Presentation and early detection of posttransplant lymphoproliferative disorder after solid organ transplantation
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CHAPTER 1

General introduction and scope of the thesis

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Immunocompromised patients are at particular risk for the development of lymphoid malignancies associated with Epstein-Barr virus (EBV). These include patients with the acquired immunodeficiency syndrome (AIDS), patients with inherited (primary) immunodeficiency's, but also haematopoietic stem cell as well as solid organ transplant recipients. This thesis will focus on lymphomas developing in solid organ transplant recipients.

EBV, the immune system and malignancy

EBV is a ubiquitous human γ-herpes virus that infects approximately 95% of the world’s adult population. It was first discovered as a new –so far unknown- human herpes virus in 1964 by Epstein and Barr, when they successfully propagated lymphoid cells in vitro from a Burkitt’s lymphoma case. EBV is the causative agent of infectious mononucleosis and is associated with several lymphoid malignancies, including Hodgkin’s lymphoma, natural killer (NK) T cell-lymphoma and Burkitt’s lymphoma.

The oropharynx is the primary site of infection and is also believed to be the preferential site of virus replication. After primary infection (mostly in early childhood, especially in underdeveloped countries), the virus persists for life in the memory B-cell compartment, with a stable number of latently infected B-cells. Healthy individuals display a vigorous humoral and cellular immune response to primary infection with EBV. After primary infection, the virus is kept under control by the cellular immune response, especially by CD8 positive T-cells specifically directed to EBV (EBV-specific cytotoxic T-lymphocytes (CTLs)). In the immunocompromised host, however, the EBV-specific T-cell response may be significantly impaired. In transplant recipients, the immunosuppressive drugs administered to suppress rejection of the transplanted organ can lead to a profound decrease in EBV-specific T-cell surveillance. In this situation, latently EBV-infected B-cells may escape host immune surveillance and expand to a polyclonal proliferation, ultimately leading to a lymphoid malignancy (posttransplant lymphoproliferative disorder, PTLD).

EBV usually does not actively replicate in B-cells but instead establishes a latent infection. The latent infection in memory B-cells is characterised by a more limited expression of a subset of virus latent genes. All EBV-positive lymphoid malignancies are associated with the virus latent cycle. The latent genes are variably expressed in different types of EBV-associated lymphoid tumours. Also non-lymphoid EBV-associated tumours, i.e. nasopharyngeal carcinoma are associated with the latent cycle. So far, three main latency types have been characterised (Table 1). Lymphomas in immunocompromised patients are almost invariably associated with latency type III, expressing the entire array of EBV latency genes.

PTLD introduction

Development of lymphoma after transplantation was first described by Doak et al. in a renal transplant recipient in 1968, whereas the term posttransplant lymphoproliferative disorder or disease (PTLD) was introduced by Starzl et al. in 1984. PTLD encompasses a heterogeneous group of lymphoproliferative diseases, ranging from EBV-driven polyclonal proliferation resembling infectious mononucleosis, to
monomorphic proliferations that may be indistinguishable from aggressive types of lymphoma such as diffuse large B-cell lymphoma\textsuperscript{9,10}. PTLD is not exclusively associated with EBV infection, and EBV negative PTLD, with a preference to develop late after transplantation, is also increasingly recognised\textsuperscript{11-13}. Most PTLD are of B-cell origin, but also T- or natural killer (NK)-cell lymphomas arising in the transplant recipient are classified as PTLD\textsuperscript{10}. Although the development of PTLD constitutes a continuing long term risk after transplantation, PTLD is most frequently observed during the first year after transplantation, especially in lung transplant recipients\textsuperscript{14}.

PTLD incidence varies significantly between different types of organ transplants, with the highest incidence (5-20%) found after lung and small bowel transplantation\textsuperscript{14,15}. In contrast, reported incidences in kidney transplant recipients are much lower (1-3%). However, as many thousands of renal transplants are performed each year, the majority of PTLD are still those observed in kidney transplant recipients\textsuperscript{14,16}.

