CHAPTER 1

General Introduction
INTRODUCTION

Renal transplant recipients
For patients with end-stage renal disease, renal transplantation provides significant survival and quality-of-life benefits over dialysis. In the last decades graft survival in adult recipients has increased, due to improved surgical techniques, development of new and more potent immunosuppressive drugs and better post-transplantation care. However, worldwide waiting lists are growing partly because people are getting older. The use of expanded criteria donors (ECD) could contribute to reducing the waiting list. ECDs are donors over 60 years of age, having a history of hypertension, creatine level >1.5mg/dL or cerebrovascular cause of death. Although the risk of graft failure in recipients from ECD is >70% higher than after living or deceased donors, it still benefits over dialysis. Since the last century, the use of ECD has become common practice.

In the Netherlands, the number of patient on the waiting list, for kidney transplantation, increased with 9.0% in 2017 (n= 650) compared to 2016 (n= 595). Before 2017 a steady decrease was observed with 622 patients on the waiting list in 2014 and 544 patients in 2015.

Immunosuppressiva
Over the last decades immunosuppressive drugs and management has improved, which has led to a transplant survival rate of 95% in the first year after transplantation. Especially, less acute rejection (<15%) is seen in the first year, while long-term survival rates and rejection episodes remained unaltered mainly due to the toxic side effects of the immunosuppressants and increased acceptance of more marginal donors and recipients.

The regimens after kidney transplantation consist of a combination of different immunosuppressive drugs, as they have synergistic efficacy leading to lower dosages of individual drugs. Most transplantation centers use quadruple therapy, with an interleukin-2 receptor antibody for induction followed by immunosuppressive maintenance therapy (i.e. calcineurin inhibitor, mycophenolate and steroids). The function of induction therapy is to reduce the risk of rejection. Mostly basiliximab is used as interleukin-2 receptor antagonist, while T cell depleting agents as ATG, thymoglobulin or alemtuzumab are being used in high risk patients. Maintenance therapy can be categorized into calcineurin inhibitors (cyclosporine A, tacrolimus), costimulation blockers (betacepts), mammalian target of rapamycin inhibitors (sirolimus, everolimus), antiproliferatives (azathioprine, mycophenolic acid derivatives) and corticosteroids.
General Introduction

To improve long-term outcome selection of appropriate immunosuppressive regimens is important and ideally should be patient tailored. Taking the patients pre-existing disease, risk of rejection, drug to drug interaction and interaction with other medication into consideration.

Non-infectious complications after kidney transplantation

Short term complications of organ and patient survival after renal transplantation have decreased over the last decade, whereas long term (beyond 5 years) complications have remained unaltered. This progress is mostly the result of the introduction and use of new and more potent immunosuppressive drugs in the last two decades. The drawbacks, however, are the side effects, such as nephrotoxicity, development of diabetes, hypertension, increased susceptibility to tumor formation and infection.

One of the major complications after renal transplantation is rejection. It can be classified in different stadia; hyper acute (occurring within minutes), acute (occurring within days to weeks), late acute (occurring after 3 months) or chronic (occurring months to years after transplantation). Besides the different stadia, rejection can be divided into antibody-mediated or T-cell mediated rejection. The last one has become less frequent through the usage of effective T-cell directed induction/immunosuppression and is mostly reversible with limited impact on long-term outcome. Antibody-mediated rejection results more in graft loss, and no adequate immunosuppressive treatment is currently available. To reduce the immunological risk of humoral rejection, it is important to identify the HLA antibodies of the recipient and donor before and after transplantation.

Whereas short term outcome has improved long-term outcome is still similar and an important long-term complication is chronic allograft dysfunction. Causes of allograft dysfunction could be immunological as highlighted above or non-immunological (chronic CNI toxicity, infection, chronic obstruction, recurrent or de novo glomerular diseases, recurrent or de novo diabetic nephropathy, hypertensive nephrosclerosis and renal artery stenosis). Chronic allograft dysfunction occurs slowly with progressive loss of renal function and is mainly diagnosed through slow rising serum creatinine levels, increasing proteinuria and worsening hypertension.

