PREVENTION, DIAGNOSIS AND TREATMENT OF ACUTE KIDNEY TRANSPLANT REJECTION

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Prevention, diagnosis and treatment of acute kidney transplant rejection

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The research described in this thesis was performed at the Department of Internal Medicine, section Nephrology and Transplantation of the Erasmus MC, University Medical Center Rotterdam, The Netherlands.

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'If you only read the books that everyone else is reading,
you can only think what everyone else is thinking'

Haruki Murakami

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INTRODUCTION

CHAPTER 1

General introduction

GENERAL INTRODUCTION

Kidney transplantation is the best treatment for patients with end-stage renal disease (ESRD). Kidney transplant recipients have a superior quality of life and life expectancy compared to patients undergoing maintenance dialysis[1-3]. In 2018, in the Netherlands, 941 patients received a kidney transplant and a total of 11,405 kidney transplant recipients were alive with a functioning allograft[4]. However, due to an imbalance between donor kidney supply and demand, 1,271 patients are currently on the waiting list for a kidney transplant in the Netherlands (1-1-2020)[5].

Kidney transplantation is a very successful therapy with reported five-year kidney transplant survival rates currently ranging between 91.8% (for deceased donor kidneys) and 95.6% (for living donor kidneys)[6]. The two main causes of kidney transplant loss are death of a patient with a functioning allograft and kidney transplant rejection. When the donor is genetically different from the recipient, immune cells of the recipient will recognize the donor kidney as foreign because of differences in human leucocyte antigens (HLA)[7]. This will trigger a robust immune response directed against the donor kidney, a process called transplant rejection. If left untreated, kidney transplant rejection will ultimately destroy the allograft.

Despite significant advances in the clinical care of kidney transplant recipients, kidney transplant rejection complicates a significant proportion of kidney transplantations[8,9]. Large randomized controlled trials report rejection rates between 8 and 16%. However, most of these trials included a highly selected patient population with a low risk of rejection[10-13]. The incidence of acute rejection is likely to be higher in the real world. Reports from major registries of kidney transplant recipients show rejection rates ranging between 9% (United States)[8] and 21.4% (Australia/New Zealand)[9,14]. In the Erasmus MC, the incidence of acute rejection in the first three months after transplantation is approximately 30% (Figure 1). This difference in acute rejection rates may reflect differences in the use of induction immunosuppression, criteria for transplantation acceptance of donor and recipient, changes in the definition of rejection, and differences in the ethnicity of patients[8,15].

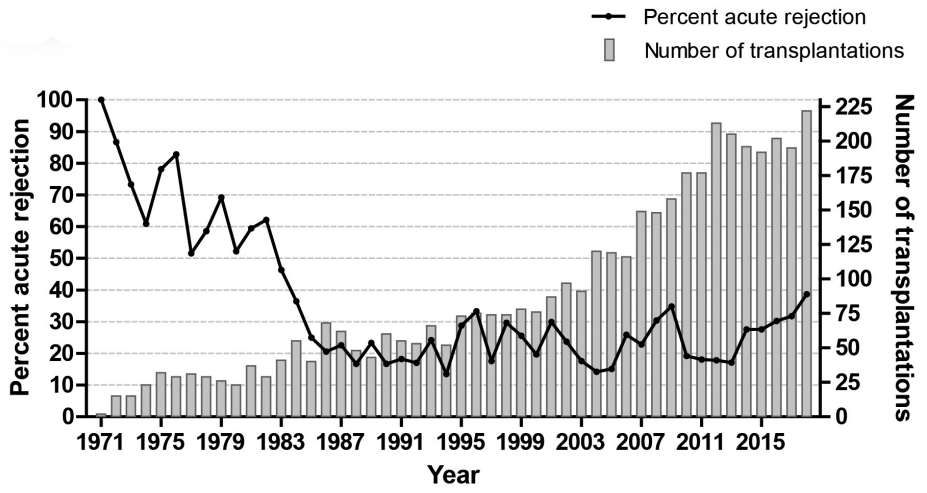

Figure 1. The number of kidney transplantations performed annually and percentage of acute rejection in Erasmus MC. The number of kidney transplantations is shown in the grey bars. The black line represents the percentage of acute rejection in the first three months after kidney transplantation. These acute rejections are presumed and/or biopsy proven acute rejections.

Kidney transplant rejection

A rejection can either be hyperacute (occurring within hours after transplantation), acute (within days to weeks), late acute (after three months) or chronic. Most rejections occur within the first weeks after transplantation[7]. The immune response to an allograft can occur via the direct, indirect and semidirect pathway of allorecognition and involves many components or the immune system (Figure 2)[7,16].

In the direct pathway, the T cell receptor (TCR) on naive T cells (both $CD4^+$ and $CD8^+$) of the recipient recognizes intact HLA molecules on donor-derived antigen presenting cells (APCs; Figure 2A). In the indirect pathway, alloantigens are taken up by the recipient's APCs and presented as processed peptides in the context of an intact HLA molecule to recipient naive $CD4^+$ T cells (Figure 2B). In the semidirect pathway, fragments of the donor cell membrane which contain HLA molecules, are transferred to the membrane of recipient's APCs. This results in presentation of intact donor HLA molecules by recipient APCs (Figure 2C)[7,16]. The direct pathway is the most important pathways directly after transplantation, while the indirect pathway is the dominant pathway later after transplantation (Figure 3)[16]. The exact role and timing of the semidirect pathway after transplantation remains to be elucidated[16,17].

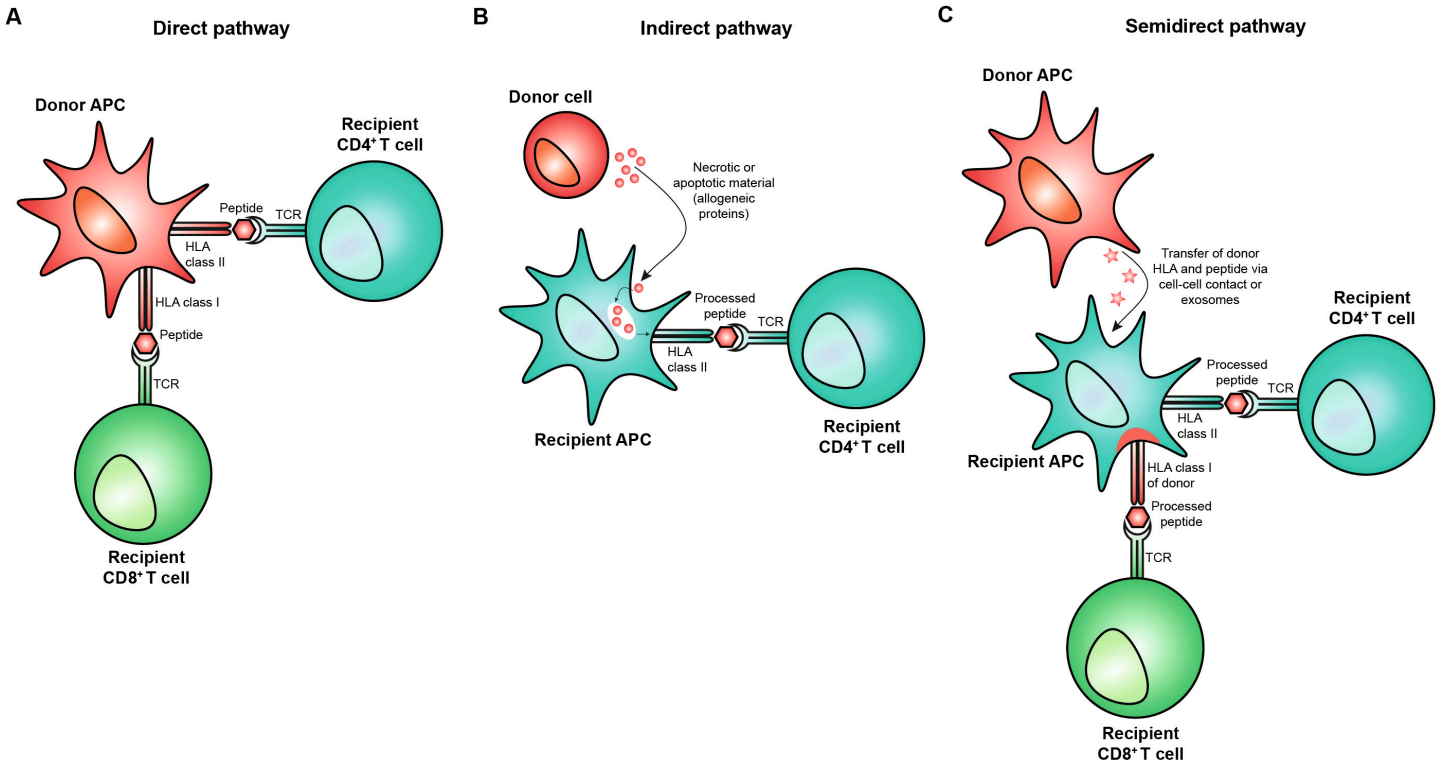

Figure 2. The direct, indirect and semidirect pathway of allorecognition in organ transplantation.

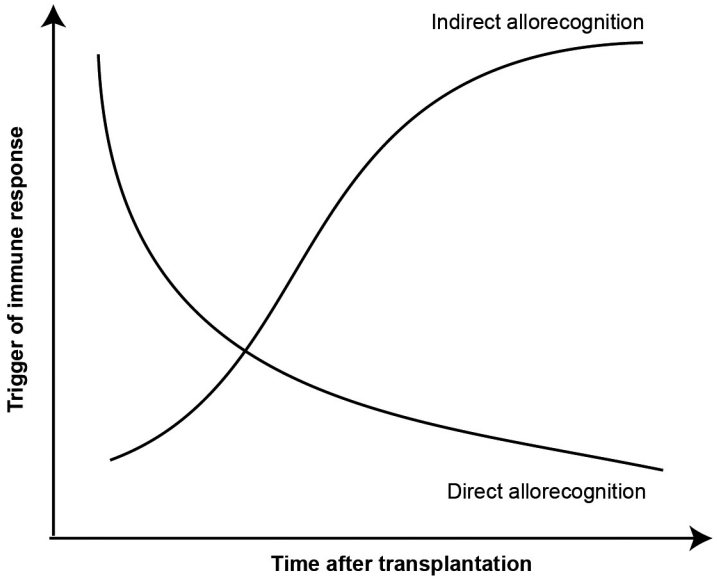

Figure 3. The occurrence of the direct and indirect pathway of allorecognition after organ transplantation.

The recognition of antigens by T cells in combination with costimulatory signals and cytokines promotes T cell proliferation and the generation of diverse subsets of $CD4^+$ and $CD8^+$ T cells which infiltrate the allograft[18]. $CD4^+$ T cells acquire helper function, while $CD8^+$ T cells are usually cytotoxic. Activated $CD4^+$ T cells secrete proinflammatory cytokines (*i.e.* interferon-γ) that allow them to provide help for activation of $CD8^+$ cells, B cells and various cells of the innate immune system.

Two types of rejection are distinguished: T cell-mediated rejection (TCMR) and antibody-mediated rejection (ABMR)[19]. In TCMR, $CD4^+$ T cells help cytotoxic $CD8^+$ T cells to release cytotoxic molecules such as perforin and granzyme that cause apoptosis (cell death) of allograft cells. In addition, $CD4^+$ effector cells can activate cells of the innate immune system (*i.e.* monocytes, natural killer [NK] cells, and macrophages), that subsequently destroy allograft cells. Infiltration of the above-mentioned mononuclear cells into renal tubular cells causes tubulitis, while invasion of these cells into arteries is called arteritis (Figure 4)[7].

In ABMR, $CD4^+$ T cells help B lymphocytes to differentiate into plasma cells that subsequently produce antibodies directed against HLA- and non-HLA antigens expressed in the transplant. These alloantibodies will bind to their target antigens and innate

immune cells (NK cells, neutrophils, and macrophages) interact with the Fc fragments of the alloantibodies. This in turn causes degranulation and release of lytic enzymes, which subsequently causes injury and cell death of the endothelial cells in the peritubular and glomerular capillaries. The microvascular injury may then lead to platelet aggregation and the formation of microthrombi in the capillaries (Figure 4)[20].

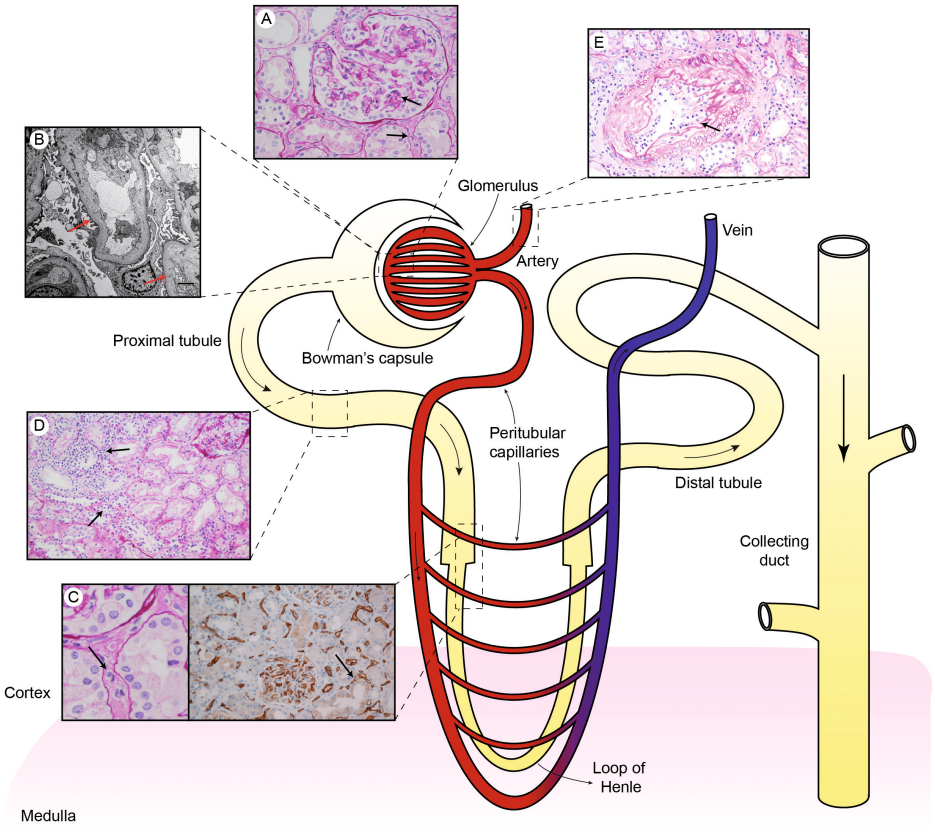

Figure 4. Histology of ABMR and TCMR. Glomerulitis (**A**), double contours of the glomerular basement membrane (**B**) and peritubular capillaritis (**C** left panel) and complement 4D positivity in the peritubular capillaritis (**C**, right panel) are features of ABMR. Interstitial inflammation (**D**), tubulitis (**D**) and arteritis (**E**) can be seen in biopsies with aTCMR.

After primary antigen exposure, such as after an infection, vaccination, pregnancy, blood transfusion or organ transplantation, memory T- and B cells are formed. These cells provide long-lasting immunity to previously encountered antigens and upon re-exposure will rapidly respond to this same antigen. Memory T- and B cells have a reduced activation threshold

and are less dependent on costimulation[21,22]. In case of an infection, memory T- and B-cells are extremely helpful in effectively fighting an infection. However, in transplantation memory T- and B cells pose a threat to the allograft[23].

Prevention of kidney transplant rejection

To prevent kidney transplant rejection, kidney transplant recipients receive life-long immunosuppressive therapy. This treatment can be divided into induction and maintenance immunosuppressive therapy. Induction therapy is administered around the time of transplant surgery. Because patients are at a high risk of acute rejection in the first months after transplantation (when direct and semidirect allorecognition are in effect) they require extra immunosuppression. Induction therapy generally consists of high-dose glucocorticoids in combination with biologicals (an interleukin [IL]-2 receptor antagonist [basiliximab], rabbit anti-thymocyte globulin [rATG] or alemtuzumab)[24]. Maintenance immunosuppressants are started at the time of transplantation and must be continued life-long. The typical maintenance immunosuppressive regimen includes glucocorticoids plus a calcineurin inhibitor (CNI; *i.e.* tacrolimus and ciclosporin), an antiproliferative agents (mycophenolic acid [MPA] or azathioprine), or a mammalian target of rapamycin (mTOR) inhibitor[10,24].

After the clinical introduction of CNIs in the 1980s (ciclosporin) and mid-1990's (tacrolimus), short-term kidney transplant outcomes improved dramatically, mainly as a result of a marked reduction in the incidence of acute rejection (Figure 1)[6]. In contrast, the long-term kidney transplant survival has only improved to a limited degree[25]. One of the factors that negatively influences the long-term allograft- and patient outcome is the toxicity of CNIs, which includes nephrotoxicity and metabolic side effects (post-transplant diabetes mellitus, hypertension, dyslipidemia). Furthermore, many kidney transplant recipients experience other side effects, like neurotoxicity (*i.e.* tremors and peripheral polyneuropathy), (opportunistic) infections, and malignancies[13,26,27]. Therefore, numerous strategies to eliminate or reduce the exposure to CNIs have been investigated over the last 25 years. These include more precise dosing of CNI by means of therapeutic drug monitoring and CNI-sparing strategies (minimization, withdrawal, conversion or avoidance). Alternative immunosuppressive drugs have been tested in CNI conversion or avoidance regimens, for instance mTOR inhibitors (everolimus and sirolimus), costimulation blockade drugs (*i.e.* belatacept), protein kinase C inhibitor (sotrastaurin), and Janus kinase (JAK)1/JAK3 inhibitor (tofacitinib). Because these drugs either had a lower efficacy (increased incidence of acute rejection as compared with CNI-based therapy) and/or serious sides effects (infections,

malignancy and toxicity), the standard of care immunosuppressive regimen still includes CNIs in 93% of kidney transplant recipients[8,10,28-33].

Belatacept, a fusion protein composed of a crystallizable fragment of immunoglobulin G1 and the extracellular domain of Cytotoxic T Lymphocyte Antigen (CTLA)-4, is an immunosuppressive drug that selectively targets the CD28-CD80/CD86 costimulatory pathway[34]. It is the only costimulation blockade drug that is currently approved for the prevention of kidney transplant rejection[35]. Further information regarding the use of belatacept in kidney transplantation is provided in **Chapter 2** of this introduction.

Diagnosis of kidney transplant rejection

Early detection of rejection is important to prevent allograft damage. Most patients with rejection of their transplant are asymptomatic and therefore clinical monitoring of allograft function is necessary. Clinical monitoring is currently based on the measurement of creatinine and urea in the blood (serum) and quantification of urinary protein excretion. However, these biomarkers are suboptimal as they are not specific for transplant rejection. Serum creatinine concentration may increase as a results of a number of clinical conditions, including urinary tract infections, hydronephrosis, drug toxicity or recurrence of primary kidney disease. Therefore, in case of an unexplained rise in the serum creatinine concentration, a rejection must be excluded. The gold standard to diagnose rejection is the histopathologic evaluation of a core needle biopsy from the allograft (Figure 4). The Banff classification is an international, standardized, histopathology-based classification that provides guidance for the diagnosis of transplant rejection[19]. Two main categories of rejection are described in the most recent Banff guideline: 1) Antibody-mediated changes (active ABMR [aABMR], chronic active ABMR [c-aABMR]); and 2) Borderline changes suspicious for acute TCMR (b-aTCMR), TCMR (acute [aTCMR] and chronic-active TCMR [c-aTMCR])[19].

A considerable number of transplant centers worldwide perform kidney transplant biopsies at predetermined time points after kidney transplantation. These so-called surveillance or protocol biopsies help to monitor the health of the allograft and identify subclinical rejection. The latter is the unexpected finding of a rejection in a clinically stable patient.

According to the Banff classification, the diagnosis of aABMR encompasses the histologic evidence of acute tissue injury (microvascular inflammation, intimal or transmural arteritis, acute thrombotic microangiopathy and/or acute tubular injury) and recent antibody

interaction with vascular endothelium (as evidenced by linear complement 4d [C4d] staining in peritubular capillaries, microvascular inflammation and/or increased intragraft expression of genes associated with ABMR; Figure 4). In addition, donor-specific anti-HLA (DSA) and non-HLA antibodies should be analyzed in the recipient's blood[19]. Evidence of chronic tissue injury (transplant glomerulopathy, multilayering of the peritubular capillary basement membrane or arterial intimal fibrosis) in combination with the afore-mentioned criteria for aABMR is diagnostic for c-aABMR[19].

Three grades of aTCMR are defined according to the Banff classification and this depends on the presence and severity of interstitial inflammation, tubulitis and/or arteritis[19]. Borderline changes suspicious for aTCMR are denoted by mild interstitial inflammation and tubulitis in transplant biopsies (Figure 4)[19]. c-aTCMR is diagnosed when inflammation is present in areas with interstitial fibrosis and tubular atrophy (i-IF/TA) in combination with moderate or severe tubulitis[19].

Although histologic examination of kidney tissue is the gold standard for the diagnosis of kidney transplant rejection, a kidney biopsy has its limitations. It is an invasive procedure with a risk for significant complications, such as bleeding. Furthermore, a biopsy is not always possible in patients with uncontrolled hypertension or a bleeding diathesis[36]. Therefore, there is an unmet need for an alternative, non- or minimally-invasive biomarker with a high sensitivity and specificity to detect kidney transplant rejection. Biomarkers may provide early detection of rejection, (*i.e.* detection at an early stage), preferentially before irreversible damage develops. It is vital that the assay is fast (short turnaround time) and inexpensive. Examples of material that can be used for the detection of minimally-invasive biomarkers are blood and urine. Various biomarkers are currently under investigation, and these include the analysis of messenger ribosomal nucleic acid (mRNA; transcriptomics), proteins (proteomics), and small deoxyribonucleic acid (DNA) fragments outside the donor cells (donor-derived cell free DNA)[37,38].

Two other limitations of a kidney biopsy are sampling error and limited reproducibility due to interobserver variation between (nephro)pathologists[39,40]. It is suggested in the Banff 2017 report that the application of gene expression analysis of a kidney transplant biopsy combined with the histopathologic evaluation by a pathologist, may improve diagnostic classification and prognosis[19]. The report offers a list of genes, many associated with ABMR and TCMR[19]. Several platforms can be used to analyze the intragraft gene expression,

such as real-time polymerase chain reaction, microarray and direct digital quantification analysis[41-44].

Treatment of acute kidney transplant rejection

Treatment of kidney transplant rejection is essential to prevent transplant failure[45]. The type (TCMR *versus* ABMR and acute *versus* chronic) and severity (tubulointerstitial rejection [aTMCR grade I] *versus* vascular rejection [aTMCR grade II and III]) of rejection determine the type of therapy[19,46].

The optimal treatment regimen for aABMR remains to be determined[24]. The empirical treatment of aABMR includes the augmentation of baseline immunosuppression in combination with removal and the suppression of the production of DSA with high-dose intravenous glucocorticoids, intravenous immunoglobulins, plasma-exchange, and/or lymphocyte-depleting antibodies[24,47,48]. No approved therapies are registered for the treatment of c-aABMR. Various combinations of immunomodulatory therapies are described in the literature, including high-dose intravenous glucocorticoids, intravenous immunoglobulins, plasma-exchange, rituximab (an anti-CD20 antibody), lymphocyte-depleting drugs, bortezomib (a proteasome inhibitor) and tocilizumab (an IL-6 receptor antibody)[49-53]. However, none of these treatments have demonstrated unequivocal benefit and some of these are not effective at all.

TCMR requires a different therapeutic intervention than ABMR. The first-line therapy for aTCMR includes high-dose intravenous glucocorticoids and intensification of the maintenance immunosuppressive therapy[24]. In case of a severe (aTCMR grade IIA or higher), recurrent or glucocorticoid-resistant aTCMR, the Kidney Disease: Improving Global Outcomes (KDIGO) guideline advises the use of rATG, a lymphocyte-depleting drug[24]. rATG is a purified polyclonal immunoglobulin preparation of sera from rabbits immunized with fresh human thymocytes[54]. Therapy with rATG leads to immunomodulation by elimination of various cell types, including T- and B cells, NK cells, macrophages, dendritic cells and other non-lymphoid cells (*i.e.* erythrocytes and platelets) that lasts for several months. Furthermore, rATG interferes with the function of regulatory T cells and NK cells and downregulates key cell-surface molecules that mediate leukocyte-endothelium interactions[55].

Alemtuzumab is another lymphocyte-depleting drug. However, it is rarely used as treatment for severe, recurrent or glucocorticoid-resistant aTCMR. It is a humanized monoclonal

antibody directed against the CD52 antigen that is present on T- and B cells, NK cells, dendritic cells, monocytes and granulocytes[56]. These cells are lysed after therapy with alemtuzumab and depletion is long-lasting (ranging between 3 months [monocytes] and three years [T cells]). Further information on the use of alemtuzumab in kidney transplant recipients is provided in **Chapter 3** of this introduction.

