionally 1–2 years after transplantation. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation and washed in 0.9% phosphate-buffered saline. One million cells were incubated in PBS containing 0.1% FCS at room temperature for 30 min with a CMV-specific tetramer developed in our lab. Cells were washed and stained with fluorescein isothiocyanate– labeled anti-CD3 monoclonal antibody (BD Biosciences, Oxford, U.K.) and allophyocyanin-labeled anti-CD8 monoclonal antibody (BD Biosciences) for 20 min at 4°C. After washing, fluorescence was measured immediately using a FACScan (BD Biosciences). Cells were analyzed using CellQuest software (BD Biosciences), measuring the percentage of CMV-specific cells in the $CD3^+/CD8^+$ living cell population.

***Statistical analysis***

Two-tailed Fisher's exact test was used to determine differences between serologic groups. Disease-free survival data were presented as Kaplan-Meier survival curves with log-rank analysis and Cox proportional hazard regression to determine differences in survival. Differences in total viral load and T-cell counts were measured using nonparametric Mann-Whitney *U* tests, assuming non-Gaussian distribution.

## RESULTS

***Donor serology is related to CMV viremia***

With regard to the pretransplantation CMV serostatus of donor and recipient among the 36 SPK transplant recipients, 9 were D+/R–, 7 were D+/R+, and 6 were D+/R–. CMV viremia was detected in 13 of 16 patients (81%) receiving seropositive

**Table 2.** Impact of donor serology on incidence of CMV viremia

| Patient group | CMV viremia | No CMV viremia | *P* value |
|---|---|---|---|
| D+ | 13 | 3 | < 0.0001 |
| D– | 2 | 18 | |
| R+ | 7 | 6 | 0.31 |
| R– | 8 | 15 | |
| D+/R– | 8 | 1 | 0.16* |
| D+/R+ | 5 | 2 | 1.0* |
| D–/R+ | 2 | 4 | 0.054* |
| D–/R– | 0 | 14 | |

*Compared with other groups at risk for CMV.

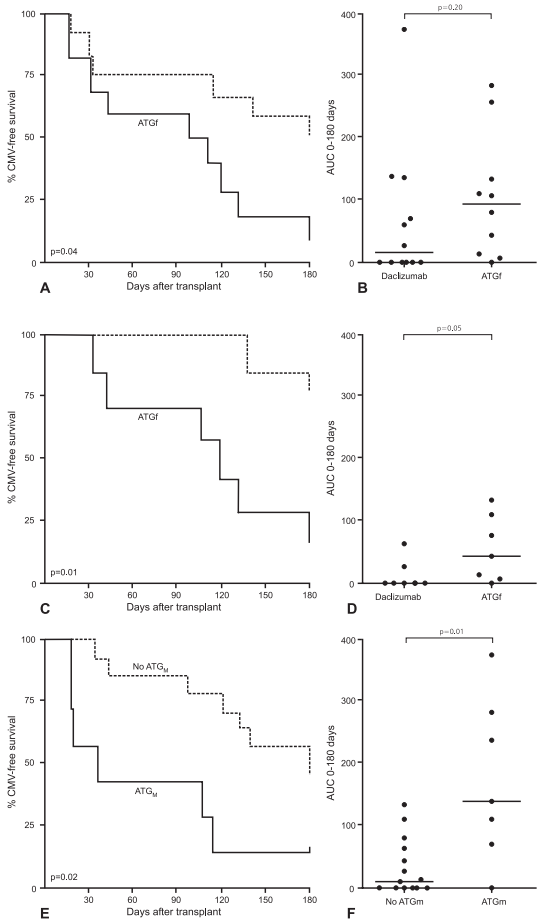
donor organs, compared with 2 of 20 patients (10%) receiving seronegative donor organs ($P < 0.0001$) (Table 2). In contrast, no significant difference was seen for the incidence of CMV viremia in seropositive recipients versus seronegative recipients (7 of 13 and 8 of 23, respectively). Regarding the serologic groups at risk for CMV, D+/R– patients tended to develop more CMV viremia, whereas D–/R+ patients showed a trend toward a reduced risk of CMV viremia compared with the other at-risk groups.

***CMV viremia occurs earlier with $ATG_F$ induction therapy***

The two different antibody induction therapies were compared with regard to the moment CMV viremia occurred. CMV viremia was defined as detection of two consecutive CMV DNA loads of more than $_{10}$log 2.7 (= 500) copies/ml plasma. In the total population, a trend was noted toward shorter CMV-free survival in the $ATG_F$-treated than in the daclizumabtreated patients ($P = 0.10$). Considering the population at risk for CMV infection ($n = 22$, D–/R– excluded), CMV-free survival was significantly shorter in the $ATG_F$ group ($P = 0.04$) (Fig. 1A). Both patient groups were comparable regarding age, sex, incidence of rejection, and CMV serostatus. The median area under the viremia curve tended to be higher in the $ATG_F$ group (Fig. 1B), indicating more severe CMV viremia.

In both groups, a number of patients received a 10-day course of $ATG_M$ rejection treatment, influencing CMV load (see rejection treatment results below). Excluding these patients from the induction group analysis did not influence patient group characteristics, and shorter CMV-free survival ($P = 0.01$) and more severe infection ($P = 0.05$) were seen in the $ATG_F$ compared with the daclizumab group (Fig. 1C and D, respectively).

**Figure 1**

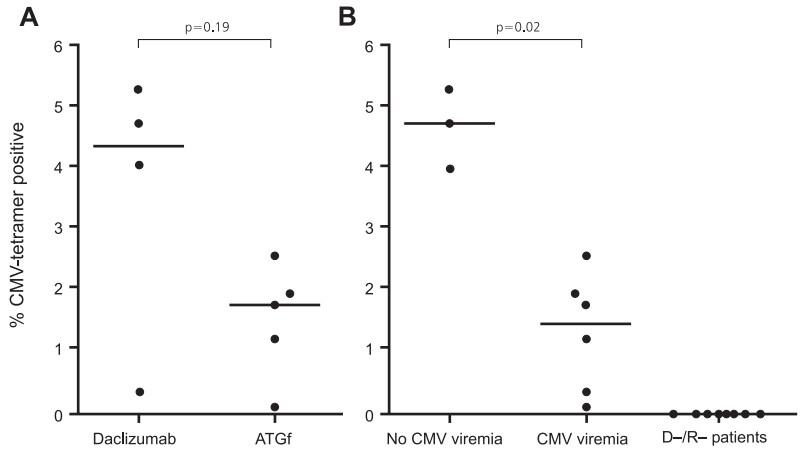



Pattern of CMV viremia of SPK transplant recipients at risk for CMV. Shown are CMV-free survival (Kaplan-Meier) and total viral load over 180 days. A and B: Differences between $ATG_F$ ($n$ = 10) and daclizumab ($n$ = 12) induction therapy for all patients at risk. First detection of CMV viremia (median ± range in days): $ATG_F$ 97 (18–180) and daclizumab 75 (20–180). C and D: Differences between ATGF ($n$ = 7) and daclizumab ($n$ = 7) induction therapy for patients at risk who did not receive $ATG_M$ rejection therapy. First detection of CMV viremia (median ± range in days): $ATG_F$ 114.5 (34–180) and daclizumab 159.5 (139–180). E and F: Differences between patients at risk who received $ATG_M$ rejection therapy ($n$ = 7) and patients who did not ($n$ = 14), regardless of induction therapy. First detection of CMV viremia (median ± range in days): $ATG_M$ 28 (18–114) and no ATGM 127 (20–180) ($P$ = 0.03, Mann-Whitney $U$ test). Median time between $ATG_M$ rejection treatment and occurrence of CMV viremia was 9 (0–75) days.

### *Rejection episodes treated with $ATG_M$ are related to earlier and more severe CMV viremia episodes*

Next, the correlation between rejection episodes treated with $ATG_M$ and CMV viremia in the patient group at risk for CMV was assessed. One patient was excluded from this analysis because he received only Solu-Medrol as rejection treatment. Fig-

**Figure 2.**




Percentage of CMV-specific cells in the $CD3^+$/ $CD8^+$ living cell population in HLA-$A2^+$ SPK transplant recipients, stratified according to induction therapy (A) and the development of CMV viremia (B).

ure 1E shows the disease-free survival curves for patients receiving $ATG_M$ rejection therapy versus patients without rejection episodes. A significantly shorter disease-free survival was seen in the $ATG_M$ rejection therapy group ($P = 0.02$). In these patients, CMV viremia occurred after administration of rejection treatment, except for one patient in whom detection of CMV coincided with rejection treatment. Total viral load as measured by the area under the curve from 0 to 180 days was higher ($P = 0.01$) than in patients without rejection episodes (Fig. 1F).

Cox proportional hazard regression identified both $ATG_M$ rejection therapy and $ATG_F$ induction therapy as independent risk factors for shorter CMV-free survival ($ATG_M$ hazard ratio 6.191 [95% CI 1.792–21.393], $P = 0.004$; $ATG_F$ 5.447 [1.598–18.564], $P = 0.007$).

***Tetramer staining shows fewer CMV specific $CD8^+$ T-cells in CMV infected patients***

To further investigate the mechanism underlying the pattern of CMV viremia in this patient group, HLA-A2–restricted CMV-specific tetramer fluorescence activated cell sorter staining was performed on PBMCs of 16 HLA-$A2^+$ patients. Several patients showed distinct populations of CMV-specific cells in the $CD3^+/CD8^+$ T-cell population. In the patients at risk, a trend was noted toward a higher percentage of CMV-specific $CD8^+$ T-cells in the daclizumab-treated group compared with the $ATG_F$-treated group (Fig. 2A). When we stratified for CMV viremia, a significantly lower percentage of CMV-specific $CD8^+$ T-cells was seen in patients who developed CMV viremia ($P = 0.01$) (Fig. 2B). To test the possibility of an ongoing infection at the time

of blood withdrawal for isolation of PBMCs, the serum samples were analyzed for CMV viremia. No CMV DNA was detected in any of the samples (not shown). As a further control, PBMCs from HLA-A2$^+$ patients not at risk for CMV infection (D–/R–) were stained, showing no CMV specificity at all (Fig. 2B).

## CONCLUSIONS

In this study, it is shown that CMV viremia not only occurred earlier but was also more severe in SPK transplant recipients receiving single-shot $ATG_F$ induction therapy compared with five-dose daclizumab and after rejection episodes treated with a 10-day course of $ATG_M$. Despite the limited number of patients included in the study, several potentially clinically relevant differences were found to be significant. In our study, we aimed to compare two different, but well-established, induction protocols. Although variations in timing and dosage conceivably affect the clinical outcome, this was not the subject of our studies because these variables are inherent to the protocols of choice.

The impact of donor pretransplant CMV serology clearly shows from these data. Patients receiving an organ from a seropositive donor had a much higher chance of developing CMV viremia than those receiving an organ from a seronegative donor. Remarkably, no direct influence of the patient's own pretransplant serology was noted. In the past, several studies have shown a higher risk for the development of CMV infection for patients who were de novo infected as a result of the transplantation (D+/R–).[17] In our patient group, only a trend in that direction was noted, conceivably due to the limited number of patients. Knowledge of pretransplant serology and subsequent adequate action could significantly decrease the risk of CMV infections. This is already being achieved by serological matching (positive organs to positive recipients and negative organs to negative recipients).[30] Unfortunately, donor shortage and limited ischemia times are restricting factors for the matching strategy. Another possible strategy would be to determine the immunosuppressive protocol individually for each patient based on CMV serology status.

Furthermore, this study stresses the need for careful monitoring of infections in patients treated with polyclonal ATG therapy. Antibody induction therapy for transplantation has become regular practice in recent years and in particular with SPK transplants.[6] We conclude that antibody induction therapy with daclizumab (anti-CD25) is safer than antibody induction therapy with $ATG_F$ regarding (re)activation of CMV in SPK transplant recipients because CMV viremia occurs later and the total viral load is lower. When patients receive ATG as rejection treatment, the effect on CMV viremia is even more pronounced. These findings are in accordance with findings in

kidney transplant recipients[14] and can be explained by the proposed mechanisms through which both agents affect the immune system. Daclizumab treatment is said to affect activated T-cells only, thus leaving memory T-cell function relatively intact, whereas ATG profoundly depletes all T-cells, conceivably leading to a longer-lasting influence than with daclizumab.[10,15] Nonetheless, in recent reports on nondepleting humanized anti-CD3 therapy in type 1 diabetes, it was suggested that modulation of T-cells can preserve β-cell function.[31,32] The latter, however, was not the subject of our present studies.

Our findings are of importance because it is known that the consequences of CMV disease for morbidity and transplant survival are strongest in the first months after transplant. Furthermore, CMV disease indirectly affects transplant survival.[33] In this study, however, none of the patients developed clinical CMV disease.

Tetramer staining for CMV-specific $CD8^+$ T-cells gives additional insight into the mechanisms underlying the noted differences. The occasional high amounts of CMV-specific cells corresponded with absence of CMV viremia both in the first 6 months and at the time of staining rather than reflecting an ongoing infection. All three patients not developing CMV viremia (and with high CMV-specific T-cell counts) were treated with daclizumab, and, interestingly, the one patient developing CMV viremia in the daclizumab group had a low CMV-specific T-cell count. These findings suggest that having high CMV-specific tetramer counts is actually beneficial, rather than a surrogate for viremia, because they are correlated with low viremic activity after transplantation. In this respect, tetramer staining might become an important tool to prospectively identify patients at high risk for CMV infection in the future.[29]

Although the number of patients limits definite conclusions, this study emphasizes the important role for cellular immunity in the prevention of CMV viremia after SPK transplantation and subsequently the impact antibody therapy has on the protective cytotoxic capacity of the immune system. With daclizumab induction therapy, this impact seems to be less vigorous than with $ATG_F$. Moreover, these results argue in favor of the use of daclizumab as induction therapy for pancreas and islet transplantation because of the reported potentiating effect of CMV on recurrent autoimmunity.[23]

CMV disease in islet transplantation has not yet been studied extensively, but because recurrent autoimmunity may be an important reason for the long-term loss of islet allografts,[34] such studies are warranted. This recommendation also applies to trials in which immunosuppressive agents are used to try to halt type 1 diabetes early in the course of the disease. For pancreas-kidney transplantation, it can be concluded that the differences between daclizumab and $ATG_F$ induction on CMV infection are relevant when choosing a certain induction or rejection therapy, considering that no difference in immunosuppressive potential has been noted.

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# 5

# Comparable incidence and severity of cytomegalovirus infections following T-cell depleted allogeneic stem cell transplantation preceded by reduced-intensity or myeloablative conditioning

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## ABSTRACT

Reports on infectious complications following reduced intensity conditioning (RIC) before allogeneic stem cell transplantation (allo-SCT) are equivocal. This prospective follow-up study compared the impact of cytomegalovirus (CMV) infections following RIC with fludarabine, ATG and busulphan or conventional myeloablative conditioning (MAC). Forty-eight RIC and 59 MAC patients were enrolled. The occurrence and severity of CMV infections within 100 days following allo-SCT were assessed, using plasma CMV DNA load kinetics. CMV DNAemia was observed in 21 RIC (60%) and in 19 MAC (44%) patients at risk for CMV. The mean CMV DNAemia free survival time was comparable following RIC and MAC: 70 days (95% (confidence interval) CI: 59–80 days) and 77 days (95% CI: 68–86 days), respectively ($P = 0.24$). Parameters indicative for the level of CMV reactivation, including the area under the curve of CMV DNA load over time as well as the onset, the peak values and duration of CMV infection episodes, the numbers and duration of CMV treatment episodes and recurrent infections, were not different in both groups. During follow-up, none of the patients developed CMV disease. RIC with fludarabine, ATG and busulphan demonstrated safety comparable to conventional MAC with regard to frequency and severity of CMV infections within 100 days following T-cell-depleted allo-SCT.

## Introduction

Allogeneic stem cell transplantation (allo-SCT) is increasingly used to treat haematological and non-haematological malignancies. Recently, conditioning regimens have been designed to exploit the graft-versus-tumour effects while reducing the intensity of the conditioning to minimize toxicities.[1–3] Results of studies demonstrate rapid allogeneic engraftment with minimal non-haematological toxicity and a significant antitumour effect. Despite the lower toxicity of the reduced intensity conditioning (RIC), acute and chronic graft-versus-host disease (GvHD) remains a significant cause of morbidity and mortality with a reported incidence of severe GvHD of 30–60%.[1]

Recently, an *in vitro* T-cell-depleted allo-SCT protocol following non-myeloablative conditioning with fludarabine, ATG, busulphan and Campath-in-the-bag was reported as a suitable platform for subsequent cellular immunotherapy.[4] It was shown that this protocol leads to durable donor engraftment, favourable response of the disease and minimal GvHD. Still, infections remain a prominent cause of transplant-related mortality following RIC.[5] As in myeloablative SCT recipients, risk factors for infections include the degree of myeloablation, GvHD and organ toxicities. However, as the timing and types of infections may differ,[5] information regarding infectious risks and outcomes are important to develop preventative strategies in allo-SCT recipients following RIC.

Cytomegalovirus (CMV) is one of the major causes of infectious complications following allo-SCT,[6] and the strategy of viral load guided pre-emptive antiviral therapy has been shown to reduce the risk of CMV disease.[7,8] Viral load kinetics has been reported to be predictive for the development of CMV disease, with the initial viral load and the initial rate of increase in viral load being independent risk factors[9] and as such this method can also be applied to assess the incidence and severity of CMV reactivation following transplantation. However, in this context, it should be considered that an episode of CMV viremia is characterized not only by its level (for example, peak load), but also by its duration;[9,10] as a consequence, long-term viremia at lower levels may have the same clinical significance as shorter episodes of high-level viremia. A novel approach has been devised previously to assess both quantities (level and duration of viremia) with a single parameter, which is based on calculating the area under the curve (AUC) of viral load over time.[10] Hence, the AUC approach is a universal means of assessing interrelated determinants, including peak viral load, initial viral load and rate of increase of viral load, parameters that have been described as independent risk factors for CMV disease.[9]

In the current prospective follow-up study, viral load kinetics were used to assess the incidence and the level of CMV reactivation in patients receiving *in vitro* T-cell de-

pleted allo-SCT following either non-myeloablative conditioning with fludarabine, ATG and busulphan or after myeloablative conditioning (MAC).

## PATIENTS AND METHODS

### *Patients*

Forty-eight consecutive patients who received allo-SCT following RIC between January 2001 and December 2004 were analysed for CMV reactivation. Patients eligible for allo-SCT were selected to receive RIC either when MAC was contraindicated (due to comorbidity or age) or in patients with an HLA identical donor who failed to respond on conventional treatment for lymphoma, multiple myeloma or chronic lymphocytic leukaemia, or in patients with solid tumours such as metastatic renal cell carcinoma or breast carcinoma. Forty-three RIC patients had haematological malignancies, four had renal cell carcinoma and one had breast carcinoma.

Additionally, 59 consecutive patients who received allo-SCT using conventional MAC regimens between August 2001 and December 2004 were included in this analysis. All conventional MAC patients had haematological malignancies.

General institutional policy with respect to patients' informed consent for inclusion into the study, approved by the ethical institutional board, was applied.

### *Transplantation*

T-cell-depleted transplantation was performed either according to a RIC protocol or a MAC regimen as described previously.[4,11] The RIC regimen consisted of fludarabine (30mg/m$^2$, intravenously, day –10 to –6), busulphan (3.2 mg/kg, intravenously, day –6 and –5) and ATG (10mg/kg/day intravenously, day –4 to –1), for both sibling and matched unrelated donor (MUD) grafts. The MAC regimen consisted of cyclophosphamide (60mg/kg/ day intravenously for 2 consecutive days) followed by single dose of total body irradiation (TBI, 9 Gy, day –1) in patients receiving sibling donor grafts. Recipients of MUD grafts, in the myeloablative regimen, received additional Campath-1G or -1 H (day –8 and –4) and cyclosporine (3 mg/kg intravenously, starting on day –1) and TBI (6 Gy, day –8 and –7). The stem cell product was infused on day 0. In all conditioning regimens, T-cell depletion of the graft was performed by *in vitro* incubation of the graft with Campath-1H (20mg).

Prophylaxis for GvHD was not administered. Assessment of acute and chronic GvHD was performed using the Glucksberg and Shulman criteria.[12,13] In the absence of GvHD or graft failure, patients received donor lymphocyte infusion (DLI) after RIC transplantation or in mixed chimerism or relapsed disease after MAC transplantation. DLI was never administered before 6 months following transplantation.

*CMV monitoring and pre-emptive treatment*

CMV DNA load was measured at least once a week for up to 100 days following transplantation. The real-time quantitative PCR for detection of CMV DNA in plasma was performed according to the method described previously.[14] The course of CMV DNA load in plasma was documented longitudinally for each patient during follow-up. Individual areas under the CMV DNAemia curve post transplant were calculated using the trapezoidal rule as described previously.[10,15]

CMV DNA load guided pre-emptive therapy was initiated according to a protocol based on criteria established in a previous study.[14] In short, CMV DNAemia episodes following transplantation treatment was initiated at a CMV DNA load level of $> 10^4$ copies/ml or at a level of $> 10^3$ copies/ml and more than one $_{10}$log increase as compared to previous measurement, without clinical symptoms of CMV disease.[14] Pre-emptive treatment consisted of 900 mg valganciclovir b.i.d. or intravenous 5 mg/kg ganciclovir b.i.d for an average duration of 2 weeks. CMV disease would be treated with intravenous 5 mg/kg ganciclovir b.i.d. Ganciclovir and valganciclovir dose were adjusted to renal function as described previously.[16] Serum creatinine levels and haematological parameters (that is, haemoglobin, leucocyte and thrombocyte counts) were monitored throughout treatment episodes.

*Study end points and statistical analysis*

The primary end point for this study was CMV infection, defined as 'detection of two consecutive positive CMV DNA loads (more than $\log_{10}$ 2.7 (= 500) copies/ml plasma) within 100 days following allo-SCT transplantation'. The level of $\log_{10}$ 2.7 copies/ml plasma as the lower detection limit of the 'real-time' quantitative CMV DNA PCR was established by earlier assessments with respect to the sensitivity and reproducibility of the assay.[14] The number of two consecutive detections of $\log_{10}$ 2.7 copies/ml as the definition of CMV infection was arbitrarily chosen to exclude incidental single positive findings.

Secondary end points were CMV DNA load-requiring antiviral treatment and recurrent infections. Definitions for CMV infection, CMV disease, CMV detection in blood and recurrent infection were adopted from internationally accepted criteria.[17]

All database entries and statistical analysis were performed with SPSS version 12.0.1.

Differences in age at transplantation, time to the first CMV DNA load detection, CMV DNA peak load, the duration of the CMV infection and the area under the DNAemia curve (AUC) were compared between groups using Mann–Whitney *U*-test and analyses of variance. For all measurements, the median and range or the 25th and 75th percentiles are presented. Differences in the distribution of CMV serostatus, underlying disease, GvHD and gender were tested using $\chi^2$ and Fisher exact-test statis-

tics. Kaplan–Meier analysis was performed to detect differences in CMV DNAemia free survival between groups during the first 100 days following transplantation and a Cox regression analysis was used to adjust for the possible confounders age and donor type. Relative risks for occurrence of CMV disease are presented with 95% confidence interval (95% CI).

## RESULTS

### *Patient characteristics*

A total of 107 patients were included in this study. The demographic and disease characteristics for patients in both conditioning groups are shown in Table 1. Distribution of the characteristics across the two groups was similar with respect to risk for CMV infections (based on donor and recipients CMV serostatus), underlying disease, GvHD and gender. However, significant differences were noted with regard to mean age at transplantation and donor type (Table 1). The mean age at transplantation was 54.5 years in the RIC patients compared with 44.0 years in the MAC patient group ($P < 0.01$). In the reduced intensity group, 31 patients were transplanted with haematopoietic stem cells from an HLA identical donor and 17 patients had mismatched unrelated donors (in the myeloablative group, 52 and 7, respectively) ($P = 0.004$). Further analyses were restricted to 78 patients who were considered to be at risk for CMV infection/reactivation (based on donor and receptor serostatus: 8 D+/R–, 40 D+/R+ and 30 D–/R+ ). This selection did not introduce significant change in the patients' characteristics.

