Cytomegalovirus Infection in Heart and Lung Transplant Patients with focus on long-term-outcome

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ABSTRACT

Cytomegalovirus (CMV) infection is a common opportunistic infection after heart and lung transplantation. The aims of this thesis were to relate the incidence and severity of CMV infection and disease to different forms of antiviral prevention and to evaluate whether CMV is a risk factor for bronchiolitis obliterans syndrome (BOS) after lung transplantation and coronary artery vasculopathy (CAV) after heart transplantation.

CMV disease had a significant negative impact on 10-year survival as compared with no CMV infection in a study of 187 lung transplant patients. CMV prevention with 14 weeks of oral ganciclovir reduced the incidence and severity and prolonged the time to onset of CMV disease, as compared with four weeks of intravenous ganciclovir in CMV seropositive patients. Our finding supports the hypothesis that a longer duration of CMV prophylaxis is beneficial to lung transplant patients (Paper I).

BOS-free 4-year survival was significantly reduced with CMV disease as compared with no CMV infection. A lower incidence of CMV infection/disease and acute cellular rejection was observed with valganciclovir (3 months) when compared with oral ganciclovir (3 months), in CMV seropositive lung transplant patients. We concluded that CMV disease reduces BOS-free survival and that CMV prevention with valganciclovir is superior compared with oral ganciclovir in lung transplant patients (Paper II).

Survival and CAV-free survival were significantly reduced in heart transplant patients with CMV disease and asymptomatic CMV infection compared with no CMV infection after a 10-year follow-up in a study of 226 patients. Our study supports the use of an aggressive strategy for reducing not only CMV disease but also asymptomatic infection after heart transplantation (Paper III).

Low-dose valganciclovir prophylaxis (450 mg daily) for 3 months to CMV seropositive heart transplant recipients prevented CMV disease and significantly reduced the number of patients with reactivated asymptomatic CMV infection when compared with a pre-emptive approach. We found that low-dose valganciclovir is safe and effective, but this has to be confirmed in prospective studies (Paper IV).

Keywords: heart transplantation, lung transplantation, cytomegalovirus, ganciclovir, valganciclovir, bronchiolitis obliterans syndrome, cardiac allograft vasculopathy

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SAMMANFATTNING PÅ SVENSKA

Cytomegalovirus (CMV) är ett herpesvirus. CMV infekterar oss vanligtvis under uppväxttiden och ger vid normalt immunförsvar inga symptom eller feber under några veckor. Viruset finns därefter kvar latent i de stamceller i benmärgen som utvecklas till monocyter i blodet och därefter till vävnadsmakrofager. Mer än 70 procent av Sveriges befolkning har antikroppar mot CMV, som tecken på en genomgången infektion.

CMV kan reaktiveras hos personer med nedsatt immunförvar och orsaka livshotande infektioner. CMV kan även överföras från donatorn vid transplantation. Läkemedel som ges för att förhindra avstötning av organ leder till ett nedsatt immunförsvar. Utan profylax debuterar CMV vanligtvis tre till sex månader postoperativt. Lungtransplanterade patienter har hög risk för att insjukna i CMV-sjukdom, medan hjärttransplanterade har en intermediär risk.

Den främsta faktorn som begränsar långtidsöverlevnaden hos hjärt- och lungtransplanterade patienter är kronisk rejektion, definierat som bronchiolitis obliterans syndrome (BOS) efter lungtransplantation och coronary artery vasculopathy (CAV) efter hjärttransplantation. BOS är en progressiv lungfunktionsnedsättning och CAV är en progressiv form av arterioskleros som drabbar hjärtats kranskärl.

Målet med avhandlingen var att utvärdera förekomst och svårighetsgrad av CMV sjukdom efter transplantation med olika profylaxregimer samt att utvärdera om CMV har betydelse för insjuknande i BOS och CAV.

Lungtransplanterade patienter som insjuknade i CMV-sjukdom, oftast lunginflammation, under det första året efter transplantation jämfördes med patienter som inte insjuknade i CMV-sjukdom. Tio års uppföljning av 187 patienter visade att de patienter som insjuknade i CMV-sjukdom drabbades av ökad förekomst av BOS. Olika profylaxregimer jämfördes. Med längre duration av CMV profylax, insjuknade färre patienter i CMV-sjukdom och själva sjukdomen blev lindrigare. Trots tre månaders valganciklovir profylax insjuknade 20 procent av de CMV-seropositiva patienterna i CMV-sjukdom.

Tio års uppföljning av 226 hjärttransplanterade patienter visade att de som insjuknade i CMV-sjukdom oftare drabbades av CAV. Även patienter utan symptom, men med påvisat CMV virus i blodet insjuknade i CAV. En ny profylaxregim med en låg dos av valganciklovir, 450 mg dagligen under tre månader till CMV-seropositiva patienter studerades. Inga patienter

insjuknade i CMV-sjukdom och enbart en låg nivå av CMV virus fanns i blodet under det första året efter transplantation.

Det är av stor betydelse att CMV profylax optimeras efter transplantation för att undvika CMV-sjukdom. Resultatet från dessa studier visar att CMV-sjukdom under det första postoperativa året har betydelse även på lång sikt när det gäller utvecklingen av BOS och CAV.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Johansson I, Mårtensson G, Andersson R. Cytomegalovirus and long-term outcome after lung transplantation in Gothenburg, Sweden. Scand J Infect Dis. 2010;42(2):129-36.

II. Johansson I. Mårtensson G, Nyström U, Nasic S, Andersson R. Lower incidence of CMV infection and acute rejections with valganciclovir prophylaxis in lung transplant recipients. BMC Infect Dis. 2013; 13:582.

III. Johansson I, Sigurdardottir V, Friman V, Selimovic N, Hanzen L, Nasic S, Nyström U, Andersson R. Cytomegalovirus infection and disease reduce 10-year cardiac allograft vasculopathy-free survival in heart transplant recipients. Submitted

IV. Johansson I. Andersson R, Sigurdardottir V, Dellgren G, Nyström U, Friman V. Low-dose valganciclovir as cytomegalovirus reactivation prophylaxis in heart transplant patients. Submitted

CONTENTS

ABBREVIATIONS

ACR	Acute cellular rejection
ACV	Aciclovir
AR	Acute rejection
ATG	Antithymocyte globulin
AZA	Azathioprine
BAL	Bronchoalveolar lavage
BO	Bronchiolitis obliterans
BOS	Bronchiolitis obliterans syndrome
CAV	Cardiac allograft vasculopathy
CMV	Cytomegalovirus
CMVIG	CMV immunoglobulin
CNI	Calcineurin inhibitor
CsA	Cyclosporine A
D+/-	Donor CMV serostatus
eGRF	Estimated glomerular filtration rate
EMB	Endomyocardial biopsy
ERL	Everolimus
FEV_1	Forced expiratory volume in one second
GFR	Glomerular filtration rate
HTx	Heart transplantation

IHC	Immunohistochemistry
IL-2	Interleukin-2
ISHLT	The International Society for Heart and Lung Transplantation
IVIG	Intravenous immunoglobulin
LTx	Lung transplantation
MDRD	Modification of diet in renal disease
mTOR	Mammalian target of rapamycin
MMF	Mycophenolate mofetil
NAT	Nucleic acid amplification testing
PCR	Polymerase chain reaction
QNAT	Quantitative nucleic acid amplification testing
R+/-	Recipient CMV serostatus
SOT	Solid organ transplantation
TAC	Tacrolimus
TBB	Transbronchial biopsy

1 INTRODUCTION

Heart and lung transplantation can be life-saving therapy for patients with severe organ dysfunction with a limited expected survival of about two years or less. The first year after transplantation, acute rejections and infections are common complications. However, the most important factors for long-term survival are the development of cardiac allograft vasculopathy (CAV) in heart transplant recipients and bronchiolitis obliterans syndrome (BOS) in lung transplant recipients. Both CAV and BOS represent manifestations of chronic rejection and are the results of an immunological response to prolonged inflammatory reactions of various kinds, including differences in HLA antigens between donor and recipient, acute rejections and viral and bacterial infections.

Cytomegalovirus (CMV) is the most common severe viral infection following the transplantation of solid organs. In the early transplantation era, CMV disease was associated with high morbidity and mortality [1, 2]. Among organ transplant recipients, especially in lung transplant, CMV pneumonitis has been the most feared infection [2, 3]. Despite CMV prophylaxis and treatment, substantial morbidity is still associated with the virus. The prevention of CMV may help to reduce the development of chronic rejection (i.e. CAV and BOS) and thus help to preserve allograft function for the long term; this is the subject of debate.

1.1 Lung transplantation

The first human lung transplantation was performed in 1963 with only 18 days' survival [4]. During the following years, only a few patients underwent lung transplants. There were some important technical advances prior to 1980, such as improved extra-corporeal circulation with improved pumps and oxygenators, together with respirators. New immonosuppression such as ATG was introduced in the 1970s and, at the beginning of the 1980s, a new era began, when cyclosporine immunosuppression was introduced. The first successful human lung transplantation was performed at Stanford University in 1981 [5]. Lung transplant and intestinal transplant recipients have a higher incidence of acute and chronic rejection compared with other solid organs, which explains why the introduction of more effective immunosuppressive treatment was vital for improved results. Monitoring lung pathology, including acute rejections using spirometry and transbronchial biopsies, was introduced during the latter part of the 1980s, resulting in additional survival benefits. As a result, survival began to improve and, in 1986, one patient survived for more than two years [6]. In Sweden, the first lung transplantations were performed in 1990 in Lund and Gothenburg. These two centres are still performing all the lung transplantations in Sweden.

There are three different types of lung transplantation; single lung, bilateral sequential lung and combined heart-lung transplantation. In the 1990s, single lung was the most frequent type of transplantation, usually performed on recipients with chronic obstructive pulmonary disease (COPD), due to alfa1 antitrypsin deficiency (A1AT) or idiopathic pulmonary fibrosis (IPF). Bilateral sequential lung transplantation was preferred for recipients with cystic fibrosis (CF) and pulmonary arterial hypertension (PAH). Combined heart-lung transplantation was reserved for patients with Eisenmenger's syndrome (e.g. congenital heart disorders combined with pulmonary hypertension) and for some patients with PAH. Today, bilateral sequential lung transplantation has become the most common surgical procedure, as this type of transplantation has been shown to be associated with better long-term survival also for patients previously selected for single lung transplantation

Patients with end-stage lung disease, where all conventional treatment has been tried and with a life expectancy of less than two years are accepted for transplantation [7]. The most common pre-transplant diagnoses are COPD, IPF, A1AT, CF, PAH [8]. Other end-stage lung diseases are fibrosis due to scleroderma, bronchiolitis obliterans due to graft-versus-host disease (GVHD) secondary to bone marrow transplantation, sarcoidosis and combined heart-lung Eisenmenger syndrome. Re-transplantation due to

severe BOS in previously lung-transplanted recipients is performed in selected patients.

The International Society for Heart and Lung Transplantation (ISHLT) has created a registry including reports from most heart and lung transplant centres worldwide. These data are analysed in annual reports that have resulted in the improved selection of suitable donors and recipients and have also improved post-transplant management. An example of these results is that bilateral sequential lung recipients appear to have a better median survival than single lung recipients (6.9 versus 4.6 years respectively) in patients where both procedures would have been possible [8]. For adult lung transplantations reported to the ISHLT between January 1994 and June 2011, the survival rate was 79% at one year, 53% at five years and 31% at 10 years. The median survival was 5.6 years. Patients who survived to one year after transplant had a median survival of 7.9 years [8].

Up to January 2014, a total of 561 lung transplantations had been performed at Sahlgrenska University Hospital in Gothenburg. Of them, 39 were re-transplantations (Figure 1).

1.2 Heart transplantation

The first heart transplantation was performed by Christian Barnard in South Africa in 1967. The patient survived for three months [9]. In 1968, Stanford University performed its first heart transplantation and the patient survived for 15 days [10]. New immunosuppression and endomyocardial biopsy (EMB) were introduced in the 1970s and made it possible to prevent, treat and verify an acute rejection. In the early 1970s, antithymocyte globulin (ATG) was introduced as immunosuppressive induction therapy and maintenance immunosuppression therapy was similarly improved by the introduction of cyclosporine A in 1981. The Stanford group recently reported that, between 1968 and 2007 (n=1,446), the one-year survival for heart transplant recipients at their centre increased from 43% to 90% [10]. In Gothenburg, the first patient was transplanted in 1984 with a donor organ from abroad, as the criteria for brain death had not been legislated on. The legislation was changed and, since 1988, Swedish heart donors have been available, resulting in an increased heart transplant programme.

The indication for transplantation is heart failure with a poor short-term prognosis. The most common pretransplant diagnoses are dilated cardiomyopathy, ischemic cardiomyopathy, restrictive cardiomyopathy,

hypertrophic cardiomyopathy, valvular heart disease and congenital heart diseases.

For all heart transplantations (both paediatric and adult) reported to the ISHLT between 1982 and June 2011, the one-year survival was 85% and the five-year survival was 69%. The median survival was 11 years; patients who survived the first year had a median survival of 13 years [11].

In January 2014, a total of 564 heart transplants had been performed at Sahlgrenska University Hospital in Gothenburg and of them 15 were re-transplantions (Figure 1).

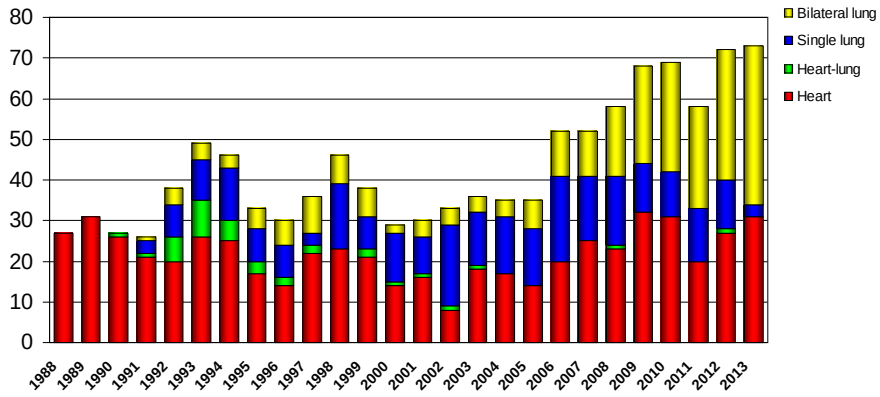


Figure 1. Heart and lung transplant patients at Sahlgrenska University Hospital from January 1988 to December 2014; a total of 1,125 transplantations were performed.

