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Opportunistic viral infections after paediatric transplantation

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OPPORTUNISTIC VIRAL INFECTIONS AFTER PAEDIATRIC TRANSPLANTATION

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ABSTRACT

Background: Opportunistic viral infections can cause considerable morbidity and mortality in organ and stem cell transplanted (SCT) patients, mainly due to iatrogenic T cell dysfunction. Whereas in SCT patients, in general the immunosuppressive treatment can be discontinued after 6-12 months, for the majority of organ transplanted patients, the need for treatment is life-long.

Aims: This thesis focuses on infections with cytomegalovirus (CMV), Epstein-Barr virus (EBV), adenovirus (AdV) and human herpes virus type 6 (HHV-6) in liver and stem cell transplanted children and on the value of quantitative viral DNA measurements. The aims were to (i) investigate the incidence and clinical picture of CMV and EBV infection the first year after liver transplantation and the usefulness of DNA quantification for identifying these infections, (ii) to compare serum and whole blood as material for the analyses and (iii) to describe infections with CMV, EBV, AdV and HHV-6 and their risk factors, identification and outcome in SCT patients during the first 6-12 months after transplantation.

Methods: Serum samples, drawn the first year after transplantation from 18 liver transplanted children were retrospectively investigated for CMV DNA by a quantitative PCR from Roche (CA Monitor), and from 24 liver transplanted children for EBV DNA by real time TaqMan PCR. In the comparison of sample materials, clinical samples (10,641 for CMV and 2,855 for EBV) drawn mainly from transplant patients, were surveyed as regards to viral DNA levels in whole blood and serum. In the study of SCT children, serum samples from 47 consecutively transplanted children were retrospectively investigated with analysis of viral DNA by TaqMan PCR and related to risk factors in a multivariate analysis.

Results: Any CMV marker was found in 83 % of the liver transplanted patients. Symptomatic infection was found in 22% and was associated with significantly higher CMV DNA levels (paper I). More than half of the liver transplanted patients in paper II were EBV naïve at transplantation, probably due to low median age, but 92 % had markers of EBV infection within 1 year. Symptomatic infection was found in 21%: 3 patients with post transplantation lymphoproliferative disease (PTLD) and 2 with hepatitis. In these 5 patients, the EBV DNA levels were significantly higher than in the patients with asymptomatic infection. In paper III, CMV DNA levels were only 0.2 log higher in WB as compared to serum, while EBV DNA levels were 1.5 log higher in WB than in serum. Out of 47 SCT children (paper IV), 47% developed CMV DNAaemia, 19% at levels > 10^4 Geq/mL, and 45% developed EBV DNAaemia, but only 6 % > 10^4 Geq/mL. CMV DNAaemia did not develop if neither donor nor recipient had CMV IgG. ATG and total body irradiation were independent risk factors for high CMV and EBV DNA levels. HHV-6 DNA and AdV DNA were each present in 28% of the SCT patients, in the majority in low or moderate levels. Three children died from CMV, EBV and AdV complications, representing 21 % of the total mortality after SCT, and all these 3 cases were retrospectively found to have very high viral DNA levels in serum.

Conclusion: Quantification of viral DNA levels contributes to a better basis of understanding of post transplant viral infections, and is critical for taking the right actions in terms of balancing immunosuppression and antiviral measures. As sample material, serum and whole blood seemed equally useful for CMV, while for EBV whole blood was more sensitive but less specific.

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LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Kullberg-Lindh C, Ascher H, Krantz M, Lindh M.
Quantitative analysis of CMV DNA in children the first year after liver transplantation.
Pediatr Transplant 2003;7: 296-301

II. Kullberg-Lindh C, Ascher H, Saalman R, Olausson M, Lindh M.
Epstein-Barr viremia levels after pediatric liver transplantation as measured by
real-time polymerase chain reaction. Pediatr Transplant 2006;10(1):83-89.

III. Kullberg-Lindh C, Olofsson S, Brune M, Lindh M.
Comparison of serum and whole blood levels of cytomegalovirus and Epstein-Barr virus DNA.
Transplant Infect Dis 2008; 10; 308-315.

IV. Kullberg-Lindh C, Mellgren K, Friman V, Ascher H, Lindh M.
Opportunistic viral infections after paediatric stem cell transplantation. Submitted.

ABBREVIATIONS

AdV	adenovirus
ATG	anti-thymocyte globulin / anti-T-lymphocyte globulin
BL	Burkitt lymphoma
CTL	cytotoxic lymphocytes
EBV	Epstein-Barr virus
EBVD	EBV disease
EHBA	extrahepatic biliary atresia
ELISA	enzyme-linked immunosorbent assay
GvHD	graft versus host disease
HCMV	human cytomegalovirus
HHV-6	human herpes virus type 6
HLA	human leukocyte antigen
HSCT	haematopoietic stem cell transplantation
IVIG	intravenous immunoglobulin
IS	immunosuppression
MUD	matched unrelated donor
MMRD	mismatched related donor
NASBA	nucleic acid sequence-based amplification
NK cell	natural killer cell
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PTLD	post transplantation lymphoproliferative disease/disorder
SCT	stem cell transplantation
SOT	solid organ transplantation
TBI	total body irradiation
TCD	T cell depletion
VAHS	virus-associated haemophagocytic syndrome
WB	whole blood
XLP	x-linked lymphoproliferative disease

CONTENTS

Key words: paediatric liver transplantation; paediatric stem cell transplantation; immunosuppression; opportunistic; CMV; EBV; AdV; HHV-6; real-time PCR; monitoring; specimen; post transplant lymphoproliferative disease

1. INTRODUCTION

In the last decades remarkable progress of transplant medicine in developing effective immunosuppressive drugs, has led to much improved results for both solid organ and stem cell transplantation in children and adults. Ten-year survival rates after solid organ transplantation (SOT) are now as high as 85-90% and for stem cell transplantation (SCT) around 55-60% (1-4). As immunosuppressants have become more effective, rejection and graft versus host reactions (GvHD) cause less problems, but instead opportunistic viral and other infections may lead to considerable morbidity and mortality in transplanted patients. The same immunological phenomena that we want to avoid in the first place, inevitably mean weaker defence against infections, and our disability to estimate the individual optimal immunosuppressive level can lead to over-immunosuppression and enhanced risk of infections. Since this problem have become more recognised, different strategies have been tried and combined. These include primary prevention by matching of host and donor, viral surveillance, antiviral prophylaxis and preemptive antiviral treatment. New techniques for viral diagnostics have been developed, which have improved our means of monitoring, but we need to learn more about how to best use them in transplanted paediatric patients in our setting.
The perfect balance is an ideal situation with just enough immunosuppression (IS) to avoid rejection or GvHD and at the same time low risk of opportunistic infections. As long as there is no way to appreciate the individual need of IS, a dysbalanced situation can appear.

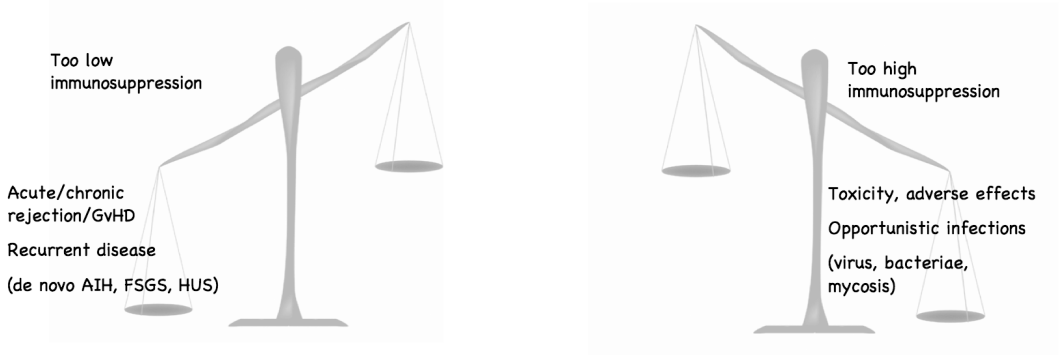


Fig.1 Consequences of under- and overimmunosuppression after transplantation.

In a setting of to low IS, rejection or recurrent disease are possible consequences, whereas if the IS is too high, opportunistic infections may cause complications.

This thesis focuses on opportunistic viral infection with cytomegalovirus, Epstein-Barr virus, human herpes virus type-6 and adenovirus in liver- and stem cell transplanted children. These viruses are all enveloped, double stranded DNA-viruses and apart from adenovirus, they belong to the herpes group. The characteristic feature of opportunistic agents is that they generally do not cause serious problems in otherwise healthy subjects, but can do that in immunocompromised patients.

2. VIROLOGY

2.1 General aspects and normal defence towards virus

Viruses are the smallest biological units that can infect living organisms, but are not considered as independent life forms since they lack metabolism and depend on host cells for their proliferation. They are usually about 100 times smaller than bacteriae with a diameter of between 20 and 300 nanometres. Viruses can be divided into RNA and DNA viruses from the type of genetic material they carry, which is protected from the environment by a capsid.
Some viruses such as herpes viruses, also have an envelope of host cell membrane origin with incorporated viral glycoproteins. Enveloped viruses are less resistant, but also more flexible in protein content than non-enveloped viruses. Some enveloped viruses have a protein layer between the capsid and the envelope called tegument, which is involved in viral replication and avoidance of the immune response (5). The virus' genetic material encodes for the proteins that constitute the capsid, but also for proteins that enable the virus to infect cells and to make them start manufacturing viral proteins. DNA viruses can either be single or double stranded and are generally more stable than RNA viruses.
The normal defence against viruses is both of innate and adaptive nature.
The innate immunity involves NK cells that exert their cytotoxic effect via interpherons, but also monocytic cells, polymorph nuclear cells and plasmacytoid dendritic cells (6). These cells have receptors binding to microbial products and this reaction is fast while the adaptive immunity is slower, partly humoral and involves B cells that carry immunoglobulin surface receptors and produce virus specific antibodies. The humoral responses towards virus are mostly T-cell-dependent. Antibodies can inhibit the binding of viruses to host cells and may also help to kill infected cells by antibody-dependent cellular cytotoxicity or antibody-complement-mediated lysis. However, specific antibodies alone may be insufficient in clearing virus or protecting against reinfection –or reactivation– of latent virus. For this, the adaptive cellular immunity is crucial, engaging both CD4+ and CD8+ T cells. CD4+ T cells produce cytokines and thereby activate CD8+ T cells, which can develop into cytotoxic lymphocytes (CTL), which may eliminate infected cells by release of cytolytic proteins.
For CMV, the cytotoxic T cell response is mainly directed against the pp65 (UL83) protein (7).

2.2 Latency

All herpes viruses (and to some extent probably also adenovirus) remain in life-long latency after primary infection, meaning that viral genomes remain in a closed circular form (episome) in latently infected host cells. The type of cell where the viruses stay latent varies from one virus to another. In the latent phase only few viral genes are expressed and few viral proteins produced, and the virus-infected cells are therefore not detected by the host's immune system. In certain situations the virus may reactivate and viral genomes start replicating again. A virus can at the same time be latent in some cells and proliferate actively in others (5). The balance between immune recognition, which could mean elimination of the virus, and infection activity, which could kill the host cell and thereby also eliminate the virus, is critical. Therefore, many of the viral proteins serve to modulate the host's cellular and immunological responses.

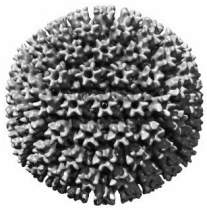
2.3 Herpes viruses

The viruses belonging to this group are highly prevalent, affecting 75-100% of humanity. There are eight different herpes viruses: Herpes simplex virus 1 or 'human herpes virus type 1' (HSV-1, HHV-1), herpes simplex virus 2 (HSV-2, HHV-2), varicella zoster virus (VZV, HHV-3), Epstein-Barr virus (EBV, HHV-4), human cytomegalovirus (HCMV, HHV-5) and human herpes viruses 6, 7, 8 (HHV-6, HHV-7 and HHV-8). The herpes viruses share important biological properties in structure and latency, but also differ in certain ways. In vitro, some have a wide host cell range, efficient replication and may destroy the cells they infect rapidly (e.g. HSV-1, HSV-2), others a more narrow host cell range (e.g. EBV, HHV-6) or a long replicative cycle (HCMV) (5). In vivo, however, during an active infection, CMV has been reported to replicate with a doubling time of approximately 1 day (7).
HHV-1-3 belong to the alpha-subfamily, HHV-5-7 to the beta-heresvirinae and HHV-4 and 8 to the gamma-herpesvirinae, an older classification based on biological properties that later has been supported by similarities in nucleotide sequences.

2.4 Cytomegalovirus

General aspects, transmission and epidemiology

Human cytomegalovirus (HCMV or HHV-5), is the largest of all the viruses in the herpes group, with a diameter of 200-300 nm. It is about 80 times bigger than for example hepatitis B virus. In the following text, when CMV is mentioned, it is HCMV that is understood.
The virion consists of three parts: the capsid with the DNA-genome of 230 kilo base pairs (kbp) arranged in a linear double strand, encoding for more than 200 proteins, the tegument and the envelope, which is a lipid bilayer of host cell origin with at least 20 embedded virus-encoded proteins (8-10).



The virus can spread transversally, by direct contact with body fluids from virus excreting individuals, and by blood transfusion, bone marrow, stem cell or organ transplantation. Latent forms as well as active CMV virions can be transferred. Virus can also be transmitted vertically, over placenta, intrapartum or via breast milk. The risk of transplacental transmission to the child if the mother experiences a primary infection during pregnancy, is as high as 20-40% (11, 12) and 10-15% of these infected children present with symptoms.
In a typical primary infection, replication is initiated in the mucosal epithelium at the port of entry and in the systemic phase, virus is shed in the highest levels in urine, saliva, breast milk and genital excretions. Viraemia and shedding can persist for a long period, in adults for months, and in children for years. Common ways of transmission in healthy individuals are contact with young children or sexual contacts (13).
Generally, the seroprevalence of CMV is greater, and CMV infection is acquired earlier in life, in developing countries. Figures of seroprevalence for CMV therefore vary with age and socioeconomic status, but have been estimated to around 20-30% of 1-year-olds, 40% of teenagers and 50-90% of adults, the higher figures in developing countries (14, 15).
After primary infection the virus remains in a life-long latency in the host, mainly in CD14+ monocytes and to some extent in macrophages and CD 34+ stem cells (but not, as during active replication, in B- or T-cells or PMNs) in a concentration of about $1/10^4$-10^5 peripheral blood cells with 2-15 Geq/cell (16, 17). Virions latently infecting donor cells can reactivate in the new host by mechanisms that are still incompletely known.

Before the understanding of how CMV transmission via leukocytes in blood transfusions could be prevented, this was a common route of infection. With the present use of leukocyte-depleted blood products this problem has been dramatically reduced.

Latent virus	Site of latency
HSV 1, 2, VZV	Neurons
EBV	Memory B cell, $1/10^4$-10^5
CMV	monocytes, macrophages
HHV-6	monocytes, macrophages, salivary glands, ?
ADV	tonsils

Fig.2 Sites of latency for herpes and adeno virus

Clinical significance of CMV infection

Acute CMV disease only occurs in a small proportion of the individuals that become infected. Severe disease develops in particular in subjects with inadequate cellular immune response, as in transplacental transmission during early pregnancy, resulting in congenital disease, or in primary infection or reactivation in immunocompromised individuals.
The severe form of congenital disease, histologically characterised by "owl's eye" inclusions, in salivary glands, liver, lung, kidney, pancreas and thyroid (in autopsy material from infants) was first described in the 1930s and the viral aetiology of the condition was discovered in the 1950s. It can cause severe neurological damage, including microcephaly, chorioretinitis and loss of hearing, but also other features, as growth impairment, hepatitis, and haemolytic anaemia.
Albeit the high frequency of vertical transmission by breast milk, few healthy full term infants present with symptoms, however, hepatitis and pneumonitis has been reported (18, 19).
Later in life, primary CMV infection is usually asymptomatic in healthy subjects, but can cause a mononucleosis-like syndrome with fever, myalgia, malaise, fatigue, headache and hepatitis, that is usually mild but sometimes includes hepatosplenomegaly and jaundice.

CMV complications in immune compromised hosts

After solid organ transplantation

The results after paediatric SOT improved dramatically when effective immunosuppressive drugs were developed in the second half of the 1980s. The 1-yr-survival is now 97% (1). Following the introduction of more potent immunosuppressive regimens, there were however reports of 30-60 % prevalence of some form of CMV infection in liver transplant recipients (20, 21) and mortality rates as high as 22% in disseminated CMV disease in liver transplanted children (22). Despite antiviral strategies of today, CMV has remained the most common of the opportunistic viral infections post transplantation. CMV affects more than 75% of SOT patients directly or indirectly (23, 24) and still contribute significantly to morbidity and mortality in both adults and children receiving solid organ transplants (22, 25).
Apart from CMV disease, CMV is believed to exert indirect effects. These include increased risk of acute and chronic rejection and of inducing a state of enhanced immune suppression with concurrent higher risk of post transplantation lymphoproliferative disease (PTLD) and bacterial and fungal infections (26-33). There are also reports about CMV causing coronary vasculopathy after heart transplantation (34) and vanishing bile ducts after liver transplantation (35, 36). A hypothesis is that these manifestations are functions of immune mediated effects, induced by longer periods of low CMV replication (23). With the exception of higher risk of PTLD and acute rejection after kidney transplantation the support for CMV's indirect effects in transplanted children is weaker than in adults (37).

After stem cell transplantation

The concept of stem cell transplantation here includes stem cells collected from peripheral blood, bone marrow and umbilical cord.
Not long ago, 10-15% of SCT-patients died from CMV infections. While overall morbidity and mortality has decreased significantly with the present strategies, 20-50 % of SCT children still develop CMV infection and 3-4% CMV disease (38, 39). CMV infection is considered an important risk factor for transplant related mortality before day 100, particularly when grafts from matched unrelated donors are used (38-40). In recent years also more late CMV disease has been observed (38, 40-42).
Children with congenital immunodeficiency syndromes as indication for SCT often have severe CMV primary infection pre-transplant, or are at high risk of developing infection after SCT (42).

Risk factors

The risk factors for CMV infections are similar in paediatric and adult transplant patients, and in solid organ and stem cell transplanted patients, although their relative importance differ somewhat. The most important factors are the CMV serostatus of donor and recipient, the overall state of IS, host factors (such as age, comorbidity, neutropenia) and the type of transplantation (which in it's turn may be related to differences in IS, age and thereby serostatus). In general, children are at higher risk of CMV complications because they are more exposed to community CMV and because they are more likely to develop a primary infection.

A high CMV DNA level is frequently named a risk factor for symptomatic CMV infection (7), but it should rather be seen as a marker for a more active infection, thus reflecting true risk factors such as poor immune status.

1. CMV-serostatus

Solid organ transplantation

For a CMV seronegative recipient to receive a CMV positive graft (D+/R-) is a main risk factor. The risk for CMV infection is then about 20 times higher, compared to a D-/R- situation, and without prophylaxis 60-100 % of these patients acquire CMV infection after SOT. Since children more often are CMV seronegative prior to transplantation, the mismatch-situation (D+/R-) is more frequent in transplanted children than in adult patients. The use of living-related (adult) donors and split grafts may contribute to a higher rate of mismatch after paediatric liver transplantation.

Stem cell transplantation

The impact of CMV serostatus after SCT is different as compared to after SOT. For SCT recipients the risk may be greater in a CMV seropositive recipient receiving a CMV seronegative stem cell graft since no immunity is then transferred with the graft, while latent recipient virus could activate uncontrolled (43). Generally, it is however not possible to estimate the risks as easily as after SOT, and all situations where either recipient or donor is CMV seropositive are considered to confer a risk for CMV disease (38, 40).

2. Immunosuppression as risk factor for CMV infection

In general, the immunosuppressive treatment is usually stronger during the first 3 months after both SOT and SCT. The most used drugs after SOT are steroids and calcineurin inhibitors such as tacrolimus. Whereas the need for IS generally is lifelong after SOT, it is stopped after 6-12 months after SCT in most cases. In SOT patients the immunosuppressive medication can, however, often be gradually reduced after 3-6 months, but if signs of acute rejection appear, intensified treatment with high-dose steroids is given. The use of such compounds as antithymocyte (ATG) or antilymphocyte globulin (ALG) and muromonab-anti-CD3 monoclonal antibodies (OKT3) is a well-documented risk factor for CMV disease (15, 44). These antibodies are used both for SOT and SCT recipients although for different reasons. In paediatric

liver transplantation, for example, ATG is commonly used as induction treatment at transplantation in steroid-free programs, and OKT3 is given in case of steroid-resistant acute rejection.
In SCT recipients, ATG is often used both as conditioning treatment before SCT and as part of GvHD treatment. Procedures that aim to achieve complete or partial T-cell depletion of the graft are known to give a higher risk of CMV disease (38, 41). Conditioning may also involve total body irradiation (TBI), which probably confers a higher risk for CMV infection than alternative regimens, based on busulfan, but this is not so well studied.