Patients with a b-aTCMR show very heterogeneous outcomes, ranging from spontaneous resolution to the development of aTCMR in up to a third of cases[57]. No clinical guideline exists for the management of b-aTCMR. Most physicians will treat patients with a b-aTCMR when they have an impaired renal function which is not explained otherwise. The type of therapy is uncertain but generally follows that of aTCMR[57]. Patients with a subclinical b-aTCMR in a protocol biopsy (b-aTCMR in clinically stable patients) should be monitored closely, including histological surveillance, and their immunosuppressive drugs should not be minimized[57].

The category c-aTCMR was for the first time incorporated into the 2015 Banff guideline[58]. The presence of i-IF/TA lesions in a kidney allograft is thought to be related to chronic underimmunosuppression, is frequently preceded by aTCMR, and is associated with adverse transplant outcomes[59-62]. However, the optimal management of c-aTCMR is currently unknown[61,62].

Outcomes after kidney transplant rejection

Despite improvement in clinical care, kidney transplant rejection still occurs in a considerable number of kidney transplant recipients. Kidney transplant rejection is associated with long-lasting consequences. First, transplant rejection is associated with a decline in renal function, proteinuria and premature transplant failure, especially in patients with a kidney function that does not return to baseline after anti-rejection treatment and in patients with a vascular- or antibody-mediated rejection[9]. Second, patients who experience an acute rejection within the first six months after transplantation have a higher risk of a recurrent rejection beyond six months (Hazard Ratio [HR], 1.85; 95%-confidence interval [CI], 1.39 to 2.46)[9]. Furthermore, aTCMR appears to be a risk factor for the formation of *de novo* DSA and subsequent development of ABMR[63,64]. Increased sensitization can also lead to a reduced likelihood to receive a subsequent solid organ transplant. Third, the potent immunosuppressive effect of anti-rejection therapy is necessary to control the rejection but also leads to an increased risk of adverse events, such as sepsis, secondary auto-immunity, and malignancy[9,65]. Fourth, the combination of an inferior transplant

function, higher risk of transplant loss and the adverse events associated with anti-rejection therapy, leads to an increased risk of death[9]. Fifth, kidney transplant rejection causes higher costs due to increased need for laboratory testing, treatment, hospital admissions, and re-transplantation[66]. Lastly, rejection impacts the psychological well-being of patients[67].

To conclude, kidney transplant rejection is a serious complication after kidney transplantation and is associated with a high burden of morbidity, mortality and higher health care-related costs. Improvement in terms of prevention, early recognition and treatment are key to improve kidney transplant- and patient survival.

REFERENCES

1. Schnuelle P, Lorenz D, Trede M, Van Der Woude FJ. Impact of renal cadaveric transplantation on survival in end-stage renal failure: evidence for reduced mortality risk compared with hemodialysis during long-term follow-up. *J Am Soc Nephrol.* 1998;9(11):2135-2141.
2. Hricik DE, Halbert RJ, Barr ML, et al. Life satisfaction in renal transplant recipients: preliminary results from the transplant learning center. *Am J Kidney Dis.* 2001;38(3):580-587.
3. Wolfe RA, Ashby VB, Milford EL, et al. Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant. *N Engl J Med.* 1999;341(23):1725-1730.
4. Nefrodata website: https://www.nefrovisie.nl/nefrodata/, asssessed on 22 March 2020.
5. Eurotransplant website: https://www.eurotransplant.org/cms/index.php?page=pat_netherlands, assessed on 22 March 2020.
6. Neuberger JM, Bechstein WO, Kuypers DR, et al. Practical Recommendations for Long-term Management of Modifiable Risks in Kidney and Liver Transplant Recipients: A Guidance Report and Clinical Checklist by the Consensus on Managing Modifiable Risk in Transplantation (COMMIT) Group. *Transplantation.* 2017;101(4S Suppl 2):S1-S56.
7. Nankivell BJ, Alexander SI. Rejection of the kidney allograft. *N Engl J Med.* 2010;363(15):1451-1462.
8. Hart A, Smith JM, Skeans MA, et al. OPTN/SRTR 2017 Annual Data Report: Kidney. *Am J Transplant.* 2019;19 Suppl 2:19-123.
9. Clayton PA, McDonald SP, Russ GR, Chadban SJ. Long-Term Outcomes after Acute Rejection in Kidney Transplant Recipients: An ANZDATA Analysis. *J Am Soc Nephrol.* 2019;30(9):1697-1707.
10. Pascual J, Berger SP, Witzke O, et al. Everolimus with Reduced Calcineurin Inhibitor Exposure in Renal Transplantation. *J Am Soc Nephrol.* 2018;29(7):1979-1991.
11. Vincenti F, Charpentier B, Vanrenterghem Y, et al. A phase III study of belatacept-based immunosuppression regimens versus cyclosporine in renal transplant recipients (BENEFIT study). *Am J Transplant.* 2010;10(3):535-546.
12. Group CSC, Haynes R, Harden P, et al. Alemtuzumab-based induction treatment versus basiliximab-based induction treatment in kidney transplantation (the 3C Study): a randomised trial. *Lancet.* 2014;384(9955):1684-1690.
13. Ekberg H, Tedesco-Silva H, Demirbas A, et al. Reduced exposure to calcineurin inhibitors in renal transplantation. *N Engl J Med.* 2007;357(25):2562-2575.
14. Australia and New Zealand Dialysis and Transplant Registry: 39th Annual Report, Chapter 7 Transplantation: https://www.anzdata.org.au/report/anzdata-41st-annual-report-2018-anzdata/. Assessed on 22 March 2020.
15. Lebranchu Y, Baan C, Biancone L, et al. Pretransplant identification of acute rejection risk following kidney transplantation. *Transpl Int.* 2014;27(2):129-138.
16. Wood KJ, Goto R. Mechanisms of rejection: current perspectives. *Transplantation.* 2012;93(1):1-10.
17. Smyth LA, Lechler RI, Lombardi G. Continuous Acquisition of MHC:Peptide Complexes by Recipient Cells Contributes to the Generation of Anti-Graft CD8(+) T Cell Immunity. *Am J Transplant.* 2017;17(1):60-68.

18. O'Shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science.* 2010;327(5969):1098-1102.

19. Haas M, Loupy A, Lefaucheur C, et al. The Banff 2017 Kidney Meeting Report: Revised diagnostic criteria for chronic active T cell-mediated rejection, antibody-mediated rejection, and prospects for integrative endpoints for next-generation clinical trials. *Am J Transplant.* 2018;18(2):293-307.

20. Colvin RB. Antibody-mediated renal allograft rejection: diagnosis and pathogenesis. *J Am Soc Nephrol.* 2007;18(4):1046-1056.

21. Sprent J, Surh CD. T cell memory. *Annu Rev Immunol.* 2002;20:551-579.

22. Seifert M, Kuppers R. Human memory B cells. *Leukemia.* 2016;30(12):2283-2292.

23. Luque S, Lucia M, Melilli E, et al. Value of monitoring circulating donor-reactive memory B cells to characterize antibody-mediated rejection after kidney transplantation. *Am J Transplant.* 2019;19(2):368-380.

24. Kidney Disease: Improving Global Outcomes Transplant Work G. KDIGO clinical practice guideline for the care of kidney transplant recipients. *Am J Transplant.* 2009;9 Suppl 3:S1-155.

25. Lamb KE, Lodhi S, Meier-Kriesche HU. Long-term renal allograft survival in the United States: a critical reappraisal. *Am J Transplant.* 2011;11(3):450-462.

26. Claes K, Meier-Kriesche HU, Schold JD, et al. Effect of different immunosuppressive regimens on the evolution of distinct metabolic parameters: evidence from the Symphony study. *Nephrol Dial Transplant.* 2012;27(2):850-857.

27. Nankivell BJ, Borrows RJ, Fung CL, et al. The natural history of chronic allograft nephropathy. *N Engl J Med.* 2003;349(24):2326-2333.

28. Tedesco-Silva H, Kho MM, Hartmann A, et al. Sotrastaurin in calcineurin inhibitor-free regimen using everolimus in de novo kidney transplant recipients. *Am J Transplant.* 2013;13(7):1757-1768.

29. Baan CC, Kannegieter NM, Felipe CR, Tedesco Silva H, Jr. Targeting JAK/STAT Signaling to Prevent Rejection After Kidney Transplantation: A Reappraisal. *Transplantation.* 2016;100(9):1833-1839.

30. Vincenti F, Silva HT, Busque S, et al. Evaluation of the effect of tofacitinib exposure on outcomes in kidney transplant patients. *Am J Transplant.* 2015;15(6):1644-1653.

31. Bouamar R, Shuker N, Osinga JAJ, et al. Conversion from tacrolimus to everolimus with complete and early glucocorticoid withdrawal after kidney transplantation: a randomised trial. *Neth J Med.* 2018;76(1):14-26.

32. Shipkova M, Hesselink DA, Holt DW, et al. Therapeutic Drug Monitoring of Everolimus: A Consensus Report. *Ther Drug Monit.* 2016;38(2):143-169.

33. Karpe KM, Talaulikar GS, Walters GD. Calcineurin inhibitor withdrawal or tapering for kidney transplant recipients. *Cochrane Database Syst Rev.* 2017;7:CD006750.

34. Larsen CP, Pearson TC, Adams AB, et al. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. *Am J Transplant.* 2005;5(3):443-453.

35. Archdeacon P, Dixon C, Belen O, Albrecht R, Meyer J. Summary of the US FDA approval of belatacept. *Am J Transplant.* 2012;12(3):554-562.

36. Morgan TA, Chandran S, Burger IM, Zhang CA, Goldstein RB. Complications of Ultrasound-Guided Renal Transplant Biopsies. *Am J Transplant.* 2016;16(4):1298-1305.

37. Eikmans M, Gielis EM, Ledeganck KJ, et al. Non-invasive Biomarkers of Acute Rejection in Kidney Transplantation: Novel Targets and Strategies. *Front Med (Lausanne).* 2018;5:358.

38. Verhoeven J, Boer K, Van Schaik RHN, et al. Liquid Biopsies to Monitor Solid Organ Transplant Function: A Review of New Biomarkers. *Ther Drug Monit.* 2018;40(5):515-525.

39. Broecker V, Mengel M. The significance of histological diagnosis in renal allograft biopsies in 2014. *Transpl Int.* 2015;28(2):136-143.

40. Schinstock CA, Sapir-Pichhadze R, Naesens M, et al. Banff survey on antibody-mediated rejection clinical practices in kidney transplantation: Diagnostic misinterpretation has potential therapeutic implications. *Am J Transplant.* 2019;19(1):123-131.

41. Suthanthiran M. Molecular analyses of human renal allografts: differential intragraft gene expression during rejection. *Kidney Int Suppl.* 1997;58:S15-21.

42. Sabek O, Dorak MT, Kotb M, Gaber AO, Gaber L. Quantitative detection of T-cell activation markers by real-time PCR in renal transplant rejection and correlation with histopathologic evaluation. *Transplantation.* 2002;74(5):701-707.

43. Halloran PF, Einecke G. Microarrays and transcriptome analysis in renal transplantation. *Nat Clin Pract Nephrol.* 2006;2(1):2-3.

44. Geiss GK, Bumgarner RE, Birditt B, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat Biotechnol.* 2008;26(3):317-325.

45. Matas AJ, Gillingham KJ, Payne WD, Najarian JS. The impact of an acute rejection episode on long-term renal allograft survival (t1/2). *Transplantation.* 1994;57(6):857-859.

46. Rekers NV, de Fijter JW, Claas FH, Eikmans M. Mechanisms and risk assessment of steroid resistance in acute kidney transplant rejection. *Transpl Immunol.* 2016;38:3-14.

47. Wan SS, Ying TD, Wyburn K, et al. The Treatment of Antibody-Mediated Rejection in Kidney Transplantation: An Updated Systematic Review and Meta-Analysis. *Transplantation.* 2018;102(4):557-568.

48. Schinstock CA, Mannon RB, Budde K, et al. Recommended Treatment for Antibody-mediated Rejection After Kidney Transplantation: the 2019 Expert Consensus From the Transplantion Society Working Group. *Transplantation.* 2020.

49. Sablik KA, Clahsen-van Groningen MC, Looman CWN, et al. Treatment with intravenous immunoglobulins and methylprednisolone may significantly decrease loss of renal function in chronic-active antibody-mediated rejection. *BMC Nephrol.* 2019;20(1):218.

50. Smith RN, Malik F, Goes N, et al. Partial therapeutic response to Rituximab for the treatment of chronic alloantibody mediated rejection of kidney allografts. *Transpl Immunol.* 2012;27(2-3):107-113.

51. Choi J, Aubert O, Vo A, et al. Assessment of Tocilizumab (Anti-Interleukin-6 Receptor Monoclonal) as a Potential Treatment for Chronic Antibody-Mediated Rejection and Transplant Glomerulopathy in HLA-Sensitized Renal Allograft Recipients. *Am J Transplant.* 2017;17(9):2381-2389.

52. Nanmoku K, Shinzato T, Kubo T, Shimizu T, Yagisawa T. Effect of Rabbit Antithymocyte Globulin on Acute and Chronic Active Antibody-Mediated Rejection After Kidney Transplantation. *Transplant Proc.* 2019.

53. Eskandary F, Regele H, Baumann L, et al. A Randomized Trial of Bortezomib in Late Antibody-Mediated Kidney Transplant Rejection. *J Am Soc Nephrol.* 2018;29(2):591-605.

54. Product monograph of Thymoglobulin; http://products.sanofi.ca/en/Thymoglobulin.pdf.

55. Mueller TF. Mechanisms of Action of Thymoglobulin. *Transplantation.* 2007;84(11S):S5-S10.

56. Hale G, Bright S, Chumbley G, et al. Removal of T cells from bone marrow for transplantation: a monoclonal antilymphocyte antibody that fixes human complement. *Blood.* 1983;62(4):873-882.

57. Nankivell BJ, Agrawal N, Sharma A, et al. The clinical and pathological significance of borderline T cell-mediated rejection. *Am J Transplant.* 2019;19(5):1452-1463.

58. Loupy A, Haas M, Solez K, et al. The Banff 2015 Kidney Meeting Report: Current Challenges in Rejection Classification and Prospects for Adopting Molecular Pathology. *Am J Transplant.* 2017;17(1):28-41.

59. Mengel M, Reeve J, Bunnag S, et al. Scoring total inflammation is superior to the current Banff inflammation score in predicting outcome and the degree of molecular disturbance in renal allografts. *Am J Transplant.* 2009;9(8):1859-1867.

60. Mannon RB, Matas AJ, Grande J, et al. Inflammation in areas of tubular atrophy in kidney allograft biopsies: a potent predictor of allograft failure. *Am J Transplant.* 2010;10(9):2066-2073.

61. Lefaucheur C, Gosset C, Rabant M, et al. T cell-mediated rejection is a major determinant of inflammation in scarred areas in kidney allografts. *Am J Transplant.* 2018;18(2):377-390.

62. Nankivell BJ, Shingde M, Keung KL, et al. The causes, significance and consequences of inflammatory fibrosis in kidney transplantation: The Banff i-IFTA lesion. *Am J Transplant.* 2018;18(2):364-376.

63. El Ters M, Grande JP, Keddis MT, et al. Kidney allograft survival after acute rejection, the value of follow-up biopsies. *Am J Transplant.* 2013;13(9):2334-2341.

64. Moreso F, Carrera M, Goma M, et al. Early subclinical rejection as a risk factor for late chronic humoral rejection. *Transplantation.* 2012;93(1):41-46.

65. van der Zwan M, Baan CC, van Gelder T, Hesselink DA. Review of the Clinical Pharmacokinetics and Pharmacodynamics of Alemtuzumab and Its Use in Kidney Transplantation. *Clin Pharmacokinet.* 2018;57(2):191-207.

66. Gheorghian A, Schnitzler MA, Axelrod DA, et al. The implications of acute rejection and reduced allograft function on health care expenditures in contemporary US kidney transplantation. *Transplantation.* 2012;94(3):241-249.

67. Timmerman L, Laging M, Timman R, et al. The impact of the donors' and recipients' medical complications on living kidney donors' mental health. *Transpl Int.* 2016;29(5):589-602.

CHAPTER 2

Costimulation blockade in kidney transplant recipients

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ABSTRACT

Costimulation between T cells and antigen-presenting cells is essential for the regulation of an effective alloimmune response and is not targeted with the conventional immunosuppressive therapy after kidney transplantation. Costimulation blockade therapy with biologicals allows precise targeting of the immune response but without non-immune adverse events. Multiple costimulation blockade approaches have been developed that inhibit the alloimmune response in kidney transplant recipients with varying degrees of success. Belatacept, an immunosuppressive drug that selectively targets the CD28-CD80/CD86 pathway, is the only costimulation blockade therapy that is currently approved for kidney transplant recipients. In the last decade, belatacept therapy has been shown a promising therapy in subgroups of kidney transplant recipients; however, the widespread use of belatacept has been tempered by an increased risk of acute kidney transplant rejection. The purpose of this review is to provide an overview of the costimulation blockade therapies that are currently in use or being developed for kidney transplant indications.

Key points:

- Multiple costimulation blockade drugs have been developed and tested in kidney transplant recipients. Belatacept, a biological that inhibits the interaction between the antigen CD28 and CD80/86, is the only costimulation blockade drug that is currently approved for the prevention of kidney transplant rejection.
- Belatacept is well-tolerated and is associated with a better allograft function compared with calcineurin inhibitors. A reason for concern is the higher risk of acute kidney transplant rejection as compared with the current standard immunosuppressive therapy.
- Optimization of the selection of patients with a low risk for belatacept-resistant rejection in combination with new treatment strategies are necessary to expand the use of belatacept in the future.
- The safety and efficacy of several other biologicals that target costimulation pathways (*i.e.* CD28 and CD40) are currently investigated for kidney transplantation.

INTRODUCTION

Kidney transplant recipients (KTR) require lifelong immunosuppressive therapy to prevent acute kidney transplant rejection (AR). Currently, the standard immunosuppressive regimen consists of induction therapy (either a T cell-depleting agent or basiliximab, an antibody directed against the interleukin [IL]-2 receptor), followed by maintenance therapy consisting of a calcineurin inhibitor (CNI; either tacrolimus or ciclosporin), mycophenolic acid (MPA) with or without glucocorticoids[1-4]. Although transplantation is a success story of modern medicine, the long-term allograft- and patient survival are influenced by the toxicity of CNIs, which include infections, malignancies, metabolic side effects, nephrotoxicity and neurotoxicity[5-7]. Another limitation of current immunosuppression is that it is a 'one size fits all' therapy and is not tailored to the individual needs of a KTR. Therefore, novel and personalized therapeutic strategies have to be developed.

Several approaches have been investigated to limit the side effects of CNI, including monitoring of CNI concentrations to guide dosing, and CNI-sparing regimens. Examples of the latter are CNI minimization, CNI withdrawal, CNI conversion to alternative immunosuppressive agents, and lastly, CNI avoidance from the time of the transplantation with substitution of an alternative immunosuppressive drug[8]. However, many such trials failed because they resulted in unacceptably high incidences of AR and toxicity, or an increased incidence of infections associated with the alternative immunosuppressants[9-15].

Costimulation is essential for the regulation of an effective alloimmune response. The costimulatory pathway is not targeted with the conventional immunosuppressive therapy. Biologicals that intervene with the costimulatory pathway may allow more precise targeting of the immune response without causing non-immune adverse events. Belatacept, a fusion protein composed of a crystallizable fragment (Fc) of immunoglobulin (Ig) G1 and the extracellular domain of cytotoxic T lymphocyte protein 4 (CTLA4), is the only costimulation blockade therapy that is currently approved for the prevention of rejection after kidney transplantation[16,17]. Belatacept is well-tolerated and its use is associated with an improved allograft function compared with CNI in certain subgroups of KTRs[18,19]; however, belatacept may not be the game changer it was hoped to be due to a high risk of AR[20]. In this review, the current applications of biologicals that target costimulation pathways in kidney transplantation are discussed, including the current status and future strategies of belatacept therapy.

COSTIMULATION

The process of T-cell activation is a complex cascade consisting of three signals. First, alloantigens from the allograft are taken up by antigen-presenting cells (APCs; dendritic cells, macrophages and B cells) which then home to the draining lymph nodes. In the lymph nodes, the alloantigens are presented on the surface of APCs by human leucocyte antigen (HLA) molecules. In humans, the T-cell receptor (TCR) on naive T cells is activated after interaction with the alloantigen/HLA complex, which is also known as signal 1 (Figure 1). A costimulatory signal (signal 2) is necessary to achieve full activation of T cells. Several cell-surface proteins (costimulatory ligands) on APCs interact with their complementary receptors on naive T cells (Figure 1). Signal 2 represents a combination of positive and negative signals that regulate the outcome of the HLA/TCR. Without this signal, naive T cells will undergo apoptotic cell death[21-23].

Two costimulatory pathways are critical for T-cell activation: 1) the Ig superfamily (*e.g.* CD28 [T-cell specific surface glycoprotein CD28] family, the CD2/Signaling lymphocytic activation molecule (SLAM) family and the T-cell/transmembrane, Ig, and mucin (TIM) family; and 2) the TNF (tumor necrosis factor)-TNF receptor superfamily (Figure 1)[21].

Signal 3 is formed by cytokines and the (increased) expression of cytokine receptors such as the IL-2 receptor a-chain (CD25; Figure 1). Activation of CD25 will activate intracellular signaling pathways downstream of the TCR, including the mitogen-activated protein kinase (MAPK), calcineurin, and PI3K pathways, followed by the activation of transcription factors that regulate the production of several cytokines (*i.e.*, IL-2 and interferon-γ)[24]. These and other cytokines promote T-cell proliferation of divers effector $CD4^+$ T-cell subsets and cytotoxic $CD8^+$ T cells[25].

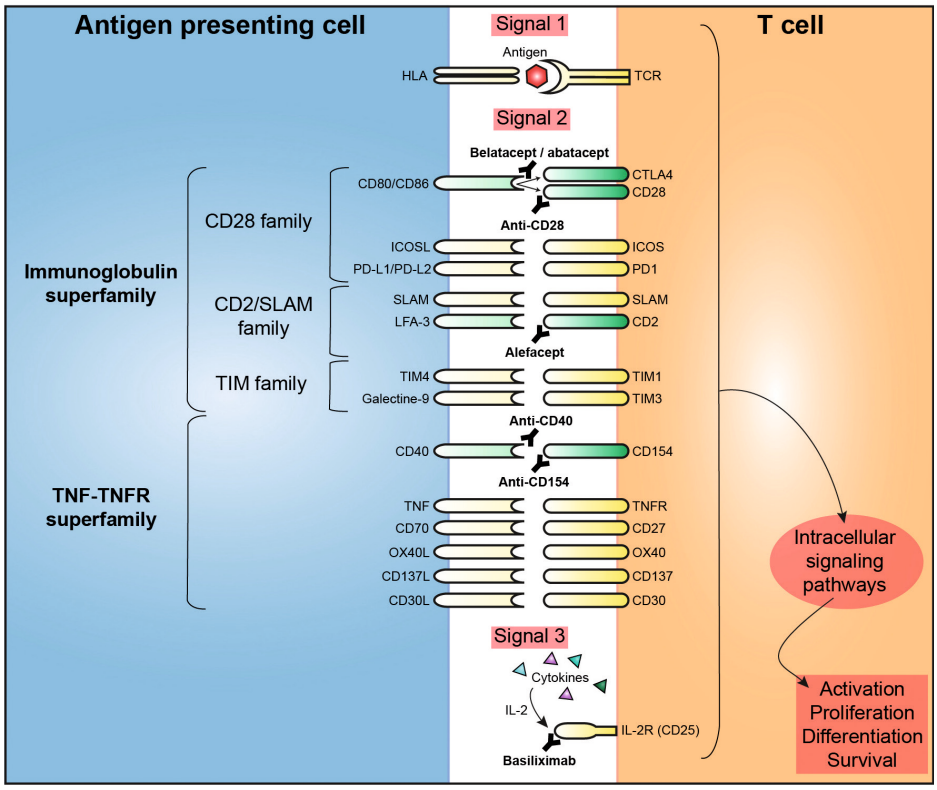

Figure 1. Costimulation between T cells and antigen-presenting cells. Schematic overview of signal 1, 2 and 3 of T-cell activation. During signal 2, costimulatory molecules on T cells and antigen-presenting cells interact to activate or inhibit T cells after alloantigen recognition. Two important groups of costimulatory molecules are presented: the immunoglobulin superfamily and the TNF-TNFR superfamily. The costimulatory molecules discussed in this review are green and the costimulatory molecules that are not discussed in are in yellow. Several biologicals are developed that interfere with the costimulatory molecules on T cells and antigen-presenting cells. CTLA 4, cytotoxic T lymphocyte protein 4; HLA, human leucocyte antigen; ICOS, inducible T cell costimulator; PD, programmed death; SLAM, Signaling lymphocytic activation molecule; TCR, T cell receptor, TIM, T cell/transmembrane, immunoglobulin, and mucin; TNF, tumor necrosis factor.