1.3 Cytomegalovirus

Cytomegalovirus (CMV) belongs to the family of human herpes viruses. CMV was identified in 1956. Viral culture was restricted to human fibroblasts, the virus slowly replicated and it was characterised by intranuclear inclusion bodies. CMV is named after the appearance of its cytopathic effect in cell culture, cytomegalia, which means a large cell. The first description of CMV disease in an adult was documented in 1965.

CMV is the largest virus that infects humans, 150-200 nm in diameter. The genome consists of 230 kilo base pair (kbp) double-stranded DNA. The genome encodes for a two to three times larger number of gene products than any other herpes virus [12]. CMV has four structural elements; core, capsid, tegument and envelope. The core contains the linear double-stranded DNA, is surrounded by a proteinaceous layer, defined as the tegument or matrix, which, in turn, is enclosed by a lipid layer containing a large number of viral glycoproteins [13] (Figure 2).

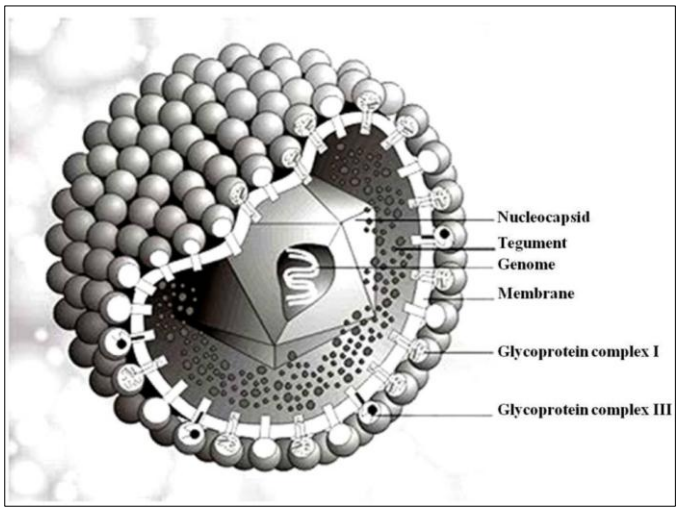


Figure 2. A schematic image of the cytomegalovirus structure. Reproduced with permission from Dr. Marko Reschke.

CMV is able to infect a large number of human cell types; fibroblasts, granulocytes, monocytes, macrophages, dendritic cells and epithelial and endothelial cells [14, 15], and causes disease in most organs, such as pneumonitis, myocarditis, gastrointestinal disease, retinitis, hepatitis, nephritis and pancreatitis. Like the other herpes viruses, CMV establishes latent infection in the host after primary infection and remains mainly in CD 34+ bone marrow progenitor cells and monocytes [16, 17]. Latent CMV is defined by the carriage of the CMV genome without active replication but with the ability of the CMV genome to reactivate under specific stimuli [18]. In the latent phase, only a few viral genes are expressed and few viral proteins are produced and the infected cell is therefore not detected by the host immune system. The exact mechanisms that control latency are unclear.

CMV pathogenesis depends on a balance of viral and host factors. Viral factors contributing to the development of CMV infection include the amount of virus to which the individual is exposed, as well as the replication dynamics of that virus. The growth rate of CMV in immune native patients is faster than the growth rate in CMV-experienced transplant patients [19]. The presence of other viral and bacterial infections also increases susceptibility to infection by CMV. Host factors are donor/recipient serostatus and the intensity of immunosuppression. CMV-specific $CD4^+$ and $CD8^+$ lymphocytes play an important role in immune protection after primary infection or the reactivation of latent disease.

1.3.1 Epidemiology

The seroprevalence of CMV in the global human population is approximately 70% [16] and the rates vary from 45-100%, depending on age, country and socio-economic conditions [20]. CMV infection is mostly acquired during early childhood and there is a peak in adolescence. Following infection, the virus is excreted in body fluid (urine, saliva, tears, semen and breast milk and cervical secretion) for a long period [13].Transmission of the virus occurs with close contact like that among family members and children in day care centres (via urine or saliva). Sexual transmission is seen between partners via semen and cervical secretion. CMV may be spread vertically; via the placenta during maternal viremia, through secretion in the birth canal or from breast milk. Transmission by blood transfusion or blood products may occur, but it is uncommon after the use of filtered blood was introduced. CMV is transferred with solid organ and bone marrow transplantation when the donor is CMV seropositive.

1.3.2 CMV infection

In an immunocompetent host, the primary CMV infection is generally asymptomatic or presents as a flu-like syndrome. Acute CMV disease, mononucleosis syndrome, only occurs in a small proportion of infected individuals. It presents with fever, pharyngitis, sometimes cervical lymphadenitis and hepatitis. The spleen may be enlarged. Atypical lymphocytes are seen in the blood. Laboratory findings usually disappear after six weeks. Fatigue usually persists for several weeks to months. Severe disease with organ-specific complications exists, but it is rare [21, 22].

1.3.3 CMV infection in solid organ transplant patients, direct effects

In organ transplant patients, CMV is the most clinically significant opportunistic infection. The virus can cause severe CMV disease, ranging from CMV syndrome to tissue invasive disease. CMV syndrome is a flu-like illness which may be characterised by fever, malaise, leucopenia, thrombocytopenia and the mild elevation of liver enzymes. The occurrence of tissue-invasive disease is different in each type of organ transplantation. The reported incidence of CMV infection/disease ranges from 38% to 75% in lung transplant patients and 9% to 35% in heart transplant patients in the absence of prophylaxis [23-25]. Despite an antiviral strategy, CMV has remained the most frequent opportunistic infection after organ transplantation [26], causing pneumonitis, gastrointestinal disease, myocarditis, nephritis, hepatitis, pancreatitis and retinitis. CMV has a predilection for invading the transplanted organ.

In lung transplant patients, the most common CMV disease is pneumonitis. CMV pneumonitis can be life threatening if not treated. In single lung transplant recipients, the disease affects the transplanted lung almost exclusively. The symptoms are fever, cough, tiredness, dyspnoea and hypoxia. The clinical symptoms of CMV pneumonitis and acute rejection are the same. A transbronchial lung biopsy (TBB) may therefore be needed to differentiate between infection and acute rejection. The treatments are opposite; acute rejection is treated with increased immunosuppressive therapy, while CMV disease is treated with antiviral drugs and reduced immunosuppressive therapy. CMV pneumonitis has been shown to be a risk factor for invasive aspergillosis following lung transplantation [27]. Gastrointestinal CMV disease is the second most common tissue-invasive disease in lung transplant recipients.

In the early era of heart transplantation, myocarditis and pneumonitis were severe complications of the CMV disease. Myocarditis is almost unique to heart transplant recipients. An endomyocardial biopsy is required to confirm the diagnosis, with viral inclusion bodies or immunohistochemistry (IHC). Myocarditis can cause arrhythmia, cardiac dysfunction and even sudden cardiac death [22, 28-30]. In heart transplant recipients CMV syndrome and gastrointestinal disease are the most common forms of CMV disease nowadays.

Gastrointestinal CMV disease is seen in all types of solid organ transplants. The symptoms related to the disease are nausea, vomiting, dysphagia, epigastric pain, diarrhea, abdominal cramps and severe gastrointestinal bleeding. CMV disease can lead to ulceration or perforation in any part of the gastrointestinal tract. The most common location is the stomach, proximal small bowel and caecum. Endoscopy shows variable lesions, from erythema to deep ulcers [31]. A biopsy and the detection of the virus with histological examination and IHC are required for the diagnosis.

Retinitis is rare in solid organ transplant patients. It causes blurring or loss of central vision, scotomata ("blind spots"), floaters, or photopia ("flashing lights"). Ophthalmologists diagnose retinitis on the basis of characteristic retinal changes. Retinitis is unusual and central nervous system (CNS) disease is extremely rare in organ transplant recipients [32].

As the CMV virus has a predilection for invading the transplanted organ, hepatitis is most frequent in liver transplant recipients, while nephtritis is most common in kidney transplant recipients [33].

1.3.4 CMV infection in solid organ transplant patients, indirect effects

In addition to the direct effects of invasive CMV infection, CMV has possible indirect effects, both general and transplant specific (Figure 3). These conditions are called indirect effects of CMV infection, as they are not directly related to viral invasion of the tissue. The possible general indirect effects include an elevated risk of bacterial, fungal and viral infection [27, 34, 35], new-onset diabetes mellitus after transplantation [36] and acute rejection [37-39].

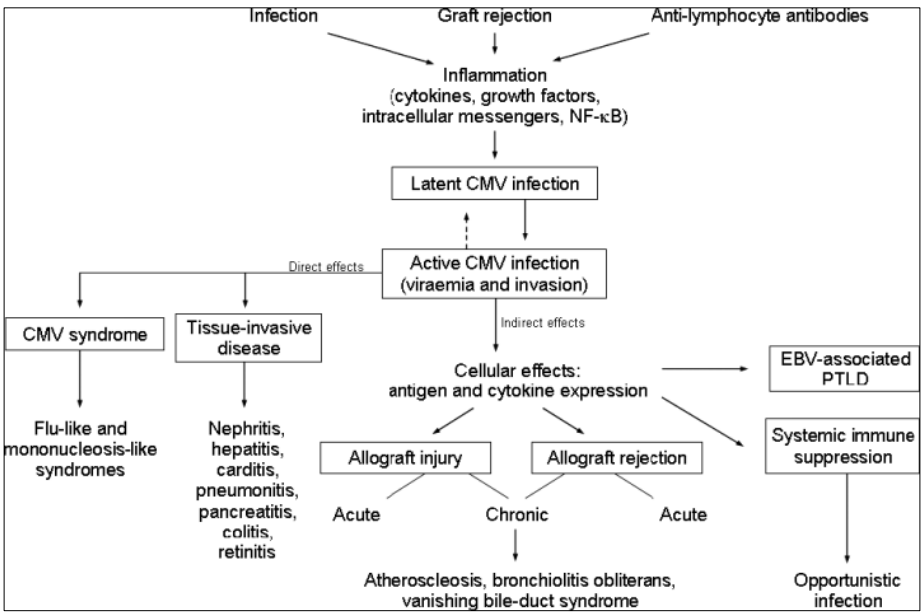


Figure 3. Overview of CMV infection; direct and indirect effects. Reproduced with permission from N Engl J Med. 1988; 338:1741. Copyright Massachusetts Medical Society.

Possible transplant-specific indirect effects that have been discussed are BOS after lung transplantation [37, 40-43] and CAV in heart transplant patients [39, 44]. The pathogenesis of BOS is the peribronchiolar infiltration of lymphocytes, leading to fibrous scarring in the bronchioles and progressive airflow obstruction [45]. The pathogenesis of CAV is an initial endothelial injury, followed by intimae hyperplasia and the proliferation of vascular smooth cells that lead to the diffuse luminal stenosis of the coronary arteries

[46]. Other possible indirect effects are chronic allograft nephropathy after renal transplantation [47-49], accelerated hepatitis C virus recurrence and vanishing bile duct syndrome after liver transplantation [50-52].

A model of CMV pathogenesis after solid organ transplantation is described by Emery [16] (Figure 4). Latent infection is transferred with the donor organ (red spots). The CMV virus becomes activated and thereafter the local spread of the virus occurs in the transplant organ over the next seven days. The virus may spread through the blood to infect other target organs. The high levels of replication, DNAemia, are associated with CMV disease. In addition, early graft infection may contribute to the indirect effects shown in the figure.

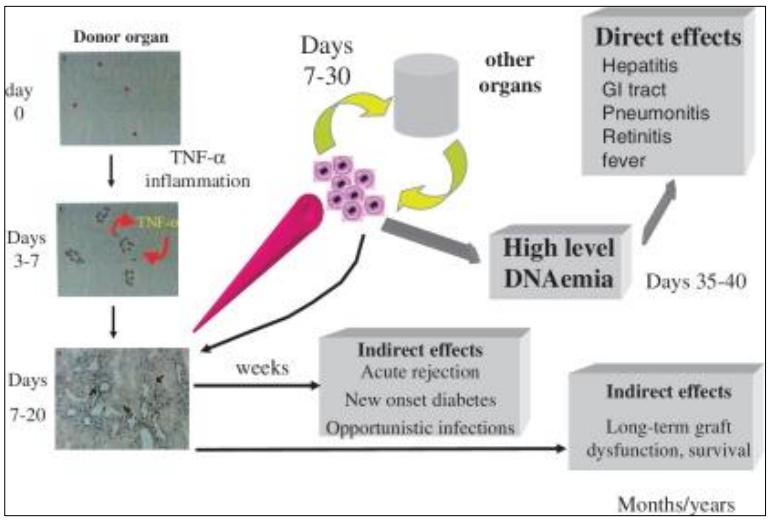


Figure 4. Cytomegalovirus: recent progress in understanding pathogeneis and control. Figure adapted with permission from Oxford University Press, OJM 2012 May: 105 (5):401-405.

1.3.5 Risk factors for CMV infection in solid organ transplantations

Serostatus

The impact of CMV serostatus is essential. Seronegative (R-) recipients who receive organs from a CMV-positive donor (D+) run the highest risk of CMV disease (as a result of the reactivation of latent virus from the transplanted organ). Seropositive (R+) recipients who receive organs from a CMV-positive donor (D+) or CMV-negative donor (D-) run a medium risk of CMV disease. Patients with D-/R- serostatus run the lowest risk of CMV infection, but they may acquire infection through natural transmission in the community settings, or by blood transfusion, if the blood is not leukocyte depleted or CMV negative.