3. Other risk factors for CMV infection

SOT: type of transplantation

The risk of developing CMV complications varies with type of graft. This can partly be explained by differences in IS, which is generally stronger after lung and kidney transplantation than after liver transplantation. There is a co-association between transplant type and mean age at transplantation (for example, many liver transplant recipients are very young). Organ tropism of the virus and, possibly, differences in amount of virus present in the transplanted allograft, may also influence the risk of CMV. The risk of CMV infection is considered to be especially high following pulmonary, intestinal and pancreas transplantation, after which 40-50% of the patients may develop CMV complications (45-47).

Liver transplant recipients can be considered as medium risk patients with the incidences of CMV disease ranging between 10 to 40%, depending on prophylactic regimen (48).
After kidney transplantation, there is a 6-40% risk of CMV infection, depending on prophylactic procedures (37). Most centres now practice universal GCV prophylaxis, due to the risk of acute rejection associated with CMV after kidney transplantation (37).

SCT

Some risk factors are specific for SCT. Firstly, the degree of HLA match between donor and recipient is important: if donor and recipient are HLA identical, the risk for CMV infection is lower than in stem cell transplantation using graft from matched, unrelated donor (MUD), because less intense immunosuppressive measures (in both conditioning and anti GvHD treatment) are required, and because the immune reconstitution in general is faster (39, 49). Secondly, diagnosis may influence the risk for infections. Immune defects as indication of SCT confers a higher risk. Thirdly, the situation after SCT differs from that after SOT in the respect that the recipients' own immune system is eradicated, to be replaced and reconstituted via the new stem cell graft.
It is not clear in what way development of acute or chronic GvHD is associated with CMV complications (41, 50, 51).

Clinical presentation

CMV infection in immunocompromised patients can be categorised, on the basis of the clinical presentation.
1) asymptomatic infection; 2) viral syndrome or CMV disease; 3) localised tissue-invasive disease of a single organ and 4) disseminated disease with tissue involvement of two or more sites, or one site in combination with CMV isolation in peripheral blood cells.
CMV disease typically presents with fever, leukopenia, thrombocytopenia, malaise, pneumonitis, hepatitis or gastrointestinal symptoms in the first 3 months post transplantation (52). Symptoms from the transplanted organ are common and it has been speculated that this could be caused by an abnormal immune response in the graft (15). For example, hepatitis and cholangitis are common presentations of CMV infection after liver transplantation (53), as is enteritis after intestinal, pneumonitis after lung and coronary artery disease after heart transplantation (54, 55). It is often difficult to distinguish CMV infection from acute rejection, both clinically and histologically. This is troublesome because the

treatment of one condition is the opposite of the other, and one can end up in a situation where you "throw both water and petrol on the fire" (anti-rejection and antiviral treatment at one time). In liver transplant recipients, the same liver function tests can be elevated from rejection, CMV (or other infection) or for that matter, also from other post transplant complications, such as vascular thrombosis, veno-occlusive disease etc.
Although the IS is critical for the emergence of CMV infection, it is possible that the pathogenesis of both acute and chronic CMV complications is immune-mediated (56). Moreover, cytokines might activate CMV replication in latently infected cells.

Explanations of expressions used in the text (adapted from Ljungman et al. 2002)

CMV infection– proof of CMV replication by CMV isolation or detection of viral nucleic acids in body fluids/tissue samples, regardless of symptoms.
CMV disease = **symptomatic CMV infection** – proof of CMV infection (culture, histopathology, immune histochemistry/ in situ hybridisation) in combination with symptoms* that cannot be explained otherwise.
* including CMV viral syndrome. Alternately, proof of end-organ involvement (colitis, hepatitis etc)
CMV syndrome – fever >38° C > 2 days in combination with cytopenia and detection of CMV in blood. (This term should be avoided in SCT recipients if not other viral causes (HHV-6 and adenovirus) have been ruled out).
CMV antigenaemia - the detection of CMV pp65 in leukocytes
CMV viraemia – isolation of CMV in blood by culture
CMV DNAaemia – detection of viral DNA in blood (plasma, whole blood, PBMC or in buffy-coat specimens) by PCR-based techniques, hybrid capture or branched chain DNA analysis
CMV RNAaemia – detection of RNA (e.g. by NASBA or reverse transcriptase-PCR) in samples of plasma, whole blood, PBMC or in buffy-coat specimens
"Recurrent infection" – detection of CMV infection in a patient who has previously documented infection and who has not had virus detected for an interval of at least 4 weeks during active surveillance. May result from reactivation of latent virus or reinfection.
"Reinfection" -–detection of a CMV strain that is distinct from the strain that was the cause of the patient's original infection. May be documented by sequencing specific regions of the viral genome.
"Reactivation" – is assumed if the 2 strains are found to be indistinguishable.

Strategies for prevention

General aspects

There are two principal strategies that can be used both for SOT and SCT, and which also may be combined, namely antiviral prophylaxis and preemptive treatment. *Prophylaxis* means administration of antiviral or immunologic compounds, immediately after transplantation, either to all transplant recipients (universal) or to those with highest risk (targeted).
Preemptive therapy refers to treatment administered for a brief period after engraftment, to asymptomatic individuals with microbiological markers that predict CMV disease (33). A prerequisite of the latter strategy is a sensitive and timely predictive test and regular monitoring in order to react prior to symptoms.
The choice of strategies differs considerably between different transplant populations and between centres, making comparison difficult. A prophylaxis regimen that is proved to be effective in one transplantation programme may not work as well in another, because of differences in immunosuppressive regimens (33). New immunosuppressive agents are being developed continuously, which adds to difficulties in comparing effectiveness of prophylaxis and preemptive strategies.
There are in fact no randomised studies comparing prophylaxis and preemptive treatment for CMV in SOT patients.

In general, prophylaxis has the advantage of effectively preventing CMV complications during the first critical period after SOT or SCT without the administration of surveillance (57). With the new sensitive and quantitative PCR methods, low levels of CMV DNA replication has been observed in many asymptomatic transplanted patients. The long-term effects of this on the outcome of the transplanted graft or on the morbidity in general are not known (33). By prophylaxis there is a theoretical possibility to prevent the indirect effects of virus, although few studies have addressed that question (23).
However, in patients receiving CMV prophylaxis, the CMV infection can be delayed. Such "late CMV disease" has become more common in both SOT and SCT patients and is observed in 5-20% of liver transplanted children after discontinuation of prophylaxis (58-60). This requires attention, since it can occur at a time when intense monitoring and follow-up has been spaced. It has been hypothesised that, when CMV is completely suppressed by a potent antiviral drug, priming of the antigen-induced host response is less effective, causing the higher incidence of late disease (7, 58). Moreover, antiviral prophylaxis has been shown to delay CMV-specific immune reconstitution after SCT (61).
Preemptive strategies are favoured by the fact that treatment is given only to patients, who need it. This reduces the risk of resistance towards antiviral compounds and the risk of adverse effects of antiviral drugs, such as myelodepression, which is especially critical in SCT recipients (62). Possibly, the preemptive strategy also allows CMV to elicit a more effective immune response, reflected by a lower incidence of late CMV disease, although this is a matter of debate (63).
On the other hand, preemptive strategy is based on rapid and sensitive diagnostics and requires extensive surveillance and administration.

	Advantages	Disadvantages
Prophylaxis	Prevents both CMV disease and indirect effects of CMV during high risk period	Risk of late CMV disease Adverse effects Lack of data on p.o. prophylaxis in children
Preemptive treatment	Treatment only to them with proven CMV infection Less risk of antiviral resistence	Administration of surveillance Limited data on p.o. treatment in children

Fig. 3 Advantages and drawbacks with anti-CMV strategies.

Strategies after solid organ transplantation

Matching of donors and recipients should be performed if possible, but this is unfortunately seldom achieved, due to shortage of grafts. However, the importance of keeping the donor/recipient (D/R) serostatus in mind during follow-up is vital. CMV hyper immuneglobulin has some prophylactic effect and is often given in case of CMV mismatch. However it is inadequate as sole prophylaxis and further studies of its role are warranted (64).
In general, there is no clear consensus regarding prophylactic or preemptive strategy in SOT children although antiviral prophylaxis for 3-6 months is preferable in mismatch patients, especially if there are other risk factors, such as use of ATG.
Preemptive treatment after SOT is probably used more often in transplanted children than in adults. This can be partly due to administration problems, because not until recently have valganciclovir (VGCV) been available in a fluid preparation. Many children cannot swallow pills and the documentation of per oral prophylaxis with VGCV for SOT children is scarce, but to keep patients hospitalised in order to treat with i.v. ganciclovir (GCV) is generally no good alternative.
I.v. GCV prophylaxis, should also be considered during anti-rejection treatment after SOT, if antiviral prophylaxis has not already been initiated (33). Finally, blood products given to transplant recipients should always be leukocyte depleted (filtered).

Strategies after stem cell transplantation

Matching of recipient and donor should be done when possible, so that CMV seronegative grafts are used for seronegative recipients. Use of seropositive grafts for seropositive recipients have also been advocated (65). Blood products should be leukocyte filtered and irradiated, which reduces risk of CMV infection as effectively as use of blood products negative for CMV antibodies (40).
For SCT children, preemptive treatment with i.v. GCV is the most commonly used strategy, provided efficient viral monitoring can be performed (38, 40). It is preferred because the myelodepressive effect of GCV and valganciclovir (VGCV) is particularly unwanted after SCT (33). However, new compounds for oral prophylaxis, hopefully with less adverse effects, are under trial (66). In some centres, CMV hyper immuneglobulin or IVIG is used, but the benefit of this is controversial.

Diagnostics

The clinical presentation of CMV disease is often unspecific, but typically it involves fever, elevated liver enzymes and sometimes respiratory symptoms. Similar symptoms may be caused by other infections or events, such as acute rejection. Therefore, accurate diagnostic methods are critical for avoiding or treating CMV complications. However, the value of methods that depend on leukocyte counts, can be limited in leukopenic patients (e.g. antigenaemia test, PCR in PBMC) and therefore PCR of serum/plasma may be advantageous in SCT patients (42).

Histopathology

Historically, CMV disease has been diagnosed by histopathology with special staining techniques. According to definitions, diagnosis of CMV disease requires histopathological detection of CMV by microscopy or in-situ hybridisation (ISH) with CMV specific DNA probes (44, 67). Applying the definitions strictly, however, is likely to lead to under-appreciation of CMV morbidity, since it has been shown that it takes a viral load of > 5 million Geq/µg DNA before inclusions can be seen by microscopy (7). Among the general drawbacks of histology are that invasive procedures are needed in order to obtain material, that histopathology is time consuming and limited in sensitivity. Altogether, histopathology is often inadequate.

Viral isolation by culture

CMV can be demonstrated in cell culture by its ability to provoke giant cells with inclusions giving them an *"owls eye"* expression. Nowadays, isolation of virus by culture is seldom practised, being a time-consuming and non-robust method.

pp65 antigenaemia assay

By this method, the CMV antigen pp65 (which is the most abundant of the structural tegument proteins) can be detected by monoclonal antibodies that attach to the antigen in peripheral blood neutrophils from patients with CMV disease. The assay is still used in many international laboratories, although not in Sweden. It is a semi-quantitative fluorescence method with the limitation of being labour-intensive and depending on neutrophil count, i.e. less sensitive for neutropenic subjects (68).

Serology

Serologic testing by CMV IgG and IgM is important before transplantation to assess the risk, and if possible, to perform CMV matching of donor-recipient.
After transplantation, it is valuable to keep track on seroconversion. In infants, maternal antibodies can be passively transferred and remain for up to 1 years of age, which is why infants should be considered CMV seronegative if there is doubt. After SCT, the usefulness of serology is limited because of frequent

administration of intravenous immunoglobulin (IVIG), blood products and antibodies conferred by the graft, as well as many patients' inability to react serologically.

Molecular methods

In most modern virus laboratories, molecular methods have become predominating and among them, real-time polymerase chain reaction (PCR) is the most valuable technique at present.
Real-time PCR can quantify CMV DNA accurately and quickly in a broad range with minimal risk of contamination (68). It is useful both for monitoring, diagnostics, to follow the course of infection and the effect of treatment. The risk of development of CMV disease can be assessed by quantification of CMV DNA, since it reflects viral replication and the specific immune response (69).

Cut-off values for initiation of preemptive treatment depend on assays used, the patients' overall immunosupression and, in SOT patients, whether it is a primary or reactivated infection (70). In SCT patients, the impact of primary infection is less clear, and cut-off values may rather depend on whether it is a first or a repeated episode of reactivation (68).
To be able to compare results between laboratories and define cut-off values for institution of treatment it is important to standardise the methods. Testing of quality control panels, QCMD (Quality Control for Molecular diagnostics), may serve to calibrate assays by comparing results from different laboratories (71).
According to the definition presented above (p. 14), symptoms/signs of disease and CMV DNA levels alone, are not considered adequate for diagnosis of CMV disease. However, in clinical practice it is often not possible to wait for results from immunohistochemistry or in-situ hybridisation before treatment for suspected CMV disease is initiated. Instead, levels of CMV DNA have become increasingly important for treatment, given preemptively or on clinical suspicion of CMV disease.

Another issue that is important when comparing results, is what sample material that should be used. Preferred sample material differs between centres and while some use plasma or serum, others prefer whole blood or PBL (42, 68, 72-74).

Monitoring

Post transplant monitoring by CMV DNA quantifications has become increasingly important. The frequencies of testing differ depending on risk factors and antiviral strategies.

- SOT patients with high risk (D+/R–) generally receive GCV prophylaxis for 3-6 months after transplantation. During this time frequent CMV DNA monitoring is not required, but surveillance is important when prophylaxis is discontinued, even if the IS by this time has been tapered.
- For medium risk SOT patients, such as D+/R+ liver recipients, preemptive strategies are common. For them, weekly monitoring of CMV DNA during the first 3 months and then with gradually longer intervals is reasonable.
- For SOT patients with low risk (D–/R–) scheduled monitoring is in general not required, but in case of suspect symptoms, CMV infection should always be ruled out, because it may be acquired from other sources than the transplanted organ, such as blood transfusions.
- For SCT patients, the preemptive strategies are usually preferred, often comprising weekly CMV DNA monitoring until day 100 after transplantation.

During monitoring, not only absolute levels of CMV DNA, but also kinetics should be considered. Emery et al. showed, that the rate of increase between the last PCR-negative and the first PCR-positive result was significantly faster in patients at risk of CMV disease (75).

Although CMV DNA is the basis for monitoring, measuring the immune status might also be of value. For example, analysing interferon-γ production by CD3+ and CD4+ T cells has been reported useful in SCT recipients (76, 77). A commercial assay for monitoring CMV specific CD8+ T cell response by measuring interferon-γ induced by a broad range of CMV proteins (QuantiFERON®-CMV) has been suggested to be

of value in combination with CMV DNA level detection for preventing CMV complications in transplant patients (78).

Treatment of CMV infection

The treatment of symptomatic CMV disease as well as preemptive treatment is in SOT patients given as intravenous ganciclovir 5 mg/kg b.i.d. for 2 weeks or longer if required to achieve CMV DNA negativity by PCR. For SCT patients, this is usually followed by GCV 6 mg/kg once daily, for a period of 2 weeks. Treatment of CMV disease with oral valganciclovir is not yet recommended in children, although it is reported to be equally effective as i.v. GCV in adult SOT recipients (79).
If there are signs or proof of GCV resistance, foscarnet or cidefovir can be used.

Antiviral resistance

Prolonged use of antiviral treatment, especially in below-therapeutic level concentrations, in combination with profound IS may predispose for drug resistance. However, GCV resistance is reported to occur in only 2-9% of transplant recipients, with the highest incidences after lung transplantation or in SCT recipients with congenital immunodeficiency syndromes (40, 62).
Resistance to GCV is caused by mutations in the viral UL97 (coding for a viral protein kinase, responsible for phosphorylation of GCV) or UL54 genes (coding for CMV DNA polymerase). Resistance should be suspected in situations of prolonged CMV DNAaemia during ongoing antiviral treatment and in particular if CMV DNA levels are increasing (40).

2.5 Epstein-Barr virus

General aspects

Epstein-Barr virus (EBV or HHV-4) was first identified by Epstein, Achong and Barr in 1964 in cultured cell lines from tumour biopsies from Burkitt's lymphoma, which is prevalent in children from malaria-endemic areas in sub-Saharan Africa (80). EBV was the first oncogenic virus to be described in humans. In 1968, Henle and co-workers were able to connect the virus to infectious mononucleosis (IM) (81). The virion is about 200 nm in diameter and consists of a nucleocapsid with a linear, double-stranded DNA-genome of 184 kbp, encoding for nearly 100 viral proteins, the tegument and an envelope with external glycoprotein spikes (82). The proteins are of importance for regulation of viral gene expression, replication of viral DNA, formation of structural components of the virion and for modulating the host immune response during viral replication (83).

Epidemiology

EBV are very common: 50% of 5 yr old children and 90-95% of adults in all human populations are infected with EBV, as demonstrated by serum IgG towards the viral capsid antigen complex (VCA). There are two types of EBV, type 1 and 2, homologous in most genes. Both types are prevalent in all populations and equally common in Africa, but type 1 is 10 times more common than type 2 in Europe.

Transmission

Infection of humans with EBV in most cases occurs by contact with saliva. The major viral envelope protein gp350, binds to the viral receptor, the CD21 molecule on the surface of the B-cell, and the virus starts a lytic infection in oropharyngeal and nasopharyngeal epithelial cells and resting (naïve) B cells. EBV has a specific tropism for B-lymphocytes and epithelial cells, but can also infect T lymphocytes and

in infectious mononucleosis (IM) also neutrophils (84). In contrast to B cells the neutrophils undergo apoptotic cell death, which may be the reason for the transient neutropenia that many IM patients develop. In short, after infection of the epithelial cells and B cells, the virus replicates in cells in the oropharynx and almost all seropositive persons actively shed virus in the saliva occasionally (85).
EBV can also be transmitted by blood products or by latently infected lymphocytes in transplanted grafts. In this case the latent (episomal) EBV in the lymphocytes may be reactivated, resulting in a lytic infection spreading to the recipients' lymphocytes.

Biology

During the lytic phase, most of the nearly 100 EBV genes are expressed, while in the latent infection, gene expression is limited to just 10 latent viral genes (82). Most studies have concentrated on latent infection, because latent genes predominate in EBV-associated malignancies. When the EBV-infected circulating B cells express all the latent genes, the normal immune system is able to react via CTLs that can eliminate the infected cells. After primary infection, the EBV genome remains as an episome in CD19+, CD23- memory B cells at a frequency of about 1 in 1.6 x 10^5-10^6 B cells (84). This corresponds to about 5,000 EBV-containing cells in the whole blood volume or 1 EBV genome per mL blood. The EBV genome can be reactivated at any time if the balance between the immune system and the latent virus is changed.
Most knowledge about the replication of EBV emanates from in vitro studies in which different phases or programs have been characterised on the basis of gene expression patterns. However, it has so far not been possible, although many have tried, to with certainty link gene expression patterns to in vivo events. This is frustrating, but it is a most interesting research field that keeps numerous scientific groups busy.
The manifestations of EBV disease have different cellular origin and vary in gene expression, which often differs from immortalised normal human B cells infected by EBV in vitro. Nevertheless, it has been reported that viral gene expression in EBV-associated diseases is limited to any of the three patterns of latency, although probably the situation in vivo is more complex (83, 86).
The following is a brief description of EBV replication and the different transcription programs known in vitro, but the mechanism for how the virus can switch between them and the actual courses in vivo are yet unknown (87).
After infecting the cell via the CD21 receptor on the B-lymphocyte, the EBV genome circularises to an extra chromosomal episome.

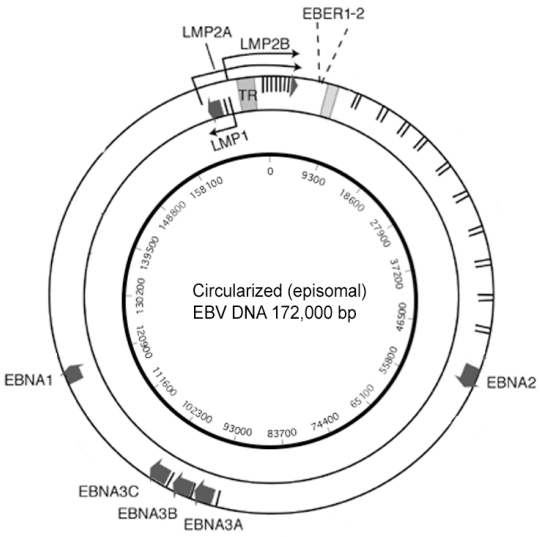


Fig. 4. The Epstein-Barr virus genome with the location of the EBV latent genes on the double-stranded EBV DNA episome are indicated by arrows. Adapted from (88).

The infection enters **type III-latency** or growth program, which is one of the four latent gene transcription programs that have been investigated by *in vitro* studies. This phase is characterized by expression of all EBV latent genes, including Epstein Barr nuclear antigens (EBNAs) 1, 2, 3A, 3B, 3C, LP, latent membrane protein (LMP1, LMP2A, LMP2B) and the polyadenylated viral RNAs (EBERs 1 and 2), of which the latter do not code for any protein (89). Expression of the genes promotes survival and transformation of infected resting B cells *in vitro* and generation of immortalised (continuously replicating) lymphoblastoid cell lines (LCL). EBNA2, but also EBNA 3A, EBNA 3C and LMP1, are believed to be essential for immortalisation of B cells (90). Many of the lymphoblasts are killed by CTLs, but some undergo a "germinal centre reaction" – type II-latency. See fig. 5, p. 21.