### Histological classification of PTLD

Histology is essential for the diagnosis of PTLD, and differentiation between rejection and PTLD involvement of the graft is necessary because episodes suggestive of rejection may in reality present allograft involvement of PTLD\textsuperscript{17,18}. An incision or excision biopsy is preferred to provide an adequate amount of tissue for evaluation of cell type and architectural background, clonality and virological studies. Needle biopsy should only be performed when larger biopsies are not possible\textsuperscript{19}. Although cytology may be helpful in the diagnosis of PTLD\textsuperscript{20}, it has a limited role and should not be used for classification.

PTLD comprises a variety of lymphoid tumours rather than one specific disease entity, and different classification systems have been applied to categorise PTLD\textsuperscript{21,22}. Currently, classification is based on the recommendations of the Society for Haematopathology\textsuperscript{9}, which identifies four major categories of PTLD (Table 2): 1. lymphoid hyperplasia’s or “early” lesions, 2. polymorphic PTLD, 3. lymphomatous or

<table>
<thead>
<tr>
<th>Viral antigens expressed</th>
<th>Tumour type</th>
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<tbody>
<tr>
<td><strong>Latency I</strong></td>
<td></td>
</tr>
<tr>
<td>EBNA\textsuperscript{a} 1</td>
<td>Burkitt's lymphoma</td>
</tr>
<tr>
<td><strong>Latency II</strong></td>
<td></td>
</tr>
<tr>
<td>EBNA 1, LMP\textsuperscript{b} 1, LMP2</td>
<td>Undifferentiated nasopharyngeal carcinoma, Hodgkin's lymphoma, T-cell lymphoma</td>
</tr>
<tr>
<td><strong>Latency III</strong></td>
<td></td>
</tr>
<tr>
<td>EBNA 1, EBNA 2, EBNA-LP, EBNA 3A, EBNA 3B, EBNA 3C, LMP1, LMP2</td>
<td>Diffuse large B-cell lymphoma, Posttransplant lymphoproliferative disorder</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Epstein-Barr virus nuclear antigen (type 1, 2, 3a, 3b, 3c) \textsuperscript{b} latent membrane protein
monomorphic PTLD including T-cell lymphoma, 4. other lymphoproliferative disorders, including myeloma and Hodgkin lymphoma. In addition, PTLD may also present with discordant lesions, in which different histological subtypes can be present in a single patient. Apart from routine histological examination, including immunophenotyping and analysis for Epstein Barr encoding RNA’s (EBER), analysis of clonality may be helpful to differentiate between (sub)categories of PTLD.

Although the association between EBV and PTLD is well established, the presence of EBV in tumour cells is not required for the diagnosis of PTLD. This implicates that according to the international classification, any lymphoma arising in the posttransplant patient is considered to be (a variant of) PTLD. However, there is increasing evidence that EBV-negative PTLD is a distinct disease entity, in that this type of PTLD tends to develop much later after transplantation and has a significantly worse outcome when compared to EBV-positive PTLD. Whether EBV-negative lymphoma in the posttransplant host is a coincidentally arising non-Hodgkin’s lymphoma or a “true” PTLD as a result of the transplant process cannot be answered with current knowledge and, until solved, remains a matter of semantics. For an extensive discussion of the pathologic work-up and classification of PTLD, the reader is referred to the review by Nalesnik et al.

**Risk factors**

**Immunosuppressive treatment**

An important risk factor for PTLD development is the intensity and amount of immunosuppression administered to the patient. In this respect, induction and
rejection treatment with anti T-cell antibodies, in particular OKT3 and ATG, may lead to an increased risk of developing PTLD. The higher incidence of early PTLD in lung and heart/lung transplant recipients supports this concept, since immunosuppressive induction therapy is more commonly applied in these patients than in other organ transplants. Furthermore, rejection treatment is more aggressively applied, probably because of the lack of alternative organ replacement therapies in this category of patients. Interestingly, induction therapy with the more recently introduced interleukin (IL)-2-receptor antibodies does not seem to lead to a higher incidence of PTLD. However, more data are necessary to confirm these findings.