BELATACEPT THERAPY IN KIDNEY TRANSPLANTATION

Development of belatacept

Belatacept targets the CD28:CD80/CD86 pathway. The costimulation molecule CD28 is a surface receptor that is constitutively expressed on T cells (Figure 1). The inhibitory receptor CTLA4 is localized in intracellular vesicles in resting T cells and is expressed on the cell surface 48-72 hours after T-cell activation. CTLA4 binds to CD80 and CD86 with a higher affinity than CD28[21]. Therefore, the binding of CTLA4 to CD80/CD86 dampens the activation of T cells[26]. At birth, almost all human T cells express CD28[27]. Aging, continuous antigenic stimulation (which can be caused by *e.g.* end-stage renal disease, human immunodeficiency virus infection and auto-immune disease) and cytomegalovirus infection lead to loss of CD28 expression of T cells[27-29]. These $CD28^-$ effector memory T cells have reduced costimulatory requirements and an impaired proliferative capacity, but are highly proinflammatory[27,30]. These cells rapidly secrete effector cytokines (*i.e.* TNF-α and interferon-γ) upon restimulation.

One of the first biologics that was designed to target the CD28-CD80/CD86 superfamily was abatacept (Figure 1), a fusion protein composed of a Fc of IgG1 and the extracellular domain of CTLA4[31]. Because CTLA4 binds with a higher affinity to CD80/CD86 than CD28, it was hypothesized that T-cell activation could be inhibited with such a CTLA4 construct. Abatacept is approved for the treatment of rheumatoid arthritis (Figure 2)[32] and has been was tested in non-human primates transplanted with a kidney or pancreatic islets; however, alloreactivity appeared to be inhibited insufficiently[33,34]. Therefore, the development of abatacept therapy for transplantation was discontinued and a new CTLA4-Ig construct (belatacept) was developed with increased avidity for CD80 and CD86 by changing two amino acids (L104E and A29Y; Figure 1 and 2)[16]. Belatacept was found to have a fourfold higher binding affinity for CD86 and a twofold higher binding affinity for CD80 compared with abatacept[16]. Although the development of abatacept in transplantation was stopped, abatacept was recently used as rescue therapy in nine KTRs with an intolerance to CNI, because belatacept was temporarily unavailable due to manufacturing problems[35,36]. None of the allografts were lost after a median period of 115 months and one patient experienced AR[35].

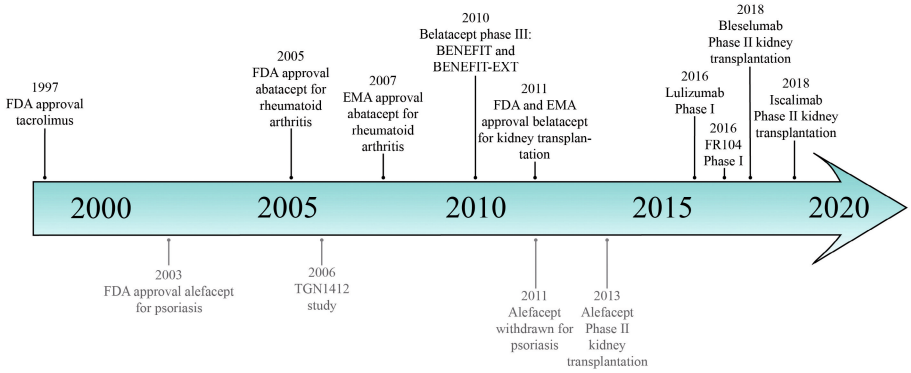

Figure 2. Timeline of the development of costimulation blockade. The costimulation blockade drugs that are currently used or tested in kidney transplant recipients are shown in black, whereas the costimulation blockade drugs that are no longer being used anymore or not developed for kidney transplantation are shown in grey. EMA, European Medicines Agency; FDA, United States Food and Drug Administration; FR104, Pegylated Monoclonal Antibody Fragment Antagonist of CD28; TGN1412, CD28 humanized antibody.

Belatacept was approved as treatment for the prevention of AR by the European Medicines Agency (EMA) and the United States Food and Drug Administration (FDA) in 2011 based on the results of two large randomized, controlled multicenter phase III trials (Figure 2): The Belatacept Evaluation of Nephroprotection and Efficacy as First-line Immunosuppression (BENEFIT) study (with standard criteria donors) and the BENEFIT-extended criteria donors (BENEFIT-EXT) study[17,37,38]. In these trials, 1,264 KTR were treated with either ciclosporin or belatacept as first-line treatment in combination with MPA and glucocorticoids. The main findings of BENEFIT and BENEFIT-EXT were that the 1-year-patient- and allograft survival of patients treated with belatacept were similar to patients treated with ciclosporin[37,38]. Although the incidence of acute T-cell-mediated rejection (aTCMR) was increased in belatacept-treated patients, the kidney function was better in these patients compared with ciclosporin-treated patients[37,38]. In addition, the use of belatacept was associated with increased risk for post-transplant lymphoproliferative disease mostly in Epstein-Barr virus seronegative KTR[37-39].

The safety and efficacy of belatacept were also tested in a phase II randomized, controlled multicenter trial in liver transplant recipients[40]. This trial randomized 260 patients between therapy with belatacept (three different belatacept regimens) or tacrolimus (two different tacrolimus regimens). The primary composite end point consisted of incidence of acute liver transplant rejection, graft loss and death at six months after transplantation. The

occurrence of the composite end point was higher in the belatacept groups (42-48%) than in the tacrolimus groups (15-38%)[40]. The results of this study were reason to discontinue further development of belatacept for liver transplantation. However, the mean estimated glomerular filtration rate (eGFR) was 15-34 mL/min/1.73 m^2 higher in the liver transplant recipients treated with belatacept[40]. Therefore, liver transplant recipients with an impaired renal function could benefit from belatacept therapy. Proper selection of patients and an adjusted treatment protocol can possibly improve the results of belatacept in liver transplantation in the future[41].

Clinical outcomes of de novo use of belatacept in KTR

A systematic review with meta-analysis was performed of five studies that compared treatment with belatacept to CNIs (including the BENEFIT and BENEFIT-EXT studies) in 1,535 KTR[42]. Of the 521 patients treated with a CNI, 478 patients used ciclosporin, and 43 patients were treated with tacrolimus. After 3 years of treatment, no difference was seen between patients treated with either belatacept or CNI regarding the risk of death (relative risk 0.75, 95%-confidence interval [CI] 0.39-1.44, $p = 0.39$), allograft loss (relative risk 0.91, 95%-CI 0.61-1.38, $p = 0.67$), and incidence of aTCMR (RR 1.56, 95%-CI 0.85-2.86, p = 0.15)[42]. However, the kidney allograft function was better in patients treated with belatacept (eGFR mean difference of 9.96 mL/min/1.73 m^2, 95%-CI 3.28-16.64, $p = 0.0035$). Furthermore, the use of belatacept was associated with a reduced incidence of post-transplant diabetes mellitus, a better blood pressure and a better lipid profile 1 year after therapy with belatacept[42].

In 2016, the 7-years follow-up results of the BENEFIT and BENEFIT-EXT were published. In these studies, the risks of death and graft loss in KTR treated with belatacept were similar to those in KTR treated with ciclosporin[18,19]. Although, the risk of aTCMR was higher in belatacept-treated patients compared with the ciclosporin-treated patients, their kidney function after 7 years was better. An explanation for the better kidney function may be that belatacept is associated with less interstitial inflammation and tubular atrophy compared with CNIs. Vitalone *et al.,* compared the 1-year protocol biopsies of KTR treated with belatacept or ciclosporin[43], and found that he biopsies of patients treated with belatacept contained less interstitial inflammation, interstitial fibrosis and tubular atrophy and gene expression analysis revealed a lower expression of genes involved in fibrosis and tubulointerstitial damage compared with the biopsies of patients treated with ciclosporin[43]. In another study, 10-year protocol biopsies were analyzed of 23 clinically stable KTRs treated with belatacept and 10 KTR treated with CNI (seven taking ciclosporin and three

taking tacrolimus)[44]. The biopsies of belatacept-treated patients contained less interstitial inflammation and tubular atrophy, less interstitial inflammation and less hyalinosis[44].

The 7-year follow-up studies also showed that the formation of *de novo* donor-specific anti-HLA antibodies (DSA) was reduced in the belatacept-treated patients compared to the patients treated with ciclosporin[18,19]. A possible explanation for this observation may be that costimulation blockade with belatacept leads to more effective prevention of DSA formation by B cells and that drug adherence is better in the patients treated with belatacept because of intravenous administration. The occurrence of post-transplant diabetes mellitus, blood pressure and lipid profile were not discussed in the long-term follow-up studies of belatacept.

To conclude, these long-term outcomes demonstrate that belatacept therapy is a safe therapy for KTR and is associated with a better kidney function and a reduced incidence of *de novo* DSA. Whether long-term belatacept therapy leads to a better metabolic profile than CNI therapy is not known[18,19,39].

A limitation of the BENEFIT studies is that belatacept therapy was compared to ciclosporin therapy. Currently, the CNI of choice in most transplant centers is tacrolimus[1,45]. No large, head-to-head randomized-controlled trials have been performed that compared the outcomes of patients treated with either belatacept or tacrolimus. In our center, a trial was performed which included 40 KTR who were randomized between first-line therapy consisting of tacrolimus or belatacept, in combination with MPA and glucocorticoids[46]. The AR incidence in the first year after transplantation was higher in belatacept-treated patients (55% versus 10%, $p = 0.006$)[46]. Another randomized-controlled trial compared three treatment regimens in KTR: alemtuzumab induction with tacrolimus, alemtuzumab induction with belatacept, and basiliximab induction with belatacept and a three-month course of tacrolimus[47]. This study was halted prematurely after inclusion of 19 patients due to a high rate of serious adverse events in belatacept-treated patients, including thrombotic complications and aTCMR[47].

The comparison between belatacept and tacrolimus therapy has also been investigated in three indirect studies[48-50]. In a single-center retrospective analysis, the outcomes of KTR treated with belatacept ($n = 97$) were compared with a historical cohort of patients treated with tacrolimus ($n = 205$)[48]. An increased rate of aTCMR was noted in patients treated with belatacept compared with tacrolimus-treated patients (50.5% *versus* 20.5%)[48]. In a retrospective propensity score-matched cohort study, the outcomes of KTR treated with

either tacrolimus or belatacept were compared[49]. The risk of AR was higher in the first post-transplant year in patients treated with belatacept (odds ratio 3.12, 95% CI 2.13-4.57, $p < 0.001$) but no difference was seen in the risk of death (hazard ratio 0.84, 95%-CI 0.61-1.15, $p = 0.28$) or allograft loss (hazard ratio 0.83, 95%-CI 0.62-1.11, $p = 0.20$)[49]. Muduma *et al*. performed a systematic review and meta-analysis with an indirect treatment comparison analysis between tacrolimus (both immediate release and prolonged-release formulations) and belatacept[50]. The AR rate was reduced in patients treated with tacrolimus compared to belatacept (risk ratio 0.22 [95%-CI 0.13-0.39] to 0.44 [0.20, 0.99])[50]. The risks of allograft loss and death were similar between both treatments.

One of the reasons for the high risk of aTCMR after belatacept may be that the immunosuppressive function of regulatory T cells (Tregs) is impaired[51-55]. Tregs are dependent on signaling via CTLA4 and binding of belatacept to CD80/86 interferes with CTLA4. Therefore, combination of belatacept with therapies that preserve Treg functionality, such as T-cell-depleting antibodies and mammalian target of rapamycin (mTOR) inhibitors could possibly lead to a reduced incidence of AR[4,56,57].

The combination of induction therapy with T-cell-depleting drugs and belatacept has been tested in several studies with various outcomes. In one study, alemtuzumab induction followed by tacrolimus or belatacept led to a similar incidence of AR[58]. In another study, patients treated with T-cell-depleting induction therapy (either rabbit anti-thymocyte globulin [rATG] of alemtuzumab) followed by belatacept were compared with patients treated with rATG induction followed by tacrolimus[59]. In all patients, glucocorticoids were withdrawn early. The AR incidence was higher in patients treated with belatacept, but the allograft- and patient survival were similar[59]. In a third study (described above), alemtuzumab induction with belatacept in KTR resulted in a high rate of serious adverse events and the study was halted prematurely[47]. T-cell-depleting induction therapy has also been tested in KTR treated with belatacept in combination with mTOR inhibitors (sirolimus or everolimus). The AR rate in patients treated with this combination of drugs is low, and a significant increase in Tregs is seen[60-63].

Possible explanations for a lower rate of acute rejection after T-cell-depleting induction therapy compared with basiliximab induction therapy are i) after T-cell-depletion therapy an increased repopulation of Tregs is seen, and ii) repopulated memory T cells in rATG-treated KTR show impaired cytokine responsiveness compared with those of basiliximab-treated KTR[57,64].

To conclude, although the studies that compare belatacept to tacrolimus therapy have their limitations (limited number of patients or indirect comparison), belatacept is associated with an increased risk of aTCMR.

Clinical outcomes after conversion to belatacept in KTR

Although the use of belatacept is associated with an increased risk of aTCMR, it has been shown to be a good alternative in KTRs with a contraindication to CNIs. Multiple studies have reported successful conversion to belatacept in KTR with CNI-induced nephrotoxicity, impaired allograft function, delayed graft function, CNI-mediated thrombotic microangiopathy, or atypical hemolytic uremic syndrome[65-82]. Furthermore, KTR with poorly controlled diabetes mellitus while receiving CNI therapy may benefit from belatacept[83,84]. In addition, since belatacept must be administered intravenously, it has the potential advantage of providing better compliance, for instance in adolescent KTRs[85].

Several approaches for conversion to belatacept have been evaluated, such as early or late conversion[77,86-89], belatacept combined with a short period of tacrolimus therapy[48], and non-invasive screening for AR after conversion to belatacept to detect AR at an early stage[90]. In a phase II prospective randomized trial, KTR with a stable kidney function were randomized 6-36 months after transplantation to maintenance therapy with either belatacept (n = 84) or CNIs (n = 89)[88]. Three years after randomization, the kidney function was better in the belatacept group[89]. The rate of acute rejection was higher in the belatacept group (8.4%) compared with the CNI-treated patients (3.6%), but this difference was not statistically significant (p = 0.2)[89]. In retrospective studies, a beneficial effect on the kidney allograft function was seen in patients with early conversion to belatacept (within 3 months post-transplantation) and in patients with low-grade proteinuria[77,86,87]. Combination of belatacept with 9 months of tacrolimus reduced the risk of aTCMR in a retrospective single center study (the 1-year aTCMR rate of belatacept therapy was 50%, of tacrolimus therapy 20.5%, and of belatacept plus nine months tacrolimus 16%), without an increased incidence of infections[48]. Malvezzi *et al.* also examined a strategy to safely convert KTR to belatacept[90]. After the start of belatacept, the dose of tacrolimus was gradually reduced and withdrawn after 2 months. Serial measurements (at time points 1-, 3-, 6-, and 12-month time points) of urine chemokine (C-X-C motif) ligand 9 (CXCL9) were used to screen for AR non-invasively. In this study, 35 KTR with a contraindication for CNIs were converted to belatacept after median 3.3 years (interquartile range 1.3–7.2) after transplantation[90]. Only one patient had a biopsy-proven AR that responded well to glucocorticoid pulse therapy[90]. The urinary CXCL9 concentration was elevated during AR. In addition to CXCL9, other

potential minimally invasive biomarkers in urine and blood of KTR, such as cell-free DNA and extracellular vesicles, may assist clinicians to identify AR at an early stage[91,92].

Currently, two studies are actively recruiting KTR for conversion to belatacept: one study will investigate the effect of conversion to belatacept on proteinuria (ClinicalTrials.gov identifier NCT02327403) and the other study will examine the outcomes of conversion to belatacept three months after transplantation (ClinicalTrials.gov identifier NCT02213068).

Belatacept therapy in sensitized kidney transplant recipients

Initially, most studies that investigated the effectiveness of belatacept included only immunological low-risk KTRs[37,42,46]. However, because belatacept therapy is associated with a reduced incidence of *de novo* DSA production, a growing number of studies on the application of belatacept (*de novo* and conversion) in sensitized KTR are available[18,19].

In the BELACOR study, 49 KTRs with preformed DSAs (maximal mean fluorescence intensity between 500 and 3000) were treated with induction therapy of rATG followed by *de novo* belatacept maintenance therapy plus MPA and glucocorticoids[93]. The outcomes were compared with a retrospective control group of patients treated with CNIs. After 1 year of follow-up, no patients in the belatacept group experienced antibody-mediated rejection, while aTCMR occurred significantly more often in the belatacept-treated patients. Complete disappearance of class II DSAs was significantly more often seen in belatacept-treated patients[93].

In a retrospective study, the efficacy of belatacept in reducing anti-HLA antibodies in highly sensitized kidney transplant (current panel reactive antibodies ≥98-100%) was investigated[94]. Sixty highly sensitized KTRs were treated with belatacept *de novo*, glucocorticoids, MPA and low-dose tacrolimus (targeted to pre-dose concentrations 5-8 ng/mL in the first six months, 3-5 ng/mL in month 6-9, followed by tapering and discontinuation at month 9-11 post-transplantation). The control group existed of 44 highly sensitized KTRs treated with the current standard-of-care therapy (tacrolimus, MPA and glucocorticoids). In the KTRs treated with belatacept a decrease in the breadth and strength of HLA class I antibodies and current panel reactive antibodies was observed compared with the control group[94].

In another retrospective single-center study, 29 DSA-positive KTRs with a contraindication for CNI therapy were converted to belatacept after median 444 days[95]. The control group consisted of 44 non-immunized belatacept-treated KTRs. After the follow-up of

median 308 days one belatacept-treated patient experienced AR and two rejections were diagnosed in the CNI-treated patients. The eGFR improved from 32 to 41 mL/min/1.73 m^2 after conversion to belatacept[95]. In a smaller retrospective study, similar results were reported in six immunized KTR (panel reactive antigen >80% or positive flow cytometry crossmatch) who were converted from tacrolimus to belatacept (median four months after transplantation)[96].

Biomarkers predicting belatacept-resistant rejection

Because of the increased risk of aTCMR, belatacept may not be the game changer it was hoped to be[20]. Possibly, the drug should be reserved for KTRs with a low risk of belatacept-resistant AR. Quantification of an individual KTR's risk of AR prior to transplantation is essential to identify those who might benefit from belatacept-based immunosuppressive therapy. Clinical tests to reliably predict the risk of belatacept-resistant AR are not yet available. The risk of AR is currently estimated with pretransplant assessment of donor-specific anti-HLA antibodies, and HLA mismatch; however, alloreactive memory T-cell responses are not measured with these assays. The presence of alloreactive T cells pre-transplantation can lead to rapid recognition of alloantigens after transplantation and early AR[97,98]. These alloreactive T cells can be measured with pre-transplantation functional assays (*e.g.* measurement donor-reactive immune cells with ELISpot)[97,99].

Several studies have been performed to elucidate the pathogenesis of AR after belatacept therapy. An immunomic analysis of biopsies with AR of KTR treated with tacrolimus or belatacept showed no difference in the intragraft gene expression and immunohistochemistry of markers that are involved in AR[100]. This implies a final common pathway of AR which is independent of the immunosuppressive regimen.

Apart from the effect of belatacept on Tregs[51-55], other T cells have been associated with belatacept-resistant AR, such as highly cytotoxic $CD28^-$ memory T cells, $CD4^+$ $CD28^+$ effector memory T cells, $CD4^+CD57^+$Programmed Death-1^- T cells, and Th17 memory cells[46,98,101-105]. However, conflicting data have been reported about the possibility to predict belatacept-resistant AR by measuring some of these T cell subsets[46,98,101-105] and currently none is a clinically reliable for AR risk.

Another reason that may contribute to the increased incidence of AR is that belatacept therapy does not inhibit the T cell activation pathway downstream of the TCR, in contrast to tacrolimus therapy[106]. In a study with 20 belatacept treated KTR, no inhibition of the

phosphorylation of three important signaling molecules (p38MAPK, extracellular signal-regulated kinases 1 and 2 [ERK1/2] and AKT8 virus oncogene cellular homolog [Akt]) was noted after treatment with belatacept[106]. Furthermore, the phosphorylation of ERK was increased in belatacept-treated patients on day 4 and day 90 in patients with an AR compared to patients without an AR[106].

Prediction of AR was not possible with a targeted proteomic analysis of pre-rejection serum samples of KTR treated with belatacept[107]. In an assay with 92 inflammation-related proteins, no difference was seen in the proteomic profile between the pre-rejection samples and samples of patients without AR[107].

To conclude, there are several explanations for the increased risk of AR associated with belatacept therapy. At present no specific tests (besides pretransplant screening for degree of sensitization) are available that can predict the risk for belatacept-resistant AR.

ALTERNATIVE APPROACHES OF COSTIMULATION BLOCKADE

CD28 antibodies

Selective targeting of the CD28 antigen on T cells might be a superior immunosuppressive therapy compared with belatacept, since this blockade leaves the inhibitory signal of CTLA-4 intact and may preserve Treg function (Figure 1)[108,109]; however, blockade of CD28 has been challenging. Most anti-CD28 antibodies bind to an epitope lying in the basolateral C''D domain of CD28. Crosslinking of this epitope with an anti-CD28 antibody results in receptor clusterization, which this leads to activation of the CD28 receptor instead of inhibition. In 2006, a CD28 humanized antibody TGN1412 was tested in a phase I study (Figure 2)[110]. This antibody was developed to cause activation and proliferation of Tregs independent of signals received from the TCR. In studies in cynomolgus macaques, TGN1412 revealed no toxic effects; however, in humans, infusion of TGN1412 led to life-threatening massive cytokine release in six healthy volunteers and all of them had to be transferred to the intensive care unit[110]. $CD4^+$ effector memory T cells appeared to be responsible for the massive cytokine release[111]. The reason that preclinical testing failed to predict this dramatic side effect was that $CD4^+$ effector memory T cells of cynomolgus macaques do not express CD28, therefore these cells cannot be stimulated with TGN1412[111].

Currently, two monovalent antibodies with only antagonistic action to CD28 are in clinical development: FR104 and lulizumab-pegol (Figure 2)[112,113]. In non-human primates

transplanted with a kidney allograft, FR104 in combination with rapamycin, a low dose of tacrolimus, or 1 month of low-dose tacrolimus, induced long-term allograft survival[114,115]. Lulizumab-pegol was tested in non-human primates and showed inhibition of T cell-dependent antibody responses and cytokine production[116]. In humans, both drugs have been evaluated in phase I clinical studies and were safe and well tolerated (Figure 2)[113]. At present, a prospective multicenter study is started to investigate the efficacy of lulizumab in combination with rATG, glucocorticoids, belatacept, tocilizumab, and everolimus in KTRs (ClinicalTrials.gov identifier NCT04066114).

CD2/SLAM family antibodies

An antibody that interferes with the CD2/SLAM family is alefacept (Figure 1). This is a fusion protein of lymphocyte function-associated antigen (LFA)-3 and the Fc part of IgG1[117]. LFA-3 is expressed on APCs and is the ligand of CD2 on T cells. CD2 is expressed on all T cells but memory T cells express the highest levels[118]. Alefacept binds to CD2 on T cells and blocks the interaction between CD2 and LFA-3. It was approved by the FDA in 2003 for the treatment of psoriasis and administration of alefacept leads to depletion of memory T cells (Figure 2)[117]. In a phase II, randomized, controlled, double-blind multicenter study, maintenance treatment with alefacept was compared with placebo in KTRs[119]. Both patient groups were also treated with tacrolimus, MPA and glucocorticoids. There was no significant difference in the rate of biopsy-proven AR (alefacept 11% *versus* placebo 7%, $p = 0.3$). Furthermore, malignancy occurred more often in the patients treated with alefacept (5.7%) compared with placebo (0.9%; $p = 0.06$)[119]. In 2011, the manufacturer decided to stop the development of alefacept[120].

CD40-CD154 (CD40 ligand) antibodies

The CD40/CD154 pathway is a promising target for immunosuppressive therapy in KTRs. CD40 (TNF receptor superfamily 5, TNFRSF5) is constitutively expressed on the surface of APCs, including B cells, macrophages and dendritic cells, and T cells (mainly $CD8^+$)[121]. CD154 is the ligand of CD40 and is expressed on activated T cells and subsets of natural killer cells, eosinophils and activated thrombocytes (Figure 1)[121]. Ligation of CD40 with CD154 leads to T-cell-dependent B-cell activation and proliferation, germinal center formation, Ig production and isotype class switching. Furthermore, stimulation of CD40 provides macrophage effector function and promotes CD28-mediated costimulation through upregulation of CD80/CD86 and HLA molecules on APCs[122,123].