Infectious complications

Adequate transplant function, hence prevention of organ rejection, depends on lifelong immunosuppression, making transplant recipients more prone for opportunistic infections, resulting in disease and mortality.
The highest risk of infections is during the first six months after transplantation, due to the peak of immunosuppression in this period. Viral and non-viral infections can occur simultaneously. Most common post-transplant opportunistic infections can be divided in viral (CMV, HSV, VZV, EBV and BKPyV), fungal (Candida species, Aspergillus species, Pneumocystis jiroveci, C. neoformans, and mucormycosis), bacterial (Nocardia species, L. pneumophila, L. monocytogenes, and mycobacteria) and parasitic (T. gondii and strongyloidiasis).

Many viral infections are the result of reactivation of latent viruses from either within the host or from the graft. Beside reactivations of latent viruses, also viral infections through community exposure may occur.

Diagnosis is mainly through testing and imaging, as clinical signs and symptoms of these infections may overlap and are often atypical, subtle or absent. Pre-transplant, all potential donors and recipients are being screened, serologically, for most common viral pathogens. These results can subsequently be used to determine the appropriate prophylaxis and preventive strategies, i.e. update vaccination status and educate patient and family about preventing measures. Post-transplant screening by real-time polymerase chain reaction (real-time PCR) for viral pathogens is performed to detect and subsequently treat active infections.

To obtain the best patient and graft outcome, it is challenging for the treating clinical physicians, to balance between over and under immunosuppression.

The most common viral infections in renal transplant recipients are with EBV, CMV and BKPyV. Therefore, the following paragraphs will focus on these viruses.

**EPSTEIN-BARR VIRUS**

Epstein-Barr virus (EBV) belongs to the gammaherpesviruses and is an ubiquitous DNA virus that infects up to 90% of the world’s population, mostly during childhood or early adolescence. Infection is often asymptomatic in immunocompetent individuals, but may result in a self-limiting, benign lymphoproliferative syndrome called infectious mononucleosis (Pfeiffer).

Following a primary infection, EBV establishes lifelong latency in memory B-cells. Asymptomatic reactivation in immunocompetent individuals is common. Cell-mediated immunity is essential for controlling primary EBV infection and latent infection.
**EBV infection**

In solid organ transplant (SOT) patients, EBV-specific cell-mediated immunity is depressed by the use of immunosuppression, which subsequently may lead to the development of uncontrolled EBV-driven B-cell proliferation and malignant transformation. In addition, suppressive cytokines such as IL-10 have also been implicated as initiating factors supporting the development of this post-transplant lymphoproliferative disorder (PTLD) after SOT. It is a serious and often fatal complication, with mortality rates up to 70%. Of the PTLDs in immunosuppressed SOT recipients, 90% are EBV positive. PTLD may present itself with a diverse spectrum of clinical symptoms and signs. Symptoms may be similar to those seen with primary infection, whereas also other organs including the central nervous system, bone marrow and internal organs may be involved. PTLD may arise at any given time after transplantation. The highest incidence, however, is in the first year after SOT, i.e. early-onset PTLD, hence during the most immunosuppressed state, and is highly associated with a primary EBV infection. Late-onset PTLD is independent of the EBV sero-status (primary infection or reactivation) or immune suppression.

Overall incidence of PTLD is 1-2%. However, this incidence strongly depends upon the pre-transplantation EBV sero-status, the type of organ transplanted, and the immunosuppressive regimen. EBV naive recipients with an EBV positive donor have a 10 to 75-fold increased incidence of developing PTLD compared to EBV sero-positive recipients. This is due to the fact that they are unable to initiate an adequate EBV-specific CTL response. The incidence of PTLD varies between different SOT recipients and is relatively low in renal transplant recipients (RTR) (1-5%) compared to heart and lung (2-10%) and in intestinal and multivisceral transplant recipients (5-20%). These percentages may be a reflection of the intensity of immunosuppressive regimens needed in these different groups and the quantity of lymphoid tissue present within the transplanted organs. As for the immunosuppressive regimen, the use of tacrolimus may give a 2 to 5 fold increase in the risk of developing PTLD. Use of OKT3 or ATG for prophylaxis against or treatment of acute rejection was associated with a 3 to 4 fold increase in the incidence of PTLD.