There is no conclusive evidence that development of PTLD is associated with a single immunosuppressive maintenance agent. Although there is some discussion on the specific effects of Tacrolimus (compared with cyclosporin A) being a risk factor for PTLD development, it seems that the recent introduction of Mycophenolate Mofetil is at least not associated with an increased risk of PTLD development. The effect on risk of PTLD development of mammalian target of rapamycin (mTOR) inhibitors (Sirolimus, Everolimus) is not clear yet. Theoretically, these compounds might play a beneficial role, because they display an inhibitory effect on PTLD-derived cells in vitro and in vivo in an animal model. The lack of prospective randomised trials assessing these different immunosuppressive regimens in relation to the risk of PTLD is a major drawback and restrains any firm conclusions regarding these agents and the risk of PTLD.

At this moment, it may be concluded that the total amount of immunosuppression -including induction and rejection therapy-, rather than a single immunosuppressive maintenance agent is associated with increased risk of PTLD.

Genetic predisposition

So far, it is unknown whether a genetic predisposition also plays a role in the development of PTLD. Given the relation between a decreased cellular immune response as a result of immunosuppression and development of PTLD, it has been suggested that patients with an inherent decreased immune capacity might be at increased risk for PTLD development. In this respect, it has been reported that cytokine polymorphisms associated with a low cellular immune response (IL-2 and interferon-γ) correlate with PTLD development.

EBV seronegativity

A special category of patients at particular risk for PTLD development (10-50 fold increased risk) are EBV-seronegative patients receiving allografts from EBV-seropositive donors, consequently leading to primary EBV infection. This is, probably, also the main cause of the higher incidences of PTLD observed in the early posttransplant period in paediatric transplant recipients, who more often are still EBV-seronegative at the time of transplantation. Because of the markedly increased risk for PTLD development in EBV-seronegative patients receiving organs from EBV-seropositive donors, pre-transplant immunisation for EBV in order to induce EBV immunity has been suggested. A vaccine against EBV
is, however, not available yet, although work is in progress\textsuperscript{40,41}. There is an anecdotic report describing the successful immunisation of two patients following donor blood transfusion before living related kidney transplantation, after which symptom free seroconversion was observed after transplantation\textsuperscript{42}. However, because of ethical and safety issues, the concept of pre-transplant iatrogenic EBV infection has not been applied yet.

Antiviral agents (acyclovir, gancyclovir), primarily used as Cytomegalovirus (CMV) prophylaxis, are also frequently applied to prevent PTLD development. Funch et al. retrospectively reported a strong association between prophylactic acyclovir or gancyclovir and freedom from PTLD in 100 PTLD patients matched with 375 controls\textsuperscript{43}. However, other reports, addressing more specifically the impact of gancyclovir, with or without CMV-intravenous immune globulins, on EBV viraemia, especially in EBV seronegative transplant recipients\textsuperscript{44}, could not show any beneficial effects on EBV-DNA load or PTLD development\textsuperscript{45}.

\textit{CMV mismatch and disease}

Whether CMV itself is associated with an increased risk of PTLD development, is debatable. CMV pre-transplantation mismatch (i.e. CMV naïve recipient transplanted with a CMV seropositive donor)\textsuperscript{46} and CMV disease after transplantation (especially in EBV naïve transplant recipients)\textsuperscript{47,48} have both been linked to an increased risk of PTLD development. However, this could not be confirmed in several large recent studies\textsuperscript{16,39,43,49,50}. Thus, although it cannot be excluded that CMV plays a role in PTLD development, it seems that CMV mismatch or disease are at least not major risk factors for PTLD development.

\textit{HLA matching}

Whether the degree of HLA matching between donor and recipient plays a role in the development of PTLD is debatable\textsuperscript{49,51}. In a recent study, increased total numbers of HLA mismatches were found to be associated with PTLD development\textsuperscript{16}. Larger cohort studies are necessary to confirm these findings. (see also chapter 3a and 3b)

