Clinical significance of asymptomatic cytomegalovirus viremia in lung transplant recipients receiving universal antiviral prophylaxis

Student: Nikta Jaamei

Tutor: PD Dr Oriol Manuel
Prof Manuel Pascual

Expert: Prof John-David Aubert

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UNIL | Université de Lausanne

Centre hospitalier universitaire vaudois

ABSTRACT

Background: Cytomegalovirus (CMV) disease has been associated with the development of chronic lung allograft dysfunction (CLAD) after lung transplantation. However, the relevance of asymptomatic CMV viremia occurring after the discontinuation of antiviral prophylaxis (late-onset CMV replication) on the development of CLAD is not fully understood. We aimed to assess the long-term clinical impact of asymptomatic CMV replication in a cohort of lung transplant recipients receiving universal antiviral prophylaxis.

Methods: We performed a single-center study including all patients who underwent lung transplantation between 2004 and 2014. Patients received valganciclovir prophylaxis for 3 to 6 months (according to CMV serostatus risk of the donor [D] and recipient [R]), followed by monitoring of CMV replication by PCR during the first year post transplant. CLAD was defined according to ISHLT definitions. Risk factors for the development of CLAD and for mortality were assessed by univariate and multivariate Cox models. A lineal regression model was used to evaluate the influence of CMV replication in the evolution of FEV1.

Results: Overall, 69 patients were included. CMV serostatus was D-/R- in 13 (19%) patients, D-/R+ in 17 (25%) patients, D+/R+ in 27 (39%) patients and D+/R- in 12 (17%) patients. Overall, 34/69 (49%) patients developed at least one episode of asymptomatic CMV replication and 8/69 (11.5%) patients developed CMV disease. Median duration of CMV replication in viremic patients was 57.5 days. After a median follow-up of 3.69 years, 25/69 (36%) patients developed CLAD and 14/69 (20%) patients died. In the univariate cox analysis, bacterial pneumonia was associated with a higher incidence of CLAD (HR 2.58, p=0.06), but asymptomatic CMV replication (HR 1.36, p=0.45), CMV disease (HR 1.00, p=0.99), and duration of CMV replication (HR 1.00, p=0.76) were not. In the multivariate model, bacterial pneumonia remained associated with CLAD (HR 2.53, p=0.06). In the mixed model of linear regression, we did not observe a correlation between CMV replication and a significant decline of FEV1 (estimate -0.162, CI 95% [-0.498 to 0.170], p=0.35). CMV replication was not associated with a higher mortality (HR 0.75, p=0.62).

Conclusion: In this cohort of lung transplant recipients receiving antiviral universal prophylaxis, asymptomatic CMV replication did not influence long-term allograft lung function and patient survival. These results suggest that the use of universal prophylaxis is protective against the indirect effects of CMV, irrespective of the development of late-onset CMV replication.

Key words: viral infection, chronic lung allograft dysfunction, lung transplantation, outcomes

1. INTRODUCTION

1.1 Lung transplantation

The first lung transplantation ever was performed in 1963 and the patient survived only 18 days. Much has changed over these last decades, as lung transplantation is today the preferred treatment for many end-stage lung diseases, such as chronic obstructive pulmonary disease (COPD), interstitial lung diseases and cystic fibrosis. Worldwide, the amount of lung transplantation procedures achieved has dramatically increased since 1990. In 2014, 56 lung transplantations were performed in Switzerland, which of 24 were done at the Lausanne University Hospital (CHUV) (1). Median waiting time for lung transplantation was 465 days, with a stable waiting list since 2010 (Figure 1). In 2014, 8 patients died waiting for a lung.

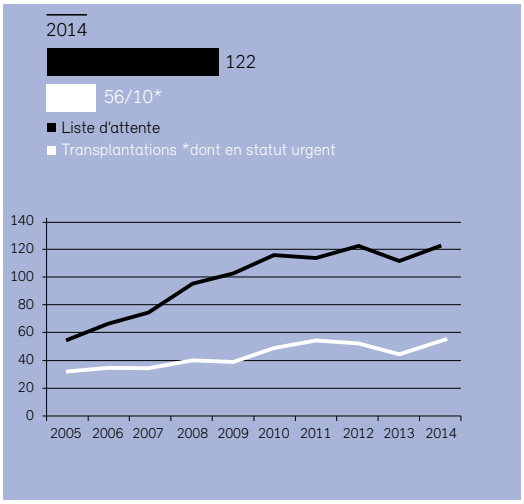

Figure 1: Lung transplantation in Switzerland and waiting list, year 2014 (Swisstransplant annual report 2014)

1.2 Outcome after lung transplantation

The survival after lung transplantation has been increasing worldwide since 1990. In the era between 1990 and 1997, the median survival post-transplantation was 4.3 years. It has now reached 6.3 years for the 2005-2012 era (2). The median survival for single and double-lung transplantation according to the data from the ISHLT registry is shown in figure 2. However, survival after transplant depends on many different factors. Better survival is correlated with younger and female recipients, as well as the underlying lung disease of the recipient. When considering adjusted survival (patients surviving at least one year), median survival was the longest for CF patients (11 years) followed by idiopathic pulmonary arterial hypertension (IPAH), sarcoidosis and COPD associated with α1-antitrypsin deficiency. Transplant

recipients receiving a double-lung transplantation have a better survival (7 years vs 4.5 years for single transplant) (2).