### *Incidence of CMV DNAemia*

CMV DNAemia occurred in 40 patients within 100 days following transplantation, which accounts for 37% of all 107 patients and 51% of patients at risk for CMV ($n = 78$). The first signs of CMV DNAemia were observed at a median of 27 days (range: 8–81) and all first episodes occurred within 90days following transplantation. None of the patients developed CMV disease during the follow-up of 100 days following allo-SCT. Among the 78 patients at risk for CMV DNAemia, the highest incidence of CMV DNAemia was observed in R+ cases; 21 (53%) D+ R+ and 18 (60%) D– R+ compared with 1 (12.5%) D+ R– patients within 100 days following transplantation.

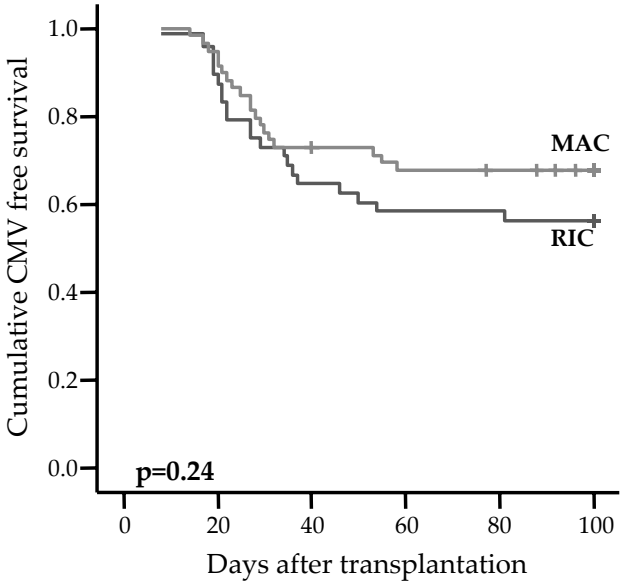
Within the group of patients at risk for CMV (35 and 43 receiving RIC and MAC, respectively), CMV DNAemia was observed in 21 (60%) patients receiving RIC and in 19 (44%) patients receiving MAC. Although the mean CMV DNAemia free survival time was shorter in RIC patients (70days, 95% CI: 59–80) then in MAC

**Table 1.** Relevant characteristics of the study population in both conditioning groups

| Characteristics | RIC ($n$ = 48) | MAC ($n$ = 59) | Statistical relevance |
|---|---|---|---|
| Age, median (range) | 54.5 (26–76) | 44.0(21–62) | $P < 0.01$ |
| Male gender (%) | 34 (71) | 43 (73) | NS |
| CMV Serostatus (%) | | | |
| D+R+ | 20 (42) | 20(34) | NS |
| D+R+ | 4 (8) | 4 (7) | |
| D–R+ | 11 (23) | 19 (32) | |
| D–R– | 13 (27) | 16 (27) | |
| Donor type (%) | | | |
| Related | 31 (65) | 52 (88) | $P < 0.01$ |
| Unrelated | 17 (35) | 7 (12) | |
| Underlying disease (%) | | | NS |
| Acute leukaemia | 10 (21) | 33 (56) | |
| CML | 5 (10) | 10 (17) | |
| CLL | 5 (10) | 1 (2) | |
| MM | 5 (10) | 7 (12) | |
| NHL | 10 (21) | 7 (12) | |
| Other | 13 (27) | 1 (2) | |
| T cell depletion (%) | 48 (100) | 59 (100) | NS |
| Acute GvHD (%) | | | $P = 0.07$ |
| Grade I/II | 4 (8) | 13 (22) | |
| Grade III/IV | 0 | 0 | |
| Chronic GvHD (%) | 0 | 5 (9) | $P = 0.07$ |
| GvHD treatment (%) (systemic) | 0 | 5 (8.5) | NS |

Abbreviations: CLL = chronic lymphocytic leukaemia; CML = chronic myelogenous leukaemia; CMV = cytomegalovirus; GvHD = graft-versus-host disease; MAC = myeloablative conditioning; MM = multiple myeloma; NHL = non-Hodgkin lymphoma; NS = not significant; RIC = reduced intensity conditioning.
No significant differences were present between the two groups, with the exception of age and donor type.
Systemic treatment of GvHD consisted of oral prednisone, intravenous methylprednisolone and/or oral cyclosporine.

**Figure 1.**




Pattern of CMV DNAemia free survival (Kaplan-Meier) during the first 100 days following allo-SCT in patients receiving reduced intensity (RIC) or myeloablative conditioning (MAC). CMV DNAemia was observed in 21 (60%) and 19 (44%) of the RIC and MAC patients, respectively. The mean CMV DNAemia free survival time in RIC patients was 70 days (95% CI: 59-80 days) compared to 77 days (95% CI: 68-86 days) in MAC patients ($P = 0.24$). Allo-SCT, allogeneic stem cell transpantation.

patients (77 days, 95% CI: 68–86), this difference was not statistically significant ($P = 0.24$; Figure 1). This was not different when a multivariate Cox regression analysis was performed to control for the possible confounders age, GvHD and donor type.

***Level of CMV reactivation following RIC and MAC***

To assess the level of CMV reactivation, the onset of the first positive CMV PCR following transplantation, the peak load of the first episodes following allo-SCT and the duration of the first CMV DNAemia episodes were evaluated in patients receiving RIC or MAC. There was no difference in the onset of the first CMV DNAemia episodes following RIC or MAC; median of 27 days (range: 8–81) and 27 days (range: 14–58) following transplant in recipients of RIC and MAC, respectively ($P = 0.36$). Also the median peak loads of the first CMV episodes following allo-SCT were comparable between the RIC and MAC patients: $\log_{10}$ 4.7 copies/ml (range: $\log_{10}$ 3.2–$\log_{10}$ 5.6) and $\log_{10}$ 4.7 copies/ml (range: $\log_{10}$ 3.5–$\log_{10}$ 6.2), respectively ($P = 0.74$). The median duration of the first CMV DNAemia episode was longer in

RIC patients (42 days (range: 7–73)) compared with MAC patients (28 days (range: 2–83)). However, this difference was not statistically different ($P = 0.72$). These findings did not change after correcting for the possible confounders age, GvHD and donor type.

Alternatively, the level of CMV reactivation was evaluated by calculating the time-adjusted area under the DNAemia curve (assessing both, the level and the duration of CMV DNAemia in mentioned time period). Although the median area under the DNAemia curve over time during the first 100 days following allo-SCT was higher in RIC patients (0.61 (range: 0.08–1.68)) compared with MAC patients (0.49 [range: 0.10–1.42]), this difference was not statistically significant ($P = 0.41$). These findings did not change after correcting for differences in age, GvHD and donor type between the two induction groups.

Another approach to assess the level of CMV reactivation in both groups was to evaluate CMV load episodes requiring antiviral treatment. (Val)ganciclovir was administered to an equal amount of RIC and MAC patients with CMV DNAemia: 17 out of 21 (81%) and 16 out of 19 (84%), respectively ($P = 0.45$). The total duration of CMV treatment was also comparable in both groups: median duration of 14 days (range: 7–53) in RIC patients and 14 days (range: 11–29) in MAC patients ($P = 0.279$). Multiple treatment episodes (with a maximum of 2) within 100 days following all-SCT were seen in 7 patients (41%) following RIC and in 4 patients (25%) following MAC. This difference did not reach statistical significance ($P = 0.458$), also not after correction for the possible confounders age, GvHD and donor type. Foscarnet was never administered within 100 days following allo-SCT. These findings also indicate equal levels of CMV reactivation in both conditioning groups.

***Recurrent CMV infections following RIC and MAC***

CMV infection recurred within 100 days following transplantation in 3 out of 21 patients (14.3%) receiving RIC and also in 3 out of 19 (15.8%) with MAC. None of the six patients with recurrent CMV infections developed more than 2 CMV DNAemia episodes within 100 days following transplant.

***Influence of donor and recipient CMV serostatus on CMV infections***

In a univariate analysis, serological status of recipient and donor appeared to be associated with the occurrence of CMV infection within 100 days following allo-SCT, when D–/R– patients were included ($P = 0.071$). Within patients at risk for CMV (donor and/or recipient seropositive), seropositive recipients were at higher risk for CMV infections compared with seronegative recipients, whereas no significant difference was observed between seropositive and seronegative donors (Table 2). Within the high-risk CMV patients (seropositive recipients), the relative risk for CMV reac-

**Table 2.** Univariate analysis of risk factors for CMV within 100 days following allo-SCT in patients at risk for CMV infection ($n = 78$)

| Risk factors | Crude RR (95% CI) | *P*-value |
|---|---|---|
| Conditioning (RIC vs MAC) | 1.50 (0.81–2.79) | 0.20 |
| Recipient age (years) (>45 vs <45) | 1.40 (0.69–2.78) | 0.35 |
| CMV serostatus | | |
| D– vs D+ | 1.40 (0.75–2.62) | 0.29 |
| R+ vs R– | 6.10 (0.84–45.50) | 0.07 |
| Donor type unrelated vs related | 1.40 (0.67–2.80) | 0.39 |

Abbreviations: allo-SCT = allogeneic stem cell transplantation; CI = confidence interval; CMV = cytomegalovirus; MAC = myeloablative conditioning; RIC = reduced intensity conditioning.

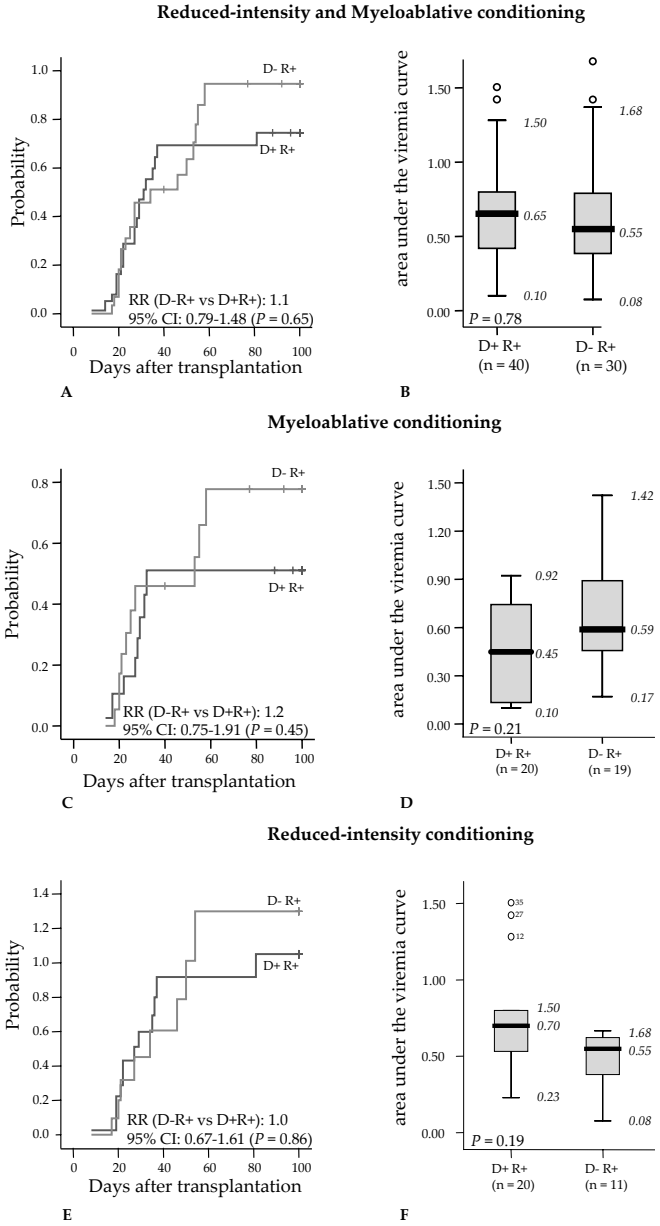
tivation was 1.1 for D–/R+ patients compared with D+/R+ patients; this difference was not statistically significant ($P = 0.65$; Figure 2a). Also, the level of CMV reactivation was comparable (Figure 2b). These findings did not change after stratification for conditioning therapy (Figure 2c–f). Donor type and recipients' age did not have significant impact on the occurrence of CMV within 100 days following transplantation.

## Discussion

It has been established that allogeneic transplantation with RIC can be successfully performed in individuals with a wide variety of different diseases and with reduced risk of transplant-related mortality.[5,18] Previously, an *in vitro* T-cell depleted allo-SCT protocol following RIC with fludarabine, ATG, busulphan and Campath-in-the-bag was reported to lead to durable donor engraftment and favourable response of the disease with no GvHD.[4] The current analysis demonstrates that there was no significant difference in incidence and severity of CMV infections within 100 days following allo-SCT preceded by RIC compared to a conventional MAC. A limitation in the current study concerns its non-randomized nature. Patients were allocated to the RIC or MAC group on clinical grounds, rather then by random selection. Therefore the possibility of confounding by indication could not be entirely excluded.

Although there was a trend towards a shorter CMV DNAemia free survival following RIC, this difference was not statistically significant. Furthermore, various

**Figure 2.**




The probability (left panels) and severity (right panels) of CMV infection in high-risk patients (that is, CMV seropositive recipients) within 100 days following allo-SCT, according to CMV serostatus of the donor and conditioning regimens. The probability and severity of CMV infection were comparable between seropositive and seronegative donors (**a** and **b**). This did not change after stratification for induction therapy (**c** and **d**, and **e** and **f**). The box plots display the median (horizontal bars), the 25th and 75th percentiles (boxes), and the smallest and largest values (whiskers). Open circles depict the outliers (values between 1.5 and 3 box lengths from the upper or lower edge of the box). Allo-SCT, allogeneic stem cell transplantation; CMV, cytomegalovirus.

parameters related to the severity of CMV infections (that is, the onset of CMV DNA detection in plasma following allo-SCT, the duration of a CMV infection, the peak load, the area under the DNAemia curve, the number and duration of pre-emptive CMV treatment episodes as well as the number of recurrent infections within 100 days following allo-SCT) were not different after RIC and MAC, supporting the conclusion of comparable severity of CMV infections in both groups.
In this study, both patient groups received T-cell depleted grafts. By itself, T-cell depletion of the graft is associated with an increased risk for CMV infections,[19] which seems to be reflected by the high overall incidence of CMV infections (51%) within 100 days following allo-SCT in this study.
Previous studies have reported variable outcomes with regard to CMV infections following RIC.[20–22] Such differences can be explained by the variable immune suppressive potentials of the RIC regimens investigated at different centres, presumably reflecting a balance between more residual immunity in the host and a higher risk for opportunistic infections either due to more persisting intracellular pathogens or an increased incidence of GvHD following RIC. A high rate of CMV infections were observed in alemtuzumab-based RIC regimen.[21] Recent reports with respect to CMV infections following fludarabine, busulphan and ATG-based RIC regimens compared to MAC have either reported no influence of conditioning protocols[23] or a significant increase of CMV infection following RIC.[22] However, limitations in these studies included analysis of CMV infections mainly using CMV antigenemia detection rather then the more sensitive and accurate CMV DNA PCR in plasma.[14] Another difference is the use of GvHD prophylaxis in these previous studies, which may be of major importance with respect to CMV infections.
The association of CMV positive serostatus of the recipients (R+) and an increased risk for CMV infections following allo-SCT is well established.[24] Recently, it has been demonstrated that a CMV seronegative donor for a seropositive patient (D–R+) in particular was found to be a risk factor for CMV infections following allo-SCT in an study including both reduced intensity as well as MAC regimens.[25] Although the previous report did not show a difference between conditioning regimens,[25] we observed increased frequency and severity of CMV infections in seropositive patients receiving a graft from seronegative donors (D–R+) compared to seropositive donor and recipient combination (D+ R+) only following MAC, presumably reflecting residual immunity following RIC. However, this difference was not statistically significant and the clinical relevance of this observation is questionable.
Another relevant conclusion resulting from the current study was that irrespective of the conditioning regimen, monitoring of CMV DNA in plasma and pre-

emptive therapy proved highly effective in preventing CMV disease following allo-SCT, as CMV disease was not seen in any patient.
In conclusion, RIC with busulphan, fludarabine and ATG demonstrated comparable safety to conventional MAC with regard to the frequency and severity of CMV infections within 100 days following T-cell-depleted allo-SCT. Moreover, with RIC, pre-emptive CMV treatment guided by CMV DNA load monitoring in plasma is highly effective in preventing CMV disease following T-celldepleted allo-SCT.

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# 6

# Management of Epstein-Barr virus (EBV) reactivation after allogeneic stem cell transplantation by simultaneous analysis of Epstein-Barr virus DNA Load and Epstein-Barr virus Specific T Cell reconstitution

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## ABSTRACT

***Background:*** Epstein-Barr virus (EBV) reactivation is a frequent event after allogeneic stem cell transplantation and may progress to life-threatening lymphoproliferative disease (EBV-LPD) in the absence of adequate EBV-specific T cell immunity. Quantification of EBV DNA load in asymptomatic individuals who are at risk is a useful (although not entirely predictive) indicator of progression to EBV-LPD and guide for preemptive treatment with CD20 antibodies.

***Methods:*** With the aim of improving the identification of patients at risk, we retrospectively analyzed, within a cohort of 25 consecutive allogeneic stem cell transplant recipients at risk for EBV-LPD, the pattern of T cell reconstitution during EBV reactivation in all preemptively treated patients (8 patients).

***Results:*** In 6 of 8 cases, a significant T cell reconstitution (i.e., a $CD3^+$ T cell count of 1300 cells/μL) was documented during EBV reactivation, which included an expansion of EBV-specific memory T cells, as shown by human leukocyte antigen class I tetramer analysis. Additional evidence for the antiviral potential of this T cell reconstitution was obtained prospectively from a cohort of 14 consecutive allogeneic stem cell transplant recipients at risk for EBV-LPD. EBV reactivation occurred in 3 patients. Preemptive treatment was successfully withheld for 2 of these patients in light of concurrent (EBV-specific) T cell recovery.

***Conclusion:*** We conclude that analysis of the level of (EBV-specific) T cell reconstitution during EBV reactivation is an important second parameter, in addition to quantification of EBV DNA load, that will be instrumental in a more accurate definition of patients at risk for EBV-LPD who, given their immunoincompetence, will be most certainly dependent on preemptive interventions.

Epstein-Barr virus (EBV) reactivation is a frequent event following receipt of an allogeneic stem cell transplant (alloSCT) that may progress to life-threatening EBV-related lymphoproliferative disease (EBV-LPD). Risk factors for EBV reactivation and subsequent EBV-LPD include the use of unrelated or mismatched family donors, T cell depletion *in vitro*, antithymocyte globulin, and nonmyeloablative stem cell transplants.[1] Clinical symptoms are frequently lacking in the early stages of EBV reactivation and are often only recognized in the later stages if they coincide with progressive EBV-LPD. The introduction of real-time PCR has provided a powerful tool to monitor EBV reactivation in alloSCT recipients who are still asymptomatic and to predict an increased risk of developing EBV-LPD.[2,3] Both prophylactic and therapeutic strategies have been reported to either prevent or treat EBV-LPD, including B cell depletion of the graft[4] and restoration of T cellular immunity by means of donor lymphocyte infusion or administration of EBV-specific cytotoxic T lymphocytes.[5,6] Recently, evidence has been provided that EBV DNA load–guided preemptive treatment with B cell–depleting CD20 monoclonal antibodies (rituximab) is effective in preventing EBV-LPD in alloSCT recipients at high risk.[2]

Although effective in preventing EBV-LPD, preemptive treatment on the basis of EBV DNA load as a single parameter definitely results in unnecessary treatment in a significant number of patients.[2] Because EBV-LPD only occurs in the absence of adequate T cell immunity, we retrospectively analyzed (EBVspecific) T cell reconstitution during EBV reactivation in pediatric alloSCT recipients who had received preemptive treatment with CD20 antibodies (rituximab). On the basis of these results and supported by the outcome in a prospectively monitored cohort, we propose that simultaneous and analysis of both EBV DNA load and T cell recovery will improve the identification of patients at high risk for EBV-LPD.

## Patients and methods

### *Patients and transplantation*

The retrospective study cohort consisted of 50 consecutive children with a hematological malignancy or genetic disease who received an alloSCT in the Leiden University Medical Center (Leiden, The Netherlands) from January 2002 through September 2003. Pretreatment of the patients was according to the disease-specific protocols of the relevant working parties of the European Group for Blood and Marrow Transplantation. Twenty-five children were regarded as at risk for the development of EBV-LPD, because they received a graft from an EBV-seropositive, unrelated ($n$ = 19) or mismatched family ($n$ = 6) donor and antithymocyte globulin during the conditioning (Imtix [Sangstat]; total dose,10 mg/kg). In 11 of 25 cases, *in vitro*

manipulation of the graft was performed by CD34 selection (CliniMACS; Miltenyi) or T/B cell depletion by immunorosetting. The prospective cohort consisted of 52 consecutive pediatric alloSCT recipients who received their transplants in the Leiden University Medical Center from October 2003 through May 2005. Of these patients, 14 were regarded as at risk for EBV-LPD, because they received a graft from an EBV-seropositive, unrelated ($n$ = 12) or mismatched family ($n$ = 2) donor and antithymocyte globulin during the conditioning. In 3 of 14 cases, *in vitro* manipulation of the graft was performed by CD34 selection (CliniMACS) or T/B cell depletion by immunorosetting. Graft-versus-host disease prophylaxis consisted of cyclosporin A and a short course of methotrexate for T cell–repleted alloSCT recipients. No antiviral prophylaxis was given, and all patients received intravenous immunoglobulin supplementation during 3–12 months after receipt of transplant, depending on the level of immune recovery.

***Monitoring and treatment of EBV reactivation***

The real-time quantitative PCR for detection of EBV DNA in plasma was performed as described elsewhere[7] and was performed on an Icycler IQ Multi-Color Real Time PCR Detection system (BioRad). EBV DNA load was prospectively monitored weekly during the first 12 weeks after stem cell transplantation and subsequently every other week until 5–6 months after stem cell transplantation or longer, until EBV DNA became undetectable. EBV reactivation was defined as occurring in patients with a PCR positive for EBV DNA in plasma. Patients with an EBV DNA load >1000 copies/mL at 2 consecutive time points were considered to be at high risk to proceed to EBV-LPD[2,3] and received a single infusion of rituximab (375 mg/m$^2$). A second infusion was administered when the reduction in viral load was <1 order of magnitude (i.e., <1log$_{10}$ copies/mL) within the first week. EBV DNA load was monitored twice a week until at least 2 negative test results were obtained.

***Immune reconstitution and tetramer analysis***

Analysis of immune reconstitution, including tetramer staining, was performed by multicolor flow cytometry, as described elsewhere.[8,9] The staining of lymphocytes was performed by incubating the cells with a pre-titrated concentration of tetramer at 37°C for 15 min. The cells were then stained for surface markers by incubation on ice for 30 min with allophycocyaninconjugated anti-human CD8 antibody (Immunotech) and fluorescein isothiocyanate–conjugated anti-human CD4 antibody (BD-Biosciences). For further phenotypic analysis, tetramer-stained cells were incubated with a fluorescein isothiocyanate–labeled CD45RO (DAKO) or an unlabeled anti-human CCR7 monoclonal antibody (BD Pharmingen) followed by detection with a goat anti-mouse IgM fluorescein isothiocyanate–labeled secondary antibody (Southern

**Table 1.** Details of major histocompatibility complex class I– restricted cytotoxic T lymphocyte epitopes from Epstein-Barr virus (EBV) lytic cycle and latent proteins.

| EBV protein | Epitope sequence | HLA restriction | Reference(s) |
|---|---|---|---|
| Lytic epitopes | | | |
| BMLF1 280–288 | GLCTLVAML | A2 | [8, 9] |
| BRLF1 109–117 | YVLDHLIVV | A2 | [10] |
| BMRF1 208–216 | TLDYKPLSV | A2 | [11] |
| BZLF1 190–197 | RAKFKQLL | B8 | [12] |
| Latent epitopes | | | |
| EBNA3B 399–408 | AVFDRKSDAK | A11 | [13] |
| EBNA3A 379–387 | RPPIFIRRL | B7 | [14] |
| EBNA3A 325–333 | FLRGRAYGL | B8 | [15] |
| EBNA3A 603–611 | RLRAEAQVK | A3 | [16] |

Biotechnology Associates). Flow cytometry was performed on a Facs Calibur (Becton Dickinson Immunocytometry Systems) using CellQuest software (Becton Dickinson). The EBV tetramers used in this study are shown in table 1 and were generated by the Institute for Cancer Studies (Birmingham, United Kingdom) and Sanguin Research (Amsterdam, The Netherlands). Written informed consent was obtained in all cases for immunological monitoring and cryopreservation of blood mononuclear cells. Approval was obtained from the local institutional review board.