Type of organ

The incidence of CMV infection and disease is different, depending on the type of organ transplanted. McDevitt reported an incidence of CMV disease in kidney transplant recipients of 8%, in liver 20%, in heart 25%, in lung or heart-lung 39% and in pancreas 50% [24]. Lung and intestinal transplant recipients run the highest risk of developing CMV disease. The reason for increased CMV disease may be the larger amount of lymphoid tissue in lung and intestinal transplant organs and also the higher immunosuppression [53].

Immunosuppression

The impact of immunosuppression in the development of CMV infection/disease depends on the type of drug, the dose and duration av the treatment. The dose is especially high during the first three to six months after transplantation. Antithymocyte globulin (ATG) has been associated with an increased risk of CMV disease [54]. New maintenance immunosuppression as a mammalian target of rapamycin (mTor) inhibitor is reported to produce a lower risk of CMV infection [55-57].

Acute rejection

There is a bidirectional relationship between CMV and acute rejection (AR), as acute rejection creates a proinflammatory environment that can reactivate CMV and the treatment for acute rejection is augmented immunosuppression. Conversely, CMV upregulates antigens, and this result in alloreactivity and increases the risk for AR [26, 58, 59].

Blood transfusion

The transfusion of blood products is a risk factor if the blood contains leukocytes. Leuko-depleted blood products have significantly reduced the risk of transfusion-transmitted CMV [60, 61].

1.3.6 Laboratory diagnosis

The laboratory tests that are available to diagnose CMV are histopathology, serology, viral culture, pp65 antigememia and nucleic acid tests (NAT). In the early days, serological testing and viral cultures from multiples sites were the cornerstone of diagnosis. Today, viral load (quantitative nucleic acid tests (QNAT)) or antigenemia are the standards for the diagnosis and monitoring of CMV infection and disease. Depending on the method used, CMV infection can be termed CMV viremia (culture), CMV antigenemia (viral antigen testing) and CMV DNAemia (NAT).

Serology

Serology detects CMV-IgM and IgG antibodies. One of the techniques most frequently used to detect CMV-specific antibodies is the enzyme-linked immunosorbent assay (ELISA). The CMV IgM antibody response following primary infection slightly precedes IgG antibody development. The CMV IgM antibody reaches a plateau in the first months after the onset of infection and then slowly declines in the following three to six months. CMV IgG antibodies persist for life and are the quickest assay to detect immunity. CMV IgG should be performed before transplantation on both the organ donor and the recipient [32]. After transplantation, CMV-IgM and IgG antibodies have a limited value for the diagnosis of CMV disease [62]. The high level of immunosuppression after transplantation results in a delayed or impaired ability to produce antibodies. The transfusion of blood products may produce a false positive test (via the passive transfer of antibodies). In the early days, seroconversion (the appearance of IgM and IgG antibodies in a previously seronegative individual) was used to diagnose CMV infection. Another possibility was to detect a fourfold increase in CMV IgG titres in paired specimens obtained at least two or four weeks apart.

Histopathology

Histopathology is the preferred method for confirming tissue-invasive CMV disease. Typical morphological changes, large cells (cytomegalia) with viral inclusion bodies ("owl's eye"), are found in a biopsy from an affected organ (Figure 5). The method is used together with IHC with monoclonal antibodies to detect CMV antigen. The histological detection of owl's eye inclusion bodies is a highly specific method for detecting CMV organ involvement, but its sensitivity is low. This method has been used since the early era of transplantation and it is still used albeit less frequently [63, 64]. Its invasive procedure has limited its use. If the transplanted organ is affected, a biopsy could be required to differentiate between acute rejection and CMV infection. A biopsy for histopathology is also needed when the symptoms persist despite the treatement of CMV disease, but CMV testing in the blood is negative, which may occur in some cases of gastrointestinal disease [65]. The detection of large cells with viral inclusion bodies and CMV antigen detection by IHC can also be used in BAL fluid [66]. In particular, alveolar macrophages appear to be the cell containing CMV.

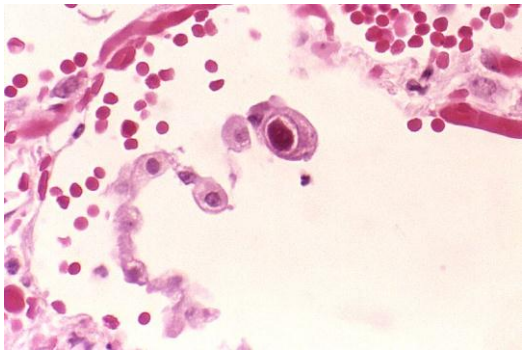


Figure 5. *Cytomegalovirus infection in the lung. Histopathology shows cytomegalic pneumocytes containing characteristic intranuclear inclusions. Downloaded from open domain, commons.wikimedia.org*

Viral culture

Viral culture is highly specific for the detection of CMV and CMV can be isolated from multiple specimen types, such as blood, urine, cerebrospinal fluid, BAL fluid and from tissue biopsy. However, the culture of human fibroblasts routinely takes two to four weeks and the sensitivity is modest. The test therefore has limited use in diagnosing infection or disease in transplant recipients. A positive blood culture is specific and predictive of CMV disease. The detection of CMV in cultures from other sites does not confirm active disease, as seropositive recipients may shed CMV in their secretions; a positive viral culture from urine is not specific for active CMV disease [67]. Viral culture is the method used when phenotypic antiviral drug resistance testing is requested. However, for the clinical diagnosis of drug resistance, the phenotypic methods are too time-consuming.

The antigenemia assay

The antigenemia assay is a semi-quantitative test that detects pp65 antigen in CMV-infected peripheral blood leukocytes [68]. The test has higher sensitivity than cultures and has been used at several centres to diagnose acute CMV infection and to guide pre-emptive therapy [69]. The main disadvantage is the need to process the clinical sample within a few hours (6-8 hours), as the test relies on leukocytes. Leucopenia is thus a limitation; an absolute neutrophil count of less than 1,000/mm^3 diminishes the performance of the assay [70].

Quantitative nucleic acid tests (QNAT)/polymerase chain reaction (PCR)

Quantitative nucleic acid tests (QNAT)/polymerase chain reaction (PCR) are the most commonly used molecular assay today [71, 72]. The method is used for the diagnosis of active disease, monitoring the response to the therapy and monitoring when pre-emptive therapy is used as a prophylactic approach. Most laboratories use real-time PCR. Compared with the previously used conventional PCR method, the advances with real-time PCR are a broader linear range, more rapid turnaround time and reduced risk of carryover contamination [73]. Both whole-blood and serum specimens are used to detect CMV viral load. It is important to use the same specimen when monitoring with quantitative real-time PCR over time. Whole blood often gives a higher viral load compared with plasma, as blood measures both cell-free and intracellular viruses [74]. There has been variability in the test results (viral load) from laboratories at different centres due to the lack of standardisation [75]. Different results could be explained, as there are differences in commercial detection reagents, primers and probes targeting different genes, methods for extracting nucleic acid and calibration, among

others. In 2010, the World Health Organisation (WHO) therefore released an international reference for the quantification of CMV nucleic acid, which enables assay calibration and standardisation among laboratories. In a recent multinational study, five different laboratories showed good reproducibility in viral load values when using a commercial test, which was calibrated to the WHO standard [76]. In earlier days, only qualitative CMV PCR was available. This is also a sensitive test, but it is unable to differentiate low-level from high-level viral replication and is therefore not a valuable test when monitoring the effect of CMV treatment. Genotypic resistance testing is now routinely used for the diagnosis of drug resistance.

1.3.7 Definition of CMV infection

The following definitions are adapted from Ljungman et al. [31].

Primary infection is the detection of CMV infection in an individual previously found to be CMV seronegative.

Reinfection or superinfection is the detection of a CMV strain that is distinct from the strain that was the cause of the patient's original infection.

Reactivation is assumed if the CMV strain detected in the previous infection is found to be indistinguishable from the strain causing the new episode.

The following definitions are in accordance with Kotton et al. [32], and Razonable et al. [77].

CMV infection: evidence of CMV replication regardless of symptoms (differs from latent CMV)

- **Asymptomatic CMV infection**: evidence of CMV infection without clinical symptoms

CMV disease: evidence of CMV infection with attributable symptoms classified as:

- **CMV syndrome**: viral syndrome with fever and/or malaise, leukopenia and/or thrombocytopenia

- **Tissue-invasive CMV disease, proven**: symptoms and signs of disease and CMV detected by immunohistochemistry (IHC) with CMV-specific antibodies in a biopsy from the affected organ. The definitive diagnosis relies on the detection of CMV in the tissue specimen, with the exception of central nervous system disease and retinitis.

1.3.8 Antiviral drugs for CMV prevention and treatment

Drugs that have been evaluated for prophylaxis in heart and lung transplant recipients are aciclovir, ganciclovir, valganciclovir and immune globulin preparations.

Aciclovir/valaciclovir

Aciclovir is a nucleoside analogue of guanosine and a homologue of ganciclovir. At the beginning of the 1990s, aciclovir was used as CMV prophylaxis in lung transplant patients. Valaciclovir is the prodrug of aciclovir. Prophylaxis is only recommended in kidney transplant patients. The recomended prophylaxis dose of valaciclovir is 2,000 mg p.o. four times daily [77], but a lower dose of valaciclovir, 1,000 mg three times daily to D+/R-, has also been shown to be effective [78, 79]. Valaciclovir is not recomended for the treatment of CMV disease.

Ganciclovir

Ganciclovir is a nucleoside analogue of guanosine and a homologue of aciclovir. The phosporylation of the drug is required to have an effect. Its mechanism of action is through the inhibition of virally encoded DNA polymerase [80]. Ganciclovir is excreted in urine and the dose has to be adjusted for renal function. Clearance is directly correlated to the glomerular filtration rate. The plasma half-life is two to four hours; the intracellular half-life of ganciclovir triphosphate is about 16.5 hours. The major toxicity is to the bone marrow and neutropenia is especially common. Granulocyte colony stimulating factor (G-CSF) can be used, together with ganciclovir, if needed, to increase the leukocyte count, if severe neutropenia occurs.

Intravenous ganciclovir has been the drug of choice for prophylaxis and treatment since the beginning of the 1990s. Oral ganciclovir has been used as prophylaxis at our transplant unit since 2000 and has made it possible to give a longer duration of prophylaxis. The bioaviability is only 6-9%. The drug has never been used as treatment for CMV disease in solid organ transplants. At our transplant unit, oral ganciclovir was replaced by valganciclovir in 2003/2004. The recommended treatment dose is 5 mg/kg of i.v. ganciclovir every 12 hours. The prophylaxis dose is 5 mg/kg i.v. once daily. Oral ganciclovir is not recommended for treatment; the prophylaxis dose is 1,000 mg three times daily.

Valganciclovir

Valganciclovir is a valine ester of ganciclovir, i.e. a prodrug of ganciclovir. The mechanism of this drug is activation via a viral protein kinase HCMV UL97 and subsequent phosphorylation by cellular kinases. It is well absorbed after oral administration and rapidly hydrolysed to ganciclovir in the intestinal wall and liver. The bioavailability of ganciclovir from valganciclovir tablets is approximately 60%. A dose of 900 mg of valganciclovir daily can achieve systemic exposure similar to 5mg/kg of i.v. ganciclovir daily [81]. The adverse effects are similar to ganciclovir and valganciclovir thus has to be adjusted for renal function and is associated with bone marrow suppression, particularly leucopenia. Valganciclovir can be used as treatment in mild or moderate CMV disease. The recommended treatment dose of valganciclovir is 900 mg twice daily and the prophylaxis dose is 900 mg once daily.

Foscarnet

Foscarnet is a pyrophosphate analogue that directly inhibits the CMV DNA polymerase [82]. In heart and lung transplant patients, the drug is principally used for the treatment of ganciclovir-resistant CMV. The most common adverse effects are renal impairment, electrolyte imbalance, anaemia and granulocytopenia. The recommended treatment dose is 60 mg/kg i.v. every eight hours. It is not recomended for prophylaxis.

Cidofovir

Cidofovir is a nucleotide analogue of cytosine. Cidofovir is converted by cellular enzymes to cidofovir triphosphate, which is an active inhibitor of viral DNA polymerase. The adverse event is dose-dependent nephrotoxicity. The treatment dose is 5 mg/kg once weekly x 2 and then every two weeks thereafter. Cidofovir is used in the event of ganciclovir resistance and is not recommended for prophylaxis. The drug is not well studied in solid organ transplant.

The recomended doses of prophylaxis and treatment is adapted from the American Society of Transplantation [77].

Intravenous immunoglobulin (IVIG)

CMV-specific immunoglobulin (CMV-IVIG) has been given as prophylaxis to lung transplant recipients, mostly in combination with intravenous ganciclovir. CMV-IVIG or IVIG is sometimes used in combination with i.v.ganciclovir in severe CMV pneumonitis. The effect is believed to be immunomodulatory and limits acute inflammatory events [59]. The efficacy of this approach is debatable.

Ganciclovir resistance

Ganciclovir resistance is observed especially after prolonged exposure to the drug, suboptimal ganciclovir levels and together with intensive immunsuppression. Gancilovir resistance is more common after lung transplantion, in CMV D+/R- and in patients with a high viral load of CMV DNA in blood or serum. Ganciclovir resistance is caused by mutations in the viral UL97 (coding for viral proteinkinase, which is responsible for the phosphorylation of ganciclovir) or UL54 genes (coding for CMV DNA polymerase). In patients treated with ganciclovir, UL97 mutations appear first in about 90% of cases, but UL54 mutations may follow later. Mutation in UL54 is associated with a higher level of resistance to ganciclovir or cross-resistance to foscarnet or cidofovir [83]. Mutations in UL97 do not affect foscarnet or cidofovir and the drugs can be used as treatement.

