Herpesvirus infections in transplant recipients

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Cover illustration: Electron microscopic image of herpesvirus by Lennart Nilsson/TT, Scale by Mikael Lindahl and T-lymphocytes imaged via electron microscope by Lennart Nilsson/TT. Used with permission.

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To my Family

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ABSTRACT

Herpesvirus infections are common and can cause serious and life-threatening conditions in transplanted individuals. In this thesis, consisting of 4 papers (**I-IV**), we investigated primary infection and reactivation of Cytomegalovirus (CMV), Human Herpesvirus type 6 (HHV-6), Varicella Zoster Virus (VZV) and Epstein-Barr Virus (EBV) in transplant patients. The overall aim was to expand our knowledge on the incidence, prophylaxis, management and long-term effects of herpesvirus infections after transplantation. The studies were all retrospective. Results from serum and whole blood analyses by quantitative polymerase chain reaction (PCR) for CMV and HHV-6 in a cohort of 97 adult allo-SCT patients (papers **I** and **II**) and CMV and EBV in 58 renal transplanted children (paper **IV**) were compiled. VZV antibodies were analyzed using ELISA assays and immunofluorescence from blood samples of 85 renal transplanted children (paper **III**).

In paper **I**, patients with CMV DNAemia had improved survival compared to CMV negative patients. There was an increased risk of CMV DNAemia with a seronegative donor to a seropositive recipient. CMV disease with debut more than 110 days after transplantation was related to steroid treatment for Graft versus Host Disease (GVHD).The morbidity associated with HHV-6 DNAemia following allo-SCT was in most cases mild. The overall one-year survival among the patients with HHV-6 DNAemia was not significantly different from the HHV-6 negative patients (paper **II**). At renal transplantation, protective VZV antibody-levels were less frequent and of lower magnitude in varicella-vaccinated children than in those with previous varicella. Vaccinated patients then lost their seropositivity to a greater extent than previously infected individuals. Herpes zoster was only seen in previously infected children (paper **III**). Long-lasting chronic high EBV load carriage (CHL) was seen in 24% of the renal transplant patients despite reduced immunosuppression. CHL carriage mainly developed in younger children. None developed post-transplant lymphoproliferative disorder (PTLD) during the median follow-up of almost 8 years (paper **IV**). To conclude, the incidence of herpesvirus DNAemia is high after transplantation. VZV-vaccination and antiviral prophylaxis against CMV and VZV as well as pre-emptive CMV treatment and surveillance of EBV DNA are life-saving and reduces the long-term effects of herpesvirus infections.

Keywords: Allogeneic stem cell transplantation, Cytomegalovirus, Epstein-Barr virus, Human Herpesvirus type 6, Renal transplantation, Varicella zoster virus.

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SAMMANFATTNING PÅ SVENSKA

Herpesgruppens virus kan ge livshotande infektioner hos transplanterade patienter. Efter den primära infektionen finns dessa virus kvar i en latent form i kroppen. Vi har valt att studera Cytomegalovirus (CMV), Humant herpesvirus typ 6 (HHV-6), Varicella Zoster Virus (VZV) och Epstein-Barr Virus (EBV) infektioner hos transplanterade patienter.

Delarbete I: CMV studerades retrospektivt hos 97 stamcellstransplanterade patienter. Sextio patienter fick CMV DNA påvisbart i blodet, varav 51% erhöll behandling mot CMV. Två av fyra patienter med CMVsjukdom avled. Patienterna med CMV i blodet hade bättre överlevnad än de som var CMV negativa.
Delarbete II: Förekomst av HHV-6 analyserades retrospektivt hos 54 patienter med virussymptom i samma patientkohort som delarbete I. HHV-6 DNA påvisades i blodet hos 15 patienter. Nio behandlades mot HHV-6 infektion. Ett-års överlevnaden hos dessa patienter var 73% och fem-års överlevnaden 67% vilket inte skilde sig signifikant från hela kohorten.
Delarbete III: VZV är det enda av herpesgruppens virus som smittar luftburet och som vi kan vaccinera mot. Retrospektivt studerades 85 njurtransplanterade barn som före transplantation hade haft vattkoppor eller vaccinerats mot vattkoppsvirus. VZV-antikroppstitrar analyserades före transplantation och följdes därefter i 5 år. Vid transplantation hade 74% antikroppar mot VZV, 94% av de som tidigare haft vattkoppor och 50% av de som vaccinerats mot VZV. Antikroppsnivån var signifikant lägre i den vaccinerade gruppen jämfört med gruppen som tidigare haft vattkoppor ($p=0.031$). De vaccinerade patienterna förlorade också antikroppar i större utsträckning än de som tidigare haft vattkoppor. Tio barn insjuknade i mild klinisk VZV infektion efter transplantation, 8 i vattkoppor och 2 i bältros. Våra resultat visar att vaccination skyddar sämre än genomgången infektion mot symptomatisk infektion men verkar skydda mot livshotande sjukdom även om antikroppsnivåerna är låga.
Delarbete IV: Nivåerna av EBV och CMV DNA i helblod och serum studerades retrospektivt hos 58 njurtransplanterade barn och korrelerades till kliniskt förlopp, infektionens svårighetsgrad, behandlingsstrategi samt utfall. Vid transplantation saknade 53% av barnen antikroppar mot EBV varav 81% utvecklade primär EBV infektion under studietiden och 74% av de som hade EBV antikroppar vid transplantation reaktiverade EBV. Totalt blev 24% av barnen bärare av särskilt höga EB virusnivåer under lång tid trots minskad immunsuppression och jämfört med de övriga 44 barnen var de yngre vid transplantation. Inget av barnen utvecklade det fruktade tillståndet ”post-transplant lymphoproliferative disorder, PTLD” (trots höga EBV DNA nivåer i blod) under den långa kliniska uppföljningstiden på nästan 8 år.

Målet med våra studier var att genom ökad kunskap om CMV, HHV-6, VZV och EBV hos transplanterade patienter, i framtiden kunna bidra till minskad sjuklighet och en ökad överlevnad i dessa infektioner.

LIST OF PAPERS

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

I. Lindahl J, Woxenius S, Brune M, Andersson, R. Cytomegalovirus DNAemia and treatment following allogeneic stem cell transplantation with focus on long-term outcome. *Scandinavian Journal of Infectious Diseases 2010; 42(9): 691-698.*

II. Lindahl J, Woxenius S, Brune M, Andersson R. Human herpesvirus type 6 DNAemia and infection following allogeneic stem cell transplantation with focus on long-term outcome. *Scandinavian Journal of Infectious Diseases 2013, 45(7): 557-61.*

III. Lindahl J, Friman V, Westphal Ladfors S, Hansson S, Andersson, R, Jertborn M, Woxenius S. Long-term study showed that vaccination protected paediatric renal transplant recipients from life-threatening varicella zoster virus. *Acta Paediatrica 2018, Dec;107(12):2185-2192. Doi: 10/1111/apa.14375. Epub 2018 May 25.*

IV. *Westphal Ladfors S, *Lindahl J, Hansson S, Brandström P, Andersson R, Jertborn M, Lindh M, Woxenius S, Friman V. Long lasting chronic high load carriage of Epstein-Barr virus is more common in young pediatric renal transplant recipients. Revisions submitted.

Note * contributed equally to this work

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CONTENTS

ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
Allo-SCT = allo-HSCT	Allogeneic hematopoietic stem cell transplantation
AML	Acute myeloid leukemia
ATG	Antithymocyte globulin
AZA	Azathioprine
BAL	Bronchoalveolar lavage
BAS	Basiliximab
BID	Twice daily
CAKUT	Congenital anomalies of the kidney and urinary tract
CD	Cluster of differentiation
CHL	Chronic high viral loads
CML	Chronic myeloid leukemia
CMV	Cytomegalovirus
CNI	Calcineurin inhibitors
CNS	Central nervous system
CS	Corticosteroids
CSF	Cerebrospinal fluid
CV	Coefficient of variation
CYA	Cyclosporine A

DNA	Deoxyribonucleic acid
D/R	Donor/recipient
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay for detection of antibodies
gE	Glycoprotein E (a VZV surface glycoprotein)
Geq	Genome equivalent
GI	Gastrointestinal
GMT	Geometric mean titer
GVHD	Graft-versus-host-disease
HHV-6	Human herpesvirus type 6
HLA	Human leucocyte antigen
HR	Hazard Ratio
IFL	Immunofluorescence
Ig	Immunoglobulin
IL	Interleukin
IQR	Interquartile range
IVIG	Intravenous immunoglobulin
Log	Log_{10}/ml
LVL	Low viral load
MMF	Mycophenolate mofetil

mTOR	Mechanistic (previously mammalian) target of rapamycin
NRM	Non-relapse mortality
NS	Not significant
OD	Optical density
PCR	Polymerase chain reaction
PTLD	Post-transplant lymphoproliferative disorder
QD	Once daily
RD	Related donor
SCT	Stem cell transplantation
SOT	Solid organ transplantation
TAC	Tacrolimus
Tx	Transplantation, transplant
URD	HLA-matched unrelated donor
UVL	Undetectable viral loads
VZV	Varicella zoster virus

DEFINITIONS IN SHORT

CMV DNAemia	Detection of CMV DNA using a qualitative or quantitative PCR method in samples of whole blood, serum or in buffy-coat specimens. The detection limit for identifying CMV using the quantitative PCR method was ≈200 CMV copies per ml (≈2.3 $\log_{10}$ genome equivalents (Geq) per ml)
CMV infection	Detection of CMV DNA or isolation of CMV in any body fluid or tissue specimen
Probable CMV disease	Clinical symptoms or radiological evidence consistent with CMV end-organ infection together with CMV DNAemia or positive CMV DNA detection by PCR from tissue biopsies without histopathological or immunohistochemical features of CMV infection or culture on tissue biopsy specimens
Proven CMV disease	Clinical symptoms or radiological evidence consistent with CMV end-organ infection together with histopathological or immunohistochemical features of CMV infection or culture on BAL, tissue biopsy specimens or positive CMV DNA in cerebrospinal fluid
HHV-6 DNAemia	Detection of HHV-6 DNA in samples of blood
HHV-6 infection	Detection of HHV-6 DNA in any body fluid or tissue specimen
Probable HHV-6 disease	Clinical symptoms or radiological signs suggestive of HHV-6 end organ infection together with HHV-6 DNAemia or positive

	HHV-6 DNA detection by PCR from tissue biopsies or any body fluid
Proven HHV-6 disease	Clinical symptoms or radiological evidence consistent with HHV-6 end organ infection together with HHV-6 DNAemia and positive HHV-6 DNA detection by PCR from tissue biopsies or any body fluid
EBV DNAemia/viremia	≈200 EBV copies per ml (≈2.3 $\log_{10}$ genome equivalents (Geq) per ml) of whole blood or serum which was the detection limit for identification of EBV using the quantitative PCR method
CHL of EBV	Presence of EBV DNA $\geq$ 4.2 $\log_{10}$ Geq per ml in whole blood in > 50% of the samples for $\geq$ 6 months

1 INTRODUCTION

Remarkable progress in transplant medicine has led to much improved results for both stem cell and solid organ transplantation in children and adults. The 10-year survival rate for allogeneic stem cell transplantation (allo-SCT) is around 55-60% and as high as 85-90% after solid organ transplantation (SOT) (1-5). These encouraging numbers illustrate an outstanding development due to improved surgery and postoperative care, but more importantly due to more effective immunosuppressive medication with less graft versus host reactions (GVHD) and graft rejections.

Still, however, opportunistic infections may cause considerable morbidity and mortality following transplantations. These infections are caused by microbes that seldom generate infections in the immunocompetent host such as viruses belonging to the group of herpesviruses, fungal infections caused by candida, aspergillus and pneumocystis, bacteria such as legionella, listeria and parasites like toxoplasmosis. A timetable illustrating opportunistic infections after transplantation is presented in Figure 1.

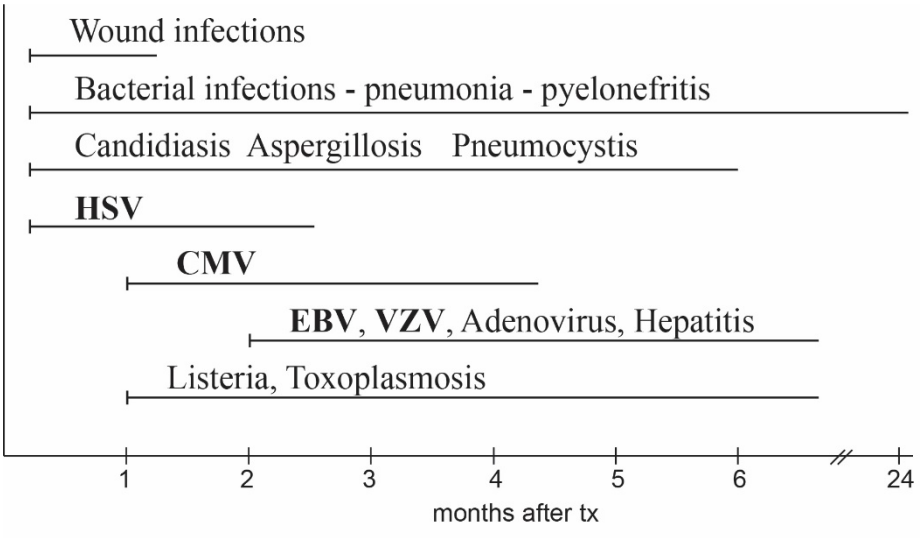

Figure 1. Timing of opportunistic infections after SOT. The first months after transplantation are crucial and infections are common.

The balance between enough immunosuppression (to avoid GVHD and rejections) and at the same time have a low risk of opportunistic infections has resulted in different treatment strategies. These strategies include primary prevention such as vaccination, matching of host and donor, more effective viral surveillance with new and improved viral diagnostic techniques and development of antiviral drugs for effective prophylaxis and preemptive treatment.