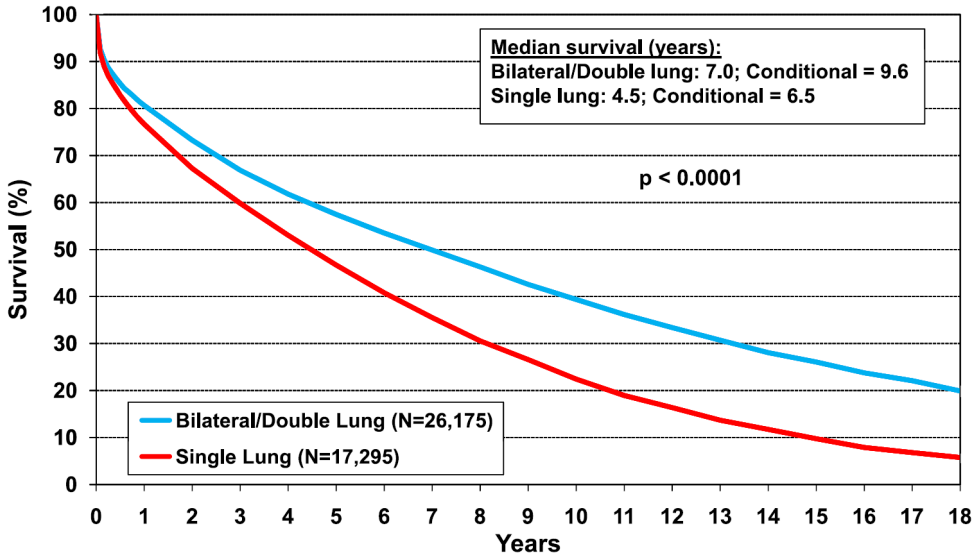

Figure 2: Median survival for single and double lung transplant recipients (ISHLT registry)

1.2.1 Causes of death

The most common causes of death within the first 30 days post-transplantation are graft failure and technical or cardiovascular causes. Infections are a major cause of death at any time. After the first year, another main cause of death is chronic lung allograft dysfunction (CLAD), which includes processes like bronchiolitis obliterans syndrome (BOS) that will be discussed later. Main causes of death are listed in table 1.

First 30 days	- Graft failure - Infectious complications - Surgical complications - Cardiovascular causes
First year	- Infectious complications
After 1st year	- Chronic lung allograft dysfunction - Infectious complications

Table 1: main causes of death following lung transplantation (ISHLT registry)

1.2.2 Risk factors for mortality

Patients with BOS or acute rejection in the first-year post-transplant have a higher risk of mortality at 5 years (2). In a study involving 23'704 lung transplant recipients, the mortality risk model at 5 years demonstrated a negative impact from advanced recipient age and lower transplant center volume. The impact of age seems to be restricted to the recipient, as age appears not to be relevant when considering donor age. In fact, it was observed that allograft from older donors (>65 years old) did not affect the survival, although there seems to be a negative effect when given to a younger recipient (3). With the aging of the population, this question must be further studied to assess the impact of transplantation including older (>60 years old) donors or recipients. Other risk factors that may influence patient survival are listed in table 2.

5-year post-transplant	- Underlying lung disease of recipient - Retransplantation - Earlier era of transplant - Increased severity of recipient illness at the time of transplantation (intensive care unit) - Donor history of diabetes - CMV mismatch (D+/R-) - Lower transplant center volume - Older recipient at age of transplant - Higher pretransplant supplemental oxygen required - Lower cardiac output

Table 2: Risk factors of death following lung transplantation (ISHLT registry)

1.2.3 Quality of life

Lung transplantation has a positive impact on the quality of life (QoL) (4). There is an improvement in many dimensions of health - as defined by the Nottingham health profile - except for the aspects of pain and mental health. However there is a clear improvement in dimensions such as social life or physical mobility.

1.3 Immunosuppressive regimen

1.3.1 Induction therapy

At the time of transplantation, induction therapy is used to prevent the immune system of the recipient, especially T-cells, to attack the allograft. Induction agents are either monoclonal agents (i.e basiliximab, daclizumab) or polyclonal agents (anti-lymphocytes, anti-thymocytes). There is some conflict in the literature about the efficacy or superiority of one agent for induction therapy. There may be some benefit using monoclonal agents to prevent from onset of chronic rejection (5), but other studies do not confirm this hypothesis (6). Of note, 50% of centers do not generally use induction therapy, and this without a clear negative impact on allograft and patient survival.

1.3.2 Maintenance therapy

The aim of maintenance therapy is to keep a balance between over-immunosuppression that will lead to infection and under-immunosuppression that will cause allograft rejection. Different agents are used low-dose, to minimize the potential toxic adverse effects. Usual treatment combines three agents:

1. Glucocorticoids, i.e prednisone: always used for immunosuppression. It inhibits both the adaptative and innate immune system and down-regulates the transcription of inflammatory genes (7)
2. Calcineurin inhibitors: main agents are cyclosporine and tacrolimus. Both agents are inhibiting T-cell activity, but tacrolimus is now more widely used, especially among lung transplant recipients (8). Tacrolimus has less nephrotoxic adverse effects and may reduce the onset of BOS (9), but is more diabetogenic.
3. Nucleotide blocking agents: usually azathioprine (AZA) or mycophenolate mofetil (MMF). While some studies have shown superiority of MMF over AZA treatment (10), this has not been confirmed in a randomized clinical trial (11).

1.4 Allograft rejection and dysfunction

1.4.1 Acute rejection

They are three types of acute rejection:

1. Cellular rejection: mediated by T-cells directed towards HLA antigens. It is the predominant type of acute rejection
2. Humoral rejection: mediated by antibodies directed towards donor HLA

3. Hyperacute rejection: occurs within first 24 hours post-transplantation and is mediated by pre-formed antibodies from the recipient

Acute rejection occurs in approximately one-third of lung transplant recipients (12). Acute rejection should be confirmed by a histological diagnosis, as an infectious process needs to be excluded. The difference between a rejection or an infectious process cannot be always done on clinical findings, as the presentation may be similar in some instances (13). Symptoms may occur mostly from A2 grade rejection and more.