## Results and discussion

***General and EBV-specific T cell immune reconstitution during EBV reactivation***

In a cohort of 50 consecutive alloSCT recipients, 25 of these patients were regarded as at risk for EBV reactivation according to the criteria defined in Patients and Methods. EBV reactivation was documented in 8 (32%) of these patients (patients 1–8; table 2). Median time to the first EBV reactivation was 59 days (range, 26–111 days) after stem cell transplantation, and preemptive therapy with rituximab was started at a median time of 67 days (range, 28–121 days) after receipt of an alloSCT (table 2). In 2 patients (patients 3 and 5) a second infusion was administered because of a lack of response after the first infusion. Retrospective analysis of general and EBV-specific T cell reconstitution was performed. In 2 of 8 cases (patients 1 and 2), clearance of EBV

**Table 2.** Characteristics of patients who underwent allogeneic stem cell transplantation (alloSCT) with Epstein-Barr virus (EBV) reactivation.

| Patient | Diagnosis | Donor | EBV serostatus (donor/ recipient) | T cell depletion ex vivo | ATG | Start of EBV reactivation,[a] days after alloSCT | EBV DNA load at rituximab initiation, $\log_{10}$ copies/mL | Clinical symptoms during EBV reactivation | Rituximab administration, days after alloSCT | Response to rituximab[b] | Cyclosporin A discontinued[c] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | JMML | MUD | +/– | Yes[d] | + | 111 | 4.6 | … | 121 | + | Yes |
| 2 | MDS | MUD | +/+ | No | + | 26 | 3.6 | … | 28 | + | No |
| 3 | ALL | MUD | +/+ | No | + | 44 | 4.5 | Fever | 51/59 | – | Yes |
| 4 | WAS | MUD | +/+ | No | + | 91 | 3.9 | … | 100 | + | Yes |
| 5 | HLH | MUD | +/– | Yes[d] | + | 46 | 5.7 | Fever/ lymphadenopathy (single node) | 52/58 | – | Yes |
| 6 | ALL | MUD | +/+ | No | + | 43 | 4.3 | … | 52 | + | Yes |
| 7 | FA | MMFD | +/+ | Yes[d] | + | 91 | < 2.7[e] | … | 99 | NA[f] | No |
| 8 | SAA | MUD | +/+ | No | + | 26 | 4.5 | … | 34 | + | No |
| 9 | ALL | MUD | +/+ | No | + | 69 | 4.7[g] | … | … | NA | Yes |
| 10 | MDS | MUD | +/+ | No | + | 25 | 4.2[g] | … | … | NA | No |
| 11 | CML | MUD | +/– | No | + | 106 | 4.1 | … | 111 | + | No |

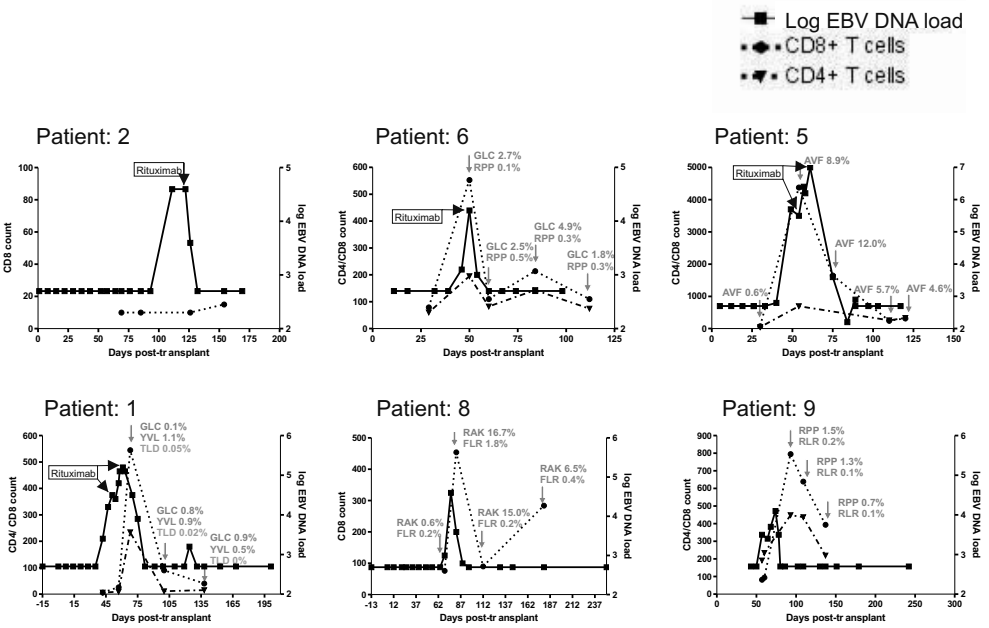
**NOTE.** ALL, acute lymphoblastic leukaemia; ATG, antithymocyte globulin; CML, chronic myeloid leukaemia; FA, Fanconi anemia; HLH, hemophagocytic lymphohistiocytosis; JMML, juvenile myeloid-monocytic leukaemia; MDS, myelodysplastic syndrome; MMFD, mismatched family donor; MUD, matched unrelated donor; NA, not applicable; SAA, severe aplastic anemia; WAS, Wiskott-Aldrich syndrome.
a Initial EBV DNA level >1000 copies/mL.
b Defined as a decrease in viral load of $\geq 1 \log_{10}$ copies/mL within the first week.
c Discontinued before or during EBV reactivation.
d $CD34^+$ selection by CliniMACS plus T cell add-back ($CD3^+$ cell count, $0.5–1.0 \times 10^5$ cells/kg).
e Two preceding values of 4.5 and 5.2. $\log_{10}$ copies/mL.
f Data NA because of reduction in viral load prior to treatment.
g No rituximab treatment (despite positive EBV DNA load) because of concurrent T cell recovery.

DNA was seen in the absence of significant T cell recovery (i.e., $CD3^+$ T cell count <20 cells/μL; figure 1 and data not shown). In the remaining 6 patients, an increase in $CD3^+$ T cell count to at least 300 cells/μL was documented during the initial 2–3 weeks of EBV reactivation, which was associated with a sustained decrease in EBV DNA load (figure 1 and data not shown). This rapid T cell reconstitution included both $CD4^+$ cells and $CD8^+$ T cells, with a predominance of $CD8^+$ T cells. The magnitude of $CD8^+$ T cell reconstitution varied between 7-fold and 50fold during the period of EBV reactivation (figure 1 and data not shown). The large majority (>95%) of the $CD8^+$ T cells were $CD45RO^+/CCR7^-$ compatible, with an effector memory phenotype (data not shown). A significant and rapid increase in EBV-specific $CD8^+$ T cell count was demonstrated during EBV reactivation in all patients in whom the HLA class I genotype and the availability of cryopreserved lymphocytes allowed us to perform an analysis with HLA class I tetramers (4 of 6 patients; figure 1 and data not shown). The peak value of the individual peptide-specific $CD8^+$ T cell populations represented 0.1%–12% of the total $CD8^+$ T cell pool for these patients and included responses to both latent and lytic EBV epitopes. Apart from biological variation, the variable number of available HLA class I tetramers per individual HLA genotype (0–3) most probably explains the quantitative differences between patients in our study. Because of these technical limitations, it remains difficult to determine the relative contribution of T cell responses against single lytic and latent epitopes in individual patients. Taking into consideration that the tetramer-based results are an underestimation of the overall EBV-specific $CD8^+$ T cell response, the cumulative EBV-specific $CD8^+$ T cell repertoire expanded from <0.5 cells/μL to 4–450 cells/μL during the EBV reactivation. The significant interindividual and peptide-dependent differences are similar to what has been reported for patients with infectious mononucleosis.[11,17]
The kinetics of these virus-induced memory T cell responses strongly suggest that these T cells might have been able to control EBV reactivation and prevent progression to EBV-LPD without rituximab. This is consistent with the observation by van Esser *et al.*[18] that, within a similar population of adult alloSCT recipients at risk and without preemptive treatment, only a minority of the patients with EBV reactivation finally progress to EBV-LPD. The observation regarding patient 7, for whom EBV DNA load had decreased prior to infusion of rituximab but in the presence of T cell reconstitution, further supports the functional relevance of T cell recovery.

***Simultaneous analysis of viral load and T cell reconstitution***

Further evidence that assessment of the level of T cell reconstitution at the time of EBV reactivation is of relevance for clinical decision making was obtained prospectively. In a cohort of 52 consecutive alloSCT recipients, 14 of these patients were regarded as at risk for EBV-LPD, according to the criteria defined in Patients and Meth-

**Figure 1.**




Epstein-Barr virus (EBV)–specific T cell reconstitution during EBV reactivation for patients who received an allogeneic stem cell transplant (alloSCT). The lower limit for reliable quantification of the EBV DNA load by real-time PCR is 2.7 log10 copies/mL. PBMCs were stained with phycoerythrin-conjugated HLA-A2.1 tetramers containing either the GLC, YVL, or TLD peptide, HLA-B8 tetramers containing the RAK or FLR peptide, HLA-A11 tetramer containing the AVF peptide, HLA-B7 tetramer containing the RPP peptide, or HLA-A3 tetramer containing the RLR peptide. The cells were also stained with anti-CD8 and anti-CD4 antibodies. The numbers of EBV tetramer–positive T cells are shown as percentage of the total $CD8^+$ T cells. Patients 1, 3, 5, and 6 received preemptive treatment with rituximab (see Patients and Methods), whereas treatment was withheld for the prospectively monitored patients 9 and 10 because of concurrent (EBV-specific) T cell reconstitution. AVF, epitope sequence AVFDRKSDAK; FLR, epitope sequence FLRGRAYGL; GLC, epitope sequence GLCTLVAML; RAK, epitope sequence RAKFKQLL; RLR, epitope sequence RLRAEAQVK; RPP, epitope sequence RPPIFIRRL; TLD, epitope sequence TLDYKPLSV; YVL, epitope sequence YVLDHLIVV.

ods. EBV reactivation was documented for 3 (21%) of these patients (patients 9–11; table 2). On the basis of a significant and rapid (EBV-specific) T cell expansion during the initial phase of EBV reactivation, rituximab was withheld for 2 of these 3 patients (patients 9–10). EBV reactivation was controlled and cleared in a fashion similar to that observed in the aforementioned patients who received additional treatment with rituximab (figure 1). Because T cell recovery was absent ($CD3^+$ T cell count <100 cells/μL during the period of EBV reactivation) for the third patient (patient 11), rituximab was administered to this patient as described for the first 8 patients and resulted in clearance of the EBV reactivation. Altogether, our results strongly suggest

that the need for preemptive intervention seems to be limited to those patients who lack an expansion of (EBV-specific) memory T cells during the initial phase of EBV reactivation. In our experience, although this threshold is arbitrary, an increase in $CD3^+$ T cell count to at least 300 cells/μL during the initial phase of EBV reactivation appears to be a practically useful threshold for determining the need for preemptive intervention. In all patients except for patients 4 and 8, $CD3^+$ T cell reconstitution was found to be exclusively of donor origin during the period of EBV reactivation; for patients 4 and 8, $CD3^+$ T cells were of mixed and autologous origin, respectively (data not shown). Notably, and in contrast to the other patients, the latter 2 patients received transplants following a nonmyeloablative conditioning regimen.

Abrogation of cyclosporin A treatment has been suggested as an effective preemptive intervention for alloSCT recipients to control EBV reactivation.[19] Unfortunately, no information was provided in this study on the characteristics of (EBVspecific) T cell reconstitution before and after this intervention.

In some of our patients, T cell reconstitution was documented while receiving continued cyclosporin A treatment, whereas in other patients, cyclosporin A was tapered. Therefore, in the presence of significant memory T cell reconstitution, the additional requirement of abrogating cyclosporin A to control EBV reactivation may be variable and needs to be weighed against the risk of eliciting graft-versus-host disease. The additional contribution of acyclovir to the control of EBV reactivation is probably limited and is difficult to assess, because it will not be administered as a single therapeutic intervention. Recently, Wagner *et al.*[20] provided evidence that prompt treatment on the basis of the combined analysis of viral load and clinical appearance of symptoms may be preferred to preemptive treatment on the basis of viral load only. However, although EBV-LPD was successfully controlled in all cases, 3 of 8 patients required treatment with EBV cytotoxic T lymphocytes. Therefore, in a significant amount of patients, the general feasibility of this approach of management of EBV reactivation may rely on the local availability of clinical-grade EBV cytotoxic T lymphocytess.

The role of virus-specific T cell responses in the control of cytomegalovirus infection in alloSCT recipients is rather similar to what we have reported for EBV infection, and assessment of these responses can be used in clinical management of cytomegalovirus infection. Cytomegalovirus-specific T cells rapidly expand during viremia and are able to prevent the recurrence or persistence of infection.[21] Similar to what we have demonstrated during EBV infection, the peak of cytomegalovirus-specific $CD8^+$ T cell responses parallels the level of viraemia. The absence of such response defines patients at risk, who may benefit from adoptive transfer of virus-specific T cells.[22]

Previous studies have provided evidence that the risk for EBV-LPD is correlated with both the general and EBV-specific immune reconstitution after stem cell transplanta-

tion.[23–26] Our data indicate that frequent analysis of T cell immune reconstitution at the time of EBV reactivation is, in combination with EBV DNA load, an important second parameter for recognition of alloSCT recipients who are at high risk for EBV-LPD and will thus further improve the identification of patients who will benefit most from preemptive interventions.

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# 7

# Role of Epstein-Barr virus DNA measurement in plasma in the clinical management of nasopharyngeal carcinoma in a low risk area

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## ABSTRACT

***Objective***: To evaluate the role of quantitative measurement of Epstein-Barr virus (EBV) DNA in the clinical management of nasopharyngeal carcinoma (NPC) in a low tumour risk area (western Europe).

***Methods:*** 22 consecutive Dutch NPC patients (11 europid) were studied. EBV DNA load in pre-treatment and post-treatment plasma samples was determined. Three patients were also sampled at frequent intervals during treatment. RNA in situ hybridisation for the detection of EBV encoded RNAs (EBERs) was carried out on tumour biopsies of all cases.

***Results***: All patients with EBER positive NPC (20/22) showed a positive EBV DNA load in plasma at the time of diagnosis (median EBV DNA level, 4.1 $\log_{10}$ copies/ml). Patients with EBER negative NPC had no detectable EBV DNA in plasma. After treatment, complete remission was achieved in all cases and concurrently EBV DNA in plasma became undetectable in all patients. In the three longitudinally evaluated cases, EBV DNA load gradually declined towards undetectable levels within three weeks after start of treatment. Two patients developed a distant metastasis with concomitant increases in EBV viral load. In addition, one EBER positive patient developed an EBER negative metastasis in the neck during follow up and in this case EBV DNA load remained undetectable at the time of recurrence.

***Conclusions***: Plasma EBV DNA load measurement appears to be useful in a low tumour risk area. However, development of local recurrences may not always coincide with raised levels of EBV DNA.

Nasopharyngeal carcinoma (NPC) is a disease with a remarkable geographical and racial distribution. It is highly prevalent in southern China, South-East Asia, and North Africa, but occurs relatively infrequently in Western areas, where it comprises merely 0.1% of all malignancies.[1–4] NPC have been classified by the World Health Organisation (WHO) into three histological types: squamous cell carcinoma (type 1), non-keratinising carcinoma (type 2), and undifferentiated carcinoma (type 3). The prevalence of these three types of tumour is different in low incidence and high tumour risk areas.[5] Furthermore, type 2 and 3 NPC are clearly associated with Epstein-Barr virus EBV), whereas virus involvement in type 1 NPC is more controversial, especially in low tumour risk areas.[6] Hence, both the prevalence and the association with EBV of the three histological types of nasopharyngeal carcinoma show different patterns in low tumour risk and high tumour risk populations.

Plasma or serum EBV DNA load is a recently developed marker for NPC in high tumour risk (Asian) populations. It was shown that the quantification of EBV DNA in plasma or serum, using real time quantitative polymerase chain reaction (PCR) technology, represents a promising tumour marker in patients from these areas.[7–12] With respect to patients in these high tumour risk regions, plasma EBV DNA quantification can be a valuable tool in monitoring response to current treatments as well as in assessing the efficacy of new forms of therapy involving neo-adjuvant chemotherapy[13] or salvage surgical treatment.[14]

However, little is known about the clinical use of EBV DNA load in plasma or serum from NPC patients in Western countries, where the tumour has a low incidence. The clinical relevance of this tumour marker in these low incidence areas may be different from that in regions where this tumour is highly prevalent, as the association of NPC with EBV also differs between these areas.

The present study was conducted in a non-endemic NPC setting, to assess the clinical use of EBV DNA load measurements in plasma from NPC patients referred to a western European (Dutch) regional centre for head and neck cancer. The EBV status of the NPC from these patients was assessed using RNA in situ hybridisation (ISH) for the detection of EBV encoded RNAs (EBERs) in the tumour specimens.

## METHODS

### *Patients*

Patients with histologically confirmed NPC referred to the department of otorhinolaryngology of the Leiden University Medical Centre (Netherlands) between January 1998 and January 2005, from whom plasma samples at time of diagnosis were available, were included after informed consent. The patients' characteristics

with respect to age, sex, and ethnic origin were recorded. All patients were investigated uniformly according to a routine diagnostic work up, comprising detailed clinical examination of the head and neck, nasopharyngoscopy, histological or cytological examination of tumour tissue, and radiological imaging techniques (computed tomography, magnetic resonance imaging, and ultrasonography). The tumours were subsequently staged according to the UICC staging criteria. Treatment consisted of radiotherapy or a combination of radiotherapy and neoadjuvant chemotherapy according to the stage. Additional blood samples were obtained during routine follow up after the completion of therapy. From three patients among the most recently referred cases, blood samples were also obtained longitudinally during treatment.

***Extraction of EBV DNA and real time quantitative EBV DNA PCR***

EDTA plasma samples were collected and stored at –80°C until further processing. Nucleic acids were extracted from 0.2 ml of the plasma or serum samples with the automated purification procedure of the MagnaPure LC (Roche Molecular Systems, Almere, Netherlands), using the Total Nucleic Acid isolation kit. Purified DNA was eluted in a final volume of 100 µl buffer. Real time quantitative PCR was carried out on these samples using the same primers and probes as previously described,[15] but amplification was done in Qiagen Hostar Master mix (Qiagen, Hilden, Germany). The cycling protocol consisted of an activation step of 15 minutes at 95°C, and subsequently 50 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C for PCR amplification. The real time PCR was carried out on an iCycler iQ Multi-Colour Real Time PCR Detection System (BioRad, Veenendaal, Netherlands). Quantification has been carried out using a purified EBV standard (Advanced Biotechnologies, Columbia, Maryland, USA). To monitor the efficiency of the DNA extraction and PCR inhibition, clinical samples were spiked with a fixed amount of phocine herpes virus (PhHV) as an internal control. This target was co-amplified in the reaction and detected by a Cy5 labelled probe as described before.[16]

***EBER in situ hybridisation***

Histological specimens were fixed in 40 g/l formaldehyde formalin and routinely processed for paraffin embedment. EBV was identified by the expression of EBV encoded small RNA-1. Histological sections (4 mm) were subjected to ISH for EBV encoded small RNA-1. ISH for EBER was carried out using the Dako EBER probe and RNA ISH detection kit (Dako, Glostrup, Denmark). The slides were counterstained for 20 seconds in nuclear fast red, mounted, and examined using a standard microscope.

## RESULTS

***Patient characteristics and clinical outcome***

We studied 22 patients with histologically confirmed NPC presenting at the Leiden University Medical Centre. Table 1 lists the characteristics of the patients and tumours. Twenty one patients had histologically proven undifferentiated nasopharyngeal carcinomas (WHO type 3) and one had squamous cell carcinoma (WHO type 1). Two non-NPC patients (with oropharyngeal carcinoma) served as negative controls. The median age at diagnosis was 45 years (range 34 to 67). Eleven patients were europid Dutch. Of the remaining 11, five were of Southeast Asian origin (Indonesia) and six of North African origin; three were first generation immigrants (born and raised in the Netherlands), while eight were immigrants. Seven of the latter had lived in the Netherlands for more than 15 years, and one for eight years. Both non-NPC patients were europid Dutch.

On completion of treatment, all patients had complete clinical responses as defined by clinical examination. However, after a median follow up of 1.5 years (range 0.3 to 5.2), one NPC patient died as a result of recurrence of his disease (patient 2: death after 10.6 months) and four other NPC patients presented with metastases: three distant (patients 11, 14, and 16) and one regional (patient 13).

***Plasma EBV DNA load by tumour cell EBER status before and after treatment***

EBER-ISH of nasopharyngeal tumour biopsy was positive in 20 of the 22 NPC patients (table 1), including the squamous cell carcinoma (WHO type 1). Both EBER negative NPCs were of the undifferentiated type (WHO type 3) and occurred in europid Dutch patients.

In all patients with EBER positive NPC, EBV DNA was detected in plasma at the time of diagnosis, with a median EBV DNA load of 4.1 $\log_{10}$ (range 2.4 $\log_{10}$ to 5.1 $\log_{10}$), whereas in both patients with EBER negative NPC, EBV DNA was undetectable at presentation (table 1). After completion of treatment, EBV DNA load in plasma became undetectable in all EBER positive NPC patients (table 1). No EBV DNA could be detected in either of the controls (table 1).