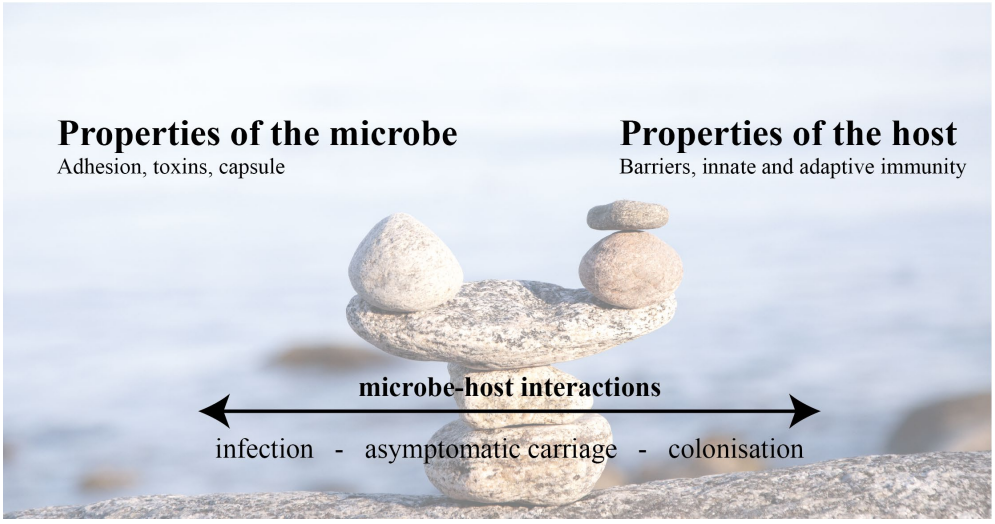

Figure 2. The balance between microbe and host interaction. Illustration: Mikael Lindahl.

This thesis focuses on opportunistic viral infections caused by cytomegalovirus (CMV), human herpesvirus type-6 (HHV-6), varicella zoster virus (VZV) and Epstein-Barr virus (EBV) in adult allo-SCT patients and pediatric renal transplant recipients.

1.1 BACKGROUND

1.1.1 TRANSPLANTATION - A PARADIGM SHIFT IN MEDICINE

In 1933, the first real attempt of a human kidney transplantation was done by Dr. Yurii Y. Voronoy, a Russian surgeon. A kidney was taken from a recently deceased individual and transplanted to a young woman who was suffering from acute mercury poisoning. The kidney was positioned in her thigh, sutured to femoral vessels and the ureter was externalized. No immunosuppression was given. Initially the kidney produced some urine but did in fact never function and the patient died two days later (6). In 1948, Sir Peter Medawar performed experiments that for the first time defined the immunology of transplantation and began to define rejection. For his pioneering work in transplant immunology, Dr. Medawar received the Nobel Prize in Physiology or Medicine in 1958.

The very first successful solid organ transplantation between humans was a kidney transplantation performed in Paris, 1952, by Dr. Jean Hamburger (7). Two years later at Brigham & Women's Hospital in Boston, a kidney transplantation involving identical twins was successfully carried out by the surgeon Joseph Murray (8). Since the donor was an identical twin, no rejection was seen even though the patient did not receive immunosuppression. This remarkable event proved several things:

1) organ replacement could cure a patient;

2) organ transplantation was technically feasible;

3) organ transplantation offered a permanent cure of the disease itself.

Present at the hospital during the time of the transplantation was a well-known hematologist, Dr. Donnall Thomas who assisted Dr. Joseph Murray caring for the kidney transplanted patient. In 1957, Dr. Thomas became the pioneer hematologist who performed the first allo-SCT at Mary Imogene Bassett Hospital in Cooperstown, New York, also involving identical twins (9). Drs. Joseph E. Murray and E. Donnall Thomas both received, in 1990, the Nobel Prize in Physiology or Medicine for the development of clinical transplantation based on their discoveries and achievements made in the 1950s. The first allo-SCT with a related sibling as a donator was performed in the late 1960s. In the

1960s liver, heart and pancreas transplantations were made, followed by lung transplantations in the 1980s.

Also in Sweden, allo-SCT and SOT were initiated early on. Following the first liver transplantation by Dr. Thomas Starzl in 1963, both Stockholm and Gothenburg sent young surgeons to him to learn from the newly started liver transplant program in Denver, USA. This international collaboration has had a great impact on organ transplantation in Sweden and the rest of Scandinavia. The international collaboration with Dr Thomas Starzl in Denver and close ties with Dr. Joseph E. Murray in Boston stimulated Dr. Lars-Erik ("Charlie") Gelin from Gothenburg to initiate the transplant program that honored him a role as the Scandinavian pioneer in organ transplantation. The first kidney transplantation made in Sweden was performed 1964 on a 17 year old adolescent, by Professor Curt Franksson, another pioneer of organ transplantation in Sweden.

The first allo-SCT made in Sweden was performed in 1975 in an adult patient and 1978 in a child.

In this thesis the focus is on adult allo-SCTs and on kidney transplantations in a pediatric population.

1.1.2 HEMATOPOIETIC ALLOGENEIC STEM CELL TRANSPLANTATION (ALLO-HSCT)

When hematopoietic stem cells are transferred from another individual to the patient it is called allogeneic hematopoietic stem cell transplantation (allo-HSCT) or allo-SCT.

Nowadays, approximately 32 000 allo-SCTs are carried out worldwide/year and in Europe approximately 12 000/year. In Sweden there are around 300 allo-SCTs/year performed at six university hospitals (Gothenburg, Stockholm, Malmö/Lund, Uppsala, Linköping and Umeå). In 2018, 46 patients were transplanted in Gothenburg, 11 were children and 35 adults (Table 1) (10).

Seventy per cent of all allo-SCTs are performed due to high-risk hematological malignancies such as leukemia, but also non-malignant diseases are treated with allo-SCT such as autoimmune diseases, severe aplastic anemia and primary immune deficiencies (11).

It is paradoxical that allo-SCT for treatment of a malignant disease causes a graft versus tumor effect which to some extent is desired for elimination of tumor cells (12). On the other hand, graft-versus-host disease (GVHD) is together with opportunistic infections, major complications after allo-SCT causing significant morbidity and mortality (13).

Acute GVHD occurs within the first 100 days post-transplant and can affect all organs such as skin, lungs, gastrointestinal tract, genitals and eyes. Sometimes GVHD gets chronic and continues or occur beyond 100 days post-transplant (14). Both acute and chronic GVHD are treated with increased immunosuppression and thereby the risk of having severe opportunistic infections increases. Opportunistic viral infections usually occur 1-4 months post-transplant. New or reactivated latent viruses are common and managements of herpesviruses are of great importance.

1.1.3 SOLID ORGAN TRANSPLANTATION (SOT)

In case of end-stage organ disease, SOT is a well-established treatment for both adults and children.

SOT is performed at four university hospitals in Sweden (Gothenburg, Stockholm, Malmö/Lund and Uppsala). A total of 14 000 kidney, 3 100 liver, 1 040 heart, 900 lung, 600 pancreas, and 30 small bowel transplantations have been performed between 1964 and 2018. In 2018 alone, 785 transplantations (448 kidney, 163 liver, 74 lung, 66 heart, 32 pancreas and 2 small bowel), were performed (Table 1).

Organ transplanted	Nos. in Sweden	Nos. in Gothenburg		
		Total	Children	Adults
Allo-SCT	300	46	11	35
Kidney tx	448	166	2	164
Liver tx	163	86	2	84
Heart tx	66	31	3	28
Lung tx	74	55	2	53
Pancreas/Islets tx	32	6	1	5
Small bowel tx	2	2	1	1

Table 1. The number of transplants made in Sweden and Gothenburg during 2018. tx=Transplantation
Source: Swedish Transplantation Registry, Scandiatransplant 2018 and Transplantation-coordinators.

1.2 IMMUNE DEFENSE

Different species have developed different strategies of defense against foreign organisms such as microbes. In humans, the defense is composed of three major levels listed below and also illustrated in Figure 3.

1) Chemical and mechanical barriers such as, an intact skin, mucus layers and stomach acidity

2) Innate immunity with an immediate activation of inflammatory cells such as neutrophils, monocytes, macrophages and dendritic cells and their signaling systems

3) Adaptive (acquired) immunity consisting of T- and B-lymphocytes that differentiate and develop during life.

Clearly, these defense systems or signals can go wrong, causing autoimmunity or auto-inflammatory diseases. In the context of transplantation-medicine it is these defense-systems (mainly adaptive immunity) that need to be reduced in order to avoid GVHD and graft rejection. A weakened immune system paves the way for infections that cause more serious illness than in an immunocompetent host.

1.2.1 ANTI-VIRAL IMMUNE RESPONSES

The immune response to a viral infection is a combination of the adaptive and the innate immune systems as illustrated in Figure 3. The viral presence triggers the innate immune system. Natural killer cells attack cells lacking the major histocompatibility complex (MHC) such as virus-infected or malignant cells. Natural killer cells are effector cells of the innate immune system and control, for instance, viral infections by secreting interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) (15). These cells bind microbial products in a fast reaction while the adaptive immunity is slower. Dendritic cells in the mucosa, lymph nodes or lymphoid tissues present viral antigens to T-lymphocytes, thus starting the activation of the adaptive immune system which is able to distinguish self- from non-self-antigens. When non-self-antigens are identified, B-cells produce antibodies and T-cells are activated to destroy foreign microorganisms. B-cells produce virus-specific antibodies that can inhibit the binding of viruses to host cells and may also help to kill infected cells by antibody-dependent cellular cytotoxicity or antibody-mediated lysis. However, specific antibodies alone may be insufficient in clearing virus or protecting against reinfection or reactivation of latent virus. The adaptive cellular immunity is crucial, engaging both CD4+ and CD8+ T cells. The CD4+ T-cells produce cytokines and thereby activate CD8+ T-cells, which then develop into cytotoxic lymphocytes. These cytotoxic T-cells release cytolytic proteins and thereby eliminate infected cells. The adaptive immune system also forms memory cells during the course of weeks. These memory plasma cells recognize foreign microorganisms and result in a more rapid response if there is another exposure. Long-lived memory plasma cells continuously secrete antibodies, immunoglobulin G (IgG), and provide long-term protection as a memory of the viral infection.

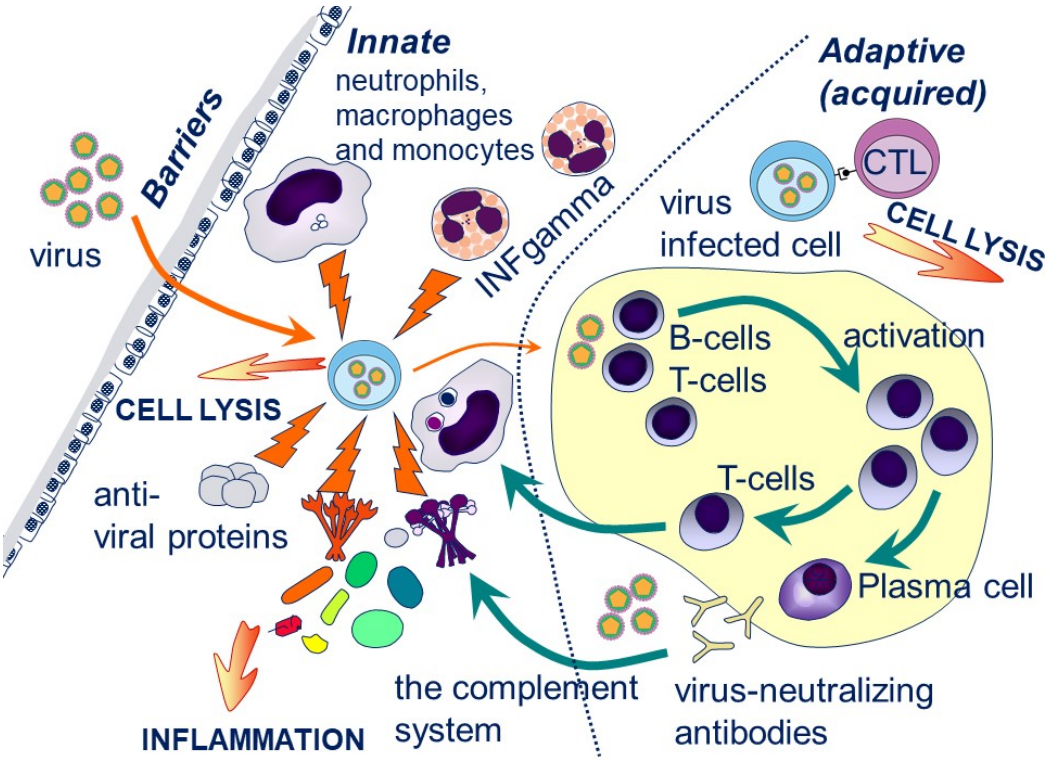

Figure 3. The three levels of defense with barriers, innate and adaptive immune systems and the immune responses against viruses. Innate immune response: neutrophils, macrophages and monocytes recognize cells infected by viruses in an antigen-independent manner, exert cytotoxic activities and rapidly produce large amounts of IFN-γ to eliminate infected cells. Adaptive immune response: antibody production directed against viral antigens are produced. CTL=Cytotoxic T-cells, or T-CD8+ cells, eliminate virus-infected cells and secrete cytokines such as IFN-γ=interferon-gamma. The innate and the adaptive immune response both result in lysis of the virus infected cell.

Source: Inflammation. Johan Mölne and Agnes Wold, 2007, Liber. Reproduced and modified by permission of Johan Mölne.

1.2.2 VIRAL VACCINES

Two major types of viral vaccines are used today, inactivated vaccines with no potential for viral replication such as subunit vaccines or virus-like particle vaccines and live-attenuated vaccines with the possibility of viral replication. In the immunocompromised host, such as transplant recipients, the risk of getting sick from viral replication after live-attenuated vaccines is high and these kinds of vaccines are therefore contra-indicated after transplantation. Examples of such live-attenuated vaccines that are not recommended for use post-transplant are varicella zoster virus vaccine, measles, mumps and rubella vaccine, rotavirus vaccine, Bacillus Calmette-Guerin vaccine against tuberculosis and yellow fever vaccine.

The live, attenuated Oka varicella vaccine was first developed about 40 years ago (16). The wild-type strain was isolated in Japan, in 1971, from the vesicle fluid of a boy called Oka who had chickenpox. Originally, the vaccine was used to prevent primary VZV infection. It was soon shown that vaccinated immunocompromised patients were also to some degree, protected against herpes zoster (17). When varicella vaccination is deemed necessary in patients scheduled for transplantation the vaccine ought to be administered prior to transplantation. But, antibody levels considered protective for healthy children may not prevent infection in children suffering from chronic renal insufficiency or in transplant recipients, in whom immunosuppression is a lifelong necessity (18). Immunosuppression reduces both humoral immunity (B-cells producing antibodies) and T-cell-mediated immunity which both are needed to eliminate intracellular pathogens such as VZV.

1.3 IMMUNOSUPPRESSION AFTER ALLO-SCT/KIDNEY TRANSPLANTATION

1.3.1 BACKGROUND

Before discussing the infections, it is important to have an understanding of the immunosuppressive agents used after allo-SCT and SOT. Immunosuppressive therapies are necessary to prevent T-cell-mediated GVHD and allograft rejection. These immunosuppressive drugs lower the activity of the immune response and thereby reduce a considerable part of the normal T-cell-mediated defenses against viruses and unfortunately, the risk for

opportunistic viral infections increases. Therefore, the risk of GVHD and rejections is always weighed against the risk of infections. In addition, chronic use of immunosuppressive drugs may also result in complications other than infections, such as malignancies, post-transplant lymphoproliferative disorder (PTLD), cardiovascular disease, diabetes and nephrotoxicity (19). Therefore, immunosuppression is tapered over time as the risk of GVHD and rejection decreases.