In **type II-latency (**default program), latently infected B-lymphoblasts express a more limited set of viral genes (EBNA1, LMP1 and LMP2A and EBERs) and provide signals for B-cell differentiation into a pool of memory B cells, whereby EBV can persist in the host permanently – type 0-1 latency.
During **type 0-latency (**latency program) no latent genes are expressed in the peripheral CD23-positive, memory B cells, which makes it possible for the virus to escape immunosurveillance by specific CTLs. Intermittently, EBNA1 is expressed in dividing B cells, allowing the episome to be transferred to each of the daughter cells (**type I-latency)**.
Most of the infected B cells remain in the memory B cell pool, but occasionally when B cells recirculate to the oropharynx, some of them switch back into the lytic cycle. Specific T-helper cells are believed to mediate B-cell differentiation to antibody producing plasma cells, which in turn triggers a EBV lytic phase with replication in lymphatic tissue and epithelial cells in the oropharynx and shedding into saliva (91). In this way, previously uninfected B cells can be recruited and new hosts may become infected (92). For the virus it is of vital importance that the level of lytic activity is balanced and optimised to ensure spreading, but still minimising exposure to the hosts' immune system (93).
LMP1 is a tumour necrosis factor (TNF) receptor and the major driving force in neoplastic transformation (84). It initiates processes that may lead to sustained proliferation and development of lymphoma (84). LMP1 appears to be required for immortalisation, but it can also induce apoptosis resistance in B cells, which thereby evade immunosurveillance and cytotoxic T cells (87).

Latent EBV has a complex survival strategy, involving (94):

1. Production of viral proteins that can transform human cells to inhibit antigen processing and apoptosis.
2. Secretion of a cytokine and soluble receptor that neutralises cellular immunity.
3. Down-regulation of its gene expression: by limiting viral gene expression during latency, EBV reduces presentation of viral proteins that are necessary for CTLs to recognise the infected cells.

In PTLD, EBV-infected cells generally express all EBV-related latent proteins (type III latency) (89).
A difference between Burkitt lymphoma (BL) and PTLD, is that whereas in PTLD, all antigens are expressed, but the immune system is inadequate (89), the expression of antigens is downregulated in BL cells, which enables the tumour to grow unrestrictedly (type 1 latency) (83). Consequently, when EBV-proliferating cells express viral antigens as in PTLD, tapering of IS can achieve successful regression of lymphoproliferation (95).

Normal control of EBV infection

Infection of humans with EBV induces both humoral and cellular immunity. The cellular defence is the most important and in healthy carriers, the EBV-specific cytotoxic CD8+ T lymphocytes (CTLs) that develop during primary infection and are sustained for life, together with natural killer (NK) cells, can control the transformed EBV-infected B cells. Many of the CTL responses directed against latent proteins are targeted to EBNA3 proteins (83). It has been described that while in immune competent individuals only $1/10^4$ -10^5 B cells are latently infected with EBV, as many as 1-5% of all CTLs are capable to react against EBV in order to keep the homeostasis (96). Additionally, neutralising antibodies and antibody-dependent cell-mediated cytotoxicity form part of the normal defence (97).

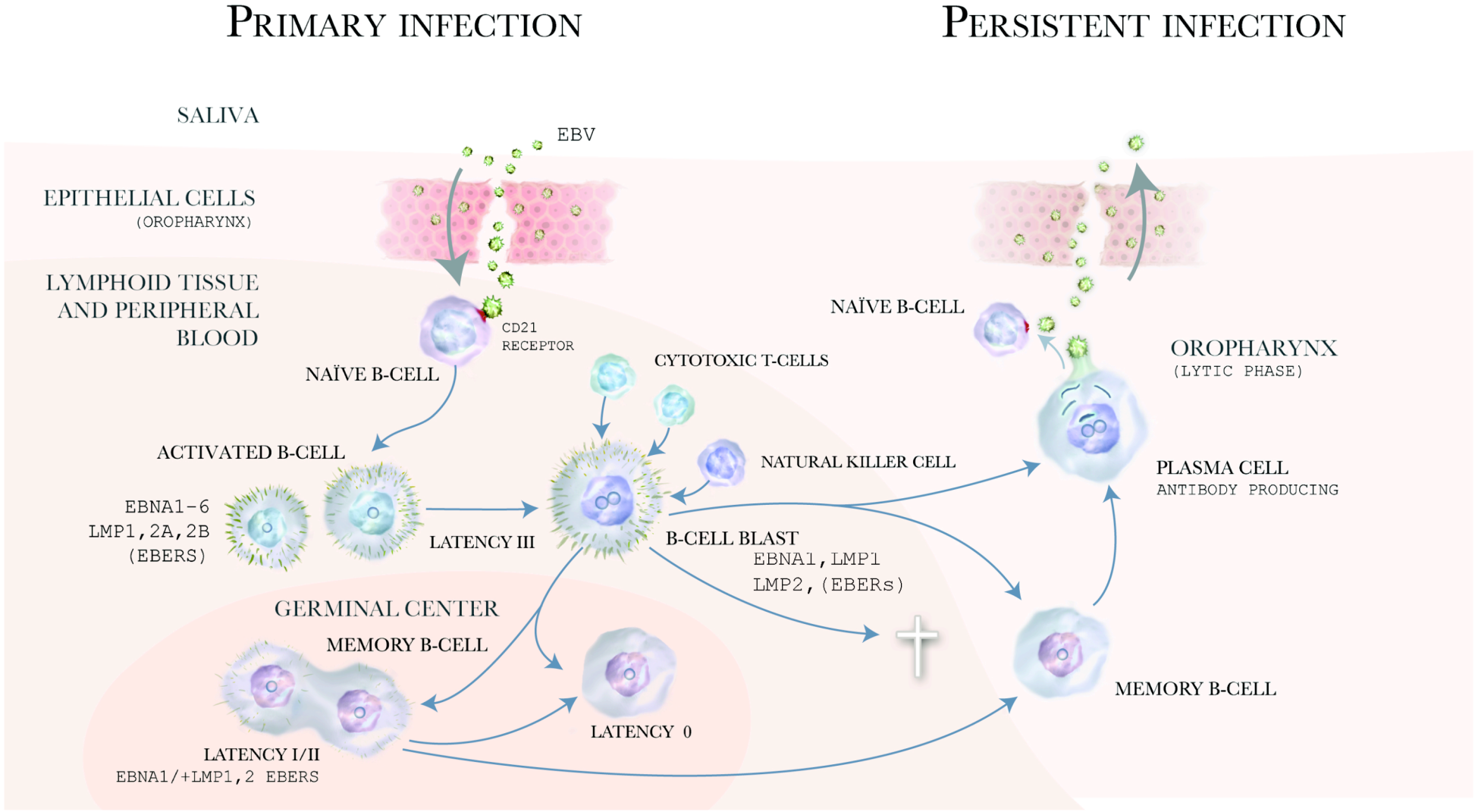


Fig. 5. Epstein-Barr virus infection and persistence in humans. The genes expressed (in vitro) in the different stages of latency (adaptation from Cohen et al. by O. Lindh).

Clinical significance of EBV infection

EBV is a ubiquitous infective agent that exclusively infects humans. In the vast majority of otherwise healthy individuals, infection with EBV is subclinical, especially if acquired in childhood. When acquired in adolescence or adulthood, it typically manifests itself as infectious mononucleosis (IM) with tonsillitis, fever, fatigue, muscular pain and hepatosplenomegaly. Complications are rare, but haemolytic anaemia, thrombocytopenia, myocarditis, pneumonitis, meningoencephalitis and Guillain-Barré have been described (83). EBV has also been associated with a wide spectrum of other diseases, including malignancies such as Burkitt lymphoma, nasopharyngeal carcinoma and post transplantation lymphoproliferative disease (PTLD). In addition, there are also non-malignant, but often fatal conditions, such as fulminant IM, virus-associated haemophagocytic syndrome (VAHS) and chronic active EBV infection (89, 98). The last three conditions are believed to be caused by exaggerated EBV non-specific immune response that can harm tissues such as liver, CNS, GI and skin seriously.
How can one virus cause so many different disease manifestations? This is quite unique and a most intriguing question.

EBV complications in immune compromised hosts

Inherited immune deficiencies

Patients with inherited immunodeficiencies, often lack or have defective T cell functions, which may be evident as an inability to control EBV infection by EBV-CTL. A special case of this, with impaired interaction of B and T cell activity, is x-linked lymphoproliferative disease (XLP), caused by a mutation in the SAP gene on chromosome Xq25, manifesting itself by severe or fatal IM with VAHS and/or hepatitis during primary EBV-infection or development to malignant lymphoma (83, 99).
In XLP, treatment with aggressive IS is sometimes effective, but often allogeneic haematopoietic SCT is required.

Explanations of expressions used in the text

EBV disease (EBVD) is defined as symptoms of active EBV disease with detection of EBV DNA in blood, but without histological proof of progression to PTLD (100).

"Pre-PTLD" clinical signs associated with PTLD (fever, impaired general condition, poor appetite, loss of weight, and irritability) proven recent primary EBV infection, increased γ-globulin production with monoclonal γ-globulin production, but without detectable lymphoid mass or histologically proven lymphocyte proliferation (101).

Post transplantation lymphoproliferative disease/disorder (PTLD) is defined as a polyclonal or monoclonal lymphoid proliferation occurring after haematopoietic or solid organ transplantation. The histological presentation can be highly diverse, from polyclonal mononucleosis-like to monomorphic B-cell or Burkitt lymphoma and in several cases also T-cell lymphoma, Hodkin-like or other types (102).

Transplanted patients

In the era of more potent immunosuppressive drugs, reports of frequent and often fatal PTLD appeared (101, 103-106). The problem, resulted in the use of milder immunosuppressive regimes and surveillance with molecular diagnostics, which made the incidence of EBVD-PTLD decrease (95). Still, an estimated 0.5-30% of all recipients of solid organs (intestinal, pancreas, lung, heart, liver and kidney) and stem cells develop PTLD (89, 99) and PTLD is the most common neoplasm in children after SOT, constituting 52%

(107). In SCT recipients the PTLD lesions are of donor origin, whereas after SOT the lesions generally are of recipient origin.
Over 90% of all PTLD cases are EBV-induced and typically evolve within the first months after SCT or during the first year after paediatric SOT (84, 108, 109). EBV-negative PTLD probably represent a different entity with a later onset, which is less responsive to treatment and more unusual in children (87, 95). The first period after transplantation, when overall IS is high, there is practically no cellular immunity directed towards epitopes of EBV antigens, predisposing uncontrolled proliferation of EBV-infected B cells. The long-term risk of PTLD in liver transplanted children with tacrolimus as primary IS is 15% (110). Even though most cases of PTLD appear early, in 80% of SOT within the first 2 years, cumulative incidence continues to increase over time. In a 10 year follow-up of liver transplanted children, 22% of the 32 surviving children, all of whom had been EBV seronegative prior to transplantation, had developed EBV-related malignancies (111).

After solid organ transplantation

PTLD represent 50% of all tumours in children after SOT, and in the majority of cases develop within 2 years after transplantation (107, 110, 112). PTLD is more common in children than in adults, mainly on account of the higher risk associated with primary infection after SOT. The overall incidence of PTLD in adults is 0.8-3% (104, 113). However, in EBV seronegative adult SOT patients it is similar to the incidence in children, and reported to appear in up to 33% depending on type of transplantation (31).
The mean age at transplantation differs between groups and is generally lower among liver transplanted children, and therefore the incidence of primary EBV infection is particularly high in these children. The incidence of EBVD is 9% and of PTLD 5-8 % after paediatric liver transplantation (109, 114, 115). Mortality in PTLD after paediatric SOT used to be 12-32 % (101, 110, 114), but has been much reduced by EBV DNA monitoring and tapered IS (102, 116).

After stem cell transplantation

EBV-related complications are more unusual after SCT than after SOT (in children 2-3%), but PTLD may evolve early and rapidly, and the mortality is high (115, 117-119). The condition is often obscure, which is demonstrated by the fact that diagnosis is made on post mortem examination in a third of the cases (119). The importance of EBV serostatus is less well understood after SCT than after SOT, and other factors such as impaired T cell function and delayed T cell reconstitution are probably of great importance.

Risk factors for EBVD/PTLD

Many of the risk factors are interconnected. For example: young age is often mentioned as the most important risk factor for EBVD/PTLD after SOT. Primary EBV-infection or EBV mismatch are also considered as important risk factors, but in fact all three are closely interrelated.

1. EBV-serostatus

Solid organ transplantation

EBV mismatch (D+/R-) is an important risk factor for EBVD and is more common in young children. About 50-80% of SOT paediatric patients are EBV seronegative prior to transplantation and up to 2/3 of these patients acquire primary EBV infection, most often transmitted by the graft, after transplantation (95, 104, 120, 121). The mean time of onset of primary EBV after SOT is 6 weeks (122). Although primary EBV infection after SOT increases the incidence of PTLD 10-76 fold, EBV seropositive recipients are not devoid of risk of PTLD (123).

Stem cell transplantation

After SCT, PTLD is not equally strongly associated with primary EBV infection as after SOT. The vocabulary with primary and reactivated infections is often inadequate since, in the clinical setting, it is often not possible to know whether an infection is reactivated or acquired from the graft or from another external source.
Positive EBV serology of either the recipient or donor, is however a risk factor (124). It is more common for the donor as well as the recipient to be EBV seropositive than to be CMV seropositive. With the graft a certain immunity can be transferred, possibly giving some protection to a recipient whose own immunological memory is wiped out by SCT.

2. Immunosuppression

Solid organ transplantation

The risk of EBVD/PTLD is highest the first 3 months after transplantation, or after intensified IS given because of rejection or GvHD. Especially treatment with anti T cell antibodies (ATG or OKT3), used as induction therapy at transplantation or as antirejection treatment are well known risk factors for development of PTLD (101, 125).

Stem cell transplantation

In addition to the general IS, T cell depleting procedures such as use of anti T cell monoclonal antibodies (OKT3), anti-lymphocyte antibodies (ALG) or ATG given as conditioning or GvHD treatment are risk factors for development of PTLD (89, 96, 118).

3. Other risk factors for EBVD/PTLD

Solid organ transplantation

TYPE OF ORGAN

The incidence of PTLD is dependent on the type of organ transplanted. The risk is especially high after small intestinal (32%), and lower after heart/lung (4-8%), liver (3-20%) and kidney transplantation (1-7%) (95, 102, 126-128). This reflects both differences in mean age of recipients, serostatus, IS and differences that are connected to the actual graft itself. For example, in kidney-transplanted children, the overall incidence of PTLD is among the lowest of all groups, but in EBV seronegative kidney recipients the incidence is reported to be as high as 15% (126)

CMV INFECTION

Concomitant CMV infection is a risk factor for EBVD/PTLD, and possibly CMV prophylaxis with GCV or IVIG may reduce the risk of PTLD, but randomized studies of this are still lacking (129, 130).

Stem cell transplantation

TYPE OF DONOR

Unrelated or human leukocyte antigen (HLA) mismatched transplants are well known risk factors for EBVD/PTLD after SCT (108). There are different mechanisms through which this can be explained. The mismatch in itself may be associated with a delayed immunological restoration. Moreover, and probably more important, is the stronger conditioning treatment, almost always involving ATG, and anti-GvHD treatment that is more often given, when MUD grafts are used (118). T-cell depletion without concomitant

B-cell depletion seems to be of special importance for development of post transplantation lymphoproliferative disorder (PTLD).
CMV disease is associated with higher risk of lymphoproliferative disease but it is not clear if this is an independent risk factor (83).

IMMUNE RECONSTITUTION

PTLD development after SCT, is different from that after SOT with an earlier and more fulminant course, which may be reflecting the importance of immune reconstitution after myeloablative procedures (117, 118, 131). It has been argued that measuring the T cell counts might be an important parameter, in addition to EBV DNA, in order to define patients at risk for PTLD (132). The immune reconstitution is more delayed, when using non HLA identical grafts, probably as a result of T-cell depleting conditioning (133).

Clinical presentation of EBVD/PTLD

Fever, lethargy, malaise, nausea, diarrhoea, protein-loosing enteropathy, occult gastrointestinal bleeding, weight loss, hepatitis, hepatosplenomegaly, peripheral lymphadenopathy, stridor, wheezing, hoarseness and exsudative/nectrotising tonsillitis can all be symptoms of active EBV disease in an immunocompromised patient (104, 118, 130, 134-137).
The highly variable clinical presentation of PTLD, often makes it hard to react in time if the awareness of the condition is not great. To quote Dr Michael Green, Pittsburgh, “a high index of suspicion at all times” is necessary to prevent PTLD (138). There are no symptoms that are specific for PTLD and the transition from EBVD to PTLD is often gradual with overlap in clinical presentation. Therefore it has been proposed that these concepts do not represent separate clinical entities, but rather a continuum with gradually increasing pathology (95, 101).
Just like CMV-hepatitis, EBV- induced hepatitis can mimic rejection by affecting liver function tests and to some extent the histology may also be similar (112).

Strategies for prevention of EBV infection

Theoretically, matching of donor and recipient with regard to EBV serostatus would be effective, but this is seldom possible because of graft shortage. An EBV vaccine is wanted, but not available as yet. Antiviral prophylactic drugs have very limited effect, see below. Filtered, leukocyte-depleted blood products are used for all patients.

Strategies for prevention of EBV disease/PTLD

General aspects

Since the development of PTLD may be very fast and the prognosis is depending on early diagnosis, a cornerstone of all strategies should be prevention. This strategy comprises finding high-risk patients and monitoring them for symptoms and signs of EBVD/PTLD as well as by EBV DNA, as first shown efficient for liver transplanted children by Mc Diarmid et al. in 1998 (95, 100). As part of this, regular physical exam should be performed to check for lymph-node, ear, nose and throat status as well as abdominal ultrasound. Anti-rejection treatment should ideally only be used in biopsy-proven acute rejection (139).
Surveillance of EBV DNA levels in plasma and whole blood is used to identify increasing levels, which may indicate impending EBVD/PTLD (84). However consensus is lacking concerning how often sampling should be performed. Since this depends on which risk group the patient belongs to, but also on the time that has passed since transplantation it is reasonable to monitor more frequently in the first 3-6

months and to diminish the frequency over time. It is documented that doubling time of EBV DNA in blood can be as short as 56 h, corresponding to a 1 log increase after 1 week (139).

Strategies after solid organ transplantation

The balancing between using as low IS as possible and at the same time avoiding rejection is a real challenge, since there are yet no adequate means of estimating the individual demand of IS. Instead plasma trough levels of immunosuppressive drugs guide the dosing in an experience-based way. Optimising IS, especially after SOT, is crucial, because PTLD is a consequence of functional over-immunosuppression (140). If the IS can be reduced in time, the chance to revert an early lymphoproliferation is very good (95).
Monitoring EBV DNA in combination with lowering of IS when indicated has been shown to reduce the incidence of PTLD in liver transplanted children from 5-16 to 1-2% and the mortality in PTLD from 30-60 to < 5% (100, 116, 141). A central part of the strategy is to high-light patients that are EBV seronegative at time of transplantation and those that have received anti-lymphocyte preparations. Monitoring them closely for EBV DNAaemia the first year after transplantation and keeping immunosuppressive treatment at a minimal level, are also parts of the proposed general strategy for prevention of PTLD in liver transplanted children along with an alertness of any suspect symptoms (48).

Strategies after stem cell transplantation

Addressing the identified risk factors such as avoiding T cell depletion and MUD or mismatched related donors is naturally done, if possible. Identification of patients at high risk of EBVD/PTLD after SCT has been more difficult than finding those at risk of CMV disease. EBV DNAaemia with high and rising levels is, however, reported to be a critical finding also in SCT patients developing PTLD (118). In order to find the rising EBV levels in time, monitoring must probably be performed rather frequently. It is therefore essential to identify the patients with highest risk and to monitor them during the high-risk period. If EBV DNA levels are significantly increasing, the IS should be minimised. Antiviral treatment is also often used, but it's value is uncertain.

Monitoring EBV DNA in plasma has been reported to predict response to treatment for PTLD after SCT, enabling rapid adjustments of inadequate therapy (142). If the effect is insufficient, rituximab and/or chemotherapy may be given.

Diagnostics of EBV/EBVD/PTLD

Serology

EBV serology (immunofluorescence assay based on IgG antibodies against VCA or EBNAs) of both recipient and donor should be done before transplantation, not so much for matching, but to enable estimation of risk. After SOT; seronegative recipients receiving seropositive graft (D+/R-) should be considered as high-risk patients for development of primary EBV infection. For SCT patients, risks are not as clearly defined by serology, but both recipient and donor should be tested prior to SCT.

Histology

Biopsies from suspected lesions should be histologically investigated and described according to standardised criteriae. A varying spectrum of patterns can be seen in PTLD: from early changes with reactive plasmacytic hyperplasia, over polymorphic or monomorphic PTLD to malignant B cell/T cell lymphoma or Hodgkin lymphoma (143). If possible, additional investigations by cell phenotyping lineage, clonality analysis, and in situ hybridisation for EBER1 and immune histochemistry detection of CD20 should be performed (110, 143). However, one should be aware of that, even when symptoms of EBVD are overt, histopathological analysis may frequently be negative and diagnosis is often not apparent until post mortem (118, 136).