***Longitudinal analysis of plasma EBV DNA load after treatment, and correlation with tumour recurrence***

After a median follow up of 1.5 years (range 0.3 to 5.2), five of the 22 NPC patients developed recurrence of their disease (patients 2, 11, 13, 14, and 16). Patient 2 died 10.6 months after initiation of his treatment. This case concerned an EBER negative tumour, and therefore additional plasma sampling during follow up was considered irrelevant for the purpose of this study. After completion of treatment, patient

**Table 1.** Characteristics of the patients and the tumours

| Patient | Age (y) | Sex | Ethnicity | Histo-logical type | EBER-ISH | Tumour classification | | | | EBV DNA load ($log_{10}$ copies/ml) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | T | N | M | Stage | Pretreat-ment | Post-treat-ment |
| 1 | 38 | F | Europid | UC | Neg | 4 | 3 | 0 | 4b | UD | UD |
| 2 | 59 | M | Europid | UC | Neg | 4 | 1 | 0 | 4a | UD | UD |
| 3 | 49 | M | Europid | UC | Pos | 3 | 1 | 0 | 3 | 4.8 | UD |
| 4 | 52 | M | Europid | UC | Pos | 1 | 1 | 0 | 2b | 4.1 | UD |
| 5 | 34 | M | Indonesian | UC | Pos | 3 | 0 | 0 | 3 | 4.2 | UD |
| 6 | 47 | M | Europid | UC | Pos | 1 | 3 | 0 | 4b | 4.7 | UD |
| 7 | 60 | M | Europid | UC | Pos | 2 | 0 | 0 | 2 | 3.9 | UD |
| 8 | 35 | M | African | UC | Pos | 1 | 1 | 0 | 2b | 4.8 | UD |
| 9 | 42 | M | African | UC | Pos | 2 | 0 | 0 | 2a | 3.2 | UD |
| 10 | 36 | M | Indonesian | UC | Pos | 4 | 2 | 0 | 4a | 5.1 | UD |
| 11 | 49 | M | Europid | UC | Pos | 3 | 2 | 1 | 4c | 4.2 | UD |
| 12 | 39 | F | African | UC | Pos | 1 | 2 | 0 | 3 | 2.6 | UD |
| 13 | 36 | M | African | UC | Pos | 1 | 2 | 0 | 3 | 4.0 | UD |
| 14 | 47 | M | Indonesian | UC | Pos | 2 | 2 | 0 | 3 | 4.6 | UD |
| 15 | 39 | M | Europid | UC | Pos | 1 | 2 | 0 | 3 | 4.0 | UD |
| 16 | 47 | M | Europid | UC | Pos | 1 | 3 | 0 | 4b | 4.3 | UD |
| 17 | 37 | M | Europid | UC | Pos | 4 | 2 | 0 | 4a | 3.9 | UD |
| 18 | 60 | M | Indonesian | UC | Pos | 4 | 2 | 0 | 4a | 3.4 | UD |
| 19 | 67 | F | Indonesian | UC | Pos | 1 | 0 | 0 | 1 | 3.3 | UD |
| 20 | 39 | F | African | UC | Pos | 2 | 1 | 0 | 2b | 2.4 | UD |
| 21 | 45 | M | African | UC | Pos | 4 | 3 | 0 | 4b | 4.2 | UD |
| 22 | 66 | M | Europid | SCC | Pos | 4 | 1 | 0 | 4a | 2.9 | UD |
| 23 | 69 | F | Europid | OC | NR | NR | NR | NR | NR | UD | NR |
| 24 | 63 | M | Europid | OC | NR | NR | NR | NR | NR | UD | NR |

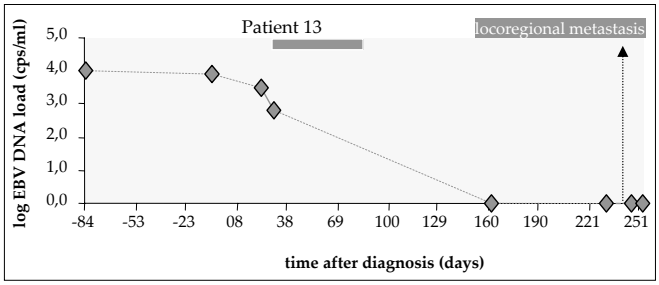
EBER-ISH, Epstein-Barr virus encoded RNA–in situ hybridisation; F, female; M, male; Neg, negative; NR, not relevant; OC, oropharyngeal carcinoma; Pos, positive; SCC, squamous cell carcinoma; UC, non-keratinising undifferentiated nasopharyngeal carcinoma; UD, undetectable; y, years.

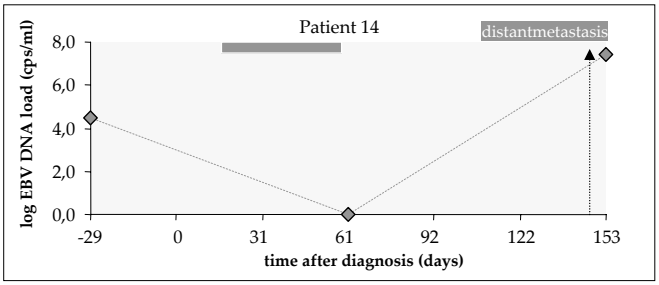
11 was lost to follow up as he presented elsewhere, and attempts to obtain blood samples were unsuccessful. The EBV DNA load in plasma was available in three EBER positive NPC patients with metastases: regional in patient 13 and distant in patients 14 and 16.
In patient 13, after the initial decrease to undetectable levels, plasma EBV DNA was found to remain negative when a metastasis in the neck was diagnosed, five months after treatment (fig 1). After surgical removal, EBER-ISH was carried out on tumour tissue from the metastasis and vital tumour cells were found to be EBER negative.
In patient 14, pulmonary metastases were detected five months after initiation of treatment. Concurrently plasma EBV DNA load had increased to a level of 7.4 $\log_{10}$ copies/ml plasma after the initial decrease to undetectable levels (fig 1).
In patient 16, one month after the completion of treatment, plasma EBV DNA load progressively increased from an undetectable level to 3.3 $\log_{10}$ copies/ml plasma (fig 1). Initially no recurrences were detected but eventually positron emission tomography indicated a lumbar metastasis; by then EBV DNA load in the plasma had reached a level of 4.0 $\log_{10}$ copies/ml plasma (fig 1).
Of the 20 EBER positive NPC patients, 16 remained disease-free after treatment and concurrently their EBV DNA load in plasma remained undetectable during follow up.

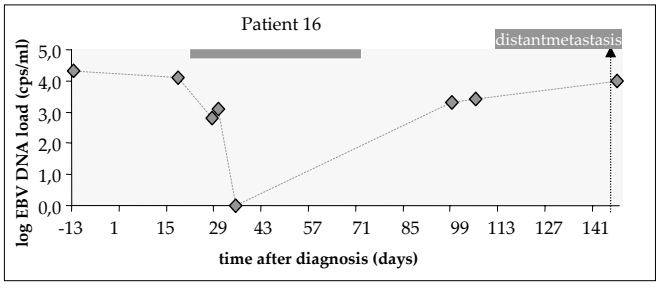
***Longitudinal analysis of plasma EBV DNA load during treatment***
In three EBER positive NPC patients (patients 15, 16, and 17), the course of the EBV DNA load in plasma was assessed throughout treatment and during follow up after treatment. In all three patients EBV DNA load decreased to undetectable levels within three weeks after the start of chemo-radiotherapy. In patient 15, EBV DNA load initially increased after one week of chemo-radiotherapy before it reached undetectable levels in the second week after treatment (fig 2). In patient 16 the EBV DNA load in plasma fell rapidly and became undetectable in the second week after initiation of chemo-radiotherapy (fig 1). In patient 17, the EBV DNA load decreased from 3.9 log10 copies/ml plasma before treatment to an undetectable level within three weeks after start of chemo-radiotherapy (fig 2).

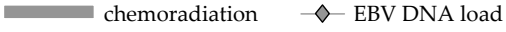
***Correlation between plasma EBV DNA load and clinical stage***
Six of 20 EBER positive NPC patients presented with stage I/II and 14 with stage III/IV disease. The median EBV DNA load in patients with stage I/II and stage III/IV disease were 3.6 log10 copies/ml plasma (range 2.4 $\log_{10}$ to 4.8 log10) and 4.2 $\log_{10}$ copies/ml plasma (2.6 $\log_{10}$ to 5.1 $\log_{10}$), respectively (fig 3). This difference did not reach statistical significance, most probably because of the small numbers.

**Figure 1.**



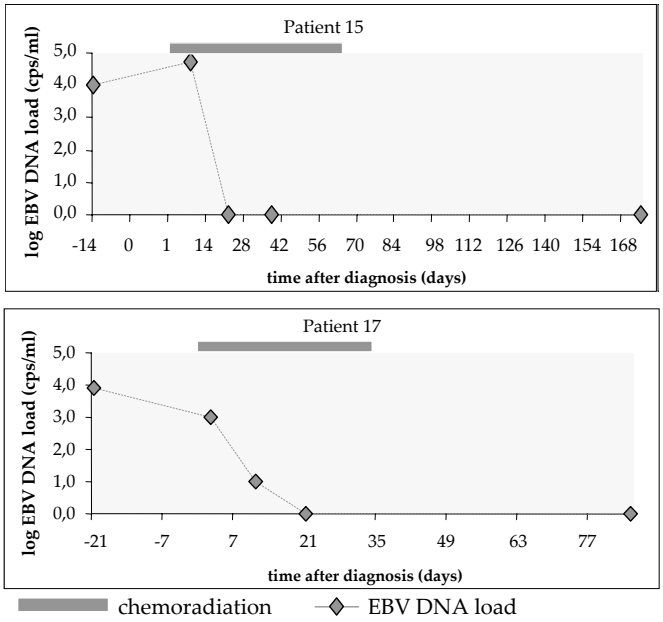



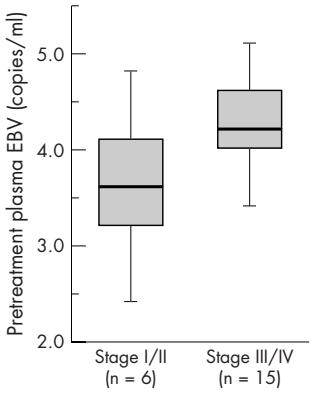

Change in plasma EBV DNA concentrations in three patients with metastases of EBER positive nasopharyngeal carcinomas. Key clinical events are indicated in the respective panels. The scale of the y axis has been optimised for the concentration range for each case. (A) Five months after chemo-radiotherapy patient 13 presented with a regional metastasis in the neck. However, EBV DNA remained undetectable during follow up and the metastatic tumour cells were found to be EBER negative. (B) After an initial decrease in the EBV DNA load from 4.5 log10 copies/ml before treatment to an undetectable level after treatment, EBV DNA load had reached a level of 7.4 log10 copies/ ml plasma at the time patient 14 presented with pulmonary metastasis. (C) Longitudinal follow up of EBV DNA load in plasma from patient 16 with lumbar spine metastasis. EBER, Epstein-Barr virus encoded RNA; EBV, Epstein-Barr virus.

**Figure 2.**




Longitudinal follow up of plasma EBV DNA load during and after chemo-radiotherapy in patients with EBER positive nasopharyngeal carcinomas. Key clinical events are indicated in the respective panels. The scale of the y axis has been optimised for the concentration range for each case. EBER, Epstein-Barr virus encoded RNA; EBV, Epstein-Barr virus.

**Figure 3.**




Plasma EBV DNA concentrations according to the stage of disease. The median load, the interquartile 50% range (box), and the range of values (whiskers) are given. Median values did not reach statistical significance.

## DISCUSSION

In NPC patients from high tumour risk regions (such as South-East Asia), the quantification of EBV DNA in plasma or serum can be of great clinical value, as it represents a promising non-invasive tumour marker.[8] Furthermore, in these patients where the association with NPC and EBV is very strong, plasma EBV DNA quantification is also useful to assess the efficacy of new treatments.[13 14 17]

A recent report by the Dutch Cancer Institute indicated that the NPC patient population in the Netherlands comprises an increasing number of patients from Asia and North Africa.[18] In the same study, 88% of the 109 evaluated NPC cases were undifferentiated carcinomas and only 6% were differentiated squamous cell carcinoma. No information regarding EBV association was included in this report, but as the undifferentiated type of NPC shows the most consistent association with EBV worldwide, and is EBV associated in nearly 100% of the cases,[4] it could be expected that plasma EBV DNA load in NPC patients in the Dutch population might be clinically relevant as a tumour marker.

In the present study, the applicability of detecting plasma EBV DNA as a tool for disease management in NPC patients in a low tumour risk area was assessed. The association between NPC and EBV was established by the detection of EBERs in tumour cells using an ISH assay. As EBERs are expressed in all forms of EBV latency, the EBER sequence is usually detectable in the nucleus of latently EBV infected cells. Furthermore, its high level of expression facilitates detection by ISH.[19–21] Therefore, EBER-ISH has served as a diagnostic gold standard of EBV presence in tissue samples.[6]

In the current study, 21 of 22 NPC (95.5%) were of the undifferentiated type and 90% (19/21) of these undifferentiated type NPC were EBV related, as they were EBER positive. Only one NPC was a histologically proven squamous cell carcinoma, which was found to be EBER positive as well. Although squamous cell NPCs from low incidence regions are not invariably EBV associated as they are in the high incidence areas, approximately one third of the cases from a low incidence regions have been reported to be EBV positive as detected by EBER-ISH.[6]

Eleven NPC patients (50%) were europid Dutch and two of these had EBER negative nasopharyngeal carcinomas (both were of the undifferentiated type). Although undifferentiated NPCs are considered to be consistently associated with EBV, EBER negative type 2 and type 3 NPCs have been described previously.[22 23] In an Italian study,[23] eight of 24 NPCs (types 2 and 3), and in a recent Israeli study,[22] three of 40 patients with types 2 and 3 NPCs, were negative by EBER-ISH.

In the current study, 11 patients (50%) were non-europid immigrants from South-East Asia (Indonesia) and North Africa, all presenting with EBER positive tumours.

Most of the non-europid patients were either long term Dutch residents or first generation immigrants. It has been reported previously that migrants from high risk NPC regions continue to show high rates of NPC irrespective of their country of migration, and this risk remains high for subsequent generations.[4] Thus the non-europid patients in the current study should be considered a high tumour risk population in a low tumour risk area. However, the patients described in this study reflect the population presently encountered in Dutch hospitals and as this study was intended to assess the clinical relevance of EBV DNA load measurements in a low risk (Dutch) area, they were included in this evaluation.

All patients (europid and non-europid) with EBER positive NPC (20 of 22) showed significant levels of EBV DNA in the plasma at time of diagnosis, and the EBV DNA load in plasma declined to undetectable levels after treatment with radiotherapy or chemo-radiotherapy. These results are consistent with those obtained from studies in high tumour risk areas[8] and with results described from one previous low risk cohort.[24 25] With regard to disease recurrence, it was found that an increased EBV DNA load in plasma correlated with the development of distant metastasis in one non-europid patient with EBER positive NPC. However, in another noneuropid patient the EBV DNA load remained undetectable despite the occurrence of regional recurrent disease (patient 13). It has previously been reported that, in contrast to distant recurrences, local recurrences of NPC in patients from high tumour risk regions cannot always be detected by EBV DNA load measurements in plasma or serum.[26 27] It is suggested that the small tumour mass present in these regional recurrences accounts for the lower detection rate compared with distant metastases,[27] or that radiation affects the release mechanism of EBV DNA without disrupting the reproductive capacity of the tumour cells.[27] Remarkably, in the case of patient 13, EBER-ISH on tumour cells from the regional metastasis was negative, suggesting that the recurrent tumour was no longer associated with EBV. To our knowledge, this is the first report of a regional recurrence of an EBV positive NPC manifesting itself without a renewed EBV association after chemo-radiotherapy. As a result, such a redifferentiation can impose limitations on the clinical applicability of the quantitative EBV DNA assay as well as on the employment of new therapeutic approaches such as adoptive immunotherapy.[17] This unusual phenomenon is currently being further examined to determine its clinical relevance.

In conclusion, plasma EBV DNA concentrations can be used as a useful molecular marker for the detection and monitoring of NPC patients in low risk regions as well as in high risk regions. No differences in the characteristics of EBV DNA concentrations were noticed between low risk patients (europid) and high risk patients (non-europid) living in a low risk area. However, unlike in high NPC incidence areas, where the association of this tumour with EBV is nearly 100%, a smaller proportion

of NPC are EBV positive in low tumour risk regions. As a result the application of plasma EBV DNA load as an NPC tumour marker in a low risk area is restricted to cases in which the association of the tumour with EBV is confirmed by EBER-ISH on tumour tissue. Thus this assay can only be used to sustain the diagnosis of NPC and not to replace routine diagnostic procedures in a low NPC risk area. Once the association of the tumour with EBV is established, the EBV DNA load in the plasma can be used to monitor treatment and recurrences. In this respect, another limitation also described for high tumour risk regions should be taken into account, which is that certain recurrences cannot be detected by the EBV DNA load. Thus routine post-treatment follow up is essential and cannot be completely replaced by plasma EBV DNA testing.

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# 8

## Clinical relevance of quantitative varicella-zoster virus-DNA detection in plasma following allogeneic stem cell transplantation

# 8a

# Clinical relevance of quantitative varicella-zoster virus (VZV) DNA detection in plasma after stem cell transplantation

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## ABSTRACT

Detection of Varicella-Zoster virus (VZV) DNA in plasma can facilitate the early recognition of complicated VZV-infection in immunocompromised hosts. The correlation of VZV-DNA in plasma with clinical presentations of VZV-infection and subsequent aciclovir treatment in allogeneic stem cell transplant (allo-SCT) recipients was studied. In 81 consecutive VZV-IgG positive allo-SCT recipients, VZV-DNA was measured at regular time points (1, 2 and 4 months) following allo-SCT and patient records were screened for VZV-related symptoms and aciclovir treatment. Subsequently, possible VZV-cases were studied in detail for the course of VZV-DNA and treatment effects. During the initial screening, VZV-DNA was detectable in seven patients. The survey of VZV-related symptoms revealed five additional possible VZV-cases. In cases where suitable plasma samples were available (10 out of 12), VZV-DNA was present almost simultaneously with the first clinical manifestations. No evidence of a preceding phase detectable by VZV-DNA only could be observed. Treatment with aciclovir was associated with a prompt reduction of VZV-DNA load. Detection of VZV-DNA in plasma in allo-SCT recipients accurately reflected the clinical presentation of VZV-infection and treatment with aciclovir. VZV-DNA detection in plasma of allo-SCT recipients appears clinically relevant as this may support early recognition and therapeutic management of VZV-infections following allo-SCT.

## Introduction

Varicella-zoster virus (VZV), one of the eight human herpes viruses, is frequently encountered in infections in immunocompromised patients, especially in HIV-infected patients and transplant recipients. Primary infection results in varicella (chickenpox), an extremely contagious acute infection that is characterized by generalized vesicular rash. Reactivation of latent VZV results in dermatomal zoster or visceral zoster, or combinations of these two, while dissemination comparable to primary infections may also occur in immunocompromised patients.[1,2] Commonly reported complications of disseminated VZV infections in severely immunocompromised patients, such as stem cell transplant (SCT) recipients, include pneumonia, encephalitis and hepatitis.[1] Another serious manifestation of herpes zoster in the immunocompromised host is 'abdominal zoster' characterized by severe abdominal pain that may precede the appearance of the cutaneous rash by hours to days.[1] In allogeneic bone-marrow transplant recipients, VZV reactivation occurs in 20–50% of the cases within the first year after transplantation and prompt therapy with antiviral agents can reduce the risk of dissemination or postherpetic neuralgia.[2–4] Still, approximately 15% of these cases subsequently develop cutaneous dissemination and 5% develop visceral dissemination.[2,5] Typically, VZV reactivation occurs between 2 and 6 months after allogeneic BMT, with the majority of the cases occurring within the first year of transplantation.[2,4] However, reactivation may occur for up to 2 years following transplantation.[4] For therapy of VZV infection or reactivation after SCT, the use of intravenous aciclovir or oral valaciclovir is the treatment of choice. VZV reactivation can be prevented in patients after SCT using oral aciclovir, but only during the period of administration. Hence, such prophylactic use of aciclovir was demonstrated to delay the onset of infection, but not to reduce the overall incidence of VZV reactivation.[4] Furthermore, long-term antiviral prophylaxis also carries the potential for the induction of VZV resistance and manifestation of side effects.[5,6]

Recent advances in molecular techniques have enabled quantification of VZV-DNA in plasma or peripheral blood mononuclear cells (PBMCs) of patients with VZV-related disease. It was shown that VZV-DNA is detectable in peripheral blood of patients with varicella as well as herpes zoster.[7–10] Therefore, this assay could be of potential value to monitor SCT recipients, with regard to the early recognition and rapid confirmation of VZV-related disease, the proper recognition of atypical presentations including visceral zoster and as a sensitive tool to evaluate the effect of therapeutic interventions.

This study evaluated the use of VZV-DNA detection in plasma in a cohort of 81 consecutive SCT recipients, employing an internally controlled quantitative PCR method. The results obtained by a prospective screening and further retrospective

detailed analysis of positive cases should provide insight into the incidence of VZV reactivation, the relation to clinical problems and the effects of treatment on the VZV-DNA load.

## PATIENTS AND METHODS

### *Patients and procedures*

Between January 2003 and December 2004, 85 consecutive patients received allogeneic stem cell transplant at the Leiden University Medical Center using a HLA identical sibling, haploid sibling or unrelated donor. Four of these patients were excluded from this study; three were not at risk for VZV reactivation (both donor and recipient were VZV seronegative before transplantation) and no plasma samples were available from one patient. The median age was 50.0 years (range 21–66 years). Data regarding pre-transplant serology for VZV, aciclovir treatment and clinical symptoms of VZV-related disease were retrospectively collected from the medical records (treatment charts, diagnosis codes and all correspondence) of the included patients. The main clinical characteristics of the patients are shown in Table 1.

Two main categories of conditioning regimen were used. Forty-six patients were transplanted with T-cell depleted peripheral blood stem cells following conditioning with a myeloablative regimen as described before.[11] Patients with a relative contraindication for conventional myeloablative allo-SCT ($n$ = 35) received a T-cell depleted allo-SCT following reduced intensity conditioning as described previously.[12]

All patients received prophylaxis with cotrimoxazole against *Pneumocystis jerovici* infections. Surveillance for CMV reactivation was performed by quantitative PCR, and pre-emptive therapy was commenced according to procedures described previously.[13] No graft-versus-host disease prophylaxis was administered.

General institutional policy with respect to patients' informed consent for inclusion into the study, approved by the ethical institutional board, was applied.

### *VZV-DNAemia and VZV reactivation*

A two-step approach was pursued to determine the relevance of VZV-DNAemia in this study. Initially, the presence of VZV-DNA in plasma was determined longitudinally in all included patients at 1, 2 and 4 months following transplantation. Patients with at least one positive plasma sample were considered possible VZV-cases. Secondly, patients' records were surveyed and patients with compatible clinical problems or aciclovir treatment, potentially associated with VZV within 4 months following transplantation were also referred to as possible VZV-cases. Subsequently, in all possible VZV-cases all available plasma samples within 4 month following trans-

**Table 1.** Patients' characteristics

| Allogeneic stem cell transplant recipients ($n$ = 81) | | |
|---|---|---|
| **Characteristics** | **Noninfected** | **Herpes zoster** |
| Number | 69 | 12 |
| Median age at SCT (range) | 52.0 (21–66) | 42.5 (24–59) |
| Sex (male/female) | 47/22 | 8/4 |
| Underlying disease, *n* (%) | | |
| AML | 23 (33) | 2 (17) |
| CML | 11 (16) | 1 (8) |
| MM/CLL | 11 (16) | 1 (8) |
| ALL | 3(4) | 1(8) |
| NHL | 10 (14) | 3 (25) |
| MDS | 2(3) | 1(8) |
| AA | 2(3) | 0 |
| Solid carcinoma | 2 (3) | 0 |
| Others | 5 (7) | 3 (25) |
| Type of conditioning, *n* (%) | | |
| RIC | 31 (45) | 4 (33) |
| MAC | 38 (55) | 8 (67) |
| Type of transplant, *n* (%) | | |
| Related | 50 (72) | 9 (75) |
| Unrelated | 19 (28) | 3 (25) |
| VZV DNA in plasma screening 1, 2 and 4 months | 0 | 7 |
| Mean (s.d.) pre-transplant VZV-IgG antibody test values | 2.1 (0.9) | 2.0 (0.6) |

Abbreviations: AA = aplastic anemia; ALL = acute lymphoblastic leukemia; AML = acute myelogenous leukemia; CLL = chronic lymphoblastic leukemia; CML = chronic myelogenous leukemia; MAC = myelo-ablative conditioning; MDS = myelodysplastic syndrome; MM = multiple myeloma; NHL = non-Hodgkin syndrome; RIC = reduced intensity conditioning.

plantation, or until death occurred, were used to describe the course of VZV-DNA in more detail.

Diagnosis of VZV infection was based on clinical presentation, considered typical by the responsible physicians (typical rash, dermatomal distribution, fever, pain, itching), and viral culture of tissue from vesicular skin lesions, was additionally included in equivocal cases only. Herpes zoster was treated with intravenous 10 mg/kg aciclo-

vir three times daily for at least 5 days. Positive VZV-DNA load was defined as the presence of more than 250 copies/ml plasma.