In 1957, azathioprine was discovered by Gertrude B. Elion and her colleague George H. Hitchings (20-22). Dr Sir Roy Calne, the British pioneer in transplantation, and Dr Joseph Murray in Boston, rapidly began to exploit this new drug but very few of their patients tolerated the doses of azathioprine that would prevent organ rejection (20, 23). This all changed when Dr. Thomas E. Starzl in Denver presented results he had achieved by using a combination of azathioprine and prednisolone. Azathioprine became in combination with corticosteroids, the standard immunosuppressive regimen into the 1980s (23, 24). Anti-thymocyte globulin (ATG) was added in the 1970s. Then in the early 1980s, cyclosporine was introduced. Thereafter, a whole range of new drugs have been introduced and greatly improved the outcome of transplant recipients.

Immunosuppressive drugs used in transplantation belong to five main groups:

1. calcineurin inhibitors
2. antimetabolites
3. corticosteroids
4. mechanistic target of rapamycin (mTOR) – inhibitors
5. mono- or polyclonal antibodies

The pharmacological mechanisms for three (groups 1, 2 and 4) of these five groups of immunosuppressive drugs used in transplantation are illustrated in Figure 4.

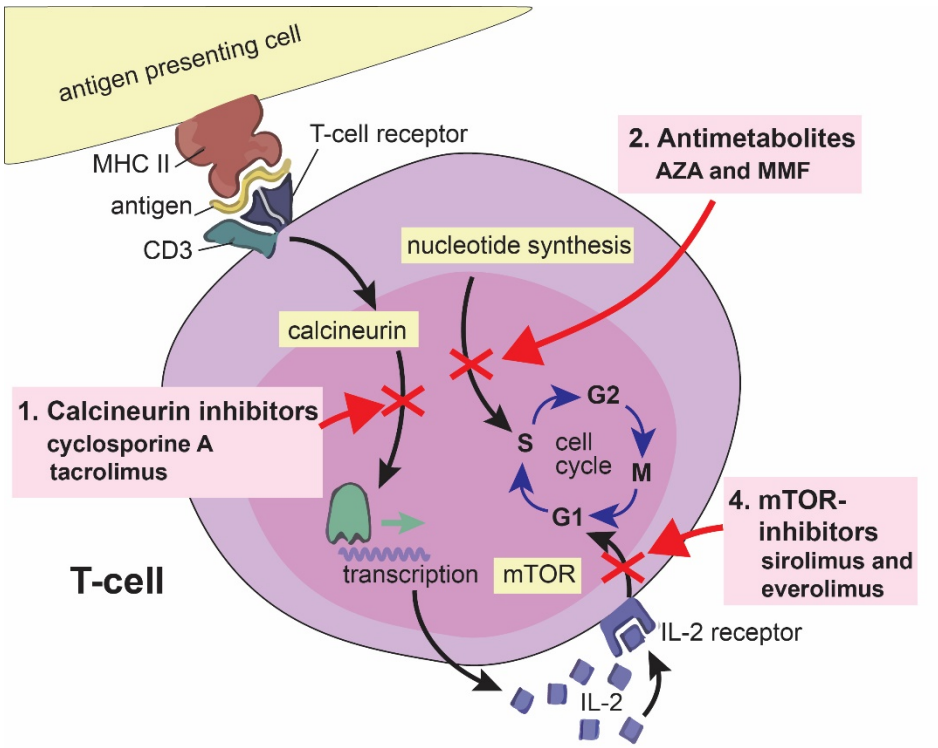

Figure 4. Illustration of the pharmacological mechanisms in T-lymphocytes, for the most commonly used immunosuppressive agents. Reproduced and modified by permission. Johan Mölne and Agnes Wold, "Inflammation" 2007, Liber.

1.3.2 INDUCTION THERAPY

Prior to the detailed presentation of the five different groups of immunosuppressant drugs it is of value to describe the place for and use of induction therapy in connection to transplantation.

Induction therapy is given in many cases to reduce the risk of early GVHD or rejection, followed by standard immunosuppressant maintenance treatments (25).

In adult allo-SCT conventional myeloablative and immunosuppressive regimens generally consists of total body irradiation in combination with

chemotherapy, usually a purine-analog, fludarabine (Fludara®). When graft from an unrelated donor or mismatched family donor is used, anti-thymocyte globulin (ATG) is often added in the conditioning.

In SOTs, ATG is also often used for induction therapy. ATG (Thymoglobuline®, Atgam®) consists of polyclonal antibodies that are generated by immunizing rabbits or horses with human thymocytes or T-cell lines to deplete the T-cell effect (26, 27). Beside the depletion of T-cells, ATG also targets antigens on B-cells, dendritic cells, macrophages, monocytes and NK cells to reduce GVHD and graft rejection.

Rituximab (MabThera®), another induction drug used, is a monoclonal anti-cluster of differentiation, CD20 antibody, that depletes CD20-positive B-cells (28). It may be used as part of a conditioning regimen for ABO-incompatible transplants (29, 30).

Intravenous immunoglobulin (IVIG) is used to reduce the level of pre-existing anti-HLA antibodies in ABO incompatible transplants and to treat antibody-mediated acute rejections (28, 29).

1.3.3 MAINTENANCE AGENTS

As mentioned above there are five major groups of immunosuppressant drugs that will be presented below. Today, most maintenance immunosuppressive regimens use the “triple drug therapy”. It consists of one calcineurin inhibitor (CNI), one antimetabolite agent and corticosteroids. Mechanistic target of rapamycin (mTOR)-inhibitors and antibody-preparations are mainly used in steroid-free immunosuppressive regimens.

Calcineurin inhibitors (CNI)

The most important of the immunosuppressive agents are the calcineurin inhibitors. Calcineurin is a protein phosphatase that activates T-cells by upregulating interleukin-2 (IL-2) expression (31). Inhibition of calcineurin results in suppressed production of IL-2, other cytokines and a suppressed T-cell activation. The dosage of the CNI is adjusted to maintain specific serum levels that are gradually reduced after transplantation. This group includes the most commonly used drugs such as cyclosporine A (CyA, Sandimmun®) and tacrolimus (TaC, Prograf®).

Cyclosporine (CyA) is a natural product, a small fungal peptide protein that binds the intracellular protein cyclophilin that inhibits calcineurin. It was the first usable CNI and has greatly improved long-term survival after transplantation (19, 28, 32). However, CyA is associated with many negative side effects such as nephrotoxicity, gingival hyperplasia, tremor, hirsutism and hypertension (25, 33).

Tacrolimus (FK506) is a natural product produced by a soil bacterium. It is a macrolide and acts by binding to an immunophilin that inhibits calcineurin similar to cyclosporine. However, tacrolimus is more potent than cyclosporine and has less pronounced side-effects (25).

Antimetabolites

Azathioprine (AZA), a purine analogue, was first used until mycophenolate mofetil (MMF) was introduced in the mid-1990s. MMF prevent the proliferation of B- and T-cells by inhibiting the guanosine base synthesis (28). Mycophenolate (Cellcept®) has largely replaced azathioprine since MMF is a more potent immunosuppressive drug and has less bone marrow toxicity than azathioprine, but it has gastrointestinal side effects and is teratogenic (25). For the proliferation inhibitors, the goal of treatment is to keep the area under the curve at a constant target value.

Corticosteriods (CS)

Corticosteroids (CS) have multiple effects on the immune systems and inhibit both innate and adaptive immune responses (34). CS have been used since the beginning of transplantation and are still one of the major corner stones both for induction and maintenance therapy after SCT and SOT (8). Due to multiple negative side effects such as osteoporosis, hypertension, weight gain and osteonecrosis, steroid-sparing protocols have been tried, albeit at the expense of more rejections (35). In acute GVHD after allo-SCT or rejection after SOT, corticosteroids are used either as pulse methylprednisolone i.v. or as prednisolone orally for 2-5 days. In maintenance immunosuppression regimens, the dosages of corticosteroids are lowered at regular intervals.

Mechanistic target of rapamycin (mTOR)

The mechanistic target of rapamycin (mTOR), previously known as mammalian target of rapamycin, is a key regulator of metabolic homeostasis. The mTOR-inhibitors impede activation of the T-cell via a kinase. Examples of drugs that inhibit the protein mTOR are sirolimus (Rapamune®) and

everolimus (Certican®). Sirolimus is a macrolide produced by a fungus and everolimus is an analog and a metabolite of sirolimus (19, 28). These agents are considered as less nephrotoxic than CNIs, but have negative effects on wound healing and haematopoiesis (36). There are some evidence that mTOR inhibitors may reduce the risk of developing malignancies after SOT, such as Kaposi's sarcoma, skin cancer and PTLD (36-38).

Antibodies

Monoclonal antibodies are directed towards exactly defined antigens, especially important are the antibodies directed against the IL-2 receptors, the T-cell receptor complex and CAMPATH-1 antigen.

Interleukin-2 is an important immune system regulator that is necessary for the clone expansion of activated T-lymphocytes. The anti-IL-2 compounds are directed against the IL-2-receptors (CD 25) and inhibit IL-2 mediated activation of T-lymphocytes. Examples of IL-2 inhibitor preparations are: basiliximab (Simulect ®) and daclizumab (Zenapax®). They are mostly used for induction treatment in kidney and liver transplantation programs and for treatment of severe GVHD after allo-SCT (39-41).

The CAMPATH-1 antigen (CD52) is a glycoprotein present on the surface of mature lymphocytes. Alemtuzumab (Mabcampath®) is an anti-CD52 monoclonal antibody preparation that induces depletion of both B- and T-cells (19). This drug is used to some extent as conditioning and anti-GVHD treatment after SCT.

Polyclonal antibody therapy affects all lymphocytes and cause general immunosuppression, possibly leading to serious infections, especially infections caused by herpesviruses such as CMV and EBV.

An example of polyclonal antibody therapy is anti-thymocyte globulin (ATG) as described above. ATG is often used for induction therapy but also in acute GVHD and graft rejection situations. Beside the depletion of T-cells, ATG also targets antigens on B-cells, dendritic cells, macrophages, monocytes and natural killer cells.

1.4 HERPESVIRUS AFTER TRANSPLANTATION

Herpesvirus infections are common in all individuals and appear at the same rate in non-transplanted and transplanted patients. The herpesviruses belong to the genus Herpesviridae and has evolved over at least 400 million years. The name herpes originates from the Greek word herpein meaning "to creep". These viruses are relatively large and they consist of a double-stranded DNA in an icosahedral capsid surrounded by an envelope of many glycoproteins. The human herpesviruses are classified into three subfamilies - α, β and γ viruses based on their biological characteristics seen in Table 2. After the primary infection, they remain in the body in a latent state. During the latent phase of replication, no or a very limited set of viral proteins are made. Currently, there are eight known viruses in the family of herpesviruses that cause disease in humans: herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), herpes zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpesvirus type 6 (HHV-6), human herpesvirus type 7 (HHV-7) and human herpesvirus type 8 (HHV-8). Herpesviruses, except for VZV, are transmitted from person to person via oral mucosa during asymptomatic shedding. Varicella zoster virus is the only herpesvirus that is airborne and is only transmitted when individuals have varicella or herpes zoster. All herpesviruses can cause life-threatening infections in immunosuppressed individuals such as transplant patients. Since the virus remain latent in the body after the primary infection, the infections in transplant patients can be caused by both reactivation of a latent infection and of a new infection. New infections may be community-acquired or transferred from the stem cell or organ donors.

In this research project, we chose to investigate and describe the primary infection and reactivation of CMV, HHV-6, VZV and EBV in transplant patients. The reason for choosing these specific viruses was that the primary infection and reactivation can cause severe disease in immunocompromised individuals and early diagnosis and prompt treatment of infections are required. In transplant recipients, blood samples are screened regularly during the first few months after transplantation regarding CMV DNAemia. When studying samples from allo-SCT recipients, we found several patients being HHV-6 DNA positive in blood. In transplanted patients EBV DNA levels are also followed in blood. When high loads of EBV DNA is seen, the immunosuppression is often reduced, to decrease the risk of EBV associated malignancies. Varicella zoster infections are also severe diseases in immunocompromised patients. Disseminated VZV disease can be seen in

transplant recipients both in the primary and the reactivated infection. Dissemination is associated with high mortality and knowledge about VZV immunity before and after transplantation is therefore of great importance.

α- herpesvirus **latent in sensory neurons**	**β- herpesvirus** **latent in T-lymphocytes**	**γ-herpesvirus** **latent in B-lymphocytes**
HSV-1 Herpes simplex virus type 1	**CMV** Cytomegalovirus	**EBV** Epstein-Barr virus
HSV-2 Herpes simplex virus type 2	**HHV-6 A** **HHV-6 B** Human herpesvirus type 6	**HHV-8** Human herpesvirus type 8
VZV Varicella zoster virus	**HHV-7** Human herpesvirus type 7	

Table 2. The human herpesviruses divided into different phylogenetic groups. The alpha-herpesviruses are latent mainly in sensory neurons, the beta- and gamma-herpesviruses in white blood cells, T-lymphocytes and B-lymphocytes respectively. Herpes simplex virus type 1 and 2 are also called Human herpesvirus type 1 and 2. Varicella zoster virus is also called Human herpesvirus type 3, Epstein-Barr virus for Human herpesvirus type 4 and Cytomegalovirus for Human herpesvirus type 5.

1.4.1 CYTOMEGALOVIRUS (CMV)

Cytomegalovirus is today the largest known virus that infects humans. Inclusion-bearing cells were first shown by Ribbert in 1881 (42). In 1921, Goodpasture and Talbert were the first to suggest that the “cytomegalia” could be due to a viral agent. Cytomegalovirus was first isolated from the salivary gland and kidney of two dying infants reported in 1956 (43). This virus usually infects individuals during early childhood and adolescence.

When CMV infects an immunocompetent individual it often gives no or only modest symptoms such as fever for a few weeks, enlarged lymph nodes and a sore throat. The virus then remains latent in white blood cells but also in various cell types such as stem cells of the bone marrow that develop into monocytes in blood and to tissue macrophages. Studies on the underlying mechanisms of CMV latency and in which human cells the virus remains latent exists but further knowledge about this is needed (44-47). Globally, the sero-prevalence of CMV is approximately 70% (48), but varies between 45-100% depending on age, country and socio-economic conditions (49).