The actual nomenclature of allograft rejection has been established by the ISHLT in 2007 (14).

A. Acute rejection: the diagnosis of acute rejection is made only on the presence of perivascular and interstitial mononuclear cell infiltrate. The grade can be between 0 (no acute rejection) to grade 4 (severe) and reflects the most advanced pattern (and not the predominant one).
B. Airway inflammation: lymphocytic bronchiolitis. This designation applies to small airways (bronchioles) and the grading is B0 (absent), B1 (low grade), B2 (high grade) or Bx (ungradable).
C. Chronic airway rejection – obliterative bronchiolitis. This definition occurs when eosinophilic hyaline fibrosis in the sub-mucosa of membranous and respiratory bronchioles is seen, resulting in a partial or complete luminal occlusion. They may be associated with destruction of the smooth muscle and elastica of the airway. Mucostasis or foamy histiocytes are commonly associated with this phenomenon even in the absence of bronchiolar occlusion.
D. Chronic vascular rejection – accelerated graft vascular sclerosis. This process is similar to coronary artery disease in the heart. It describes a fibrointimal thickening of arteries and veins.

1.4.2 Chronic lung allograft dysfunction (CLAD)

Surgical techniques and immunosuppressive regimens have greatly improved since 1963, however lung transplantation is still the solid organ transplantation (SOT) with least success, since 5 years survival is only 53% (15). The major factors that explain this disparity compared to other SOT is the highest incidence of chronic allograft rejection in lung transplant recipients, which is now defined as chronic lung allograft dysfunction (CLAD) (16).

CLAD is not an etiological definition, but indicates that the lung is not achieving its full function. It is not irreversible per se, although it is usually a permanent condition. Depending on the spirometrical parameters, CLAD may present as a restrictive or an obstructive pattern.

- *Bronchiolitis Obliterans Syndrome (BOS)*: decreased FEV1 (FEV1) <80% from baseline for > 3 weeks without any confounding condition and evidence of airflow obstruction (17). FEV1 baseline is defined as "the average of the two highest values for each measurement that were obtained at least three weeks apart post-transplant without the administration of a bronchodilatator" (17).
- *Restrictive Allograft Syndrome (RAS)*: FVC or TLC <80% baseline FVC or TLC for > 3 weeks.

We will discuss the pathogenesis and management of BOS in the next section.

1.5 Bronchiolitis Obliterans Syndrome

BOS is the clinical correlate of obliterative bronchiolitis (OB). OB is a histological diagnosis presenting as progressive obliteration of small airways. Because it was difficult to make this diagnosis via transbronchial lung biopsy, the actual consensus is to use BOS as the clinical marker of chronic allograft dysfunction. BOS is a major issue in lung transplantation as it represent the main cause of death adjusted for conditional survival of one year (18). The incidence reaches 50% within 5 years after transplantation (19) and the median survival after diagnosis is 3 years.

1.5.1 Pathogenesis of BOS

The first step of BOS is onset of lymphocytic infiltrates in the submucosa, with processes like lymphocytic bronchiolitis (LB) leading to epithelial injury. This injury will thus create the following sequence: necrosis → ulceration → inflammation that will end up by fibroblastic proliferation. Finally, the intraluminal polypoid granulation will eventually occlude totally or subtotally airways lumen (20). This is a complex process including immune and non-immune mechanisms. The molecular pathways are not fully understood yet, but we will shortly summarize the role of some cytokines in figure 3.

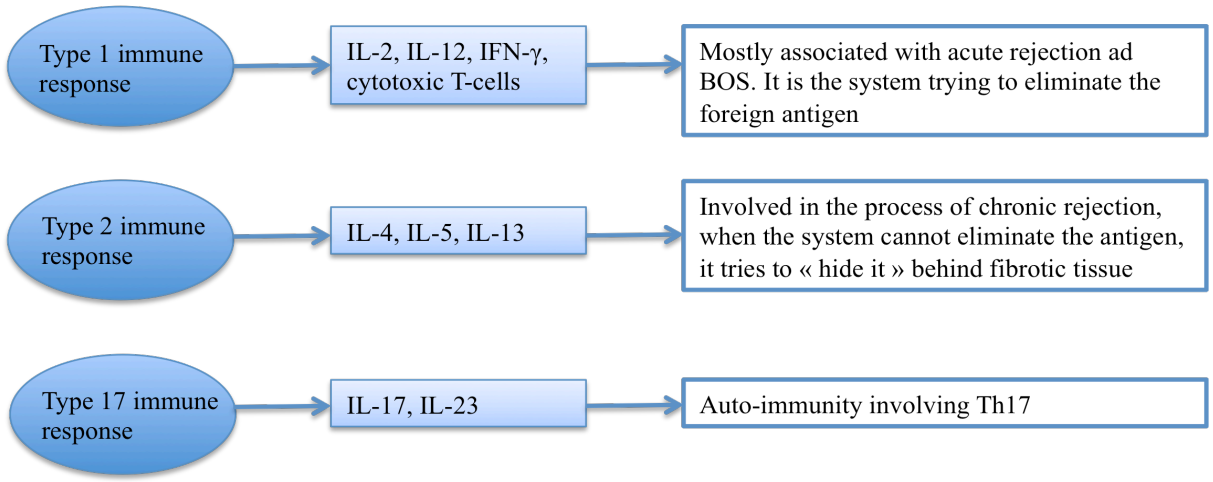

Figure 3: cytokines involved in BOS pathogenesis (Adapted from Weigt and al, 2010)

1.5.2 Risk factors of BOS

Here are listed some risk factors for onset of BOS.

