Importance of molecular diagnostic of viral infections in renal transplant recipients
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CHAPTER 8

Summary, discussion and future perspectives
SUMMARY

Renal transplantation after end-stage kidney disease has significant survival and quality of life benefits, compared to dialysis. Since the introduction of new immunosuppressive drugs, survival in the first year after transplantation increased up to 95% 1,2. The long term outcome, however, is unchanged and clinical physicians still have to deal with the side effects of these drugs 1,3. One of the major side effects are viral infections, which still lead to more disease and mortality. Viral infections can caused by reactivation of the recipient or a latent infection from donor organ resulting in primary infections in the recipient 4.

CMV, BKPyV and EBV are most common viral infections after renal transplantation and do cause various complications. CMV infections mostly causes tissue-invasive disease, which can lead to graft rejection or dysfunction 5,6. BKPyV viremia can progress to BKPyV nephropathy, resulting in renal transplant loss 7, 8 and EBV can cause post-transplant lymphoproliferative disorder (PTLD), a potential fatal complication 9.

The presented studies in this thesis focus on the impact of BKPyV, CMV and EBV infections after renal transplantation, with the focus of the necessity in diagnostic screening for these viruses to improve patient management and transplantation outcome.

Various BKPyV genotypes have different cellular tropisms and pathogenic potentials, which means that not all BKPyV and subgroups may contribute equally in the pathogenesis towards disease 10,11. In Chapter 2 we first developed a real time genotyping PCR assay for BKPyV. With this assay, the different genotypes of BKPyV could be detected fast and easily compared to sequencing. In our study the distribution of the different genotypes, was comparable with the literature. Genotype I was the main, followed by genotype IV and then genotype II. Genotype III was not found at all in our cohort. The use of this assay is helpful to obtain more insight in the role of BKPyV genotypes.

Recipients and donors having BKPyV viremia at time of transplantation, may be more prone to develop BKPyVAN after transplantation 12,13. However, a prognostic marker to predict patients at risk for developing BKPyVAN is still lacking. MicroRNA’s could play a role in this as they assist the virus to escape immune elimination by downregulation of the large-T expression and targeting host factors 14.

BKPyV miRNA’s circulate in blood, urine and cerebrospinal fluid, but little is known about the expression of BKPyV miRNA’s from an early latent infection to BKPyVAN.
In **chapter 3**, retrospectively, we measured longitudinally BKPyV genomic DNA and miRNA and compared this to clinical outcome in patients with either BKPyV viruria, viremia or BKPyVAN. We demonstrated that BKPyV 5p and 3p miRNA PCR curves are comparable to the measurement of BKPyV DNA in the different patient groups (viruria, viremia and BKPyVAN), with an exception in patients with persistent viremia. After the decline of BKPyV DNA in the persistent viremia group, the 3p and 5p BKPyV miRNA levels continue to rise. More studies are necessary to investigate whether the rising miRNA levels could serve a biomarker for patients at high risk for developing BKPyVAN.

Currently BKPyV viral load monitoring is used to indicate patients at risk for BKPyVAN. The usage of immunosuppressive drugs play a crucial role in the development of BKPyVAN, as the incidence of BKPyVAN has increased since the introduction of new and improved immunosuppressive drugs. **Chapter 4 and 5** describe studies investigating the usage of different immunosuppressive regimens at the UMCG in relation with the outcome in incidence of BKPyV and BKPyVAN in RTRs.

The focus in **Chapter 4** was on the frequency of BKPyV complications between a group treated with tacrolimus and mycophenolate acid (MPA) (TacM) versus treatment with cyclosporine A and MPA (CsAM). In this single center study, we demonstrated that the occurrence of BKPyV viremia between the TacM and CsAM group was comparable, whereby a significantly difference, in BKPyV loads, was found at 6 and 12 months. The group treated with TacM had higher BKPyV loads in comparison to the CsAM group, which resulted in more BKPyVAN in the TacM group. On the other hand, the incidence of BPAR was higher in recipients treated with CsAM. The diagnosis of BKPyVAN as well as BPAR was mostly before adjustment of treatment. These findings are in line with the literature, in which it is described that treatment with CsA is related with a higher incidence of BPAR.