Infections caused by CMV can arise as a community acquired infection, reactivation of latent CMV or as an infection transmitted from the transplanted stem cells or organ. CMV infections have long been one of the most feared infections after allo-SCT (50). In this population, the incidence of CMV infection in seropositive patients, without prophylaxis, is approximately 15-60% and for CMV disease 20-35% (12, 51). The most common symptoms of CMV infection in immunocompromised patients are fever, bone marrow failure, pneumonitis, gastrointestinal disease and infection of the transplanted organ. Without antiviral prophylaxis the initial symptoms usually occur three to six months after transplantation but with antiviral prophylaxis, infection and illness is sometimes postponed and often diminished. The number of CMV copies in the blood is checked regularly by PCR (polymerase chain reaction) and CMV DNAemia is used to guide the antiviral treatment.

1.4.2 HUMAN HERPESVIRUS TYPE 6 (HHV-6)

Human herpesvirus type 6 was isolated in 1986 from patients with lymphoproliferative diseases (52). There are two different types of HHV-6, type A and B. Of these, type B is the most common. More than 90% of the world population, over the age of two years, is HHV-6 seropositive (53). Transmission of HHV-6 is generally horizontal from mother-to-child or child-to-child, and occurs early in life. Human herpesvirus type 6 A and B differ from other human herpesviruses because of the unique ability of their genomes to integrate in a persistent latent state in the chromosomes and because of this ability they can be transmitted from parent to child in the germ line (54-58). This causes diagnostic pitfalls since such an integration of viral sequences in every leukocyte easily is identifiable and persistent high levels of HHV-6 DNA in both whole blood and serum is detected in

asymptomatic patients (59-61). In immunocompetent individuals, primary HHV-6B infection cause relatively mild symptoms such as exanthema subitum (roseola) and fever in young children (62), but it can also cause enlarged lymph nodes, leukopenia and hepatomegaly (52). Virus has also been detected in the cerebrospinal fluid of children with febrile seizures (63). Latent HHV-6 can be reactivated in immunocompromised patients (64). In these individuals, HHV-6 can cause fever and rash (65) but also life-threatening disease in the liver (66), lung (67) and brain (66, 68, 69). Human herpesvirus type 6 can also cause long lasting bone marrow suppression (66, 70) that makes it easier for other viral infections to infect or to reactivate, (including reactivation of CMV) (71). In patients undergoing allo-SCT, reactivated HHV-6 is seen in 33-48% (71-73). 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 as well as after pediatric renal transplantation leading to a higher rate of kidney rejection (74-76). A causative relationship between HHV-6 and these complications is, however, not well established.

1.4.3 VARICELLA ZOSTER VIRUS (VZV)

In 1875, Steiner demonstrated that chickenpox was caused by an infectious agent by inoculating volunteers with vesicular fluid from a patient with acute varicella (77). Clinical observations of the relationship between varicella and herpes zoster were made in 1888 by von Bokay, when children without evidence of varicella immunity acquired varicella after contact with herpes zoster. Varicella zoster virus was isolated from vesicular fluid of both varicella and zoster lesions in cell cultures by Thomas Weller in 1954 (78). This virus causes chickenpox as its primary infection while reactivation of latent VZV causes herpes zoster (shingles) and is the only virus in the group of herpesviruses that is air-borne transmitted. The infectiousness is high and immunity against this virus is extremely important, especially for immunocompromised individuals. In Sweden, approximately 98% of children are immune against VZV at the age of 12 years (79). Both in the primary and reactivated form, VZV infection is potentially life threatening for the immunocompromised. Disseminating VZV infection may cause significant morbidity and mortality in immunocompromised renal transplanted patients (80, 81). The live attenuated VZV vaccine can provide protective immunity against VZV in immunocompetent individuals.

Generally live vaccines are not recommended after organ transplantation due to the risk of disseminated infection (82, 83). Therefore, susceptible patients, if possible, are vaccinated before the transplantation. The vaccine response is examined by measuring IgG antibodies against VZV in serum. In many of these patients, VZV-specific antibodies are reduced and sometimes even disappear after transplantation (84, 85). The knowledge of how immunity is subject to change over time and depending on the immunosuppression is limited. The mechanisms for these immunity changes are unclear and it is often difficult to determine if the patient is immune or not.

1.4.4 EPSTEIN-BARR VIRUS (EBV)

Epstein-Barr virus was discovered in 1964 by Anthony Epstein and Yvonne Barr (86). Around 95% of the world's adult population is latent carriers of this virus (87). It was shown to be the causative agent of infectious mononucleosis, kissing disease, in 1968. In immunocompetent individuals mononucleosis is a self-limited lymphoproliferative disorder accompanied by variable clinical manifestations such as fever, tonsillitis, lymphadenomegaly and splenomegaly (88). The virus is spread between individuals through saliva or other body fluids. Epstein-Barr virus stays latent in the B-cells, mucosal cells, T-cells, NK-cells and muscle cells. This was the first virus implicated in human cancer (86). The virus is able to immortalize B-lymphocytes and this oncogenic potential can particularly in transplant recipients be developed into EBV-associated complications such as Hodgkin's lymphoma, non-Hodgkin's lymphoma (for example Burkitt's lymphoma), nasopharyngeal carcinoma and PTLD (89-94). EBV-associated PTLD is a feared complication after transplantation, especially in children. Monitoring EBV viral load by EBV DNA PCR is important for diagnosis of EBV infection and PTLD. PTLD develops due to uncontrolled proliferation of lymphocytes within the context of post-transplant immunosuppression after SCT or SOT and the vast majority are EBV-associated (95-98). EBV-associated PTLD can be responsible for graft loss and even death (99). The overall pediatric incidence of PTLD after SOT is 6-20% and the mortality is as high as 20% (95, 100).

Risk factors for developing PTLD have previously been described (90, 97, 101-103). High EBV DNA replication is recognized as a large risk factor (104). However, whether a long term high level of EBV load, called chronic high load (CHL), constitutes a valid predictive marker for the later development of EBV-related PTLD remains unclear. It is therefore of great interest to better

understand the relationship between the dynamics in EBV viral load and the occurrence of PTLD after transplantation (92, 105).

1.5 ANTIVIRAL THERAPY - A PARADIGM SHIFT

Gertrude B. Elion together with George H. Hitchings discovered many life-saving drugs such as merkaptopurin against leukemia, allopurinol against gout, pyrimetamin against malaria, trimethoprim against bacterial infections, azatioprin – the first immunosuppressive drug used after transplantation and also acyclovir – the first antiviral drug. In 1967, Gertrude Elion turned her attention to the antiviral activity of purines. Testing the compound arabinosyldiaminopurine, Elion and her assistants altered sidechains to produce a more active compound to interfere with the replication of the herpesvirus. The approach proved successful with the synthesis of acycloguanosine, also known as acyclovir (Zovirax®) (106). This work proved that drugs can be selective and almost atoxic to human cells (107). Based on this principle, her colleagues later developed the drug azisothymidine (AZT) used against the human immunodeficiency virus (HIV). Gertrude B. Elion, George H. Hitchings, and Sir James W. Black received the 1988 Nobel Prize in Physiology or Medicine for discovering important principles for drug treatment, leading to reduced mortality and morbidity in many diseases and for many individuals (Figure 5).

Figure 5. In 1988, Gertrude Elion receives the Nobel Prize in Physiology or Medicine from his Majesty the King. Together with colleagues, she discovered the smart mechanisms of action in antiviral therapy leading to the antiviral paradigm shift. Acyclovir and ganciclovir are guanosine analogues used against some herpesviruses. Photographer/source: Anders Holmström/TT.

1.5.1 ANTIVIRAL THERAPY – MECHANISMS

Antiviral drugs inhibit the virus either by blocking:

1) adsorption and penetration into the cell

2) the viral DNA/RNA polymerase or

3) transcription of viral proteins.

Antiviral polymerase inhibitors can be divided into three groups: nucleoside-, nucleotide- and pyrophosphate analogues. The DNA molecule consists of four different nucleic acids (deoxyadenosine-, deoxyguanosine-, deoxycytidine- and deoxytymidinetriphosphate). Each nucleic acid is made up of phosphate, sugar and a purine or pyrimidine fundament.

Acyclovir is a synthetic acylic purine nucleosid analogue. It is first phosphorylated to acyclo-guanosine monophosphate by viral thymidine kinases and then into the active triphosphate form, acyclo-guanosine triphosphate, by cellular kinases (108). As the active triphosphate form is incorporated into viral DNA, the chain is terminated because of a premature structure and the activity the viral DNA polymerase is inhibited. Synthesis of the viral DNA is irreversibly stopped (109). The viral polymerase has greater affinity to acyclovir triphosphate than to the human cellular polymerase, hence the toxicity of acyclovir is very low. Renal toxicity may occur after high doses of intravenous administration and accumulation of metabolites from acyclovir in the central nervous system (CNS) is associated with neuropsychiatric side effects (110).

Antiviral agents for herpesvirus were among the first to be registered. In 1981, acyclovir was approved for the treatment of herpes simplex virus (HSV-1 and -2) infections.

Valacyclovir is a prodrug in the form of a valine ester of acyclovir with a greater oral bio-availability than acyclovir resulting in significantly higher serum acyclovir levels (111). Valacyclovir is converted by esterases to active

acyclovir via hepatic metabolism and the toxicity and side effects are similar to those of acyclovir.

Some examples of approved antiviral agents for different herpesviruses and their mechanism of action, administration route and important side effects are shown in Table 3.

Antiviral drug	Mechanism of action	Active against herpesvirus	Adm. route	Important side effects
Acyclovir/valacyclovir	nucleoside analogue	HSV 1, HSV 2, VZV, CMV	iv, oral and topical	Renal failure, neurological and psychiatric
Ganciclovir/valganciclovir	nucleoside analogue	CMV, HHV-6, HSV 1[1], HSV 2[1]	iv, oral and intravitreal	Bone marrow suppression
Foscarnet	pyrophosphate analogue	CMV, HHV-6, HSV 1, HSV 2, VZV resistant to acyclovir	iv	Nephrotoxic, electrolyte disorders
Cidofovir	nucleotide analogue	CMV, HSV 1[1], HSV 2[1]	iv	Nephrotoxic, uveitis
Letermovir	terminase inhibitor	CMV	oral	Gastro-intestinal

Table 3. Some approved antiviral agents for different herpesviruses and their mechanism of action, administration route and important side effects.
Adm.=Administration and iv=Intravenous
[1] *Ganciclovir and cidofovir are active against HSV 1 and 2 but not fully approved for routine clinical treatment*

1.5.2 ANTIVIRAL THERAPY OF CMV IN IMMUNOSUPPRESSED INDIVIDUALS

Since CMV is one of the most important infections in transplanted patients, causing death and significant organ manifestations, it is important to have a prophylactic CMV infection strategy in all transplanted patients. The choice of strategy depends on the patient's risk of developing CMV infection. CMV infection risk is weighed against side-effects and costs. The CMV infection risk depends on many factors such as: 1) patient and donor CMV serological status, 2) grade of immunosuppression, or 3) type of organ transplanted.

CMV is susceptible to ganciclovir (Cymevene®), valganciclovir (Valcyte®), foscarnet (Foscavir®), cidofovir (Vistide®) and letermovir (Prevymis®).

Antiviral prophylaxis: Antiviral prophylaxis against herpesviruses are routinely given to patients at high risk of CMV infection; i.e. D+R- (donor positive, recipient negative for CMV IgG antibodies before transplantation), D-R+ and D+R+ patients. The drugs recommended for antiviral prophylaxis have changed over the years and differ between different centra and transplantations. Prophylaxis against CMV after SOT in Gothenburg has changed from acyclovir (1992-1997), to ganciclovir (1998-2005) and valganciclovir (2005 and onwards). Hence, valganciclovir is currently the most recommended and commonly used drug for prophylaxis (112). Letermovir has been studied in CMV positive allo-SCT recipients and since it is active only against CMV either acyclovir or valacyclovir has to be added to prevent herpes simplex and VZV infections (113). The prophylaxis is started seven days post-transplantation and is given until at least six months after transplantation for D+R- and at least three months after transplantation for D+R+ and D-R+ patients (114-116).

Antiviral pre-emptive therapy: Effective pre-emptive therapy involves monitoring by PCR for CMV in blood at regular intervals to detect early viral replication. Once a predetermined threshold is achieved (optimally before the development of symptoms), antiviral treatment is begun, to prevent progression to clinical disease. A universal threshold for starting therapy has not been defined. It is likely that optimal thresholds are different among different risk groups (115, 116). Ganciclovir is the most commonly used drug for pre-emptive antiviral therapy. Valganciclovir is as effective and safe as

ganciclovir (117-119). Foscarnet has been shown in a randomised trial to be as effective as ganciclovir for pre-emptive treatment (120)

Antiviral treatment: Ganciclovir and valganciclovir are the most commonly used drugs for antiviral therapy. Foscarnet and cidofovir are usually used as a second or third-line therapy because of its renal toxicity (120, 121). Maribavir, an inhibitor of viral kinase, is under investigation as a treatment for resistant or refractory CMV infection in allo-SCT patients (122). However, its efficacy for the treatment of refractory or resistant CMV disease in SOT has been reported with higher doses (123, 124). Occurrence of resistance has been reported (125). Letermovir, a novel non-nucleoside CMV inhibitor targeting the viral terminase complex, was approved by the U.S. Food and Drug Administration in 2017 for the prevention of CMV infection in bone marrow transplantation. In this population, a phase 3 randomized trial is showing a superior efficacy of letermovir compared with placebo in preventing CMV disease with myelotoxicity and nephrotoxicity rates similar to those of placebo (113). Letermovir has also shown to be effective in treating CMV viremia in renal transplant recipients (126).

The lipid-conjugated analogue of cidofovir, brincidofovir, has high oral availability and less nephrotoxicity than cidofovir. Efficacy has been low in prevention in hematopoetic stem cell transplant patients, and few data are available in SOT recipients (127). Moreover, Faure et al reported two cases of acute renal injury in SOT patients who received brincidofovir (128).

There are no formally controlled trials made for treatment of CMV disease but the standard therapy for CMV pneumonitis has been a combination of iv ganciclovir and high-dose iv immunoglobulin. At the end of 1980s, mortality in CMV pneumonitis was more than 90%. Three uncontrolled studies has shown that high doses of ganciclovir and high doses of intravenous immunoglobulin reduced the mortality rate in CMV pneumonitis to 50% (129-131). This combination therapy is still standard regimen for treatment of CMV pneumonitis even though the additional immunoglobulin treatment has been discussed during past years (116, 132, 133). If standard treatment against CMV pneumonitis seems to fail, the second-line treatment of CMV disease with either cidofovir or foscarnet or the combination of full dose iv ganciclovir and foscarnet might be an alternative (116, 121). Maribavir, letermovir and brincidofovir needs further studying before recommendations can be given.