X-ray

Computerised tomography, MRI or PET-CT of chest, abdomen and CNS should be performed on suspicion of PTLD (102, 127).

EBV DNA levels

Measuring EBV DNA levels by PCR is a method that has a strong negative predictive value for PTLD and accumulating data underline the importance of viral monitoring for preventing PTLD both after SOT and SCT (95, 100, 118, 135, 139, 144, 145). Most (>50%) published studies have used whole blood, with the arguments that this material better reflects the viral burden and clonal expansion of EBV infected cells and that EBV DNA can be detected earlier. However, low levels of EBV DNA may be detected in whole blood in approximately 25 % of healthy blood donors and in up to 50% of transplant recipients without EBV complications, while serum/plasma testing is generally negative in these subjects. Whole blood being more unspecific, in combination with frequent cytopenia in transplanted patients, are arguments for serum/plasma as preferred sample material instead of whole blood (72, 118, 146-148).

A drawback of serum/plasma could be that a variable degree of cell lysis might give overestimation of EBV DNA or irreproducible results.
When EBV DNA is detected in immunosuppressed SOT patients without symptoms of EBV-associated disease, the plasma copy number is generally about 10^2-10^3Geq/ml, while patients with infectious mononucleosis have 10^3-10^4 Geq/ml (84). In PTLD patients, the plasma levels are typically above 10^4, but equally high levels can be detected in asymptomatic SOT patients with primary EBV infection (144, 149, 150).
The so called high-load carriers, of some reported to constitute as many as 30% of SOT patients, may have EBV DNA levels in WB >50,000 Geq/ml during several months, without signs of EBVD (144, 151). By measuring EBV DNA in plasma a higher specificity has been reported, and applying a treshold of $>10^4$ Geq/ml might help to discriminate PTLD in these patients (118, 148). It has been suggested that the high serum/plasma levels reflect a lytic infection that is paralleled by a clonal expansion of EBV infected cells (95, 118, 135, 146). In SCT recipients with PTLD, EBV DNA level monitoring in plasma has been reported to be a better predictor of the response to therapy than measurements in WB (142). However, whether serum/plasma or whole blood is superior to the other is still unclear and few studies have used both specimens, which makes comparisons more difficult (see table below).

Table.1 Conversion for different units of EBV DNA levels in whole blood (adapted from Rowe et al. (144))

Units	Conversion factor	Low load	High load
Copies/10^5 PBL	1	8 - 200	>200
Copies/µg DNA	3	24 - 600	>600
Copies/mL blood	20	160 - 4000	>4000
Copies/10^7 B cells	1000	10^4 -10^5	$>10^5$

PBL, peripheral blood lymphocytes

To further increase the benefit of EBV DNA quantification in monitoring transplanted patients at risk of EBVD, it is important to standardise sampling frequency and sampling material, and to establish cut-off levels (139). Also in order to compare studies, standardisation is of high priority. Although probably kinetics are more informative than are single values, cut-off values for different patient categories would be helpful, but has not yet been defined (149, 150, 152). It is generally accepted that in paediatric SOT patients, EBV DNA should be monitored regularly and more frequently if EBV mismatch is present, if the

EBV DNA levels are high or rising, or if a patient develops symptoms suspicious for EBVD (102, 127). An example of flow-chart is shown in fig. 16, p.54, adapted from Smets et al. (95).

Gene expression

Some studies have tried to find gene expression markers, specific for EBVD/PTLD (153), but it has not yet been shown that EBV mRNA detection in blood is clinically useful. Possibly, rather than isolated markers, characteristic EBV gene expression patterns should be sought (154).

In situ hybridisation

Biopsies can be examined by in situ hybridisation. By targeting EBV RNA, e.g. EBER, rather than EBV DNA, it may be possible to distinguish cells with active EBV transcription (112, 115, 155).

Cellular immunity

There are different methods to study EBV specific immunity, such as detecting cytokine (interferon-γ) secretion induced by EBV-specific T cells by Elispot, or by intracellular staining and flow cytometry analysis (95, 132, 156). Some studies report high predictive values regarding progression to PTLD by such tests, which might motivate their use in high load carriers.
There are also commercial tests of functional (not EBV-specific) T cell activity as an option to assess immunoreactivity and appreciate the risk of acute rejection, for example in a setting of tapered IS, or to detect overimmunosuppression (157-161). Using the method (ImmuKnow™), is a way to assess the activity in the patients' cell mediated immune system by measuring adenosine triphosphate (ATP) concentration in the phytohemagglutinin stimulated (CD 4+) T lymphocytes.

Treatment

What is now the main principle for both preemptive and curative treatment of PTLD, i.e. to reduce or stop immunosuppressive medication, was first suggested by Thomas Starzl in 1984 (162). This strategy is effective in up to 60 % of PTLD cases after SOT, but an obvious problem is the risk of rejection or GvHD (114, 163, 164). However, Hurwitz et al. reported that rejection after discontinuation of IS in liver transplanted children with EBVD/PTLD was rare and did not cause graft loss and, moreover, 20 % (8/38) of the patients had developed tolerance (114). The response to reduced IS should be closely followed clinically, by monitoring EBV DNA and possibly by immune function tests, and should be apparent within 2 weeks (157, 158). If signs of rejection appear, IS need to be restored. If there is no or insufficient response to reduced IS, additional treatment must be considered.

Antiviral drugs

Antiviral drugs, i.e. GCV, is often used, both preemptively in combination with tapered IS, and in overt PTLD. However, antiviral drugs exert their effect on viral polymerases only, and during latent infection host cellular polymerases are used for DNA replication so antivirals should not limit expansion of EBV infected cells (48). Still, recent studies demonstrate a potential benefit, probably through blocking of lytic gene expression or by limiting the virus' switch to a lytic cycle, and hence spreading of EBV to previously uninfected B cells and recruitment of new latent clones (87, 95, 165).

Antibody-based immunotherapy

Since most PTLDs are of B cell origin, treatment with monoclonal antibodies directed against CD20, CD21 or CD24 can be effective in up to 50% (166, 167). The humanised monoclonal antibodies towards CD20 (rituximab) is the only commercially available preparation and is recommended (in a dose of 375 mg/m^2 i.v. weekly x 4 weeks) if CD20 is expressed on tumour tissue (168, 169). Rituximab depletes practically all B cells for a long time period (up to 6 months). However, severe CMV reactivation has been described as a side effect and long-time effects awaits further study (87, 139). Rituximab is not effective

in CNS-involvement of PTLD (if not administrated intrathecally) since the antibodies can not pass the blood-brain barrier (163).

Cellular immunotherapy

Immunotherapy with donor-derived EBV-specific CTLs has been successful in SCT patients with imminent or overt PTLD (170, 171). In SOT patients, autologous EBV-specific cytotoxic T cells can be grown in vitro after appropriate stimulation and have been described to be effective for treatment of PTLD, but its use is limited by the time-consuming technique (172, 173).

Chemotherapy

Conventional chemotherapy, like CHOP (cyklophosfamide, hydroxydaunorubicin, oncovin, prednisolone) can be used for PTLD that does not respond to a period of 2-3 weeks of IS withdrawal, particularly in EBV-negative monoclonal lymphomas (174).

Interleukin-6 blockers

Still on an experimental stage, there are reports of effect of monoclonal antibodies towards IL-6, which promotes growth of EBV-infected cells and is present in high levels in patients with PTLD (175).

Other options

Possibly, shifting the immunosuppressive therapy to rapamycin or everolimus may prevent progression of or even retard the growth of PTLD-associated lymphomas, but whether this has a place in clinical practice remains to be shown (87).

2.6 HHV-6

General aspects, transmission and epidemiology

Human herpes virus type 6 is a ubiquitous virus, that appears in two different types, A and B. Transmission of HHV-6 is generally horizontal from mother-to-child or child-to-child, early in life. No distinct illness has been clearly attributed to HHV6-A, but HHV-6B causes exanthema subitum (roseola) in young children, an illness that is generally mild and self-limiting, but quite often causes febrile convulsions. Also other less benign conditions, such as hepatitis, meningitis and encephalitis have been associated to HHV-6 type B, but are more rare presentations (176). Alike CMV and HHV-7, HHV-6 is a beta herpes virus with a similar virion structure, but the virus is smaller than CMV, with a diameter of 160-200 nm and the DNA consists of 161-170 kbp, coding for a yet unknown number of proteins. HHV-6 can infect cells in many different tissues, including liver, central nervous system and salivary glands. Most individuals have been exposed to HHV-6 and seroconverted by 2 years of age. The virus may remain latent mainly in monocytes, macrophages, CD34+ progenitor stem cells and salivary glands and is found in saliva at low levels in most subjects. A special form with chromosomally integrated HHV-6 DNA has been reported in rare cases, but the importance of this phenomenon is not known (69, 176-178). Transplant recipients can develop HHV-6 infection by reactivation of latent virus or from external sources, such as the graft itself or blood products, but although the virus is believed to cause clinical disease, data are limited. It is known that asymptomatic reactivation is common after SCT, but HHV-6 replication has also been linked to bone marrow suppression, pneumonitis, encephalitis, myelitis, and gastrointestinal symptoms and after paediatric renal transplantation to a higher rate of kidney rejection (179-181).

A causative relationship between HHV-6 and these complications is, however, not well established. The risk factors for complications caused by HHV-6 are less well known than for the other opportunistic viruses, but is probably greater if MUD or MMFD grafts are used in SCT (69).

Diagnostics and treatment

Quantitative PCR detection of HHV-6 DNA in whole blood or plasma is the method of choice (69). On suspicion of HHV-6 encephalitis, cerebrospinal fluid should be examined. In HHV-6 encephalitis after SCT, treatment with foscarnet and GCV has been reported to be effective, either alone or in combination (182). As a second-line therapy, cidefovir is recommended (69).

2.7 Adenovirus

General aspects, transmission and epidemiology

Adenovirus is a linear, double-stranded DNA virus of about 35 kbp, coding for more than 30 structural and non-structural proteins. The virion consists of an icosahedral protein capsid with antenna-like projections around the DNA core, but unlike herpes viruses it has no envelope. There are six genogroups of AdV (A-F) and 51 known serotypes. The classification is based partly on their ability to agglutinate red blood cells and of clinical interest in the respect that organ tropism and disease patterns appear to be similar in certain subgroups (183). For example, serogroup 40 and 41 of subgroup F cause most cases of gastroenteritis and C is the predominant subgroup in immunocompromised patients (184).
AdV is spread via respiratory, faecal-oral or ocular conjunctival routes and probably also via the graft after liver and SCT transplantation. The principal site of replication is in the oropharynx, but many adenoviruses can replicate in the gastrointestinal tract. After infection, lifelong immunity to the specific causative serotype probably develops in immune competent hosts (183). Neutralising antibodies to the most common strains, protecting against reinfection with the same serotype, develop in most children before 5 years of age (185). Accordingly, AdV is most important as a common (5-8%) cause of respiratory and gastrointestinal infections in young children, although infants are often protected by maternal antibodies (185, 186). Probably AdV can stay latent after primary infection, and mucosal lymphocytes have been proposed as a reservoir, since viral DNA have been detected at high levels in T lymphocytes from 80% of tissues removed in routine tonsillectomy or adenoidectomy (185). Shedding of AdV, demonstrated by AdV positive cultures from stools and throat for prolonged time periods, is common and reported by Lion et al. in 27% of SCT children, while only 8% were positive by PCR in samples of peripheral blood (187).

Complications and risk factors

The importance of AdV has been particularly emphasized for paediatric stem cell recipients, since AdV among these patients, can cause pneumonitis, haemorrhagic cystitis and encephalitis, but also gastrointestinal symptoms, including hepatitis and often fatal disease (187-191). AdV DNAaemia has been detected in 8-15% of children undergoing SCT, and AdV related death has been described in up to 2-6% of all paediatric SCT (192, 193).
After SOT, the primary site of AdV disease has a predilection for the transplanted organ and this, similar to the case in CMV or EBV infections, holds a risk for symptoms being mistaken as rejection and treated as such (47, 183, 194). In lung transplant recipients pneumonitis, in renal transplant recipients haemorrhagic cystitis, and in intestinal transplant recipients deleterious enteral infections, can be caused by AdV (184, 194, 195). In liver transplanted children AdV seem to be the third most important viral pathogen with incidence rates of invasive disease of 2.7-4% (196-199). Symptoms range from self-limiting fever, gastroenteritis or cystitis to severe disease with pneumonia or hepatitis (184, 196, 198,

199). Risk factors for AdV are similar to the other opportunistic viruses. After SCT, unrelated donors and T cell depleting procedures and after SOT, surgical complications and high IS are among the most important risk factors (199, 200).

Diagnostics

Different subtypes of AdV vary in disease patterns and importance for transplanted patients (183, 184). Possibly, the importance of mismatch between donor and recipient is equal to that of CMV and EBV in regard to risk for disease, but the use of serology is limited due to the many different serotypes of AdV and subtyping is not routinely performed in the clinical setting.
Historically, diagnosis of AdV infection has relied on detection of cytopathogenic effect in virus culture. This method is time-consuming and too sensitive since viral shedding can be persistent in asymptomatic individuals and when applied on faeces, is not proof of symptomatic infection (201). Therefore, importance of AdV as a causative agent should be supported by viral load measurements. The development of quantitative AdV PCR has greatly improved the possibilities of correctly linking AdV to disease and more importantly of predicting AdV disease (189, 193, 195, 197, 199, 202). A low (<1,000 Geq/ml) AdV DNA level in serum can be seen in asymptomatic patients, whereas in disseminating disease, AdV DNA levels of 10^5-10^{10} Geq/ml serum have been described (193, 197, 202). By PCR assays with group and type specific primers, diagnostics can be further improved (183).

Surveillance and treatment

Also for AdV, it is possible to monitor viral DNA levels in order to taper or withdraw IS if AdV DNA levels are high or rapidly rising. This strategy is practiced in some centres and is reasonable at least in high risk patients, such as after T cell depleted SCT or intestinal transplantation. Testing for AdV by PCR in blood and urine is, however, important in all transplanted patients with signs and symptoms of viral disease (199, 200, 203).
Unfortunately, few antiviral drugs have effect on AdV in vivo. Ribavirin is reported to be of use in AdV disease, but relapse is frequent (204). Treatment with cidofovir i.v. is reported to be efficient (in inhibiting viral replication), but is restricted by nephrotoxicity (184, 199, 205). By careful hydration and spacing of administration of cidofovir, the risk of renal side effects can, however, be limited (204, 205).
As lymphocyte reconstitution appears to be of utmost importance for clearance of AdV viraemia after SCT, interventions such as use of adoptive immunotherapy (donor leukocytes) in SCT recipients has been tried (190, 200, 206).

3. IMMUNOSUPPRESSION

General principles and groups of drugs

Immunosuppressive therapies are necessary to prevent T cell-mediated allograft rejection after SOT and graft vs. host reactions (GvHD) after allogeneic SCT. Since T cells mediate a considerable part of the normal defence against viruses, the balance gets shifted and the risk for opportunistic viral infections increases for patients on immunosuppressive treatment.
The immunosuppressive drugs used in transplantation belong to 5 main groups:
Corticosteroids, calcineurin inhibitors, purine synthesis-inhibitors (mycophenolate mofetil), mTOR-inhibitors and mono- or polyclonal antibodies. The most important of the immunosuppressive agents are the calcineurin inhibitors: cyclosporine A and tacrolimus, which are part of most of the used immunosuppressive regimens.

Corticosteroids

Historically corticosteroids have constituted the basis of IS after transplantation and are still commonly used during the first time period after SOT and SCT, in combination with other immunosuppressive drugs, i.e. calcineurin inhibitors. For liver transplant recipients, corticosteroids are often given in a single dose at transplantation and then in gradually lowered doses, to be weaned after 3 months. In the last years steroid-free IS regimens including induction therapy with ATG or IL-2 inhibitors have become widely used in liver transplantation programs without higher incidence of acute or chronic rejection or graft loss (207-209). A higher incidence of 'de novo-autoimmune hepatitis' has been reported and it is not clear whether this is associated to steroid-free regimens. In acute rejection after SOT or aGvHD after SCT, corticosteroids are used either as pulse methylprednisolone i.v. or as prednisolone orally for 2-5 days.

Calcineurin inhibitors

This group includes cyclosporine A (Sandimmun Neoral®) and tacrolimus (Prograf®).
These are highly efficient immunosuppressive compounds that block T cells by binding to cytoplasmic receptors, thereby inactivating calcineurin, which is an enzyme with a central role for T cell function.

mTOR-inhibitors

mTOR is short for 'mammalian target of rapamycin'. The mTOR-inhibitors: Sirolimus (Rapamune®) and Everolimus (Certican®) inhibit activation of the T cell via a kinase. These agents are considered as less nephrotoxic than the calcineurin inhibitors, but have disadvantages, such as negative effects on wound healing and haematopoiesis.

Antilymfocyte antibodies

Anti-T-lymphocyte globulin (ATG-Fresenius®, Thymoglobuline®), is used both as induction therapy in steroid-free immunosuppressive regimens and as anti-rejection therapy after SOT. After SCT, ATG can be used both for conditioning and as anti- GvHD treatment. Monoclonal antibodies towards T cell CD3-antigen (Orthoclone OKT3®) are mainly used in steroid-resistant acute rejection in SOT patients.

Monoclonal antibodies to interleukin-2, (IL-2) inhibitors (basiliximab (Simulect®), daclizumab (Zenapax®))

These preparations are monoclonal antibodies to the IL-2-receptor, expressed in T lymphocytes, inhibiting IL-2 mediated activation of T lymphocytes.

These agents are mostly used as induction treatment in combination with tacrolimus +/– steroids in liver or kidney transplantation programs and daclizumab also sometimes for treatment of severe GvHD after SCT (207-209).

Campath (alemtuzumab (Mabcampath®)

Alemtuzumab is a humanised antibody preparation towards CD52, the most abundant antigen expressed in B and T lymphocytes that induces long-time elimination of both lymphocyte populations. This drug is used to some extent as conditioning and anti GvHD treatment after SCT.

SCT: myeloablative and immunosuppressive regimen

Conventional myeloablative regimen consists either of total body irradiation (TBI) or busulfan, in combination with cyclophosphamide. In general, patients older than 4 years of age with leukaemia receive TBI-based regimens, while the other patients get busulfan-based conditioning. When graft from an unrelated donor or mismatched family donor is used, ATG is often added, after individual assessment. Many different non-myeloablative regimens exist, but of them, the Jerusalem scheme with fludarabin and TBI/cyclophosphamide is frequently used. For GvHD prophylaxis in both myeloablative and non-myeloablative regimens, the patients receive tacrolimus for 3-6 months (prolonged if GvHD), and in general, with addition of a short course of methotrexate (except for most of the patients with leukaemia, receiving a graft from an HLA-identical sibling).

4.1 ANTIVIRAL REGIMENS

Antiviral procedures of paediatric liver transplantation program in Göteborg

The procedures for surveillance and treatment of CMV and EBV have been modified during the last 10 years, but the present strategies are here related in brief: Since 2000, GCV prophylaxis 5 mg/kg b.i.d., i.v. is given for 2 weeks, followed by 3 months of oral VGCV for liver transplanted children when CMV D+/R- mismatch is at hand (medium risk patients). These children also receive CMV hyperimmune globulin (Cytotect®) 50 U/kg in repeated doses (day 0, 7, 14, 35, 56 and 77 post transplant). Filtered, leukocyte depleted blood products are used, when needed. All liver transplanted children are monitored for CMV and EBV and treated preemptively with GCV i.v. when CMV DNAaemia is detected. The effect of treatment is followed by CMV DNA measurements and the length of treatment is guided by these results. In case of rejection treatment in patients that are not on GCV prophylaxis, GCV i.v. is considered. When EBV DNAaemia with high or rapidly rising viral levels is detected, IS is tapered/discontinued in a first line of action. If the patient is symptomatic or has high viral levels, treatment with GCV i.v., is also initiated. If a CD20 positive lymphoproliferative process is diagnosed, treatment with rituximab (Mabthera®) is given.
Children younger than 1 years of age, are considered as CMV and EBV seronegative, even if IgG is detected, since IgG positivity can be maternally transferred.

Antiviral procedures of paediatric stem cell transplantation program in Göteborg

The present regime includes antiviral prophylaxis with acyclovir/valacyclovir to all patients for a minimum of 6 months post transplantation. Children with primary immune deficiencies or total IgG levels < 4.0 g/l before SCT, are substituted with intravenous immunoglobulin (IVIG), once weekly until discharge, then once a month until day 90 post SCT.
Preemptive therapy with GCV (5 mg/kg b.i.d. for ≥ 2 weeks, followed by 6 mg/kg x I, for 2 weeks) is started if CMV DNA is above 300 Geq/mL serum and rising in subsequent samples, or if the level is ≥1,000 Geq/mL in first sample. The efficacy of the treatment is followed by CMV DNA measurements and treatment is prolonged or exchanged to foscavir if there are signs of refractory infection.
CMV DNA in serum is followed once a week from day 0 to day 100 and on clinical suspicion EBV and AdV is also included in the analysis.