- Alloimune mechanisms: BOS may be the clinical consequence of repeated acute rejections episodes (21). There is an increased risk of BOS when there are more HLA mismatches (22). Acute rejection may present with peri-airway infiltration of activated lymphocytes, which is described as lymphocytic bronchiolitis (LB). LB is recognized as the most significant risk factor for developing BOS (23).
- Primary graft dysfunction (PGD): occurs typically within the first 24 hours after transplantation and is caused by events during the transplantation procedure, such as pulmonary ischemia or donor tissue preservation (20). There is evidence that not only it does affect the survival on a short term, but influences also the long-term survival and is hence considered a BOS risk factor.
- Respiratory infections: community-acquired respiratory virus (CARV) have been identified as risk factor for BOS (24), and this risk was tripled when the lower respiratory tract was implicated. In smaller studies, *Chlamydia pneumophilae* and HHV-6 were also recognized as risk factors. The airway colonization by *Pseudomonas aeruginosa* and *Aspergillus* may be an independent risk factor for BOS as well (25)
- Others: gastro-intestinal reflux, air pollution.

CMV role will be discussed later in a separate section

1.5.3 Prevention and detection of BOS

BOS is a clinical diagnosis mainly based on FEV1 evolution from baseline. However it needs some expertise by the transplant physician. There is an overall good agreement regarding the presence or absence of BOS diagnosis (26). There may be some variability when performing the spirometry, due to patient's performance. One issue with BOS diagnosis is that it is retrospective by definition therefore the impact on the clinical management is limited. However there is some literature about how to predict or prevent onset of BOS. In a retrospective study including 121 patients, the measurement of CRP and neutrophilia in the broncho-alveolar lavage (BAL) at 90 days after transplantation was a predictive factor for determining the outcome of the allograft (27). Patients with BOS had a higher BAL CRP (cutoff >173 pg/ml) than the BOS-free group (BAL CRP baseline: 50 in stable group and 540,4 in BOS group, p=0.002). CRP stimulates alveolar macrophages and alters surfactant function in the lungs which indicates a role during acute respiratory distress syndrome and could be used as a marker of lung injury (28). The use of azithromycine, a macrolide antibiotic with immunomodulatory effect, as prophylaxis (29) or treatment (30) is useful by inhibiting the neutrophilic airways inflammation pathway. When used as prophylaxis, it increases the BOS-free interval of 2.3 years and the pulmonary functions were significantly better (p=0.051). As a treatment, azithromycine given during 12 weeks increases the FEV1 significantly (FEV1 difference between azithromycine and placebo group was 0.035 l and 39% of patients in azithromycine group had >10% gain in FEV1 compared to 0% in the placebo group).

1.6. Infections in a lung transplant recipients

Infections are one of the most common causes of death after transplantation (2), usually presenting as pneumonia (31). The pathogens are mostly bacteria, with gram-negative rods such as *Pseudomonas* and *Burkholderia* being the most frequent causes of pneumonia in lung transplant recipients (31).

1.7. Human cytomegalovirus

Cytomegalovirus (CMV) is a double-stranded DNA virus in the family of Human herpes virus (HHV) and is also called HHV-5. CMV is transmitted by contact, blood transfusion or organ transplantation. In the immunocompetent host, the primary infection is usually

asymptomatic and CMV remains latent. However the virus may reactivate in case of stress or immunocompromise.

Cytomegalovirus disease is the most important viral infection developing after SOT (32). CMV disease usually presents as a syndrome with fever, fatigue and body ache. Regardless of the type of transplant, CMV tissue-invasive disease usually involves the gastro-intestinal tract, with complications such as hemorrhage or perforation. It can however also affect lungs, kidney, liver or any other organ (33). These effects are called the "direct effects" of CMV. Regarding lung transplantation, the main concern is about CMV pneumonitis, that causes alterations of the allograft.

Moreover, CMV infection leads to many "indirect effects" - which cannot directly be related to a viral invasion of tissue - like an increased rate of bacterial and fungal infections or acute rejection (34). A relationship between kidney transplant, late-onset CMV infection and the concurrent post-transplant diabetes mellitus has been observed as well (35), suggesting a broad spectrum of indirect CMV effects. Direct and indirect effects are summarized in table 3.

Direct	**Indirect**
- CMV syndrome - Tissue-invasive disease (mostly GI tract)	- ↑ Bacterial, fungal infections - Acute and chronic rejection - Diabetes - More aggressive HCV infection after liver transplantation - Post transplant lymphoproliferative disorder - Death

Table 3: Direct and indirect effects of CMV

1.7.1. Risk factors for CMV infection

The incidence of CMV infection depends on different factors. The most important one is the serostatus at transplantation. Naïve recipients receiving organs from seropositive donors (D+/R-) are considered at high-risk. Patients with either D-/R+ or D+/R+ seroconstellation are considered intermediate risk and finally D-/R- low risk (32). The state of immunosuppression also influences the risk of CMV disease (36). The risk also depends on the organ transplanted: lung, small intestine and pancreas have the highest risk of CMV, whereas liver and kidney have the lowest (36). Risk factors are summarized in table 4.

Risk factors for CMV disease
- Serostatus - State of immunosuppression - Lung, small intestine or pancreas transplantation

Table 4: Risk factors for CMV disease

1.7.2. Immunomodulation and CMV

To be able to maintain latency in the immunocompetent individual, CMV had to find ways to hide from the host immune system. For example, it interferes in the antigen presentation mechanism by producing HLA class I homologue that will block recognition by natural killers and other T cells (37) and also downregulates expression of antigen-presenting cells (APCs) (38). CMV also modulate the humoral immune mechanisms by producing its own FC receptor homolog (39). These receptors will bind circulating IgG and thus allow the infected cell to escape other immunoglobulin directed towards viral proteins. By altering cellular and humoral immune pathways, CMV inhibits the immune system in a non-specific manner, which would be likely to increase infection susceptibility in the host.