In case of CMV infection, treatment with ganciclovir (5 mg/kg BID for 7 days, followed by 5 mg/kg QD for 7 days) or valganciclovir is recommended (134). If pulmonary CMV disease is diagnosed, ganciclovir treatment is

prolonged (5 mg/kg BID for 14 days, followed by 5 mg/kg QD for 7 days up to 3 weeks).

CMV immune globulin: CMV immune globulin has been used, for prophylaxis, in patients with prolonged neutropenia who are intolerant to ganciclovir and in patients with refractory CMV disease and hypogammaglobulinemia (135). For other types of CMV disease than pneumonitis, such as gastroenteritis, existing data shows that the additional treatment with immunoglobulin has no improved effect but controlled studies are lacking (136). CMV immune globulin is not recommended for use, although there may be specific circumstances, when used in combination with antivirals, in which some benefit has been demonstrated.

CMV immunotherapy: Cytomegalovirus-specific T-cell lines and clones derived from the donor, the patient's own or from a third party can be life-saving in isolated cases when antiviral treatment alone does not seem effective (137-143). However, it is very time consuming and labour intensive to obtain CMV-specific T-cell lines and high-dose steroids (>1 mg prednisolone per kg) might interfere with the CMV-directed cytotoxic T-cell function.

1.5.3 ANTIVIRAL THERAPY OF HHV-6 IN IMMUNOSUPPRESSED INDIVIDUALS

Antiviral prophylaxis: Two small non-randomized studies of SCT recipients suggest that prophylactic ganciclovir can prevent recurrent HHV-6 infection (144, 145) but given the low risk of HHV-6 disease together with the toxicity of ganciclovir, antiviral prophylaxis against HHV-6 cannot be recommended (146).

Antiviral treatment: In patients with HHV-6 DNAemia and clinical symptoms consistent with HHV-6 disease such as HHV-6 encephalitis after allo-SCT or SOT, treatment with either ganciclovir (10-18 mg/kg/day) or foscarnet (180 mg/kg/day) has been reported to be effective (69, 146-148). Ganciclovir and foscarnet are reported to be effective against HHV-6, either alone or in combination (149). If treatment failure is noted or ganciclovir resistance present, a second-line therapy with cidofovir is recommended (146).

1.5.4 ANTIVIRAL THERAPY OF VZV IN IMMUNOSUPPRESSED INDIVIDUALS

Untreated primary infection with VZV has high mortality rate in transplant recipients. Treatment with acyclovir (10 mg/kg x 3 to adults and children > 12 years and 500 mg/m^2 body surface x 3 to children < 12 years) has dramatically improved the prognosis. Varicella in immunocompromised patients must therefore always be treated initially with antiviral drugs. Intravenous treatment is recommended for 7-10 days and there-after should oral treatment be considered (150-152).
Even reactivated VZV, herpes zoster, should always be treated with antivirals in transplanted patients as there are a risk that the disease may become disseminated. Valacyclovir is as effective as acyclovir in treating herpes zoster in immunocompromised patients (153).

Prophylaxis against VZV infection is recommended to VZV seronegative patients waiting for SOT. Before transplantation, varicella vaccination with a live attenuated varicella vaccine is recommended. To seronegative transplant recipients post exposure prophylaxis with varicella zoster immunoglobulin (VZIG) within 96 hours of exposure as well as antiviral drugs are recommended (154). Antiviral prophylaxis with acyclovir against herpes zoster up to 6 months after allo-SCT or SOT reduces the risk (155). Vaccination with an inactivated zoster-vaccine pre-transplant has been shown to reduce the risk of herpes zoster and will hopefully be available in Sweden soon (156).

1.5.5 THERAPY FOR EBV IN IMMUNOSUPPRESSED INDIVIDUALS

There is no recommended antiviral treatment available for EBV today. Acyclovir and ganciclovir has proven to be ineffective in EBV infection and early phase of PTLD (157, 158). Prophylactic (val)ganciclovir has been studied but more investigations and controlled studies are needed (159). Reduction of immunosuppression is today the recommended preemptive strategy when rising EBV DNA levels are noted. Using this strategy a 50% decline of PTLD lesions is described (157, 158, 160). When PTLD is diagnosed, a step-by-step strategy is recommended with further reduction of immunosuppression, treatment with anti-CD 20 monoclonal antibodies, chemotherapy, and in occasional cases immunotherapy with EBV specific cytotoxic T-cells, surgery and radiotherapy might be considered (142, 161-165).

2 AIMS

The overall aim of this thesis was to expand our knowledge on the incidence, prophylaxis, management and long-term effects of herpesvirus infections after transplantation, and more specifically:

- To study the incidence of CMV DNAemia, infection and disease along with prognostic factors and their importance for morbidity, mortality and long-term outcome after adult allogeneic haematopoetic stem cell transplantation (allo-SCT, paper **I**).

- To describe the clinical picture associated with HHV-6 infection and to follow the outcome associated with HHV-6 in adult allo-SCT patients (paper **II**).

- To analyse and follow VZV antibody levels in pediatric renal transplant recipients who had a pre-transplant history of varicella infection or vaccination and to determine the outcome of varicella infection and herpes zoster in the two cohorts during follow up (paper **III**).

- To evaluate the incidence, time of occurrence, risk factors and outcome of EBV CHL carrier state after pediatric renal transplantation (paper **IV**).

3 PATIENTS AND METHODS

3.1 PATIENTS

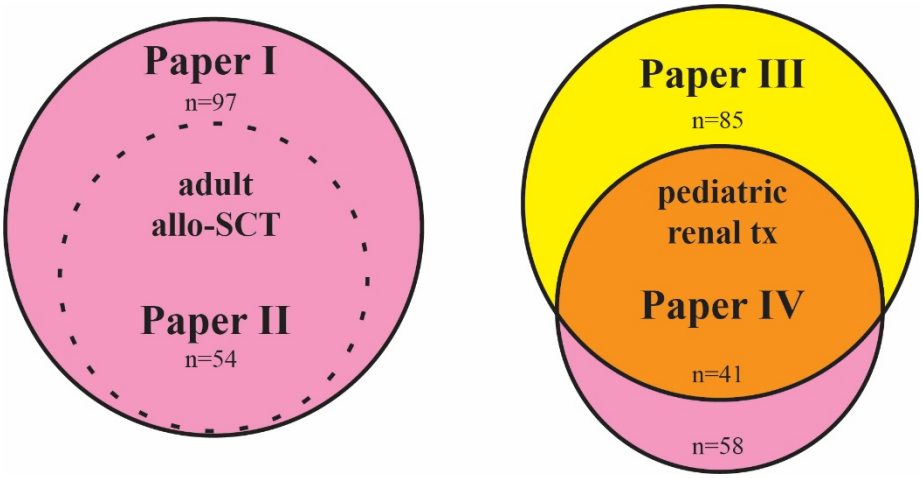

Figure 6. The number of patients and relationship between the four cohorts in Papers I-IV. Paper I included all adult allo-SCT patients, whereas 54 of them also were studied in paper II. In papers III and IV there were 41 individuals appearing in both.

Four different study cohorts were used in this thesis. The number of patients and relationship in the four different investigations are illustrated in Figure 6.

All four studies were retrospective. The cohort used for paper I consisted of 97 adult allogeneic stem cell recipients, transplanted at the Section of hematology at Sahlgrenska University Hospital between January 1997 and December 2001. Fifty-four of them participated also in paper II. The cohorts used for papers III and IV were pediatric renal transplant recipients, transplanted and followed at Queen Silvia Children's Hospital, Sahlgrenska University Hospital. In paper III the cohort consisted of 85 pediatric renal transplant recipients who were transplanted between 1986 and 2014. In paper IV, 58 children transplanted between 2004 and 2017 were included (41 of these patients were included in paper III as well). The additional 17 patients were transplanted between 2014 and 2017.

Compilation of already analysed samples were made. No additional samples or analyses were carried out. Approval for all four studies were given by the Regional Ethical Review Board in Gothenburg (Dnr S 649-01 for Papers I and II and Dnr 549-13 for Papers III and IV).

3.2 POLYMERASE CHAIN REACTION (PCR)

In all four studies, viral DNA were analysed in whole blood, serum and/or "buffy coat" (i.e. purified white blood cells) using polymerase chain reaction (PCR). PCR was developed by Kary Mullis in 1983 and for this he and Michael Smith were awarded the Nobel Prize in Chemistry 10 years later (in 1993) (166, 167). The method is illustrated in Figure 7.

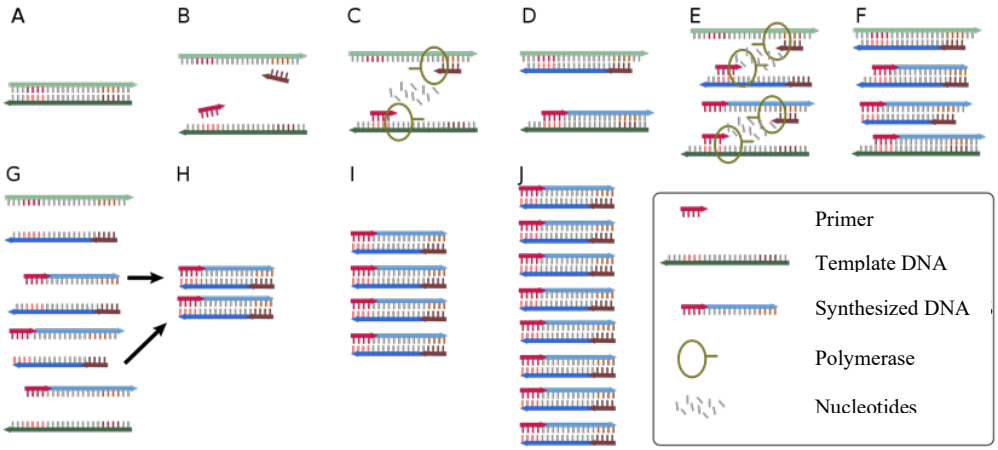

Figure 7. The polymerase chain reaction (PCR) amplification process: A. Extraction of genetic material from the sample. B. Denaturation by heating the reaction chamber, yielding two single-stranded DNA molecules. C. Annealing of the primers, hybridization of the primers to the strands and the enzyme polymerase binds to the primer-template hybrid and begins DNA formation by using the added nucleotides. D. Elongation where the polymerase copies the DNA, extension of the strands ending the first cycle. E. Denaturation and annealing in the second cycle. F. Elongation of the second cycle using the synthesized DNA as templates as well. G. Denaturation, annealing and elongation constitutes another cycle and when reaching the set number of cycles, the reaction chamber is cooled for short-term storage of the PCR products.

Source: https://upload.wikimedia.org

Patients transplanted between 1986 and 2000 were analysed for viral DNA by a qualitative PCR that presented the outcome as + or –. Hence, it was difficult to establish the role that the herpesvirus played for the individual patient. Between 2000 and 2003, the samples were analysed by a quantitative PCR Amplicor monitor (Roche), but this assay was available only for CMV DNA. From 2004 and onward, the real-time PCR method was used. Serum and whole

blood samples were analysed for CMV, EBV and HHV-6 DNA with real-time PCR (168). The viral loads were calculated from the slope and intercept of the standard curve, and results were expressed in genome equivalents/ml. The lower detection limit for the assays is ≈2.3 $\log_{10}$ genome equivalents (Geq)/ml (≈200 EBV or CMV DNA copies/ml). Amplification cycles were run in an ABI Prisma 7900 HT Fast Real-time instruments (Applied Biosystems, 7900) (169). The real-time PCR (qPCR) has a wider quantification range and a higher sensitivity than the Amplicor assay. But, the units are not equivalent: 1 unit by Amplicor corresponds to approximately 3 Geq by real-time PCR.

3.3 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

In all four studies, enzyme-linked immunosorbent assay (ELISA) was used for detection of CMV, HHV-6 and VZV IgG antibody titers in serum. To detect VZV antibodies of IgG class, whole virus antigen was used for coating between 1986 - 2011 and recombinant VZV glycoprotein E antigen was used for coating from 2012 to 2015 (170, 171).

In short, ELISA is a rapid immunochemical test that is used to detect an antigen or antibody. For detection of antibodies the corresponding antigen is coated on the surface of reaction plates. The test material is applied, and then all unbound material is washed away. Antibodies (monoclonal or polyclonal), specific for human antibodies, and with an enzyme bound to them are then added. The excess is again washed away and a substrate is added to the plates and the enzyme converts the substrate to a color that can be detected using a spectrophotometer. The antibody level can be estimated either by the signal strength or as the greatest dilution (titer) that is reactive.

3.4 IMMUNOFLUORESCENCE (IFL)

Antibodies of IgM class against CMV, HHV-6 and VZV IgM as well as IgM and IgG antibodies against the viral capsular antigens of EBV were analysed by immunofluorescence. In paper III this method was also used to evaluate discordant samples of VZV IgG antibodies. The method is illustrated in Figure 8.

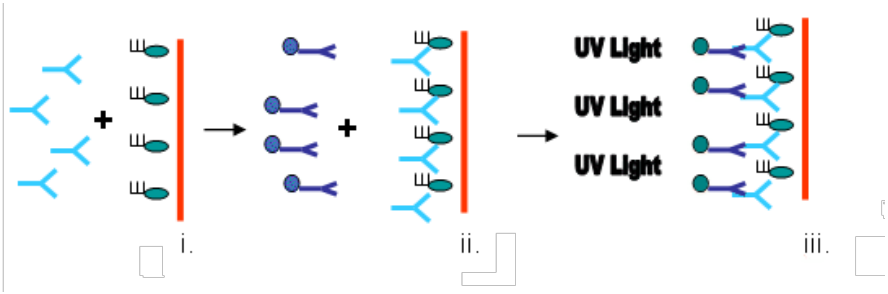

Figure 8. The immunofluorescence method. ***i.*** *Infected cells present antigens that specific antibodies in a sample can bind to.* ***ii.*** *A secondary antibody carrying a fluorophore then binds to the antibody/antigen complex and can* ***iii.*** *be detected by an immunofluorescence microscope.*

Source: http://www.biobest.co.uk/assets/images/diagnostics/Immunofluoresence1.gif

3.5 SAMPLINGS AND ANALYSES

Detailed description of sampling and analysis of specimens have been presented in papers I – IV, and below follows a brief summary. All analyses were performed by accredited diagnostic assays at the Department of Clinical Microbiology, Sahlgrenska University Hospital.