*Quantification of VZV-DNA in plasma*

Plasma samples were collected and stored at –20°C until the PCR assay was performed. DNA for VZV real-time PCR analysis was isolated from 200 µl serum using the MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche Molecular Diagnostics, Penzberg, Germany). Amplification and quantitative detection of VZV-DNA was performed on a BioRad i-cycler IQ™ real-time PCR detection system (BioRad, Veenendaal, The Netherlands). Primers (sense: 5′-CTCGCC-CGTCTCTATCTC-3′, antisense: 5′-GATGAGGTGGTTGTTATTGTTC-3′) and probe (FAM5′CGCGATCGAC GAAGCGTGCCAG-TTGAATCGCG-3′DABCYL) were selected to detect a 139 bp fragment from the glycoprotein gene (ORF 31) of the VZV genome. The sensitivity and the specificity of the real-time VZV-DNA PCR were evaluated. In order to quantify the VZV-DNA in clinical samples, $\log_{10}$ dilutions of the QCMD standard for VZV-DNA starting at 5.10e5 IU/ml were tested and used as an external standard curve.
Co-amplification of Phocine herpesvirus (PhHV), spiked to each sample before DNA extraction, was used as an internal control to check for the DNA isolation and inhibition in the PCR as described previously.[13] The VZV real-time PCR assay has been used in our laboratory as a routine diagnostic assay for swabs for 3 years. The assay was validated in comparison to the previously used conventional PCR. Quality control is performed by monitoring Ct-values of positive controls in time and by participating in the QCMD proficiency panels. In the QCMD 2005 panel a 100% score was achieved. This panel contained a sample (VZV05-07) with 260 GEq/ml, which is 5 GEq in the PCR.

*VZV IgG detection*

Pre-transplant VZV-IgG antibody titres were routinely performed for all transplant recipients using the ELFA VZV-IgG assay (Biomerieux, Boxtel, The Netherlands) according to the manufacturer's instructions. Patients with pre-transplant negative VZV-IgG test values were not considered at risk for VZV-disease following transplantation and were excluded from this study. Additionally, in patients with VZV-disease, VZV-IgG antibody values were determined at the end of the episodes and at 4-month following transplantation. No prophylaxis with immunoglobulin, acyclovir or ganciclovir was administered.

*Statistical analysis*

All database entries and statistical analysis were performed with SPSS version 12.0.1. For comparison of noncategorical data we used Mann–Whitney *U*-test.

## Results

*Detection of possible VZV-cases after initial screening*

In total, 81 patients with positive pre-transplant VZV-IgG antibody titres were included as they were considered to be at risk for VZV disease following transplant. Twelve out of these 81 transplant recipients (15%) were considered possible VZV-cases. In seven of these cases, VZV-DNA was detectable in at least one plasma sample during the regular screening at 1, 2 and 4 months following transplantation (Figure 1a–g). A survey of clinical problems potentially associated with VZV infection revealed five additional possible VZV-cases, not detected in the initial screening; three of which are represented by Figure 1h–j.

The course of VZV-DNA was analyzed in more detail in 10 possible VZV-cases; in the remaining two possible VZV-cases, no plasma samples were available during the VZV disease episodes. Median time to the first detectable VZV-DNA load in plasma following transplantation was 36 days (range; 14–83 days), and the median peak load was 3.85 log10 copies/ml plasma (range: 2.6 log10–5.3 log10). The median time to reach the peak load was not different from the median time to the first detectable load (36 days) as in most cases the initial load was also the peak load.

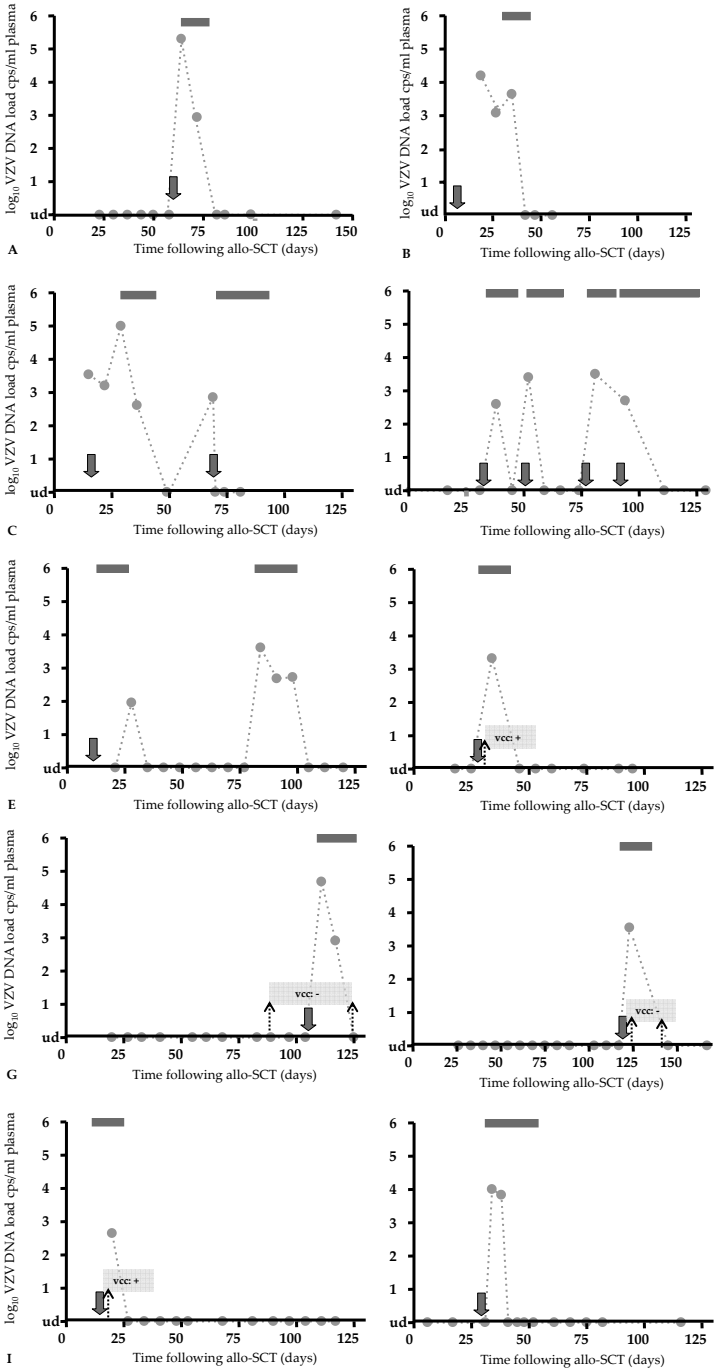
*Correlation of VZV-DNA in plasma with clinical course of VZV-related disease*

Correlation with clinical data revealed that all seven possible VZV-cases from the initial VZV-DNA screening presented with herpes zoster-like skin lesions and were treated with aciclovir at time of detectable VZV-DNA in plasma (Figure 1a–g). In five out of these seven cases (Figure 1a–e), diagnosis of VZV infections was based on typical clinical presentation and in the two remaining cases (Figure 1f and g), confirmation using viral culture were considered necessary. However, viral cultures could provide confirmation in only one case (Figure 1f). The patient represented by Figure 1d showed progression to disseminated disease and bilateral progressive retinal necrosis.[14]

In five possible VZV-cases from the clinical data survey, diagnoses of VZV infections were based on typical clinical presentation. Confirmation by viral culture was considered necessary in three out of these five cases (Figure 1h–j) and could only be provided in one case (Figure 1i). In three out of five possible VZV-cases from the clinical data survey, from whom plasma samples were available, VZV-DNA was detectable at time of herpes-zoster skin lesions (Figure 1h–j).

In total, 12 out of 80 transplanted patients presented with herpes-zoster skin lesions within 4 months after transplantation. The sensitivity and specificity of regular screening (at 1, 2 and 4 months following transplant) for VZV DNA for actual VZV disease was 58 and 100%, respectively (Table 2). Viral culture was considered necessary for

**Figure 1.**




Course of VZV-DNA load in 12 allo-SCT recipients. Solid circles indicate the VZV-DNA load. Horizontal bars indicate treatment with aciclovir and bold arrows indicate the moment of first clinical signs of VZV disease. The outcome and timing of viral cell cultures (VCC) of vesicular tissue, are indicated by the dotted arrows: '+' indicate positive VZV cultures and '–' indicate negative VZV cultures. UD = undetectable VZV-DNA load.

**Table 2.** Correlation between detection of VZV DNA at 1, 2 and 4 months following transplant and VZV disease

| | VZV disease status | | |
|---|---|---|---|
| | **Positive** | **Negative** | **Total** |
| VZV DNA detection in plasma (at 1, 2 and 4 months) | | | |
| Positive | 7 | 0 | 7 |
| Negative | 5 | 69 | 74 |
| Total | 12 | 69 | 81 |

The calculated sensitivity and specificity of VZV-DNA detection for actual VZV disease were 58 and 100%, respectively (positive predictive value: 100%; negative predictive value: 93%).

the confirmation of the diagnosis of VZV infection in five out of 12 cases and this confirmation could only be provided in two cases. No clear associations were observed between the occurrence of VZV infection and conditioning regimen (VZV infection occurred in four out of 35 (11.4%) and eight out of 46 (17.4%) patients receiving reduced intensity and myeloablative conditioning, respectively) or donor type (VZV infection was observed in nine out 59 (15.3%) and in three out of 22 (13.6%) patients receiving grafts from related donors and unrelated donors, respectively). If suitable specimens were available (as in 10 of these 12 patients), no VZV-related clinical conditions were observed without simultaneous VZV-DNA detection in plasma and conversely, all VZV-DNA positive cases concurrently presented with VZV-related disease. Furthermore, VZV-DNA was detectable in plasma in cases where viral culture could not confirm the diagnosis of VZV infection (Figure 1g, h and j). VZV-DNA in plasma was present almost simultaneously with the first clinical manifestation and no evidence of preceding phase of positive VZV-DNA could be observed. Except for the VZV retinitis case (Figure 1d), no other atypical cases or visceral zoster cases were detected in this cohort.

***Effect of aciclovir treatment on the course of VZV-DNA load in plasma***

Treatment with aciclovir resulted in a rapid resolution of herpes zoster skin lesions in all patients. This clinical response to aciclovir treatment was consequently reflected by a prompt decrease of VZV-DNA load upon treatment with aciclovir in all cases where suitable plasma samples were available (Figure 1a–j).

***Course of VZV-IgG antibody titres in patients with VZV disease and VZV-DNA in plasma***

In 10 patients with VZV-disease and concurrent positive VZV-DNA load within 4 months following transplantation the course of VZV-IgG test values was analyzed.

In accordance with manufacturer's instructions a test value of at least 0.9 is considered positive. The mean (s.d.) VZV-IgG test value pre-transplantation, during the VZV disease episode and at 4 months following transplantation were not statistically different: 2.0 (0.6), 2.0 (0.8) and 2.0 (0.7) respectively. The analysis of VZV-IgG titres following transplantation was limited to the 10 patients clinically positive for VZV disease and VZV DNA in plasma, since no effect on VZV-IgG titres were seen in relation to VZV disease.

## DISCUSSION

This study was performed to evaluate the use of VZV-DNA detection in plasma from SCT recipients. Initially, VZV-DNA was measured at regular time points and patient records were screened for VZV-related clinical problems. Subsequently, positive cases resulting from both these approaches were analyzed in more detail.
Twelve patients with VZV-disease, occurring within 4 months following transplantation were recognized. It should be noted that the incidence of 12 cases with VZV infection within 4 months following allo-SCT is a minimal estimate, since possible interference with CMV therapy by ganciclovir in this group cannot be excluded. In this respect, the study was designed to evaluate the use of VZV-DNA detection in plasma following allo-SCT, rather then to perform a systematic epidemiological survey. It appeared that the timing of VZV-DNA detection as used in this study was not able to uncover all clinical cases of VZV-disease, as in five out of 12 VZV-cases, no VZV-DNA was detected in plasma at 1, 2 and 4 months following transplant. However, in all cases with VZV-related clinical symptoms, concomitant VZV-DNA detection in plasma was observed, if suitable specimens were available. Furthermore, all VZV-DNA positive cases concurrently presented with VZV-like disease and treatment resulted in prompt reductions of VZV-DNA as clinical symptoms resolved. Attempts to confirm a clinical diagnosis by viral culture remarkably were largely unsuccessful, probably due to the relative low sensitivity of this procedure in routine clinical practice. These findings are indicative of a high sensitivity and specificity of VZV-DNA detection in plasma following allo-SCT, and are in agreement with a previous report.[5]
Analysis of the course of VZV-IgG antibody values clearly demonstrated no effect of VZV-IgG titres in relation to VZV disease and VZV-DNA detection in this group of patients who are apparently incapable of mounting an adequate humoral immune response against VZV within 4 months following allo-SCT.
It has previously been reported that severe complications of disseminated zoster infections appear to be associated with elevated VZV-DNA levels in sequential plasma specimen,[8,9,15] enabling early diagnosis and treatment of these severe manifestations

of VZV infections. In the current study, all 12 VZV-cases presented with cutaneous lesions and except for one patient presenting with unusual involvement of the eyes, as described previously in more details,[14] no severe complications of VZV infection were detected. By determining VZV-DNA at time points as used in this study no evidence of a preceding phase of positive VZV-DNA could be observed in these patients with uncomplicated cutaneous VZV-disease. As a consequence, any attempt to design pre-emptive policies to prevent VZV disease, as have been described for CMV and EBV, will encounter severe limitations because of the nearly coincidental rise in VZV-DNA and clinical symptoms. If still a pre-emptive approach would be pursued, more intensive sampling (e.g. one or twice a week) might be required.

In conclusion, this study demonstrated that the use of VZV-DNA detection in plasma could be useful in the diagnosis of VZV-related disease in SCT recipients, allowing an early and specific confirmation of clinical suspicion and reflecting accurately the clinical course, including the effects of treatment. The potential benefit of regular screening, enabling pre-emptive therapeutic strategies with regard to VZV-diseases, could not be demonstrated in the present study and would therefore at least require more intensive sampling.

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# 8b

## Varicella zoster virus (VZV)-related progressive outer retinal necrosis (PORN) after allogeneic stem cell transplantation

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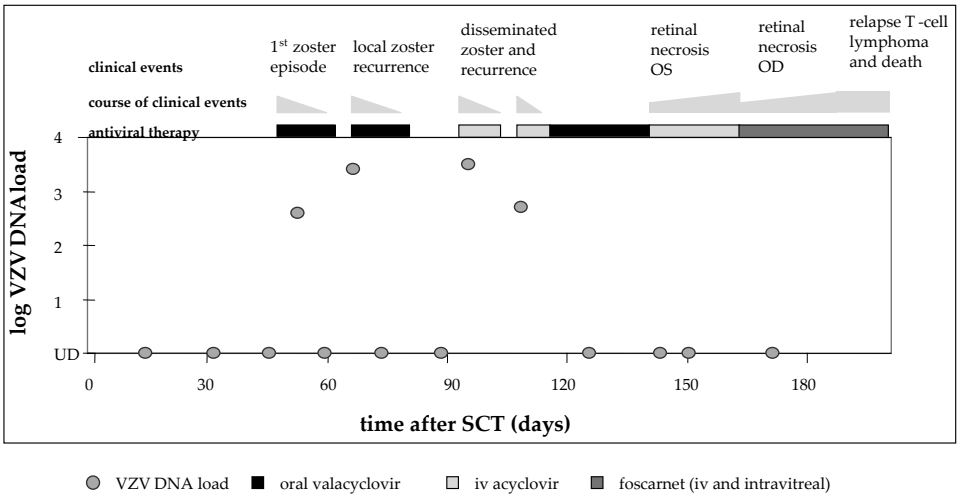
Commonly reported complications of disseminated varicella zoster virus (VZV) infections in severely immunocompromised patients (eg stem cell transplant recipients) include VZV pneumonia, encephalitis and hepatitis. Progressive outer retinal necrosis (PORN) is described as a distinct form of VZV necrotizing chorioretinitis and is found almost exclusively in patients with acquired immunodeficiency syndrome (AIDS).[1–4] However, a few cases in non-AIDS patients have been reported. These patients were immunocompromised due to therapy for idiopathic thrombocytopenic purpura (ITP), cutaneous non-Hodgkin's lymphoma, rheumatoid arthritis and renal transplantation.[1] To our knowledge no case of PORN, with laboratory confirmation of VZV involvement, occurring after allogeneic stem cell transplantation (allo-SCT) has been reported until now. Here, we report a laboratory confirmed case of PORN following disseminated VZV infection in an allo-SCT patient.

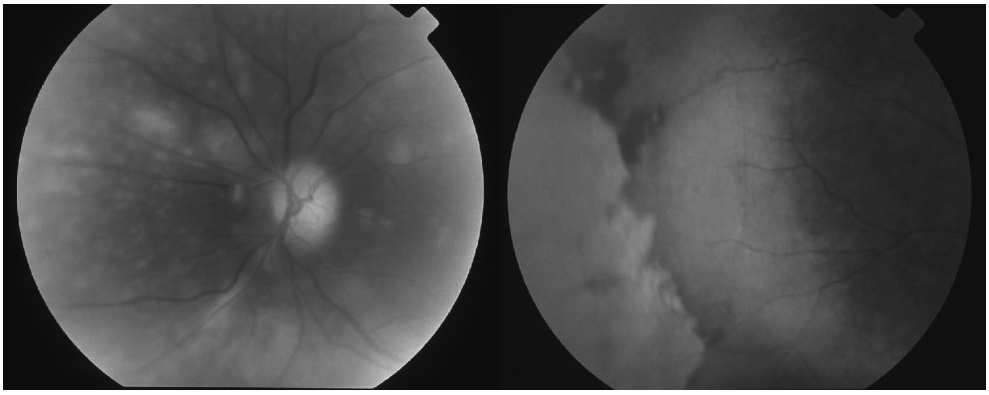
A 24-year-old patient with relapse of T-lymphoblastic lymphoma received a haploidentical allo-SCT with peripheral stem cells from his brother. The VZV serostatus prior to transplantation for both donor and recipient was positive (D+ /R+); Cytomegalovirus (CMV) serostatus for donor and recipient was positive and negative, respectively, (D+ /R–) and human immunodeficiency virus (HIV) serostatus was negative for the donor and recipient. The conditioning regimen consisted of total body irradiation (9 Gy), thiotepa (5 mg/kg for 2 days), fludarabine (40 mg/m$^2$ for 4 days) and ATG (10 mg/kg for 4 days). Neutrophil and platelet engraftment was rapid and no signs of graft-versus-host disease (GVHD) were observed. Starting at 40 days after transplantation the patient presented with recurrent episodes of generalized cutaneous herpes zoster, initially with a dermatomal distribution of skin lesions on the right lateral chest and subsequently with dissemination of these lesions to the trunk and arms. The episodes of localized herpes zoster were treated with oral valaciclovir 1000 mg t.i.d for 10 days resulting in the disappearance of all lesions. The disseminated infections were treated with intravenous aciclovir 10 mg/kg t.i.d. for 8 days, after which all lesions resolved. After recovery from the last episode of generalized zoster, the patient was discharged with oral valaciclovir 1000 mg t.i.d. as maintenance therapy (see Figure 1 for an overview of treatment episodes). After 4 weeks, the patient reported visual loss in the left eye while still on oral valaciclovir. Ophthalmic examination of the left eye revealed a visual acuity of 0.2, faint aqueous flare and the presence of cells in the anterior segment, and a very few vitreous cells. Fundus examination of the left eye showed small yellow chorioretinal inflammatory dots and an exudative retinal detachment extending from the inferior retina towards the papilla and macula; no optic disc edema was noticed (Figure 2a). Ophthalmic examination of the right eye was without any abnormalities. Nucleic acid detection with PCR performed on left anterior chamber fluid was positive for VZV DNA and negative for HSV, CMV and EBV DNA. Intravenous aciclovir (10 mg/kg

## Figure 1.




The course of plasma VZV DNA load in relation to relevant clinical events after SCT. On the lower horizontal axis, the time (in days) after SCT is depicted. The upper horizontal axis shows data concerning the course of relevant clinical events and antiviral treatment. The vertical axis shows the log VZV DNA load in plasma. VZV DNA load was detectable in plasma at presentation of zoster skin lesions and dropped to undetectable levels after each treatment episode. VZV DNA was undetectable in plasma during ophthalmic involvement. However, VZV DNA was detected in anterior chamber and vitreous fluids of both eyes during ophthalmic involvement. UD = undetectable; SCT = stem cell transplantation; i.v. = intravenous.

## Figure 2.



(a) Left eye: fundus at the first ophthalmic examination. (b) Right eye: peripheral retinal necrosis with retinitis progression towards the center.

t.i.d.) was resumed and tobradex (oc gtt qid ODS) was administered. Trans-pars-plana vitrectomy was performed to facilitate retinal attachment by means of silicone oil-vitreous exchange as well as endolaser coagulation, lensectomy and 360° retinotomy were performed. In the left vitreous fluid again VZV DNA was detected; HSV, CMV and EBV DNA were not detected. During follow-up after surgery, visual acuity of left eye was 1/300, the retina remained attached with silicon oil tamponade, but the yellow/white retinitis showed progression despite intravenous treatment with aciclovir.

After 4 weeks of the start of symptoms in the left eye the patient complained of decreased vision with the right eye and ophthalmic examination of the right eye revealed a visual acuity of 1.0 and an unremarkable anterior segment without vitreous cells. Fundus examination showed coalescing peripheral retinal lesions with temporal opacifying retinal necrosis (Figure 2b). Again the presence of VZV DNA was demonstrated in right anterior segment fluid and HSV, CMV and EBV DNA were undetectable. Aciclovir was replaced by foscarnet: intravenous (24 mg/ml t.i.d.) and intravitreal in the right eye (1.2 mg/0.05 ml three times a week). Laser coagulation was performed to separate necrotic from healthy retina. After 1 week, signs of recovery (retinal traction) were seen and surgery as carried out for the left eye, was performed. After the operation visual acuity of the right eye dropped to 0.4 but no progression was seen with regard to the retinitis. At 6 months after transplantation, T-cell lymphoma relapsed and after 4 weeks the patient died of progressive lymphoma.

The nature of the retinal lesions (multifocal, coalescing and beginning in the peripheral retina), their rapid progression, the lack of response to treatment, the lack of more prominent inflammatory reaction in vitreous and anterior chambers as well as the involvement of VZV but not other herpes viruses (HSV, CMV, EBV) are typical of PORN.[1]

In a majority of previously reported patients, generalized herpes zoster infections were found prior to or coincident with PORN.[2,3] As such, hematogenous dissemination during herpes zoster eruptions has been suggested as a predisposing factor.[5] It has also been suggested that resistance of VZV to aciclovir may contribute to the pathogenesis of PORN, since the occurrence of aciclovir resistance is known to be relatively common in the course of repeated VZV infections in immunocompromised patients.[5]

With regard to the present case, VZV DNA load in plasma was determined longitudinally by a quantitative real-time PCR using dilution series of reference materials of the QCMD (quality control for molecular diagnostics) VZV proficiency panel as standard. Plasma VZV DNA load in relation to relevant clinical events and aciclovir therapy are shown in Figure 1. At the time of ocular presentation, no VZV DNA was

detectable in peripheral blood. Additionally, no mutations in VZV TK-genes, associated with aciclovir resistance[6] were encountered in DNA extracted from plasma samples and anterior chamber fluid of both eyes. These findings indicate that it is unlikely that either persisting VZV-viraemia, with continuous dissemination to the eye, or aciclovir resistance contributed to the pathogenesis of PORN in this case. Therefore, the 'relative immune privileged' site of the eye must be considered and as such the possible role of local VZV recurrence with local ocular and neural dissemination cannot be ruled out.

In severely immunocompromised patients, such as SCT recipients, herpes zoster can present with unusual manifestations and can cause potentially life-threatening complications such as atypical generalized zoster or abdominal zoster. This case report implies that, although uncommon, VZV necrotizing chorioretinitis should be recognized as another potentially severe complication of disseminated VZV infection after allo-SCT. Awareness of such atypical herpes zoster presentation and appropriate diagnostic studies including ophthalmic examination can facilitate early recognition and initiation of aggressive (antiviral) treatment in order to maintain useful vision for the duration of the patients' life.

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# 9

# Assessment of disseminated adenovirus infections using quantitative plasma PCR in adult allogeneic stem cell transplant recipients receiving reduced intensity or myeloablative conditioning

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## ABSTRACT

***Objectives:*** Disseminated adenovirus (AdV) infections following allogeneic stem cell transplantation (allo-SCT) are increasingly recognised, particularly in children. This study evaluated the clinical relevance of disseminated AdV infections in adult allo-SCT recipients, after different conditioning regimens.