1.7.3. CMV and allograft rejection

There is a question whether CMV reactivation would increase the risk of allograft rejection or the allograft rejection would lead to CMV reactivation. The answer is that the mechanism is bidirectional, as summarized in Figure 8. Allograft rejection produces a pro-inflammatory cytokine (TNF) that will induce the reactivation of CMV (40). When CMV reactivates, it stimulates infected macrophages and AECs to produce molecules molecules that will increase the number of inflammatory cells in the lung. These molecules are endothelial adhesion molecules such as VCAM, ICAM, LFA-1 and VLA-4. It does also activates MHC class I antigen by molecular mimicry (41). Figure 4 shows this bidirectional interaction. There are some genetic factors affecting the susceptibility to CMV recurrence. Polymorphism in recipient's genes coding for IFN-λ3 and IFN-λ4 influence the susceptibility to CMV replication in SOT recipients (42). This correlates with previous studies finding that these polymorphisms were associated with a reduced hepatitis C virus clearance. There is also an association between SNP polymorphisms of IFN-γ and CMV disease and high CMV viremia (43).

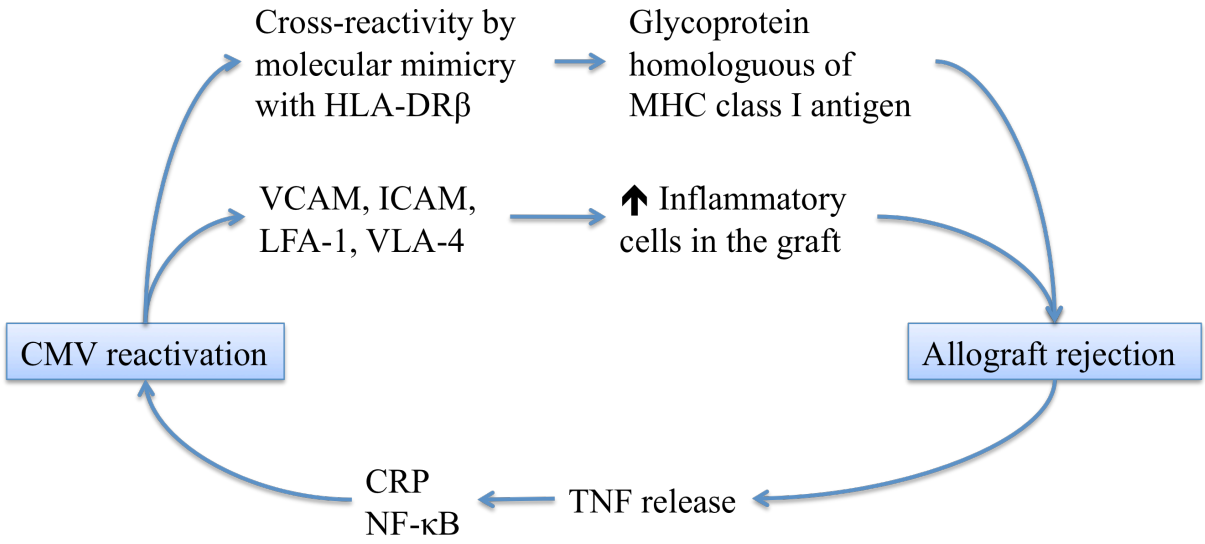

Figure 4: Bidirectional interaction between CMV reactivation and allograft rejection (Adapted from Freeman and al, 2009)

1.7.4. Management and prevention of CMV replication/disease in lung transplant recipients

Strategies have been set to minimize the impact of CMV replication/disease on lung transplant recipients. The actual guidelines recommend a prophylactic approach over a preemptive one for high-risk (D+/R-) group with a 6 months therapy (44). Intermediate risk group (D-/R+, D+/R+) should be receiving 3 months of prophylaxis and there is no indication for prophylactic treatment regarding low-risk group (D-/R-). The use of universal prophylaxis with valganciclovir or ganciclovir has decreased overall the incidence of CMV infection and acute rejection in the current era (45).

1.7.5. CMV infection and BOS

CMV pneumonitis is a well-known risk factor for developing a BOS. With the development of antiviral prophylaxis for high-risk (D+/R-) group, the incidence has consequently diminished. However, CMV replication remains associated with graft rejection and graft loss (46) and D+/R- recipients have still a higher mortality than the other groups despite the prophylaxis, which means they should be followed more carefully and proactively (47). Nowadays, we have more technical tools that allow us to identify CMV DNAemia in the blood and it has been therefore noticed that there may be a subclinical CMV replication in the bloodstream. There is still a debate about the clinical relevance of these subclinical DNAemia. Some studies showed an correlation between CMV DNAemia episodes and onset of BOS (48), which was not confirmed by other studies (49).

1.8. Filling a literature gap

The impact of CMV disease on survival and allograft function is nowadays well studied. CMV disease, especially CMV pneumonitis, occurring at an early or late stage is known as a risk for graft dysfunction and mortality (50). But the impact of asymptomatic CMV replication is not well defined yet and how to manage it in clinical practice. The aim of this study is to examine the potential clinical consequences of asymptomatic CMV replication in blood on allograft outcomes in a cohort of lung transplant recipients.

2. MATERIAL AND METHODS

2.1 Study design and patient population

We performed a single-center retrospective cohort study including all patients who underwent lung transplantation between 2004 and 2014 in the University Hospital of Lausanne (CHUV). Patients that were transplanted in Lausanne but had a follow-up in another transplant center (Basel, Bern of Geneva) were not included in the analysis.