Furthermore, more intense immunosuppression is a risk factor for the development of BKPyVAN, whereby the combination of Tac with MPA results in more intense immunosuppression than CsA with MPA. This raised the question if one immunosuppressive agent or the combination is responsible for the increased risk of BKPyVAN. Retrospectively, we investigated this aspect in a randomized controlled, prospective multicenter trial with de novo renal transplant recipients (**chapter 5**). In this study, the maintenance therapy, after 6 months, consisted of prednisolone with either CsA, mycophenolate sodium (MPS) or everolimus. We demonstrated that the incidence of BKPyV viruria and BKPyVAN was significantly higher in the group treated with prednisolone and MPS. Furthermore, recipients with active BKPyV replication and treated with prednisolone and CsA cleared faster BKPyV replication. In vitro Some studies showed a suppressive effect of CsA on BKPyV infected cells. This could be an explanation for the earlier
clearance of BKPyV viremia which we demonstrated in recipients that received treatment with CsA (chapter 4 and 5). Concluding from the studies performed in chapter 4 and 5, recipients with high risk for developing BKPyVAN, may benefit from, an immunesuppressive regimen consisting CsA. Although this can only be done in recipients with low risk of BPAR and graft loss.

Besides BKPyV replication after renal transplantation, Cytomegalovirus (CMV) replication is a common opportunistic infection and associated with CMV disease 21, end-stage renal allograft dysfunction and reduced graft survival 22. Most studies focus on the risk of a primary CMV infection and its effect on transplant survival and BPAR. It has been demonstrated that renal function decline is predictive for graft failure 23. To gain more insight in the detected CMV load and the effect on renal function after transplantation, we retrospectively studied in chapter 6 the influence of peak CMV load (PVL), in the first three months after renal transplantation, and outcome in renal function. Of note, this population was treated pre-emptively for CMV and therefore didn’t received any CMV prophylaxis treatment after transplantation.

RTR were categorized in three groups based on the median PVL (6310 IU/ml) in the first three months post-transplantation. We demonstrated that a CMV PVL >6310 IU/ml in the first three months is associated with lower and irreversible renal function up to 36 months post-transplantation. As currently, most transplant institutes start with CMV prophylaxis after renal transplantation, it would be interesting to study the effect of CMV prophylaxis and the outcome in PVL in the first three months. Especially in the group where the donor has CMV antibodies (D+) and the recipient is negative for CMV antibodies (R-) before transplantation, since these recipients are prone to develop a CMV primary infection. After a primary infection the immune response will be activated to clear the infection. RTR, however, receive immunosuppressive therapy which affect the initiation of the immune response which may lead to more severe infection 24. Intensive screening for CMV load in the first months after transplantation, can help to identify patients at risk to develop complications upon a CMV infection (chapter 6).

Finally, we studied Epstein Barr virus (EBV), another virus that may have a clinical impact after renal transplantation. Up to now, EBV load monitoring after transplantation, is used to predict the development of post-transplant lymphoproliferative disorder (PTLD). The incidence in RTRs, especially compared to other SOT recipients, is low (1-5%), and the implications other than predicting PTLD development of EBV load monitoring in RTR remains unclear. We demonstrated, retrospectively, in our single center study that the incidence of viremia was common in a population of 384 RTR (67.5%), with only one proven case of PTLD. However, recipients with EBV viremia had a significantly lower eGFR after 48 months, but viremia did not resulted in more graft loss, BPAR or mortality (chapter 7).
Patients with a primary EBV infection are prone for the development of PTLD in the first year after transplantation. For this population, EBV DNA monitoring could have an added value to predict PTLD. Although in this group not only EBV viral load is important, but also clinical symptoms. In our study, 25% of the recipients had a detectable EBV load, above $1.0 \times 10^4$ cp/ml. In this group, however, only one recipient (with no EBV IgG antibodies before transplantation), proven by histopathology, developed PTLD. Ten other recipients (3 with no EBV IgG antibodies before transplantation), did not get the disease PTLD, by rising EBV loads and specific clinical symptoms and signs. Our study (chapter 7) demonstrated that EBV load monitoring had an added value for recipients that are EBV seronegative before transplantation. In contrast, for the other RTR, screening for symptoms and clinical examination is preferred.