**Diagnosing EBV infection**

There are several non-invasive diagnostic assays which can be used in diagnosing of EBV infection or PTLD development in renal transplant recipients. First, serological assays are most useful for determining the pre-transplantation sero-status. For this, enzyme-linked immunosorbent assays (ELISAs) or commercially available EBV enzyme immunoassays (EIAs) are available. Secondly, after transplantation the detection of EBV DNA viral load in whole blood, plasma or EBV genome in peripheral blood mononuclear cells by real-time PCR has been
developed to aid in diagnosing an EBV infection and monitor the development of PTLD. The best assays use targets in a highly conserved ORF with no or few false-negatives.  

The implications of EBV viremia, however, after the first year of renal transplantation are not clear. Retrospective studies reporting on the prevalence, patterns and long term outcome of RTR demonstrate divergent associations of EBV viremia with graft loss, biopsy proven acute rejection (BPAR), renal function and other infections. Recent studies focus on the progress on how EBV utilizes miRNA’s for immune invasion. These insights could help in the developing of using EBV miRNA’s as a new prognostic marker for risk stratification for PTLD.  

Besides EBV-diagnostics, 18F-FDG-PET scanning is now standard for PTLD staging by visualizing malignant lymphoma’s. The final diagnosis, however, is always based on histopathological examination. PTLD can be classified into four histological types (early lesions, P-PTLD, monomorphic PTLD and classical Hodgkin lymphoma), whereby the first three are most common and develop the typical morphology.  

Treatment of EBV infection

Once a primary infection with EBV or reactivation is determined, several interventions could be considered. First-line treatment involves reduction of immunosuppressive therapy to restore the cell-mediated immune response, with the unfortunate consequence of increasing the risk of graft rejection. The immunosuppressive protocol can be switched to other immunosuppressive drugs, with potentially less lymphoma outgrowth. Several studies suggest that mTOR inhibitors-based immunosuppression, i.e. everolimus and sirolimus, displays potent inhibitory effect on PTLD-derived cells with maintenance of potent immunosuppressive properties.  

The treatment strategy which is standard of care in treating PTLD is the use of a chimeric human/mouse anti-CD20 monoclonal antibody, Rituximab. Since most of the PTLD is of B-cell lineage, the use of monoclonal antibodies specific for B-cells is a logical therapeutic approach. Rituximab binds to the CD20 antigen present on malignant B-cells, resulting in profound and long-lasting depletion of all B-cells (up to 12 months) and it is also able to elicit strong anti-B-cell tumor responses. Mostly, it is given in adjunction to reduction of immunosuppression or chemotherapy, but also as first line treatment of PTLD. Results of treatment demonstrate a high efficacy.  

Although conclusive evidence of clinical utility of antiviral prophylaxis or therapy is lacking, anti-viral drugs are often considered, especially in recipients with a primary infection with EBV. Acyclovir and ganciclovir are capable of inhibiting lytic viral replication, suggesting that by diminishing the EBV viral load, the risk of PTLD may be reduced in EBV-naïve recipients.
Another way to restore the cell-mediated immune response is to administer either allogeneic or autologous cultured in vitro EBV-specific CTLs. Several preliminary clinical studies show that treatment with autologous EBV-specific CTLs may well be a very promising strategy, especially in EBV-seropositive recipients. These studies have demonstrated a considerable fall in EBV viral loads, a temporary increase in EBV-specific CTL precursors, and up to complete regression of established PTLD \(^{48-50}\). More recently, Haque et al. demonstrated encouraging results in a phase II multicenter clinical trial using HLA-matched allogeneic CTLs for the treatment of PTLD. Upon treatment, a 52% response rate at six months was shown \(^{51}\).