We reviewed patient's records to collect demographic parameters, immunosuppressive regimens, FEV1 values, as well as infectious complications and episodes of acute rejection. The "Comission d'éthique de la recherche du canton de Vaud" approved the study protocol.

2.2 Definitions in the study

CMV infection was defined according to standard guidelines (44) by the evidence of CMV replication regardless of symptoms. CMV disease was the evidence of CMV infection with attribuable symptoms. CMV disease was further categorized as viral syndrome (fever, fatigue, myalgia) or end-organ disease (pneumonitis, colitis, gastro-enteritis or other). CLAD, BOS and RAS were classified according to ISHLT definitions. The term BOS is used when a persistent decline of FEV1 with no potential reversible cause found (30). Acute rejection was also defined following ISHLT definitions (14) which means the diagnosis of acute rejection is made only on the presence of perivascular and interstitial mononuclear cell infiltrate.

2.3 Immunosuppressive regimen and antiviral prophylaxis

The induction treatment was done with basiliximab. Maintenance therapy regimen used was tacrolimus, prednisone and mycophenolate mofetil (MMF). Patients at an intermediate risk for CMV serostatus (D+/R+ and D-/R+) received valganciclovir prophylaxis during 3 months and patients at high-risk (D+/R-) during 6 months. Patients with D-/R- serostatus received no anti-CMV prophylaxis, but anti-herpes prophylaxis with valaciclovir for 3 months. After discontinuation of prophylaxis, patients were monitored for CMV replication by PCR every 2 weeks. The detection of CMV viral load in blood was done by PCR according to standard method (51).

Lung function tests, including FEV1, FVC and 6 minutes walking test were measured at 3, 6, 12 months post-transplant and then each year and they were used to calculate the development of BOS and RAS.

2.4 Statistical analysis

We aimed to assess the long-term clinical impact of asymptomatic CMV replication in a cohort of lung transplant recipients receiving universal antiviral prophylaxis.
Descriptive statistics were used to describe the demographics and clinical characteristics of the population. Risk factors for the development of CLAD and for mortality were assessed by univariate and multivariate Cox models, including variables such as CMV replication, CMV diseasem CMV serostatus, other infectious complications (bacterial pneumonia, invasive fungal infection) and acute rejection. A lineal regression model was used to evaluate the influence of CMV replication in the evolution of FEV1.

3. RESULTS

3.1. Characteristics of the study population

Overall, 69 patients were included in the study. The mean age was 52 years old and 61% of patients were female. Most patients received double-lung transplantation. The main indications for transplantation were COPD (40%) and cystic fibrosis (26%). The median duration of follow-up was 3.67 years. Overall, 17% of patients had a high-risk CMV serostatus (D+/R-). 81% of patients received antiviral prophylaxis with valganciclovir for a mean duration of 3.29 months and 14% received valaciclovir. Baseline characteristics and main outcomes are listed in tables 5 and 6.

Demographic variables	**All recipients**
Number of patients	69
Time of follow-up in days, median (range)	1341 (34-3806)
Age, years mean (SD)	52.25 (14)
Male sex (%)	27 (39%)
Type of lung transplant (%) - single - double	 7 (11%) 62 (89%)
Underlying lung disease (%) - COPD - Cystic fibrosis - Other (IPF, alpha1AT deficiency, sarcoidosis)	 28 (41%) 18 (26%) 23 (33%)

Immunosuppressive regimen	
- Tacrolimus	68 (98%)
- MMF	64 (93%)
- Steroids	69 (100%)
CMV serostatus (%)	
- D-/R-	13 (29%)
- D-/R+	17 (25%)
- D+/R+	27 (39%)
- D+/R-	12 (17%)
Antiviral prophylaxis (%)	
- valganciclovir	56 (81%)
- valaciclovir	10 (14%)
Median duration of antiviral prophylaxis in days (range)	91 (0-1464

Table 5: Baseline characteristics of the study population

Outcomes	**All recipients**
CMV asymptomatic replication n (%)	34 (49%)
Duration of CMV replication, days, median (range)	57.5 (1-506)
CMV disease, n (%)	8 (11.5%)
Pneumonia episodes (%)	41 (59%)
Acute rejections (A1-A3) episodes (%)	36 (52%)
CLAD (%)	25 (36%)
CLAD free interval, median (range)	957 (237-2182)
Death (%)	14 (20.3%)

Table 6: Main outcomes of patients included in the study

3.2. CMV infection and disease

Overall, 34/69 (49%) patients developed at least one episode of asymptomatic CMV replication and 8/69 (11.5%) developed CMV disease (3 patients presented with colitis, 2 with pneumopathy, 1 with gastro-enteritis and 2 with other presentation). Median duration of CMV replication in viremic patients was 57.5 days.

3.3. Risk factors for the development of chronic lung allograft dysfunction

After a median follow-up of 3.67 years, 25/69 (36%) patients developed CLAD. Of the 25 cases of CLAD, 22 were considered to be BOS and 3 were considered to be RAS.

Table 7 shows the univariate Cox analysis for risk factors associated with CLAD. Asymptomatic CMV replication (HR 1.36, p=0.45) and CMV disease (HR 1.00, p=0.76) were not associated with a higher risk for the development of CLAD. We observed a lower proportion of CLAD in the D-/R+ group (HR 0.289, p=0.123), but this was not statistical significant. The duration of valganciclovir prophylaxis did not have an influence on the development of CLAD. Bacterial pneumonia was associated with a higher incidence of CLAD (HR 2.58, p=0.06). Kaplan-Meier curves of CLAD-free survival according to CMV replication, CMV serostatus, acute rejection and bacterial pneumonia are shown in Figure 5.