Multiple antibodies that target the CD40/CD154 pathway have been developed and tested in patients with autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, psoriasis, inflammatory bowel disease and idiopathic thrombocytic purpura[124]. Several CD154 monoclonal antibodies (*e.g.* hu5C8, IDEC-131, ABI793 and H106) were tested in non-human primates and showed prolonged kidney allograft survival[33,125-128]. However, in humans, administration of anti-CD154 antibodies led to an unanticipated, higher incidence of thrombotic complications, possibly because of activation of the coagulation cascade through CD154 activation on thrombocytes[129]. Therefore, the clinical development of anti-CD154 antibodies was terminated. Since CD40 is not expressed on thrombocytes, antagonistic anti-CD40 antibodies might not evoke thrombotic events. At present, many antagonistic anti-CD40 antibodies (*e.g.* ASKP1240, CFZ533, HCD122, Chi220, 3A8, 2C10R1, 2C10R4, BI-655064, FFP104, ch5D12) are under investigation[124,130]. Two of these anti-CD40 antibodies are tested in KTRs, namely CFZ533 (iscalimab) and ASKP1240 (bleselumab).

Bleselumab is a fully humanized non-depleting anti-CD40 IgG4 antibody. In non-human primates, bleselumab prolonged kidney allograft survival[131]. In a phase Ib study in KTRs, bleselumab was well tolerated and no thrombotic events occurred[132]. A more extensive (phase II) trial examined the efficacy and safety of bleselumab in KTR (ClinicalTrials.gov identifier NCT01780844; Figure 2). Preliminary data presented at a conference showed that the incidence of AR three years of therapy was 13% in KTRs treated with standard therapy (tacrolimus), 11% in patients treated with bleselumab with low-dose tacrolimus (p = 1.00 *versus* standard therapy), and 41% in patients treated with bleselumab (p = 0.02 *versus* standard therapy)[133]. Furthermore, an increased incidence of CMV and BKV infections was seen in patients treated with bleselumab[132,133].

Iscalimab is a non-B-cell-depleting anti-CD40 antibody and iscalimab induced prolonged survival and function of kidney allografts in cynomolgus monkeys [134]. Furthermore, iscalimab led to complete absence of splenic germinal center formation and formation of *de novo* DSA[134]. Iscalimab is currently tested in 325 KTRs in a phase II trial (ClinicalTrials.gov identifier NCT02217410) comparing standard-of-care therapy (tacrolimus, MPA and glucocorticoids) with subcutaneous iscalimab every 2 weeks in combination with MPA and glucocorticoids (Figure 2). Data from a proof-of-concept trial performed in 2016-2017 demonstrated a comparable efficacy on the composite endpoint of AR, graft loss, or death (21.2% *versus* 22.2%), better kidney function (55.8 *versus* 45.5 mL/min/1.73 m^2) and a reduced incidence of post-transplant diabetes mellitus (14.7 *versus* 38.9%) in patients treated with iscalimab compared with tacrolimus after 6 months of treatment[135].

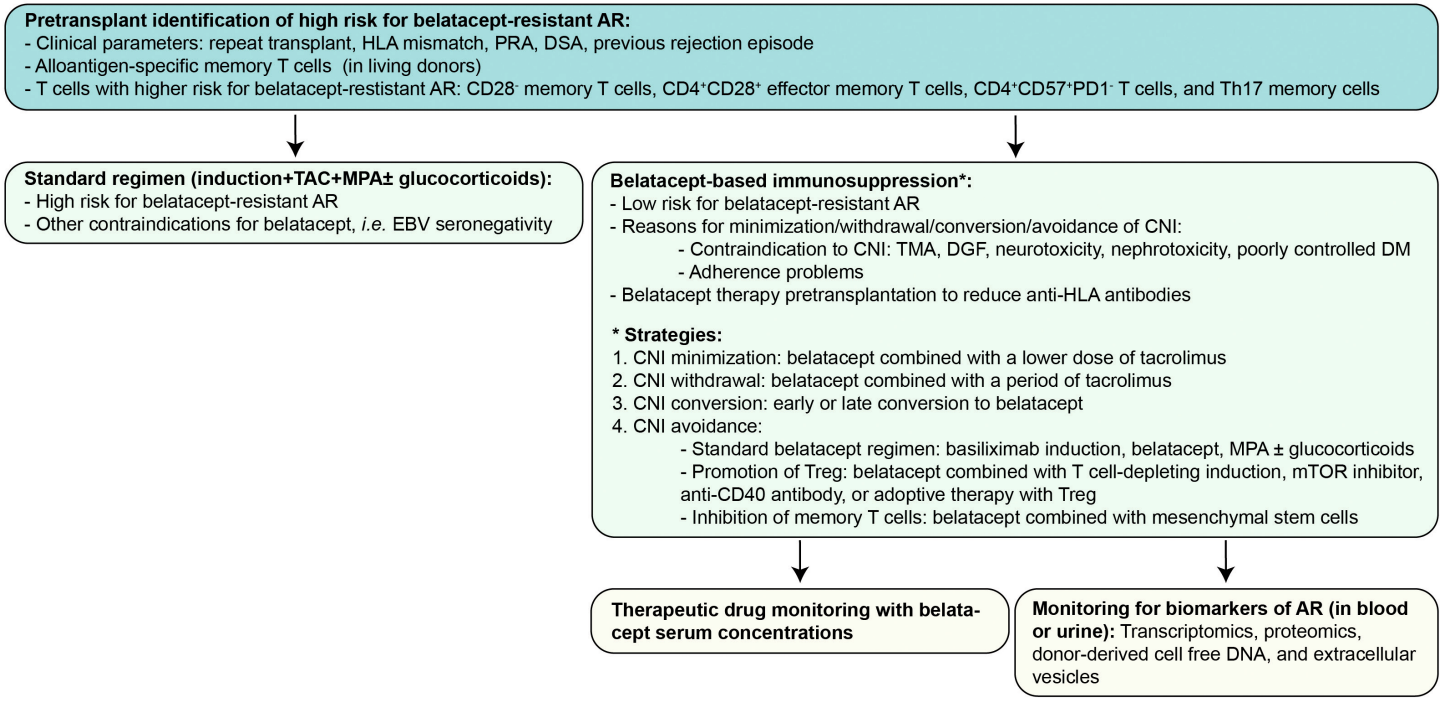

Figure 3. Future directions for belatacept treatment in kidney transplant recipients. A more tailored approach in the selection, treatment strategy and postconversion monitoring might be a way to expand the use of belatacept in kidney transplant recipients. AR, acute kidney transplant rejection; CNI, calcineurin inhibitors; DGF, delayed graft function; DM, diabetes mellitus; DSA, donor-specific anti-HLA antibodies; EBV, Epstein-Barr virus, HLA, human leucocyte antigen; MPA, mycophenolic acid; mTOR, mammalian target of rapamycin; PD-1, programmed death-1; PRA, panel reactive antigens; TAC, tacrolimus; Th17, T helper 17 cell; TMA, thrombotic microangiopathy; Treg, regulatory T cells

FUTURE DIRECTIONS

Modulation of the costimulation pathway with biologicals remains a promising strategy for the prevention of AR because it is more specific than traditional pharmacologic immunosuppression and appears to have the advantage of having only limited non-immune toxicity. To date, belatacept is the only costimulation blockade therapy approved for the prevention of rejection. Although belatacept has been shown to be a promising therapy in subgroups of patients, its widespread use has been limited because of 1) the increased risk of aTCMR compared with tacrolimus; 2) concerns regarding its safety (increased risk of post-transplant lymphoproliferative disorder); and 3) logistic aspects including the need for regular infusions and the temporarily unavailability due to production shortages.

The use of belatacept could be expanded in the future when it is possible to reliably identify patients who are at a low risk for belatacept-resistant AR (Figure 3). Belatacept-based therapy may be given to these low-risk patients, while the standard of care (tacrolimus-based therapy) should be offered to KTRs with a high risk for belatacept-resistant AR (Figure 3). Belatacept may also be an attractive alternative for patients with contraindications for CNIs (Figure 3).

Another strategy to expand the use of belatacept is to combine belatacept with tacrolimus or other immunosuppressive therapies (Figure 3). The risk of belatacept-resistant AR is reduced when it is used in combination with a short period or low-dose of tacrolimus[48,90]. Belatacept therapy influences the immunosuppressive function of Tregs[51-55]; therefore, combination of belatacept with therapies that preserve Treg functionality, such as mammalian target of rapamycin (mTOR) inhibitors, T -cell-depletion therapy, anti-CD40 antibodies, and adoptive therapy with Tregs could possibly lead to a more precise control of alloimmunity (Figure 3)[56]. The combination of CTLA4-Ig and blockade of CD40-CD154 has not yet been tested in humans; however, in several animal transplant models, this combination produced long-term allograft survival[136-140].

$CD28^-$ memory T cells are insensitive to belatacept therapy; therefore, belatacept should be avoided in KTRs with a high number of these cells, or should be combined with drugs that effectively control $CD28^-$ memory T cell immunity (Figure 3). Mesenchymal stem cell therapy has immunomodulatory properties and, *in vitro,* these cells are shown to inhibit $CD28^-$ memory T cells[141,142]. The combination of alemtuzumab induction followed by infusion with mesenchymal stem cells, belatacept and sirolimus is currently being tested (ClinicalTrials.gov identifier NCT03504241).

More intensive monitoring of KTRs after conversion to belatacept might lead to a better prevention or earlier recognition of AR. Belatacept dosing is administered at fixed intervals and is based on the body weight of the patient. According to the manufacturer of belatacept, it is not advisable to perform therapeutic drug monitoring of belatacept[143]. However, in the BENEFIT studies a more intensive regimen was associated with an increased incidence of malignancies and infections, without an increase in efficacy[37,38]. An automated assay to determine belatacept serum concentrations was recently developed[144]. The authors found reduced peak concentrations of belatacept in patients with a lower bodyweight[144]; however, whether this reduced exposure to belatacept leads to an increased risk of AR is unknown, but certainly a reason for further investigation (Figure 3).

Belatacept is currently dosed every 4 weeks in the maintenance phase. In a 10-year follow-up study, the belatacept 4-weekly regimen was compared to administration of belatacept every 8 weeks[145]. After 10 years, kidney function and the risk of allograft loss or death was similar between the two groups; however, the risk of AR was higher in patients who received belatacept every 8 weeks[2]. With further investigation, such as the above-mentioned measurement of belatacept serum concentrations, the 8-weekly dosing regimen could offer logistical advantages in subgroups of KTRs in the future.

Early recognition and treatment of AR leads to less allograft damage; therefore, minimal-invasive screening for (preclinical) AR in KTRs could lead to a better allograft survival (Figure 3). Potential biomarkers for minimal-invasive screening of AR in blood or urine are now entering the clinic[91,107,146]. Applying these minimal-invasive screening tools to belatacept-treated patients could be a way to expand the use of belatacept.

To conclude, targeting the costimulation pathway is a complex but exciting task. Belatacept is a promising immunosuppressive therapy for KTRs, but a more tailored approach in the selection of patients, treatment protocol and posttransplant monitoring is necessary to expand the use of belatacept.

REFERENCES

1. Kasiske BL, Zeier MG, Chapman JR, et al. KDIGO clinical practice guideline for the care of kidney transplant recipients: a summary. *Kidney Int.* 2010;77(4):299-311.
2. Brunet M, van Gelder T, Asberg A, et al. Therapeutic Drug Monitoring of Tacrolimus-Personalized Therapy: Second Consensus Report. *Ther Drug Monit.* 2019;41(3):261-307.
3. van Gelder T, Hesselink DA. Mycophenolate revisited. *Transpl Int.* 2015;28(5):508-515.
4. van der Zwan M, Baan CC, van Gelder T, Hesselink DA. Review of the Clinical Pharmacokinetics and Pharmacodynamics of Alemtuzumab and Its Use in Kidney Transplantation. *Clin Pharmacokinet.* 2018;57(2):191-207.
5. Claes K, Meier-Kriesche HU, Schold JD, et al. Effect of different immunosuppressive regimens on the evolution of distinct metabolic parameters: evidence from the Symphony study. *Nephrol Dial Transplant.* 2012;27(2):850-857.
6. Nankivell BJ, Borrows RJ, Fung CL, et al. The natural history of chronic allograft nephropathy. *N Engl J Med.* 2003;349(24):2326-2333.
7. Ekberg H, Tedesco-Silva H, Demirbas A, et al. Reduced exposure to calcineurin inhibitors in renal transplantation. *N Engl J Med.* 2007;357(25):2562-2575.
8. Neuberger JM, Bechstein WO, Kuypers DR, et al. Practical Recommendations for Long-term Management of Modifiable Risks in Kidney and Liver Transplant Recipients: A Guidance Report and Clinical Checklist by the Consensus on Managing Modifiable Risk in Transplantation (COMMIT) Group. *Transplantation.* 2017;101(4S Suppl 2):S1-S56.
9. Tedesco-Silva H, Kho MM, Hartmann A, et al. Sotrastaurin in calcineurin inhibitor-free regimen using everolimus in de novo kidney transplant recipients. *Am J Transplant.* 2013;13(7):1757-1768.
10. Baan CC, Kannegieter NM, Felipe CR, Tedesco Silva H, Jr. Targeting JAK/STAT Signaling to Prevent Rejection After Kidney Transplantation: A Reappraisal. *Transplantation.* 2016;100(9):1833-1839.
11. Vincenti F, Silva HT, Busque S, et al. Evaluation of the effect of tofacitinib exposure on outcomes in kidney transplant patients. *Am J Transplant.* 2015;15(6):1644-1653.
12. Bouamar R, Shuker N, Osinga JAJ, et al. Conversion from tacrolimus to everolimus with complete and early glucocorticoid withdrawal after kidney transplantation: a randomised trial. *Neth J Med.* 2018;76(1):14-26.
13. Shipkova M, Hesselink DA, Holt DW, et al. Therapeutic Drug Monitoring of Everolimus: A Consensus Report. *Ther Drug Monit.* 2016;38(2):143-169.
14. Pascual J, Berger SP, Witzke O, et al. Everolimus with Reduced Calcineurin Inhibitor Exposure in Renal Transplantation. *J Am Soc Nephrol.* 2018;29(7):1979-1991.
15. Karpe KM, Talaulikar GS, Walters GD. Calcineurin inhibitor withdrawal or tapering for kidney transplant recipients. *Cochrane Database Syst Rev.* 2017;7:CD006750.
16. Larsen CP, Pearson TC, Adams AB, et al. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. *Am J Transplant.* 2005;5(3):443-453.
17. Archdeacon P, Dixon C, Belen O, Albrecht R, Meyer J. Summary of the US FDA approval of belatacept. *Am J Transplant.* 2012;12(3):554-562.

18. Durrbach A, Pestana JM, Florman S, et al. Long-Term Outcomes in Belatacept- Versus Cyclosporine-Treated Recipients of Extended Criteria Donor Kidneys: Final Results From BENEFIT-EXT, a Phase III Randomized Study. *Am J Transplant.* 2016;16(11):3192-3201.

19. Vincenti F, Rostaing L, Grinyo J, et al. Belatacept and Long-Term Outcomes in Kidney Transplantation. *N Engl J Med.* 2016;374(4):333-343.

20. Van Gelder T, Hesselink DA. Belatacept: A Game Changer? *Transplantation.* 2016;100(7):1390-1392.

21. Sharpe AH. Mechanisms of costimulation. *Immunol Rev.* 2009;229(1):5-11.

22. June CH, Ledbetter JA, Gillespie MM, Lindsten T, Thompson CB. T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. *Mol Cell Biol.* 1987;7(12):4472-4481.

23. Mueller DL, Jenkins MK, Schwartz RH. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu Rev Immunol.* 1989;7:445-480.

24. Nakayama T, Yamashita M. The TCR-mediated signaling pathways that control the direction of helper T cell differentiation. *Semin Immunol.* 2010;22(5):303-309.

25. O'Shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science.* 2010;327(5969):1098-1102.

26. Linsley PS, Greene JL, Tan P, et al. Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. *J Exp Med.* 1992;176(6):1595-1604.

27. Weng NP, Akbar AN, Goronzy J. CD28(-) T cells: their role in the age-associated decline of immune function. *Trends Immunol.* 2009;30(7):306-312.

28. Betjes MG, Huisman M, Weimar W, Litjens NH. Expansion of cytolytic CD4+CD28- T cells in end-stage renal disease. *Kidney Int.* 2008;74(6):760-767.

29. Pawelec G, Derhovanessian E. Role of CMV in immune senescence. *Virus Res.* 2011;157(2):175-179.

30. Sprent J, Surh CD. T cell memory. *Annu Rev Immunol.* 2002;20:551-579.

31. Linsley PS, Brady W, Urnes M, et al. CTLA-4 is a second receptor for the B cell activation antigen B7. *J Exp Med.* 1991;174(3):561-569.

32. Kremer JM, Westhovens R, Leon M, et al. Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig. *N Engl J Med.* 2003;349(20):1907-1915.

33. Kirk AD, Harlan DM, Armstrong NN, et al. CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. *Proc Natl Acad Sci U S A.* 1997;94(16):8789-8794.

34. Levisetti MG, Padrid PA, Szot GL, et al. Immunosuppressive effects of human CTLA4Ig in a non-human primate model of allogeneic pancreatic islet transplantation. *J Immunol.* 1997;159(11):5187-5191.

35. Badell IR, Karadkhele GM, Vasanth P, et al. Abatacept as rescue immunosuppression after calcineurin inhibitor treatment failure in renal transplantation. *Am J Transplant.* 2019;19(8):2342-2349.

36. Gabardi S, van Gelder T. Causes and Consequences of the Worldwide Belatacept Shortage. *Transplantation.* 2017;101(7):1520-1521.

37. Vincenti F, Charpentier B, Vanrenterghem Y, et al. A phase III study of belatacept-based immunosuppression regimens versus cyclosporine in renal transplant recipients (BENEFIT study). *Am J Transplant.* 2010;10(3):535-546.

38. Durrbach A, Pestana JM, Pearson T, et al. A phase III study of belatacept versus cyclosporine in kidney transplants from extended criteria donors (BENEFIT-EXT study). *Am J Transplant.* 2010;10(3):547-557.

39. van den Hoogen MW, Pipeleers L. Three-year outcomes of belatacept studies; reason to be optimistic? *Am J Transplant.* 2012;12(8):2259; discussion 2260.

40. Klintmalm GB, Feng S, Lake JR, et al. Belatacept-based immunosuppression in de novo liver transplant recipients: 1-year experience from a phase II randomized study. *Am J Transplant.* 2014;14(8):1817-1827.

41. Knechtle SJ, Adams AB. Belatacept: is there BENEFIT for liver transplantation too? *Am J Transplant.* 2014;14(8):1717-1718.

42. Masson P, Henderson L, Chapman JR, Craig JC, Webster AC. Belatacept for kidney transplant recipients. *Cochrane Database Syst Rev.* 2014(11):CD010699.

43. Vitalone MJ, Ganguly B, Hsieh S, et al. Transcriptional profiling of belatacept and calcineurin inhibitor therapy in renal allograft recipients. *Am J Transplant.* 2014;14(8):1912-1921.

44. Furuzawa-Carballeda J, Uribe-Uribe NO, Arreola-Guerra JM, et al. Tissue talks: immunophenotype of cells infiltrating the graft explains histological findings and the benefits of belatacept at 10 years. *Clin Exp Immunol.* 2019.

45. Webster AC, Woodroffe RC, Taylor RS, Chapman JR, Craig JC. Tacrolimus versus ciclosporin as primary immunosuppression for kidney transplant recipients: meta-analysis and meta-regression of randomised trial data. *BMJ.* 2005;331(7520):810.

46. de Graav GN, Baan CC, Clahsen-van Groningen MC, et al. A Randomized Controlled Clinical Trial Comparing Belatacept With Tacrolimus After De Novo Kidney Transplantation. *Transplantation.* 2017;101(10):2571-2581.

47. Newell KA, Mehta AK, Larsen CP, et al. Lessons Learned: Early Termination of a Randomized Trial of Calcineurin Inhibitor and Corticosteroid Avoidance Using Belatacept. *Am J Transplant.* 2017;17(10):2712-2719.

48. Adams AB, Goldstein J, Garrett C, et al. Belatacept Combined With Transient Calcineurin Inhibitor Therapy Prevents Rejection and Promotes Improved Long-Term Renal Allograft Function. *Am J Transplant.* 2017;17(11):2922-2936.

49. Cohen JB, Eddinger KC, Forde KA, Abt PL, Sawinski D. Belatacept Compared With Tacrolimus for Kidney Transplantation: A Propensity Score Matched Cohort Study. *Transplantation.* 2017;101(10):2582-2589.

50. Muduma G, Hart WM, Patel S, Odeyemi AO. Indirect treatment comparison of belatacept versus tacrolimus from a systematic review of immunosuppressive therapies for kidney transplant patients. *Curr Med Res Opin.* 2016;32(6):1065-1072.

51. Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med.* 2000;192(2):295-302.

52. Takahashi T, Tagami T, Yamazaki S, et al. Immunologic self-tolerance maintained by CD25(+) CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med.* 2000;192(2):303-310.

53. Wing K, Onishi Y, Prieto-Martin P, et al. CTLA-4 control over Foxp3+ regulatory T cell function. *Science.* 2008;322(5899):271-275.

54. Alvarez Salazar EK, Cortes-Hernandez A, Aleman-Muench GR, et al. Methylation of FOXP3 TSDR Underlies the Impaired Suppressive Function of Tregs from Long-term Belatacept-Treated Kidney Transplant Patients. *Front Immunol.* 2017;8:219.

55. Riella LV, Liu T, Yang J, et al. Deleterious effect of CTLA4-Ig on a Treg-dependent transplant model. *Am J Transplant.* 2012;12(4):846-855.

56. Zwang NA, Leventhal JR. Cell Therapy in Kidney Transplantation: Focus on Regulatory T Cells. *J Am Soc Nephrol.* 2017;28(7):1960-1972.

57. Bouvy AP, Klepper M, Kho MM, et al. The impact of induction therapy on the homeostasis and function of regulatory T cells in kidney transplant patients. *Nephrol Dial Transplant.* 2014;29(8):1587-1597.

58. Sparkes T, Ravichandran B, Opara O, et al. Alemtuzumab induction and belatacept maintenance in marginal pathology renal allografts. *Clin Transplant.* 2019;33(6):e13531.

59. Woodle E, Kaufman D, Shields A, et al. The BEST Trial: A Prospective Randomized Multicenter Trial of Belatacept-Based CNI- and Corticosteroid-Free Immunosuppression [abstract]. https://atcmeetingabstracts.com/abstract/the-best-trial-a-prospective-randomized-multicenter-trial-of-belatacept-based-cni-and-corticosteroid-free-immunosuppression/. Accessed August 15, 2019.

60. Kirk AD, Guasch A, Xu H, et al. Renal transplantation using belatacept without maintenance steroids or calcineurin inhibitors. *Am J Transplant.* 2014;14(5):1142-1151.

61. Ferguson R, Grinyo J, Vincenti F, et al. Immunosuppression with belatacept-based, corticosteroid-avoiding regimens in de novo kidney transplant recipients. *Am J Transplant.* 2011;11(1):66-76.

62. Bestard O, Cassis L, Cruzado JM, et al. Costimulatory blockade with mTor inhibition abrogates effector T-cell responses allowing regulatory T-cell survival in renal transplantation. *Transpl Int.* 2011;24(5):451-460.

63. Wojciechowski D, Chandran S, Yang JYC, Sarwal MM, Vincenti F. Retrospective evaluation of the efficacy and safety of belatacept with thymoglobulin induction and maintenance everolimus: A single-center clinical experience. *Clin Transplant.* 2017;31(9).

64. Bouvy AP, Klepper M, Kho MM, et al. T cells Exhibit Reduced Signal Transducer and Activator of Transcription 5 Phosphorylation and Upregulated Coinhibitory Molecule Expression After Kidney Transplantation. *Transplantation.* 2015;99(9):1995-2003.

65. Ebcioglu Z, Liu C, Shapiro R, et al. Belatacept Conversion in an HIV-Positive Kidney Transplant Recipient With Prolonged Delayed Graft Function. *Am J Transplant.* 2016;16(11):3278-3281.

66. Kumar D, Yakubu I, Cooke RH, Halloran PF, Gupta G. Belatacept rescue for delayed kidney allograft function in a patient with previous combined heart-liver transplant. *Am J Transplant.* 2018;18(10):2613-2614.