5. AIM OF THE STUDY

The aim of this thesis was to:

Investigate the frequency and symptomatic manifestations of cytomegalovirus and Epstein-Barr virus infections during the first year after paediatric liver transplantation.

Evaluate the clinical relevance of quantification of CMV DNA and EBV DNA in serum for identification of symptomatic infections in liver transplanted children.

Compare serum and whole blood as specimen for quantification of CMV DNA and EBV DNA.

Describe the incidence and clinical outcome of opportunistic, viral infections during the first year after paediatric stem cell transplantation by quantification of viral DNA in saved serum samples.

6. PATIENTS AND METHODS

Three papers (I, II and IV) have a clinical, descriptive approach and are based on patient material.

I. In paper I, we focused on the incidence of CMV infections after paediatric liver transplantations and the value of quantitative PCR for identifying the symptomatic patients. CMV infections during the first year after liver transplantation were retrospectively investigated in 18 consecutive children by examination of patient files and analysis of CMV DNA in available, saved serum samples by quantitative PCR Amplicor monitor (Roche). Six patients were transplanted because of extrahepatic biliary atresia (EHBA) and had ages ranging between 0.4 and 1.3 years, while 12 patients had a wide spectrum of diagnoses and ages ranging between 0.5 and 15.7 years (median 3.0 years). Until 2003, amplification of CMV DNA was performed by a qualitative "in-house", nested PCR using the primers CMVie_ouF (GAGCACCCTCCTCCTCTTCCTCAT) and CMVie_ouR (GCGCCGCATTGAGGAGATCTGCAT) in a first round of 30 cycles, and CMVie_inF (GCCGATCCTCTGAGAGTCTGCTCTC) and CMVie_inR (CAGCCACAATTACTGAGGACAGAGG) in the second round of 40 cycles, followed by agarose electrophoresis. This method was used to amplify CMV from either serum or from "buffy coat", i.e., purified white blood cells. Evaluations prior to the introduction of real-time PCR, showed that the real-time PCR had higher sensitivity than nested PCR on serum, while real-time PCR on whole blood and nested PCR on buffy coat, showed similar sensitivities.
The result of the qualitative test was only given as + or –, and it was difficult to establish the role that CMV played for the individual patient. This was a significant problem because after transplantation the children often presented with elevated liver functions tests, fever, leukopenia and a concurrently positive CMV PCR.
The Amplicor method is a competitive traditional PCR with post-PCR detection of the amplicon by ELISA, with a relatively narrow detection range, between 400 and 100,000 Geq/ml. After the study was performed, real-time PCR methods have been developed and implemented. The real-time PCR method that is now used, has a wider quantification range and a higher sensitivity than the Amplicor assay, and the units are not equivalent: 1 Geq by Amplicor corresponds to approximately 3 Geq by real-time PCR.

Detection of CMV IgG by ELISA and IgM by immunofluorescence was also recorded or performed retrospectively. From patient files, information about infectious episodes (fever and other symptoms, results from bacterial and viral cultures, treatment with antibiotics and antiviral drugs), liver function tests, immunosuppressive treatment, rejection episodes and treatment, as well as other complications during the first year after liver transplantation was extracted.

The patients were classified on the basis of clinical symptoms in combination with donor/recipient CMV serostatus, and the results from CMV DNA detection by PCR. See Fig 6, page 39.

Definitions, of CMV infections.

Symptomatic CMV infection was defined as patients with symptoms/signs of CMV disease, in combination with CMV DNA in serum, or IgM, emerging after transplantation, positive in a titre of ≥1/64. These were divided in primary or reactivated infections, depending on donor/recipient serostatus.

Asymptomatic CMV infection was present when, without symptoms, CMV DNA was detected in serum, or if IgM emerged after transplantation and was positive in a titre of ≥1/64.

Latent CMV infection was defined as CMV IgG positivity or when CMV DNA was only detected in buffy coat.

During the study period the only prophylaxis consisted of hyper CMV immunoglobulin, given to the CMV seronegative patients that received a graft from a seropositive donor. GCV treatment was only given preemptively when CMV DNAaemia was detected (by qualitative test).

One patient, that was CMV seropositive, received GCV, because of a preceding fulminant EBV infection (as indication for transplantation).

II. In paper II, serum samples from the first year post transplant from 24 consecutively liver transplanted children 1995-2002, were analysed for EBV DNA. The group of patients from paper I was expanded with the 6 children, transplanted January 2001-May 2002. Previous results from routine surveillance by qualitative "in-house", nested PCR of EBV DNA in serum and buffy coat, and serological results of EBV IgG by ELISA and IgM by immunofluorescence were recorded. The mean age of the added children were 3.3 years. The median age of all the 24 children was 1.7 years. In 18 children, when earlier results indicated possible EBV infection, EBV DNA levels in saved serum samples were analysed by real-time TaqMan PCR. The method, as described by Niesters, has a detection range of 10^2-10^7 Geq/ml (147). Every sample was run in duplicate. The mean number of samples, analysed per patient were 7.5 (range 1-16).

The results were related to clinical picture, IS, rejection and other infections. From patient files, information about infectious episodes (fever and other symptoms, results from bacterial and viral cultures, treatment with antibiotics and antiviral drugs), liver function tests, immunosuppressive treatment, rejection episodes and treatment as well as other complications during the first year after liver transplantation were recorded.

The 18 patients, of whom 10 were ≤ 1.3 years of age, were classified on the basis of clinical symptoms in combination with recipient EBV serostatus and the results from EBV DNA detection by PCR. The criteria for classification of EBVD, EBV hepatitis and PTLD constituted a combination of clinical and histological parameters, in accordance with definitions given on page 22. Please, see fig. 7, page 40.

III. Both for CMV and EBV analyses, the used sample material differs between centres.
An obvious advantage of serum as sample material for PCR is the option to use serum originally taken for serological purposes. In most studies of EBV, whole blood is used, with EBV DNA expressed in relation to number of white blood cells or µg DNA. Theoretically, whole blood could be the more relevant sampling material, since it may better reflect clonal expansion of EBV infected cells. Rowe et al. have described patients with long-standing high EBV DNA levels in PBLs, in which changes were not reflected in plasma (144), but several other studies have shown that elevated EBV DNA also in serum accompanies such cellular expansion, and that rapidly rising levels in serum/plasma is more specific (118, 147, 148).

We wanted to compare serum and whole blood, in order to gain more information about which material is preferable. In paper III, we therefore retrospectively compared levels of CMV and EBV DNA in serum and whole blood, in all real-time PCR analyses for CMV and EBV performed in Göteborg 2002 till 2006, to provide a background for interpretation of viral DNA levels in these sample materials.
Please, see fig. 8 A and B page 41-42.
For CMV the forward primer was CMV_UL123_F (TGATCACTGTTCTCAGCCACAAT), the reverse primer CMV_UL123_R (TCCTCTGATTCTCTGGTGTCACC), and the probe CMV_UL123_P (CCCGCACTATCCCTCTGTCCTCA). For EBV primers and probes described by Niesters were used (147). The probes for both CMV and EBV were labelled with 5'FAM (6-carboxyfluorescein) and 3'TAMRA (6-carboxytetramethyl-rhodamine). Quantification was obtained from a plot of Ct values for quantification standards. The assays have ranges of quantification spanning from 200 Geq/mL to 10 million Geq/mL as determined by testing serial dilution of plasmids with viral inserts.

IV. In paper IV, we retrospectively investigated 47 consecutive patients, who had undergone allogeneic haematopoietic stem cell transplantation (SCT) performed 2001-2005 in order to describe the incidence and clinical outcome of opportunistic, viral infections. Saved serum samples were analysed by real-time PCR for infections with CMV, EBV, HHV-6 and AdV during the first 12 months for CMV and 6 months for the other viruses. Serology for CMV and EBV, but not for AdV or HHV-6, was included in the survey. Please, see fig. 9, page 43.

We specifically studied the known risk factors: donor-recipient serostatus, donor category, use of anti-thymocyte globulin (ATG) and other conditioning treatment and presence of acute or chronic graft versus host disease (GvHD). These data were collected from patient files along with information of GvHD prophylaxis, antiviral prophylaxis and treatment, and infectious and other complications. The potential impact on viral DNA levels of age, gender, transplant type (MUD/HLA identical), ATG (yes/no), TBI (yes/no), and acute or chronic rejection was evaluated by multiple linear regression with backward elimination.

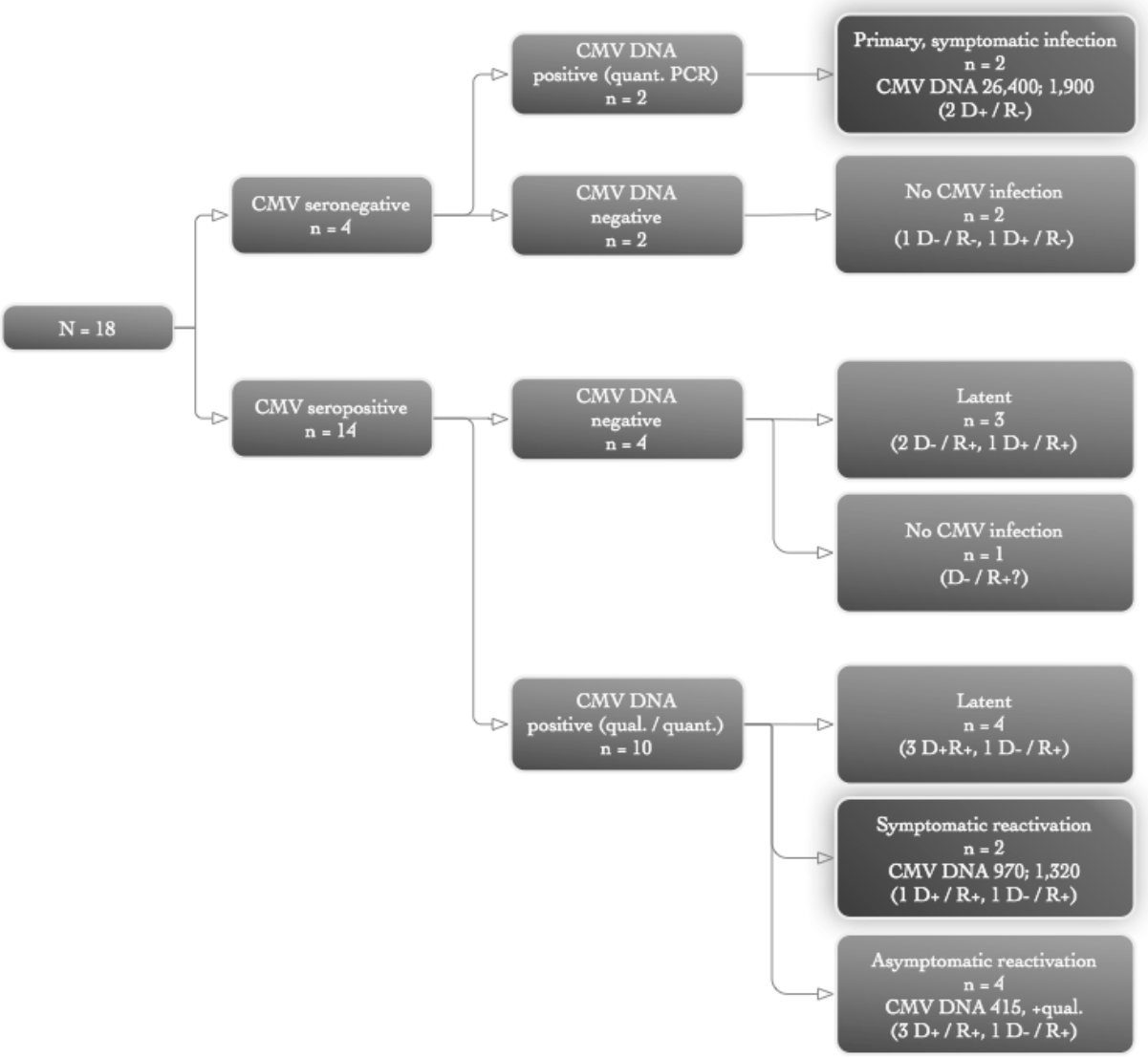


Fig. 6. Number of patients, transplanted between January 1995 and December 2000, and results from serology and CMV DNA detection (paper I). Viral levels in Geq/mL, should be multiplied with a factor of ≈3 to be comparable with values by TaqMan PCR.

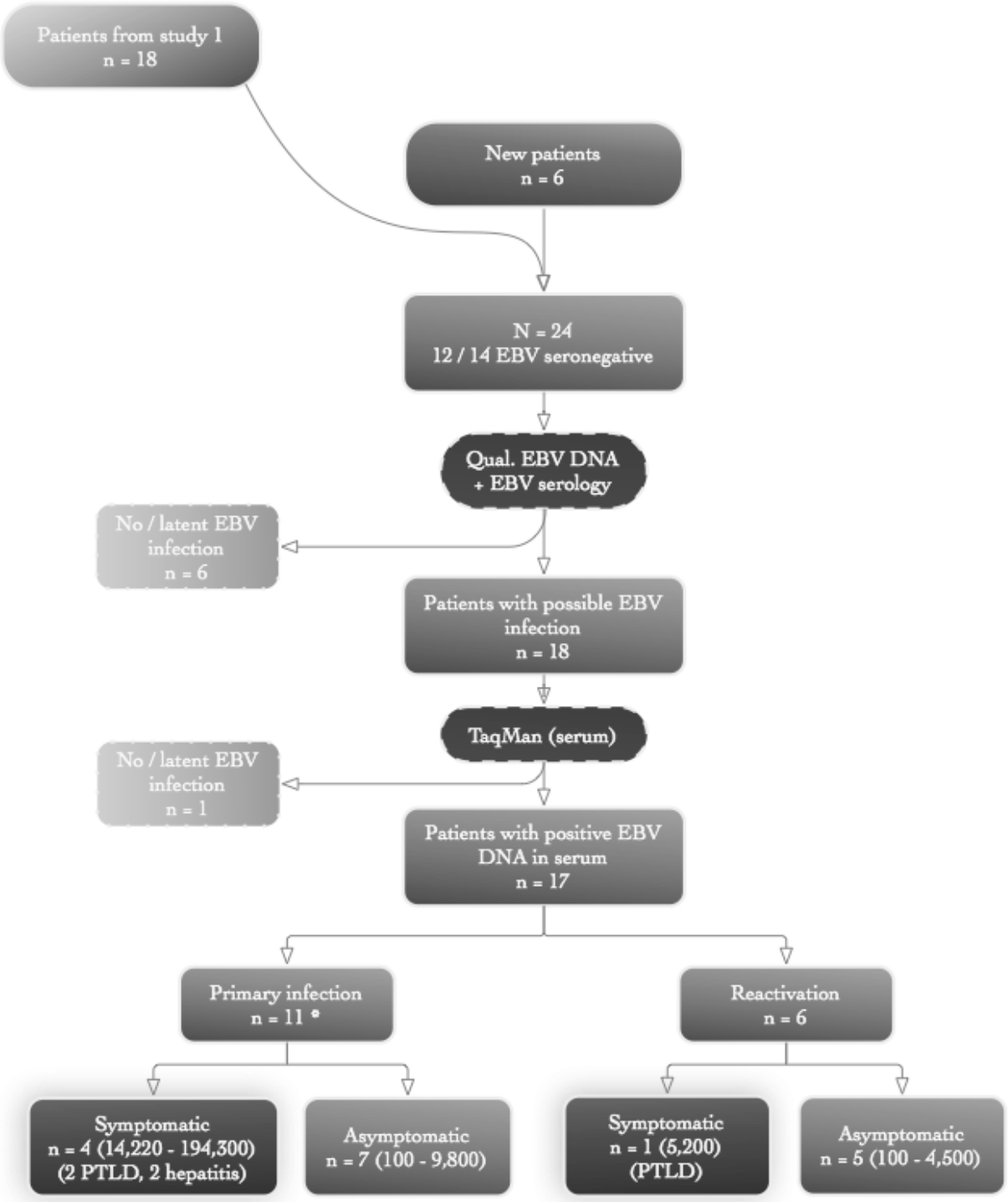


Fig. 7. Number of patients (addition of 6 patients, transplanted between January 2001 and May 2002) and results from serology and EBV DNA analysis in the patients (paper II). Viral levels in Geq/mL.
*Median age 1.0 years.

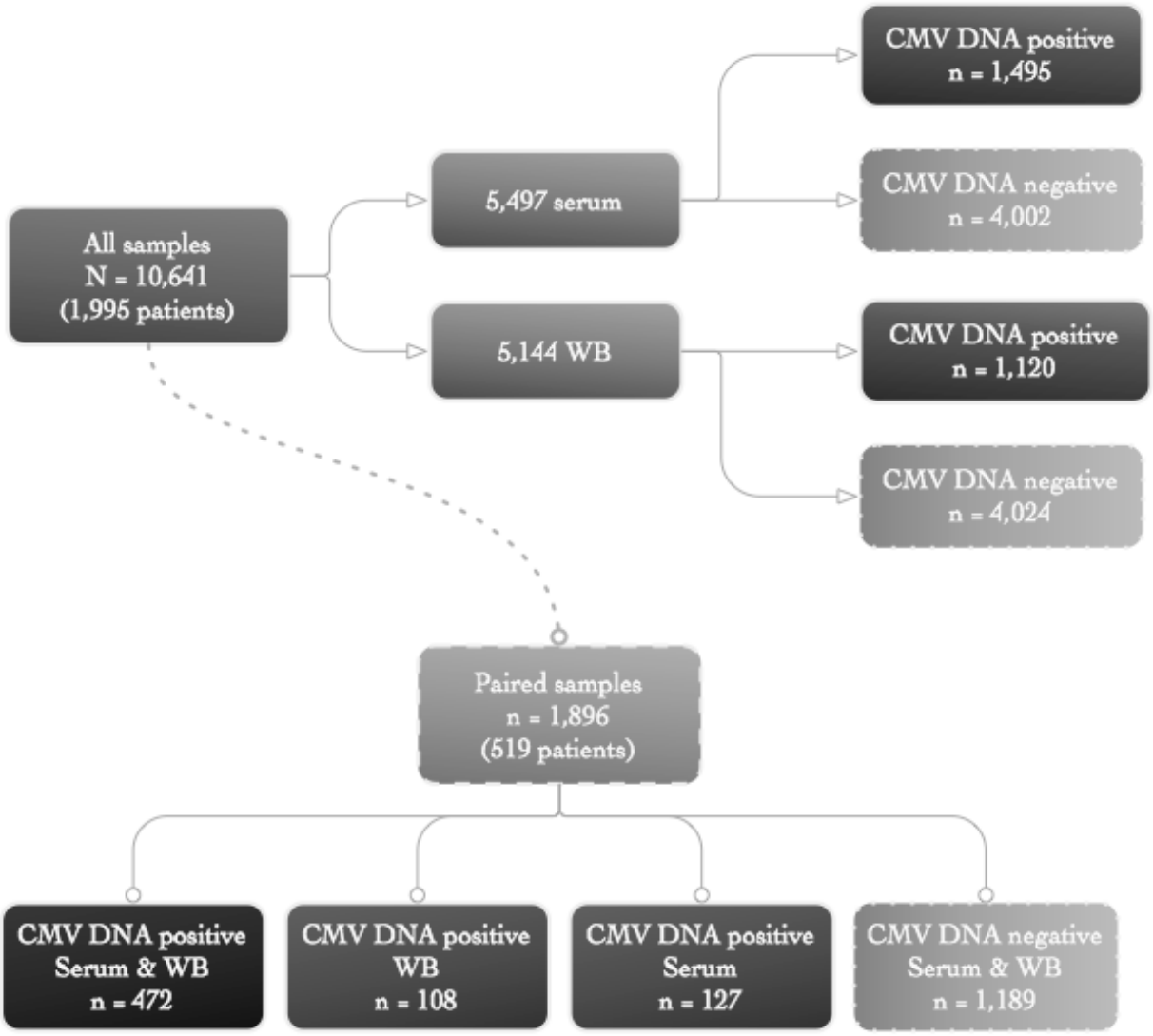


Fig. 8 A. Samples analysed for CMV DNA and results in whole blood and serum (paper III).