**EBV prevention strategies**

A way to prevent EBV infection would be vaccination. Especially in EBV seronegative recipients, mostly children, exposure to EBV prior to transplantation is currently under investigation \(^{52-54}\). An EBV vaccine is not yet available for general use and whether these vaccines are able to induce a sufficient immune-response in often already immunocompromised patients prior to transplantation, remains to be investigated \(^{55,56}\).

**CYTOMEGALOVIRUS**

Cytomegalovirus (CMV) is the fifth member of the human herpesvirus family and belongs to the betaherpesviruses. Seroprevalence of CMV in the adult population approaches 60% and increases with age \(^{57,58}\). Most commonly, CMV is acquired during childhood to early adulthood. In immune-competent individuals, a primary infection usually manifests as a benign disease. After a primary infection, CMV establishes latency and is able to persist in numerous cellular sites, leukocytes, endothelial cells, renal epithelial cells and salivary glands \(^{59}\). CMV infection is the most common opportunistic infection after renal transplantation. In absence of preventive measures, 40%-100% will develop CMV infection and 67% will develop CMV disease.

**CMV infection**

Lack of an effective CMV specific immunity predisposes RTR to develop CMV infection and CMV disease. Depending on the immunologic status of the transplant recipient, infection either occurs by reactivation of latent virus in response to immunosuppression or as primary infection due to donor-positive-recipient-negative mismatch \(^{60}\).

CMV infection comprises both direct and indirect effects. The direct effects, also known as CMV disease, are associated with high viral loads and can be further categorized as CMV syndrome or as tissue-invasive disease. CMV syndrome may manifest as mononucleosis-like syndrome with fever, malaise and myelosuppression. In about half of the cases, CMV invades an organ system and subsequently causes tissue-invasive disease. This may present as a gastrointestinal
disease (colitis), pneumonitis, hepatitis, myocarditis, retinitis, with a predilection for the transplanted organ. As opposed to the direct effects, the indirect effects are a result of the immunomodulatory effects of CMV infection and independent of active CMV disease. These effects may present, in RTR, as graft rejection or dysfunction, opportunistic infections, co-infection with other viruses, development of cancer or PTLD and increased risk of mortality 61,62.

Most frequently, CMV disease occurs within the first three months post-transplant 63. However, the current use of antiviral prophylaxis against CMV in patients at risk, has resulted in a delayed onset of CMV disease. This so-called late-onset CMV infection occurs after the discontinuation of prophylaxis 61,64. It is most significant in seronegative recipient in combination with a seropositive donor; up to 30% develop late-onset CMV disease at a median of five months post-transplant 65,66.

**Diagnosing CMV infection**

The diagnosis of CMV infection and disease has improved over the years, first by the introduction of the pp65 antigenemia assay and subsequently the quantitative real-time PCR assay. The antigenemia assay is a semiquantitative assay and can provide an estimate of the magnitude of viral load by counting the number of infected cells 67. However, the real-time PCR assays represent a major advance in the diagnosis of CMV, through the ability to quantify the amounts of CMV in a broad range of clinical samples which can be stored beforehand, the short turnaround time, and the reduced risk of contamination using a closed system. Results demonstrate that real-time PCR from whole blood is superior, for detection, monitoring and also for initiation of treatment, to either real-time PCR in plasma or peripheral blood leukocytes 66,68.

CMV serology is useful to determine the CMV status during pre-transplantation screening of recipients and donors to determine the risk of either primary CMV infection or reactivation. Furthermore, seroconversion, in a formally seronegative RTR, can serve as a marker for immune protection against CMV, and may lower the risk of later CMV disease 69. Yet, due to the immunosuppressive medication, the role of serologic testing post-transplant is debated.