67. Wojciechowski D, Chandran S, Vincenti F. Early post-transplant conversion from tacrolimus to belatacept for prolonged delayed graft function improves renal function in kidney transplant recipients. *Clin Transplant.* 2017;31(5).

68. Ashman N, Chapagain A, Dobbie H, et al. Belatacept as maintenance immunosuppression for postrenal transplant de novo drug-induced thrombotic microangiopathy. *Am J Transplant.* 2009;9(2):424-427.

69. Cicora F, Paz M, Mos F, Roberti J. Use of belatacept as alternative immunosuppression in three renal transplant patients with de novo drug-induced thrombotic microangiopathy. *Case Rep Med.* 2013;2013:260254.

70. Koppula S, Yost SE, Sussman A, Bracamonte ER, Kaplan B. Successful conversion to belatacept after thrombotic microangiopathy in kidney transplant patients. *Clin Transplant.* 2013;27(4):591-597.

71. Merola J, Yoo PS, Schaub J, et al. Belatacept and Eculizumab for Treatment of Calcineurin Inhibitor-induced Thrombotic Microangiopathy After Kidney Transplantation: Case Report. *Transplant Proc.* 2016;48(9):3106-3108.

72. Dedhia P, Govil A, Mogilishetty G, et al. Eculizumab and Belatacept for De Novo Atypical Hemolytic Uremic Syndrome Associated With CFHR3-CFHR1 Deletion in a Kidney Transplant Recipient: A Case Report. *Transplant Proc.* 2017;49(1):188-192.

73. Midtvedt K, Bitter J, Dorje C, Bjorneklett R, Holdaas H. Belatacept as immunosuppression in patient with recurrence of hemolytic uremic syndrome after renal transplantation. *Transplantation.* 2009;87(12):1901-1903.

74. Tatapudi VS, Lonze BE, Wu M, Montgomery RA. Early Conversion from Tacrolimus to Belatacept in a Highly Sensitized Renal Allograft Recipient with Calcineurin Inhibitor-Induced de novo Post-Transplant Hemolytic Uremic Syndrome. *Case Rep Nephrol Dial.* 2018;8(1):10-19.

75. Gupta S, Rosales I, Wojciechowski D. Pilot Analysis of Late Conversion to Belatacept in Kidney Transplant Recipients for Biopsy-Proven Chronic Tacrolimus Toxicity. *J Transplant.* 2018;2018:1968029.

76. Snyder HS, Duhart BT, Jr., Krauss AG, Rao V. Belatacept conversion in African American kidney transplant recipients with severe renal dysfunction. *SAGE Open Med Case Rep.* 2016;4:2050313X16674865.

77. Le Meur Y, Aulagnon F, Bertrand D, et al. Effect of an Early Switch to Belatacept Among Calcineurin Inhibitor-Intolerant Graft Recipients of Kidneys From Extended-Criteria Donors. *Am J Transplant.* 2016;16(7):2181-2186.

78. Bertrand D, Cheddani L, Etienne I, et al. Belatacept Rescue Therapy in Kidney Transplant Recipients With Vascular Lesions: A Case Control Study. *Am J Transplant.* 2017;17(11):2937-2944.

79. Belliere J, Guilbeau-Frugier C, Del Bello A, et al. Beneficial effect of conversion to belatacept in kidney-transplant patients with a low glomerular-filtration rate. *Case Rep Transplant.* 2014;2014:190516.

80. Abdelwahab Elhamahmi D, Heilman RL, Smith B, et al. Early Conversion to Belatacept in Kidney Transplant Recipients With Low Glomerular Filtration Rate. *Transplantation.* 2018;102(3):478-483.

81. Nair V, Liriano-Ward L, Kent R, et al. Early conversion to belatacept after renal transplantation. *Clin Transplant.* 2017;31(5).

82. Brakemeier S, Kannenkeril D, Durr M, et al. Experience with belatacept rescue therapy in kidney transplant recipients. *Transpl Int.* 2016;29(11):1184-1195.

83. de Graav GN, van der Zwan M, Baan CC, Janssen J, Hesselink DA. Improved Glucose Tolerance in a Kidney Transplant Recipient With Type 2 Diabetes Mellitus After Switching From Tacrolimus To Belatacept: A Case Report and Review of Potential Mechanisms. *Transplant Direct.* 2018;4(3):e350.

84. Vanrenterghem Y, Bresnahan B, Campistol J, et al. Belatacept-based regimens are associated with improved cardiovascular and metabolic risk factors compared with cyclosporine in kidney transplant recipients (BENEFIT and BENEFIT-EXT studies). *Transplantation.* 2011;91(9):976-983.

85. Lerch C, Kanzelmeyer NK, Ahlenstiel-Grunow T, et al. Belatacept after kidney transplantation in adolescents: a retrospective study. *Transpl Int.* 2017;30(5):494-501.

86. Durr M, Lachmann N, Zukunft B, et al. Late Conversion to Belatacept After Kidney Transplantation: Outcome and Prognostic Factors. *Transplant Proc.* 2017;49(8):1747-1756 e1741.

87. Darres A, Ulloa C, Brakemeier S, et al. Conversion to Belatacept in Maintenance Kidney Transplant Patients: A Retrospective Multicenter European Study. *Transplantation.* 2018;102(9):1545-1552.

88. Rostaing L, Massari P, Garcia VD, et al. Switching from calcineurin inhibitor-based regimens to a belatacept-based regimen in renal transplant recipients: a randomized phase II study. *Clin J Am Soc Nephrol.* 2011;6(2):430-439.

89. Grinyo JM, Del Carmen Rial M, Alberu J, et al. Safety and Efficacy Outcomes 3 Years After Switching to Belatacept From a Calcineurin Inhibitor in Kidney Transplant Recipients: Results From a Phase 2 Randomized Trial. *Am J Kidney Dis.* 2017;69(5):587-594.

90. Malvezzi P, Fischman C, Rigault G, et al. Switching renal transplant recipients to belatacept therapy: results of a real-life gradual conversion protocol. *Transpl Immunol.* 2019.

91. Verhoeven J, Boer K, Van Schaik RHN, et al. Liquid Biopsies to Monitor Solid Organ Transplant Function: A Review of New Biomarkers. *Ther Drug Monit.* 2018;40(5):515-525.

92. Hurkmans DP, Verhoeven J, de Leur K, et al. Donor-derived cell-free DNA detects kidney transplant rejection during nivolumab treatment. *J Immunother Cancer.* 2019;7(1):182.

93. Leibler C, Matignon M, Moktefi A, et al. Belatacept in renal transplant recipient with mild immunologic risk factor: A pilot prospective study (BELACOR). *Am J Transplant.* 2019;19(3):894-906.

94. Parsons RF, Zahid A, Bumb S, et al. The impact of belatacept on third party HLA alloantibodies in highly sensitized kidney transplant recipients. *Am J Transplant.* 2019.

95. Ulloa CE, Anglicheau D, Snanoudj R, et al. Conversion From Calcineurin Inhibitors to Belatacept in HLA-sensitized Kidney Transplant Recipients With Low-level Donor-specific Antibodies. *Transplantation.* 2019;103(10):2150-2156.

96. Gupta G, Regmi A, Kumar D, et al. Safe Conversion From Tacrolimus to Belatacept in High Immunologic Risk Kidney Transplant Recipients With Allograft Dysfunction. *Am J Transplant.* 2015;15(10):2726-2731.

97. Crespo E, Bestard O. Biomarkers to assess donor-reactive T-cell responses in kidney transplant patients. *Clin Biochem.* 2016;49(4-5):329-337.

98. de Graav GN, Hesselink DA, Dieterich M, et al. An Acute Cellular Rejection With Detrimental Outcome Occurring Under Belatacept-Based Immunosuppressive Therapy: An Immunological Analysis. *Transplantation.* 2016;100(5):1111-1119.

99. van Besouw NM, Yan L, de Kuiper R, et al. The Number of Donor-Specific IL-21 Producing Cells Before and After Transplantation Predicts Kidney Graft Rejection. *Front Immunol.* 2019;10:748.

100. van der Zwan M, Baan CC, Colvin RB, et al. Immunomics of Renal Allograft Acute T Cell-Mediated Rejection Biopsies of Tacrolimus- and Belatacept-Treated Patients. *Transplant Direct.* 2019;5(1):e418.

101. Cortes-Cerisuelo M, Laurie SJ, Mathews DV, et al. Increased Pretransplant Frequency of CD28+ CD4+ TEM Predicts Belatacept-Resistant Rejection in Human Renal Transplant Recipients. *Am J Transplant.* 2017;17(9):2350-2362.

102. Espinosa J, Herr F, Tharp G, et al. CD57(+) CD4 T Cells Underlie Belatacept-Resistant Allograft Rejection. *Am J Transplant.* 2016;16(4):1102-1112.

103. Krummey SM, Cheeseman JA, Conger JA, et al. High CTLA-4 expression on Th17 cells results in increased sensitivity to CTLA-4 coinhibition and resistance to belatacept. *Am J Transplant.* 2014;14(3):607-614.

104. de Graav GN, Hesselink DA, Dieterich M, et al. Down-Regulation of Surface CD28 under Belatacept Treatment: An Escape Mechanism for Antigen-Reactive T-Cells. *PLoS One.* 2016;11(2):e0148604.

105. Kraaijeveld R, de Graav GN, Dieterich M, et al. Co-inhibitory profile and cytotoxicity of CD57(+) PD-1(-) T cells in end-stage renal disease patients. *Clin Exp Immunol.* 2018;191(3):363-372.

106. Kannegieter NM, Hesselink DA, Dieterich M, et al. Differential T Cell Signaling Pathway Activation by Tacrolimus and Belatacept after Kidney Transplantation: Post Hoc Analysis of a Randomised-Controlled Trial. *Sci Rep.* 2017;7(1):15135.

107. van der Zwan M, Hesselink DA, Clahsen-van Groningen MC, Baan CC. Targeted Proteomic Analysis Detects Acute T Cell-Mediated Kidney Allograft Rejection in Belatacept-Treated Patients. *Ther Drug Monit.* 2019;41(2):243-248.

108. Poirier N, Blancho G, Vanhove B. A more selective costimulatory blockade of the CD28-B7 pathway. *Transpl Int.* 2011;24(1):2-11.

109. Liu D, Krummey SM, Badell IR, et al. 2B4 (CD244) induced by selective CD28 blockade functionally regulates allograft-specific CD8+ T cell responses. *J Exp Med.* 2014;211(2):297-311.

110. Suntharalingam G, Perry MR, Ward S, et al. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *N Engl J Med.* 2006;355(10):1018-1028.

111. Eastwood D, Findlay L, Poole S, et al. Monoclonal antibody TGN1412 trial failure explained by species differences in CD28 expression on CD4+ effector memory T-cells. *Br J Pharmacol.* 2010;161(3):512-526.

112. Shi R, Honczarenko M, Zhang S, et al. Pharmacokinetic, Pharmacodynamic, and Safety Profile of a Novel Anti-CD28 Domain Antibody Antagonist in Healthy Subjects. *J Clin Pharmacol.* 2017;57(2):161-172.

113. Poirier N, Blancho G, Hiance M, et al. First-in-Human Study in Healthy Subjects with FR104, a Pegylated Monoclonal Antibody Fragment Antagonist of CD28. *J Immunol.* 2016;197(12):4593-4602.

114. Poirier N, Dilek N, Mary C, et al. FR104, an antagonist anti-CD28 monovalent fab' antibody, prevents alloimmunization and allows calcineurin inhibitor minimization in nonhuman primate renal allograft. *Am J Transplant.* 2015;15(1):88-100.

115. Ville S, Poirier N, Branchereau J, et al. Anti-CD28 Antibody and Belatacept Exert Differential Effects on Mechanisms of Renal Allograft Rejection. *J Am Soc Nephrol.* 2016;27(12):3577-3588.

116. Suchard SJ, Davis PM, Kansal S, et al. A monovalent anti-human CD28 domain antibody antagonist: preclinical efficacy and safety. *J Immunol.* 2013;191(9):4599-4610.

117. Krueger GG. Selective targeting of T cell subsets: focus on alefacept - a remittive therapy for psoriasis. *Expert Opin Biol Ther.* 2002;2(4):431-441.

118. Sanders ME, Makgoba MW, Sharrow SO, et al. Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN-gamma production. *J Immunol.* 1988;140(5):1401-1407.

119. Rostaing L, Charpentier B, Glyda M, et al. Alefacept combined with tacrolimus, mycophenolate mofetil and steroids in de novo kidney transplantation: a randomized controlled trial. *Am J Transplant.* 2013;13(7):1724-1733.

120. https://www.nanostring.com/products/gene-expression-panels/gene-expression-panels-overview/human-organ-transplant-panel, assessed on 2 October 2019.

121. Grewal IS, Flavell RA. CD40 and CD154 in cell-mediated immunity. *Annu Rev Immunol.* 1998;16:111-135.

122. Bingaman AW, Pearson TC, Larsen CP. The role of CD40L in T cell-dependent nitric oxide production by murine macrophages. *Transpl Immunol.* 2000;8(3):195-202.

123. Larsen CP, Pearson TC. The CD40 pathway in allograft rejection, acceptance, and tolerance. *Curr Opin Immunol.* 1997;9(5):641-647.

124. Karnell JL, Rieder SA, Ettinger R, Kolbeck R. Targeting the CD40-CD40L pathway in autoimmune diseases: Humoral immunity and beyond. *Adv Drug Deliv Rev.* 2018.

125. Kirk AD, Burkly LC, Batty DS, et al. Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. *Nat Med.* 1999;5(6):686-693.

126. Pearson TC, Trambley J, Odom K, et al. Anti-CD40 therapy extends renal allograft survival in rhesus macaques. *Transplantation.* 2002;74(7):933-940.

127. Schuler W, Bigaud M, Brinkmann V, et al. Efficacy and safety of ABI793, a novel human anti-human CD154 monoclonal antibody, in cynomolgus monkey renal allotransplantation. *Transplantation.* 2004;77(5):717-726.

128. Kanmaz T, Fechner JJ, Jr., Torrealba J, et al. Monotherapy with the novel human anti-CD154 monoclonal antibody ABI793 in rhesus monkey renal transplantation model. *Transplantation.* 2004;77(6):914-920.

129. Boumpas DT, Furie R, Manzi S, et al. A short course of BG9588 (anti-CD40 ligand antibody) improves serologic activity and decreases hematuria in patients with proliferative lupus glomerulonephritis. *Arthritis Rheum.* 2003;48(3):719-727.

130. Zhang T, Pierson RN, 3rd, Azimzadeh AM. Update on CD40 and CD154 blockade in transplant models. *Immunotherapy.* 2015;7(8):899-911.

131. Song L, Ma A, Dun H, et al. Effects of ASKP1240 combined with tacrolimus or mycophenolate mofetil on renal allograft survival in Cynomolgus monkeys. *Transplantation.* 2014;98(3):267-276.

132. Vincenti F, Klintmalm G, Yang H, et al. A randomized, phase 1b study of the pharmacokinetics, pharmacodynamics, safety, and tolerability of bleselumab, a fully human, anti-CD40 monoclonal antibody, in kidney transplantation. *Am J Transplant.* 2019.

133. Harland R, Klintmalm G, Jensik S, Yang H, Bromberg J, Holman J, Kumar MAnil, Santos V, Larson T, Wang X. Efficacy and Safety of Bleselumab in Kidney Transplant Recipients: A Phase 2, Randomized, Open-Label Study [abstract]. https://atcmeetingabstracts.com/abstract/efficacy-and-safety-of-bleselumab-in-kidney-transplant-recipients-a-phase-2-randomized-open-label-study/. Accessed July 4, 2019.

134. Cordoba F, Wieczorek G, Audet M, et al. A novel, blocking, Fc-silent anti-CD40 monoclonal antibody prolongs nonhuman primate renal allograft survival in the absence of B cell depletion. *Am J Transplant.* 2015;15(11):2825-2836.

135. Nashan B, Tedesco H, van den Hoogen MW, et al. CD40 Inhibition with CFZ533 - A New, Fully Human, Non-Depleting, Fc Silent mAB - Improves Renal Allograft Function While Demonstrating Comparable Efficacy vs. Tacrolimus in De-Novo CNI-Free Kidney Transplant Recipients. *Transplantation.* 2018;102:S366.

136. Page A, Srinivasan S, Singh K, et al. CD40 blockade combines with CTLA4Ig and sirolimus to produce mixed chimerism in an MHC-defined rhesus macaque transplant model. *Am J Transplant.* 2012;12(1):115-125.

137. Gilson CR, Milas Z, Gangappa S, et al. Anti-CD40 monoclonal antibody synergizes with CTLA4-Ig in promoting long-term graft survival in murine models of transplantation. *J Immunol.* 2009;183(3):1625-1635.

138. Larsen CP, Elwood ET, Alexander DZ, et al. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature.* 1996;381(6581):434-438.

139. Zhu P, Chen YF, Chen XP, et al. Mechanisms of survival prolongation of murine cardiac allografts using the treatment of CTLA4-Ig and MR1. *Transplant Proc.* 2008;40(5):1618-1624.

140. Pinelli DF, Wagener ME, Liu D, et al. An anti-CD154 domain antibody prolongs graft survival and induces Foxp3(+) iTreg in the absence and presence of CTLA-4 Ig. *Am J Transplant.* 2013;13(11):3021-3030.

141. Engela AU, Baan CC, Litjens NH, et al. Mesenchymal stem cells control alloreactive CD8(+) CD28(-) T cells. *Clin Exp Immunol.* 2013;174(3):449-458.

142. Hoogduijn MJ, Betjes MG, Baan CC. Mesenchymal stromal cells for organ transplantation: different sources and unique characteristics? *Curr Opin Organ Transplant.* 2014;19(1):41-46.

143. Belatacept Product Monograph: https://www.ema.europa.eu/en/documents/product-information/nulojix-epar-product-information_en.pdf, assessed on 8 August, 2019.

144. Klaasen RA, Egeland EJ, Chan J, et al. A Fully Automated Method for the Determination of Serum Belatacept and Its Application in a Pharmacokinetic Investigation in Renal Transplant Recipients. *Ther Drug Monit.* 2019;41(1):11-18.

145. Vincenti F, Blancho G, Durrbach A, et al. Ten-year outcomes in a randomized phase II study of kidney transplant recipients administered belatacept 4-weekly or 8-weekly. *Am J Transplant.* 2017;17(12):3219-3227.

146. Eikmans M, Gielis EM, Ledeganck KJ, et al. Non-invasive Biomarkers of Acute Rejection in Kidney Transplantation: Novel Targets and Strategies. *Front Med (Lausanne).* 2018;5:358.

CHAPTER 3

Review of the clinical pharmacokinetics and pharmacodynamics of alemtuzumab and its use in kidney transplantation

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ABSTRACT

Background

Alemtuzumab is a humanized monoclonal antibody against CD52 and causes depletion of T and B lymphocytes, monocytes, and NK cells. Alemtuzumab is registered for the treatment of multiple sclerosis (MS) and is used off-label in chronic lymphocytic leukemia (CLL). Alemtuzumab is used off-label in kidney transplantation as induction and anti-rejection therapy. The objective of this article is to review the pharmacokinetics, pharmacodynamics, and use of alemtuzumab in kidney transplantation.

Methods

We conducted a systematic literature search using Ovid MEDLINE, EMBASE, and the Cochrane Central Register of controlled trials.

Results

No pharmacokinetic or dose-finding studies of alemtuzumab exist for kidney transplantation. Although such studies were conducted in patients with CLL and MS, these findings could not be directly extrapolated to transplant recipients because CLL patients have a much higher load of CD52-positive cells and therefore target-mediated clearance will differ between these two indications. Alemtuzumab used as induction therapy in kidney transplantation results in a lower incidence of acute rejection compared to basiliximab therapy and comparable results as compared with rabbit anti-thymocyte globulin. Alemtuzumab used as anti-rejection therapy results in a comparable graft survival rate compared with rabbit anti-thymocyte globulin, although infusion-related side effects appear to be less.

Conclusion

There is a need for pharmacokinetic and dose-finding studies of alemtuzumab in kidney transplant recipients to establish the optimal balance between efficacy and toxicity. Furthermore, randomized controlled trials with sufficient follow-up are necessary to provide further evidence for the treatment of severe kidney transplant rejection.

Key points:

- Alemtuzumab, a monoclonal antibody against CD52, is registered for multiple sclerosis, but is used off-label in patients with chronic lymphocytic leukemia and as induction- and anti-rejection therapy after kidney transplantation.
- Alemtuzumab causes a rapid and profound depletion of T and B lymphocytes, as well as various cells of the innate immune system. Reconstitution of cells form the innate immune system is faster (within 6 months) than that of T and B lymphocytes, which may take more than 1 year.
- No pharmacokinetic studies of alemtuzumab exist for kidney transplant recipients. The results of the pharmacokinetic studies performed in patients with chronic lymphocytic leukemia could not be extrapolated directly to the kidney transplant population because patients with chronic lymphocytic leukemia have a much higher load of CD52-positive (tumor) cells.

INTRODUCTION

Alemtuzumab (Campath-1H) is a humanized, rat monoclonal IgG_1 antibody with a molecular weight of approximately 150 kDa, directed against CD52. The depletion of donor T lymphocytes from stem cell transplants to eliminate graft-vs.-host disease was developed in the laboratory of Herman Waldmann and Geoff Hale at the university of Cambridge, UK[1]. The first anti-CD52 antibody developed was of the IgM class (Campath-1M), which was very effective in eliminating T lymphocytes *in vitro*. *In vivo*, there was depletion of blood lymphocytes in stem cell transplant recipients, but there was no depletion of lymphocytes in the bone marrow and no effect on solid lymphoma masses or splenomegaly[1,2]. This fueled further research and led to the development of a new IgG1 antibody (Campath-1G) which was found to result in long-lasting depletion of lymphocytes from both blood and bone marrow. A few years later, this antibody was humanized (Campath-1H) to reduce the anti-globulin responses (Fig. 1)[2-4].

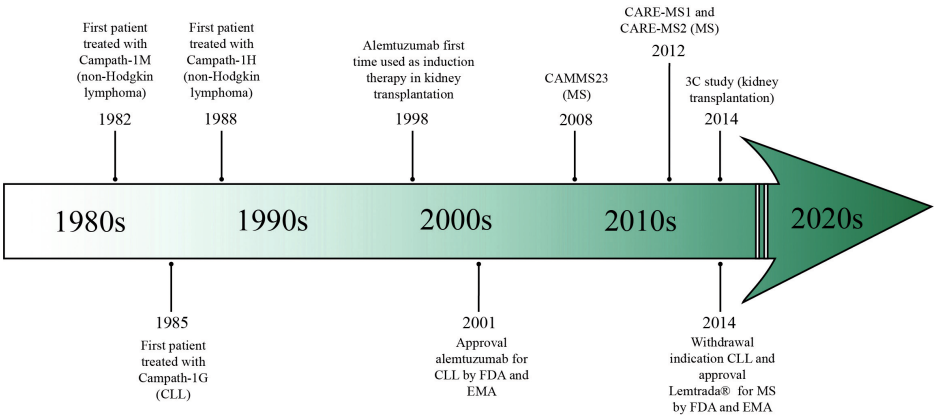

Figure 1. Timeline of alemtuzumab. In the 1980s, alemtuzumab was called Campath and was mainly used in hematology patients. Around 20 years later, alemtuzumab was approved for the treatment of chronic lymphocytic leukemia (CLL) and for the first time in kidney transplantation. A decade later, the registration of alemtuzumab for CLL was withdrawn and alemtuzumab was approved as Lemtrada® for the treatment of multiple sclerosis (MS). In 2014, a large randomized controlled trial compared alemtuzumab induction therapy with basiliximab induction therapy. EMA European Medicines Agency, FDA US Food and Drug Administration.

In 2001, the US Food and Drug Administration and the European Medicines Agency approved alemtuzumab for the treatment of chronic lymphocytic leukemia (CLL)[5]. Later, alemtuzumab was also approved by the Food and Drug Administration (2014) and European

Medicines Agency (2013) for the treatment of remitting-relapsing multiple sclerosis (RRMS) and is currently marketed for this indication under the name Lemtrada® (Sanofi-Genzyme, Cambridge, Massachusetts, United States)[6]. Following the market approval of Lemtrada®, the approval for the treatment of CLL was withdrawn (Fig. 1). However, alemtuzumab remains available for patients with CLL via the world-wide Campath Distribution Program[7]. In addition, alemtuzumab has also been used off-label for a variety of other diseases and conditions, including the prevention and treatment of acute rejection after solid organ transplantation (SOT).

In recent years, there has been a renewed interest in the use of alemtuzumab in SOT. In this review, we discuss the pharmacokinetics and pharmacodynamics of alemtuzumab, its use as induction and anti-rejection therapy in kidney transplantation, and strategies to improve the outcomes of alemtuzumab therapy.