**DISCUSSION**

**General discussion and diagnostic implications**

Many improvements, such as new immunosuppressive drugs, new surgical techniques and the identification of risk factors (recipient characteristics and donor characteristics) have increased transplant and patient survival after renal transplantation.

However, complications are still a challenge and can be categorized into non-infectious (rejection, allograft dysfunction, side effects immunosuppressive drugs) and infectious (viral, bacterial, parasitic).

In this thesis, we focus mainly on viral infections with BK Polyomavirus (BKPyV), Cytomegalovirus (CMV) and Epstein Barr virus (EBV). Insight was gained into the developments of improved diagnostic tests, the effect of immunosuppressive treatment and the frequency of monitoring viral load.

**Development of improved diagnostic tests**

Serology is used to identify antibodies against viral pathogens, indicative for the presence of latent pathogens in both donor and recipient. The pre-transplantation serostatus for CMV and EBV of renal transplant recipients (RTR) and donors, is used to determine the a–priory risk of a primary infection or whether reactivation after transplantation may occur. Most serological tests used are commercially available and can be performed rapidly. These tests, however, have a limited value post-transplantation due to the impaired immune response of recipients caused by the immunosuppressive medications used.

Currently, serological assays against BKPyV are not performed routinely before transplantation, mainly because the added value is not clear. However, it has recently been shown that donor
BKPyV IgG levels at pre-transplantation are strongly associated (p<0.001) with the risk of BKPyV viremia and BK Polyomavirus nephropathy (BKPyVAN) after-transplantation. Thus, screening of donors and recipients before transplantation for the presence of these antibodies could be a predictor to identify recipients who are at risk for developing BKPyV viremia as well as BKPyVAN. Performing these serological assays however, is currently labour-intensive and time-consuming, which makes routine implementation less attractive. A commercial available test for routine use would be preferable.

Viral load screening, is commonly used post-transplantation to determine whether CMV, EBV and BKPyV replication occurs. These viral load levels are indicative for decisions on treatment. The introduction of WHO international standards for CMV, EBV and BKPyV DNA, measured in International units (IU/ml), ensured that identical levels are measured, whether commercial or lab-developed assays are used.

Cell-mediated immune response tests, currently commercially available only for CMV, like the Elispot or Quantiferon Gamma Interferon-releasing assay, may also be helpful to support decisions on treatment options. However, these assays are not implemented routinely in most laboratories. With these assays, CMV specific T-cell responses can be measured. As soon as a good T-cell response is measured, CMV prophylaxis could be stopped. Combining this immunological test with viral load monitoring could more personalize the use of prophylaxis treatment, and thus reduces the chance of developing resistance, side effects and cost of antiviral therapy.

Currently, testing for CMV drug resistance is only performed in recipients when antiviral treatment does not lead to a more than 10-fold reduction in viral load or when there is a progress in clinical symptoms. These methods are time-consuming and often viral load measurements are repeated to get more insight in the viral replication. If it takes too long to get this information, it is always possible to switch to another antiviral therapy. However, sequence information of the genes involved in resistance provides information on which antivirals could be used.

For BKPyV it is unknown if or what role BKPyV genotypes have in both the recipient and the donor in relation to the development of BKPyV viremia and BKPyVAN. To obtain more information, we developed a specific real time PCR assay by which the BKPyV genotypes can be determined within a few hours (chapter 2).

For BKPyV, there are assays available to measure miRNAs, however, their added value is under discussion whether they can be used as biomarkers for patients with a high risk to develop
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BKPyVAN. We demonstrated in chapter 3 that BK virus miRNA levels are mostly a reflection of viral replication measured with BKPyV DNA, yet in recipients with persistent viremia miRNA levels continue to rise, whereas BKPyV DNA levels declined. More studies are needed to investigate the role of miRNA’s.

In the case of EBV, the development of PTLD is the main serious complication. However, there is besides monitoring the viral load levels and clinical screening (MRI), no method is available to predict this serious complication 36.

In summary, the implementation of diagnostic assays to detect the above mentioned viruses CMV, EBV and BKPyV, surely improves the identification of patients which are at a high risk of pre- and post- transplantation complications.