***Methods:*** In a cohort of 107 adult allo-SCT recipients, receiving either reduced intensity conditioning (RIC, $n = 48$) or myeloablative conditioning (MAC, $n = 59$), AdV DNA levels in plasma were determined retrospectively at 1, 3 and 6 months following transplantation. Results of this screening regimen were compared with a cohort of 58 paediatric allo-SCT recipients, in whom AdV DNA load was monitored prospectively, as part of a pre-emptive treatment strategy. In positive cases, the course of AdV DNA load and clinical outcome were assessed.

***Results:*** AdV DNA levels ≥ 1000 copies/mL were detected in five adults (4.7%) and eight children (13.8%). Screening for AdV viraemia at 1, 3 and 6 months would have detected seven of eight paediatric patients. One adult, receiving MAC, died with disseminated AdV disease and in four (three RIC and one MAC) AdV viraemia was transient without clinical symptoms specifically attributable to AdV. Seven paediatric patients with AdV viraemia were pre-emptively treated with ribavirin or cidofovir and in three of them disseminated AdV infection was related to a fatal outcome.

***Conclusions:*** Disseminated AdV infections following allo-SCT was a rare event in the adults and cause morbidity in a minority of these patients. In four of five adult patients, spontaneous clearance of AdV viraemia occurred. Results did not differ between the conditioning regimens that were applied in the adult cohort.

## Introduction

Infections with adenoviruses (AdV) are increasingly observed after allogeneic stem cell transplantation (allo-SCT) resulting in high mortality upon dissemination of the virus, particularly in paediatric patients.[1] In these patients initially mild symptoms, including fever and diarrhoea, can progress to haemorrhagic cystitis, pneumonia, hepatitis, eventually leading to death.[1] Reports using conventional viral tissue culture of excreted AdV, have shown incidences in SCT recipients of 23–32% in children and 3–14% in adults.[2–5] Besides young age, other reported risk factors for AdV infections include SCT with grafts from mismatched or unrelated donors, T-cell depletion of the graft, anti-thymocyte globulin (ATG) or Alemtuzumab in the conditioning regimen, delayed immune recovery following transplant and the occurrence of acute graft-versus-host disease (GvHD).[5,6] One of the major difficulties in the clinical diagnosis of AdV infections in SCT patients is the fairly similar presentation of post-transplant complications. Combined with the moderately sensitive, laborious and time-consuming conventional culture approach to diagnose AdV infections, this renders management decisions rather difficult. Recently, it was shown that monitoring of AdV DNA load in peripheral blood samples is a sensitive and specific tool for the recognition of patients at risk of a potential fatal, disseminated AdV infection.[7–9] Similar to Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) infections, monitoring of AdV DNA load in plasma can potentially guide the clinical management of AdV infections, as demonstrated in paediatric allo-SCT recipients.[10–12] Although children undergoing SCT are at particularly high risk, it has been suggested recently that AdV is also emerging as a post-transplantation pathogen in adult patient following either myeloablative conditioning (MAC) or reduced intensity conditioning (RIC).[13,14] Noteworthy, these studies primarily used viral cell culture assays to detect AdV infections. Based on these prospective studies, active surveillance for AdV infections in T-cell depleted SCT and pre-emptive intervention comprising either withdrawal or reduction of immune suppressive treatment or administration of antiviral treatment, have been proposed.[13] In addition, conditioning regimens could be relevant to the occurrence and outcome of this infection. The present study retrospectively analysed the impact of AdV infections in adult allo-SCT recipients. A real-time quantitative AdV polymerase chain reaction (PCR)[15] was employed to evaluate the occurrence and outcome of disseminated AdV infections following T-cell depleted allo-SCT in adult patients receiving either a fludarabine, busulphan and ATG-based RIC or conventional MAC regimen.

**Table 1.** Characteristics of adult allo-SCT recipients ($n$ = 107) according to conditioning regimen. Systemic treatment of GvHD consisted of oral prednisone, intravenous methylprednisolone and/or oral cyclosporin

| Characteristics | RIC ($n$ = 48) | MAC ($n$ = 59) |
|---|---|---|
| Age (median/range) | 54.5 (26–76) | 44.0 (21–62) |
| Male gender (%) | 34 (71) | 43 (73) |
| Donor type (%) | | |
| Matched sibling | 31 (65) | 52 (88) |
| Matched unrelated | 17 (35) | 7 (12) |
| Underlying disease (%) | | |
| Acute leukaemia | 10 (21) | 33 (56) |
| CML | 5 (10) | 10 (17) |
| CLL | 5 (10) | 1 (2) |
| MM | 5 (10) | 7 (12) |
| NHL | 10 (21) | 7 (12) |
| Other | 13 (27) | 1 (2) |
| Acute GvHD (%) | | |
| Grade I/II | 4 (8) | 13 (22) |
| Grade III/IV | 0 | 0 |
| GvHD treatment (%) (systemic) | 0 | 5 (8) |

Allo-SCT, allogeneic stem cell transplantation; CLL, chronic lymphocytic leukaemia; CML, chronic myelogenous leukaemia; GvHD, graft-versus-host disease; MAC, myeloablative conditioning; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; RIC, reduced intensity conditioning.

## PATIENTS AND METHODS

### *Patients*

Forty-eight consecutive adult patients who received allo-SCT following RIC and 59 consecutive adult patients receiving allo-SCT using conventional MAC regimens between January 2001 and January 2005 were included in this study. Forty-three RIC patients had haematological malignancies, four had renal cell carcinoma and one breast carcinoma. All MAC patients had haematological malignancies. The main characteristics of these patients are shown in Table 1. Data related to antiviral treatment, clinical symptoms potentially caused by disseminated AdV infections, lymphocyte counts and CMV infections were retrospectively collected from the medical records of the patients included.

**Table 2.** Characteristics of paediatric allo-SCT recipients ($n = 58$)

| Characteristics | Children ($n = 58$) |
|---|---|
| Age (median/range) | 11.0 (1–18) |
| Male gender (%) | 44 (76) |
| MUD | 34 (59) |
| ORD | 10 (17) |
| IRD | 14 (24) |
| Acute leukaemia | 37 (64) |
| MDS | 10 (17) |
| CML | 6 (10) |
| JMML | 4 (7) |
| Others | 1 (2) |
| T-cell depletion (%) | 21 (36) |

Allo-SCT, allogeneic stem cell transplantation; CML, chronic myelogenous leukaemia; IRD, identical related donor; JMML, juvenile myelomonocytic leukaemia; MDS, myelodysplastic syndrome; MUD, matched unrelated donor; ORD, other related donor.

A cohort of 58 paediatric patients receiving allo-SCT for haematological malignancies between January 2001 and January 2005 were included as a reference group. As this cohort was frequently monitored for AdV infection (see below), it was included to assess the efficacy of screening for AdV DNA at 1, 3 and 6 months following allo-SCT as applied for the adult patients. The main characteristics of these patients are shown in Table 2. General institutional policy with respect to patients' informed consent for inclusion into the study, approved by the ethical institutional board, was applied.

***Transplantation***

In adult patients, T-cell depleted transplantation was performed either according to a RIC protocol or to a conventional MAC protocol as described previously.[16,17] The RIC regimen consisted of fludarabine (30 mg/m$^2$, intravenously, day –10 to –6), busulphan (3.2 mg/kg, intravenously, day –6 and –5) and ATG (Horse, Genzyme) (10 mg/kg/d intravenously, day –4 to –1), for both matched sibling and matched unrelated donor (MUD) grafts. Prophylaxis for GvHD was not administered. The MAC regimen consisted of cyclophosphamide (60 mg/kg/d intravenously for two consecutive days) followed by a single dose of total body irradiation (TBI, 9 Gy, day –1) in patients receiving matched sibling donor grafts. In the MAC regimen, recipients of MUD grafts received additional Campath-1G or -1H (5 mg/d, day –8 and –4) and cyclosporin A (CsA) (3 mg/kg intravenously, starting on day –1) and fractionated TBI

(6 Gy, day –8 and –7). The stem cell product was infused on day 0. In all conditioning regimens, T-cell depletion of the graft was performed by *in vitro* incubation of the graft with Campath-1H (20 mg).
Assessment of acute and chronic GvHD was performed using the Glucksberg and Shulman criteria respectively.[18, 19] In the absence of GvHD or graft failure, patients received donor lymphocyte infusion (DLI) 6–9 months after RIC transplantation or at the occurrence of mixed chimerism or relapsed disease after MAC transplantation. DLI was never administered before 6 months following transplantation.
In paediatric patients, the sources of stem cells were Human leukocyte antigen (HLA) matched related donors, other related donors and MUDs. Pretreatment of the graft recipient was performed in accordance with disease-specific protocols of the relevant working parties of the European Group for Blood and Marrow Transplantation. Rabbit ATG (IMTIX; total dose 10 mg/kg given in 4 d) or Campath-1H (total dose 1 mg/kg given in 5 d) was given shortly before the transplantation date to all SCT recipients of a graft of another than a matched related donor. GvHD prophylaxis consisted of CsA (trough level, 100–200 mg/L). For AdV viraemia, ribavirin (60 mg/kg/d; starting dose, 30 mg/kg) or cidofovir (1 mg/kg/3 times a week) was given to paediatric patients. Intravenous immunoglobulin substitution was given to patients with grafts from unrelated donors for 3–6 months after SCT. In adult as well as in paediatric patients, pre-emptive ganciclovir treatment was administered for proven (i.e. real-time quantitative PCR based) CMV viraemia.

***Monitoring the occurrence and course of adenovirus infection***
In all adult patients AdV DNA load in plasma was initially measured at 1, 3 and 6 months following transplantation. From patients positive in this initial screening (>500 copies AdV DNA/mL), all available plasma samples during the first 180 d following transplantation were subjected to quantitative AdV PCR according to the method previously described.[15]
From 2001 onwards, all paediatric allo-SCT recipients in the paediatric transplantation unit of the Leiden University Medical Centre were prospectively analysed for the occurrence of AdV infection. Screening methods during 6 months following SCT included quantitative AdV PCR on serum or plasma samples. Samples were obtained weekly during the first 8–12 wk and every 2–4 wk thereafter.
Disseminated AdV infection was defined as the presence of >1000 copies/mL AdV DNA in $\geq$2 consecutive plasma samples. This definition is based on results from a previous study.[8] AdV disease was considered to be present when clinical signs and symptoms such as fever, haemorrhagic cystitis or enteritis, upper-or lower-tract infection, possibly confirmed by X ray (i.e. localised disease) or manifestation of infection in other organs such as the liver and the central nervous system (i.e. disseminated disease) were pres-

ent in patients with an AdV infection and without an alternative explanation for the disease. The identification of AdV in tissue specimens taken at biopsy or at autopsy was the definitive proof of AdV-disease and AdV-related death respectively.

## Results

*Patient characteristics and adenovirus infection*

A total of 107 adult patients were included in this study. Relevant characteristics of the patients included are shown in Tables 1 and 2. In five of 107 adult patients (4.7%), AdV DNA was detected in at least one sample during the initial screening at 1, 3 and 6 months following transplantation. Further analysis showed that disseminated AdV infections, as demonstrated by the presence of >1000 copies AdV DNA/mL in $\geq$2 consecutive plasma samples, occurred in all five patients (Table 3).

From five adult patients with AdV viraemia, three received RIC and two MAC (Table 3). Also the duration of AdV viraemia as well as peak AdV loads was comparable in both conditioning groups. However, AdV viraemia was detected early (before 100 d) following transplantation in the patients receiving RIC compared with MAC (Table 3). The onset of AdV infection in adults was at a median of 49 d (range: 22–148 d) following transplantation. Median AdV peak load was 7.0 $\log_{10}$ copies/mL (range: 4.5 $\log_{10}$–10.3 $\log_{10}$) and the median duration of AdV infection was 44 d (range: 21–98 d).

*Efficacy of AdV screening at 1, 3 and 6 months after transplant*

In the paediatric cohort, disseminated AdV infections were observed in eight of the 58 (13.8%) allo-SCT recipients (Table 4). Screening for AdV DNA at 1, 3 and 6 months following allo-SCT would have detected seven of these eight paediatric patients with AdV infection, only missing patient 3 (Table 4). This patient died before 90 d following allo-SCT, with a disseminated AdV infection despite treatment with ribavirin (Table 4).

*Clinical and virological findings in adults*

One 60-yr-old male patient (patient 4) receiving allo-SCT for chronic lymphocytic leukaemia died with severe hepatitis, enterocolitis and concurrent high AdV DNA levels in plasma (Table 3). AdV serotype 1 was recovered from cell cultures of intestinal biopsies and stool samples and GvHD were absent.

These complications occurred following the second allo-SCT from his HLA identical sister, after RIC; the first allo-SCT in this patient, following MAC, was performed 57 wk prior to the second, and was unsuccessful because of inadequate engraftment.

**Table 3.** Characteristics and outcome of adult SCT patients with AdV viraemia

| Patient | Age (yr)/ sex | Donor type | Conditioning | AdV viraemia | | | | Clinical outcome | Course of AdV viraemia |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Onset after SCT (d) | Duration (d) | Peak load (log cps/mL) | Antivirals | | |
| 1 | 59/F | Related | RIC | 46 | 56 | 5.5 | No | No symptoms | Resolved |
| 2 | 40/M | Unrelated | RIC | 49 | 98 | 7.0 | No | No symptoms | Resolved |
| 3 | 55/F | Unrelated | RIC | 22 | 21 | 8.0 | No | No symptoms | Resolved |
| 4 | 60/M | Related | MAC | 148 | 27 | 10.3 | CDV | Died of adenohepatitis | Increase |
| 5 | 47/M | Related | MAC | 137 | 44 | 4.5 | No | No symptoms | Resolved |

AdV, adenovirus; CDV, cidofovir; MAC, myeloablative conditioning; RIC, reduced intensity conditioning; SCT, stem cell transplantation.

**Table 4.** Characteristics and outcome of paediatric SCT patients with AdV viraemia

| Patient | Age (yr)/ sex | Donor type | TCD/Cam-path/ATG | AdV viraemia | | | Antivirals | Clinical outcome | Course of AdV viraemia |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Onset after SCT (d) | Duration (d) | Peak load (log cps/mL) | | | |
| 1 | 1/F | MUD | Yes/no/yes | 10 | 21 | 6.7 | No | No symptoms | Resolved |
| 2 | 3/F | MUD | No/no/yes | 19 | 37 | 7.9 | CDV | No symptoms | Resolved |
| 3 | 13/F | MUD | No/no/yes | 47 | 26[1] | 5.5 | RBV | Multiple causes of death including AdV | Increase |
| 4 | 11/F | MUD | No/no/yes | 33 | 23[1] | 7.8 | CDV and RBV | Died of AdV infection | Increase |
| 5 | 4/F | MUD | Yes/yes/no | 19 | 71 | 7.0 | CDV | Died of GvHD grade IV | Resolved after DLI |
| 6 | 1/M | MUD | No/no/yes | 14 | 40[2] | 6.0 | CDV | Died of AdV infection | Increase |
| 7 | 1/F | MUD | Yes/no/yes | 19 | 37 | 5.6 | CDV | Haemorrhagic cystitis | Resolved |
| 8 | 17/M | ORD | Yes/yes/yes | 19 | 56[2] | 4.1 | CDV | No symptoms | Resolved |

1 AdV presentation still present at decease.
2 Censored at day of second transplantation.
AdV, adenovirus; ATG, anti-thymocyte globulin; CDV, cidofovir; DLI, donor lymphocyte infusion; GvHD, graft-versus-host disease; MUD, matched unrelated donor; ORD, other related donor; RBV, ribavirin; SCT, stem cell transplantation; TCD, T-cell depletion of graft.

Treatment with cidofovir was initiated at a late stage and 1 wk later the patient succumbed because of AdV hepatitis. No symptoms particularly related to AdV infections were present in the four other adult patients with transient AdV viraemia and none of these four patients was treated for AdV infections with cidofovir or ribavirin (Table 3). The overall mortality at 6 months following allo-SCT was not significantly different for patients with compared with patients without AdV viraemia (57.8% vs. 40.0% respectively).

***Lymphocyte counts and AdV DNA load in plasma from adult patients***

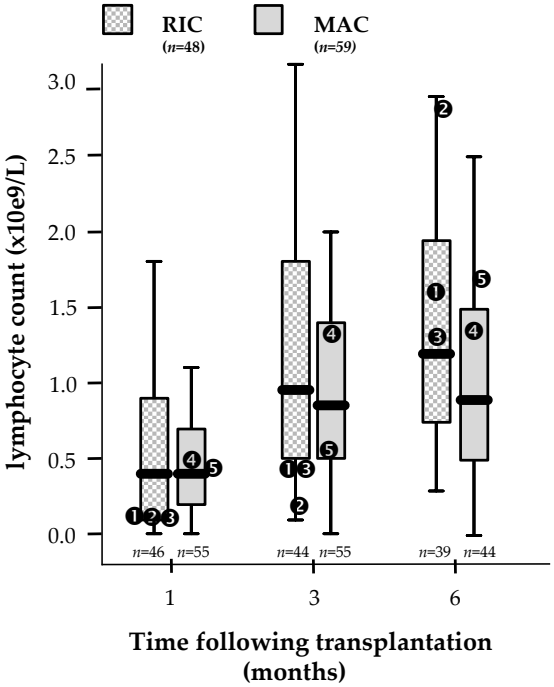
Lymphocyte recovery in adult patients receiving allo-SCT following either RIC or MAC was evaluated by assessing cell counts at 1, 3 and 6 months following allo-SCT. No significant difference in lymphocyte recovery following transplantation was observed with respect to both conditioning groups (Fig. 1).

Patients 1, 2, and 3 developed AdV viraemia early (within 100 d) following allo-SCT (Fig. 2). Concurrently, lymphocyte cell counts in these patients were substantially lower compared with the group of RIC patients, until after 3 months following allo-SCT (Fig. 1); AdV viraemia had spontaneously been resolved by then (Fig. 2). Patients 4 and 5 showed no significant different pattern in lymphocyte recovery following allo-SCT compared with the adult patients (Fig. 1); these two patients developed AdV viraemia late (beyond 100 d) following transplantation despite apparently normal lymphocyte counts. Furthermore, in four of five patients without clinical symptoms related to AdV infection, clearance of AdV DNA coincided with a rise in blood lymphocytes (Fig. 2, panels 1, 2, 3 and 5). In one patient with disseminated AdV disease no increase in blood lymphocyte counts was seen, and shortly after the initiation of treatment with cidofovir the patient died because of severe hepatitis (Fig. 2, panel 4).

## Discussion

This study demonstrated a comparably low incidence of AdV-related complications, following allo-SCT in adult patients after two different conditioning regimens. Unlike previous reports on adult SCT recipients,[14,20] we report AdV viraemia in only five of 107 (4.7%) patients using quantitative real-time AdV PCR in plasma. Furthermore, in four of these five patients AdV viraemia was transient without clinical signs of AdV infection and only one patient developed AdV disease. With respect to this difference, it should be noted that in contrast with these previous reports[14,20] alemtuzumab *in vivo* was only used prior to SCT in recipients with MUDs and in MAC conditioning regimen. Hence, it has been reported that Alemtuzumab plays a role in delaying immune reconstitution following allo-SCT.[13] Accordingly, the high inci-

**Figure 1.**



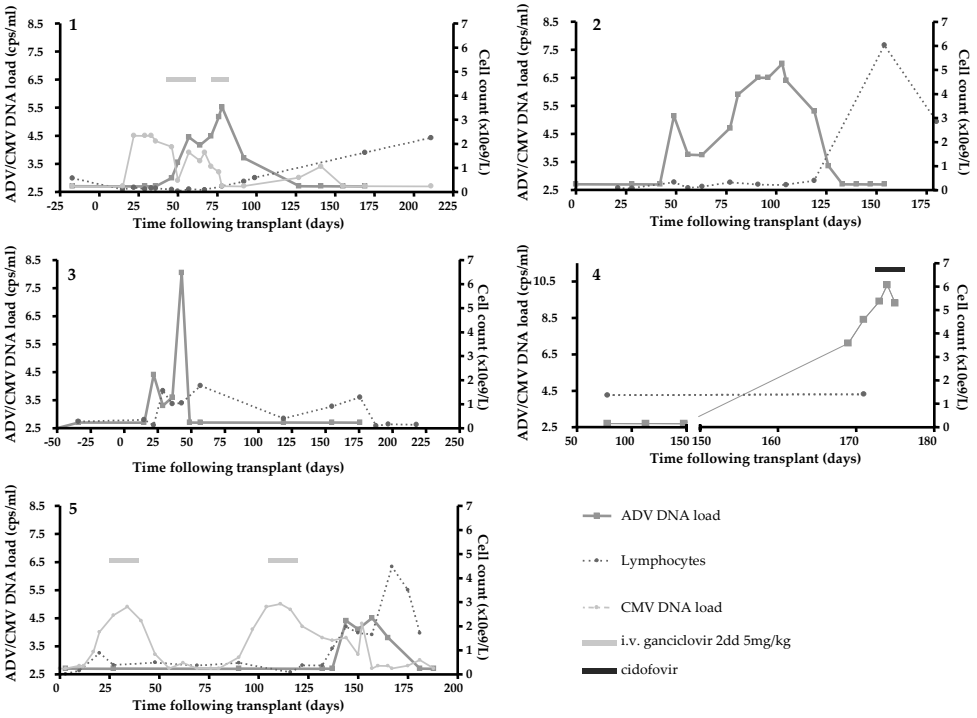

Lymphocyte recovery in adult allogeneic stem cell transplantation (allo-SCT) recipients following either fludarabine-based reduced intensity conditioning (RIC) or conventional myeloablative conditioning (MAC). Cell counts at 1, 3 and 6 months following transplantation are shown. The box plots display the median, the 25th and 75th percentiles (box), and the smallest and largest values (whiskers) in allo-SCT recipients without adenovirus (AdV) viraemia. Solid circles depict the values of individual patients with AdV viraemia and the patient numbers refer to Table 3.

dence of AdV infections in these previous studies is consistent with earlier reports on the increased risk for other viral infections (i.e. CMV and respiratory infections) after RIC using Alemtuzumab.[13,21]

As the association between detection of AdV DNA in plasma or serum and AdV disease following allo-SCT is well established,[9,22] we systematically used this approach to assess the occurrence and course of disseminated AdV infection in allo-SCT recipients. In contrast, the incidence of severe AdV infections in adult patients as reported in previous studies was primarily based on less sensitive viral cell culture assays, which partially may account for the difference with our findings. In these previous reports, AdV DNA PCR, performed solely on some patients with positive culture results, was only positive in a minority of the cases.

The efficacy of monitoring for AdV DNA in plasma at 1, 3 and 6 months was validated using a reference group of paediatric allo-SCT recipients treated for haema-

**Figure 2.**




Adenovirus (AdV) and CMV DNA load (left y-axis in log copies/mL), lymphocyte reconstitution (right y-axis) and antiviral treatment data in adult patients with AdV viraemia following allo-SCT. The scale of the left y-axis was adjusted to the high-ADV load in patient 4, represented in panel 4. Patients 1 and 5 also had CMV viraemia following allo-SCT which was pre-emptively treated with i.v. ganciclovir.

tological malignancies. In this paediatric population, the applied screening protocol was able to identify nearly all cases. This would therefore rule out any substantial underestimation of the incidence of AdV infection in our adult population, because of infrequent monitoring at 1, 3 and 6 months. The incidence and the impact of disseminated AdV infection seemed to be higher in the paediatric cohort. However, there are major differences between the adult and paediatric cohort, for instance with respect to the percentage of matched family donors, the conditioning regimens, the use of post-transplant immune suppression, the use of Campath and pre-emptive AdV therapy. Therefore, any comparison of data with respect to AdV infection between the two cohorts in this study would be dubious. However, previous studies, have also described a higher incidence of AdV infections in children compared with adults.[3,12,23] It was speculated that this observation is a reflection of age-de-

pendent exposure to AdV.[24,25] Alternatively, it can be hypothesised that tonsils and adenoids represent a reservoir for AdV from which the virus can reactivate, as it has been shown that T cells in these organs contain AdV DNA.[26] Preceding removal of these organs for medical reasons or possible atrophy would protect preferably adult patients for this complication. A lack of reliable data on this aspect has hampered confirmation of this hypothesis until now.