Variable	**Hazard ratio**	**IC 95%**		**P-value**
Sex (male)	0.775	0.345	1.741	0.538
Age at Tx	0.998	0.970	1.027	0.897
CMV replication	1.368	0.601	3.118	**0.455**
Duration of CMV replication	1.004	0.979	1.029	0.758
CMV disease	1.001	0.297	3.374	0.998
Duration of valganciclovir	0.895	0.737	1.086	**0.259**
CMV serostatus				
D-/R- (ref.)	1			
D-/R+	0.289	0.060	1.402	0.123
D+/R-	0.556	0.176	1.756	0.317
D+/R+	0.818	0.313	2.137	0.681
Acute rejection	1.561	0.668	3.645	0.304
Bacterial pneumonia	2.583	0.959	6.958	0.060
Fungal infection	2.020	0.756	5.396	0.161

Table 7: Univariate Cox regression model of risk factor for the development of CLAD

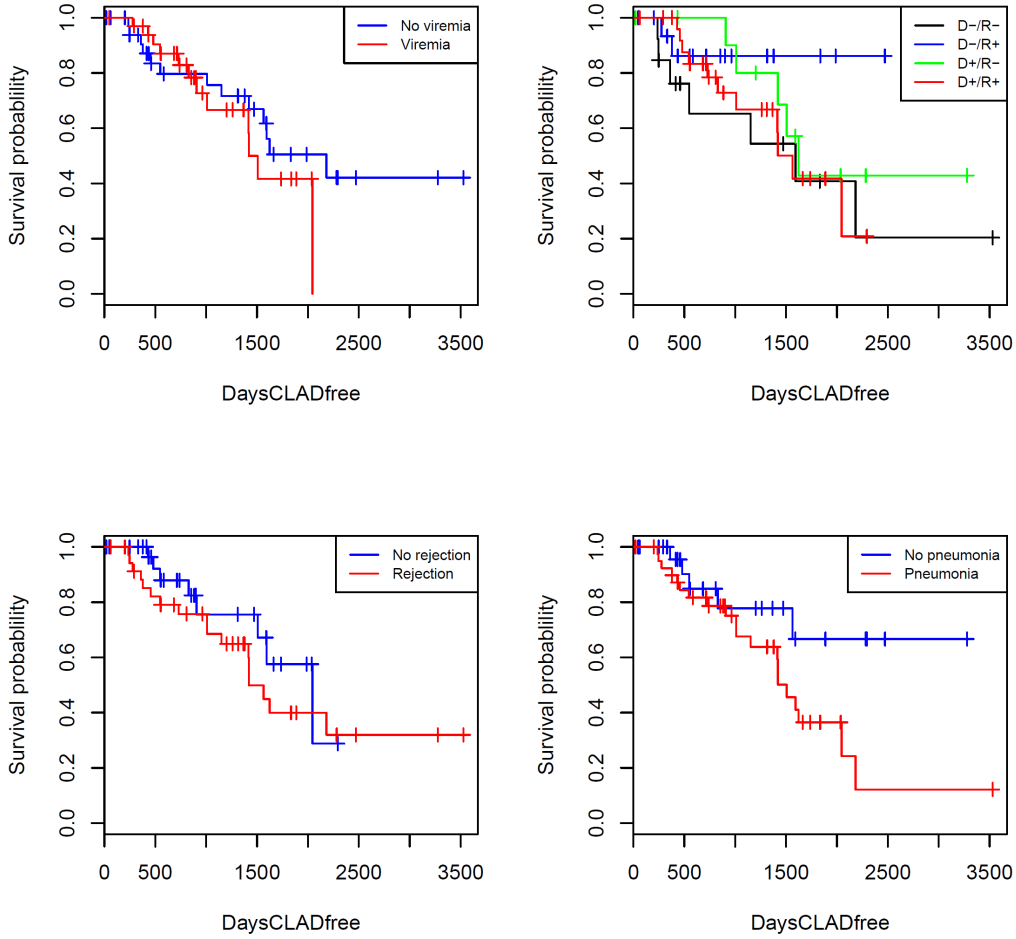

Figure 5: Kaplan-Meier curves on CLAD-free survival according to CMV replication (above, left), CMV serostatus (above, right), acute rejection (below, left), and bacterial pneumonia (below, right)

In the multivariate model (table 8), bacterial pneumonia remained associated with CLAD (HR 2.53, p=0.06) but CMV replication did not (HR 1.16, p=0.71).

Variable	Hazard ratio	IC 95%		P-value
CMV replication	1.162	0.511	2.639	0.719
Bacterial pneumonia	2.531	0.934	6.862	0.067

Table 8: Multivariate Cox regression model of risk factors for the development of CLAD

In the mixed model of linear regression (Table 9), we did not observe a correlation between CMV replication and a significant decline of FEV1 (estimate -0,162, CI 95% [-0.498 to 0.170], p=0.35).

Coefficient	Estimate	CI 95%		p-value
(Intercept)	2.370	2.112	2.629	0.000
viremia	-0.162	-0.498	0.175	0.346
period	0.052	-0.134	0.239	0.583
I(period^2)	-0.021	-0.075	0.033	0.446
I(period^3)	0.001	-0.004	0.005	0.808
viremia :period	0.216	-0.169	0.601	0.271
viremia :I(period^2)	-0.080	-0.207	0.047	0.214
viremia :I(period^3)	0.007	-0.004	0.018	0.197

Table 9: Linear regression on the evolution of FEV1 over time

3.4. Risk factors for mortality

During the study period 14 out of the 69 patients died. In the univariate Cox model (Table 10), CMV replication was not associated with a higher mortality (HR 0.75, p=0.62). The presence of CLAD was not significantly associated with a higher mortality (HR 2.814, p=0.085). Kaplan-Meier curve of patient survival according to the presence of CLAD is shown in Figure 6.