1.3.9 Strategies for CMV prevention and treatment

Universal prophylaxis

Universal prophylaxis is the administration of an antiviral drug to all patients at risk of CMV infection (i.e. all patients except for D-/R-) during a fixed period of time. Antiviral medication starts immediately or very early after transplantation and most often continues for three to six months [32] and even longer for lung transplant recipients [84]. The advantages of universal prophylaxis are that it is easy to administer, less monitoring with QNAT is needed and the drug also protects from other herpes viruses, such as the herpes simplex virus and varicella zoster virus [32]. The disadvantages are increased drug toxicity, drug-related cost and the risk of resistance emergence. After the cessation of the antiviral drug, there is a risk of developing CMV disease (i.e. late-onset disease) [85].

Targeted prophylaxis

Targeted prophylaxis involves the administration of antiviral drugs in clinical circumstances when patients are at high risk of CMV disease, such as lymphocyte-depleting induction immunosuppression [26].

Pre-emptive therapy

Using the pre-emptive therapy approach, patients are monitored at regular intervals to detect early viral replication. Once viral replication reaches a certain threshold, antiviral treatement is initiated. These laboratory methods include the CMV pp65 antigenemia assay and QNAT for the detection of CMV DNA from blood or serum. Treatment is thus given to prevent the progression of asymptomatic infection to disease. The pre-emptive approach requires frequent monitoring and can be difficult to practise if the patients live far away from the hospital or laboratory. Once CMV is reactivated, the viral load may increase very rapidly [86]. Emery et al. reported a doubling time of approximately 24 hours [87]. Only one assay and one specimen type, either whole blood or plasma, should be used to compare the difference in viral load. Whole blood often gives a higher viral load compared with plasma. The advantages are reduced toxicity, drug cost and a lower rate of late-onset disease.

Treatment

Antiviral therapy is given to patients with symptomatic CMV disease, most often i.v. ganciclovir with two to three weeks' duration. In mild or moderate CMV disease, valaganciclovir is an alternative. The treatment should be continued until CMV is undetectable; today, this is often monitored once a week with CMV DNA from blood or serum.

1.4 Acute rejection

All solid organ transplant recipients are at risk of acute cellular rejections (ACR). The histopathology of an ACR is characterised by the infiltration of mononuclear white blood cells, predominantly activated lymphocytes and monocytes/macrophages. The lymphocytotoxic activity causes tissue damage. The risk factors for ACRs are differences in antigens between donor and recipient (HLA, ABO), to low doses of immunosuppressive treatment, infections and other specific or unspecific inflammatory reactions**.**

1.4.1 Acute cellular rejection in lung transplant recipients

Acute cellular rejection (ACR) in the initial post-operative phase is often associated with clinical symptoms such as breathlessness, chest tightness and subfebrility. Chest radiographs show parenchymal infiltrates or pleural effusion and laboratory analyses show leukocytosis. After the initial post-operative phase, ACRs may be asymptomatic at the time of pathological diagnosis. When patients have symptoms, they vary from subfebrility, dyspnea and cough or sputum production to acute respiratory distress. ACRs are associated with a reduction in lung function tests, above all FEV_1. Regular daily measurements of lung function, using various microspirometers and performed by the patients at home, have proven to be a useful tool in predicting episodes of ACR [88]. These tests do not distinguish between ACR, infections or other lung disorders but are indicators of a pathological disorder in the lung. When ACR is suspected, it can be verified by performing bronchoscopic transbronchial lung biopsies (TBBs). Many centres perform scheduled surveillance biopsies at various time points and then in addition when ACR or other lung pathology is suspected. Microscopic examination shows lymphocytic perivascular or peribronchiolar infiltrate in the lung tissue [89]. The majority of these mononuclear cells are T-cells and CD8+ T cells are most common [90]. Rejections in the lung are graded according to ISHLT guidelines into type A, B, C and D; A represents ACR, B represents bronchial inflammation, C represents chronic bronchial rejection including bronchiolitis obliterans (BO) and D chronic vascular rejection. Type A is in turn divided according to severity into grade 0, 1, 2, 3 and 4, where 0 represents no signs of ACR, 1 represents minimal signs of ACR, 2 mild signs of ACR, 3 moderate signs of ACR and 4 represents severe signs of ACR. Normally, only ACRs of category ≥ 2 need to be treated [89]. The incidence of ACR is most frequent during the first three to six months. ACR decreases with time and is rare after three years post transplantion.

Differential diagnoses during this time include CMV infection and pneumonia with pneumocystis jirovecci, among others.

1.4.2 Acute cellular rejection in heart transplant recipients

Symptoms of ACR in the heart-transplanted recipient vary from none to malaise, low-grade fever, dyspnea, weight gain and palpitations. ACRs are more frequent during the first three to six months. There is no reliable non-invasive method for monitoring the occurrence of an ACR and myocardial biopsies are therefore often performed according to a fixed time schedule during the initial post-operative months and, in addition, as needed due to symptoms and signs. Microscopic examination usually shows lymphocytic perivascular, interstitial and muscular tissue infiltration. CD3-reactive T-lymphocytes and macrophages are involved [91]. Until 2004, ACR was graded according to ISHLT into grade 0, 1, 2, 3 and 4, but a new grading system was introduced in 2004 and, since 2007, it has been applied at our transplant centre. According to the new classification, grade 0 represents no signs of ACR, grade 1 mild, grade 2 moderate and grade 3 severe ACR [91].

1.5 Chronic rejection

In lung transplantion chronic rejection is called bronchiolitis obliterans syndrome (BOS). In heart transplantation chronic rejection is called cardiac allograft vasculopathy (CAV). Other names for CAV are graft coronary artery disease, graft coronary vascular disease, transplant coronary artery disease and accelerated graft arteriosclerosis.

1.5.1 Bronchiolitis obliterans syndrome

Bronchiolitis obliterans (BO), also called obliterative bronchiolitis (OB), is the histological diagnosis of chronic allograft rejection; the peribronchiolar infiltration of lymphocytes, leading to fibrous scarring in the bronchioles. The histological confirmation of BO is difficult because transbronchial biopsy specimens are often not sensitive enough and BOS based on pulmonary function tests has therefore been introduced and is used as a surrogate marker of BO [45, 92].

The definition of BOS is chronic allograft dysfunction/chronic rejection defined as a progressive airflow obstruction not explained by acute rejection, infection or other confounding complication [45]. Spirometry is a standard method for monitoring lung transplant recipients. In order to monitor for the new onset of impaired allograft function, a baseline value for FEV1 is assessed shortly after transplantation. A baseline value is used for comparison with FEV1 values measured later and to calculate a patient's BOS grade. BOS grade 1 has an FEV1 of 65-80%, BOS grade 2 has an FEV1 of 50%-65% and BOS grade 3 an FEV1 of less than 50% of the baseline value.

Clinically, progressive airflow limitation develops with symptoms of dyspnea and non-productive cough. The more advanced stages of BOS are associated with dyspnea at rest and with a productive cough if bronchiectasis has developed. BOS has remained a major source of morbidity and mortality in lung transplant recipients. It is present in 49% of recipients five years after lung transplantation and, at 10 years, the rate reaches 75% [93]. The disease has an unpredictable course; some patients develop rapid loss of pulmonary function, whereas other patients have a slow or intermittent loss of function. In most patients, BOS is a progressive process for some years.

Many factors have been reported as risk factors for BOS [94, 95]. The role of antibody-mediated rejection is a source of ongoing investigation and debate [96]. Alloimmunological injury directed against the endothelial and epithelial

structures has been thought to mediate BOS. Probable risk factors for BOS are acute rejection, lymphocytic bronchitis/bronchiolitisis, CMV pneumonitis and medication non-compliance [45]. Acute rejections predispose for BOS, especially if they are frequent, long lasting or severe [97, 98]. Gastroesophageal reflux is common in and may contribute to BOS via acid aspiration [99, 100]. Different definitions of CMV infection and disease, CMV pneumonitis and different prophylactic strategies among institutions have made it difficult to interpret CMV as a risk factor for BOS. The following studies report that CMV infection is a reason for the development of BOS [41, 42, 101-104], but other studies have not found any impact of CMV infection on the onset of BOS [105, 106]. Community-acquired respiratory viruses (CARV) as a risk factor for BOS are discussed. Gottlieb et al. report that symptomatic CARV infection increases the risk of the new onset of BOS but not the progression of BOS [107]. Kumar et al. found that, in some patients, CARV infection is a trigger for AR and BOS [108]. Transplant type, single more than bilateral lung transplantation, may be a risk factor for BOS [109]. Bronchoscopy with BAL is valuable in excluding other reasons for airflow limitaton on spirometry, such as AR, infection, malignancy and stenosis at the anastomotic site, before the diagosis of BOS is made. The potential prevention for BOS includes aggressive initial immunosuppression to eliminate AR during the first year(s), prophylaxis against CMV infection, the treatment of gastroesophagal reflux to reduce acid aspiration and long-term azitromycin (generally 250 mg orally three times weekly) [110, 111]. No effective treatment for BOS exists.

1.5.2 Cardiac allograft vasculopathy

Cardiac allograft vasculopathy (CAV) is a major limiting factor for long-term survival following heart transplantation. CAV is a rapidly progressive form of atherosclerosis unique to transplant recipients. It is prevalent and, within one year, about 10% and, by 10 years, more than 50% of recipients are diagnosed with CAV [112].

The classical description of CAV is a diffuse concentric narrowing with luminal stenosis [113]. The pathogenesis is initial endothelial injury, followed by intimae hyperplasia and the proliferation of vascular smooth cells that lead to a diffuse luminal stenosis of the coronary arteries [46]. There are histological difference between CAV and coronary arteriosclerosis. Coronary arteriosclerosis is non-circumferential, focal and often presents proximally within the epicardial arteries. CAV is concentric, longitudinal and involves both intima and media (Figure 6). The whole length of the artery is commonly affected. Both epicardial and intramural coronary arteries are involved. CAV occurs in the arteries of the donor but not in the recipient [114].

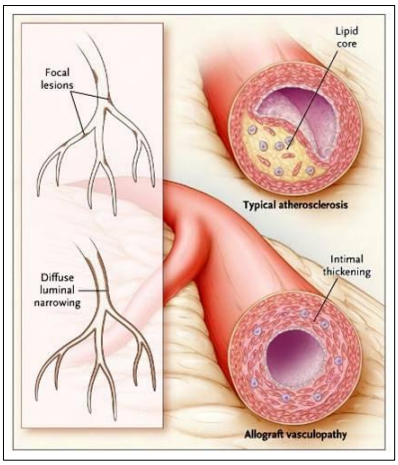


Figure 6. Atherosclerosis and allograft vasculopathy. Reproduced with permission from N Engl J Med. 2003;349:829.Copyright MassachusettsMedical Society

CAV is a complex, multifactorial process. Both immunological and non-immunological risk factors have been implicated in the pathogenesis of CAV [112, 115-117]. Immunological factors associated with CAV are the development of donor-specific human leukocyte antigen (HLA) antibodies [118, 119] and acute rejection [120, 121]. Non-immunological risk factors include donor or recipient history of hypertension, increasing donor age, hyperlipidemia and hyperglycaemia, among others [112].

CMV may play an essential role in CAV progression [122, 123]. Following primary infection, CMV remains latent in CD34+ bone marrow progenitor cells and monocytes and frequently reactivates [16]. The endothelial cell appears to be a target for CMV. Evidence of a link between CMV and CAV has been presented [39, 124, 125], whereas other studies have not confirmed these findings [126, 127].

Coronary angiography is the method for diagnosing CAV and arteriosclerosis. In the mid-1990s, intravascular ultrasound (IVUS) was used to detect silent CAV [128]. Although IVUS is more sensitive for diagnosing early CAV, coronary angiography is still the most commonly used method. The classification of angiographic CAV has to include a description of the maximum stenosis of the following vessels in the heart; left main artery, primary vessels and secondary branch vessels. The recently recommended classification of CAV from ISHLT is from 2010 and is reported by Mehra et al. [129].

Most patients are unable to experience typical angina associated with myocardial infarction or ischemia because of denervation of the donor heart and, as a result, CAV typically presents as a silent myocardial infarction, severe arrhythmia or sudden death. Once CAV is established, the therapeutic options are limited and are only palliative to slow the progression of the disease [130].

1.6 Immunosuppression

Induction

Antithymocyte globulin (ATG) is a preparation created from rabbits or horses with antibodies against human T cells and it acts to deplete T cells. It is given together with glucocorticoids and antihistamine to prevent or reduce infusion-related symptoms. Lymphocyte subsets (CD3) may be followed to determine whether to administer the following dose.

Other drugs used internationally are basiliximab and alemtuzumab. Basiliximab is a chimeric murine/human monoclonal antibody preparation that is specific to and binds with high affinity to the alpha subunit of the interleukin-2 receptor (IL-2R, CD25) on activated T cells. This agent thus inhibits the IL-2-mediated proliferation and differentiation of T cells but does not deplete them. Alemtuzumab is an antibody directed toward the CD52 antigen that is present on virtually all lymphocytes, both T and B cells. Alemtuzumab leads to the depletion of T cells through complement-mediated and direct cellular cytotxicity.

Maintenance immunosuppression

Most maintenance immunosuppressive regimens are three-drug regimens consisting of a glucocorticoid, a calcineurin inhibitor (cyclosporine, tacrolimus) and an antimetabolite agent (mycophenolate or azathioprine).

Glucocorticoid

Glucocorticoid inhibits both cell-mediated and humoral immunity. The majority of lung transplant recipients stay on prednisone for life, while heart transplant recipients stay on prednisone for at least one year.

Azathioprine

Azathioprine (AZA) was the first successful immunosuppressive agent. It is a purine analogue and it is thought to act by inhibiting DNA replication and thus blocking the proliferation of lymphocytes [131]. A common side-effect is myelosupression, especially leucopenia. The drug is rarely used today.