Another method is the detection of CMV specific cell-mediated immunity (CMI), as this has the ability to control viral replication. Detection of CMI can help to determine individuals at risk of an infection and to use as an indicator in therapeutic decisions. Several tests are available for measuring CMI, but only the Enzyme-linked Immunosorbent Spot (Elispot) and the Quantiferon Gamma Interferon-releasing assay are practical for diagnostic use. These tests are standardized, cost-effective and rapid, compared to the other tests, which are only used in specialized laboratories 70,71.
For diagnosing tissue-invasive disease histopathological examination of biopsy specimens can be used. By using a hematoxylin-eosin stain the presence of giant cells with typical intracellular viral inclusions, characteristic “owl’s-eye” inclusion bodies, may be demonstrated. This technique has a high specificity for disease, and hence a low sensitivity.

**Treatment of CMV infection**
Current therapy for CMV disease consists of ganciclovir, foscarnet, cidofovir, valganciclovir, hyperimmune globulin, or combinations thereof. Foscarnet and cidofovir are being considered second-line therapies due to their associated toxicities. The first line of treatment for CMV disease remains ganciclovir intravenously, while in mildly asymptomatic cases of CMV disease and also in a prophylactic setting, valganciclovir has shown to be an effective alternative.

Upon treatment, the viral load of CMV should decline. A “nonresponding” viral load may be a marker of drug-resistant CMV. To date, the incidence of ganciclovir-resistant CMV is low 0%-3% in recipients with CMV prophylaxis. In the population with a positive CMV donor and negative for CMV recipient the incidence is 5-12%. Resistant strains can be confirmed using genotypic and/or phenotypic methods. Most commonly, a mutation is found within the CMV UL97 (protein kinase) gene, making CMV resistant to ganciclovir, however, the virus remains susceptible to foscarnet and cidofovir. In the case of a mutation in UL54 (DNA polymerase) gene, CMV is resistant to all before mentioned drugs.

**Prevention of CMV**
There are two main strategies to prevent CMV disease, universal prophylaxis and preemptive therapy. Universal prophylaxis is usually directed in patient where baseline risk of disease is high. The antiviral drugs are given from the time of transplant onwards, mostly for duration of three to six months.

The second strategy, preemptive therapy, is mainly reserved for patients at imminent risk for developing CMV disease. Peripheral blood samples are monitored on a weekly basis for the presence of CMV. Treatment is initiated after the first detectable CMV viral loads and before the onset of clinical symptoms.

Most transplant institutes give CMV prophylaxis after renal transplantation. Both strategies have proven to be effective in preventing CMV disease. However, there is increasing evidence that prophylactic treatment is beneficial and improves long-term graft and patient outcomes. In addition CMV prophylaxis with ganciclovir or valganciclovir suppress HHV6 replication.
Another issue that should be addressed is the optimal duration of prophylaxis. There is accumulating evidence that extension of the duration (200 days instead of 100 days) of prophylaxis may protect against this late onset CMV disease, by facilitating the development of CMV-specific immune responses. 

**BK POLYOMAVIRUS**

BK Polyomavirus (BKPyV) is a non-enveloped double-stranded DNA virus that belongs to the Polyomavirus family. It was discovered in 1971 and has been associated primarily with nephropathy in RTR.

After a primary infection, which is usually asymptomatic or associated with mild upper respiratory tract symptoms, it remains latent in the epithelial cells of the reno urinary tract. The transmission route is still unknown, but likely involves the oral or respiratory route.

BKPyV is ubiquitous and widely spread in adult human population, whereby infection results in lifetime persistence of the virus, with seropositivity of up to 80%. It is divided into 4 subtypes. Subtype I is the most prevalent throughout the world, while subtype IV can mostly be found in Asia and in Europe. Subtype II and III are more rare spread.

**BKPyV infection**

Intermittent active replication and asymptomatic viral shedding occurs in up to 20% in immunocompetent individuals and up to 60% in immunocompromised. In immunocompromised individuals, reactivation of BKV can switch from subclinical replication to a lytic infection, which result in viruria in up to 50% and viremia in up to 20%.