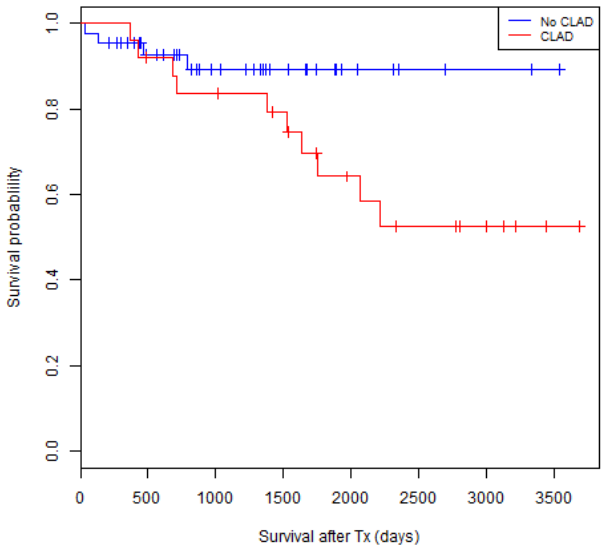

Figure 6: Survival after lung transplantation according to the presence of CLAD

Univariate Cox model of the risk factors for mortality are shown in Table 10.

Exposition	HR	HR CI 95%		p-value
CLAD	2.814	0.866	9.148	0.085
CMV replication	0.758	0.250	2.299	0.625
Duration of CMV replication	0.991	0.953	1.031	0.667
CMV disease	1.219	0.272	5.471	0.796
Valganciclovir	1.087	0.964	1.225	0.174

Sex (male)	0.433	0.135	1.385	0.158
Age at Tx	1.010	0.980	1.060	0.346
CMV serostatus				
D-/R- (ref.)				
D-/R+	1.302	0.254	6.663	0.751
D+/R-	1.733	0.413	7.271	0.452
D+/R+	0.874	0.191	3.994	0.863
Acute rejection	0.958	0.337	2.728	0.937
Bacterial pneumonia	1.443	0.458	4.545	0.531
Fungal infection	0.436	0.057	3.336	0.424

Table 10: univariate Cox model of the risk factors for mortality

4. DISCUSSION

In our study, we aimed to assess the impact of late-onset CMV replication on allograft and patient outcomes in a single-center cohort of lung transplant recipients. We found that asymptomatic CMV replication occurring after the discontinuation of antiviral prophylaxis (i.e late-onset CMV replication) was not associated with a significant decrease in lung function and survival. The incidence of CLAD was similar in patients that developed CMV replication and there was no correlation between duration of CMV replication and the onset of CLAD. Moreover the decline of FEV1 was not affected by the development of CMV replication and there was no significant decrease of patient survival in patients with CMV replication. Finally, our results showed a decrease of patient survival in the CLAD group, which has already been widely accepted in the literature as the main cause of death in lung transplant recipients (17).

These data suggest that asymptomatic CMV replication occurring after the discontinuation of antiviral prophylaxis does not have a major clinical impact in our population, as lung function and survival were not altered in patients with or without replication. However, the influence of CMV replication on allograft outcomes is still debated. It appears that the administration of antiviral drugs may have an impact on reducing the indirect effects of CMV, as most of the studies that found a relationship between CMV and allograft dysfunction were performed before the introduction of universal antiviral prophylaxis protocols. Another issue is whether detection of CMV in the allograft may be associated with different outcomes as respect to detection of CMV in blood. Some studies observed an association between CMV replication in the lung (CMV detected in BAL samples) and the occurrence of CLAD (48), which was not confirmed by another study (49). Because the CMV surveillance protocol was done more intensively in blood than in the BAL in our study, we decided to analyze only the presence of CMV in blood as the primary

endpoint. Our results showing a rather benign effect of CMV replication in lung transplant recipients may be useful for the management of these patients in the routine practice, as it might be not necessary to systematically monitor for CMV replication in asymptomatic patients.

In addition to CMV disease, bacterial pneumonia and respiratory virus infections (52) have been associated with development of CLAD in the literature. In our results, bacterial pneumonia episodes were the only risk factor identified by our analysis that was associated with a higher incidence of CLAD, which suggests an association between infectious episodes and the development of allograft dysfunction. Although this has previously been reported, a clear mechanism of the association between bacterial infection and allograft dysfunction is not fully understood yet (53). Overall, these data suggest an important interaction between mechanisms of host defense against infection and rejection. The balance between on one hand too high immunosuppressive state and development of infection or, on the other hand not enough immunosuppressive state and development of rejection is a subtle adjustment, which need to be more explored to improve lung transplant recipients care.

Our study has some limitations. First, this is a single-center study with the inherent biases of a retrospective study. Moreover, the modest sample size limited the strength of the analysis, especially regarding the multivariate analysis. However, we did not observe any trend towards a deleterious effect of CMV replication or CMV disease in terms of allograft or patient survival; so that we do not think that a larger sample size would have changed the results of our study. The main strength of our study is the relatively long-term follow-up, with a median follow-up duration of 3.67 years. Moreover, the immunosuppressive regimens, antiviral prophylaxis protocols and the management of infection were homogenous during all the study period, which have potentially diminished the bias associated with a different transplant care over time. CMV detection by PCR was hence performed in a single laboratory, which is relevant since CMV detection has a poor inter-center agreement (54).

5. CONCLUSION

Asymptomatic CMV replication in the blood did not affect the lung function or survival of lung transplant recipients receiving anti-CMV universal prophylaxis. These results suggest that universal prophylaxis given to patients according to their serostatus is protective regarding CMV as a risk factor for the onset of CLAD.

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