METHODS

A systematic literature search was performed (8 February 8, 2017) of Ovid MEDLINE, EMBASE and the Cochrane Central Register of controlled trials. The search terms included 'alemtuzumab', 'campath', 'pharmacokinetics', 'pharmacodynamics', 'induction therapy', 'rejection therapy', and 'adverse effects' (see Electronic Supplementary Material). The search revealed 1668 articles. After exclusion of irrelevant articles (after reading the title and abstract), 730 articles remained, of which the relevant were included in this review. Examination of the reference list of the included studies identified further studies. There were no restrictions with regard to publication date. Only papers published in English were included.

PHARMACODYNAMICS OF ALEMTUZUMAB

CD52 is a 21-28 kDa cell surface glycoprotein that is attached to the cell membrane by a glycosylphosphatidyl-inositol anchor of 12 amino acids. CD52 is one of the most abundant membrane glycoproteins on T and B lymphocytes and is also expressed on natural killer (NK) cells, monocytes, macrophages, dendritic cells, eosinophilic granulocytes and to a lesser extent on neutrophilic granulocytes[1,8]. CD52 is not expressed on erythrocytes, platelets and hematopoietic progenitor cells[9]. The exact function of CD52 is unknown but it is suggested that the molecule may be involved in T lymphocyte co-stimulation, the induction of regulatory T lymphocytes and T lymphocyte migration and adhesion[10,11].

Administration of alemtuzumab causes a profound depletion of T and B lymphocytes, NK cells, dendritic cells, granulocytes and monocytes by three mechanisms: complement-dependent cytotoxicity (through C1q activation and subsequent generation of the membrane attack complex), antibody-dependent cellular cytotoxicity (after the activation of NK cells and macrophages through their IgG fragment C receptor), and induction of apoptosis (Fig. 2)[12,13]. Depletion of peripheral lymphocytes occurs within 1 h after alemtuzumab administration. Lymphocyte depletion from secondary lymphoid tissues occurs over 3-5 days[14]. Alemtuzumab administration significantly depletes peripheral monocytes and NK cells[15].

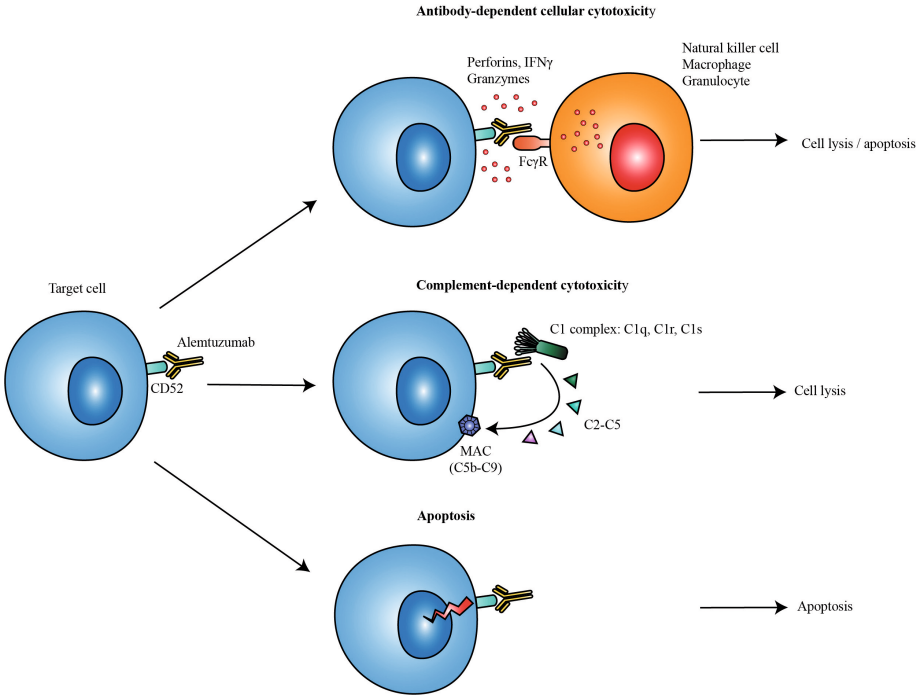

Figure 2. Mechanism of action of alemtuzumab. Alemtuzumab binds to CD52 on target cells (T and B lymphocytes, natural killer (NK) cells, monocytes, granulocytes and dendritic cells) and via three pathways depletion of the target cells occur. The antibody-dependent cellular cytotoxicity involves the IgG fragment C receptor (FcγR) on NK cells, macrophages and granulocytes. The FcγR recognizes the Fc region of alemtuzumab and binds to it. The NK cell, macrophage, or granulocyte releases perforins and granzyme B which causes lysis and apoptosis of the target cell. In complement-dependent cytotoxicity, the C1 complex (consisting of C1q, C1r and C1s), binds to alemtuzumab and this initiates the complement activation cascade and subsequently the formation of the membrane attack complex (MAC). Finally, binding of alemtuzumab to CD52 induces apoptosis directly. IFN interferon.

Alemtuzumab has a long-lasting depletional effect. In kidney transplant recipients receiving alemtuzumab as induction therapy (40-mg total dose), B lymphocytes recovered after 12 months. In contrast, T lymphocyte numbers recovered to approximately 50% of baseline 36 months after alemtuzumab administration. $CD8^+$ T lymphocytes repopulated more rapidly than $CD4^+$ T lymphocytes[16]. Cells of the innate immune system reconstitute faster than cells of the adaptive immune system. After 1 month, more than half of the peripheral lymphocytes consists of NK cells and the number of NK cells returns to 60-80% of baseline by 6 months[17]. Monocytes are only mildly depleted and recover after 3 months[16]. Dendritic cells recover to baseline levels 6 months after alemtuzumab treatment[18].

Immunological reconstitution of T lymphocytes, either partial or complete, appears to occur predominantly trough homeostatic proliferation of residual $CD4^+CD25^+$Forkhead box $P3^+$ ($FoxP3^+$) regulatory lymphocytes, as well as memory T lymphocytes and not by thymopoiesis[19]. Normally, levels of $FoxP3^+$ regulatory T lymphocytes in kidney transplant recipients make up 3-4% of the total $CD4^+$ population. After alemtuzumab treatment, a relative increase of $FoxP3^+$ regulatory T lymphocytes is seen (up to 12%) which persists for 2 years[20]. During immunological reconstitution skewing of the immune system to a more anti-inflammatory pattern is observed: an increase in the percentage of the anti-inflammatory cytokines interleukin (IL)-10 and transforming growth factor-β1 (produced by $CD4^+$ and $CD8^+$ cells), an increased percentage of IL-4-producing T-helper 2 cells, and decreased levels of the pro-inflammatory cytokines IL-17 and interferon (IFN)-γ[21].

Anderson *et al.* described the reconstitution of T lymphocytes 12 years after treatment with alemtuzumab because of rheumatoid arthritis[22]. Twenty patients treated with alemtuzumab were compared to 13 age-matched rheumatoid arthritis patients. Total $CD4^+$ lymphocyte counts were lower in the alemtuzumab group compared with the controls (median 0.55 $x10^9$/l *vs.* 0.85 x 10^9/l; $p = 0.0014$). The naïve and central memory $CD4^+$ lymphocytes were significantly reduced in the alemtuzumab-treated patients (0.09 $x10^9$/l *vs.* 0.21 $x10^9$/l ($p = 0.0007$) and 0.1$x10^9$/l *vs.* 0.36 $x10^9$/l ($p < 0.0001$), respectively). However, effector memory $CD4^+$ lymphocyte counts were not different. Total $CD8^+$ lymphocytes were similar in both groups, but the naïve and central memory $CD8^+$ lymphocytes were significantly lower in the alemtuzumab treated patients (0.05$x10^9$/l *vs.* 0.07$x10^9$/l (p = 0.0061) and 0.02$x10^9$/l *vs.* 0.04$x10^9$/l ($p = 0.0342$)[22].

B lymphocyte reconstitution in patients treated with alemtuzumab coincides with a high level of the cytokine B lymphocyte activating factor (BAFF, also known as TALL-1, BLyS,

THANK and zTNF4), that persists for over 12 months[23,24]. From the second month after alemtuzumab administration, B lymphocytes start to repopulate. First the transitional B lymphocytes dominate, followed by Bm2' (mature naïve) B lymphocytes[25]. Differentiation to memory B lymphocytes is slow and reaches 25% of baseline after 12 months[23,24]. After alemtuzumab induction therapy there is an increased risk of formation of *de novo* donor-specific anti–HLA antibodies (DSA) compared with basiliximab or anti-thymocyte globulin (ATG), which can lead to chronic humoral immune responses against graft alloantigens and subsequent graft failure[25,26]. The authors hypothesized that the spared alemtuzumab-resistant memory cells in presence of alloantigens can rapidly convert to plasmablasts and secrete donor-specific antibodies[25].

PHARMACOKINETICS OF ALEMTUZUMAB

Administration

Alemtuzumab is available as a solution for intravenous or subcutaneous administration. A vial contains 30 mg in 1 mL, or in case of Lemtrada® 12 mg in 10 mL. The recommended dose depends on the indication for alemtuzumab. In RRMS, the initial treatment is 12 mg/day intravenously for 5 consecutive days (cumulative dose of 60 mg) followed at 12 months by a second treatment course with 12 mg/day for 3 consecutive days (cumulative dose of 36 mg)[27]. For the indication CLL, it is advised to start with a maximum dose of 3 mg, intravenously, a second dose which is increased to 10 mg, which is followed by a third dose of 30 mg. Thereafter, the recommended alemtuzumab dose is 30 mg/day administered three times weekly for a maximum of 12 weeks (maximum cumulative dose 1080 mg)[28]. Dose recommendations have also been made for the reduced-intensity hematopoietic stem cell transplantation setting for non-malignant hematologic disease[29]. A typical dosing scheme of alemtuzumab in SOT is 1 or 2 gifts of 30 mg intravenously or subcutaneously[30-32]. This dose is empirical and has been deducted from the maximum dose used in hematology. No formal dose-finding studies have been performed in SOT recipients. It is recommended that patients are pre-medicated with glucocorticoids, acetaminophen and anti-histamines immediately prior to the administration of alemtuzumab to diminish infusion-related reactions[33,34].

Absorption

No pharmacokinetic studies of alemtuzumab have been performed in SOT recipients, whereas in patients with CLL and MS only a few such studies have been conducted. By definition, the bioavailability of alemtuzumab is 100% after intravenous administration. In

one study, the maximum concentration (C_{max}) of intravenously administered alemtuzumab was evaluated in 216 patients with RRMS[34]. Administration of 12 mg per day for 5 consecutive days resulted in a mean C_{max} of 3014 ng/mL directly after the last administration on day 5. In patients with CLL, C_{max} of 2800-26,400 ng/ml (mean 10,700 ng/mL) were measured after intravenous administration of 30 mg three times a week for 8 weeks[35].

Alemtuzumab can also be administered subcutaneously. Subcutaneous administration is more convenient and causes less infusion-related reactions as compared to intravenous administration[36,37]. The bioavailability of subcutaneously administered alemtuzumab was studied in cynomolgus monkeys. Doses of 1, 2 and 3 mg/kg were slowly absorbed from the site of injection and the time to reach C_{max} was around 48 h. The bioavailability after subcutaneous administration was approximately 47%[28]. In humans, Hale *et al.*[35] compared blood concentrations from patients with CLL who were treated either intravenously or subcutaneously (30 mg three times weekly). The highest measured pre-dose concentrations were similar between the two routes of administration (mean 5400 ng/mL). To reach a pre-dose concentration of 1000 ng/mL (an arbitrary threshold known to be potentially lympholytic), a higher cumulative dose was required when the drug was given subcutaneously as compared with intravenous administration (1106 mg and 146 mg, respectively).

Induction therapy with alemtuzumab in simultaneous pancreas-kidney transplant (SPKT) recipients showed no clinical difference between subcutaneous or intravenous therapy. Total lymphocyte and $CD3^+$ lymphocyte depletion were not significantly different and the incidence of acute rejection episodes, as well as patient survival, were comparable in the two groups[31].

Distribution

Because of its size, alemtuzumab is not likely to cross cell membranes and is therefore expected to distribute between the plasma and interstitial space. In patients with MS the volume of distribution was reported to be 14.1 L[34]. To measure the volume of distribution in CLL patients, Mould *et al.*[38] pooled the data of 67 patients from four studies. This resulted in a steady-state volume of distribution of 11.3 L.

In addition to being expressed on the cell surface, CD52 also exists in soluble form. Soluble CD52 can bind alemtuzumab, form immune complexes, and thereby reduce the amount of free and bioactive drug. Soluble CD52 levels are likely to be lower in patients with MS and recipients undergoing SOT compared with CLL patients[39]. Higher plasma levels of

soluble CD52 may require higher doses of alemtuzumab for sufficient efficacy[40]. There are no data on binding of alemtuzumab to other plasma proteins.

Metabolism and elimination

The half-life of alemtuzumab depends on the concentration of its target. In the case of a high concentration of CD52, such as in patients with CLL with a large tumor burden, the half-life of alemtuzumab is short, because binding of alemtuzumab to CD52 leads to cytotoxicity of malignant cells and rapid receptor-mediated clearance from the blood. When CD52 levels decrease (following successful treatment), the half-life of alemtuzumab increases. Therefore, patients with CLL will require a higher cumulative dose than patients treated for another indication. The half-life of alemtuzumab in patients with CLL is 6.1 days and in stem cell transplant recipients 8-21 days[35,41,42]. The half-life of alemtuzumab in patients with RRMS (12 mg on 5 consecutive days) was approximately 4-5 days and low or undetectable serum concentrations were measured within 30 days after completion of the course[34].

The mechanism of clearance of alemtuzumab from the circulation and interstitial space is not well understood. In a study of patients with CLL, alemtuzumab showed time- and concentration-dependent pharmacokinetics with (non-linear) clearance with large inter-patient variability[38]. This is probably explained by a difference in tumor burden. It is not known whether individual variations in factors such as hepatic function or macrophage activity affect the elimination rate of alemtuzumab[43]. No studies of the pharmacokinetics of alemtuzumab have been performed in patients with renal insufficiency or hepatic impairment.

It is also unknown if alemtuzumab binds to the neonatal Fc-receptor like some other monoclonal antibodies. The Fc-receptor is expressed on endothelial cells and influences the half-life of IgG_1 by internalization of immunoglobulins and protection from lysosomal degradation[44].

The expected metabolic pathway of alemtuzumab is degradation to small peptides and individual amino acids by widely distributed proteolytic enzymes. Classical biotransformation studies have not been conducted but are unlikely to be relevant for alemtuzumab clearance[34].

There is no known antidote available in case of an accidental overdose and treatment consist of supportive measures[34]. The effect of hemodialysis on the plasma concentration

of alemtuzumab is unknown. However, it is unlikely that alemtuzumab is removed with hemodialysis because of its size (150 kDa). Likewise, no studies investigated if alemtuzumab is removed by plasmapheresis. For the monoclonal antibody rituximab, it is known that plasmapheresis removes an important proportion of the drug if performed within the first 72 h after administration[45]. Like rituximab, alemtuzumab has a small volume of distribution and it is therefore likely that plasmapheresis can reduce the plasma concentration of alemtuzumab. However, the depletional effect on peripheral lymphocytes is already seen in the first hour after alemtuzumab administration.

Immunogenicity

Alemtuzumab is a recombinant humanized protein with a variable framework with constant regions from a human IgG_1 immunoglobulin and six complementarity-determining regions from a rat IgG_{2a} antibody. The humanization of alemtuzumab has reduced the risk of antiglobulin responses[46]. Anti-drug antibodies are still observed after administration of alemtuzumab[35,46].

In patients with CLL, no patient developed anti-alemtuzumab antibodies in the group treated with intravenous alemtuzumab (n = 30), whereas two patients in the group given subcutaneous alemtuzumab developed such antibodies (n = 32). The antibodies likely inactivated alemtuzumab, because these two patients did not show a significant reduction in lymphocyte count following alemtuzumab administration[35].

The phase III studies CARE MSI (Comparison of alemtuzumab and Rebif® efficacy) and CARE MSII (trials performed in patients with MS) showed a much higher percentage of anti-alemtuzumab antibodies. These antibodies were detectable in 29% of patients just before the second course of alemtuzumab (12 months after the last alemtuzumab gift) and in 81-86% of patients 1 month after the second course. Although the presence of anti-alemtuzumab antibodies was associated with a lower alemtuzumab concentration after the second course, the clinical outcome, lymphocyte depletion and repopulation were not influenced[47,48]. Rebello *et al.* described 12 patients treated with alemtuzumab because of kidney transplant rejection. No anti-alemtuzumab antibodies were detected[46].

Many factors possibly influence the immunogenicity of alemtuzumab including the dose and length of treatment, the route of administration, prior exposure to chemotherapy, and the concomitant use of other immunosuppressive drugs[46,49]. Additionally, the incidence of

anti-alemtuzumab antibodies is dependent on the sensitivity and specificity of the assay that is used.

THERAPEUTIC DRUG MONITORING

Pharmacokinetic monitoring is performed by three assays to measure alemtuzumab concentrations: an enzyme-linked immunosorbent assay, an indirect immunofluorescence method with flow cytometry detection, and liquid chromatography mass spectrometry[50-52].

For enzyme-linked immunosorbent assay, serum samples are added to microtiter plates that contain rabbit anti-rat IgG antibodies that recognize the remaining rat sequence in the alemtuzumab molecule[50]. After incubation the plates are washed and incubated with peroxidase-conjugated, affinity purified rabbit anti-human Fc. After washing, the substrate (3,3'-5,5'-tetramethylbenzidine; DAKO, Carpinteria, CA, USA) is added. The reaction is stopped with hydrochloride and the signal is measured with a spectrophotometer. No significant difference was seen between serum or plasma. The lower limit of detection of the assay is 0.05 μg/mL[50].

Alemtuzumab can also be measured by means of flow cytometry. For this technique a HUT-78 cell line is used. This $CD8^+$ T- cell line is derived from a patient with Sézary syndrome and expresses high levels of CD52[53]. The cell line is incubated with the serum of the patient treated with alemtuzumab. After washing, fluorescein isothiocyanate-labelled polyclonal anti-human Ig Fc antibodies are added and fluorescence is measured by flow cytometry. The lower limit of detection is 0.15 μg/L and the lower limit of quantification is 0.25 μg/L[51]. Recently, Marsh *et al*. used flow cytometry with normal donor peripheral blood mononuclear cells instead of the HUT-78 cell line to measure alemtuzumab concentrations[54]. The lower limit of detection was 0.02 μg/mL which is lower than that of the HUT-78 cell line-based assay[54].

Mass spectrometry has been described as a method to measure alemtuzumab[52]. It is currently not frequently used for the measurement of alemtuzumab. However, liquid chromatography-tandem mass spectrometry might become an important method to measure the blood concentrations of monoclonal antibodies in the future[55]. Pharmacodynamic monitoring is mainly done by flow cytometry to quantify the numbers of circulating T and B lymphocytes and NK cells.

From the above, it is clear that measuring the serum or plasma concentration of alemtuzumab is possible. However, these assays are not widely available, technically demanding and difficult to standardize. In SOT, no formal dose finding studies exist and at present there are no tests that support specific alemtuzumab target concentrations, with an optimal balance between efficacy and toxicity. Such studies have been performed in patients undergoing hematopoietic stem cell transplantation, and based upon pharmacokinetic-pharmacodynamic model target concentrations for this specific population have been proposed (personal communication, R. Admiraal, Leiden University Medical Center, The Netherlands).

CLINICAL USE OF ALEMTUZUMAB IN KIDNEY TRANSPLANTATION

Alemtuzumab was never registered for SOT indications. The drug has been used off-label for both the prevention and treatment of acute allograft rejection in kidney, pancreas, intestinal and lung transplantation.

Alemtuzumab as induction therapy

Kidney transplantation

In many transplant centers, induction therapy is used to reduce early rejection rates. Two types of induction therapy are recognized: T lymphocyte-depleting antibody therapy and antibody therapy directed against the IL-2 receptor. Basiliximab is a non-depleting monoclonal antibody directed against the IL-2 receptor, whereas ATG and alemtuzumab are depleting antibodies. Alemtuzumab was first used as induction therapy in 1998 in a case series of 13 kidney transplant recipients. The patients received induction therapy with alemtuzumab (two doses of alemtuzumab 20 mg intravenously on day 0 and 1) followed by low-dose ciclosporin as maintenance therapy. In the 6- to 11-month follow-up, only one patient experienced acute rejection[56].

Following this initial experience, the efficacy of alemtuzumab to prevent acute rejection following kidney transplantation was compared to IL-2 receptor antibodies in randomized-controlled trials (RCTs). A systematic review of five of these RCTs described a reduced risk of acute rejection using alemtuzumab as compared with an IL-2 receptor antagonist at 12 months after kidney transplantation (659 patients; relative risk = 0.54; 95% confidence interval (CI) 0.37-0.79; p <0.01)[57]. No significant difference was seen in graft loss, delayed graft function, or patient survival.

Recently, the results of the first phase of the 'Campath, calcineurin inhibitor reduction and chronic allograft nephropathy' (3C) study were published. The hypothesis of this RCT was that a more potent induction therapy at the time of transplantation allows for minimization of tacrolimus exposure without an increased risk of acute rejection. An immunosuppressive regimen with reduced exposure to the nephrotoxic tacrolimus could potentially lead to better renal function and longer graft survival. In the 3C study, induction therapy with alemtuzumab (30 mg on days 0 and 1, subcutaneously or intravenously) was compared with basiliximab (20 mg intravenously on days 0 and 4). A total of 852 patients were included (n = 426 in the alemtuzumab and n = 426 in the control arm). Patients in the alemtuzumab arm were co-treated with low-dose tacrolimus (aiming for pre-dose concentrations of 5-7 ng/mL) and mycophenolate sodium (360 mg twice daily) without glucocorticoids. In the control arm, basiliximab-treated patients were co-treated with a standard-dose tacrolimus (target pre-dose concentrations 5-12 ng/mL), mycophenolate sodium (540-720 mg twice daily) and glucocorticoids (15 to 20 mg prednisone, withdrawn in accordance with local practice).

The primary endpoint of the 3C study was the incidence of biopsy-proven acute rejection (BPAR) at month 6 after transplantation. Induction therapy with alemtuzumab in combination with low-dose tacrolimus and mycophenolate sodium without glucocorticoids significantly reduced the incidence of BPAR: 26 (6.1%) *vs.* 65 (15.3%; (p <0.0001, hazard ratio 0.37 (95% CI 0.23-0.58)), for the alemtuzumab and control arms, respectively. No significant difference was seen in the occurrence of biopsy-proven antibody-mediated rejection (ABMR): 8 (1.9%) *vs.* 5 (1.2%) (p = 0.41, hazard ratio 1.59 (95% CI 0.52-4.86). There was no difference 6 months after randomization between the two groups in terms of graft function (mean eGFR 50.1 mL/min per 1.73 m^2 in the alemtuzumab-treated patients *vs.* 49.8 mL/min per 1.73 m^2 in the basiliximab group), the incidence of graft failure, mortality, or serious infection[58]. Limitations of the 3C study were the short follow-up duration of 6 months and no blinding of the induction therapies. In addition, the difference in tacrolimus exposure was limited: The average pre-dose concentration of tacrolimus in the alemtuzumab treated patients was 6.9 ng/mL and in patient treated with basiliximab 8.3 ng/mL[59].

Hanaway *et al.* compared alemtuzumab induction therapy (a single shot of 30 mg, intravenously) with basiliximab induction therapy (in patients with low risk of acute rejection) or with rabbit ATG (rATG) induction therapy in high risk patients. A high risk of acute rejection was defined as panel-reactive antibody (historical or current) above 20%,

repeat transplantation or black ethnicity. There were 139 high risk patients; 70 received alemtuzumab and 69 received rATG. In the low risk group, 335 patients were included; 164 received alemtuzumab and 171 patients received basiliximab. Basiliximab was given on day 0 and day 3,4, or 5 (20 mg per gift). The total dose of rATG was 6 mg/kg (divided over four gifts). All patients received tacrolimus (target pre-dose concentration of 7-14 ng/mL in the first 3 months after transplantation, and 4-12 ng/mL after month 3), mycophenolate mofetil (1000 mg twice daily), and glucocorticoids (withdrawn on post-operative day 5). The rate of BPAR at 12 months in the alemtuzumab group was lower than in the basiliximab-treated patients (3% *vs.* 20%, p <0.0001). No significant difference in BPAR after month 12 was observed between alemtuzumab and rATG in the high risk group (10% *vs.* 13%, p = 0.53)[60].