\textit{Time of onset after transplantation and site of PTLD presentation}

PTLD may arise at any time after transplantation and present as early as 15 days after transplantation\textsuperscript{52}. The risk of PTLD development is significantly higher in the early posttransplant period (<1 year after transplantation), especially in heart/lung and lung transplant recipients. This is generally attributed to higher doses of immunosuppression and more intensive use of induction therapy with anti-T-cell antibodies in these categories of patients. In a large series comprising more than 150,000 patients, Opelz et al. showed that almost half of all PTLD following lung and heart/lung transplantation developed in the first year post transplantation after which the risk of developing PTLD levelled off\textsuperscript{14}. This is in sharp contrast to kidney transplant recipients in whom only 20 percent of all PTLD developed within the first year following transplantation after which the incidence stabilised at lower rates in subsequent years\textsuperscript{14}. 
The site of PTLD presentation seems to be closely related to the time elapsed after transplantation. In lung transplant recipients, more than 50 percent of all PTLD during the first posttransplant year develop in the allograft\textsuperscript{14,53}, whereas allograft localisation is rarely observed after the first posttransplant year\textsuperscript{54}. Although not as evident as in lung transplant recipients, PTLD development in the allograft is also more frequent in the first posttransplant year in kidney transplants\textsuperscript{53,55}.

Apart from allograft involvement, the most commonly affected extranodal sites of PTLD are observed in the gastrointestinal tract\textsuperscript{54,56}. There seems to be no relation between time of onset and development of PTLD in the gastrointestinal tract. Other commonly affected sites of PTLD include the sinonasal cavity\textsuperscript{57} and isolated involvement of the central nervous system (CNS), which like in other patients with impaired T-cell function, e.g. humane immunodeficiency virus (HIV) infection, are more frequently observed when compared to non-Hodgkin’s lymphoma in patients without apparent immune deficiency\textsuperscript{58}. Isolated lymph nodes may also be affected in up to 25 percent of all PTLD cases\textsuperscript{55}. Skin involvement is observed in approximately 5-10 percent\textsuperscript{59}, and must be differentiated from other coetaneous malignancies, given the fact that organ allograft recipients have an increased risk for development of coetaneous malignancies such as squamous cell carcinoma.

Clinical symptoms of PTLD

Because PTLD often presents in a non-specific way in asymptomatic patients, it is a major challenge to diagnose PTLD at an early stage. Keeping in mind that PTLD often presents at extranodal sites, including the allograft and digestive tract, there may be early signs and symptoms for which PTLD should at least be included in the differential diagnosis, and which often directly point to possible sites of involvement. This is especially true for allograft involvement of PTLD. Kidney transplant recipients with allograft involvement of PTLD often present with renal dysfunction, hydronephrosis due to ureteral obstruction, and fever\textsuperscript{55,60}. An ultrasound can quickly reveal adenopathy or an ill-defined mass\textsuperscript{60}. Lung transplant recipients may present with organ dysfunction after which a plain chest X-ray or CT scan of the thorax may be helpful in the diagnostic process\textsuperscript{54,61,62}.

Because the gastrointestinal tract is also frequently involved, gastrointestinal symptoms, e.g. diarrhoea and bleeding or even bowel perforation, may also lead to a diagnosis of PTLD. Other signs that should trigger awareness of PTLD may be more subtle, such as unexplained fever or lymphadenopathy, but also more localised symptoms such as headache or confusion in case of CNS involvement\textsuperscript{58}, nasal airway obstruction in sinonasal PTLD involvement\textsuperscript{57}, or orbital symptoms in orbital PTLD\textsuperscript{63}. Finally, PTLD may also present with disseminated disease in asymptomatic patients.

Methods for early detection of PTLD

Much effort has been put in developing methods that can identify patients at risk for developing PTLD by measuring the amount of circulating EBV-DNA in the peripheral blood. After the first reports claiming a quantitative difference in circulating EBV-DNA load and Epstein-Barr virus nuclear antigen (EBNA) antibodies between transplant
recipients with and without PTLD\textsuperscript{64,65}, this relation has been investigated to establish its significance and clinical relevance for the identification of the patient at risk.