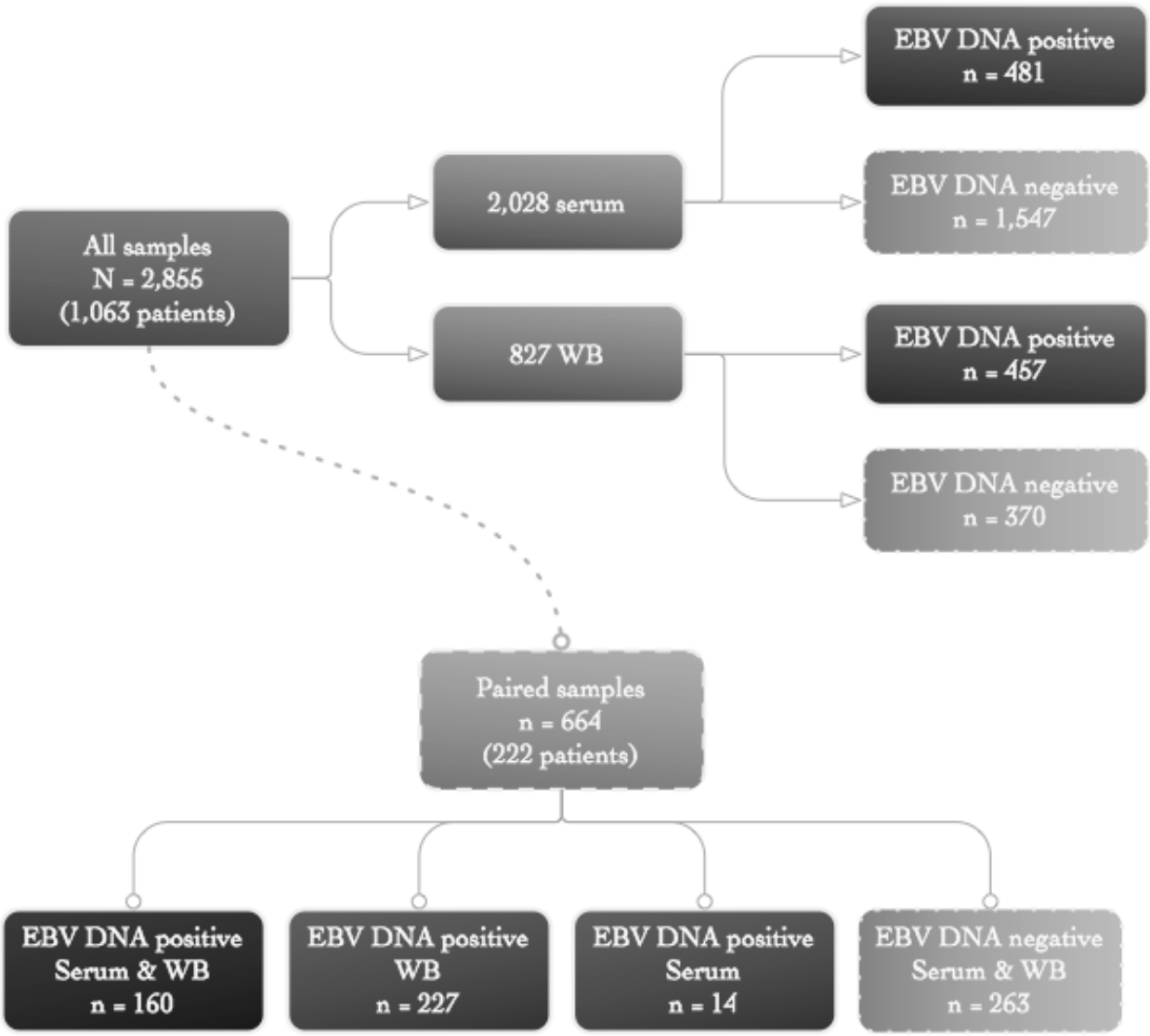


Fig. 8 B. Samples analysed for EBV DNA and results in whole blood and serum (paper III).

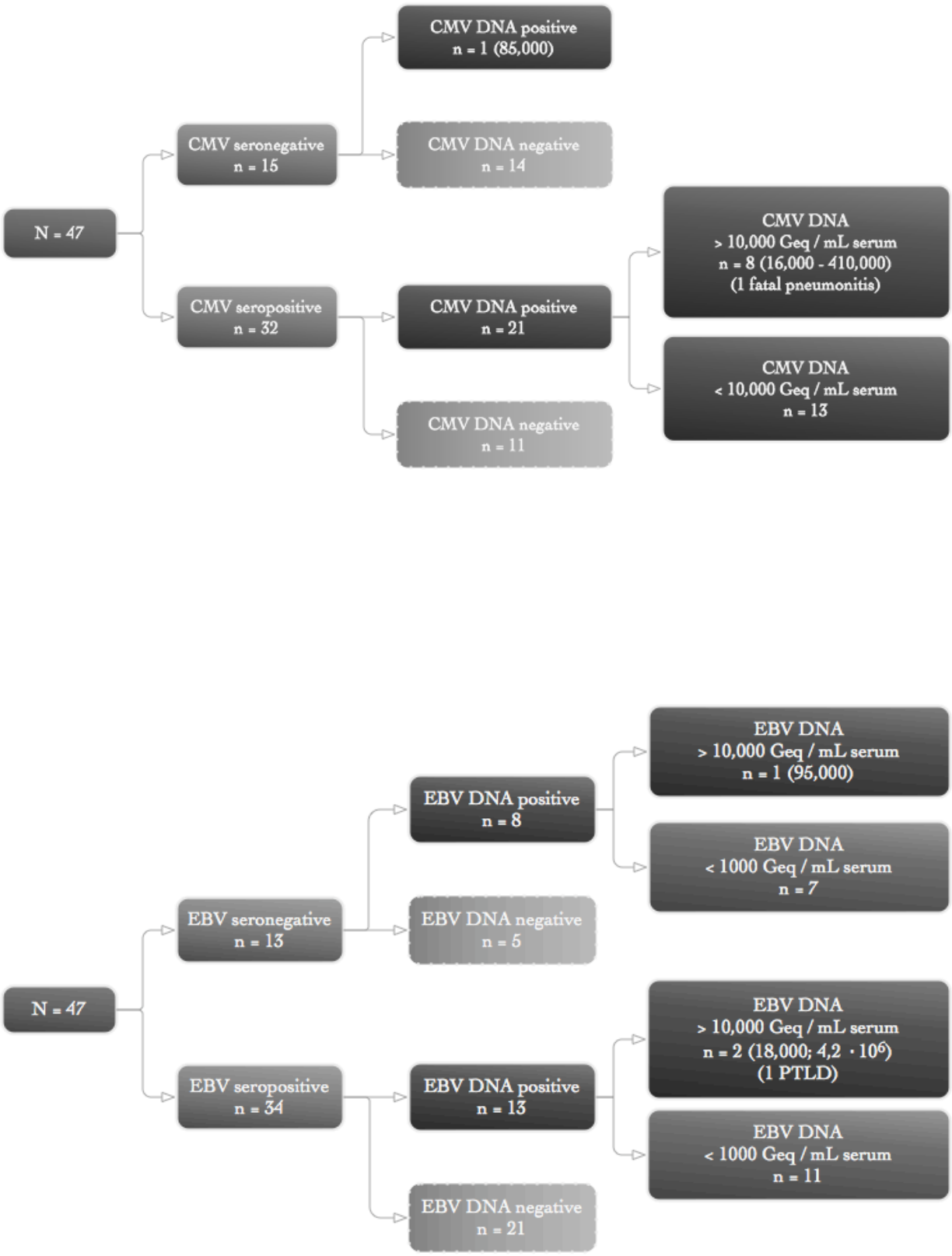


Fig. 9. Number of SCT recipients, (January 2001 to December 2005) and results from serology, CMV and EBV DNA analysis (paper IV). Viral levels in Geq/mL.

7. RESULTS AND DISCUSSION

In the following text, we report and discuss the results from the different papers for each of the investigated viruses at a time. The results from each publication are summarised in the section below.

Summary of findings in paper I-IV.

Paper I

- CMV markers (CMV DNA, IgG or IgM) was detected in 83% (15/18) of the patients
- Symptomatic CMV infection was found in 4 patients (22 %), in 2 of them primary
- 2 out of 3 CMV mismatched patients developed symptomatic infection at a mean of 1.7 months after transplantation
- CMV DNA levels were higher in primary and symptomatic CMV infections
- CMV DNA was 970 - 26,400 Geq/mL in 4 of the symptomatic patients, but was < 500 Geq in all the 14 asymptomatic patients
- Preemptive GCV was given to 6 patients (33%)

Paper II

- 12/24 patients were considered EBV seronegative at transplantation, but 6 of them had maternally transferred IgG
- EBV markers (EBV DNA, IgG or IgM) was detected in 92% (22/24) of the patients
- EBV DNA was positive in serum in 71% (17/24) of the patients
- 11/24 developed primary EBV infection, at a mean of 4.8 months after transplantation
- 6/24 developed reactivated EBV infection, at a mean of 4.0 months after transplantation
- Symptomatic EBV infection was found in 21% (5/24) of the patients: in 3 of them as PTLD, in 2 as hepatitis
- 4/5 of the symptomatic patients had primary infections and all of them had received a graft from a EBV positive donor
- The EBV DNA levels in the 4 patients with symptomatic primary infection were higher than in the patients with asymptomatic primary infection, median 50,550 vs. 2,890 Geq/mL (p=0.011)
- The EBV DNA levels in the 3 patients with PTLD and the 2 with hepatitis were in the same range
- 1/6 patients with reactivated EBV infection was symptomatic (PTLD), and had a higher EBV than the 5 patients with asymptomatic reactivation, 5,200 vs. median 790 Geq/mL
- PTLD was found in 12% (3/24) of the patients

Paper III

- CMV DNA was detected in 27% (1495/5497) of serum samples with a median level at 2.53 log Geq/mL in the positive samples
- CMV DNA was detected in 22% (1120/5144) of WB samples with a median level at 2.52 log Geq/mL in the positive samples
- EBV DNA was detected in 24% (481/2028) of serum samples with a median level of 2.48 log Geq/mL in the positive samples
- EBV DNA was detected in 55% (457/827) of WB samples with a median level of 3.36 log Geq/mL in the positive samples
- In 1896 paired serum and WB samples, drawn on the same day, CMV DNA was detected in both specimen types in 472 samples with 0.18 log higher levels ($P<0.001$) in WB than in serum (median level 2.73 vs. 2.56 Geq/mL) and in only either serum or WB in 127 and 108 samples, respectively, generally at levels below 1000 Geq/mL
- In 664 paired serum and WB samples, drawn on the same day, EBV DNA was detected in both specimen types in 160 samples with 1.5 log higher levels ($P<0.001$) in WB than in serum, (median 4.2 vs. 2.4 log Geq/mL) and in only either serum or WB in 14 and 227 samples.
- 16% of WB as compared to 2% of serum samples had EBV DNA >4.0 log Geq/mL

Paper IV

- CMV DNA was detected in serum in 23 recipients (49%), in all within 8 weeks after SCT
- CMV DNA reached levels > 10^4 Geq/mL in 9 patients: 1 with fatal CMV pneumonitis and 3 with other possibly CMV associated symptoms
- The CMV DNA levels did not differ significantly between the D+/R+ and D–/R+ groups
- All D–/R– cases remained CMV DNA negative
- ATG and TBI conditioning were independently associated with higher CMV DNA levels

- EBV DNA was detected in serum in 21 recipients (45%) within 6 months after SCT
- EBV DNA reached levels > 10^4 Geq/mL in 3 patients, one of whom developed and died of PTLD
- ATG and TBI conditioning were independently associated with higher EBV DNA levels

- HHV-6 DNA was detected in 13 recipients (28%), 10 never reaching levels > 10^3 Geq/mL, 3 reaching levels > 10^4 Geq/mL

- AdV DNA was detected in 13 recipients (28%), in all except 1 case at levels < log 3 Geq/mL
- One patient, who had been conditioned with TBI and ATG, developed a fatal, disseminated AdV infection and had a maximum AdV DNA level of 7.15 log Geq/mL

- 21 % of the total mortality was caused by viral complications

7.1 CMV infections

CMV DNAaemia

In our studies, we have focused on the presence of CMV DNA in serum. CMV DNA was detected in serum on some occasion during the first year after transplantation in 28% (5/18) of the liver recipients (paper I) and in 47% (23/47) of the stem cell transplanted children (paper IV). In the clinical samples described in paper III, most of which were drawn from organ or stem cell transplant recipients, 27% (1495/5497) were CMV DNA positive in serum. See flow-charts pages 39, 41 and 43. These findings demonstrate that some degree of CMV activity is present and detectable in serum in many patients after transplantation. By quantifying the CMV DNA levels, this activity could be characterised more accurately, which is essential for decisions regarding IS and antiviral treatment.
In paper I, as many as 67% (12/18) of the patients were CMV DNA positive at some time point if qualitative results in buffy coat were also considered. A positive qualitative test led to GCV treatment on 10 occasions in 6 (33%) of these patients, but in 3 of the patients the CMV DNAaemia retrospectively was found to be low and transient. In all, 8 patients (44%) in study I received GCV. At the time for study IV, quantitative PCR had been introduced. This may explain that the number of stem cell transplanted children in study IV that received GCV (in single or repeated courses) was lower, 36% (17/47), despite the higher proportion with detectable CMV DNA, 47% vs. 28% in study I, as mentioned above.

Antiviral prophylaxis was not generally used for CMV, neither to liver transplanted nor to stem cell transplanted children. Instead, preemptive GCV treatment was initiated quite frequently. This could have affected the CMV DNA levels in many cases, and makes it difficult to describe the course of CMV infection in these patients, or to identify CMV DNA levels associated with symptoms.

In paper I, 4 of the 18 patients were classified as symptomatic. Two of them had primary CMV infections with maximum CMV DNA at 26,000 and 1,900 Geq/mL, respectively, in serum as measured by the Cobas Amplicor assay. The other 2 had reactivations with maximum CMV DNA at 1,320 and 970 Geq/mL, respectively. Only one of the 4 patients classified as having asymptomatic reactivation, had CMV DNAaemia, at a level of 400 Geq/mL serum. CMV DNA levels obtained by the Cobas Amplicor assay need to be multiplied by a factor of ≈3 to be comparable with today's values by TaqMan PCR. Such a conversion would give levels ranging from 2,900 to 79,000 Geq/mL in the 4 patients with symptomatic infections, as compared to ≤1200 Geq/mL for the asymptomatic infections.

In paper IV, 47% (22/47) of all patients, and 66% (21/32) of the patients that were CMV IgG positive at the time of SCT, were CMV DNA positive at least once during the year after transplantation. In all these 22 cases, CMV DNAaemia appeared within 8 weeks after SCT. Nine of these 22 patients reached CMV DNA levels above 10,000 Geq/mL at some point, and 4 of these 9 had symptomatic infection (including one patient that died in CMV pneumonitis), with concomitant CMV DNA of ≥37,000 Geq/mL. Six patients reached levels between 1,000 and 10,000 Geq/mL, and 7 had levels that were never above 1,000 Geq/mL, but none of these 13 patients had symptoms of CMV disease. These findings show that quantification of CMV DNA in serum can help to distinguish symptomatic and asymptomatic CMV infections after transplantation. They indicate that levels below 1,000 Geq/mL may be interpreted as benign, because they are often transient and not associated with symptoms. Levels above 10,000 Geq/mL seem to be associated with a high risk for symptomatic infection, while levels between 1,000 and 10,000 Geq/mL represent a grey zone, with intermediate risk for symptomatic infection. Levels above 1,000 Geq should however always be considered a warning sign, because if levels increase, complications may rapidly develop, as seen in Case A in paper IV (fig.10). Even levels below 1,000 Geq/mL may be alarming if they represent the start of a primary infection shortly after transplantation when the patient is vulnerable.

In total, 4/47 (8.5%) were categorized as symptomatic, which is in concordance with incidence of CMV disease reported by others (38, 39, 42).

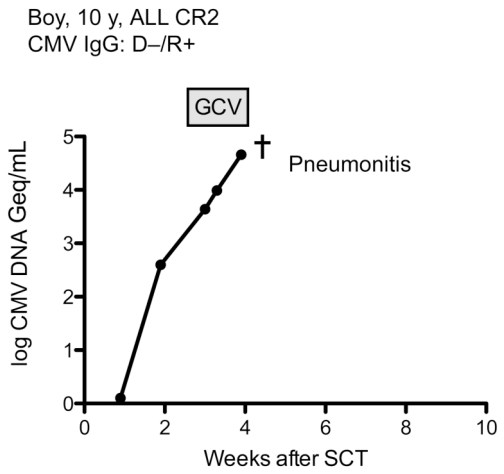


Fig.10. CMV DNA levels in serum in a patient after SCT (case A).

The finding of higher CMV DNAaemia levels in transplanted children with symptomatic infection is in agreement with other reports. Bai et al. found higher whole blood levels of CMV DNA in symptomatic vs. asymptomatic children after SOT, and suggested a cut-off at 10,000 copies/mL (210). Leruez-Ville et al. observed CMV DNAaemia in plasma in 37% of stem cell transplanted children with median levels at 15,000 Geq/mL in symptomatic vs. 3,000 Geq/mL in asymptomatic patients (42). Kalpoe et al. compared CMV DNA levels with the pp65 assay and found that detection of CMV DNA in plasma occurred earlier than pp65 antigen in leukocytes. They defined cut-off levels for antiviral therapy for SCT patients at 1,000 Geq/mL for the first episode of CMV, but at 10,000 Geq/mL for recurrent CMV DNAaemia. For SOT patients, they suggested that therapy was indicated in any detectable level in primary CMV infection, while the cut-off was set to 5,370 Geq/mL for reactivations (68). Gor et al. suggested a cut-off between 3,500 and 35,000 Geq/mL in whole blood for identifying patients with higher probability of CMV disease (43).

CMV serology

It is well known, that CMV serostatus is of great importance for the risk of CMV complications, but the conditions are different for solid organ and stem cell transplanted children. For SOT recipients, the risk is greatest if a seronegative recipient receives a graft from a seropositive donor. After SCT, CMV seropositivity in either recipient or donor confers a risk for CMV complications (38, 39, 50, 211), although the D–/R+ setting is by some considered to be especially risky (43, 65, 212). In Göteborg, 40% of all liver transplanted children 1995-2008, were CMV seronegative and 28% of them received CMV seropositive grafts. For comparison, 66% of the kidney-transplanted children during the same period, were CMV seronegative and more than 40% received CMV seropositive grafts.

Of the liver transplanted children in paper I, only 22% (4/18) were CMV IgG negative despite a low median age: 2.5 years (range 0.4-15.8). The explanation for the high seroprevalence might be that they, as compared to children in general, had been exposed to CMV to a higher degree by blood products or long-term hospital stays. In paper IV, 68% (32/47) of the recipients, were CMV IgG positive with a median age of 8.6 years (range 0.9-18).

Out of the 4 children in paper I, who were CMV seronegative, 3 received a liver graft from a CMV seropositive donor and of them, 2 developed primary symptomatic CMV infection at a mean of 4 weeks after transplantation. Six of the 14 CMV seropositive recipients developed CMV reactivation (or reinfection), which in 2 cases was classified as symptomatic. In all, symptomatic CMV infection was found in 22% (4/18) of the patients, all of them younger than 2 years of age at transplantation. The

definition of symptomatic CMV infection was symptoms or signs of CMV disease, i.e. fever or hepatitis, (where there was no more plausible explanation for these symptoms), in combination with CMV DNA in serum or IgM≥1/64. Interestingly, despite IS, CMV IgM was positive in all symptomatic patients with very high titres in the patients with primary infection. The mortality among the 18 patients was 16.7 % (3/18), but in no case attributable to CMV-infection.
In study IV, CMV DNAaemia was only found if either the donor or recipient was CMV seropositive. Out of the 15 patients that were CMV seronegative, 3 received grafts from seropositive donors, but only 1 patient demonstrated CMV DNA positivity or symptoms of CMV infection. In all, 7 patients seroconverted during the first year post transplant, but all the others were asymptomatic and never had CMV DNAaemia identified by PCR. Of the 32 recipients that were CMV seropositive at SCT, 3 demonstrated symptoms likely due to CMV infection post transplant. In the multivariate analysis of factors that might be of importance for CMV DNAaemia, donor/recipient CMV IgG showed a strong and independent association with CMV DNAaemia (was therefore excluded from the final multiple regression analysis).

Sample material

In order to investigate if serum is an adequate sample material we performed in study III a survey of 10,641 clinical samples, which had been analysed for CMV DNA by TaqMan PCR from September 2002-March 2006. Samples from patients < 1 months of age were excluded. In all, there were 5,497 serum and 5,144 whole blood samples. Seventy-five percent of the samples were negative, probably reflecting that the majority were taken for monitoring of asymptomatic patients or during antiviral treatment. CMV DNA was detected in 2,615 (24%) samples: in 1,495 of the 5,497 serum samples (27%) and in 1,120 of the 5,144 WB samples (22%).
On 1,896 occasions serum and WB samples had been taken the same day from 519 patients with a median age of 34.8 year and a mean of 3.5 samples per patient.
CMV DNA was detected in 108 occasions in WB only (with a median of 560 Geq/mL), in 127 in serum only (with a median of 340 Geq/mL) and in 472 in both materials (fig. 11). In the latter 472 samples we found a good correlation between values in serum and whole blood for CMV with $R^2 = 0,74$ with WB levels 0.18 log higher than serum levels. This is in concordance with observations by other groups (42). Kalpoe et al. also found a good correlation and that WB levels were 0.15 log higher than plasma values (R^2=0.962) (68). Von Müller et al. observed that 43% of plasma samples from adult SCT patients were CMV DNA positive, and reported that CMV DNA appeared slightly earlier in blood cells than in plasma (74).