The occurrence of transient high loads of AdV DNA in plasma without clinical symptoms is interesting. It has previously been reported that some patients carrying high loads of AdV DNA are able to resolve this situation in the absence of antiviral treatment. The strong correlation between lymphocyte recovery and clearance of AdV in previous reports strongly suggests a potential role of immunological reconstitution.[7,11,12] We also observed that clearance of AdV DNA in four patients coincided with an increase of lymphocyte counts in peripheral blood. In contrast, in the single adult patient who succumbed to AdV disease (patient 4), lymphocyte counts did not increase. Because of unsuccessful engraftment following the first allo-SCT, this patient received a second transplant, which accounted for a prolonged period of immune suppression. Analysis of immune reconstitution was confined to lymphocyte counts, as adequate samples for more detailed examination of lymphocyte subsets were not available. Although the small numbers in this study do not allow us to draw firm conclusions, these findings are in keeping with previous reports on the relevance of immune reconstitution with respect to clearance of AdV DNA in peripheral blood.

Ribavirin and cidofovir are the antiviral drugs most often used for the treatment of AdV infections. The efficacy of these two drugs has not been demonstrated unequivocally and has been questioned in the case of ribavirin.[27,28] It has also been suggested previously that ganciclovir, given for CMV prevention, may have a protective effect on AdV infection following allo-SCT.[14,29,30] We observed AdV viraemia during and following ganciclovir treatment in two adult allo-SCT recipients but small numbers prevented definite conclusions on the potential effect of ganciclovir on disseminated AdV infections.

In summary, viraemia occurred rarely and generally has a mild course following allo-SCT in adult patients either following a fludarabine, busulphan and ATG-based RIC regimen or conventional MAC. However, severe infections leading to a fatal outcome do occur in adult allo-SCT recipients, most likely because of a prolonged immunocompromised state. Unlike in paediatric patients, systematic monitoring of AdV DNA load in plasma of adult allo-SCT recipients is unlikely to be beneficial. However, clinical awareness of this complication is mandatory, and upon clinical suspicion it should lead to early identification of these patients at risk, by using plasma monitoring for AdV DNA.

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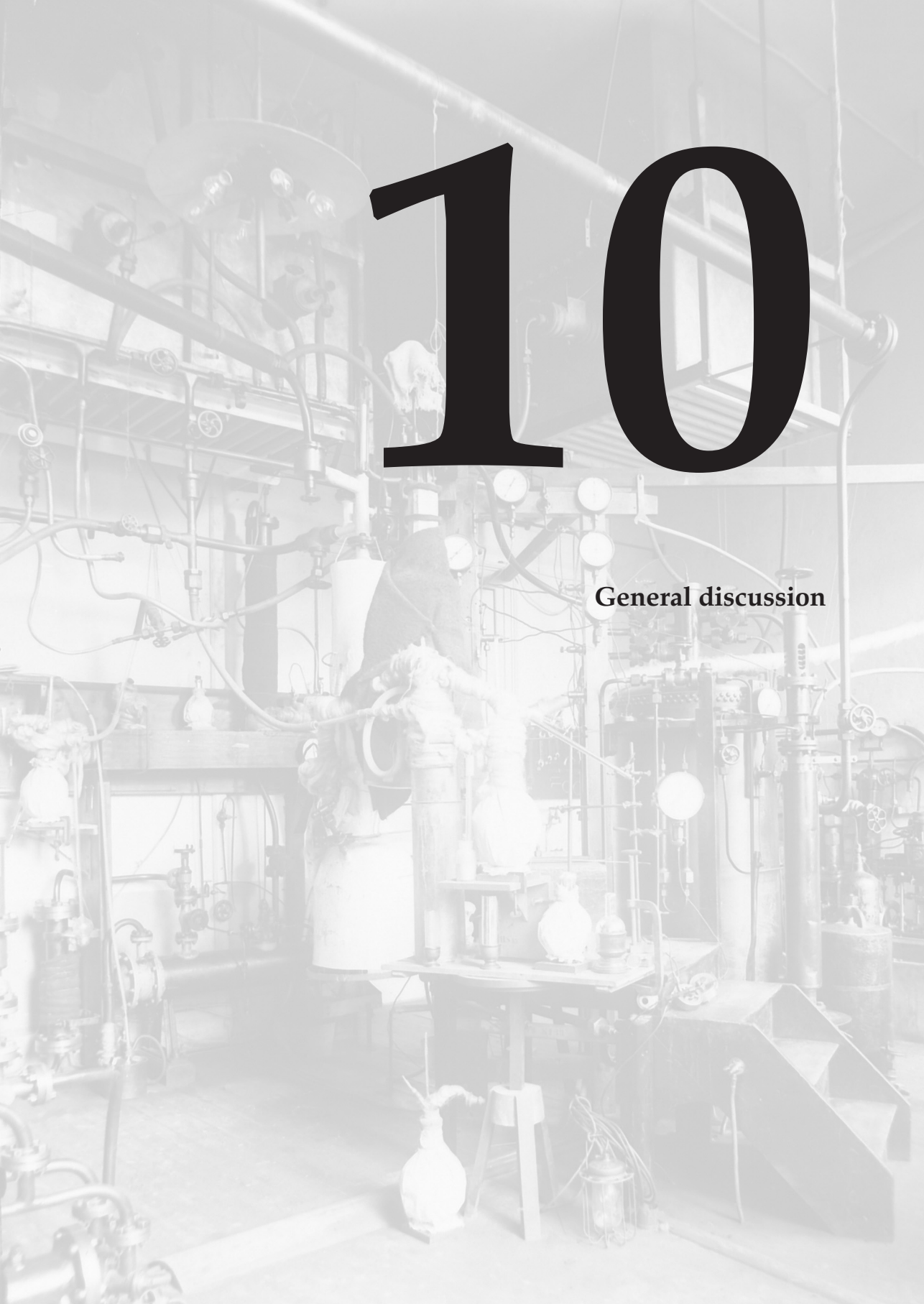

# 10

## General discussion

The concept of quantification is widely applied and has become indispensable in diagnostic microbiology, particularly as it enables the distinction between clinically relevant infection and commensal flora. Bacterial colony counts, for example, are instrumental in differentiating between infected urine and urine from normal persons contaminated by urethral flora.[1] Similar approaches based on quantitative differences are used for a wide range of diagnostic specimens in bacteriology, such as respiratory samples,[2] successfully reducing false positive results and, consequently unwarranted and undesired antibiotic usage.
Likewise, in chronic, or reactivating viral infections, merely demonstrating the presence of a virus in diagnostic samples does not necessarily indicate virus-associated disease. In addition, the clinical relevance of the quantitative detection of a virus without any information on time-dependent changes may be limited. Hence, viral kinetics rather than qualitative information allows the identification of the relevant infection and subsequent early intervention and monitoring of its effects. These quantitative principles have already been proven to be of great value in the clinical management of chronic viral diseases, particularly HIV, but also hepatitis B and hepatitis C virus infections. Advances in molecular technology have established improved PCR-based methods for viral quantification such as real-time quantitative PCR. The advantages of real-time PCR technology, which include excellent quantification, low contamination risk, ease of performance and speed, potentially warrant widespread routine clinical application of quantitative viral assays. Particularly in situations where a disturbed balance between host immunity and pathogen activity favors opportunistic viral infections, diagnostic approaches reflecting the status of this balance by the use of viral kinetics in time are essential. The studies described in the preceding chapters have demonstrated the wide range of applications of real-time quantitative PCR technology in clinical virology. The general implications of these studies will be discussed in the next sections.

**Cytomegalovirus (CMV)** has long been recognized as the most significant opportunistic pathogen in transplant recipients. Prevention of CMV-associated disease has been attempted using two strategies: general prophylaxis and pre-emptive therapy.[3-5] In prophylaxis, an antiviral agent is administered to all patients considered to be at risk, for a prolonged period, usually 90 to 100 days after transplantation. Pre-emptive therapy, on the other hand, is targeted towards a subset of patients identified by laboratory tests indicating early stages of viral replication, in an attempt to prevent the progression of asymptomatic infection to CMV disease. Pre-emptive therapy for CMV is started based on the detection of CMV in the blood and relies on rapid and sensitive diagnostic assays. The CMV pp65 antigenemia test has been widely employed to guide pre-emptive treatment of CMV infection following transplanta-

tion.[6-8] Quantitative real-time PCR has several advantages over the antigenemia test, including the increased sensitivity for early detection of CMV infection or reactivation, the applicability for patients with neutropenia, the stability of target DNA in blood specimens, the wide detection range of CMV DNA, the ability to process large number of specimens, the flexibility of time of transport and processing of specimens, and the increased accuracy of results. The results in chapter 2 demonstrated the positive correlation between the two assays and established optimal cut-off values and kinetic criteria for CMV DNA load in plasma. This enabled the definition of new guidelines for pre-emptive treatment in stem cell transplant (SCT) and solid organ transplant (SOT) recipients. Consequently, the pp65 antigenemia assay could safely be replaced by the real-time quantitative CMV PCR for the purpose of monitoring for CMV infection and guidance of antiviral treatment in transplant recipients.

The issue of prophylaxis versus pre-emptive CMV therapy following transplantation has been debated extensively and is still considered to be a matter of controversy.[9-11] As a basic principle in the management of infectious diseases, prophylactic administration of drugs should be limited to patients at the highest risks of infection and for a minimal duration. However, in the prophylactic CMV strategy, antiviral agents are administered to all patients in a broad category of transplant-associated risks and for a prolonged duration, although, in general, a vast majority of these patients will not need them. While this debate cannot be resolved easily, the studies described in this thesis demonstrate that the pre-emptive CMV treatment approach is at least technically feasible and practically effective.

Intravenous ganciclovir is generally used for the pre-emptive treatment of CMV infections following transplantation. With the introduction of valganciclovir, the oral formulation of ganciclovir, effective oral treatment for CMV infections became available as demonstrated for the prevention and treatment of CMV retinitis in AIDS patients and for prophylactic use in high risk solid organ transplant recipients.[12,13] In chapter 3, quantitative analysis of CMV DNA in plasma demonstrated a similar efficacy of pre-emptive CMV treatment using oral valganciclovir and intravenous ganciclovir following both allo-SCT and solid organ transplantation. Contrary to intravenous ganciclovir treatment, pre-emptive administration of oral valganciclovir enables treatment in an outpatient setting, avoiding unnecessary hospitalization. Recurrent CMV infections after treatment constitute a persistent problem, particularly in allogeneic stem cell transplant (allo-SCT) recipients. It is important to identify markers of such recurrent infections, also by careful studies of viral kinetics, to enable early recognition of patients who are likely to show a poor response to treatment.

The actual incidence of antiviral-resistant CMV and its impact on mortality and morbidity following transplantation is, as yet, unclear. However, with the increasing ease of ganciclovir administration, the accelerated development of ganciclovir-resistant

CMV mutants should be anticipated. Indeed, ganciclovir-resistant CMV is already increasingly recognized, particularly in solid organ transplant recipients.[14-17] Genotypic assays can be employed to detect specific mutation in the CMV genome, conferring antiviral resistance.[15,18] In order to enable early recognition of ganciclovir-resistant CMV and to guide treatment adjustment, the combined use of viral load monitoring and these genotypic assays should be evaluated.

The risk for opportunistic infections such as CMV following transplantation strongly depends on the level of immune suppression or the rate of immune recovery. Induction therapy in solid organ transplant recipients and conditioning regimens in allo-SCT recipients, contribute significantly to the risk for CMV infections post transplant. Accurate quantification of CMV DNA concentrations in blood, using the highly sensitive real-time quantitative PCR technology, can be applied as a marker with respect to the occurrence of opportunistic infections in general after various conditioning or induction regimens. This application was the subject of the studies described in chapters 4 and 5. The sequential quantification of DNA load in plasma was used to calculate the area under the curve of viral load over time (AUC). In this way, the AUC represents an integrated approach which includes various parameters that have been described as independent risk factors for CMV disease, such as peak viral load, initial viral load and rate of increase of viral load.[19] Using this approach, accurate viral quantification will enable an efficient comparison of the various regimens.

Furthermore, the evaluation of novel immunosuppressive approaches could benefit greatly from the precise monitoring of CMV reactivation. From the results in chapter 4 it can be concluded that with respect to (re)activation of CMV in solid organ transplant recipients, antibody induction therapy with daclizumab (anti-CD25) is safer than antibody induction therapy with polyclonal antithymocyte globulin (ATG), as CMV viremia was shown to occur later and less severe with daclizumab. As a consequence, careful monitoring for CMV infections in transplant protocols using induction or rejection therapy with ATG is indicated, as these patients are at a significantly high risk for CMV reactivation. Additionally, patients with type I diabetes are at a higher risk for recurrent autoimmunity following simultaneous pancreas-kidney transplantation.

The same approach including CMV DNA quantification and calculation of the area under the viremia curve was applied to assess the safety of reduced intensity and conventional myeloablative conditioning with respect to viral infections. Chapter 5 demonstrated the comparable severity of CMV reactivation following T-cell depleted allo-SCT preceded either by a fludarabine, ATG and busulphan-based reduced intensity or a conventional myeloablative conditioning regimen. Thus, patients receiving T-cell depleted allo-SCT preceded by reduced intensity conditioning do not

incur an increased risk for CMV infection. Conversely, when ATG is administered as induction or rejection therapy in solid organ recipients, earlier and more severe occurrence of CMV infections post-transplant should be anticipated.

**Epstein-Barr virus (EBV)** reactivation and subsequent progression to life-threatening EBV-related lymphoproliferative disease (EBV-LPD) is a much-feared complication, which is particularly frequent after allogeneic stem cell transplantation (allo-SCT). It has previously been shown that monitoring of EBV DNA in plasma in allo-SCT recipients at risk for EBV-LPD enables the recognition of the early stage of EBV-LPD development, facilitating pre-emptive treatment and, consequently, the prevention of EBV-LPD.[20-22] Pre-emptive treatment solely guided by EBV DNA monitoring inevitably results in some degree of overtreatment. Theoretically, this could be reduced if it were possible to employ any marker of specific immunological recovery. Combining the monitoring of the viral load of the infectious agent with the corresponding T-cell response capacity of the host, provides an attractive approach in the management of EBV reactivation (chapter 6) and presumably also in the management of CMV and other persisting viral infections, such as VZV, adenovirus and BKV, in transplant recipients. Evidently, the combined application of quantitative tools to analyze viral infections and quantitative tools to recognize different qualities of T cell reconstitution, also provides a means to study the relationship between the infectious agent and the immune system. Further evaluation of the interrelationship between viruses and specific T cells, can provide more detailed insights into these processes, potentially enabling the design of innovative future strategies with respect to the prevention and treatment of viral diseases.
Besides its application in the monitoring of transplant recipients, real-time quantitative EBV PCR proved to be of value in patients with nasopharyngeal carcinoma (chapter 7), which also constitutes a life-threatening EBV-associated disorder. In these patients it was demonstrated that EBV DNA load measurement can be applied for diagnostic as well as prognostic purposes and also accurately reflected treatment efficacy. This marker is therefore relevant in the management of all cases of this malignant disorder, also in areas with relatively low endemicity. Furthermore, besides its application in monitoring response to current therapeutic options for nasopharyngeal carcinoma, EBV DNA quantification can also be applied to develop and to assess the efficacy of new therapeutic options.[23-25]

**Varicella-zoster virus (VZV)** represents another herpesvirus that is known to reactivate in immunocompromised hosts. To explore the potential value of viral DNA detection in blood with regard to the risks associated with VZV infection, a study was designed combining regular virus monitoring and clinical surveillance (chapter 8).

In contrast to EBV and CMV, no preceding phase of detectable VZV DNA in plasma was recognized prior to disease development following allo-SCT. Apparently, clinical presentation coincides with VZV DNA detection in plasma upon viral reactivation following allo-SCT, and a pre-emptive strategy based on viral DNA quantification in plasma does not seem feasible. However, in contrast to cell-culture or immunofluorescence-based diagnostic assays, real-time VZV PCR can be used for reliable and rapid confirmation upon clinical suspicion of VZV reactivation. Quantification of VZV DNA can subsequently be applied to monitor the efficacy of treatment with aciclovir. The response as observed in individual patients could well be relevant with regard to the risk of repeated viral reactivations, which are potentially associated with further organ-specific complications. One such sequence of events is illustrated by the case described in chapter 8. The identification of the subset of patients at risk of serious and recurring reactivation of VZV is as yet very difficult. This should be approached by the combined application of accurate virological and specific immunological monitoring.
While viruses such as EBV and CMV have been known for many years to cause complications following transplantation, **adenoviruses (AdV)** have only recently emerged as important pathogens, particularly in the pediatric allo-SCT population.[26] Clinical presentations range from asymptomatic viremia to severe disseminated illness.[27] In contrast to pediatric allo-SCT recipients, adenovirus (AdV) reactivation is of limited clinical impact in adult patients regardless of the conditioning regimens described in chapter 9. However, disseminated AdV infections resulting in a fatal outcome may occur in adult patients, possibly due to a prolonged severe state of immune suppression as illustrated in this thesis. Also, the use of alemtuzumab as a part of conditioning regimens has been reported to impose a significant risk for severe disseminated AdV infections following allo-SCT.[28,29] Therefore, clinical awareness of this complication in adult allo-SCT recipients is essential. Early identification of these high risk adult patients can be achieved by using plasma monitoring of AdV DNA, which has already demonstrated its usefulness in the pediatric population.[26,27] Antiviral treatment options for AdV reactivation following transplant constitute a matter of controversy,[26,27] and studies that aim to identify novel drugs as well as studies to assess the efficacy and tolerability of currently available drugs are required. The use of quantitative diagnostic approaches has also proven to be of great value for this purpose.[30]

These examples from the field of clinical virology illustrate some interesting principles with regard to the value of a quantitative approach to describing the interactions between a virus and its infected host more precisely. In general, quantitative scientific research relies on the process of measurement which provides the funda-

mental connection between any empirical observation and a mathematical expression of relationships. Using quantitative methods, it is possible to derive precise and testable expressions from qualitative ideas. For that reason, quantitative research is widely used, particularly in natural sciences. The Dutch physicist Heike Kamerlingh Onnes was particularly concerned with the value and the importance of quantitative research in physics throughout his scientific career. In his inaugural lecture at Leiden University on 11 November 1882, he described this high regard for quantitative physical research, as it enabled the formulation of fundamental laws in physics, as well as the development of more precise instruments to enhance further research. This approach also relied on the development of a practical metric system to enable standardization. Interestingly, he expressed his appreciation of quantitative measurements in the form of a now well-known Dutch motto "Door meten tot weten" (Knowledge through measurement).[31] In 1913 Kamerlingh Onnes received the Nobel Prize in physics for "his investigations on the properties of matter at low temperatures which also led to the production of liquid helium". Obviously, the significance of Kamerlingh Onnes' famous expression is not restricted to physics. In clinical virology, the value of quantitative results, complementing a qualitative diagnosis, has also been established.

The results as discussed in the previous paragraphs demonstrate the various ways in which quantitative information on viral infections can be applied to clinical problems. In general, the interactions between the different factors that determine the occurrence and the course of the infectious disease can now be studied more precisely, when compared to the days of conventional qualitative results. This applies specifically to the effects of treatment as well as to the influences of immunological status and immunosuppressive regimens. Evidently, the applications described serve as examples to illustrate the broad potential of this quantitative approach in the care for patients with impaired immunity. In particular, this thesis underlines the general value of extending observations to the level of precise measurements in order to uncover additional relevant information from the data, which can then be applied successfully to the care of the patients threatened by viral infections. To underline the particular role of this approach, which in some ways constitutes a new paradigm, one could consider the designation 'quantum virology', in accordance with the title of this thesis.

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# 11

**Nederlandse samenvatting**

**Quantumvirologie,**

Verbetering van het klinische beleid bij virale infecties door middel van kwantitatieve virusmetingen

Virussen verschillen fundamenteel van andere verwekkers van infectieziekten zoals bacteriën, schimmels en protozoën. Een virus is geen levende cel zoals een bacterie dat wel is. Een virus kan het best omschreven worden als een partikel bestaande uit genetisch materiaal verpakt in een eiwitomhulsel en is als levensvorm gereduceerd tot het strikt noodzakelijke voor vermenigvuldiging. Een virus is erg klein, vele malen kleiner dan bacteriën en het kleinste infectieuze agens tot nu toe bekend, met uitzondering van infectieuze eiwitten (prionen). Echter, een virus is in staat een levende cel binnen te dringen en daar processen uit te voeren met als uiteindelijk doel nieuwe viruspartikels te produceren. Dit proces is essentieel voor de overleving van het virus maar kan gelukkig niet ongestoord plaats vinden. Immers, het afweersysteem van de mens is er altijd op gericht virussen te herkennen en te elimineren waardoor een infectie onder controle gehouden kan worden. Het menselijk lichaam kan dus gezien worden als een slagveld, waarbij virussen de aanval, en het afweersysteem de verdediging vormen. Door de jaren heen hebben zowel virussen als de mens zich steeds aan elkaar aangepast. De één (het virus) met als voornaamste doel zo efficiënt mogelijk het afweersysteem te omzeilen, en de ander (de mens) om het virus zo goed mogelijk te bestrijden. Deze aanpassingen hebben er mede toe geleid dat een virale infectie uiteindelijke verschillende uitkomsten kan hebben. Een infectie kan, voordat het virus opgeruimd wordt door het afweersysteem, leiden tot destructie van de geïnfecteerde cel en acute ziekte veroorzaakt door de afweerreactie, zoals dat bij influenza virussen en andere virussen van het ademhalingssysteem gebeurt. Als na een infectie het virus niet volledig geëlimineerd wordt, dan kan er een persisterende infectie ontstaan. Persisterende virale infecties kunnen chronisch-actief of latent zijn. Bij chronische-actieve virale infecties blijven er actieve virussen aanwezig in de geïnfecteerde cellen en wordt er gedurende een lange periode nieuwe virussen geproduceerd. Dit ziet men onder andere bij Hepatitis B (HBV) en Hepatitis C (HCV) virussen die de lever infecteren, maar ook bij infecties met het Humaan Immunodeficiëntie virus (HIV). Een latente virale infectie kenmerkt zich door de aanwezigheid van inactieve virussen in de cel waarbij het virus zich niet meer vermenigvuldigt. Dit is typisch voor herpesvirussen, zoals het herpes simplex virus (HSV), de veroorzaker van een “koortslip”. Een typisch kenmerk van deze virussen is dat zij wel in staat zijn te reactiveren vanuit een latente infectie om zo opnieuw infecties te veroorzaken. Dit ziet men vooral als de balans tussen afweer en het virus verstoord raakt bijvoorbeeld door ziekte of door medisch handelen. Naast acute en persisterende infecties kan een virale infectie leiden tot veranderingen in het gedrag van de geïnfecteerde cel wat uiteindelijk (na vele jaren) kan resulteren in het ontstaan van tumoren. Dit is onder andere bekend van het humaan papilloma virus (HPV) welke geassocieerd is met baarmoederhalskanker en het Epstein-Barr virus (EBV) welke geassocieerd is met tumoren van bloedcellen (lymfomen) en van de mond- en keelholte (nasofarynxcarcinomen).