Paper I. Ninety-seven patients were monitored at least once weekly from engraftment until day 100 after allo-SCT. Following this period, patients who had experienced CMV reactivation or had severe GVHD continued to be monitored weekly during the GVHD period. The qualitative CMV DNA analyses of serum and white blood cells (buffy coat) of all 97 patients were carried out routinely using an in-house nested PCR (172) that had a lower detection limit of approximately 200 CMV DNA copies/ml. In addition, CMV DNA analyses were initiated when infections were clinically suspected. Specimens chosen for these analyses could include buffy coat, serum, bronchoalveolar lavage (BAL) fluid, or biopsies from any symptomatic organ. Quantitative CMV DNA analyses were performed in 25 available serum samples from 11 patients that previously had been analysed and tested positive for CMV DNA content in serum using the qualitative PCR method. The frozen samples were analysed retrospectively by quantitative real-time PCR, modified from Yun et. al. (173), with a similar sensitivity as the nested qualitative PCR.

Paper II. Fifty-four patients who belonged to the same cohort as those in paper I, were tested for HHV-6 DNA at the discretion of the treating clinicians. If a HHV-6 infection was suspected, mostly due to severe or unclear clinical symptoms or prolonged fever, HHV-6B DNA was then analysed in peripheral blood using a real-time PCR assay with a qualitative read-out (174).

Paper III. We retrospectively followed 85 pediatric patients who were consecutively transplanted with renal grafts between 1986 and 2014. Five patients were excluded since one patient lacked a proven history of varicella infection or vaccination prior to transplant and the VZV serostatus was missing from four patients before their transplant. Due to lack of serum samples, 13 patients were excluded from the serological follow-up, but were followed for the clinical outcome.

All children had routinely been tested for the presence of serum IgG antibodies against VZV before transplantation and then at various time points for a median time of five (range 0-21) years post-transplant until the age of 18 years. The patients were, however, excluded from the follow-up of VZV serology if

they developed a VZV infection, were re-transplanted, lost to follow-up or died. An ELISA was used to detect VZV antibodies of IgG class using whole virus antigen (1986-2011) or recombinant VZV glycoprotein E antigen (2012-2015) for coating (170, 171). The cut-off level was set as an optical density value of a negative serum control diluted 1:200 plus 0.200 optical density units. A VZV IgG antibody titer of $\geq$ 200 was considered seropositive, indicating prior VZV antigen exposure (170). In addition, in specimens with a VZV IgG titer of 200 against whole virus antigen, IFL analyses were carried out and a titer of eight was regarded as positive (171). Changes in antibody titers were considered significant when a four-fold or greater titer increase or decrease was seen.

Paper IV. We partly used the same cohort as in paper III and retrospectively studied all 58 children who had their first renal transplant between January 2004 and June 2017. Measurements of EBV and CMV DNA load in blood samples were performed at least every week during the first three months, once monthly up to one year after transplantation and thereafter according to EBV and/or CMV PCR-status. When the levels of EBV or CMV DNA increased, when EBV or CMV infection were suspected or when the patient was treated for rejection, samples were taken more often.

Serological analyses of donors and recipients regarding EBV and CMV antibodies were performed, along with post-transplant serial measurements of EBV and CMV DNA levels. EBV IgG, IgM and CMV IgM antibodies were analysed using IFL, whereas CMV IgG antibodies were analysed by an ELISA.

Serum and whole blood samples were analysed for EBV and CMV DNA with a real-time quantitative PCR assay using primers and probes designed by Niesters et al. in 2002 (169). The same assay was used during the whole study period.

4 STATISTICS

The different statistic methods used in this thesis are summarized in Table 4.

All tests were two-tailed and conducted at a significance level of 0.05. Analyses were performed using SAS (statistical software) version 9.4 (Cary, NC, USA). In paper I, SPSS version 15.1 (IBM, Armonk, NY) software package was also used. In paper II there were no need for statistical methods being used.

Papers: **Methods:**	**Paper I**	**Paper III**	**Paper IV**
Chi-square test	**X**		**X**
Fisher's exact test	**X**	**X**	**X**
Mantel-Haenszel Chi-square test			**X**
Mann-Whitney U-test		**X**	**X**
Wilcoxon's signed rank test		**X**	
Kaplan-Meier	**X**	**X**	**X**
Cox proportional hazard	**X**	**X**	**X**

Table 4. Summary of the different statistic methods used in paper I, III and IV.

5 RESULTS

Detailed results have been presented in papers I – IV, and below follows a description of the findings in general.

5.1 CYTOMEGALOVIRUS AFTER ALLO-SCT (PAPER I)

In the cohort of 97 allo-SCT patients, 60 (61%) developed CMV DNAemia. There was an increased risk of CMV DNAemia in CMV seronegative donors to seropositive recipients (D-R+) whereas the lowest risk was found in the D-R- group. A tendency towards earlier CMV DNAemia was seen in the CMV D-R+ group. In 29 patients CMV DNAemia developed after day 100 post-transplant. CMV disease with debut more than 110 days after transplantation was related to steroid treatment for GVHD in three out of four patients.

Sixty-seven patients developed acute and 58 chronic GVHD. A total of 71 patients received steroid treatment for GVHD and 54 patients received prophylactic treatment with ATG.

CMV treatment was given to 50 (51%) of the patients and was initiated at a median of 63 days after transplantation. Many patients developed repeated episodes of CMV reactivation and therefore, received repeated treatment episodes, in total 93, for proven or suspected CMV infection.

The overall one-year survival was 75% and the five-year survival 55%. The patients with diagnosed CMV DNAemia ($\geq$ 200 CMV DNA copies/ml) showed an improved survival compared to patients without detectable CMV DNAemia (p=0.026) as shown in Figure 9.

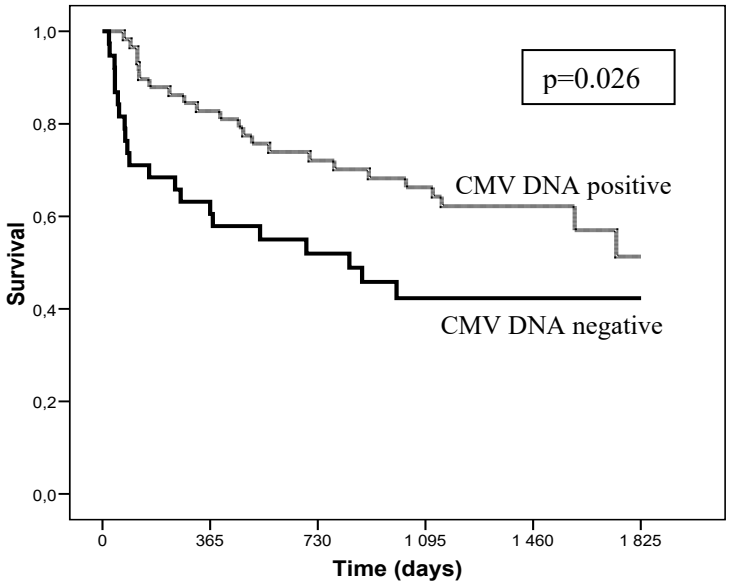

Figure 9. Probability for survival between patients that were CMV DNA positive and CMV DNA negative after allo-SCT.

Two patients had proven CMV disease, and two additional patients, in whom adequate laboratory samples were missing, had probable CMV disease (Table 5). In two of these patients CMV contributed to the patients' death. Yet another patient in whom CMV DNA was detected in BAL fluid and buffy-coat died of respiratory disease 476 days after transplantation.

Diagnosis	CMV DNAemia days after tx	D/R	Underlying diagnosis	Donor source	Condi-tioning	GVHD	Prednisolone (mg/day)	Ad mortem, days after tx
Probable CMV pneumonitis	21	+/+	AML/ALL	URD	Full	0	0	1741
Proven CMV pneumonitis	111	-/+	Lymphoma	URD	Reduced	Chronic in skin, GI and liver	25	117
Proven CMV retinitis	151	+/+	AML	RD	Full	Chronic in skin, liver, GI and lung	75	-
Probable CMV pneumonitis	468	+/+	CML	RD	Full	Chronic in liver and mouth	40	476

Table 5. Characteristics of the four patients with CMV disease. GI=Gastrointestinal; RD=Related donor and URD=HLA-matched unrelated donor

5.2 HUMAN HERPESVIRUS TYPE 6 AFTER ALLO-SCT (PAPER II)

In the cohort of 97 patients studied in paper I, 54 patients (56 %) had at least one sample analysed for HHV-6 DNAemia. HHV-6 DNAemia was detected in 15 of 54 tested patients at a median of 76 (24-387) days after allo-SCT. These 15 patients are described in Table 6, with regard to HHV-6 treatment, co-infection with CMV, antiviral prophylaxis, relapse and mortality. Clinical symptoms leading to assessment of HHV-6 included fever, skin exanthema, CNS symptoms, gastrointestinal (GI) disease, elevated liver enzymes, and pain in joints and muscles. Based on clinical symptoms associated with HHV-6 DNAemia, 9 patients received empirical antiviral treatment for proven or probable HHV-6 infection. Three patients who received HHV-6 antiviral treatment (nos. 1, 2 and 9 in Table 6) died but it could not be established to what extent HHV-6 contributed to their death. Another two patients died after relapse in malignancy and one patient died after rejecting the graft. One patient with mild symptoms such as skin exanthema and diarrhoea had only one positive HHV-6 DNA sample from serum and did not receive treatment against HHV-6.

As many as thirteen (87%) of 15 patients with HHV-6 DNAemia had at the same time GVHD.

Eight out of 15 (53%) HHV-6 DNAemia patients were treated with antivirals against CMV infection or disease, compared with 50 patients (51%) in the whole cohort of 97 allo-SCT patients. Two patients in the whole cohort were treated for EBV-related PTLD. One of these patients died of probable severe VZV encephalitis. None of these two patients had HHV-6 DNAemia.

The overall one-year survival after transplantation was 75% and the five-year survival 55% in the 97 patients studied. Among the 15 patients with HHV-6 DNAemia, the one-year survival was 73% and the five-year survival 67% compared to the 39 HHV-6 DNA negative patients who had a one-year survival of 74%. Hence, no reduction of survival because of HHV-6 DNAemia was seen.

No	HHV-6 treatment	CMV			Relapse	Ad mortem	HHV-6 follow-up after tx	Follow-up of survival
		D/R sero status	DNAemia	Treatment				
							(days/years)	(days/years)
1	Yes	+/-	Negative	No	No	Yes	264 days	264 days
2	Yes	-/+	Negative	No	Yes	Yes	74 days	74 days
3	Yes	-/-	Negative	No	Yes	Yes	157 days	157 days
4	Yes	-/-	Negative	No	No	No	3.5 years	13 years
5	No	+/-	Positive	Yes x 1	No	Yes	5.7 years	5.7 years
6	Yes	-/+	Positive	Yes x 1	No	No	3 years	10 years
7	Yes x 2	+/+	Positive	Yes x 1	No	No	5 years	13 years
8	No	-/-	Negative	No	Yes	No	5 years	13.3 years
9	Yes	+/+	Negative	No	No	Yes	41 days	41 days
10	No	+/+	Positive	Yes x 2	Yes	Yes	2.5 years	2.5 years
11	Yes	+/+	Positive	Yes x 1	No	No	4.3 years	12 years
12	Yes x 2	+/+	Positive	Yes x 2	Yes	No	2 years	13.5 years
13	No	+/+	Positive	Yes x 1	No	No	5 years	11.5 years
14	No	+/+	Positive	Yes x 3	No	No	4 years	11.5 years
15	No	+/-	Negative	No	No	No	3.4 years	9 years

Table 6. Characteristics of patients with HHV-6 DNAemia.

5.3 VARICELLA ZOSTER VIRUS AFTER PEDIATRIC RENAL TRANSPLANTATION (PAPER III)

In 85 pediatric renal transplant recipients, 47 children had a pre-transplant history of varicella infection and 38 were vaccinated. The vaccinated children were significantly younger ($p=0.0001$) than the previously infected.

At transplantation the frequency of VZV seropositivity was significantly higher in patients with a history of varicella infection (94%) than in those who had been vaccinated pre-transplant (50%; $p<0.0001$). Among the seropositive patients the median antibody titer was significantly higher in those with a history of varicella infection compared with those being vaccinated ($p=0.031$, Table 7).

Due to the lack of serum samples after transplantation, seven patients in the infection group and six in the vaccination group were excluded from the serological follow-up. Of the 72 children who were followed serologically until censoring at VZV infection, loss to follow-up, re-transplantation or death, 52 children were seropositive at transplantation; 38 of them had a history of varicella and 14 had been vaccinated. The patients with a history of varicella infection had significantly higher VZV antibody titers than those who had been vaccinated, when studied at the time points of one, two and five years after transplantation ($p<0.0001$; Table 7 and Figure 10).

Only one of the children who was seropositive at transplantation after a previous varicella infection, became seronegative, but 37% of them had a four-fold or greater reduction in antibody levels during the follow-up period. In contrast, 71% of the vaccinated patients who were seropositive at transplantation became seronegative at a median time of 1.7 (IQR 1-4) years after transplantation. After five years' follow-up of the seropositive individuals, significantly more patients in the infection group (97%) remained seropositive than in the vaccination group (28%; $p<0.0001$; Figure 10).

Of the 85 studied patients, 10 developed symptomatic VZV disease during a median follow-up period of 10 (range 0.5-28) years post-transplant. Clinical varicella infection affected eight patients; seven of them had been vaccinated before transplantation. All eight patients presented mild varicella infection with no or low grade fever, a moderate amount of skin lesions and no other complications. Two patients experienced herpes zoster during follow-up and they both belonged to the pre-transplant varicella infection group.

Time after transplantation	History of varicella infection	Vaccinated before transplantation	p-value between groups
	VZV IgG titer median (min; max)	VZV IgG titer median (min; max)	
At start	n=38	n=14	
	3200 (200; 12800)	1200 (200; 6400)	0.031
Year 1	n=28	n=12	
	3200 (400; 6400)	150 (50; 1600)	<.0001
Year 2	n=29	n=13	
	3200 (200; 12800)	100 (50; 800)	<.0001
Year 5	n=19	n=10	
	1600 (200; 6400)	150 (50; 800)	<.0001

n= number of patients.
For comparison between groups the Mann-Whitney U-test was used.

Table 7. Comparisons of VZV IgG values measured by ELISA at transplantation and then at one, two and five years post-transplant between the two groups: previous varicella infection and VZV vaccinated pre-transplant. Only the patients that are seropositive at transplantation (at start) are serologically followed. Due to lack of serum samples at the different time-points after transplantation, the number of patients vary. The patients are lost to serological follow-up for different reasons such as VZV infection, re-transplantation or death.