A systematic review with meta-analysis compared induction therapy with alemtuzumab to rATG. A total of 446 patients was included and a comparable incidence of BPAR (relative risk = 0.79; 95% CI 0.52-1.21; p = 0.28) was seen. There was also no significant difference in graft loss and overall survival[57]. A recent Cochrane systematic review also showed comparable rates of BPAR between the alemtuzumab and rATG in a total of six studies (446 patients; RR 0.68 (95% CI 0.44-1.05; p = 0.66). However, rates of BPAR after alemtuzumab induction were lower in four studies with early glucocorticoid withdrawal (360 patients; RR 0.57; 95% CI 0.35-0.93; p = 0.025). Rabbit ATG plus glucocorticoid continuation *vs.* alemtuzumab plus early glucocorticoid withdrawal showed no difference between the two groups (2 studies; 86 patients, RR 1.27, 95% CI 0.5-3.19; p = 0.57)[61].

Although no higher rejection rate was seen after alemtuzumab induction therapy in the studies described above, higher rates of acute ABMR have been described in a few studies[62-64]. LaMattina *et al.*[64] compared in a retrospective study induction therapy with either alemtuzumab (n = 632), basiliximab (n = 690) or rATG (n = 125). Alemtuzumab was given one or two times (30 mg), basiliximab was administered on postoperative day 0 and 4 (20 mg) and the total dose of rATG was 6-8 mg/kg. Maintenance immunosuppression consisted of tacrolimus or ciclosporin in combination with mycophenolate mofetil and glucocorticoids (tapered to 5-10 mg/day after the first post-operative month). No significant difference was seen in overall frequency of BPAR; however, ABMR was significantly increased in the group of patients treated with alemtuzumab induction therapy compared to the group treated with rATG or basiliximab induction therapy. The 1-, 3- and 5- year cumulative incidence of alemtuzumab treated patients was 18.8, 23.8 and 26.5% respectively, *vs.* 11.3, 15.2 and 17.6% for the group receiving rATG or basiliximab (p<0.0001). The higher incidence of

ABMR could have been caused by a higher incidence of DSA after alemtuzumab treatment; however, this study did not test for the presence of DSA.

Simultaneous pancreas-kidney transplantation

Adding a pancreas allograft to a kidney transplant seems to increase the risk of acute rejection. Over 90% of SPKT receive antibody induction, with nearly 80% receiving a T lymphocyte depleting antibody[65].

In a single-center RCT, 28 SPKT recipients treated with alemtuzumab induction were compared to 18 SPKT patients treated with rATG. Alemtuzumab induction consisted of a single dose of 30 mg intravenously or rATG (cumulative dose 5-6 mg/kg). All patients received maintenance immunosuppression consisting of tacrolimus, mycophenolate mofetil, and glucocorticoids (with complete withdrawal on post-operative day 5). Patients identified as being high immunological risk remained on maintenance glucocorticoids. In this underpowered study, no significant difference was seen in the frequency of rejection after 1 year (18 and 39%, respectively for alemtuzumab and rATG; $p = 0.17$)) and 5 year (21 and 44%; $p = 0.12$). Total patient survival after 5 years was not significantly different (82 *vs.* 89% for alemtuzumab and rATG, respectively). Also, after 5 years, no significant difference was seen in kidney graft survival (78.6 *vs.* 66.7%) and pancreas graft survival (64.3 *vs.* 55.5%)[66].

Alemtuzumab induction therapy was compared with basiliximab in a retrospective cohort study of 136 SPKT recipients. All patients received maintenance immunosuppression with tacrolimus, mycophenolate mofetil and glucocorticoids. Basiliximab was given to 39 patients and alemtuzumab (30 mg, on days 0 and 1) was given to 97 patients. Acute cellular rejection of the kidney was significantly less frequent in the alemtuzumab-treated patients (3.1 *vs.* 15.4%, $p = 0.017$). The occurrence of ABMR was not different between the two groups (18% *vs.*14.4%, $p = 0.6$, for alemtuzumab and basiliximab, respectively). After 3 years, no significant difference was seen in patient survival, allograft survival of the kidney (86.2% for alemtuzumab and 91.8% for basiliximab) or pancreas (88.6% for alemtuzumab and 81.8% for basiliximab)[67].

Taken together, alemtuzumab is frequently used as an induction agent in SOT. Compared with basiliximab induction therapy, alemtuzumab results in a lower incidence of acute rejection. However, when compared to rATG no difference in the risk of acute rejection

was observed. Graft survival and patient survival are mostly comparable between induction therapy with alemtuzumab and basiliximab or rATG.

Alemtuzumab as anti-rejection therapy

In most centers, the first line treatment of BPAR of a kidney transplant is pulse therapy with glucocorticoids. In case of glucocorticoid-resistant rejection or in case of severe (histological grade) rejection, depleting antibody therapy is indicated[68]. The standard depleting antibody is rATG[69]. However, treatment with ATG has limitations. First, ATG must be administered via a high-flow intravenous access (often a central venous catheter) or an arteriovenous fistula to avoid thrombophlebitis. Second, administration of ATG can cause cytokine release syndrome immediately after infusion. Cytokine release syndrome is characterized by fever, hypotension, pulmonary edema, nausea, tachycardia, rash or chills. Furthermore, anti-rabbit antibodies can form after rATG administration. In case of subsequent exposure to rATG, this can lead to diminished activity and adverse reactions like serum sickness[70]. An alternative treatment would be necessary in these patients.

Alemtuzumab has incidentally been used as treatment of BPAR after kidney transplantation[30,32,46,71-73]. No RCTs investigating this application have been performed. Clatworthy *et al* [73] described the long-term outcome of first line treatment of BPAR with alemtuzumab. Of the 15 patients described in this retrospective case series, 12 patients were diagnosed with an acute cellular rejection, one with an ABMR and two with a mixed-type rejection. Alemtuzumab was administered intravenously and the first six patients were treated with 10 mg per day for 7 days (cumulative dose of 70 mg). The remaining nine patients received alemtuzumab in a dose of 6 mg/day for 4-10 days. The control group consisted of 25 patients with an acute rejection treated in the same period with intravenous methylprednisolone (1000 mg/day for 3 consecutive days). Of the 25 biopsies, 22 showed acute cellular rejection and three mixed-type rejections. Maintenance immunosuppressive therapy consisted of ciclosporin, azathioprine, and glucocorticoids. Baseline characteristics were comparable in both groups. All rejection episodes were treated successfully, as shown by a fall in serum creatinine within 3-10 days of treatment. Long-term transplant survival and allograft function were similar in both groups. There was no excess rate of cytomegalovirus infection, malignancy, autoimmunity, or post-transplant lymphoproliferative disorder in the alemtuzumab-treated patients. Serious infections during the first year were noted in 47% of patients treated with alemtuzumab and three patients died in the first year because of infection[73]. In conclusion, this study demonstrates that treatment of acute rejection with alemtuzumab results in comparable long-term outcomes as with methylprednisolone

pulse treatment; however, with an excess of infection-related death in the first year after treatment[73].

Alemtuzumab has also been used as second line treatment in glucocorticoid-resistant or severe acute rejection. Basu *et al.* described 40 patients with glucocorticoid-resistant rejection (29 patients) or severe rejections (Banff 1B or worse, 11 patients). No control group was included. The patients were treated with alemtuzumab intravenously (30 mg, one to four doses). All patients had previously received induction therapy consisting of rATG or alemtuzumab followed by tacrolimus monotherapy as maintenance immunosuppression. Graft survival after a mean duration of 453 ± 163 days was 62.5%. In 14 patients, infectious complications occurred. Two patients died: one patient developed post-transplant lymphoproliferative disorder and the other patient died because of an intraabdominal abscess[72]. The authors concluded that the outcome after treatment with alemtuzumab is comparable to the outcome of other antibody preparations (indirect comparisons with RCT). However, infectious complications were frequent[72].

Another retrospective study compared alemtuzumab to rATG for the treatment of glucocorticoid-resistant rejection. Eleven patients were treated with 15-30 mg alemtuzumab (subcutaneously) for 1-2 consecutive days. The reason for treating these patients with alemtuzumab were as follows: fluid overload, positive test for anti-rabbit IgG antibodies, treatment with ATG after previous transplantation and cardiac ischemia. Three patients had no contra-indication for ATG. The control group consisted of 20 patients who were treated with rATG (2.5-4.0 mg/kg for 10-14 days). These historical controls consisted of patients with a glucocorticoid-resistant rejection and were matched for date after transplantation. The endpoint of this small study was a composite endpoint named 'treatment failure' after three months which was defined as either graft loss, the need for additional anti-rejection therapy or the lack of improvement of renal allograft function (drop of less than 25% of serum creatinine at 3 months after treatment with alemtuzumab or rATG). The incidence of treatment failure was comparable in both groups (alemtuzumab 27% *vs.* rATG 40%, $p = 0.89$)[30].

Taken together, anti-rejection therapy with alemtuzumab results in a comparable graft survival compared with rATG. However, head-to-head RCTs with an rATG control and with longer follow-up are necessary to support this conclusion.

Alemtuzumab in pediatric kidney transplantation

Reducing the toxicity of immunosuppressive drugs is of paramount importance in pediatric kidney transplant recipients. In particular, the minimization of glucocorticoids, which can cause, among others growth retardation, post-transplant diabetes mellitus and weight gain, is an important goal in this population. Induction therapy with alemtuzumab has been used incidentally to avoid glucocorticoids and reduce calcineurin inhibitor exposure but no prospective, randomized controlled clinical trials comparing different induction therapies have been performed in children[74-83]. Several reasons may exist why limited studies have been performed with alemtuzumab in children. First, most children are unsensitized at the time of transplantation because most patients did not have prior kidney transplantations or pregnancies. Second, physicians may be concerned for the development of primo-cytomegalovirus and Epstein–Barr virus (EBV) infections and EBV-related post-transplant lymphoproliferative disease (PTLD) after alemtuzumab administration.

The first experience with alemtuzumab as induction therapy in pediatric kidney transplant recipients was described in 2005[75]. Four patients ranging from 20 months to 16 years of age received alemtuzumab intraoperatively (one dose of 30 mg in three patients and two doses of 30 mg in one patient). Three patients also received a calcineurin inhibitor, mycophenolate mofetil with or without corticosteroids as maintenance immunosuppressive therapy. In the fourth patient, calcineurin inhibitor therapy was withheld because of concerns for recurrence of Factor H, deficiency-associated hemolytic uremic syndrome. In the short follow-up period of 5-12 months, three children experienced acute rejection (of which two were C4d-positive suggesting an antibody-mediated rejection) without graft loss. No serious infections or PTLD occurred[75]. White blood cell counts were measured by flow cytometry in one patient and demonstrated that $CD3^+$, $CD4^+$, $CD8^+$ and $CD20^+$ lymphocyte counts had recovered to 50% of baseline one year after administration. Monocytes recovered to baseline level by month 3[75].

After this initial and disappointing experience, better results were obtained in a larger case series of 42 pediatric kidney transplant patients (mean age 9.0 years) treated with alemtuzumab induction therapy (in a dose of 0.4 - 0.5 mg/kg intravenously) followed by tacrolimus monotherapy[76]. The mean follow-up was 24.1 months. The aim of tacrolimus dosing was a pre-dose concentration of 8-12 ng/mL in the first 6 months. In case of no rejection and in the absence of the development *de novo* DSA and graft dysfunction, the tacrolimus dose was lowered to every other day. This strategy was successful in 12 patients. Only two patients experienced an episode of an acute cellular rejection and no cases of acute

antibody-mediated rejection were observed. The 4-year graft survival rate was 85.4%. No cases of cytomegalovirus infection were seen and two patients were diagnosed with BK viremia. No PTLD or serious infections occurred. Two children died: one of unknown cause and one because of a disconnected tracheostomy at home[76].

A larger case series of 101 pediatric kidney transplant patients (mean age 10.7 years) described a different outcome regarding the incidence of rejection and infection[77]. The patients were treated with two 30-mg doses of alemtuzumab: the first dose 12-29 days before transplantation and the second dose on the day of transplantation. The mean follow-up was 3.8 years. Maintenance therapy consisted of a calcineurin inhibitor (tacrolimus or ciclosporin) in combination with mycophenolate mofetil. Glucocorticoids were discontinued around day 5 if the graft function was acceptable and target calcineurin blood concentrations were reached. The incidence of acute rejection (including subclinical rejections) was 37%. In four patients rejection led to graft loss. Overall graft survival was 89.1% after three years. Cytomegalovirus and BK viremia occurred mostly during the first three months (30% and 25%, respectively). Twenty percent of patients experienced EBV viremia by year 2. No patients developed PTLD. Eight patients died (range 26-1457 days) of which five because of an infection[77].

In a phase II multicenter prospective analysis 35 pediatric kidney transplant patients were treated with one gift of alemtuzumab (0.3 mg/kg, maximum 20 mg) as induction therapy[84]. The primary aim of this study was to characterize the reconstitution of lymphocyte subsets in pediatric renal transplant recipients after alemtuzumab induction therapy followed by calcineurin inhibitor withdrawal. The patients were unsensitized and were first-time recipients with living donors. Maintenance immunosuppressive therapy consisted of tacrolimus and mycophenolate mofetil. Tacrolimus was switched to sirolimus after 2-3 months. In the follow-up period of 2 years six patients developed acute rejection. Two patients experienced graft loss: one to focal and segmental glomerulosclerosis and one to non-adherence of medication. Fourteen children experienced infectious episodes. The reconstitution of the lymphocytes in these patients mimicked the pattern seen in adults. $CD8^+$ T lymphocytes recovered faster than $CD4^+$ lymphocytes: after 24 months, $CD8^+$ T lymphocytes recovered to 60% of baseline and $CD4^+$ lymphocytes to 25% of baseline ($p = 0.014$). No significant difference was seen in the recovery of $CD4^+$ naïve and memory lymphocyte subsets and $CD8^+$ naïve and memory lymphocytes. In the $CD4^+$ memory lymphocyte population, the effector memory lymphocytes recovered faster than the central memory lymphocytes (44% *vs.* 24% after 24 months, respectively ($p = 0.027$)). No significant difference was seen in

the recovery of $CD8^+$ central memory and effector memory lymphocytes. At baseline, 4% of $CD4^+$ lymphocytes were $CD4^+CD25^+FoxP3^+$ regulatory T lymphocytes. Three months after alemtuzumab, there was relatively less depletion of regulatory $CD4^+$ lymphocytes (around 10% of the $CD4^+$ cells had a regulatory T lymphocyte phenotype) and this effect persisted until month 12 months alemtuzumab treatment[84].

Alemtuzumab has also been successfully used as part of the induction therapy in highly sensitized pediatric kidney transplant patients in two small case series[85,86]. To our knowledge, only one paper has described the use of alemtuzumab as anti-rejection therapy in pediatric kidney transplant recipients[87]. Three patients were treated with alemtuzumab (0.3 mg/kg, intravenously) because of five episodes of a late (*i.e.* more than 3 months after transplantation) glucocorticoid-resistant acute rejection. All patients were treated with ATG on two previous occasions. The first 14-year old patient suffered from recurrent rejection because of non-adherence. The first two episodes (acute cellular rejection (ACR) Banff type 1B) responded well to alemtuzumab. The third episode (ACR Banff type 1A) did not respond and the patient experienced graft loss soon thereafter. The second patient (14 years old) received one gift of alemtuzumab because of a ACR Banff type 1B. The serum creatinine concentration dropped from 292 μmol/L to 150 μmol/L 1 week after the administration of alemtuzumab. Two months after the alemtuzumab treatment, the patient experienced a borderline rejection with good response to high-dose glucocorticoids. The serum creatinine concentration stabilized around 175 μmol/L. The absolute lymphocyte count recovered to baseline level after 23 months. After 10 months there was an asymptomatic rise in serum EBV load with spontaneous resolution. The third patient (5 years old) experienced an ACR Banff type 1B-2A. He was treated unsuccessfully with methylprednisolone, ATG, rituximab, intravenous immunoglobulins, and finally alemtuzumab after which he lost his graft. In the year after the anti-rejection treatment this patient suffered from multiple serious infections probably related to the severe leukopenia, which required treatment with granulocyte colony-stimulating factor[87]. In conclusion, alemtuzumab reversed three of five rejection episodes in pediatric patients with a late glucocorticoid- and ATG-resistant rejection. However it did not prevent graft loss in two of the three patients.

In summary, alemtuzumab is sometimes used as induction therapy and rarely as anti-rejection therapy in pediatric renal transplant recipients. The results are variable and different dosing schemes (some are weight adjusted and some not) of alemtuzumab are used. Prospective, randomized controlled trials comparing different induction therapies

(like basiliximab, ATG and alemtuzumab) are needed to establish the efficacy and long-term safety of alemtuzumab in pediatric renal transplant recipients.

COMPLICATIONS OF ALEMTUZUMAB ADMINISTRATION

Infusion associated reactions

Acute infusion-related reactions occur in 70-80% of patients during treatment with alemtuzumab when given intravenously. These reactions are caused by cytokine release from lysed immune cells. These reactions are mostly mild to moderate and include headache, rash, nausea, hypotension, rigors and pyrexia. Following subcutaneous administration, infusion-related reactions occur less frequently, although local injection site reactions do occur[34,37] .

Infections

Alemtuzumab results in a prolonged depletion of T- and B lymphocytes (usually for over 12 months). This profound immunosuppression predisposes patients to infections. However, no depletion of the neutrophilic granulocytes typically occurs and reconstitution of the cells of the innate immune system is faster: monocytes typically recover after 3 months (although repopulation may occur in as little as 1 month) and NK cells return to 60-80% of baseline after 6 months).

Prophylaxis with an oral anti-herpes agent and prophylaxis against P*neumocystis jirovecii* should be started directly after administration of alemtuzumab and be continued for a minimum of 2 months after the last alemtuzumab gift or until the $CD4^+$ T lymphocyte count is ≥ 200 cells /μL[33,34]. In our center, we do not routinely screen kidney transplant recipients for adenovirus or EBV, whereas we do for BK virus.

Published data on the occurrence of opportunistic infections after alemtuzumab treatment are limited. BK virus infection is more common after alemtuzumab induction in kidney transplantation compared to ATG induction[32]. Cytomegalovirus and opportunistic and non-opportunistic infections were not more common when comparing alemtuzumab with ATG induction therapy[32]. In contrast to induction therapy with alemtuzumab, anti-rejection therapy with alemtuzumab is associated with a higher risk of opportunistic infections (4.5% *vs* 21% $p<0.001$). The higher incidence of opportunistic infections may be directly related to the alemtuzumab treatment, but could also be owing to the fact that after rejection the maintenance immunosuppressive therapy is also intensified[88].

Malignancy

Long term data linking alemtuzumab treatment with malignancy are scarce and the risk of developing of malignancy is poorly defined. In a single-center retrospective analysis among 1350 kidney transplant recipients no increased cancer incidence 4 years after induction therapy with alemtuzumab (2.8%) compared with ATG (5.4%) or no induction therapy (3.3%), was seen (across all groups; $p = 0.234$). This study did not include non-melanoma skin cancer[89].

In contrast, another study which used US transplantation and cancer registries data to explore the relationship between induction therapy and cancer after transplantation came to a different conclusion[90]. A total of 111,857 kidney transplant recipients were available for inclusion with a median follow-up of 3.5 years. Of the total group, 3394 patients received alemtuzumab induction therapy. Alemtuzumab induction, compared to no induction therapy, was associated with an increased risk of non-Hodgkin lymphoma ($n = 15$, adjusted incidence rate ratio (aIRR), 1.79; 95% CI, 1.02–3.14; $p = 0.04$) and all virus-related tumors like non-Hodgkin lymphoma, Hodgkin lymphoma, human papilloma virus-related cancers, Kaposi sarcoma, and liver cancer ($n = 19$, aIRR 1.84; 95% CI, 1.11– 3.03; $p = 0.02$). Alemtuzumab induction was also associated with increased colorectal cancer ($n = 7$, aIRR 2.46; 95% CI, 1.03–5.91; $p = 0.04$) and thyroid cancer ($n = 10$, aIRR 3.37; 95%-CI, 1.55–7.33; $p = 0.002$). Alemtuzumab induction was not associated with an increased risk of lung or kidney cancer, or melanoma[90]. No direct comparison between alemtuzumab and polyclonal depleting induction therapy was made.

Three RCTs compared alemtuzumab with interferon-β-1a in RRMS. In both the phase II (CAMMS223) and III trials (CARE-MSI and CARE-MS-II), malignancy was not more frequent after alemtuzumab compared with IFN-β-1a[47,48,91]. In the CAMMS223 trial, malignancy was observed in 2.8% of patients treated with alemtuzumab (one patient with cervical cancer and one patient with breast cancer) and 0.9% of patients taking IFN-β-1a (colon cancer) after a follow up of 3 years. In the extension part of this trial, one patient in the alemtuzumab group died of sepsis following chemotherapy for Burkitt's lymphoma[91]. In CARE-MSI, two patients (0.5%) in the alemtuzumab group developed thyroid papillary carcinoma. It is not clear whether these cases were induced by alemtuzumab or were an incidental finding on ultrasound investigation of patients with thyroid dysfunction after screening. No patients in the IFN-β-1a group developed a malignancy[47]. In CARE-MSII, malignancy rates for alemtuzumab- *vs.* IFN-β-1a treated patients were 0.6% *vs.* 1.5%, respectively, after 24 months of follow-up. These malignancies included one case of papillary

thyroid cancer, basal cell carcinoma (two patients), cervical cancer (one patient) and colon cancer (one patient) in the alemtuzumab-treated group. In the IFN-β-1a group, two malignancies were observed (one patient with a basal cell carcinoma and one case of acute myeloid leukemia)[48]. No further malignancies were observed in the long-term open-label follow-up (median 7 years, range 33-144 months)[92].

Occurrence of EBV-positive large-cell lymphoma has been described after administration alemtuzumab in patients with CLL. In a study to investigate the efficacy and safety of alemtuzumab in patients with CLL with residual disease, 3 of 41 patients developed EBV-positive large-cell lymphoma. Two of three patients had spontaneous resolution without therapy and one patient was treated with immunoglobulins and anti-viral medication[93]. A case report described the development of an EBV-positive lymphoma in an 80-year old patient with CLL treated with chemotherapy and alemtuzumab[94].

In conclusion, alemtuzumab results in an increased risk of malignancy as compared with no induction therapy in kidney transplantation. In contrast, no increased risk of malignancy was seen associated with the use of alemtuzumab in patients with MS when compared to IFN-β-1a.

Autoimmunity

Secondary autoimmune events have been reported after alemtuzumab treatment. Interleukin-21 seems to play a role in the development of this autoimmunity. Interleukin-21 is involved in the proliferation of cytotoxic T lymphocytes, the inhibition of regulatory T lymphocytes, and the differentiation of B lymphocytes into antibody-producing plasma cells[95]. Pre-treatment concentrations of IL-21 in patients with MS were two-fold higher in patients developing autoimmunity after alemtuzumab treatment compared to patients without autoimmunity[96].

Most commonly, the thyroid gland is affected. Autoimmune thyroid disorders, especially hypothyroidism and hyperthyroidism (Graves' disease) tend to occur between 6 and 61 months, peaking in the third year post-treatment in patients with MS. In kidney transplantation, Graves' disease has also been observed after alemtuzumab administration[97]. The total incidence of thyroid events, described in CAMMS223, CARE-MSI and CARE-MSII, ranged between 16 and 30%[47,48]. In the patients treated with IFN-β-1a the incidence of thyroid events was 3-6%. It is advised that thyroid function tests should be obtained

prior to initiation of treatment and tested on a regular basis until 48 months after the last infusion[34].

Immune thrombocytopenia (idiopathic thrombocytopenic purpura) as a side effect of alemtuzumab treatment was first described in the CAMMS223 study. A patient presented with intracranial hemorrhage and died. The incidence of idiopathic thrombocytopenic purpura was 1-2% in the CAMMS223 and CARE-MS studies[47,48,91]. Furthermore, four cases of glomerulopathy (0.3%) were described after alemtuzumab treatment in the CAMMS223, CARE-MSI and CARE-MSII trials. Two patients developed anti-glomerular basement membrane disease and two patients membranous glomerulopathy. The onset of kidney disease ranged from 4 to 39 months after alemtuzumab administration[47,48,91]. One case of Guillain-Barre syndrome was reported in a patient who was treated with alemtuzumab because of a T lymphocyte prolymphocytic leukemia[98].

Fertility and pregnancy

Alemtuzumab has been assigned to pregnancy category C by the Food and Drug Administration, meaning that animal production studies have shown an adverse effect on pregnancy outcomes but that no adequate studies have been performed in humans[34]. Immunoglobulin G molecules, such as alemtuzumab, are known to cross the placental barrier and may potentially affect the fetus.

Six months after delivery, levels of infliximab and adalimumab can be detected in the baby[99]. The administration of live vaccines (such as Bacillus Calmette-Guerin, rotavirus, varicella zoster, mumps, measles and rubella) in the first six months after delivery to babies of mothers treated with infliximab can be life threatening[100]. It is not known whether alemtuzumab can cause fetal harm when administered to pregnant women or whether it can affect reproductive capacity. In the Cambridge long-term follow-up study of MS patients a total of 15 babies were born to 12 women treated with alemtuzumab after a median interval from most recent treatment of 26 months (range 13–86 months). All deliveries and births were uncomplicated[92].