BKPyV reactivation is most common in RTR, where BKPyV infection could result in BKPyV nephropathy (BKPyVAN). BKPyV viruria and viremia due to reactivation are found in, respectively, 80% and 40% of renal transplant patients, mainly in the first year after transplantation, and 1-10% of patients progress to BKPyVAN. Several risk factors are known for the developing of BKPyV replication and BKPyVAN, whereby immunosuppression plays an important role.

**Diagnosing BKPyV**

Screening for BKPyV replication can be done by using the detection of decoy cells in urine or by screening urine and plasma viral load using real-time PCR. The last method is widely implemented in the laboratory as screening plasma for BKPyV replication is clinical relevant.
BKPyV serology is not performed routinely before transplantation, as about 90% of the human population is seropositive. Recent studies demonstrated that BKPyV IgG seroreactivity is stable over time and is associated with BKPyV replication after transplantation \(^{101,102}\). This insight gives new opportunities to predict patients at risk for developing BKPyVAN.

For the diagnosis of BKPyVAN, a biopsy is taken and by using SV40 staining the characteristic cytopathic changes in the tubular epithelial cells are visualized. This is necessary to differentiate between acute mediated rejection or polyomavirus infection.

A negative result cannot rule out BKPyVAN, therefore it is also necessary to screen the plasma and urine for BKPyV viral load. Urine viral loads of more than log 10 cp/ml and plasma loads of more than log 4 cp/ml are predictive values for developing BKPyVAN.

Recently, it has been demonstrated that BKPyV microRNA’s (miRNA) may play a role in induction of reactivation and the development of disease \(^{103}\). These findings are interesting as they could help in identifying new antiviral strategies. Until now, a few clinical studies showed that BKPyV miRNA levels in renal transplant patients can be detected in plasma and urine and in recipients with BKPyVAN \(^{104,105}\). However, other studies are necessary to find out the added value of measuring BKPyV miRNA’s above BKPyV viral load.

**Treatment and or prevention of BKPyV**

In recipients with BKPyV viremia and risk of BKPyVAN, the first step in treatment is reduction, reduction of the immunosuppressive load. This could be done by reducing first the calcineurin inhibitors followed by reduction of the anti-metabolite or reduce/discontinuous the anti-metabolite and thereafter the calcineurin inhibitors or reduce the anti-metabolite as well as the calcineurin inhibitors \(^{106}\). Although these strategies can lead to reduction of BKPyV plasma loads, the risk of transplant rejection increases and it is not effective in all patients \(^{107,108}\).

Antiviral agents, such as cidofovir, leflunomide, intravenous immunoglobulin and/or fluoroquinolones have been described.\(^{108-111}\) To date, however, the role of these specific antiviral therapy remains unclear. Adequately powered trials to define optimal treatment are therefore necessary.

Recent studies demonstrated screening pre-transplantation donors for BKPyV IgG seroreactivity and BKPyV replication could contribute to identify recipients at high risk. This could be used for the choice of the immunosuppressive regimen post-transplantation \(^{101,112}\). Taking into consideration that an everolimus based regimen with reduced CNI exposure results in less CMV and BKPyV infection \(^{113}\).
SCOPE OF THIS THESIS

The UMCG is a tertiary University Medical Centre which performs around 200 kidney transplantations per year. The implementation of new techniques in transplantations and the introduction of new immunosuppressive drugs, contribute to transplant kidneys not only under less optimal conditions, but also in older, more frail, recipients. This trend is, at one hand, good for the recipients. But at the other hand, infectious related complications increase, which makes screening pre- and post-transplantation more important.

The main focus of this thesis is to gain better insight in the clinical importance of regularly molecular monitoring of BKPyV, CMV and EBV DNA in adult RTR. Several research questions will be addressed in this thesis.

1. Development of additive diagnostic tools to gain more insight in BKPyV infection.

2. What is the optimal strategy and frequency for screening on BKPyV, CMV and EBV viral load after RTR?

3. What is the association between different immunosuppressive regimens and the detection of BKPyV, CMV and/or EBV?

The goal of this thesis is to improve patient management and transplantation outcome, which subsequently contributes to a better outcome of rental transplant recipients.
REFERENCES