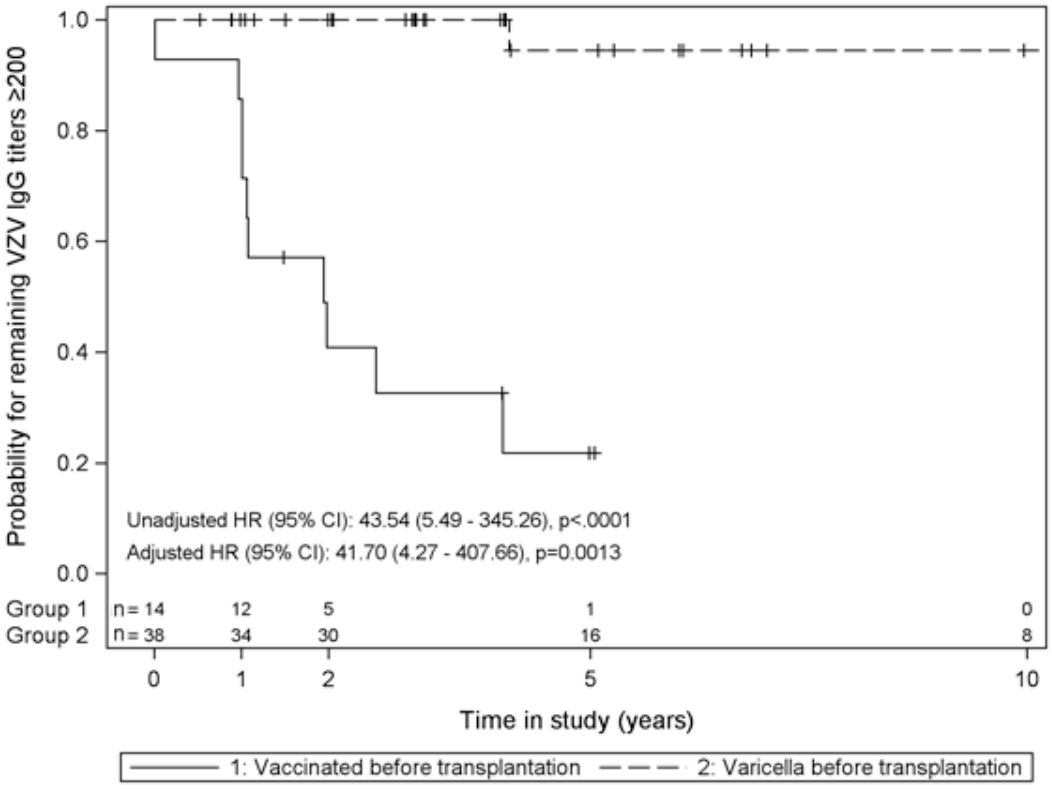

Figure 10. Persistence of a VZV antibody titer of ≥ 200 after renal transplantation.

Only seropositive individuals were included at start. indicates censored patients at re-transplantation, symptomatic or asymptomatic VZV infections, death and loss to follow up.

n=Number of patients at risk of becoming seronegative at each time point; CI=Confidence interval and HR=Hazard ratio

5.4 EPSTEIN-BARR VIRUS AFTER PEDIATRIC RENAL TRANSPLANTATION (PAPER IV)

The 58 renal transplant children included in this study were divided into two groups; chronic high EB viral load (CHL) and non-chronic high load (non-CHL). The non-CHL group was further divided into two groups; low EB viral load (LVL) and undetectable viral load (UVL). LVL included children not meeting the criteria for neither UVL nor CHL. At transplantation, 31 (53%) of the recipients lacked EBV IgG and 25 (81%) of them developed primary EBV infection after transplantation. Of the 27 (47%) seropositive patients, 20 (74%) experienced reactivation of EBV after 12 (0-64) days.

Altogether, 14 (24%) patients developed CHL, 31 (53%) LVL and 13 (22%) UVL. The development of CHL started at a median of 69 (0-278) days post-transplant. The children in the CHL group had a median follow-up time of 7.4 (0.6-12) years after high EBV load was diagnosed. Even though the immunosuppressive treatment were reduced they had a median CHL duration of 2 (0.5-6.5) years. They were younger at transplantation (HR 0.74 [95% CI 0.63 to 0.87], p=0.0002) and had a higher rate of congenital anomalies of the kidney and urinary tract (CAKUT) as underlying renal diagnosis (HR 3.92 [95% CI 1.23 to 12.51], p=0.021) compared to children that did not develop CHL (Figure 11 and Table 8). When adjusting for age the difference between CHL and non-CHL group was not significant regarding CAKUT (p=0.16).
The clinical presentation of EBV infection in the CHL-group was in most cases asymptomatic or unspecific. With immunosuppression kept at a minimum level, three out of 14 patients were treated for rejection and one was re-transplanted because of graft failure. No patient developed PTLD during the post-transplant follow-up time of 7.8 (0.7-13) years.

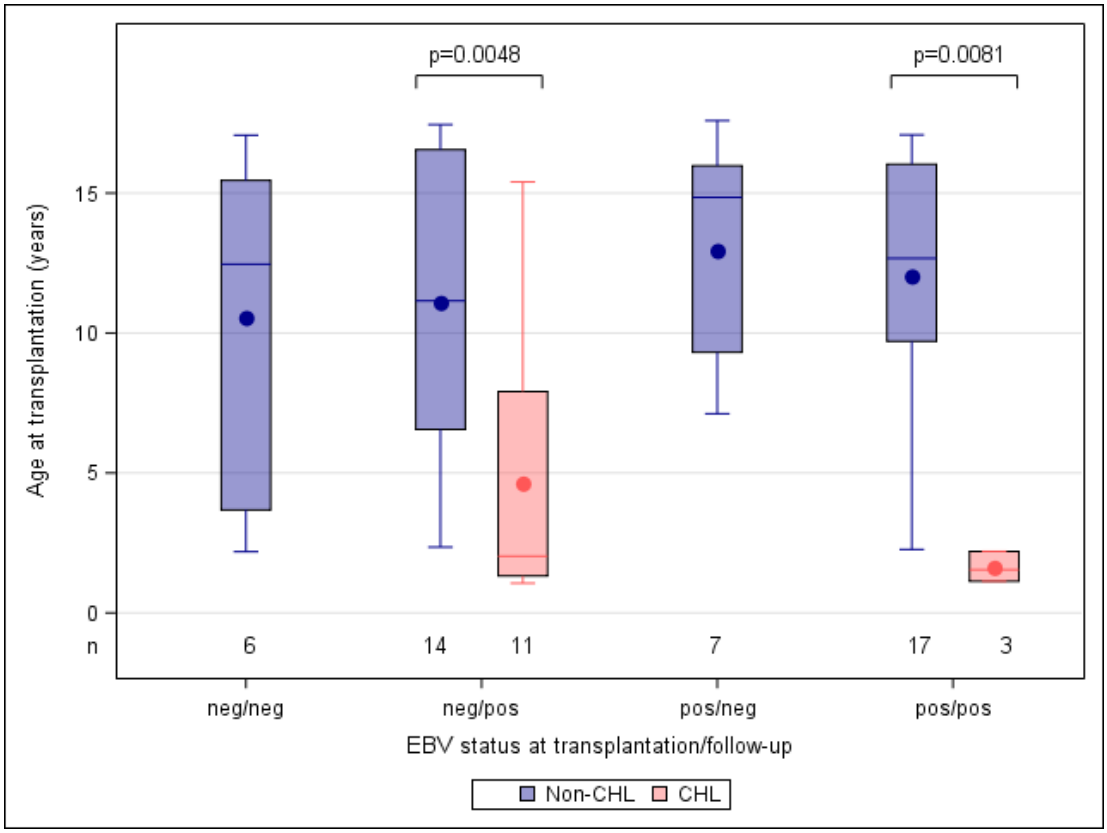

Figure 11. Distribution of age at transplantation for kidney recipients with different EBV status.
Younger age and more EBV naive children at transplantation (tx) are seen in the CHL group in red compared to the non-CHL group in blue. Fourteen non-CHL and 11 CHL patients had a primary EBV infection. Seventeen non-CHL and three CHL patients had a reactivated EBV infection. Seven non-CHL patients that were EBV seropositive at transplantation stayed negative in EBV DNA measured by PCR post-transplant.
neg/neg means seronegative at transplantation/EBV DNA negative during follow up
neg/pos means seronegative at transplantation/EBV DNA positive during follow up
pos/neg means seropositive at transplantation/EBV DNA negative during follow up
pos/pos means seropositive at transplantation/EBV DNA positive during follow up

n=Number of patients, **-** *in columns indicates median and* ● *in columns indicates mean*

Variable	Category	n (%)	HR (95% CI)	p-value
Age at transplantation (years)	≤10	13 (44.8)		
	>10	1 (3.4)	0.74 (0.63:0.87)	**0.0002**
Sex	Male	10 (34.5)		
	Female	4 (13.8)	0.34 (0.11:1.10)	0.072
CAKUT	No	4 (12.1)		
	Yes	10 (40.0)	3.92 (1.23:12.51)	**0.021**
HLA mismatch	0-2	5 (19.2)		
	3-4 (vs 0-2)	8 (34.8)	1.89 (0.62:5.79)	ns
	5-6 (vs 0-2)	1 (11.1)	0.56 (0.07:4.82)	ns
Living donor	No	1 (7.1)		
	Yes	13 (29.5)	4.61 (0.60:35.30)	ns
Dialysis before tx	No	5 (21.7)		
	Yes	9 (25.7)	1.28 (0.43:3.81)	ns
Diagnosis	CAKUT	10 (40.0)		
	Hereditary disorders vs CAKUT	3 (16.7)	0.35 (0.10:1.28)	ns
	Acquired diseases vs CAKUT	1 (7.7)	0.16 (0.02:1.27)	0.084
EBV mismatch (D+R-)	No	3 (15.0)		
	Yes	10 (35.7)	2.56 (0.70:9.32)	ns
EBV serology in recipients	Positive	3 (11.1)		
	Negative	11 (35.5)	3.55 (0.99:12.76)	0.052

Table 8. Univariate analysis of risk for CHL in cohort of renal transplant recipients. CAKUT=Congenital anomalies of the kidney and urinary tract, CHL=Chronic high load, HLA=Human leucocyte antigen, n=Number of patients and tx=Transplantation

Antiviral prophylaxis was given for 6 months post-transplant to 21 children and for 3 months to 24 children. Among the 58 patients, 35 (60%) of the recipients were CMV seronegative prior to transplantation. In the 25 patients who experienced CMV DNAemia, nine episodes (36%) of CMV DNAemia were recorded during the prophylaxis period but in 16 (64%) patients the CMV DNAemia started 7-9 months after transplantation, soon after the antiviral prophylaxis was ceased. CMV DNAemia was more common in the CHL group (57%) compared to the LVL (28%) and the UVL (22%) groups. Primary CMV infection before or at the same time as EBV DNAemia (+/- one month) was seen in three patients (2 CHL and 1 non-CHL) in the cohort.

Twenty-three patients remained CMV sero- and CMV DNA negative during follow up.

Clinical symptoms that could be caused by CMV infection/disease developed in six CHL and four non-CHL patients. Of these, only three patients received antiviral treatment against CMV infection, two had primary infections and one reactivated, all belonging to the non-CHL group. One of these patients had leukopenia, diarrhea and proctitis, and therefore, CMV tissue invasive disease was highly suspected. In addition, three patients had EBV and CMV related symptoms. These patients presented only mild forms of infection except for the third patient who died 9 months after transplantation due to bacterial pneumonia and multi-organ failure while having high EBV DNA levels in blood and concomitant CMV DNAemia.

6 DISCUSSION

Transplantations of stem cells and solid organs have been established during the latest 70 years as active and often curable treatments of patients suffering from serious and frequently life-threatening diseases. The main goal with this thesis was to improve the knowledge of how to protect transplanted patients from infections caused by some of the viruses belonging to the herpes family.

6.1 CYTOMEGALOVIRUS AFTER ALLO-SCT (PAPER I)

This retrospective, single-center study of 97 adult allogeneic SCT patients describes the morbidity and mortality of 60 patients that developed CMV DNAemia post-transplant compared to 37 patients that stayed CMV DNA negative. A total of 50 patients received CMV treatment, 4 of these because of proven or probable CMV disease.

It is well known that CMV infection and disease cause morbidity and mortality both early and late after allo-SCT (175-177). We found, however, that patients with diagnosed CMV DNAemia showed an overall improved survival ($p=0.026$), suggesting a positive effect of the CMV monitoring and the pre-emptive treatment these patients received to reduce the risk of CMV disease.

Some studies have presented a protective effect of CMV reactivation on relapse of the underlying malignancy, a virus versus leukemia effect with lower relapse mortality in CMV reactivating patients (178-180). However, this CMV benefit was nullified by the increased non-relapse mortality (NMR) in patients with CMV viremia (177, 179, 180). In agreement with another study (181), we could not find a significant difference in relapse mortality or NMR between patients with CMV DNAemia and those who stayed CMV DNA negative. However, there was a tendency towards statistical significance ($p=0.06$) in NMR between the different D/R groups with the highest incidence of NRM in the D+R- group with the patients who are at highest risk of CMV viremia.

Our retrospective quantitative CMV DNA analyses performed on 25 available serum samples from 11 patients were inconclusive but the association of a high

level of CMV viremia with CMV disease and even death would have been interesting to study if possible. Others have demonstrated that high viral load is associated with an increased risk of disease but only if the patients are not receiving pre-emptive therapy. These results confirm our findings of the efficacy of pre-emptive therapy (182-184).

Also, CMV DNAemia has been associated with the pathogenesis of invasive bacterial and fungal infections as well as with graft versus host disease (GVHD) (185, 186). The role of GVHD as a risk factor for CMV disease is well known (187-189). The data in our cohort are in line with these studies and confirm the association between GVHD and CMV DNAemia, infection and disease. Proven and probable CMV disease was diagnosed in four of our patients and in three of them, the debut of CMV disease was related to cortisone treatment for GVHD.

When new treatments against malignant diseases arise, new challenges in CMV management strategies are needed and new diagnostic techniques including monitoring of the CMV-specific immune response needs to be studied. Additionally, the management and treatment of CMV infection and disease with viruses resistant to the first-line treatment (ganciclovir and valganciclovir) is particularly challenging, as alternative drugs (foscarnet and cidofovir) carry significant toxicities. New drugs for refractory CMV infection and disease possessing a better toxicity profile are eagerly awaited in transplanted patients.

6.2 HUMAN HERPESVIRUS TYPE 6 AFTER ALLO-SCT (PAPER II)

Human herpesvirus type 6 was not monitored regularly in the cohort studied in paper I and is not recommended in guidelines for management of patients after allo-SCT. In 54 of the 97 patients, at least one sample was analysed for HHV-6 DNA because of symptoms or because of the treating clinician's suspicion of HHV-6 reactivation. HHV-6 DNAemia was detected in 15 patients and 9 of these received empirical antiviral treatment for proven or probable HHV-6 infection. Three patients with antiviral treatment against HHV-6 died but HHV-6's role could not be established retrospectively. There was no difference

in the overall survival between the HHV-6 negative and HHV-6 positive patients.