Mycophenolate mofetil

Mycophenolate mofetil (MMF) was introduced in the mid-1990s and it is an antimetabolite agent. MMF is converted in the liver to its active form, mycophenolic acid (MPA), which depletes guanosine nucleotides in T and B lymphocytes and the proliferation of T- and B-lymphocytes is thus inhibited [132]. The main toxicity is from the gastrointestinal tract and myelosuppression.

Cyclosporine

Cyclosporine (CsA) was introduced at the beginning of 1980s and it has been the cornerstone of immunosuppression for many years. CsA is a lipophilic cyclic peptide of 11 amino acids, isolated from fungi. CsA is a calcineurin inhibitor. Calcineurin is a protein phosphate that is critical for T-cell activation. The effect is exerted through binding to cyclophilins; it inhibits the transcription of interleukin 2 in T cells and thus prevents the proliferation of T cells. Nephrotoxicity is the most common and also the most important clinically adverse effect [131, 133]. Other side-effects are dyslipidemia, hypertension, gingival hyperplasia and hirsutism.

Tacrolimus

Tacrolimus (TAC) was introduced in the mid-1990s and it is currently the most widely used calcineurin inhibitor. It is a macrolide antibiotic isolated from fungi. TAC binds to the cytoplasmic immunophilin and inactivates calcineurin. This leads to the inhibition of interleukin 2 and the inhibition of T-cell activation and proliferation. Nephrotoxicity is a common side-effect. TAC is associated with less dylipedemia and hypertension compared with CsA, but new-onset diabetes mellitus is observed more frequently in TAC compared with CsA.Nephrotoxicity in CsA and TAC manifests as an acute increase in serum creatinine. It is mostly reversible after dose reduction, but it may be chronic and progressive. Both drugs have narrow therapeutic windows and careful monitoring of blood levels is necessary.

Mammalian target of rapamycin (mTor) inhibitor

The mammalian target of rapamycin (mTor) inhibitors, everolimus and sirolimus, exert their effect by binding to FKBP12, a member of the immunophilin protein family. The mTOR inhibitor FKBP12 complex blocks the mTOR, thereby interrupting DNA and the protein synthesis and proliferation of T, NK, and B cells. Both everolimus and sirolimus inhibit fibroblast proliferation and this may result in poor wound healing.

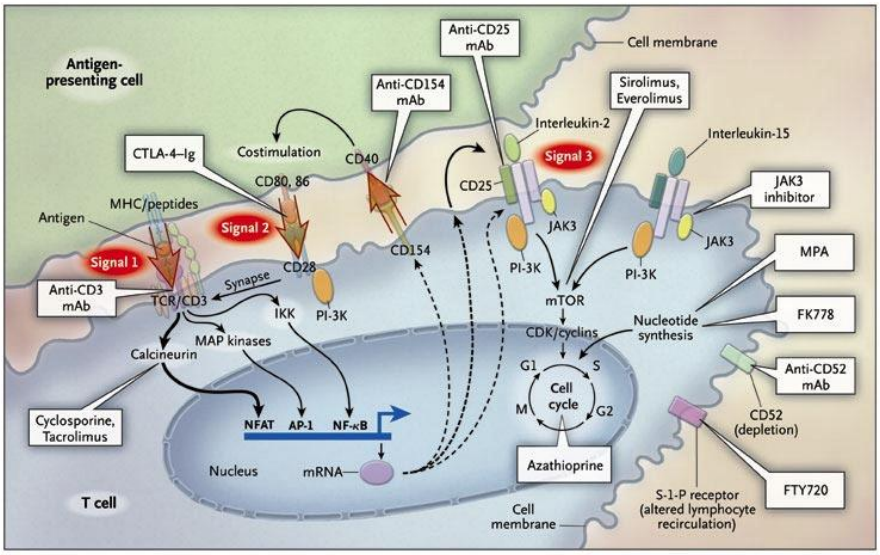


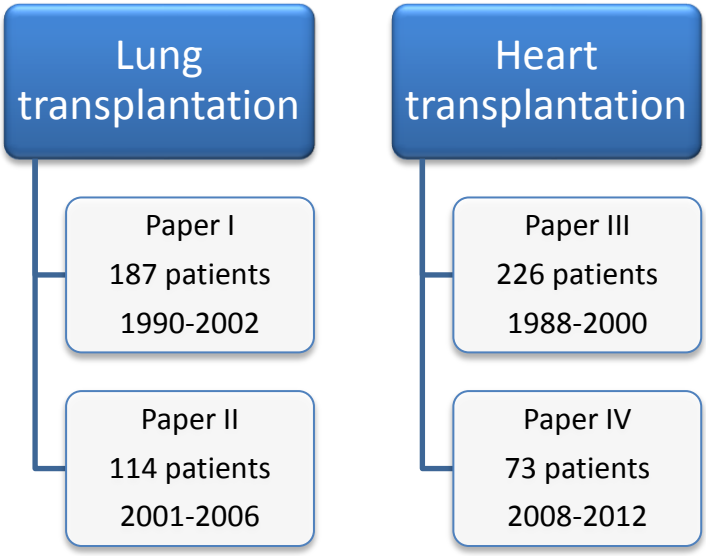
Figure 7. *Immunosuppressive drugs and sites of action.*
MPA denotes mycophenolic acid (MMF). CTLA 4-Ig denotes betalacept. Anti-CD 25 mAb denotes simulect. Anti CD52 mAb denotes alemtuzumab. The following drugs are not used for solid organ transplants today: anti-CD3 mAb, JAK3 inhibitor, FK778 and FTY 720. Reproduced with permission from N Engl J Med. 2004; ***351****: 2715. Copyright Massachusetts Medical Society*

2 AIMS

The overall aims of this thesis were to study different aspects of CMV infection in lung and heart transplant patients. The specific aims were to:

- Investigate the impact of CMV infection and disease on survival and BOS-free long term survival in lung transplant recipients
- Relate the incidence and severity of CMV infection and disease in lung transplant patients in relation to different drugs and durations of antiviral prevention
- Compare oral ganciclovir with valganciclovir with respect to incidence, severity of CMV infection or disease and acute rejection in lung transplant recipients
- Investigate the impact of CMV, both asymptomatic infection and disease, on survival and CAV-free long-term survival in heart transplant recipients
- Compare pre-emptive treatment and low-dose valganciclovir prophylaxis in heart transplant patients.

3 PATIENTS AND METHODS



All the patients in the studies were transplanted at Sahlgrenska University Hospital in Gothenburg. Re-transplants and patients who died within 30 days after transplantation were excluded from the studies.

3.1 Lung transplant patients and study design

Paper I

A retrospective study of 187 lung and heart-lung transplant patients transplanted between January 1990 and December 2002. The majority were women, 61% (n=114). Their mean age was 45 years (range 7-68 y). The type of transplantations was single lung in 58% (n=109), bilateral lung in 26% (n=49) and heart-lung in 16% (n=29).

The pre-operative diagnoses were chronic obstructive pulmonary disease (COPD) in 29%, α-1-antitrypsin deficiency with emphysema in 23%, Eisenmenger's syndrome in 12%, idiopathic pulmonary arterial hypertension in 9%, idiopathic pulmonary fibrosis in 9%, cystic fibrosis in 8% and others in 10% of patients

The incidence and severity of CMV infection or disease with different CMV prevention and the impact of CMV as such on the development of BOS were studied. Medical records were reviewed. Signs and symptoms of CMV infection were registered. Tissue-invasive disease, such as CMV pneumonitis and gastrointestinal CMV, was detected with typical morphological changes and IHC with monoclonal antibodies to CMV. Retinitis was confirmed by an ophthalmologist. Pulmonary function was followed with spirometry.

Paper II

A retrospective study of 114 lung and heart-lung transplant patients transplanted between January 2001 and December 2006. The majority of the patients were women, 63% (n=72), and their mean age was 49 years (range 10-68y). The type of transplantation was single lung in 70% (n=80), bilateral lung in 27% (n=31) and heart-lung in 3% (n=3).

The pre-transplant diagnoses were chronic obstructive pulmonary disease in 38%, idiopathic pulmonary fibrosis in 20%, alpha-1 antitrypsin deficiency with emphysema in 18%, cystic fibrosis in 6%, pulmonary arterial hypertension and pulmonary hypertension in 6%, graft-versus-host disease in 3.5%, scleroderma in 3.5%, Eisenmenger's syndrome in 2% and others in 3% of patients

The impact of CMV on the development of BOS was studied. Pulmonary function was registrered (spirometry with FEV1). The patients were followed for four years or until death. In a subcohort of 88 CMV seropositive patients, oral ganciclovir (3 months) and valganciclovir (3 months) were compared. The incidence and severity of CMV infection/disease and acute rejection within the first 12 months after transplantation were registered and compared for the drugs.

3.2 Heart transplant patients and study design

Paper III

A retrospective study of 226 heart transplant patients transplanted between January 1988 and December 2000. The majority were male, 78% (n=176). Their mean age was 45 years (range 14-65y).

The pre-transplant diagnoses were dilated cardiomyopathy in 53% (n=119), ischemic heart disease in 32% (n=73), myocarditis in 4% (n=9), congenital heart disease in 4% (n=8), valvular heart disease in 3% (n=7), arrhythmogenic right ventricular dysplasia in 2% (n=5), hypertrophic cardiomyopathy in 2% (n=5) and restrictive cardiomyopathy in 0.4% (n=1) of patients

The incidence of CMV infection and disease during the first year and acute rejection, defined as the total or any rejection score at three, six, nine and 12 months after transplantation, was studied. Data were collected from medical records. The results of coronay angiography were re-evaluated by a cardiologist. CMV infection and disease was diagnosed with laboratory methods described in the next section. Survival and CAV-free survival within 10 years after transplantation were analysed.

Paper IV

A retrospective study of 73 adult CMV seropositive heart transplant patients transplanted between January 2008 and December 2012.

The pre-transplant diagnoses were dilated cardiomyopathy in 58% (n=42), hypertrophic cardiomyopathy in 4% (n=3), restrictive cardiomyopathy in 3% (n=2), ischemic heart disease in 16% (n= 12), congenital heart disease in 8% (n= 6) and others in 11% (n=8) of patients

They were divided into two cohorts, an historical cohort with pre-emptive therapy (n=31) and a cohort with three months of low-dose VGCV prophylaxis (n=42). In the pre-emptive cohort, 71% were male and the mean age was 50 (±15 y) and, in the valganciclovir cohort, 74% were male, with a mean age of 51 (±14 y). No significant difference in pre-transplant diagnosis was found in the two cohorts.

The incidence and severity of CMV infection and disease was compared in the two cohorts. Myelosuppression, especially leucopenia and kidney function, was evaluated.

3.3 Definitions in lung transplant patients

CMV pneumonitis

To identify CMV pneumonitis, clinical signs and symptoms such as fever, cough, dyspnea and hypoxia were recorded. CMV pneumonitis was verified from a biopsy with typical morphological changes, with viral inclusion bodies or IHC using monoclonal antibodies to identify early and late CMV antigens, together with parenchymal diffuse or perivascular inflammation. The grading of the severity of pneumonitis was mild, moderate and severe, for details see the method section in Papers I and II. Broncoscopies with TBB and BAL were monitored regularly at 0.5, 1, 2, 3, 4.5, 6, 9 and 12 months after transplantation. A control biopsy was taken four weeks after CMV pneumonitis was treated. Biopsies were analysed with histopathology and IHC with CMV-specific antibodies (Papers I and II).

Non-pulmonary CMV infection and disease

To identify non-pulmonary infection and disease, quantitative or qualitative CMV PCR from blood or serum were recorded, together with clinical signs and symptoms. A biopsy from tissue, from the gastrointestinal tract, for example, was diagnosed with morphological changes and/or IHC (Papers I and II).

Acute rejection

Acute cellular rejection was diagnosed by the presence of perivascular and/or interstitial mononuclear infiltrates.A bronchoscopy with TBB was assessed at 0.5, 1, 2, 3, 4.5, 6, 9 and 12 months. TBB was repeated four weeks after episodes of acute rejection and also when rejection was suspected. The grading of the severity of acute rejction is based on the degree of inflammation, according to the ISHLT pathological scoring system (A1 = minimal AR, A2 = mild AR, A3 = moderate AR, A4 = severe AR) [89]. The method used to compare acute rejection was the CAR score divided by the number of evaluable TBBs. The method based on CAR score and CAR score divided by the number of evaluable TBBs has been used elsewhere and reported in other studies [37, 41, 134, 135]. We also compared at least one episode of mild AR (i.e. AR grade $\geq$ 1) and one or two treatable acute rejections (i.e. AR grade $\geq$ 2) within three and 12 months after transplantation (Paper II).

Bronchiolitis obliterans syndrome

Pulmonary function tests consisted of spirometry with FEV1. A baseline FEV1 value was calculated and this baseline value was used to compare

FEV1 values, to calculate a patient's BOS grade every year. Classification was made according to the classification from 1993: BOS 0: FEV1 80% or more of baseline, BOS 1: FEV1 66-80% of baseline, BOS 2: FEV1 51-65% of baseline, BOS 3: FEV1 < 50% of the baseline value [45] (Papers I and II).

3.4 CMV prevention and treatment in lung transplant patients

Prior to November 1992, only oral aciclovir was given as CMV prophylaxis, after which all R+ were given four weeks of i.v. ganciclovir. In January 2001, the prophylaxis for R+ was switched to oral ganciclovir for 14 weeks and, since December 2003, valganciclovir for three months has been used.

In 1997, the first D+/R- lung transplantation was carried out. Antiviral prevention was given for a minimum of six weeks with i.v. ganciclovir, followed by oral ganciclovir for at least eight additional weeks. From May 2002, oral ganciclovir was given for 14 weeks and, in December 2003, the prophylaxis was changed to valganciclovir for six months. Between 1997 and 2006, CMV IG was added on day 0, 7, 14, 35, 56 and 77 after transplantation (Papers I and II).