Immunosuppressive treatment

In most transplant centers, immunosuppressive treatment after renal transplantation consist of a quadruple therapy. The treatment regimen is often initiated with an induction agent consisting of an interleukin-2 receptor antibody (mostly basiliximab) followed by a maintenance regimen containing a combination of a calcineurin inhibitor (CNI) (tacrolimus, Cyclosporine A) with mycophenolate and steroids 2. Currently, tacrolimus is more used as CNI compared to Cyclosporine A. It has been shown that treatment with tacrolimus leads to less acute rejection and better kidney function in the first years post-transplantation 37,38. However, in the long run, it is also associated with drug side-effects, such as nephrotoxicity, and new onset of diabetes. Due to the narrow therapeutic window, it is important to measure tacrolimus trough levels, as under-immunosuppression this may contribute to de novo donor specific antibodies (DSA) formation, which in turn can lead to chronic antibody-mediated rejection 38. In chapter 4, we confirmed that recipients treated with a tacrolimus based regimen, had less acute rejection compared to recipients treated with a Cyclosporine A based regimen. Whereas, on the other hand, higher viral BKPyV loads and more BKPyVAN was seen in RTR treated with a tacrolimus based regimen.

Recent studies focus on minimization of CNIs to reduce the drug related nephrotoxicity. It has been shown that combination therapy of low dose CNI and mycophenolate, results in good long-term graft outcome 39. An alternative is combining low dose CNI with everolimus instead of mycophenolate. In the recently published TRANSFORM study, this regimen was not associated with more rejection or de novo DSA formation. Moreover, CMV and BKPyV infection were lower in a regimen combining everolimus and tacrolimus, supporting our observations from chapter 5 40,41.
Regimens with mTOR immunosuppression (sirolimus or everolimus) have the benefit of an antiviral effect. In contrast, a higher frequency of rejection has been associated with treatment with an mTOR-based regimen without CNI as compared to regimens including CNI. Therefore, the combination of mTOR and CNI potentially interesting as maintenance therapy in recipients at higher risk for CMV and BKPyV induced disease. Using such a regimen would make it possible to avoid CMV prophylaxis, which could be useful when antiviral therapy is contra-indicated or resistance has been determined.

For now, physicians who have to make a decision about immunosuppressive therapy, must balance between rejection and infection.

**Viral monitoring: who, when and how to act**

Serological screening and viral monitoring pre- and post-transplantation, is important to identify recipients at risk for viral replication and to manage the related complications after renal transplantation. One important risk factor for a primary infection is a serological mismatch between donor and recipient. It is well known that a recipient with a negative serostatus for CMV and/or EBV, is more prone to develop a primary infection after receiving a kidney from a donor with a positive serostatus for CMV and/or EBV. Recently, new insights have been gained about the added value of BKPyV serostatus pre-transplantation, as described above, but this has not yet been implemented into diagnostic standards.

Viral load monitoring is the method of choice to identify decision thresholds, as viral infections can cause serious complications, such as renal rejection, graft loss, patient death and more.

For CMV, there are internationally accepted guidelines regarding the frequency of viral load monitoring. It is recommended to screen at least once weekly in the first three months especially for pre-emptive monitoring, as start of antiviral treatment depends on the viral load measured in combination with clinical symptoms and serostatus. In chapter 6 we demonstrated that, recipients without prophylaxis, a peak viral load above 6310 IU/ml within the first three months, is associated with an irreversible lower renal function. Through the introduction of the international WHO standard, this viral load threshold should be used routinely in transplant centers.

However, CMV prophylaxis after transplantation is currently standard clinical practice. This prophylaxis is given for a period of three months post-transplantation for recipients with a positive serostatus pre-transplantation and six months post-transplantation for recipients with a negative serostatus, to reduce the chance of developing late onset CMV disease. Another
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Advantage of using prophylaxes is that it will prevent infections caused by other herpesviruses as HSV, VZV and to a lesser extent by HHV6.

For EBV monitoring after renal transplantation, formal guidelines are absent. It has been recommended in recipients with a serological-mismatch or with T-cell depletion, to screen for EBV viral load weekly or biweekly during the first months after transplantation followed by a monthly screening during the first year post-transplantation, when the patients are more prone for developing PTLD. In addition, it is important to screen for clinical symptoms and signs by a physician to adjust therapy in time. Reduction in immunosuppression is the initial intervention in recipients with an EBV viremia and suspected for developing PTLD.