There are previous investigations where HHV-6 has been monitored regularly after allo-SCT. These reports all indicate that HHV-6 is a common pathogen (65, 71, 190-193). Furthermore, reactivated HHV-6 is reported in 33 to 48% of patients after allo-SCT (71-73). Although the virus is believed to cause clinical disease, data are limited and a clear-cut syndrome has not yet been established (64, 192). In our patients, HHV-6 reactivations were associated with clinical symptoms such as fever, skin exanthema, CNS symptoms, GI disease, elevated liver enzymes, and pain in joints and muscles leading to assessment of HHV-6 infection. With the exception of encephalitis, criteria for initiating antiviral therapy for HHV-6-related manifestations are not well established (194). It is known that asymptomatic reactivation is common after SCT, but HHV-6 replication has also been linked to fever and rash (65) as well as to life-threatening disease in liver (66), lung (67), brain (66, 68, 69) and to cause bone marrow suppression (66, 70). These organ diseases and the long duration of bone marrow suppression make it easier for other viral infections to infect or to reactivate, including reactivation of CMV (71). We found that eight of 15 (53%) HHV-6 DNAemia patients were also treated with antivirals against CMV infection or disease, compared with 50 patients (51%) in the whole cohort (n=97). Like Dulery et al, we found no association between HHV-6 and CMV reactivation (193), in contrast to a few small series of allo-SCT patients that have suggested associations between reactivations of HHV-6 and CMV (71, 195, 196).

It is also reported that HHV-6 can cause bone marrow suppression, pneumonitis, encephalitis, myelitis, GI symptoms and even cause a higher rate of kidney rejection as well after pediatric renal transplantation (74-76). A causative relationship between HHV-6 and these complications is, however, not well established.

HHV-6 differ from other human herpesviruses due to its unique ability to integrate genome into human chromosomes resulting in a persistent latent state. This chromosomal integration enables HHV-6 to be transmitted from parent to child in the germ line (54-58). This causes diagnostic pitfalls since such an integration of viral sequences in every leukocyte easily is identifiable and persistent high levels of HHV-6 DNA in both whole blood and serum is detected in asymptomatic patients (59-61).

So, HHV-6 is a recognized pathogen in transplant recipients despite the considerable problems in the interpretation of available data. Antiviral

prophylaxis or pre-emptive treatment to prevent HHV-6 disease after transplantation is not recommended given the low risk of HHV-6 disease together with the toxicity of antiviral drugs such as ganciclovir (146). The role of HHV-6 reactivation in morbidity and mortality after allo-SCT remains unclear and further studies are needed. HHV-6 reactivation, clinical manifestations and efficacy of available therapeutic approaches are also important future projects.

6.3 VARICELLA ZOSTER VIRUS AFTER PEDIATRIC RENAL TRANSPLANTATION (PAPER III)

In our investigation of 85 children subjected to renal transplantations, 38 had been vaccinated against VZV before transplantation. Vaccinated children were significantly younger and had a lower VZV IgG titer at transplantation than children with natural varicella infection pre-transplant. Interestingly, we could show a significant difference in VZV seropositivity 5 years after transplantation, when 97% in the group with previous varicella infection still were seropositive compared to only 28% in the vaccinated group.

Other studies have also described the occurrence of varicella in pediatric renal transplant patients with a previous history of either varicella infection or vaccination prior to transplant (197-201). Broyer et al. studied pediatric renal transplant candidates who were seropositive after the natural varicella infection and found that they lost VZV antibodies after grafting: 0.4% after one year, 2.8% after two years and 4.5% after five years (84). These numbers are in agreement with what was found in our study, where only one of 38 children lost seropositivity about four years post-transplant. Broyer et al. also reported that among varicella-vaccinated children who underwent renal transplantation and were seropositive at transplantation, 7% had lost VZV IgG after one year, 11% after two years and 24% after five years (84).

VZV is an airborne virus and therefore highly contagious. Varicella infections are common in children where routine varicella vaccination is not introduced and hence, difficult to avoid for our vulnerable immunosuppressed patients. It is well documented that vaccination, prophylactic antiviral treatment after exposure and antiviral treatment of VZV infections are very important and life-

saving strategies (202-205). Even though we found that VZV vaccination protected from symptomatic disease to a lesser extent than natural infection, it seemed to provide protection from life-threatening disease.

In addition, antibody levels considered protective for healthy children may not prevent infection in children suffering from chronic renal insufficiency or in immunosuppressed transplant recipients, where the immunosuppressive treatment is a lifelong necessity (18, 84, 206). This treatment reduces both humoral immunity and T-cell-mediated immunity. A T-cell-mediated immunity is needed to eliminate intracellular pathogens such as VZV. However, B-cells producing antibodies, appear to supplement protection by the cell-mediated immunity, as demonstrated by the success of passive immunisation with specific immunoglobulin against VZV (207). Another aspect has been presented by Gershon et al. who studied three patients that died of VZV disease. They were seropositive but showed poor or no cellular immunity against VZV, hence, the importance of cellular immunity (208).

After transplantation the live attenuated varicella Oka vaccine can cause disseminated disease and even death in immunosuppressed individuals (82, 83, 209). There are, however, previous studies on both liver and renal transplanted children who received varicella vaccine post-transplant (85, 210-215). Because of the risk of giving live attenuated vaccines to immunosuppressed individuals, one possibility is then to vaccinate family members who are VZV seronegative in order to prevent immunosuppressed individuals from becoming infected. When more countries introduce routine varicella vaccination to children, the majority of children undergoing renal transplants will be vaccinated as well as their siblings and schoolmates. In a study published in 2017, the VZV infection was demonstrated to produce lifelong immunity in healthy individuals, whereas seropositivity after VZV vaccination was estimated to decrease by 8% for each year that had elapsed since vaccination (216). The effect of the varicella vaccine as a routine immunisation of healthy children world-wide, also in Sweden, is still however, under consideration.

When the promising new glycoprotein E subunit varicella zoster vaccine now becomes available, more opportunities arise in the future. Comparing the new gE subunit zoster vaccine with the Oka varicella vaccine Leroux-Roels et al. found that both humoral and cell- mediated responses were higher for up to 42 months after administration in healthy adults, in favour of the subunit vaccine (217). These results of Leroux-Roels et al. from 2012 have been confirmed in two investigations by Chlibek et al. from 2014 and 2016, where the subunit vaccine was found to be immunogenic and well tolerated in healthy adults aged $\geq$60 years with good humoral and cellular response persisting for 6 years after two-dose vaccination (218, 219). Although the cellular and humoral responses

decreased over time, they remained substantially above pre-vaccination levels after 6 years (219). Larger investigations and clinical efficacy studies will be needed. Also, investigations regarding the possibilities to use the gE subunit zoster vaccine in children and in immunosuppressed transplanted individuals would be desirable and most valuable.

6.4 EPSTEIN-BARR VIRUS AFTER PEDIATRIC RENAL TRANSPLANTATION (PAPER IV)

Studies during the latest two decades have reported EBV-associated complications after transplantations. In these situations when cellular and humoral responses are reduced due to immunosuppressive treatment the much feared PTLD has been associated with EBV (95-98).

The three main risk factors linked to PTLD in pediatric graft recipients are:

1/ EBV-seronegative recipient when receiving a kidney from an EBV seropositive donor and later acquiring a primary EBV infection, **2/** presence of a concomitant primary cytomegalovirus (CMV) infection and **3/** the overall burden of immunosuppressive therapy (90, 97, 101-103).

In our study of 58 renal transplanted children, 53% were EBV IgG seronegative at transplantation and in 81% of them a primary EBV infection developed post-transplant. Altogether, we found that CHL carriage after renal transplantation occurred frequently (24%), was often long lasting and developed mainly in younger children. No child developed PTLD during a median clinical follow-up of almost 8 years.

In a similar study of 30 liver transplanted children with a median age of 2 years at transplantation, primary EBV infection occurred in 87% during the first year post-transplant among the children who were EBV seronegative at transplantation (220). In the whole cohort, 42% developed CHL during the year following transplantation and they did not report any case of PTLD (220).

In our material, the frequency of CHL carriers was higher than the 8% described by Yamada et al. (221), which to some extent might be due to the relatively small study group but also to our more frequent EBV DNA measurements undertaken during the first years post-transplant. The median

time to onset of CHL in our cohort was 69 days post-transplant which is shorter than the 104 days and 228 days in previous studies (221, 222). The median CHL duration of 2 years is similar to the results of other studies, but the follow-up time of almost 8 years is longer than other comparable studies (221, 223, 224). In a previous multicentre-study, 2% of PTLD was reported following renal transplantation (225). In our study of 58 children, there was no case of PTLD, which also might be due to the limited study size.

Green et al. (226, 227) have described the chronic high EB viral load as occurring more often after primary EBV infection. Accordingly, in our study, out of 28 EBV mismatch (D+/R-) patients, 25 (89%) developed a primary EBV infection within 2 months after transplantation and 11 (44%) progressed to CHL. In our CHL group, these 11 out of the 14 patients were seronegative at transplantation and had a primary EBV infection post-transplant. The remaining three children who developed CHL had their first positive test for EBV at or shortly before transplantation. They were classified as EBV reactivation according to protocol, but might have had a prolonged primary EBV infection at the time of transplantation. Thus, most children who developed CHL experienced a primary EBV-infection post transplantation, but this was not a statistically significant independent risk factor in our material when adjusting for age.

Eight of the 14 CHL patients were also co-infected with CMV. A similar frequency was seen in the non-CHL group. Primary CMV-infections were seen in 36 % in the CHL-group compared to 18% in the non-CHL group. This is consistent with the results of previous investigations where primary CMV infection is described as a risk factor for CHL and PTLD (90, 97, 102).

In addition to the three main risk factors for developing PTLD presented above a high EBV DNA replication has also been recognized (104). However, whether a long term high level of EBV load, CHL, constitutes a valid predictive marker for the later development of EBV-related PTLD remains unclear. In previous presentations of renal transplant recipients with high EB viral load a development of PTLD was not reported (105, 228). This is in agreement with a report by Qu et al, who noted that some SOT children with rising EB viral load remained in a CHL carrier state without developing PTLD (229). Hence, the presence of a high or persistent EBV load alone in pediatric renal transplant recipients does not appear to be predictive for later development of PTLD. Therefore, other possible risk factors ought to be considered such as intensity of immunosuppressive therapy (230), the EBV virulence (231), the nature of EBV-infected B-cells (232), EBV-specific T-cell

response (233), and genetic predisposition (234). However, we were not able to retrospectively evaluate these proposed additional risk factors.

Obviously, despite the association between EBV infection after renal transplantation in children and the risk of developing PTLD, there is still no consensus on viral load monitoring or the benefits of specific EBV chemoprophylaxis/treatment in this population. In other words, there is no universally accepted approach for the management of post-transplant EBV infections. Reduction of the total burden of immunosuppression is one therapeutic option. In the present study, immunosuppression was reduced in all 14 patients in the CHL group and also in several of the other patients due to short term high EBV loads. The use of antiviral agents to prevent EBV infection in pediatric patients with EBV seroconversion is a controversial option (96, 159, 226). Our patients did not receive any antiviral therapy.

As the number of young renal transplant recipients are increasing and EBV-associated PTLD has been reported to be higher in children than in adults (235, 236), there are concerns for the future. Higher rates of EBV-seronegative individuals receiving transplants might result in an increased prevalence of EBV-associated PTLD (237). Another related aspect is that primary EBV infection after transplantation, as previously described in young individuals, increases the risk of chronic EBV-associated diseases such as PTLD, Burkitt and Hodgkin lymphomas, non-Hodgkin lymphomas, stomach and nasopharyngeal cancers (238-241). The risk of EBV causing malignant diseases is low but increases in immunosuppressed individuals. Chemotherapy, anti-B-cell treatment and reduction of the immunosuppressive regimen remain the main treatments for EBV-associated malignancies. Infusions of human leukocyte antigen-matched EBV cytotoxic T-cells as a strategy for prophylaxis and treatment of EBV-induced lymphoproliferative disorders has been tried, but is very time consuming and labor intense. Identifying factors responsible for acquisition of the virus as well as EBV vaccine development would be important steps to improve public health. Researchers from Fred Hutchinson Cancer Research Center, Joost Snijder and Andrew T. McGuire et al published an article in 2018 where the first human antibody found to block EBV was presented (242). The finding of the antibody, along with its target sites, opens a new pathway for developing an effective vaccine against EBV. This is an important field and a fertile area for future research in prevention of EBV-associated diseases.

7 CONCLUSIONS

Based on the results in this thesis it was concluded that:

- An increased risk for CMV DNAemia after allo-SCT was found in patients with a seronegative donor to a seropositive recipient. Patients with diagnosed CMV DNAemia had improved survival suggesting a positive effect of CMV monitoring and pre-emptive antiviral treatment (paper **I**).

- The survival rate in allo-SCT patients with HHV-6 DNAemia was comparable to the HHV-6 DNA negative patients and to the whole group of allo-SCT patients. Hence, the indication for routine screening of HHV-6 DNAemia seems to be weak (paper **II**).

- Varicella vaccination protected from symptomatic VZV disease to a lesser degree than natural infection, but provided effective protection from life-threatening disease in pediatric renal transplant recipients (paper **III**).

- Previously varicella-infected patients more often reactivated herpes zoster while those who were vaccinated developed varicella (paper **III**).

- CHL was frequent (24%), long lasting and occurred mainly in younger renal transplant recipients (paper **IV**).

- The absence of PTLD after renal transplantation suggests that monitoring of EBV DNA to guide immunosuppression may be effective but additional markers to identify patients at risk for PTLD are warranted (paper **IV**).

8 FUTURE PERSPECTIVES

Although this thesis adds knowledge on certain herpesvirus infections after allo-SCT and kidney transplantation, there is still much to explore in the field. Various questions remain to be answered regarding interactions between microorganisms and the host. It would be of great interest to further investigate which host genetic markers and immune parameters that predispose for severe opportunistic herpesvirus infections. This kind of new information might perhaps increase the possibilities to prevent complications and to offer our patients the best of treatments.

Further studies that are of certain interest regarding new treatment and/or prophylaxis management against herpesviruses are already in the pipeline. Examples of such planned investigations are: multicenter studies on CMV and EBV after transplantation, new vaccines against CMV, VZV and EBV and perhaps most importantly the development of new antiviral drugs against herpesviruses. In particular, new drugs for refractory CMV infection and disease possessing a better toxicity profile are eagerly awaited for transplanted patients.

In addition, the powerful genome editing technology CRISPR-Cas9 has increasingly been used to treat human diseases. Applying this technique also in the field of immunology will most likely, in the not too distant future, result in innovative, novel and improved possibilities for monitoring and treatment.

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