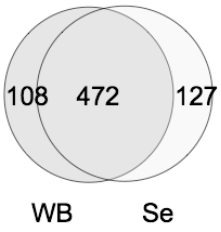


Fig. 11. CMV DNAaemia in 707 paired WB and serum samples: 472 samples were positive in both materials, 108 in WB only and 127 in serum only. †

Immunosuppression and conditioning

In the study of the liver transplanted children (paper I) the material was too small to assess the impact of immunosuppression on the risk for CMV infection. In study IV, however, we included ATG, TBI and presence of GvHD together with age, gender, transplant type and serostatus in a multivariate analysis of factors that might influence CMV DNAaemia. We found significantly higher CMV DNA levels in patients that had received ATG (given to 23/25 of the patients that received MUD grafts and in total to 26 patients) or TBI conditioning, as compared to when ATG or TBI was not used.

Thus, ATG (p=0.017) – or HLA mismatch – and TBI (p=0.021) acted as independent, additive risk factors for higher CMV DNA level (fig. 12). Acute or chronic GvHD, age or gender showed no association to CMV DNA levels.
In a special report for the European Conference on infections in Leukaemia, Ljungman et al. reviewed 76 studies for CMV and concluded that seropositivity, CMV disease prior to transplantation, and presence of acute or chronic GvHD were associated with higher risk for CMV disease (69). Gor et al. found that recipient seropositivity, presence of aGvHD and elevated CMV DNA levels were independent risk factors for CMV disease in multivariate analysis, and that viral load was the factor most strongly associated with CMV disease (43). HLA-mismatch and T cell depletion has also been described as risk factors for CMV complications, (38, 39, 51), but to our knowledge there is no report that has taken into account all the above mentioned factors in relation to CMV DNA levels like in study IV. We could show that the risk for higher CMV DNA levels increased additively if either recipient or donor were CMV seropositive and if ATG and/or TBI were used in the conditioning regimen. Conversely, if neither recipient nor donor were CMV seropositive the risk was very small regardless of conditioning procedure. Therefore preventive efforts by monitoring of viral levels and preemptive therapy or antiviral prophylaxis could be targeted to the patients with the highest risk for CMV complications.

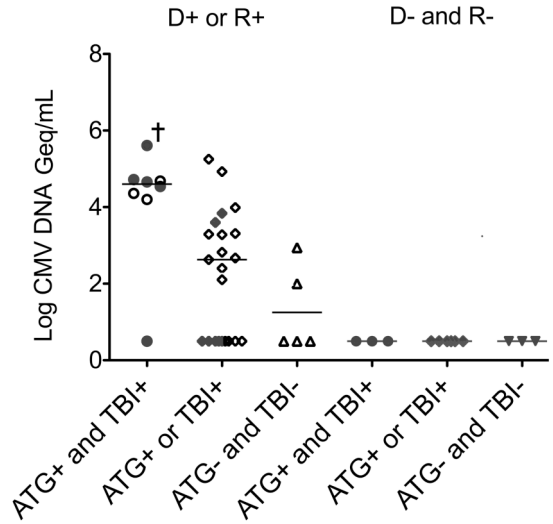


Fig 12. Maximum DNA levels of CMV in relation to risk factors ATG and TBI. Filled grey dots represent cases with seronegative donors. †=fatal case.

7.2 EBV infections

EBV DNAaemia

De novo infections and reactivations of EBV are common events both after SOT and SCT in children. We found that 70% of liver transplanted and 45% of stem cell transplanted children were EBV DNA positive in serum on at least one occasion during the first 6-12 months after transplantation. In healthy subjects, EBV DNA is almost never detected in serum (148), but low levels may be found in whole blood: we observed that 25% of blood donors had detectable EBV DNA in WB, all with levels below 1,000 Geq/mL (paper III).

EBV serology

De novo or primary EBV infections are more common in liver transplanted than among other transplanted children, as a consequence mainly of the low mean age in this patient group. The median age in the stem cell transplanted children in paper IV, was 8.6 years (range 0.9-18), which is higher than in the liver transplanted children in paper II: 1.7 years (0.25-15.8). Of the liver transplanted children, as many as 50% were EBV seronegative, while only 28% of the stem cell recipients were EBV seronegative at transplantation. After SOT, primary EBV infections are more often complicated than reactivations, and a mismatch setting with an EBV seropositive donor and an EBV seronegative recipient is a high risk situation. It is therefore essential that EBV serostatus of recipient and donor is kept in mind. Infants should be considered as probably seronegative even if they are EBV IgG positive, because most babies have maternally transferred antibodies. In 11 of the 17 children in paper II (with a median age of 1.0 years), the EBV infection was classified as primary infection, presenting at a mean time of 4.8 months after transplantation. Six other patients had a reactivated EBV infection a mean of 4.0 months after transplantation. Reactivated infection presented with significantly lower EBV DNA levels, as compared to primary infections (median 800 vs. 5,900 Geq /mL), probably because of a recalled immune response in reactivated infections. In the multivariate analysis of paper IV, significant association between EBV DNA levels and EBV IgG in recipient and donor could not be seen, but this could be due to the low number of patients. Sundin et al. have reported mismatch in EBV serology as a risk factor for PTLD post SCT (124).

Symptoms and EBV DNAaemia levels

In liver transplanted children with elevated liver function tests or unspecific but worrisome symptoms, interpretation of positive EBV DNAaemia by qualitative tests used to constitute a clinical problem. We set out to see if quantitative PCR of serum could help us to estimate the clinical significance of EBV infection.

Five patients in study II had symptoms judged to be caused by EBV, including 4 of the 11 patients with primary, and 1 of the 6 patients with reactivated EBV infection, (i.e. 21% of the whole group). Two patients had EBVD hepatitis and 3 had PTLD. See fig.7, page 42. Although they all received GCV as prophylaxis for, or as treatment of, CMV, the EBV DNA levels in serum reached high levels, in particular in the patients with primary symptomatic EBV infection, who showed significantly higher levels (median 50,550 Geq/mL, range 14,200-194,300) than observed in the patients with primary asymptomatic infection (median 2,900 Geq/mL, range 100-9,780), p= 0.011. However, the 3 patients with PTLD could not be separated by EBV DNA levels from the patients with primary EBV infection and hepatitis. Since the EBV DNA levels were retrospectively analysed in the available samples, it is possible that the absolute maximum could have been missed, but some other studies have also reported EBV DNA levels to be equally high in EBVD as in PTLD (135, 150, 213).

The symptoms of EBVD, prePTLD and PTLD are overlapping and these conditions should be considered to represent a continuum of lymphoproliferative disorders. We described three liver transplanted children, who all presented with gastrointestinal symptoms, such as melaena, diarrhoea and hypoalbuminaemia. They also displayed more unspecific symptoms such as intermittent fever, nausea and feeding problems. Easily, such symptoms can become misinterpreted and treated with antibiotics on suspicion of bacterial infections or lead to investigations that can delay the proper actions. As has been highlighted earlier, histological changes are often unspecific, even in patients with pronounced symptoms and high EBV DNA levels. In the described PTLD cases, repeated endoscopy with multiple biopsies for histological examination had to be performed before histological support for the suspicion of EBVD-PTLD could be obtained. When IS was stopped, the EBV DNA levels decreased and the condition was reversed in all cases. This illustrates that suspicious symptoms or high EBV DNA levels should be indication to initiate reduction of IS in order to curtail progression of disease.

The total mortality among the patients in study II was 4/24 (17%). All the deceased patients belonged to the primary EBV infection category, but in no case was the cause of death directly attributable to EBV

complications. The incidence of PTLD was 12%, which is comparable to the incidence in most transplantation centres during this time period.
The immunosuppressive regimens of the liver transplantation program have undergone changes during the study period and thereafter. During the first years of study I, cyclosporine A was the most common basal IS, with addition of ATG and azathioprine in many of the patients. After tacrolimus had been introduced and taken the place of cyclosporine A, the incidence of PTLD was reported to increase markedly (109, 214). During the last years, however (after study II), the aimed trough levels for tacrolimus have been reduced and the immunosuppressive protocol in most cases become steroid-free. At the same time, more patients have received induction therapy by use of ATG, which is a known risk factor for viral complications (101, 102). All these changes make comparisons of risk factors and between centres difficult, but in general the lately modified immunosuppressive protocols have again reduced the risk for PTLD. Still, the value of EBV DNA monitoring remains, in particular for high risk patients.

EBV DNA was detected at least once during the first 6 months, in 21/47 (45%) of the stem cell transplanted patients (paper IV), but the EBV DNA levels were above 10^4 Geq/mL serum only in 3 patients (6.4%). The relatively low levels could partly be explained by the nature of the transplantation itself: the recipient of an EBV (or CMV) positive stem cell graft also get a certain amount of transferred immunity as opposed to SOT recipients (68). Since the donors more often are EBV seropositive than CMV seropositive (83% vs. 49% in our series), the putative protective effect should be more notable for EBV than for CMV.
The incidence of EBV induced PTLD is lower after SCT than after SOT (1-3%), but the onset is often more rapid and (possibly) less responsive to reduction of immunosuppressive drugs (117). This is illustrated by case 2 in paper IV, who died in EBV associated PTLD 10 weeks after SCT. In this patient, retrospectively analysed serum samples showed rapidly rising levels of EBV DNA that did not decrease even though IS was discontinued and treatment with GCV and rituximab was given (fig. 13).

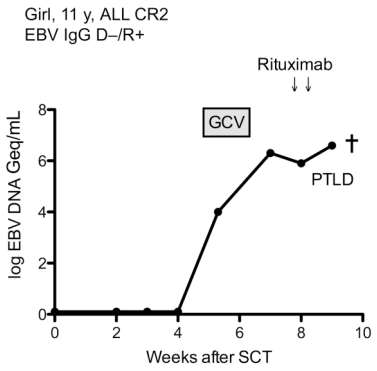


Fig. 13. EBV DNA levels in a patient developing PTLD shortly after SCT.

Sample material

In our studies, we had used serum as sample material. Many centres, however, prefer peripheral blood cells or whole blood, at least for EBV, but there is no consensus as to which is the best. In paper III, we recorded the EBV DNA levels in 2855 clinical samples that had been analysed during the period from January 2003 to March 2006. Fifty-five percent of the WB samples, as compared to 24% of serum samples, were EBV DNA positive, and among the samples that were positive in serum or WB, the levels were significantly higher in WB than in serum (median 16,000 vs. 250 Geq/mL). In a control group of healthy blood donors, EBV DNA was detected in 25% of WB samples, but then at low levels (range 100-700 Geq/mL).
There were 664 paired WB and serum samples, taken on the same day from 222 SOT or SCT patients, both adults and children (median age 14.5 years) (Fig. 14). As compared to CMV, EBV DNA was detected much more frequently, but the correlation between EBV levels in serum and WB was weaker (R^2 = 0.31). The weaker correlation between the two materials as regards EBV DNA suggests that the distribution of EBV DNA between serum and WB can shift, possibly reflecting variation of replication,

variable residual number of leucocytes in serum or variable degree of lysis of EBV infected cells. Of the 664 paired samples, 160 (24%) were positive in both materials.

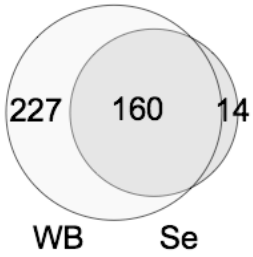


Fig. 14. EBV DNAaemia in 401 positive paired WB and serum samples: 160 samples were positive in both materials, 227 only in WB and 14 only in serum.

For the samples. that were positive in both materials, the EBV DNA levels in WB were at a mean 1.5 log higher than in serum, suggesting that more than 97% of the EBV particles in the blood reside within lymphocytes.

The higher EBV DNA levels in transplanted patients as compared to controls (blood donors) could be an effect of an enlarged pool of EBV infected cells, or of a higher number of EBV genomes in each infected cell. An interesting question, which is not yet clear, is to what extent EBV DNA in serum originates from episomal DNA in infected cells or from active replication (146).

When longitudinal comparisons of serum and whole blood levels in applicable cases were done, the 1.5 log higher level in whole blood was evident also in individual patients. In general, the EBV DNA levels increased somewhat earlier in whole blood, but the curves were to great extent parallel (fig. 15).

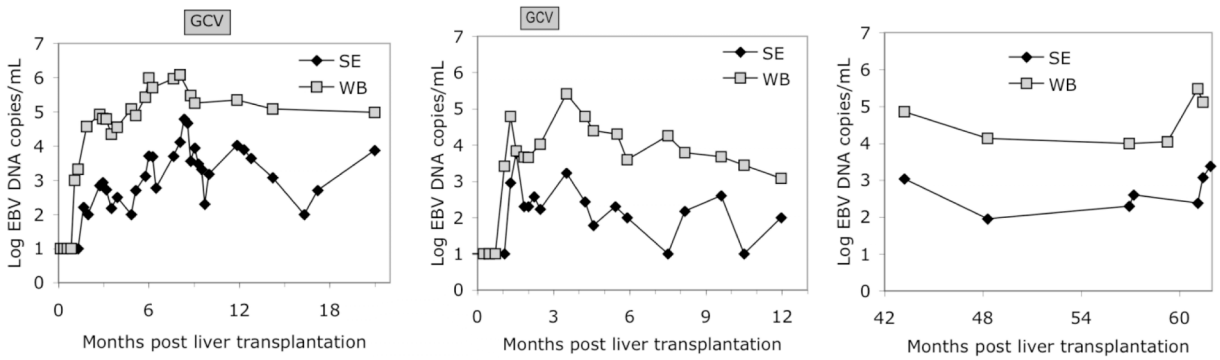


Fig. 15. EBV DNA monitored in both serum and WB in 3 patients after transplantation (paper III).

'Chronic high load carriers'

The so called 'chronic high load carriers', constitute a special problem. These may have long-standing high EBV DNA levels in whole blood without symptoms as has been reported by Green et al. (151). By fluorescence in situ hybridization (FISH) analysis, a distinct population of CD19+ memory B cells with an increased number of viral genomes (20-30/cell), has been described in these patients (215). However, among the 5 liver transplanted patients with sustained high EBV DNA levels in paper II, none was completely well but instead, they all demonstrated various symptoms, which faded in parallel with tapered IS and decreasing EBV DNA levels. The presence of symptoms in all these patients may be related to the fact that their EBV DNA levels were clearly higher than the definition of 'chronic high load carriers' proposed by the Pittsburgh group, which is >200 viral copies/10^5 leucocytes (corresponding to around

4,000-16, 000 Geq/mL in WB). The difficulty to evaluate this kind of patients was illustrated by a heart transplanted child (patient J in paper III, fig 16), who had high EBV DNA levels in whole blood (and serum) for more than two years without clear symptoms. The serum levels then increased with 1.5 log further one month before diagnosis of PTLD, while whole blood levels were unchanged. The rising serum levels in serum could reflect a lytic infection, which has been proposed by others (146, 147), or perhaps in this case, viral DNA being released from tumour tissue. The transient peaks of high serum EBV DNA levels observed after rituximab therapy support the existence of such a phenomenon.

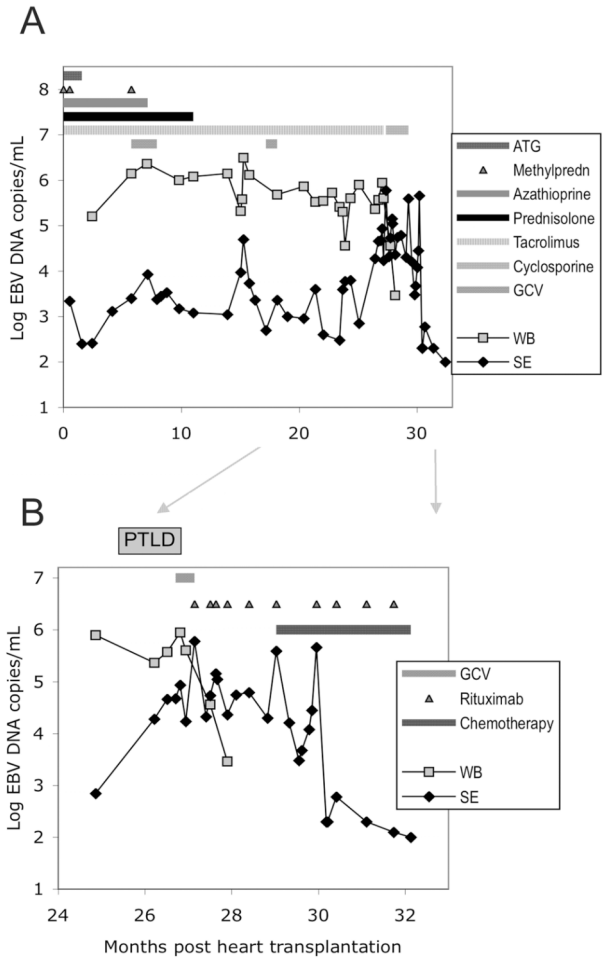


Fig. 16. EBV DNA levels in serum and WB in a child (J), who developed PTLD (paper III).

Despite the difficulty to interpret the results in some cases, monitoring of EBV DNA levels in WB and serum in solid organ or stem cell transplanted children has become an established tool to discern the patients at risk for EBV complications. However, from previous studies and the data presented above, one has to conclude that neither material is optimal or sufficient in itself. WB is often preferred because the sensitivity is higher, and therefore the negative predictive value (regarding PTLD) of undetectable EBV DNA is higher for WB than for serum. On the other hand, the levels in WB may be influenced by white blood cell count, and the interpretation of EBV DNA in WB can be difficult because thresholds have not been established. We used only serum in the studies in paper II and IV, and found that all cases of EBVD or PTLD developed relatively high EBV DNA levels. This observation, and reports by others (71, 72, 118, 146, 148, 216), support that serum measurements may be sufficient for identifying PTLD. Serum has the advantage of being more specific, as EBV DNA in the normal case should be negative in serum, thereby making it easier to react even to low levels.

Many groups have tried to define threshold levels for EBV DNAaemia in serum or WB. Campe et al. used 120,000 Geq/mL in WB, or serum positivity at any level, to differentiate between symptomatic and asymptomatic patients (135). With these limits they found a good (80%) agreement between serum and WB as regards identification of symptomatic infection. However, they concluded that continuous surveillance of EBV DNA levels, i.e. kinetics, may be the only way to distinguish pathological EBV levels (135). Even though kinetics, rather than single values, is a preferred way to use viral DNA measurements, it would be of great value to define a critical level, at which one has to decide to take action. However, setting such a threshold is difficult because is may differ between transplantation categories, depending on for example the degree of IS or whether it is a primary or a reactivated infection.

In our setting in Göteborg, in liver transplanted children with normal WBC count, EBV DNA levels below 4,000 Geq/mL in WB are considered as low. Higher levels in an asymptomatic patient, without concomitant EBV DNA detection in serum, only motivate continued monitoring. However, if the levels in WB are increasing or if EBV DNA in serum is >1,000 Geq/mL in repeated samples, we interpret this as a signal to reduce IS also in an asymptomatic patient. In fig.17, a suggestion for surveillance of EBV DNA in liver transplanted children is shown.

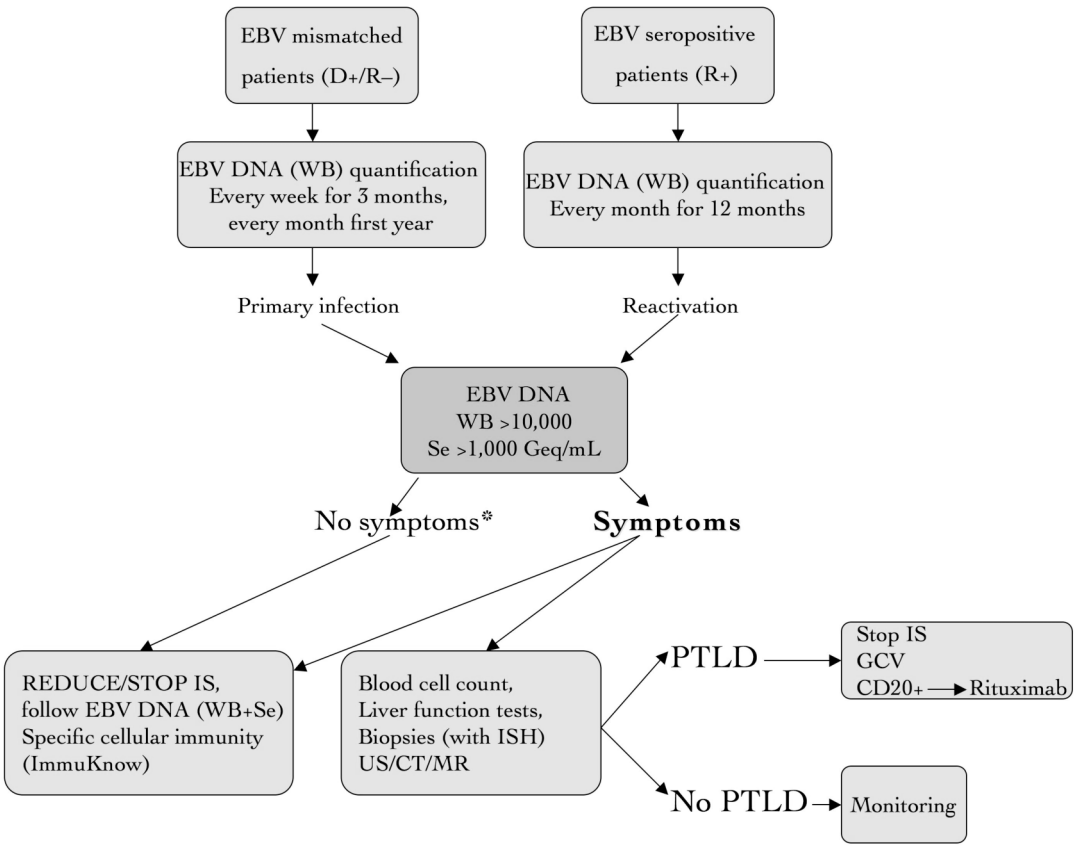


Fig. 17. Flow-chart for surveillance of liver transplanted children as regards to EBV DNA monitoring. *If no suspicious symptoms are present, but EBV DNA in serum >1,000 Geq/mL in repeated samples – proceed to: reduce IS.