CMV pneumonitis was treated with 5 mg/kg of i.v. ganciclovir twice daily for at least 14-21 days. A control biopsy was performed four weeks after the start of treatment and, if CMV was found, the patients received additional treatment. Patients with hypoxia also received 0.5 g/kg of IVIG every other day until an improvement was seen (maximum five doses). Foscavir was an alternative when patients did not respond to ganciclovir or ganciclovir resistance was found. In recent years, 900 mg x 2 of VGCV has been an alternative for treating a mild infection (Papers 1 and II).

3.5 Immunosuppression in lung transplant patients

Induction therapy: since 1993/1994, ATG has been the standard induction therapy, initially 2.5 mg/kg/day for three to 10 days. From 1998, the ATG doses were based on daily CD3-positive T lymphocyte cell counts. An initial ATG dose of 2.0 mg/kg body weight/day was given, followed by 1.5 mg/kg once daily when the CD3-positive T lymphocyte count exceeded 0.05×10^9/l.

In most cases, two to four doses of ATG were given. Methylprednisolone was given together with ATG (Papers I and II).

Maintenance therapy: triple immunosuppression therapy with a calcineurin inhibitor (CsA or TAC), an antimetabolite (AZA or MMF) and a corticosteroid was standard. AZA was regulary replaced with MMF from December 1997. Immunosuppressive treatment in 2000-2006 is described in detail in Paper II.

3.6 Definitions in heart transplant patients

CMV infection and disease

The methods for detecting CMV replication were serology (seroconversion post-transplantation), viral culture, qualitative PCR for CMV DNA, biopsies with histopathology and IHC with CMV-specific antibodies (Table 1). Clinical signs and symptoms were recorded. The diagnoses were CMV disease, asymptomatic CMV infection and no CMV infection in accordance with Ljungman et al. [31]. Between 1988 and 1997, serological analyses were repeated once monthly for the first four months post-heart transplantation, then after six, nine and 12 months and thereafter annually and when infection was suspected. Qualitative PCR has been evaluable since 1992 (Paper III).

Table 1. Laboratory tests to detect CMV infection in heart transplant patients in different time periods

Period	CMV serology	Viral culture	Biopsy tissue	Qualitative CMV PCR	Quantitative CMV PCR
1988-1991	+	+	+	-	-
1992-1997	+	(-)	+	+	-
1998-2000	(-)	(-)	+	+	-
2001-2014	(-)	(-)	+	-	+

The table shows the laboratory test used in our studies of heart transplantations

Between 2008 and 2012, QNAT from serum was monitored weekly during the hospital stay and thereafter at three, four, five, six and 12 months. The method is described in detail by C. Kullberg-Lindh et al. [136] (Paper IV).

Acute rejection

All endomycardial biopsies were reclassified according to the ISHLT Classification from 2004 [91]. ISHLT Standardised Cardiac Biopsy Grading from 2004 is adopted from Stewart et al. [91].

- Grade 1R, mild AR: interstitial and/or perivascular infiltrate with up to one focus of myocyte damage.
- Grade 2R, moderate AR: two or more foci of infiltrate with associated myocyte damage
- Grade 3R, severe AR: diffuse infiltrate with multifocal myocyte damage ± oedema ± haemorrhage ± vasculitis

At our centre, endomyocardial biopsies to detect AR (and also CMV myocarditis) were performed weekly according to the protocol during the first six weeks, thereafter at two-week intervals until three months, monthly from three to six months and then every three months or on clinical indication until 12 months after transplantation. A control biopsy was taken seven to 10 days after a treated rejection (Paper III). For a description of the total rejection score and any rejection score, see the method section in Paper III.

Coronary artery vasculopathy

Angiographic CAV is defined as mild (≤ 50% stenosis), moderate (50%-70% stenosis) and severe (> 70% stenosis) stenosis of the left main coronary artery [137]. The patients were followed with angiography annually for 10 years (with a few exceptions) or until death.

3.7 CMV prevention and treatment in heart transplant patients

Different approaches to CMV prophylaxis were used during the study years; no CMV prophylaxis, targeted prophylaxis, pre-emptive therapy and universal prophylaxis.

For treatment 5 mg/kg of i.v. ganciclovir twice daily was given for two to three weeks. In recent years, 900 mg of valganciclovir twice daily has been used for mild disease. Foscarnet was an alternative.

3.8 Immunosuppression in heart transplant patients

Induction therapy: in 1988-1993, CsA was given as induction therapy, apart from the first nine patients transplanted in 1988 who received 100 mg/day of prednisone for three weeks. From 1993/1994, 2.5 mg/kg/day of ATG for three to five days was the standard. In 2008, the doses were based on daily CD3-positive T lymphocyte cell counts. An initial ATG dose of 2.0 mg/kg body weight/day was given, followed by 1.5 mg/kg once daily when the CD3-positive T lymphocyte count exceeded 0.05 x 10^9/l. Since 2010, standard induction has been reduced to 1 mg/kg/day of ATG for three days. Methylprednisolone is administered together with ATG.

Maintenance therapy: since 1994, at the very least, standard immunosuppression treatment was a calcineurin inhibitor (CsA or TAC), an antimetabolite (AZA or MMF) and a corticosteroid. In the late 1990s, AZA was replaced by MMF. CsA was switched to TAC if patients had repeated rejections. Immunosuppresive therapy for 2001-2006 is described in detail in Paper II.

Statistical analyses

Descriptive statistics were calculated for continuous variables, frequencies and proportions for categorical variables. The chi-square test was used to compare proportions and occurrences between the groups. The Mann-Whitney test, as we were dealing with ordinal data (Paper II), and comparisons with respect to continuous variables, as most of them had a skewed distribution deviating from normal distribution, were used (Paper IV). Confidence intervals were calculated using a normality approximation algorithm. Survival analysis was performed using the Kaplan–Meier procedure and statistical comparisons of survival distributions between different categories were made using the log rank test (Papers I, II and III). Cox's regression was used to confirm results in a multiple model after including possible confounding variables (Paper III). Statistical significance was set at the 5% level, i.e. $p < 0.05$. The data were analysed using SPSS 15-22 (SPSS Inc., Chicago, IL, USA).

Ethics

This research was approved by the local ethics committee at Sahlgrenska University Hospital, Gothenburg, Sweden.

4 RESULTS

4.1 Lung transplant patients

Incidence of CMV disease

Of the 187 lung transplant (LTx) patients transplanted between 1990 and 2002, CMV pneumonitis verified by TBB (with or without symptoms) was found in 58% (n=109) of the patients. Six per cent (n=11) of the patients were diagnosed with gastrointestinal (GI) CMV. One per cent (n=2) suffered from retinitis. Between 2001 and 2006, CMV disease was found in 29% (n=33) of the 114 LTx patients. Eight per cent (n=9) had GI CMV. No retinitis was found (Papers I and II).

Severe CMV pneumonitis

Severe CMV pneumonitis was seen in 10% (n=19) of the patients in 1990 to 2002. Three patients died of CMV pneumonitis. One additional patient died of CMV pneumonitis, together with a co-infection with the fungi Aspergillus fumigatus (Paper I). In the next study of 114 LTx patients transplanted from 2001-2006, 4% (n=5) were diagnosed with severe CMV pneumonitis and one of them died (Papers II).

Impact of CMV serostatus

The impact of CMV serostatus was significant. CMV disease was found as follows: for D+/R- 88%, for D+/R + 40%, for D-/R + 28% and for D-/R- 0%. In the more recent period, 2001-2006, the impact of CMV serostatus from donor and recipient was still high. The incidence of CMV infection/disease was 65% for D+/R-, 39% for D+/R+, 27% for D-/R + and 11% for D-/R- (Papers I and II).

Outcome of different prophylaxis to R+

During the study period, four different prophylaxis regimens were given to CMV seropositive patients. With aciclovir for at least four weeks, 38% of patients were diagnosed with CMV disease, with four weeks of i.v. ganciclovir, 39% on average, with oral ganciclovir for threee months, 32%, and, with valganciclovir for three months, 20% of patients were diagnosed with CMV disease (Table 2). The incidence of CMV infection/disease was lower in the valganciclovir cohort compared with the oral ganciclovir cohort (24% vs. 54%, p=0.003). There was a trend towards a lower incidence of CMV disease in the valganciclovir cohort (20% vs. 33%, p=0.17) (Papers I and II).

Table 2. *Episodes of CMV disease and asymptomatic CMV infection during the first 12 months in 209 R+ lung transplant recipients (Papers I and II)*

	Disease %		Disease %		Infection %	No CMV %
Prophylaxis	Total	Severe	Moderate	Mild		
Oral ACV	38	19	5	14	33	29
i.v. GCV	39	13	19	7	22	39
Oral GCV	32	0	22	11	22	46
VGCV	20	4	10	6	4	76

Aciclovir (ACV) was given to 21 patients. From November 1992 to December 2000, four weeks of i.v. ganciclovir (GCV) was given to 100 patients. In January 2001, the CMV prophylaxis was changed to oral GCV for three months and, since 2004, valganciclovir (VGCV) has been given for three months. Oral GCV was given to 37 patients and VGCV to 51 patients.

The onset of CMV disease in the aciclovir cohort was a mean of 41 days (range: 23-65) post-transplantation and, with four weeks of i.v. ganciclovir, a mean of 75 days (range: 40-177). The onset of CMV infection/disease with oral ganciclovir for three months was 163 days (range: 100-270) and, with valganciclovir for three months, 136 days (range: 23-201) (Papers I and II).

Outcome of different prophylaxis to D+/R-

Seventeen D+/R- patients were transplanted in 2001 to 2006. Between 2001 and 2003, oral ganciclovir for three months was given to eight patients and, in 2004 to 2006, nine patients received valgancilovir for six months. In addition, all patients received six doses of CMV IG. The demographics were homogeneous for type of transplantation, age and gender. Six of the eight (75%) patients with oral ganciclovir prophylaxis developed CMV pneumonitis as compared with four of nine (45%) with valganciclovir prophylaxis. With oral ganciclovir, four of eight had GI CMV disease, while there were none in the valganciclovir cohort. One patient was diagnosed with ganciclovir resistance in the valganciclovir cohort, while there were none in the ganciclovir cohort (unpublished data).

Acute rejection with different prophylaxis to R+

Three months of oral ganciclovir prophylaxsis was compared with three months of valganciclovir prophylaxis in 88 R+ patients. Acute rejection was less frequent in the valganciclovir group during the entire first year. The cumulative acute rejection score (CAR score) divided by the number of evaluable TBBs decreased significantly in the valganciclovir group. At least one episode of acute rejection grade 1 or acute rejction ≥ 2 was significantly decreased in the valganciclovir group. Minimal or no changes were made in the immunosuppressive regimens in 2001-2003, when oral ganciclvir was used as prophylaxis, and from 2004 to 2006, when valganciclovir was used as prophylaxis. The incidence of AR is shown in Table 4 in Paper II.

Survival at 10-year follow-up in lung transplant patients

The overall one-, five- and 10-year survival rates in the 30-day survivors of LTx patients, in our study group of 187 patients, were 89%, 66% and 47% respectively. CMV disease had a significant negative impact on survival, with a 10-year survival of only 32% as compared with 53% after asymptomatic CMV infection and 57% with no CMV ($p < 0.001$) (Figure 8) (Paper I)

Between 2001 and 2006, the six-year survival was lower among patients with CMV disease (64%, $p = 0.042$) and asymptomatic CMV infection (55%, $p = 0.018$) as compared with patients with no CMV infection (84%) (Paper II).

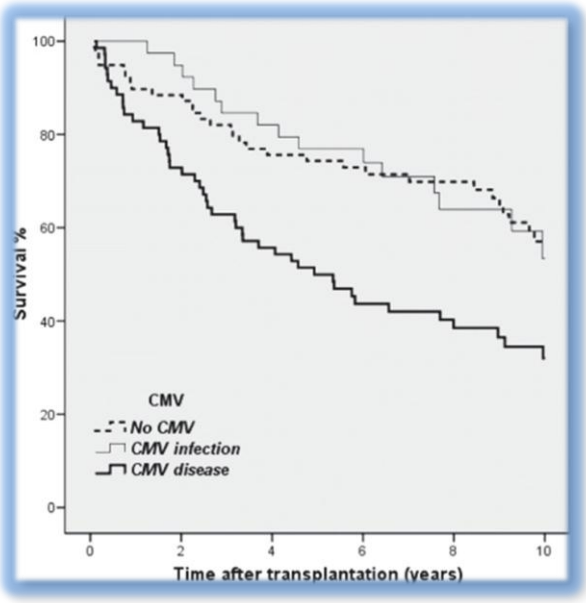


Figure 8. Survival related to CMV infection among 187 patients followed for 10 years. The difference in survival among patients with CMV disease (n = 70), asymptomatic CMV infection (n = 39) and no CMV infection (n = 78) was statistically significant; p = 0.001 by log rank test, Kaplan-Meyer survival curve.

BOS-free survival at four-year follow up

BOS-free survival was reduced in patients with CMV disease compared with patients with no CMVinfection (Papers I, II)

Between 1990 and 2002, CMV disease was associated with a statistically significant increase in BOS-free survival (p=0.037) in 168 LTx patients at both one and two years after transplantation (Figure 2 in Paper I). BOS-free four-year survival in 107 LTx patients, between 2001 and 2006, is illustrated in Figure 9. FEV_1 was followed for the entire four years (or until death) for all patients. BOS-free four-year survival for patients with CMV disease was 32%, (p = 0.005), for asymptomatic CMV infection 36%, (p = 0.061) as compared with patients without CMV infection (69%). BOS-free survival was 2.9 (95% CI 2.6-3.2) years on average for the total group (Paper II).