Through the years, different methods for the detection of EBV-DNA have been used, including comparative polymerase chain reaction (PCR) assays with endpoint dilution, quantitative competitive PCR assays as well as real time quantitative PCR assays. The latter is considered to be sensitive, precise, reproducible and suitable for widespread application\textsuperscript{66-69}, and is now commonly used as the detection method of choice for the detection of EBV-DNA. EBV-DNA load can be measured by real time PCR in plasma, in peripheral blood mononuclear cells (PBMCs) as well as in whole blood. Which of these compartments is preferable to determine the EBV load is still a matter of debate\textsuperscript{70,71}. (see also chapter 5)

Despite the consensus that PTLD patients have a significantly higher EBV-DNA load compared to healthy EBV seropositive donors or non-PTLD transplant recipients\textsuperscript{64,65,72}, it is still unclear which threshold values are predictive for PTLD\textsuperscript{73}. Many studies have tried to define a threshold value corresponding with the best sensitivity and specificity for PTLD development and different threshold values have been reported, all with different sensitivity (60-100\%) and specificity (71-100\%)\textsuperscript{74-78}. A limitation of EBV-DNA load monitoring is the observation that PTLD developing late after transplantation is not necessarily associated with primary or secondary (i.e. reactivation of already present endogenous EBV of the recipient) EBV infection, and, presumably, may therefore develop without concomitant rises in EBV-DNA load\textsuperscript{76,79}.

Because of the shortcomings of EBV-DNA load measurements as a single parameter for predicting PTLD development, and the supposed relation between high EBV-DNA loads and overimmunosuppression\textsuperscript{80}, it has been suggested that concomitant combined monitoring of EBV-DNA load and EBV-specific CTL responses (as a marker for possible overimmunosuppression) might better identify the individual patient at risk\textsuperscript{81}. Especially the positive predictive value of a high EBV-DNA load as predictor for PTLD development might be improved by this method\textsuperscript{82}. Some preliminary reports, indeed, suggest that this may be the case. Smets et al. showed that high EBV-DNA loads in patients who underwent primary EBV infection were indicative for PTLD development only if there was a low concomitant EBV-specific cellular immune response\textsuperscript{83}. More recently, a strong correlation between a lymphocyte activation assay closely measuring the immunosuppressive status of paediatric liver transplant recipients and EBV-DNA loads was identified, which might be useful for interpretation of persistently high EBV loads detected in the absence of symptoms and PTLD development\textsuperscript{82}.

**Imaging of PTLD**

Because different signs and symptoms may reflect PTLD activity, imaging modalities are frequently applied to differentiate between PTLD involvement, infection or other abnormalities. Conventional diagnostic methods to visualise PTLD include ultrasound, endoscopy, MRI (particularly in case of CNS involvement) and CT scanning\textsuperscript{84-87}. In addition, fluorodeoxyglucose (FDG)-positron emission tomography (PET) scanning is increasingly recognized as an important tool in the visualisation of
malignant lymphoma, especially for the detection of extranodal localisations. FDG-PET has shown to be superior compared with conventional diagnostic methods in these types of patients. Because PTLD frequently presents at extranodal localisations, FDG-PET may also be very useful for the visualisation of PTLD. So far, two small studies have evaluated the use of FDG-PET in the visualisation of PTLD and have shown excellent results. (see also chapter 4 of this thesis)

Treatment of PTLD

Because of lack of randomised controlled trials comparing different treatment modalities, there are thus far no commonly applied guidelines for the treatment of PTLD. As PTLD development is generally considered to be the result of clonal expansion of EBV-infected B-cells due to decreased T-cell surveillance, the initial step in the treatment of PTLD always consists of reduction of immunosuppression. Especially early, polyclonal, lesions may respond well to the reconstitution of EBV-specific T-cell control in this way.

Antiviral therapy is frequently instigated in patients with PTLD. Although some reports show a beneficial result of antiviral agents, the value of these agents remains at least doubtful. Antiviral agents such as acyclovir and gancyclovir only limit productive viral replication and do not affect the latent virus, whereas PTLD is associated with the latent cycle of EBV infection, in which B-cell proliferation is independent of spontaneous viral replication.