Immunosuppression and conditioning

As for CMV, we found higher EBV DNA levels in SCT patients that had received ATG or TBI as compared to conditioning without ATG or TBI. In multivariate analysis, ATG (p=0.004) – or HLA mismatch – and TBI (p=0.001), were independently associated with higher EBV DNAaemia levels, and the highest levels were seen if both ATG and TBI were given (fig. 18). To our knowledge, risk factors have not been investigated in relation to EBV DNA levels in this way before, and the risk associated with TBI has not been much discussed. Other reports have however identified use of graft from unrelated donor, use of ATG for prophylaxis or treatment of GvHD, T-cell depletion or treatment with OKT3 for aGvHD as the most important risk factors for development of PTLD after SCT (108, 124, 217-219). Curtis et al. found the risk for PTLD after SCT to be as high as 22 % if ≥ 3 risk factors were present (MUD/MMRD, TCD, ATG/OKT3 and aGvHD) (108). Monitoring EBV DNA in SCT patients with several risk factors has also been suggested by others as a means of initiating preventive measures (217). Cesaro et al. reported that the preemptive strategy comprising reduced immunosuppressive therapy was effective and feasible in paediatric SCT recipients, and did not increase the risk for GvHD or transplant related mortality (220). Meijer et al. are also in favour of a preemptive strategy with initial reduction of IS, followed by rituximab if necessary (217), while Wagner et al. have proposed a 'prompt' strategy for high-risk SCT recipients (221).

We conclude that it is reasonable to focus the EBV monitoring after SCT on patients with multiple risk factors in order to allow preemptive modulation of IS if EBV DNA levels increase. Our findings clearly indicate that particularly patients given both ATG and TBI should be monitored more closely in order to prevent emergence of PTLD.

In addition to ATG and TBI, we found that also absence of cGvHD was independently associated with higher EBV DNA levels (p=0.023). We believe that the explanation is that patients without cGvHD may have received stronger immunosuppressive treatment, which might have resulted in higher EBV DNAaemia levels. The association may have relevance for the discussion that EBV DNA levels (in WB) might be useful as a means to optimise the dosing of immunosupppression.

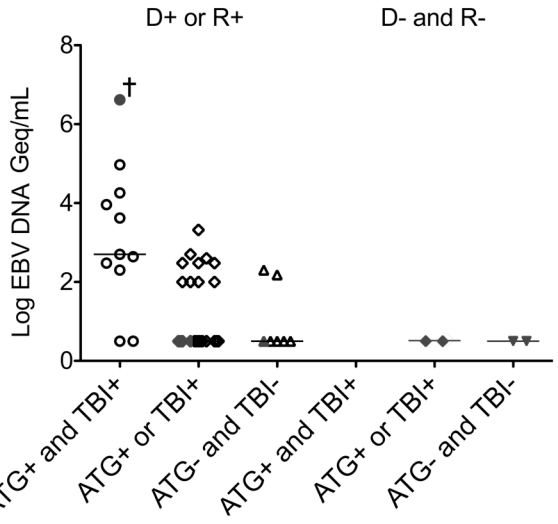


Fig 18. Maximum DNA levels of EBV in relation to risk factors ATG and TBI. Filled grey dots represent cases with seronegative donors. †=fatal case.

Quantification of EBV is valuable for identifying patients at risk of developing PTLD, especially during the first 6 months after transplantation, and in particular after SOT with EBV mismatched (D+/R-) setting and after SCT in patients with multiple risk factors. Serum and WB may both be used, but need to be interpreted differently. One might use WB as initial material and add serum if EBV DNA in WB increases above 10,000 Geq/mL. The initial action to be taken when high or increasing EBV DNA levels are detected after SOT or SCT, is to reduce or withdraw the IS and continue to follow viral levels. However, tapering of IS may be hazardous, in particular in heart transplanted patients where rejection may be fatal. A critical issue, especially among SOT patients with prolonged elevated EBV DNAaemia, is therefore how to further recognise patients at risk, and to find a better marker of an imminent threat of PTLD. EBV gene expression patterns have been suggested to be more specific of PTLD, but this is not well established as yet and there has been no generally accepted such marker.

7.3 Adenovirus and HHV-6 infections

The presence of AdV and HHV-6 infection was only investigated in the stem cell transplanted children of paper IV. AdV DNA was detected in serum in 28% (13/47) of the SCT patients during the first 6 months after transplantation, but in all except one case, the levels were low (<10^3 Geq/mL) and transient. One patient who had received conditioning with ATG and TBI, developed a fatal adenoviral pneumonitis and demonstrated increasing AdV levels during 5 months time, reaching a maximum level of 14 million (log 7.15) Geq/mL serum, shortly before death 6 months after transplantation (fig. 19). Multivariate analysis showed that aGvHD (p=0.037) and female gender (p=0.022) were associated with AdV levels, but the validity of these associations is uncertain (fig. 20).
The finding that the patient with fatal AdV infection (but no others) had high and rising AdV DNA levels during 4 months before death, supports the earlier claims that quantitative real-time PCR of plasma is a highly specific and sensitive marker for adenovirus disease (187, 189, 200, 222). Kalpoe et al. advocate surveillance by AdV DNA measurement in plasma in SCT children (190) and Erard et al. suggest a cut-off for initiation of cidefovir treatment of 1000 Geq/mL plasma and AdV DNA analysis in all patients with symptoms compatible with AdV infection to prevent disseminated disease (189) .

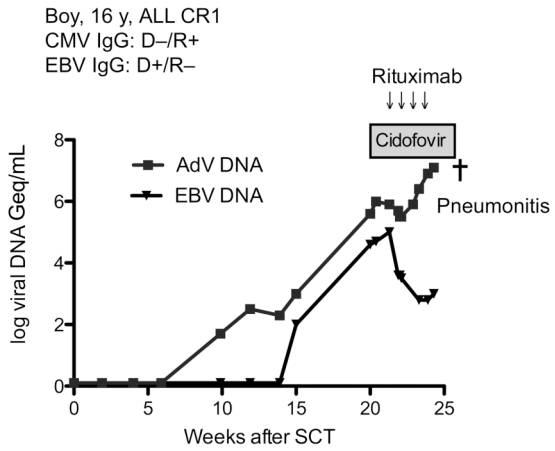


Fig.19. Viral DNA levels in serum in a patient with AdV (and EBV) infection after SCT (paper IV).

HHV-6 was also detected in 13/47 patients (28%), including 10 cases in levels below 10^3 Geq/mL serum and in 3 cases in levels between10^4 and 10^5 Geq/mL. None of the patients had symptoms suspicious of HHV-6 infection and HHV-6 DNAaemia was less common and the levels are lower than earlier described (181, 223).

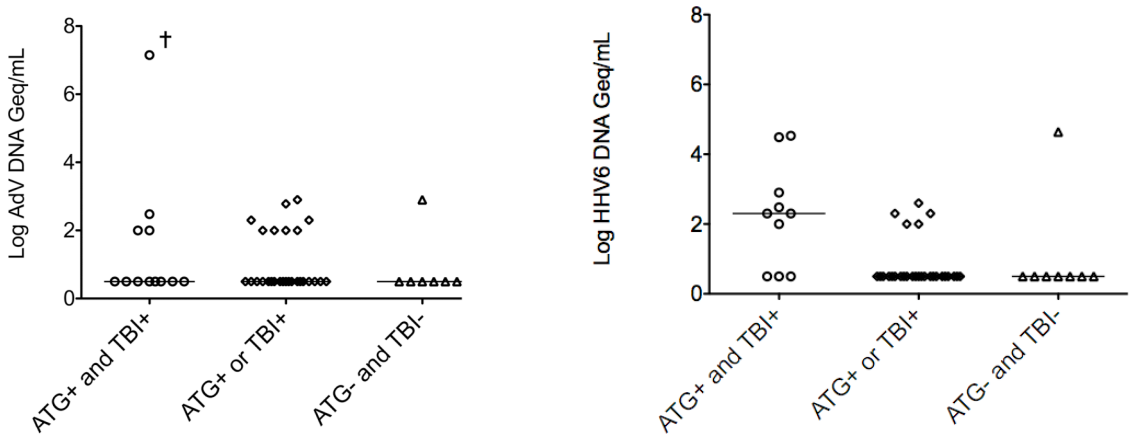


Fig 20. Maximum DNA levels of AdV and HHV-6 in relation to risk factors ATG and TBI.
†= fatal case.

8. CONCLUDING REMARKS

Viral complications cause significant problems after solid organ and stem cell transplantation. Out of the liver transplanted patients (Paper I and II), 22% developed symptomatic CMV infection and 21 % symptomatic EBV infection. Thus, CMV and EBV significantly contributed to post-transplant morbidity, but in no case clearly to the overall mortality of 17%.
CMV infections were observed in nearly 50% of the children after SCT (Paper IV). CMV DNA levels above 1,000 Geq/mL were frequent (34%), but not clearly associated with symptoms. Levels above 10^4 Geq/mL were seen in 19%, but only half of these patients presented probable CMV symptoms, including one fatal CMV pneumonitis. We found that the risk for CMV infections is low if neither donor or recipient is CMV seropositive (D–/R–), but significant for SCT patients who receive ATG or TBI. Our results support that CMV DNA quantifications are valuable for monitoring children after SOT or SCT. CMV DNA levels help to identify infections that may become symptomatic and should be preemptively treated, and to confirm (or reject) presumed symptomatic infections that require prompt antiviral treatment. Moreover, CMV DNA levels are essential for assessing the effect of treatment. Our results in Paper III suggest that either serum or whole blood may be used for monitoring CMV DNA, because the levels were similar in paired samples (only 0.18 log higher in WB).

EBV DNAaemia in serum was observed in 71% of the children after liver transplantation (Paper II) and in 49% after SCT. In the liver transplanted children, EBV DNA levels were higher in primary infections and if EBV symptoms were present. In Paper IV we observed higher EBV DNA levels if ATG (or HLA mis-match) or TBI was given. Our results support that quantification of EBV DNA is valuable for identifying patients at risk of developing PTLD, especially after SOT with EBV mismatch (D+/R–) and after SCT in patients with multiple risk factors, in particular if both ATG and TBI is given. Serum and WB may both be used, but need to be interpreted differently, because WB levels are on average 1.5 log higher than serum levels, reflecting that EBV infects a significant number of B cells in peripheral blood, as shown in Paper III. We conclude that it is rational to use WB as initial material and add serum if EBV DNA in WB increases above 10,000 Geq/mL. The initial action to be taken when high or increasing EBV DNA levels are detected after SOT or SCT, is to reduce or withdraw the IS and continue to follow viral levels. However, tapering of IS may be hazardous, in particular in heart transplanted patients where rejection may be fatal. A critical issue, especially among SOT patients with prolonged elevated EBV DNAaemia, is therefore how to further recognise patients at risk, and to find a better marker for an imminent threat of PTLD. EBV gene expression patterns have been suggested to be more specific of PTLD, but this is not well established as yet and there has been no generally accepted such marker.
Monitoring of EBV-DNA levels in serum and whole blood should be valuable to predict and avoid serious EBV-associated complications and to follow the effect of preemptive modulation of IS and other treatment of PTLD both in solid organ and stem cell transplanted patients at risk. Serum and whole blood are equally useful materials for surveillance of CMV-DNA levels. For analysis of EBV-DNA levels, whole blood is safer for ruling out EBV-associated PTLD, but the specificity is restricted, and for the so called "chronic high load carriers", serum might be more informative in identification of transition to PTLD.

Complications caused by adenovirus or HHV-6 were only studied in paper IV and were less common as assessed by AdV DNA and HHV-6 DNA real-time PCR. However, one of the three patients with fatal viral complication after SCT died from AdV pneumonitis, and developed very high AdV DNA levels, indicating that quantification of AdV DNA is valuable for identifying significant AdV infections. Analysis of AdV and HHV-6 DNA in patients with suspicious symptoms can enable timely and accurate diagnosis of these complications.

In conclusion, molecular diagnostic methods enable fast and correct quantification of viral DNA that can aid clinicians to the right decisions concerning the balance of immunosuppression and antiviral therapy.

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11. SVENSK SAMMANFATTNING

Virusinfektioner som hos för övrigt friska individer i regel inte orsakar allvarlig sjukdom, men som hos patienter som behandlas med immundämpande medel kan orsaka svåra och i bland livshotande infektioner, brukar kallas för opportunistiska. Efter transplantationer är de normala försvarsmekanismerna försvagade av läkemedel som behövs för att undvika avstötning av det transplanterade organet eller efter benmärgstransplantation för att undvika s.k. graft-mot-värd-reaktioner (GvHD).
För de flesta organtransplanterade patienter är medicineringen livslång, medan för benmärgs-transplanterade patienter störst risk för infektioner föreligger de första 3-6 månaderna efter transplantationen eftersom den immundämpande medicineringen i de flesta fall därefter kan sänkas och så småningom avslutas.

Under de senaste decennierna har resultaten efter organ- och benmärgstransplantation förbättrats på ett fantastiskt sätt, till stor del på grund av mer effektiva immundämpande läkemedel. Samtidigt har dock problemen med opportunistiska virusinfektioner ökat. Denna avhandling fokuserar på opportunistiska virusinfektioner med cytomegalovirus (CMV), Epstein-Barr-virus (EBV), herpes virus typ 6 (HHV-6) och adenovirus (AdV) hos lever- och benmärgstransplanterade barn och på användandet av nya tekniker för att diagnosticera dessa infektioner. Gemensamt för dessa virus är att de är mycket vanliga och att de efter genomgången infektion finns kvar i kroppen i inaktiv (latent) form, som kan reaktiveras om värdens immunsvar försvagas.

CMV orsakade under organ- och benmärgstransplantationernas inledande skede stor sjuklighet och dödlighet upp mot 15-20%. Efterhand utvecklades dock strategier och läkemedel som kraftigt förbättrade resultaten, men fortfarande är CMV ett allvarligt hot mot transplanterade patienter, både mer direkt genom att kunna orsaka sjukdomssymtom, ofta ifrån det transplanterade organet, och genom förmodade indirekta effekter, såsom ökad infektionskänslighet och ökad risk för avstötning. EBV, som hos för övrigt friska kan orsaka körtelfeber, kan hos transplanterade orsaka feber, leverpåverkan men också förstadier till eller fullt utvecklade lymfom med hög dödlighet (‘post transplantation lymphoproliferative disease’-PTLD). Adenovirus och HHV-6 kan, särskilt hos benmärgstransplanterade, ge svåra, för adenovirus ofta dödliga, infektioner. Risken för virusorsakade komplikationer avgörs bl a av tidigare immunitet mot virus (serostatus), grad av immundämpande medicinering och typ av transplantation och i allmänhet löper barn större risk än vuxna.

Opportunistiska virus kan diagnosticeras med molekylära metoder, främst s.k. PCR-teknik som påvisar virusarvsmassa (DNA eller RNA), och numera kan också mängden virus noggrant bestämmas, vilket har stor betydelse för att kunna avgöra om åtgärder för att bromsa virusförökningen behöver vidtagas. När det gäller vilket provmaterial som är mest lämpat att påvisa olika virus och vilka gränsvärden för behandling man bör sätta (cut-off) saknas klara riktllinjer.

Mot CMV finns effektiva antivirala läkemedel, som kan användas både i förebyggande syfte (profylax) och som behandling. Dessa läkemedel kan dock, särskilt hos benmärgstransplanterade, ha bieffekter som gör att man inte vill använda dem förebyggande utan hellre sätta in behandling när det behövs, t ex när man konstaterar förekomst av CMV i viss mängd (preemptiv=blockerande behandling) eller tidigt vid symtom. Organtransplanterade patienter med hög risk, särskilt de som saknar immunitet för CMV och mottagit ett organ från en donator som har genomgått infektionen, får i regel profylaktisk behandling under 3-6 månader. När denna behandling avslutas finns dock en risk att patienten får en förstagångs-(primär) CMV-infektion och det är då extra viktigt att övervaka virusnivåer. Också för EBV är risken störst (hos organtransplanterade) vid primärinfektion, men mot EBV saknas ännu specifik behandling. I stället kan man om stigande mängder av EBV konstateras, sänka den immundämpande behandlingen för

att ge immunförsvaret möjlighet att bekämpa virus. För adenovirus och HHV-6 finns antivirala läkemedel, men behandling måste startas i tid för att ha effekt.

Syftet med avhandlingsprojektet var:

- att undersöka hur vanlig den är och vilka symtom CMV- och EBV-infektion ger hos barn första året efter levertransplantation
- att utvärdera nyttan av koncentrationsbestämning (kvantifiering) av CMV- och EBV-DNA i serum för att kunna identifiera symtomgivande infektion
- att jämföra serum och helblod som material för dessa analyser
- att beskriva förekomst och konsekvenser av virusinfektioner under det första året efter benmärgstransplantation hos barn, med hjälp av kvantifiering av virus i sparade serumprover

I de första två delarbetena undersöktes provmaterial från 18 respektive 24 levertransplanterade barn med avseende på CMV- respektive EBV-infektioner.

Studie I: CMV-infektion är vanlig – 83% hade tecken på genomgången eller aktuell infektion – och trots användande av antivirala läkemedel i hög utsträckning utvecklade 22 % av patienterna symtomgivande infektion. Risken var särskilt stor för små barn, som utsattes för CMV-smitta för första gången (primär CMV-infektion). Kvantifiering av CMV-DNA är värdefullt för att kunna identifiera patienter med symtomgivande CMV-infektion, men också för att kunna undvika onödig behandling.

I studie II, fann vi att hälften av patienterna inte var immuna mot EBV vid transplantationen, men att 92 % visade tecken till genomgången eller aktuell EBV-infektion under första året efter transplantationen, och att 21 % utvecklade symtomgivande infektion: 3 patienter med PTLD och 2 patienter med leverinflammation (hepatit). Nivåerna av EBV-DNA var högre hos de 5 patienter som hade symtomgivande infektion än hos dem utan symtom, vilket ger stöd för att kvantifiering av EBV är användbart för att övervaka patienter med risk att utveckla PTLD. Samtliga patienter med PTLD blev friska då den immundämpande behandlingen sänktes eller sattes ut.

I studie III, jämfördes resultaten från tidigare analys av ett stort antal prover som kvantifierats avseende CMV- och EBV-DNA i helblod och serum. För CMV var serum och helblod i stort likvärdiga som provmaterial. För EBV låg nivåerna i genomsnitt 35 gånger (1,5 log) högre i helblod än i serum och ökningar uppträdde tidigare i helblod än i serum. För att utesluta EBV-orsakad PTLD förefaller helblod säkrare, men för en grupp av patienter som uppvisar förhöjda nivåer av EBV-DNA i helblod under en längre tid (‘high load carriers’) kan det vara svårt att veta när man ska reagera. I dessa fall kan mätningar av virusmängd i serum möjligen förbättra identifiering av övergång till PTLD.

I **studie IV** undersöktes virus-DNA-nivåer i serum avseende CMV, EBV, AdV och HHV-6 hos 47 benmärgs-transplanterade barn. Tjugotre (49%) hade förekomst av CMV-DNA i serum någon gång under de första 8 veckorna efter transplantationen. Av dem hade 9 patienter höga nivåer (>10,000 viruskopior/mL): samtliga behandlades med antivirala medel, men en patient avled trots detta i CMV-orsakad lunginfektion. Antikroppsförekomst tydande på tidigare CMV-infektion hos patient eller donator i kombination med användande av anti-T-cellsantikroppar (ATG) eller strålning som förbehandling inför transplantationen var förknippat med ökad risk för höga CMV-DNA-nivåer.

EBV-DNA påvisades i serum hos 24 (49%) av patienterna under de första 6 månaderna efter transplantationen. Höga nivåer (>10,000 viruskopior/mL) påvisades hos 3 av dem, varav en avled i PTLD. Också för EBV utgjorde ATG och strålning oberoende riskfaktorer för höga EBV-DNA-nivåer. HHV-6-DNA och AdV-DNA förekom hos 28 % av de benmärgstransplanterade patienterna, i majoriteten i måttliga nivåer, men i ett fall av AdV i mycket höga (14 millioner viruskopior/mL) nivåer. Denna patient dog i AdV-orsakad lunginfektion. Totalt orsakade virusinfektioner 21% av dödsfallen hos de 47 patienterna i studie IV.

Sammanfattningsvis orsakar virus betydande komplikationer efter organ- och benmärgstransplantation hos barn. Molekylär diagnostik möjliggör snabb och korrekt kvantifiering av virus-DNA, vilket kan förbättra underlaget för att fatta rätt beslut som rör balansen mellan behovet av immundämpande och antivirala läkemedel.

12. APPENDIX PAPERS I-IV