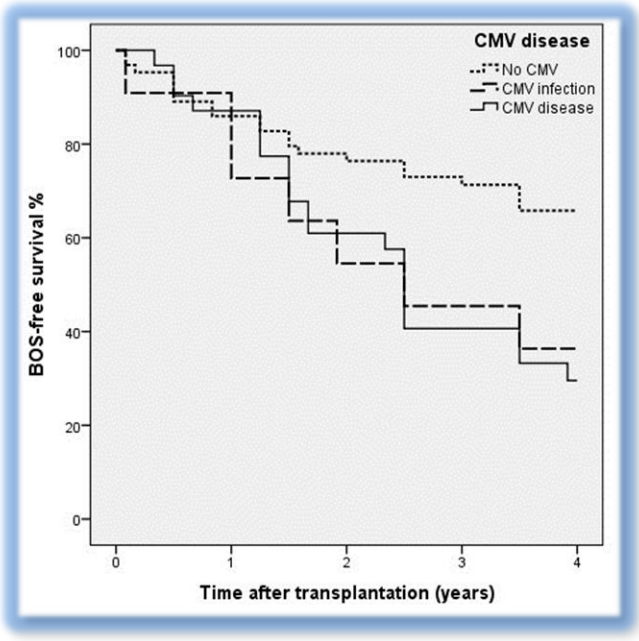


Figure 9. *BOS-free survival in 107 lung transplant recipients related to CMV disease. No CMV infection (n = 65), asymptomatic CMV infection (n = 11), CMV disease (n = 31). BOS-free four-year survival for patients with CMV disease was significantly reduced as compared with no CMV (p = 0.005). Kaplan-Meyer survival curve.*

4.2 Heart transplant patients

Incidence of CMV disease

CMV disease was found in 28% of patients and, of them, 12% (n=26) were diagnosed with tissue-invasive disease and 17% (n=38) with CMV syndrome (Paper III).

CMV disease

Tissue-invasive disease was found in 26 of 226 (12%) of the patients. Myocarditis was diagnosed in 12 patients by histopathology and/or IHC with CMV-specific antibodies. It was possible to establish the diagnosis as endomyocardial biopsies (EMB) were performed frequently during the first three months after transplantation (i.e. weekly according to the protocol during the first six weeks, thereafter every two weeks until three months). Two of the patients with myocarditis had additional symptoms; one patient had symptoms from the upper gastrointestinal tract (verified by IHC from the stomach) and one patient had symptoms of pneumonia (with positive qualitative CMV PCR from BAL fluid).

Pneumonitis was found in five patients. Two of them had CMV verified from TBB or BAL by typical morphological characteristics or IHC for CMV. The third patient had a positive viral culture from BAL fluid. There were also two patients with probable CMV pneumonitis. These patients had symptoms from the respiratory tract and qualitative CMV PCR from BAL fluid was found. One of them also had a positive culture from blood.

Gastro-intestinal disease was found in seven patients. The diagnosis was verified in four patients in biopsies and IHC from the gastro-intestinal tract. An additional three patients had severe symptoms from the gastrointestinal tract and positive qualitative PCR in serum.

Nephritis (n=1) was diagnosed in a biopsy from the kidney and verified with IHC.

Retinitis (n=1) was found in one patient, diagnosed by an ophthalmologist.

Hepatitis was not diagnosed as a liver biopsy was lacking. Some of the patients with CMV syndrome (with fever and leucopenia) had elevated liver enzymes.

CMV syndrome was diagnosed in 17% of the patients (38/226).

Impact of CMV serostatus

In the total group of 226 patients, the incidence of CMV disease was 65% in D+/R-, 21% in D+/R+, 17% in D-/R+ and 13% in D-/R-. It is worth noting that only 5% (11 of 226) of patients received universal CMV prophylaxis in 1988-2000.

Outcome of different CMV prophylaxis to D+/R-

We found that, without prophylaxis and pre-emptive treatment, an average of 70-73% of the patients suffered from CMV disease. Asymptomatic CMV infection was diagnosed in 25-20% and no CMV infection in 5-6%. A dose of 1,000 mg of ganciclovir three times daily reduced the incidence of CMV disease to 45% (Table3) (Paper III).

The onset of CMV disease in D+/R- without prophylaxis occurred a mean of 57 (range 22-178) days after transplantation. With oral ganciclovir for 14 weeks, CMV disease occurred a mean of 103 (range 64-156) days post-heart transplantation (Paper III).

Table 3. Incidence of CMV infection and disease in 46 D+/R- heart transplant patients between 1988 and 2000 (Paper III)

Period	Prophylaxis	CMV disease	CMV infection	No CMV
1988-1991 n=20	No prophylaxis	14 (70%)	5 (25%)	1 (5%)
1992-1997 n=15	Pre-emptive therapy	11 (73%)	3 (20%)	1 (6%)
1998-2000 n=11	Oral ganciclovir 14 weeks	5 (45%)	3(27%)	3(27%)

D+/R-: CMV seropositive/recipient negative patients. Pre-emptive therapy: when CMV DNA from serum was detected, patients received treatement with 5 mg/kg of i.v. ganciclovir for at least 10 days. CMV infection = asymptomatic CMV infection.

Outcome of different prophylaxis to R+

The incidence or CMV disease was 19-20% on average with no prophylaxis or with targeted prophylaxis, while asymptomatic CMV infection was detected in 13-22% of the patients (Table 4).

The onset of CMV disease in the R+ group (n=11) occurred without prophylaxis a mean of 45 (range 19-86) days and, with targeted prophylaxis (n=21), a mean of 51 (range 17-151) days post-transplantation (Paper III).

Table 4. Incidence of CMV infection and disease in 165 R+ heart transplant recipients between 1988 and 2000 (PaperIII)

Period	Prophylaxis	CMV disease	CMV infection	No CMV
1988-1991 n=59	No prophylaxis	11 (19%)	8 (13%)	40(68%)
1992-2000 n=106	Targeted prophylaxis[1]	21 (20%)	23 (22%)	62(58%)

[1]Targeted prophylaxis consisted of 5 mg/kg of i.v. ganciclvoir twice daily for 10 days in association with the first anti-rejection treatment with ATG/OKT3 and the second anti-rejection treatment with high-dose corticosteroids within the first four months post-transplantation.

In the more recent period between 2008 and 2012, the result was as follows. With pre-emptive therapy, CMV disease was found in 10% of the patients (3 of 31); one case of non-invasive disease (CMV syndrome) and two cases of tissue-invasive disease presenting as gastritis and colitis. With 450 mg of valganciclovir daily for three months, no CMV disease was found during the first year after transplantation (Table 5). The peak of viral load was detected after a mean of 68 days (range 18-184) in the pre-emptive cohort, while the corresponding figure was 96 days (range 10-251) in the prophylaxis cohort.

Table 5. Incidence of CMV infection and disease in 73 CMV seropositive heart transplant patients between 2008 and 2012 (Paper IV)

Period	Prophylaxis	CMV disease	CMV infection	No CMV
2008-2010 n=31	Pre-emptive therapy	3 (10%)	24 (77%)	4 (13%)
2010-2012 n=42	VGCV 3 months	0	24 (57%)	18(43%)

VGCV: 450 mg of valganciclovir daily for three months

Survival at 10-year follow-up in heart transplant patients

Survival for patients with CMV disease was 7.0 years (95% CI 6.0-7.9), with asymptomatic CMV infection 7.5 years (95% CI 6.4-8.5) and with no CMV infection 8.7 years (95% CI 8.2-9.2). The mean follow-up for survival was 9.9 years (Figure 10).

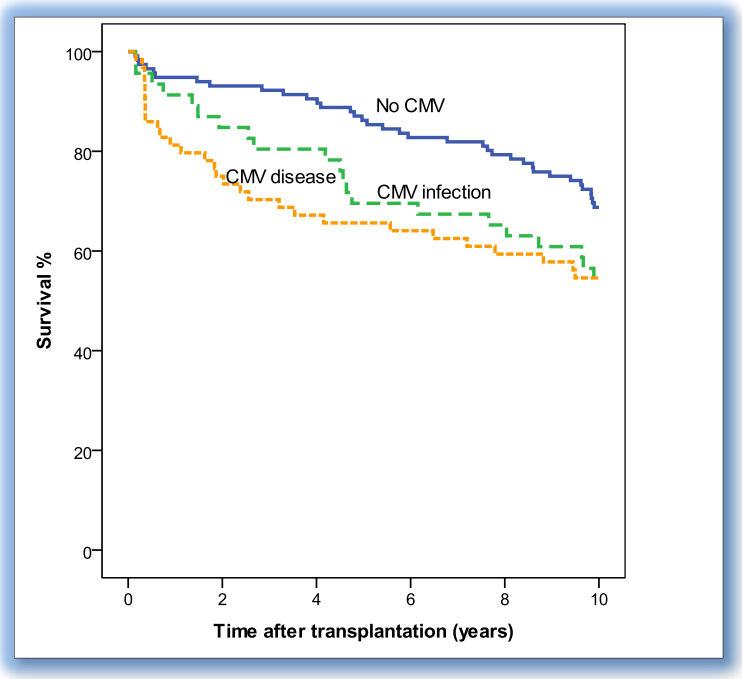


Figure 10. Survival was significantly higher for patients with no CMV infection, n=116 (69%), compared with patients with CMV disease, n=64 (55%; p=0.018), and asymptomatic CMV infection, n= 46 (54%; p=0.053). Kaplan-Meyer survival curve.

CAV-free survival at 10-year follow up

The CAV-free survival in heart transplant patients was 6.4 years (95% CI 5.7-7.0) for the 116 patients with no CMV infection, 5.1 years (95% CI 4.1-6.2) for the 46 patients with asymptomatic CMV infection and 4.1 years (95% CI 3.1-5.1) for the 64 patients with CMV disease. The CAV-free survival for the total group was a mean of 5.5 years (95% CI 4.96-5.98). When tested in a multiple Cox-regression model, CMV disease, asymptomatic CMV infection and donor age (but not recipient age, gender and HTx due to ischemic heart disease) were significant predictors of CAV-free survival 10 years after transplantation.

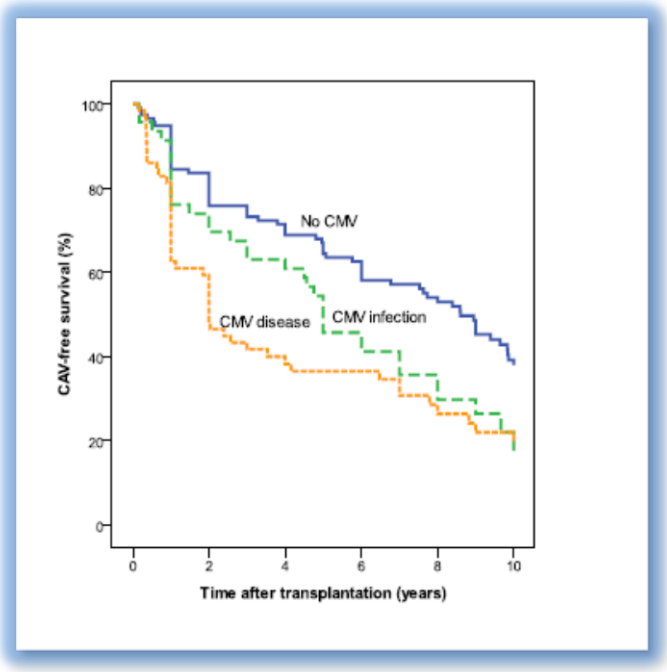


Figure 11. *CAV-free survival during a follow-up of 10 years in 226 heart transplant patients was significantly higher for patients with no CMV infection (n =116), compared with patients with CMV disease (n =64, p =<0.0001) and asymptomatic CMV infection (n =46; p =0.013), Kaplan-Meyer survival curve.*

5 DISCUSSION

A transplanted lung is a high-risk organ for CMV infection and heart transplantation entails an intermediate risk of CMV infection. We studied the incidence and severity of CMV infection and disease with different prophylactic regimens and the impact of CMV on the development of BOS in lung transplant recipients and CAV in heart transplant recipients. The discussion is divided into two parts, beginning with CMV in lung transplant (LTx) recipients, followed by CMV in heart transplant (HTx) recipients.

CMV in lung transplant recipients

What are the optimal drug, dose and duration of CMV prophylaxis to LTx recipients? Universal prophylaxis, not pre-emptive therapy, has always been the prophylactic approach at our centre. Oral aciclovir was the first drug to be used; late in 1992, prophylaxis was changed to i.v. ganciclovir with four weeks' duration. Oral ganciclovir was introduced in 2001, followed by valgancilovir in 2003; both drugs were given for three months.

In our study a dose of 1,000 mg of oral ganciclovir three times daily was compared with 900 mg of valganciclovir once daily in R+ patients. All patients received prophylaxis for three months. CMV infection and disease were registered for the first 12 months. We found a significantly lower incidence of CMV infection/disease in the valganciclovir cohort compared with the ganciclovir cohort. The drugs were compared in a prospective study by Paya et al. on effect of CMV prophylaxis in D+/R- solid organ transplant recipients. The authors found a higher viral suppression and a delay to viremia in the patients with valganciclovir prophylaxis as compared with oral ganciclovir. They concluded that valganiclovir was as clinically effective as oral ganciclovir. However, LTx patients were not included in that study [138]. To our knowledge, there is no prospective clinical study comparing the drugs in LTx patients. One possible explanation of the different effects found in our study is that oral ganciclovir results in a lower blood concentration of the drug. Valganciclovir is a prodrug of ganciclovir, with 60% bio-availability compared with six per cent for oral ganciclovir. Pescovitz et al. concluded that 1,000 mg of oral ganciclovir three times daily is comparable to 450 mg of valganciclovir once daily [139].