De mens draagt dus vele virussen bij zich waar men gelukkig meestal niet veel van merkt. Echter, dit verandert als ziekte of medische ingrepen leiden tot een verandering in de balans die er bestaat tussen afweer en virus. Virale verwekkers krijgen en grijpen dan veelal die kans en veroorzaken infecties. In de laatste decennia is het aantal patiënten met afweerstoornissen sterk gegroeid. Hiertoe hebben de voortschrijdende wereldwijde HIV epidemie, intensievere en succesvolle chemotherapie voor kanker en in het bijzonder de beschikbaarheid van zeer potente afweer verlagende middelen voor orgaantransplantatiepatiënten, een significante bijdrage geleverd.
Het succes van orgaan- en stamceltransplantaties is voornamelijk te danken aan het feit dat afstotingsreacties en infecties beter voorkomen en bestreden kunnen worden. Echter, virale infecties vormen bij deze patiënten nog steeds een ernstige bedreiging vooral als gevolg van langdurige afweer verlagende therapie. Immers, het risico op reactivatie van chronische virale infecties neemt toe naarmate de duur en intensiteit van de afweerverlaging toeneemt. Voornamelijk de herpesvirussen, zoals Cytomegalovirus (CMV), Epstein-Barr virus (EBV), Varicella-zostervirus (VZV) en Herpes Simplex virus (HSV), maar ook adenovirussen kunnen, vooral in de eerste maanden na transplantatie, voor ernstige complicaties zorgen.
Echter, sterke verbeteringen in de diagnostiek, in het bijzonder door moderne moleculair-biologische technieken maken het mogelijk om deze persisterende infecties te volgen. Veel nauwkeuriger dan ooit is men nu in staat te volgen hoe de balans tussen afweer en virussen uit evenwicht raakt en virusinfecties tijdig te detecteren om daar iets aan te doen. In de praktijk ligt hier het belang van de kwantitatieve diagnostiek in de klinische virologie. Immers, hierdoor kan naast het vaststellen dat er sprake is van een infectie vooral bepaald worden hoe actief die is en welke gevolgen die zou kunnen hebben. Aldus krijgt men door het kwantitatief benaderen van virusinfecties inzicht in zowel het klinische beloop, de besmettelijkheid en de resultaten van therapie. Bij deze kwantitatieve benadering gaat het meestal om het meten van virusdeeltjes in het bloedplasma. Het zijn vooral recente technische ontwikkelingen geweest die het uitvoeren van deze metingen op het niveau van virale nucleïnezuren (DNA of RNA) eenvoudig uitvoerbaar hebben gemaakt en de praktische toepasbaarheid daarvan sterk hebben bevorderd. Het voornaamste principe van deze technieken berust op de “Polymerase Chain Reaction” (PCR), een techniek om uit zeer kleine hoeveelheden DNA of RNA specifiek één of meer gedeeltes te vermeerderen tot er genoeg van is om het te analyseren. Hiermee is betrouwbare kwantificering haalbaar. Een belangrijke vooruitgang op het gebied van de PCR betreft de mogelijkheid om het gevormde product tijdens de reactie zelf en niet pas na afloop te detecteren. Deze benadering, ook wel “real-time” PCR of kinetische PCR genoemd, is relatief eenvoudig uitvoerbaar en bied de mogelijkheid nauwkeurig te kwantificeren. De “real-time” PCR heeft een aantal belangrijke voordelen ten opzichte van de oudere

kwantificering technieken, zoals de hogere gevoeligheid en specificiteit, waardoor er minder kans bestaat op fout positieve of fout negatieve resultaten. Verder verkort deze benadering de duur van de totale bepaling in sterke mate en is het mogelijk om meerdere virussen tegelijk te detecteren en nauwkeurig te kwantificeren. De klinische toepassingen van de moderne kwantitatieve virusdiagnostiek zijn nog lang niet voldoende geëvalueerd. De interpretatie van de metingen van verschillende virussen en in verschillende situaties en de relevantie ervan voor de klinische praktijk zullen nog duidelijk moeten worden.

Dit proefschrift exploreert en beschrijft diverse potentiële toepassingen van "Real-time" kwantitatieve PCR in de klinische virologie en richt zich voornamelijk op de volgende aspecten. Enerzijds op het ontwikkelen van "real-time" kwantitatieve PCR's voor specifieke virussen. Daarnaast op de toepassing van deze PCR's in relevante patiëntenpopulaties en waar mogelijk de vergelijking met al bestaande virus kwantificeringsmethoden. Tevens wordt nadruk gelegd op de klinische relevantie van deze kwantitatieve real-time virus PCR's. Anderzijds richt dit proefschrift zich op virussen die in staat zijn persisterende infecties te veroorzaken waarbij er dus een delicaat evenwicht bestaat tussen het virus en het afweersysteem van de gastheer; een evenwicht dat indien verstoord, kan leiden tot vermeerdering van het virus en een hernieuwde infectie.

Cytomegalovirus (CMV), een herpesvirus, is één van de meest beruchte veroorzakers van ernstige infecties na stamcel- of orgaantransplantaties. **Hoofdstuk 2** beschrijft de ontwikkeling en de klinische evaluatie van een real-time PCR voor het detecteren en kwantificeren van CMV DNA in bloedplasma. Aan de hand van de klinische evaluatie in stamcel- en orgaantransplantatiepatiënten, en een vergelijking met de reeds bestaande kwantificeringsmethode (de pp65 antigeentest), werden criteria gedefinieerd voor zogenoemde pre-emptieve CMV therapie op basis van CMV DNA concentraties in bloedplasma. Pre-emptieve therapie duidt op het beginnen van behandeling op het moment dat er significante virusvermeerdering wordt gesignaleerd, in dit geval aan de hand van virusconcentratie in het bloedplasma, nog voordat er symptomen van een (lastig behandelbare) infectie aanwezig zijn.

Voor pre-emptieve CMV therapie in transplantatiepatiënten is intraveneuze toediening van het antivirale middel ganciclovir de eerste keus. Een nadeel van ganciclovir is dat het voor een effectieve toepassing, per se intraveneus toegediend moet worden, waarvoor vaak een opname in het ziekenhuis vereist is. Recent is valganciclovir, een orale vorm van ganciclovir, beschikbaar gekomen. Echter, de effectiviteit van pre-emptieve CMV therapie met het oraal toegediende valganciclovir ten opzichte van het intraveneus toegediende ganciclovir dient nader vastgesteld te worden. In **hoofdstuk 3** worden de effectiviteit en de veiligheid van pre-emptieve therapie met intraveneus ganciclovir en oraal valganciclovir geëvalueerd en met elkaar vergele-

ken. Door CMV DNA concentratie in bloedplasma te gebruiken als een marker van het effect van therapie werd aangetoond dat beide middelen voor pre-emptieve CMV therapie vergelijkbaar effectief en veilig zijn in zowel stamcel- als orgaantransplantatiepatiënten.
Om de kans op een afstotingsreactie na stamcel- of orgaantransplantatie te beperken worden patiënten voorafgaand aan een transplantatie behandeld met afweerremmende middelen (immuunsuppressiva). Een keerzijde van deze immuunsuppressieve behandelingen is het verhoogde risico op met name CMV infecties. De mate waarin afweerremmende middelen het risico op en het beloop van deze virale infecties beïnvloeden, kan voor de verschillende combinaties waarin deze middelen gebruikt worden verschillen. De **hoofdstukken 4 en 5** beschrijven de toepassing van CMV DNA concentratiemeting in bloedplasma als een veiligheidsmarker voor virusinfecties na verschillende afweerremmende behandeling schema's in transplantatiepatiënten. Deze methode werd in **hoofdstuk 4** toegepast voor de afweerremmende middelen daclizumab en ATG bij gecombineerde nier- en pancreastransplantaties. Het bleek dat CMV infecties vaker optreden na behandeling met ATG en ook een ernstiger beloop hebben; een complicatie waarop nu geanticipeerd en adequaat gereageerd kan worden bij het gebruik van ATG. In **hoofdstuk 5** werd voor stamceltransplantatiepatiënten aangetoond dat afweerremmende therapie met een "gereduceerde intensiteit" een vergelijkbaar risico oplevert op CMV infecties, vergeleken met de conventionele immuunsuppressieve behandeling.

Het Epstein-Barr virus (EBV), een ander herpesvirus, is bij gastheren met een normale afweer ook wel bekend als de veroorzaker van de ziekte van Pfeiffer. EBV veroorzaakt, evenals alle herpesvirussen, een latente infectie en kan bij patiënten met een afweerstoornis reactiveren en voor ernstige complicaties zorgen. Vooral in stamceltransplantatiepatiënten leidt reactivatie van EBV tot een potentieel kwaadaardige aandoening van bepaalde witte bloed cellen (B-lymfocyten) waarin het virus aanwezig is. Deze aandoening wordt ook wel posttransplantatie lymfoproliferatieve ziekte genoemd (EBV-Post-Transplant Lymphoproliferative Disease; EBV-PTLD). Het ontstaan van EBV-PTLD wordt gekenmerkt door een stijging van de EBV DNA concentratie in het bloedplasma. Echter niet alle patiënten bij wie na transplantatie EBV-DNA in het bloed gemeten wordt zullen een PTLD ontwikkelen; bij een deel wordt deze virusreactivatie spontaan onderdrukt door het (herstellende) afweersysteem van de patiënt. **Hoofdstuk 6** beschrijft, aan de hand van een studie in kinderen die een stamceltransplantatie hebben ondergaan, hoe patiënten met een risico op EBV-PTLD met een hoge mate van precisie kunnen worden opgespoord en behandeld. Dit werd bewerkstelligd door de EBV DNA concentratie in het bloedplasma te volgen en gelijktijdig het afweersysteem van de patiënt te analyseren. Hierdoor kan een gebalanceerde en

effectieve zorg voor patiënten met risico op EBV-PTLD worden gerealiseerd doordat alleen patiënten die dat echt nodig hebben blootgesteld worden aan therapie.
Een andere EBV-geassocieerde kwaadaardige ziekte betreft het nasofarynxcarcinoom (nasopharyngeal carcinoma, NPC) dat vooral voorkomt in Zuid-oost Azië. Bij deze patiënten kan de EBV DNA concentratie in plasma gebruikt worden als een tumormarker voor diagnostische, therapeutische en prognostische doeleinden. **Hoofdstuk 7** illustreert hoe EBV DNA concentratie bepalingen in bloedplasma ook in de Nederlandse situatie effectief toegepast kunnen worden als een tumormarker bij patiënten met nasofarynxcarcinomen.

Varicella-zoster virus (VZV, ook wel waterpokken-virus) is een derde herpesvirus dat verantwoordelijk is voor infectieuze complicaties in transplantatiepatiënten. **Hoofdstuk 8** beschrijft een studie naar de klinische relevantie van VZV DNA concentratiemetingen in bloedplasma van stamceltransplantatiepatiënten. Uit deze studie kwam naar voren dat de klinische symptomen van een VZV infectie geheel samenvallen met de detectie van viraal DNA in bloedplasma. In tegenstelling tot CMV en EBV infecties was er geen fase van detecteerbaar VZV DNA, voorafgaand aan de klinische symptomen. Hierdoor wordt pre-emptieve therapie, op basis van VZV DNA detectie in plasma, zoals dat toegepast wordt bij CMV en EBV infecties, dus lastig. Echter, de real-time VZV PCR bleek zeer geschikt om de diagnose bij klinische verdenking op VZV gerelateerde ziekte te bevestigen en voor het monitoren van het effect van VZV therapie met het antivirale middel aciclovir.

In tegenstelling tot de herpesvirussen zijn ernstige infecties met adenovirussen (AdV) na stamceltransplantatie een recent fenomeen, dat vooral optreedt bij kinderen. Adenovirussen veroorzaken bij gezonde kinderen veelal slechts een luchtweginfectie, maar kunnen fataal zijn na een stamceltransplantatie. **Hoofdstuk 9** beschrijft het voorkomen van adenovirusinfecties bij volwassen stamceltransplantatiepatiënten. In deze studie werd met behulp van een kwantitatieve real-time PCR de AdV DNA concentratie in plasma op regelmatige tijdstippen na transplantatie gemeten. In tegenstelling tot bij kinderen komen AdV infecties na stamceltransplantatie bij volwassen zelden voor. Daarom is het systematisch monitoren van AdV DNA in plasma bij alle volwassen stamceltransplantatiepatiënten, gezien de lage incidentie van AdV infecties, niet zo efficiënt als dat bij kinderen is. Echter, uit hoofdstuk 9 blijkt ook dat fatale AdV infecties, hoewel zeldzaam, wel degelijk in de volwassen populatie kunnen optreden. Voor deze volwassen stamceltransplantatiepatiënten met een verhoogd risico op adenovirusinfecties kan vroegtijdige opsporing met behulp van AdV DNA detectie in plasma en pre-emptieve interventie wellicht uitkomst bieden.

De implicaties van de onderzoeken beschreven in de hoofdstukken 2 tot en met 9 worden besproken in **hoofdstuk 10**. Deze voorbeelden illustreren de interessante toepassingen en de waarde van een kwantitatieve benadering van virale infecties in de klinische virologie. Over het algemeen staat het proces van meten centraal in wetenschappelijk onderzoek. Kwantitatieve metingen maken het mogelijk kwalitatieve (empirische) observaties nauwkeurig en verifieerbaar te beschrijven. Kwantitatieve meetmethoden worden voor wetenschappelijk onderzoek daarom veelvuldig gebruikt, voornamelijk in de natuurwetenschappen. Zo heeft de Nederlandse natuurkundige Heike Kamerlingh Onnes (1853-1926) gedurende zijn wetenschappelijke carrière het belang en de waarde van kwantitatief onderzoek in de natuurkunde veelvuldig benadrukt. Zijn waardering voor kwantitatief onderzoek is terug te vinden in zijn beroemde motto: "Door meten tot weten". Het mag duidelijk zijn dat de relevantie van Kamerlingh Onnes zijn beroemde uitdrukking niet beperkt is tot de natuurkunde.

Dit proefschrift benadrukt de meerwaarde van kwantitatieve metingen ten opzichte van kwalitatieve waarnemingen in de klinische virologie. In het bijzonder illustreert dit proefschrift de mogelijkheden van de kwantitatieve benadering van virale infecties ter verbetering van de zorg voor immuungestoorde patiënten met virale infecties of andere virusgerelateerde aandoeningen zoals PTLD. Om deze specifieke rol te accentueren is die kwantitatieve benadering in de klinische virologie hier ook aangeduid met "quantumvirologie", evenals in de titel van dit proefschrift.

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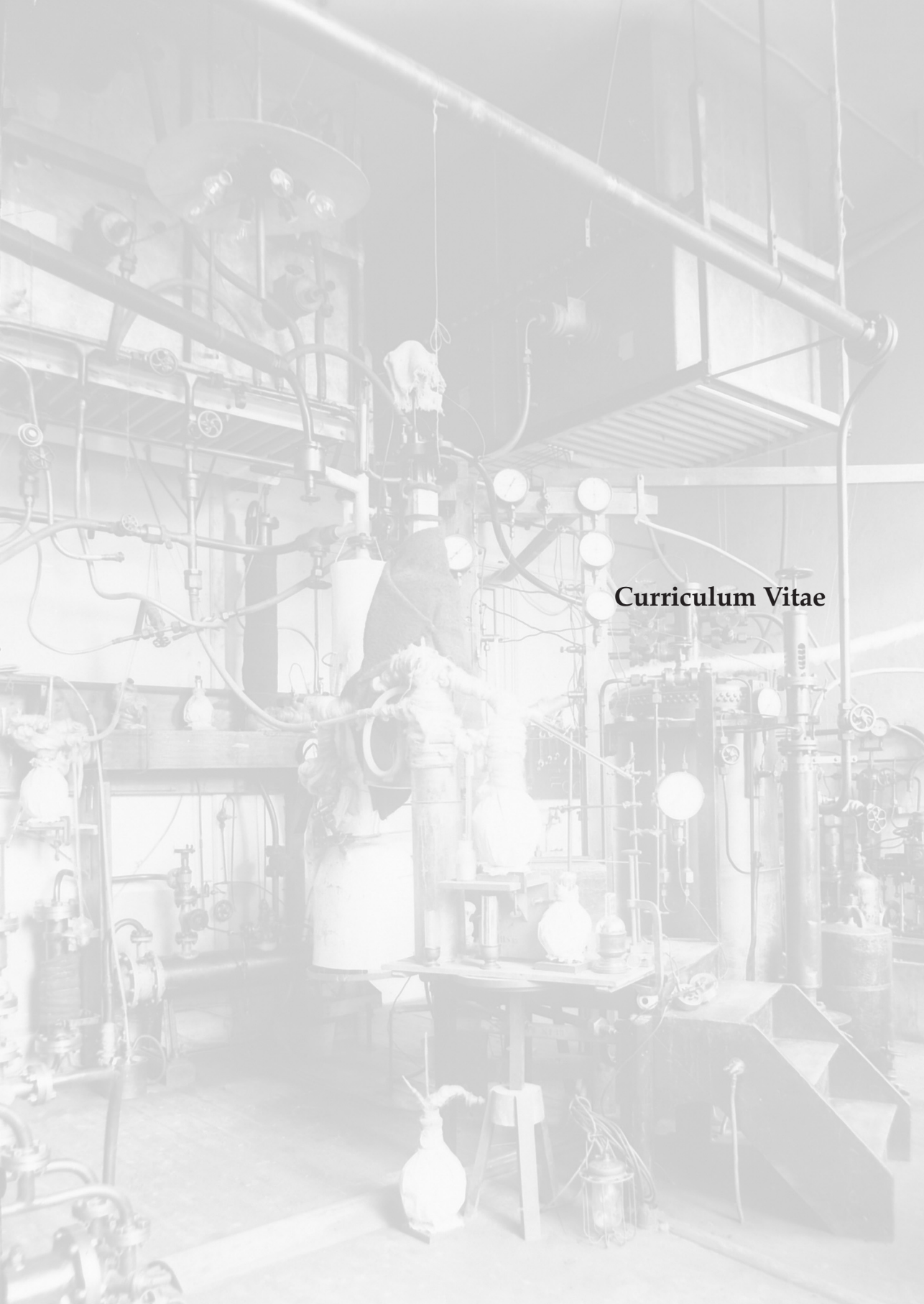
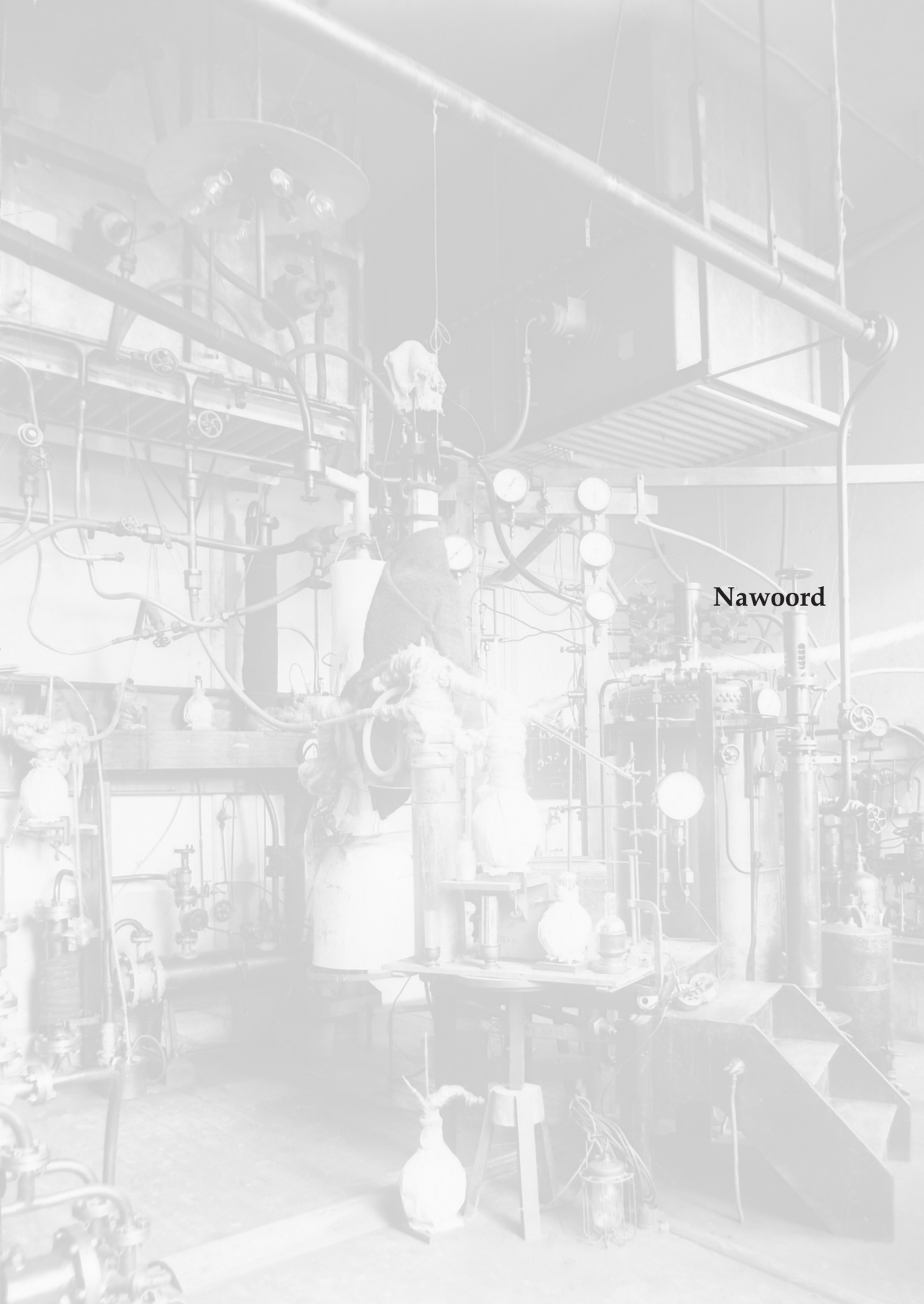


# Curriculum Vitae

## CURRICULUM VITAE

Jayant Kalpoe werd geboren op 23 juli 1973 te Wageningen in Suriname. Hij groeide op in Suriname en doorliep zijn middelbare school van 1985 tot en met 1991 aan het Mr. Dr. J.C. de Miranda Lyceum in Paramaribo. Na het behalen van het eindexamen VWO vertrok hij in 1992 naar Nederland en begon in hetzelfde jaar aan zijn studie Biomedische Wetenschappen aan de Rijksuniversiteit Leiden. Hij liep stage op de afdeling Moleculaire Virologie (LUMC, Leiden) bij dr. E.J. Snijder en prof. dr. W.J.M. Spaan. In 1995 participeerde hij in een uitwisselingsprogramma met de Universiteit van Oxford en deed hij onderzoek naar matabotrope glutamaat receptoren aan de Anatomical Neuropharmacology Unit van het Medical Research Council in Oxford, UK bij R.A.J. McIlhinney, BSc. D.Phil. Eenmaal terug uit Oxford begon hij daarnaast met de studie Geneeskunde aan de Rijksuniversiteit Leiden. Tijdens zijn studies deed hij eerst onderzoek naar de diagnostiek van tractus-digestivus tumoren en Ewing's sarcomen met behulp van moleculair-biologische methoden op de afdeling Pathologie van het LUMC bij prof. dr. C.J. Cornelisse en prof. dr. P.C.W. Hogendoorn en daarna op het gebied van malaria bij prof. dr. A.P. Waters en Dr. C.J. Janse van de afdeling Parasitologie in Leiden. In 1998 legde hij de doctoraalexamens af voor Geneeskunde en Biomedische Wetenschappen en in 2000 behaalde hij het artsexamen. Daarna ging hij als AGNIO werken op de afdeling Chirurgie/Traumatologie in het Amphiaziekenhuis in Breda en behaalde in dat jaar het Advance Trauma Life Support (ATLS) certificaat. In september 2001 begon hij met zijn promotieonderzoek naar de kwantitatieve virologie bij prof. dr. A.C.M. Kroes op de afdeling medische microbiologie van het Leids Universitair Medisch Centrum, welk onderzoek hij combineert met de opleiding tot arts-microbioloog op dezelfde afdeling. Hij is momenteel werkzaam als arts-assistent in opleiding tot arts- microbioloog op de afdeling medische microbiologie in het LUMC, bij prof. dr. A.C.M. Kroes.



# Nawoord

# Nawoord

Het is vanzelfsprekend dat het werk in dit proefschrift er heel anders zou hebben uitgezien zonder de inzet van veel andere mensen zowel op het werk als daarbuiten.
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