During the last years, monoclonal antibody therapy (especially Rituximab, directed against the B-cell receptor CD20) is frequently applied and is now widely regarded as first line treatment in CD20 positive PTLD. This therapy has proven to be effective and safe in terms of toxicity. Polychemotherapy, often with CHOP (cyclophosphamide, adriamycin, vincristine, and prednisone), which used to be associated with significant treatment-related morbidity and mortality, is nowadays reserved for patients in whom other treatment options have failed or when PTLD is CD20 negative. Infusion of recipient-derived EBV-specific CTLs as treatment for PTLD may also be helpful. However, results are still preliminary and more studies are necessary to further investigate this time consuming and laborious approach.

More recently, pre-emptive strategies guided by EBV-DNA load, e.g. reduction of immunosuppression or treatment with autologous EBV-specific CTLs, have been evaluated.

Outcome of PTLD

Survival rates of PTLD vary significantly between transplant centres. This may be partly explained by the fact that, in small studies, small numbers of patients have been studied, different treatment strategies have been applied, all during various periods. Moreover, different types of transplanted organs have often been grouped together, which probably also could have influenced survival rates.

In the Rituximab era, since 1998, complete remission rates range from 28 up to 75%, and one year overall survival rates range from 44 up to 73%. However, there may be subgroups of patients that differ in their response to treatment. Especially
late onset PTLD carries a worse prognosis when compared with early onset PTLD\textsuperscript{12,13}. This might be attributed to the larger percentage of EBV negative PTLD in late onset disease.

In non-Hodgkin’s lymphoma, the international prognostic index (IPI) (based upon five adverse prognostic factors (age >60 years, performance status >1, serum lactate dehydrogenase (LDH) > upper normal limit, Ann Arbor stage\textsuperscript{102} >II and number of extranodal sites >1) distinguishes patients at the time of their initial diagnosis in terms of likelihood of response to treatment, progression and overall survival\textsuperscript{103}. The IPI has never been validated for PTLD, however, and questions remain which factors have prognostic value for PTLD outcome\textsuperscript{53,104}.

A multivariable model for survival, using three adverse factors, including poor performance status, monomorphic disease, and graft involvement, was recently developed in a study on 104 PTLD patients collected between 1970 en 2004\textsuperscript{105}. Incidence of each of the three factors was counted as 1 point and the sum of the points was used as a prognostic score. The scoring was dichotomised into patients with two or more factors and patients with one or none of the risk factors (hazard ratio for 10 year overall survival in patients with two or more factors: 5.31). This model turned out to be a more reliable prognostic model than the IPI. However, larger homogeneous cohort studies are necessary to confirm this model.

**Conclusion**

Despite the recent advantages in the possibilities of identifying the patient at risk for development of PTLD by means of EBV-DNA load monitoring and treatment with Rituximab, PTLD is still one of the most severe and often fatal complications after solid organ transplantation. It remains a major challenge to get more insight in risk factors and pathophysiologic mechanisms involved in development of PTLD, and find ways for better prevention, early detection and treatment.

**Scope of the thesis**

We aimed to gain more insight in the presentation of PTLD after solid organ transplantation and to identify new risk factors possibly involved in PTLD development. In addition, we explored the feasibility of EBV-DNA load guided reduction of immunosuppression for prevention of PTLD.

In **chapter 2**, we analysed incidence, patient characteristics, clinical presentation and prognostic factors of importance for treatment outcome and survival of PTLD in patients transplanted at our centre between January 1985 and December 2002, during which more than 1200 kidney and 200 lung transplants have been performed. In **chapter 3a and 3b**, risk factors for PTLD development in kidney and lung transplant recipients and the association with the degree of human leukocyte antigen (HLA) mismatching were analysed.

In **chapter 4**, the value of FDG-PET in the staging and treatment evaluation of PTLD in kidney transplant recipients was analysed.

In **chapter 5**, we compared the value of measurements of EBV-DNA in whole blood and plasma in terms of sensitivity for the detection of EBV-DNA.
In chapter 6, we analysed the frequency and dynamics of EBV re-activations occurring late after lung transplantation. In addition, we evaluated the feasibility of EBV-DNA load monitoring and subsequent reduction of immunosuppression in these patients. In chapter 7, the results of these studies as well as the value and future development of methods for early detection are discussed.
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