Our studies on CMV R+ recipients support a longer duration of CMV prophylaxis. An intravenous dose of 5 mg/kg of ganciclovir twice daily for two weeks, followed by 5 mg/kg once daily for an additional two weeks, was compared with oral ganciclovir for 14 weeks. Oral ganciclovir, which was

given in a dose resulting in a lower blood concentration but given for a longer duration as compared with i.v ganciclovir, was more effective in preventing of late onset CMV infection and disease. In addition, we found that 14 weeks of oral ganciclovir resulted in a delay in the onset of CMV infection/disease and also a trend towards less severe CMV pneumonitis. Valganciclovir given in a dose of 900 mg daily for three months to R+ LTx patients reduced the incidens of CMV infection and disease, but the burden of CMV disease was still high, 20%. A high incidence of late-onset CMV disease in LTx recipients (D+/R-and R+) with three months of valganciclovir has also been found by others [140]. CMV disease has to be reduced further and our findings support the hypothesis that a longer duration of prophylaxis to R+ LTx patients is beneficial. Zuk et al. reported in 2010 that the duration of prophylaxis varies at different centres from three months to indefinitely, with three to 12 months as the most common duration [141]. Benefits of a longer duration of CMV prophylaxis with valganciclovir to LTx recipients have been reported in different time periods [42, 103, 134, 142, 143]. A randomised, controlled trial compared three months of valganciclovir with 12 months of valganciclovir to all at-risk LTx recipients (D+/R- and R+) and demonstrated the efficacy of a longer duration of CMV prophylaxis. One comment is that they only followed the incidence of late-onset CMV for six months after the cessation of 12 months of prophylaxis [84]. A longer follow-up is needed and, as a result, 38 patients from the previously mentioned study were followed longer, an average of four years, in a single-centre study. They reported a sustainable effect of prophylaxis with a reduced risk of pneumonitis for patients who received 12 months of valganciclovir [144]. Two studies from 2013 highlighted the impact of donor/recipient CMV serostatus and recommended six months of valganciclovir to R+ and 12 months to D+/R- LTx patients [85, 145]. The international guidelines for solid organ transplant recipients (Transplantation Society International CMV Consensus Group [32, 70] and the Americ an Society of Transplantation) recommend six to 12 months of CMV prophylaxis to R+ and 12 months to D+/R- LTx patients [77].

Comparing studies from different centres may be difficult due to various definitions of CMV infection and disease, various diagnostic methods, various frequencies of monitoring and follow-up times**.** The strengths of our studies when we compared different types of prophylaxis are that we included the same definition of CMV disease and infection, the same CMV serostatus (R+), the same frequency of monitoring and the same diagnostic methods used with TBB/BAL during the whole study time, from 1990 to 2006.

The optimal way to compare CMV prophylaxis in retrospective studies is to compare patients who are treated with the same immunosuppression. This is done in Paper II. In Paper I, there was a change of immunosuppressive therapy in 1997, when the triple immunosuppression of AZA, CsA and steroids was changed to MMF, CsA and steroids. Induction with ATG has been used throughout the whole study period, but the dose was reduced in 1998.

CMV disease was not detected during the prophylaxis period if an adequate dose of ganciclovir or valganciclovir was given. Late-onset CMV disease (i.e. disease after the prophylactic period ends) occurred on average 1.5-2.5 months after the cessation of ganciclovir/valganciclovir with three months' prophylaxis. It is essential to monitor frequently during this time period to detect CMV infection/disease when three months of prophylaxis is given. Future studies may identify the optimal length of the monitoring period and the intensity of monitoring.

Bronchiolitis obliterans syndrome

We found that CMV disease had a significant negative impact on survival. After 10 years 32% of the patients were alive and 57% of those without CMV infection. This impact of CMV disease on survival could be attributable to the infection as such, but our results also suggest that CMV disease increases the risk of the development of BOS. The results from both our studies support the idea that CMV disease is a risk factor for the development of BOS. We found a tendency towards lower BOS and histologically verified BO with valganciclovir as compared with oral ganciclovir. However, no significant difference between the two drugs in terms of 4-year survival and BOS-free 4-year survival was seen.

Preventing BOS is essential, as no effective cure exists. The median survival after the onset of BOS is three to four years [146, 147]. BOS is manifested by a progressive decline in pulmonary function. The histopathological features of BO are that injury to and inflammation of epithelial cells and sub-epithelial structures of small airways lead to excessive fibroproliferation, including ineffective epithelial regeneration. The result is a partial or complete obstruction into the airway lumen [148]. The pathogenesis of BOS is complex and the mechanisms underlying the possible association with CMV infection and BOS are unclear. CMV has both immunosuppressive and inflammatory properties [48]. Several cytokines, chemokines and growth factors are suggested to be involved in the pathogenesis [149]. The risk factors for BOS are said to be multifactorial and different medical conditions can lead to the same microscopic, physiological and clinical results [150].

Several other studies have addressed the possible relationship between CMV infection and BOS [37, 40-42, 103, 104, 151, 152], but, on the other hand, some authors present conflicting results [105, 106, 153]. The strongest evidence of an association between CMV infection and BOS dates back to the pre-ganciclovir era.

Acute rejection in lung transplant recipients

Is there an association between CMV and acute rejection? We found that CMV infection/disease and acute rejection were significantly lower in the valganciclovir cohort, as compared with the ganciclovir cohort. Both cohorts had the same protocol for immunosuppression. One explanation of the lower rate of acute rejection among those receiving the valganciclovir prophylaxis could be the lower incidence of CMV infection/disease during the first year post-transplant. A lower CMV burden results in less inflammatory response in the transplanted lung(s) and fewer episodes of acute rejection may therefore be triggered.

It is assumed that there is a bidirectional relationship between CMV and acute rejection, with several mechanisms involved in this effect. Cytokines, chemokines and growth factors are induced in response to both CMV infection and acute rejection and result in the activation of the vascular endothelium and inflammatory cells [26, 58]. The tumour necrosis factor (TNF) is released during acute rejection and is one of the most important proinflammatory cytokines in the reactivation of CMV from latency [58, 59]. As a result of same proinflammatory mediators, the histological features of acute rejection and CMV pneumonitis are almost the same. Distinguishing histologically between the two diagnoses is difficult and requires the detection of the CMV virus.

If CMV in fact induces acute rejection, the antiviral prophylaxis should be able to reduce/influence the incidence of acute rejection. Our result supports this concept. Other authors have also found that CMV prophylaxis influences the incidence of acute rejection. Paraskeva et al. observed a significantly lower incidence of acute rejection within the first 12 months when valganciclovir for five months was compared with ganciclovir for three months, with no difference in the immunosuppression protocols between the two groups [37]. Jaksch et al. found that there was a positive trend towards a lower acute rejection score when D+/R- LTx patients received 12 months of valganciclovir as compared with three months [134].

CMV in heart transplant recipients

Our studies range from 1988 to 2012. The spectrum of CMV disease, the diagnostic tools and CMV prevention have changed during this period. In the 1990s, myocarditis, pneumonitis, gastrointestinal disease and CMV syndrome were the most common forms of CMV disease. Today, the most common manifestation of CMV disease in HTx recipients is CMV syndrome, followed by gastrointestinal disease, but the myocarditis and pneumonitis are seldom seen. Nephritis, hepatitis and retinitis were represented at a low frequency during the time period.

Viral culture and CMV-specific IgG and IgM antibodies were the available diagnostic laboratory methods during the late 1980s and the early 1990s. These laboratory methods confirmed the CMV infection/disease first when the patients had developed severe or moderate symptoms or had already recovered from a mild CMV infection. Regular, frequent endocardial myocardial biopsies made it possible to verify CMV myocarditis. A high incidence of CMV disease occurred during this period, as no prophylaxis was given. Qualitative PCR, an in-house nested PCR, has been available at our transplant centre since 1992 [154]. Quantitative PCR, Cobas Amplicor Monitor (Roche), has been available at our centre since 2001 [155, 156]. A few years later, the method was changed to quantitative real-time TacMan® PCR, which is the method used today [136].

We found that both CMV disease and asymptomatic CMV infection during the first year after heart transplantation had a significant influence on CAV-free survival after 10 years of follow-up. In agreement with our results, other studies have shown that CMV infection may play an essential role in CAV progression [39, 124, 157, 158]. In some studies, however, the effect of CMV on CAV development has been questioned [126, 127]. The reason for this difference may be different definitions of CAV and the fact that only the influence of CMV serology, not CMV infection or disease, on CAV development was studied [126, 127]. CAV is a rapidly progressive form of arteriosclerosis unique to transplant patients. It is a complex multifactorial process, with both immunological and non-immunological risk factors. The pathogenesis is endothelial injury, followed by intimae hyperplasia and the proliferation of vascular smooth cells that lead to diffuse luminal stenosis of the coronary arteries. Histologically, a diffuse concentric longitudinal intimal hyperplasia in the epicardial coronary arteries is seen. It is diagnosed with coronary angiography or intravascular ultrasound (IVUS). The structural changes occur in the coronary artery during the first year after heart transplantation and this adversely affects long-term survival [159-161]. CMV is capable of infecting endothelial cells

and smooth-muscle cells in the vascular wall, thereby causing cell damage [115, 122, 162]. This is just one of the characteristics of the CMV that explain how the virus may play a role in CAV progression. The exact role of CMV in CAV development, however, remains unclear [115].

In 2010, our transplant centre changed CMV prophylaxis for CMV seropositive HTx patients from pre-emptive to universal prophylaxis. The reason was to reduce CMV disease and the possible negative impact of CMV in the long term. There have been different opinions about the best prophylactic approach to HTx recipients [163]. There is no randomised trial comparing the two different approaches to HTx. Pre-emptive therapy has the advantage of fewer incidences of late-onset CMV infection, theoretically more opportunity to develop CMV-specific immunity in addition to lower drug cost, reduced drug exposure and a reduced risk of developing resistant CMV. Universal prophylaxis is easy to implement, has a lower monitoring cost, may prevent other opportunistic infections, acute rejection and improve graft survival. CMV prophylaxis guidelines recommend a 900-mg daily dose of valganciclovir to prevent disease in solid organ transplant recipients [32]. A well-known adverse event of valganciclovir is myelosuppression, especially leucopenia. A low dose of valganviclovir, 450 mg daily, has been reported to provide effective CMV prophylaxis in liver and kidney transplant recipients [164, 165] Valganciclovir, 450 mg daily, is the current prophylactic dose for liver and kidney transplant recipients at our centre. It is unclear which dose of valganciclovir is needed in heart transplant patients in order to achieve optimal antiviral activity with minimal adverse effects. Only one study has been published on 450 mg of valganciclovir daily to HTx patients [166]. Our centre introduced low-dose valganciclovir for three months to CMV seropositive patients in April 2010. We compared 450 mg of valganciclovir daily with pre-emptive therapy in CMV seropositive HTx patients. None of the patients in the valganciclovir cohort was diagnosed with CMV disease within the first year post-transplantation. The CMV burden (viral load) was significantly lower as compared with the pre-emptive cohort. The drug was well tolerated. One concern is that the use of a prolonged low dose of valganciclovir may be a risk factor for the development of ganciclovir resistance. Some studies suggested that the treatment of CMV infection with sub-therapeutic doses of antiviral medication should be avoided [167, 168]. However, none of our patients developed clinical valganciclovir-resistant CMV disease. It is too early to evaluate the possible advantages of valganciclovir prophylaxis in the long term.

6 CONCLUSIONS

- CMV disease reduced 10-year survival and BOS-free 4-year survival compared with no CMV infection. Aggressive CMV prevention is essential for pulmonary function and long-term survival.

- CMV prevention with 14 weeks of oral ganciclovir reduced the incidence and severity and prolonged the time to onset of CMV disease, as compared with 4 weeks of intravenous ganciclovir, in CMV seropositive lung transplant patients. Our study supports a longer duration of CMV prevention.

- A lower incidence of CMV infection/disease and acute cellular rejection was observed with valganciclovir prophylaxis when compared with oral ganciclovir, both administered for 3 months to CMV seropositive lung transplant patients. Valganciclovir is more effective in reducing the CMV burden and might even have an impact on acute rejection in lung transplant patients.

- Ten-year survival and CAV-free ten-year survival was significantly reduced in patients with CMV disease and asymptomatic CMV infection in heart transplant patients. Aggressive CMV prevention within the first year post-transplant is essential to reduce CAV and increase long-term survival.

- A low dose of valganciclovir prophylaxis (450 mg daily) to CMV seropositive heart transplant patients prevented CMV disease and significantly reduced the number of patients with reactivated asymptomatic CMV infection as compared with a pre-emptive approach. Prospective, controlled studies are needed to confirm these results.

7 FUTURE PERSPECTIVES

During the last decade, significant advances have been made in the management of CMV infection. Effective drugs and viral monitoring have reduced the frequency of CMV disease.

The optimal duration of CMV prevention to LTx and HTx recipients is, however, not known, even though a longer duration of CMV prevention to LTx recipients is beneficial. Low-dose valganciclovir, 450 mg, in HTx recipients needs to be evaluated in prospective studies, with frequent monitoring with QNAT to evaluate the efficacy and safety of this prophylaxis. Futhermore, long-term studies are needed to access the possible/probable causal relationship, and not only the association, between CMV reactivation and graft injury, such as BOS and CAV.

There is a need for novel oral antiviral drugs for both prevention and treatment CMV infection and disease. Several drugs are in various stages of clinical development: Maribavir, an oral inhibitor of CMV UL97 kinase [169]. Brincidofovir, an oral bio-derivative of cidofovir, with less or no renal toxicity [170], Letermovir, a CMV UL56 terminase inhibitor [171] and Cyclopropavir, a nucleoside analogue [172].

The development of CMV vaccine is an area of research [173, 174]. The goal is to find a vaccine able to prevent or modulate CMV infection and/or disease. Clinical trials phase II and III are ongoing. TransVax DNA vaccine will enter a phase 3 trial [175].

Immunological monitoring may be performed in the future. Assays commercially available today are QuantiFeron®-CMV (only detects CD8+ response) and ImmuKnow® (not specific for CMV). The assays may help to individualise CMV prophylaxis in the future, but it remains to be proved whether these tests are sensitive enough for LTx and HTx recipients [176-178].

These are just a few of the never-ending questions that need to be answered when it comes to this complex and interesting virus.

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