Our findings in chapter 7 are in line with the above mentioned guidelines. We demonstrated that only EBV load monitoring in seronegative EBV recipients has an added value. Whereas in recipients with a low risk (seropositive), screening for symptoms and clinical examination is preferred.

In case of BKPyV, it is recommended to screen all RTR in plasma samples monthly in the first 6 months post-transplantation, followed by three-monthly screening until two years after transplantation. BKPyV viral load above 10,000 copies/ml in plasma is associated with an increased risk of developing BKPyVAN. Reduction of immunosuppression is the first method of choice since an antiviral treatment is currently not available.

**FUTURE PERSPECTIVES**

As described before, insights have been gained in the clinical importance of molecular monitoring for BKPyV, CMV and EBV. Independent of the initial immunosuppressive regimen, it is important and necessary to perform the right diagnostic test at the right time, allowing timely regimen adjustments and to act more patient-tailored to prevent either toxic or viral complications.

The greatest challenge after transplantation remains the long-term graft survival and prevention of infections and related complications. Specific diagnostics and therapy is required to provide the best care for patients after their renal transplantation. Diagnostic stewardship serves as a key in patient-tailored transplantation. The risk profile of each patient can be mapped before transplantation, ensuring that, individually, at the right time, on the appropriate specimens, state-of-the-art diagnostic will be performed in a patient-tailored way. Physicians will get help in selecting and interpreting the diagnostic tests, so that appropriate therapy can be initiated.
or changed 49,50. This is important and crucial for transplant patients in general as the quality of life depends on the success of the transplantation.

To take steps towards more patient tailored diagnostics, assays should be used concerning the measurement of T-cell immunity. Those have the property to be a marker for the reduction of immunosuppression or even stopping antivirals. As mentioned above, for CMV already T-cell immunity tests are available. For BKPyV and EBV, more studies are needed and diagnostic tests should be developed for easily implementation in routine diagnostic.

Furthermore, research is needed to gain more insight into the role of the donor kidney in relation to virus replication and related complications. This has a greater impact than previously thought, as demonstrated by a recent study where recipients had a higher risk of developing BKPyV replication after transplantation when they received a kidney from a donor with BKPyV viruria 51.

To date, specific markers to determine when immunosuppression is too high, are lacking. For CNI, trough levels are now being used, yet it is not clear whether this correlates more with the risk of drug-related toxicity or the immunosuppressive efficacy. A specific marker would make it possible to reduce the risk of infections and at the same time not to reduce the immune suppressive efficacy too much, which increases the chance of rejection, hence keeping the right balance.

Recent research has been focused on the measurement Torque Teno Virus (TTV) levels as a marker to predict over-immunosuppression. TTV is a small, non-enveloped, single stranded DNA virus that belongs to the Anelloviridae family. It is prevalent in about 90% of the human population and has not been linked to a disease 52,53. It seems that TTV viral load levels in blood can be related to the immune-competence of the patient 53,54, as peripheral blood levels of TTV might mirror the overall strength of innate and specific immunity 55. A few studies have been performed using TTV as a predictor for over-immunosuppression after transplantation 56-58 Results were contradicting and prospective studies are needed to provide insight into the outcome in terms of rejection and infection after adjustment of immunosuppression by TTV viral load levels compared with adjusting the immunosuppression dose by CNI trough levels.

For this, the use of international standards for virus quantification (EBV, CMV, BKPyV, TTV) are essential as only then it is possible to use the cut-off values universally in the different transplant centers. Taking into consideration that the various available tests and different platforms on which these tests can be carried out, also contribute to the differences in quantification. Ideally, to reach a general cut-off value, all transplant centers should use and perform the same test in
same materials on the same systems. However, the reality shows that this is not possible, which makes the use of international standards even more important. The test performance should be monitored through external quality assessment proficiency testing. Provided by independent organizations that monitor the test results, such as QCMD, SKML or Nequas. Nevertheless, a general cut-off value can be used as a guideline for actions related to the adaptation of therapy and decisions for additional diagnostics.

In conclusion, the developments in diagnostics and treatment are of great importance to provide patients with end-stage renal disease a better and longer quality of life. But even more important is the shortening of the waiting list due to the availability of kidney donors. For this, it is necessary to inform the general public more about the importance of transplantation.
REFERENCES

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