CD52 is expressed in the male reproductive system, including the epididymis, vas deferens, seminal vesicles and mature spermatocytes[101]. Although CD52 antibodies agglutinate and inactivate sperm *in vitro*, reproductive problems have not been reported following therapy with alemtuzumab, although available data are limited. A long term follow up study reported six males fathering seven live births, a median of 14 months (range 8–44 months)

from most recent treatment to conception[92]. Another (sub)study (n = 13) showed that at baseline, and 1, 3, and 6 months post alemtuzumab treatment, there was no evidence of aspermia, azoospermia, motility disorders, or depressed sperm counts[102].

It is unknown if alemtuzumab is excreted in human breast milk, but it has been detected in the milk of lactating mice. Therefore, breastfeeding should be discouraged to women for at least 4 months following treatment[34].

SUMMARY AND FUTURE DIRECTIONS

Alemtuzumab is frequently used off-label in kidney transplantation as induction therapy and less frequently as anti-rejection therapy. No pharmacokinetic studies have been performed in SOT recipients, probably because alemtuzumab never has been registered for this indication. Most pharmacokinetic and pharmacodynamics studies have been performed in patients with CLL and MS. However, the pharmacokinetics of alemtuzumab in the latter two patient populations may be very much different from SOT recipients. The alemtuzumab dose used in induction and anti-rejection therapy (30 mg one to two times) is not based on formal dose finding studies in SOT recipients, but is based on experience in CLL and MS. The duration of depletion of immune cells of the innate and the adaptive immune system is much longer after alemtuzumab treatment compared to rATG[103]. It may therefore be possible that lower doses of alemtuzumab will result in the same effect on graft survival, though with less toxicity. Subcutaneous administration showed the same outcomes compared to intravenous administration, but with less adverse events, although anti-alemtuzumab antibody formation may be more frequent.

Induction therapy with alemtuzumab in kidney transplantation shows comparable results in terms of graft and patient survival as compared with basiliximab and rATG. However, induction therapy with alemtuzumab is more effective in preventing acute rejection as compared to induction therapy with basiliximab. Results are comparable with induction therapy with rATG. The use of alemtuzumab induction therapy may facilitate minimization of the exposure to nephrotoxic immunosuppressive drugs, which may possibly lead to better long-term graft survival. Alemtuzumab used as anti-rejection therapy has shown some promising results. Replacement of rATG by alemtuzumab for this indication could lead to less infusion-related adverse events, shorter hospital stay and reduction in costs. However, long-term adverse events like infection, autoimmunity, malignancies and a higher frequency

of ABMR might be more frequent among alemtuzumab-treated patients compared with rATG.

Although alemtuzumab is used off-label in kidney transplantation it can be an additional treatment option to the drugs now used as induction or anti-rejection therapy. We should start the discussion with the pharmaceutical company to expand the indication for alemtuzumab to SOT, and thus more clinical studies can be performed. There is an unmet need to optimize alemtuzumab dosing in SOT patients, and we believe dose-finding studies are needed. Furthermore, RCTs are required to compare the effectiveness and long-term results of alemtuzumab with rATG for the treatment of acute rejection.

REFERENCES

1. Hale G, Bright S, Chumbley G, et al. Removal of T cells from bone marrow for transplantation: a monoclonal antilymphocyte antibody that fixes human complement. *Blood.* 1983;62(4):873-882.
2. Dyer MJ, Hale G, Hayhoe FG, Waldmann H. Effects of CAMPATH-1 antibodies in vivo in patients with lymphoid malignancies: influence of antibody isotype. *Blood.* 1989;73(6):1431-1439.
3. Hale G, Dyer MJ, Clark MR, et al. Remission induction in non-Hodgkin lymphoma with reshaped human monoclonal antibody CAMPATH-1H. *Lancet.* 1988;2(8625):1394-1399.
4. Riechmann L, Clark M, Waldmann H, Winter G. Reshaping human antibodies for therapy. *Nature.* 1988;332(6162):323-327.
5. Demko S, Summers J, Keegan P, Pazdur R. FDA drug approval summary: alemtuzumab as single-agent treatment for B-cell chronic lymphocytic leukemia. *Oncologist.* 2008;13(2):167-174.
6. Genzyme Submits Applications to FDA and EMA for Approval of LEMTRADA™ (alemtuzumab) for Multiple Sclerosis, http://news.genzyme.com/press-release/genzyme-submits-applications-fda-and-ema-approval-lemtrada-alemtuzumab-multiple-sclero. Published on June 12, 2012.
7. Campath Distribution Program, http://www.campath.com/. Assessed on May 22, 2017.
8. Ambrose LR, Morel AS, Warrens AN. Neutrophils express CD52 and exhibit complement-mediated lysis in the presence of alemtuzumab. *Blood.* 2009;114(14):3052-3055.
9. Elsner J, Hochstetter R, Spiekermann K, Kapp A. Surface and mRNA expression of the CD52 antigen by human eosinophils but not by neutrophils. *Blood.* 1996;88(12):4684-4693.
10. Watanabe T, Masuyama J, Sohma Y, et al. CD52 is a novel costimulatory molecule for induction of CD4+ regulatory T cells. *Clin Immunol.* 2006;120(3):247-259.
11. Rowan WC, Hale G, Tite JP, Brett SJ. Cross-linking of the CAMPATH-1 antigen (CD52) triggers activation of normal human T lymphocytes. *Int Immunol.* 1995;7(1):69-77.
12. Hu Y, Turner MJ, Shields J, et al. Investigation of the mechanism of action of alemtuzumab in a human CD52 transgenic mouse model. *Immunology.* 2009;128(2):260-270.
13. Stanglmaier M, Reis S, Hallek M. Rituximab and alemtuzumab induce a nonclassic, caspase-independent apoptotic pathway in B-lymphoid cell lines and in chronic lymphocytic leukemia cells. *Ann Hematol.* 2004;83(10):634-645.
14. Kirk AD, Hale DA, Mannon RB, et al. Results from a human renal allograft tolerance trial evaluating the humanized CD52-specific monoclonal antibody alemtuzumab (Campath-1H). *Transplantation.* 2003;76(1):120-129.
15. Lundin J, Porwit-MacDonald A, Rossmann ED, et al. Cellular immune reconstitution after subcutaneous alemtuzumab (anti-CD52 monoclonal antibody, CAMPATH-1H) treatment as first-line therapy for B-cell chronic lymphocytic leukaemia. *Leukemia.* 2004;18(3):484-490.
16. Bloom DD, Hu H, Fechner JH, Knechtle SJ. T-lymphocyte alloresponses of campath-1H-treated kidney transplant patients. *Transplantation.* 2006;81(1):81-87.
17. Sageshima J, Ciancio G, Guerra G, et al. Prolonged lymphocyte depletion by single-dose rabbit anti-thymocyte globulin and alemtuzumab in kidney transplantation. *Transpl Immunol.* 2011;25(2-3):104-111.

18. Kirsch BM, Haidinger M, Zeyda M, et al. Alemtuzumab (Campath-1H) induction therapy and dendritic cells: Impact on peripheral dendritic cell repertoire in renal allograft recipients. *Transpl Immunol.* 2006;16(3-4):254-257.

19. Bouvy AP, Klepper M, Betjes MG, et al. Alemtuzumab as Antirejection Therapy: T Cell Repopulation and Cytokine Responsiveness. *Transplant Direct.* 2016;2(6):e83.

20. Bloom DD, Chang Z, Fechner JH, et al. CD4+ CD25+ FOXP3+ regulatory T cells increase de novo in kidney transplant patients after immunodepletion with Campath-1H. *Am J Transplant.* 2008;8(4):793-802.

21. Zhang X, Tao Y, Chopra M, et al. Differential reconstitution of T cell subsets following immunodepleting treatment with alemtuzumab (anti-CD52 monoclonal antibody) in patients with relapsing-remitting multiple sclerosis. *J Immunol.* 2013;191(12):5867-5874.

22. Anderson AE, Lorenzi AR, Pratt A, et al. Immunity 12 years after alemtuzumab in RA: CD5(+) B-cell depletion, thymus-dependent T-cell reconstitution and normal vaccine responses. *Rheumatology (Oxford).* 2012;51(8):1397-1406.

23. Thompson SA, Jones JL, Cox AL, Compston DA, Coles AJ. B-cell reconstitution and BAFF after alemtuzumab (Campath-1H) treatment of multiple sclerosis. *J Clin Immunol.* 2010;30(1):99-105.

24. Heidt S, Hester J, Shankar S, Friend PJ, Wood KJ. B cell repopulation after alemtuzumab induction-transient increase in transitional B cells and long-term dominance of naive B cells. *Am J Transplant.* 2012;12(7):1784-1792.

25. Todeschini M, Cortinovis M, Perico N, et al. In kidney transplant patients, alemtuzumab but not basiliximab/low-dose rabbit anti-thymocyte globulin induces B cell depletion and regeneration, which associates with a high incidence of de novo donor-specific anti-HLA antibody development. *J Immunol.* 2013;191(5):2818-2828.

26. Mao Q, Terasaki PI, Cai J, et al. Extremely high association between appearance of HLA antibodies and failure of kidney grafts in a five-year longitudinal study. *Am J Transplant.* 2007;7(4):864-871.

27. Dörr J, Baum K. Alemtuzumab in the treatment of multiple sclerosis: Patient selection and special considerations. *Drug Des Dev Ther.* 2016;10:3379-3386.

28. Product monograph Mabcampath: http://shoppers-healthcare-portal-a88a1f4b.s3.amazonaws.com/AgilityUGC/da4d4219-085f-41c6-83c0-d09ee5bebe4f/MabCampath_EN_PM00010074.PDF. Published on March 22, 2010.

29. Marsh RA, Lane A, Mehta PA, et al. Alemtuzumab levels impact acute GVHD, mixed chimerism, and lymphocyte recovery following alemtuzumab, fludarabine, and melphalan RIC HCT. *Blood.* 2016;127(4):503-512.

30. van den Hoogen MW, Hesselink DA, van Son WJ, Weimar W, Hilbrands LB. Treatment of steroid-resistant acute renal allograft rejection with alemtuzumab. *Am J Transplant.* 2013;13(1):192-196.

31. Clatworthy MR, Sivaprakasam R, Butler AJ, Watson CJE. Subcutaneous administration of alemtuzumab in simultaneous pancreas-kidney transplantation. *Transplantation.* 2007;84(12):1563-1567.

32. Friend P. Alemtuzumab-based induction treatment versus basiliximab-based induction treatment in kidney transplantation (the 3C Study): A randomised trial. *Lancet.* 2014;384(9955):1684-1690.

33. Keating M, Coutre S, Rai K, et al. Management guidelines for use of alemtuzumab in B-cell chronic lymphocytic leukemia. *Clin Lymphoma.* 2004;4(4):220-227.

34. Lemtrada Summary of product characteristics, http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/003718/WC500150521.pdf. Assessed on May 22, 2017.

35. Hale G, Rebello P, Brettman LR, et al. Blood concentrations of alemtuzumab and antiglobulin responses in patients with chronic lymphocytic leukemia following intravenous or subcutaneous routes of administration. *Blood.* 2004;104(4):948-955.

36. Bowen AL, Zomas A, Emmett E, et al. Subcutaneous CAMPATH-1H in fludarabine-resistant/relapsed chronic lymphocytic and B-prolymphocytic leukaemia. *Br J Haematol.* 1997;96(3):617-619.

37. Patel K, Parmar S, Shah S, et al. Comparison of Subcutaneous versus Intravenous Alemtuzumab for Graft-versus-Host Disease Prophylaxis with Fludarabine/Melphalan-Based Conditioning in Matched Unrelated Donor Allogeneic Stem Cell Transplantation. *Biol Blood Marrow Transplant.* 2016;22(3):456-461.

38. Mould DR, Baumann A, Kuhlmann J, et al. Population pharmacokinetics-pharmacodynamics of alemtuzumab (Campath) in patients with chronic lymphocytic leukaemia and its link to treatment response. *Br J Clin Pharmacol.* 2007;64(3):278-291.

39. Vojdeman FJ, Herman SE, Kirkby N, et al. Soluble CD52 is an indicator of disease activity in chronic lymphocytic leukemia. *Leuk Lymphoma.* 2017:1-7.

40. Albitar M, Do KA, Johnson MM, et al. Free circulating soluble CD52 as a tumor marker in chronic lymphocytic leukemia and its implication in therapy with anti-CD52 antibodies. *Cancer.* 2004;101(5):999-1008.

41. Morris EC, Rebello P, Thomson KJ, et al. Pharmacokinetics of alemtuzumab used for in vivo and in vitro T-cell depletion in allogeneic transplantations: relevance for early adoptive immunotherapy and infectious complications. *Blood.* 2003;102(1):404-406.

42. Rebello P, Cwynarski K, Varughese M, et al. Pharmacokinetics of CAMPATH-1H in BMT patients. *Cytotherapy.* 2001;3(4):261-267.

43. Elter T, Molnar I, Kuhlmann J, Hallek M, Wendtner C. Pharmacokinetics of alemtuzumab and the relevance in clinical practice. *Leuk Lymphoma.* 2008;49(12):2256-2262.

44. Suzuki T, Ishii-Watabe A, Tada M, et al. Importance of neonatal FcR in regulating the serum half-life of therapeutic proteins containing the Fc domain of human IgG1: a comparative study of the affinity of monoclonal antibodies and Fc-fusion proteins to human neonatal FcR. *J Immunol.* 2010;184(4):1968-1976.

45. Puisset F, White-Koning M, Kamar N, et al. Population pharmacokinetics of rituximab with or without plasmapheresis in kidney patients with antibody-mediated disease. *Br J Clin Pharmacol.* 2013;76(5):734-740.

46. Rebello PR, Hale G, Friend PJ, Cobbold SP, Waldmann H. Anti-globulin responses to rat and humanized CAMPATH-1 monoclonal antibody used to treat transplant rejection. *Transplantation.* 1999;68(9):1417-1420.

47. Cohen JA, Coles AJ, Arnold DL, et al. Alemtuzumab versus interferon beta 1a as first-line treatment for patients with relapsing-remitting multiple sclerosis: a randomised controlled phase 3 trial. *Lancet.* 2012;380(9856):1819-1828.

48. Coles AJ, Twyman CL, Arnold DL, et al. Alemtuzumab for patients with relapsing multiple sclerosis after disease-modifying therapy: a randomised controlled phase 3 trial. *Lancet.* 2012;380(9856):1829-1839.

49. Schnitzer TJ, Yocum DE, Michalska M, et al. Subcutaneous administration of CAMPATH-1H: clinical and biological outcomes. *J Rheumatol.* 1997;24(6):1031-1036.

50. Jilani I, Keating M, Giles FJ, et al. Alemtuzumab: validation of a sensitive and simple enzyme-linked immunosorbent assay. *Leuk Res.* 2004;28(12):1255-1262.

51. Rebello P, Hale G. Pharmacokinetics of CAMPATH-1H: assay development and validation. *J Immunol Methods.* 2002;260(1-2):285-302.

52. Ashton DS, Beddell CR, Cooper DJ, et al. Mass spectrometry of the humanized monoclonal antibody CAMPATH 1H. *Anal Chem.* 1995;67(5):835-842.

53. Gootenberg JE, Ruscetti FW, Mier JW, Gazdar A, Gallo RC. Human cutaneous T cell lymphoma and leukemia cell lines produce and respond to T cell growth factor. *J Exp Med.* 1981;154(5):1403-1418.

54. Marsh RA, Fukuda T, Emoto C, et al. Pretransplant Absolute Lymphocyte Counts Impact the Pharmacokinetics of Alemtuzumab. *Biol Blood Marrow Transplant.* 2017;23(4):635-641.

55. Qu M, An B, Shen S, et al. Qualitative and quantitative characterization of protein biotherapeutics with liquid chromatography mass spectrometry. *Mass Spectrom Rev.* 2016.

56. Calne R, Friend P, Moffatt S, et al. Prope tolerance, perioperative campath 1H, and low-dose cyclosporin monotherapy in renal allograft recipients. *Lancet.* 1998;351(9117):1701-1702.

57. Morgan RD, O'Callaghan JM, Knight SR, Morris PJ. Alemtuzumab induction therapy in kidney transplantation: a systematic review and meta-analysis. *Transplantation.* 2012;93(12):1179-1188.

58. Group CSC, Haynes R, Harden P, et al. Alemtuzumab-based induction treatment versus basiliximab-based induction treatment in kidney transplantation (the 3C Study): a randomised trial. *Lancet.* 2014;384(9955):1684-1690.

59. Kuypers DRJ. Alemtuzumab induction therapy in kidney transplantation. *Lancet.* 2014;384(9955):1649-1651.

60. Hanaway MJ, Woodle ES, Mulgaonkar S, et al. Alemtuzumab induction in renal transplantation. *N Engl J Med.* 2011;364(20):1909-1919.

61. Hill P, Cross NB, Barnett AN, Palmer SC, Webster AC. Polyclonal and monoclonal antibodies for induction therapy in kidney transplant recipients. *Cochrane Database Syst Rev.* 2017;1:CD004759.

62. Knechtle SJ, Pirsch JD, H. Fechner J J, et al. Campath-1H induction plus rapamycin monotherapy for renal transplantation: results of a pilot study. *Am J Transplant.* 2003;3(6):722-730.

63. Noureldeen T, Albekioni Z, Machado L, et al. Alemtuzumab induction and antibody-mediated rejection in kidney transplantation. *Transplant Proc.* 2014;46(10):3405-3407.

64. LaMattina JC, Mezrich JD, Hofmann RM, et al. Alemtuzumab as compared to alternative contemporary induction regimens. *Transpl Int.* 2012;25(5):518-526.

65. Kandaswamy R, Stock PG, Gustafson SK, et al. OPTN/SRTR 2015 Annual Data Report: Pancreas. *Am J Transplant.* 2017;17 Suppl 1:117-173.

66. Stratta RJ, Rogers J, Orlando G, et al. 5-year results of a prospective, randomized, single-center study of alemtuzumab compared with rabbit antithymocyte globulin induction in simultaneous kidneyepancreas transplantation. *Transplant Proc.* 2014;46(6):1928-1931.

67. Pascual J, Pirsch JD, Odorico JS, et al. Alemtuzumab induction and antibody-mediated kidney rejection after simultaneous pancreas-kidney transplantation. *Transplantation.* 2009;87(1):125-132.

68. Kidney Disease: Improving Global Outcomes Transplant Work G. KDIGO clinical practice guideline for the care of kidney transplant recipients. *Am J Transplant.* 2009;9 Suppl 3:S1-155.

69. van den Hoogen MW, Hoitsma AJ, Hilbrands LB. Anti-T-cell antibodies for the treatment of acute rejection after renal transplantation. *Expert Opin Biol Ther.* 2012;12(8):1031-1042.

70. Hardinger KL. Rabbit antithymocyte globulin induction therapy in adult renal transplantation. *Pharmacotherapy.* 2006;26(12):1771-1783.

71. Csapo Z, Benavides-Viveros C, Podder H, Pollard V, Kahan BD. Campath-1H as rescue therapy for the treatment of acute rejection in kidney transplant patients. *Transplant Proc.* 2005;37(5):2032-2036.

72. Basu A, Ramkumar M, Tan HP, et al. Reversal of acute cellular rejection after renal transplantation with Campath-1H. *Transplant Proc.* 2005;37(2):923-926.

73. Clatworthy MR, Friend PJ, Calne RY, et al. Alemtuzumab (CAMPATH-1H) for the treatment of acute rejection in kidney transplant recipients: long-term follow-up. *Transplantation.* 2009;87(7):1092-1095.

74. Knechtle SJ. Present experience with Campath-1H in organ transplantation and its potential use in pediatric recipients. *Pediatr Transplant.* 2004;8(2):106-112.

75. Bartosh SM, Knechtle SJ, Sollinger HW. Campath-1H use in pediatric renal transplantation. *Am J Transplant.* 2005;5(6):1569-1573.

76. Tan HP, Donaldson J, Ellis D, et al. Pediatric living donor kidney transplantation under alemtuzumab pretreatment and tacrolimus monotherapy: 4-year experience. *Transplantation.* 2008;86(12):1725-1731.

77. Kaabak MM, Babenko NN, Samsonov DV, et al. Alemtuzumab induction in pediatric kidney transplantation. *Pediatr Transplant.* 2013;17(2):168-178.

78. Ellis D, Shapiro R, Moritz M, et al. Renal transplantation in children managed with lymphocyte depleting agents and low-dose maintenance tacrolimus monotherapy. *Transplantation.* 2007;83(12):1563-1570.

79. Ona ET, Danguilan RA, Africa J, et al. Use of Alemtuzumab (Campath-1H) as Induction Therapy in Pediatric Kidney Transplantation. *Transplant Proc.* 2008;40(7):2226-2229.

80. Shapiro R, Ellis D, Tan HP, et al. Alemtuzumab pre-conditioning with tacrolimus monotherapy in pediatric renal transplantation. *Am J Transplant.* 2007;7(12):2736-2738.

81. Sung J, Barry JM, Jenkins R, et al. Alemtuzumab induction with tacrolimus monotherapy in 25 pediatric renal transplant recipients. *Pediatr Transplant.* 2013;17(8):718-725.

82. Supe-Markovina K, Melquist JJ, Connolly D, et al. Alemtuzumab with corticosteroid minimization for pediatric deceased donor renal transplantation: A seven-yr experience. *Pediatr Transplant.* 2014;18(4):363-368.

83. Velez C, Zuluaga G, Ocampo C, et al. Clinical description and evolution of renal transplant pediatric patients treated with alemtuzumab. *Transplant Proc.* 2011;43(9):3350-3354.

84. De Serres SA, Mfarrej BG, Magee CN, et al. Immune profile of pediatric renal transplant recipients following alemtuzumab induction. *J Am Soc Nephrol.* 2012;23(1):174-182.

85. Kim IK, Choi J, Vo AA, et al. Safety and Efficacy of Alemtuzumab Induction in Highly Sensitized Pediatric Renal Transplant Recipients. *Transplantation.* 2016.

86. Pirojsakul K, Desai D, Lacelle C, Seikaly MG. Management of sensitized pediatric patients prior to renal transplantation. *Pediatr Nephrol.* 2016;31(10):1691-1698.

87. Upadhyay K, Midgley L, Moudgil A. Safety and efficacy of alemtuzumab in the treatment of late acute renal allograft rejection. *Pediatr Transplant.* 2012;16(3):286-293.

88. Peleg AY, Husain S, Kwak EJ, et al. Opportunistic infections in 547 organ transplant recipients receiving alemtuzumab, a humanized monoclonal CD-52 antibody. *Clin Infect Dis.* 2007;44(2):204-212.

89. Puttarajappa C, Yabes J, Bei L, et al. Cancer risk with alemtuzumab following kidney transplantation. *Clin Transplant.* 2013;27(3):E264-271.

90. Hall EC, Engels EA, Pfeiffer RM, Segev DL. Association of antibody induction immunosuppression with cancer after kidney transplantation. *Transplantation.* 2015;99(5):1051-1057.

91. Investigators CT, Coles AJ, Compston DA, et al. Alemtuzumab vs. interferon beta-1a in early multiple sclerosis. *N Engl J Med.* 2008;359(17):1786-1801.

92. Tuohy O, Costelloe L, Hill-Cawthorne G, et al. Alemtuzumab treatment of multiple sclerosis: long-term safety and efficacy. *J Neurol Neurosurg Psychiatry.* 2015;86(2):208-215.

93. O'Brien SM, Keating MJ, Mocarski ES. Updated guidelines on the management of cytomegalovirus reactivation in patients with chronic lymphocytic leukemia treated with alemtuzumab. *Clin Lymphoma Myeloma.* 2006;7(2):125-130.

94. Ghobrial IM, Otteman LA, White WL. An EBV-positive lymphoproliferative disorder after therapy with alemtuzumab. *N Engl J Med.* 2003;349(26):2570-2572; discussion 2570-2572.

95. Parrish-Novak J, Dillon SR, Nelson A, et al. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature.* 2000;408(6808):57-63.

96. Jones JL, Phuah CL, Cox AL, et al. IL-21 drives secondary autoimmunity in patients with multiple sclerosis, following therapeutic lymphocyte depletion with alemtuzumab (Campath-1H). *J Clin Invest.* 2009;119(7):2052-2061.

97. Kirk AD, Hale DA, Swanson SJ, Mannon RB. Autoimmune thyroid disease after renal transplantation using depletional induction with alemtuzumab [1]. *Am J Transplant.* 2006;6(5 I):1084-1085.

98. Abbi KK, Rizvi SM, Sivik J, et al. Guillain-Barre syndrome after use of alemtuzumab (Campath) in a patient with T-cell prolymphocytic leukemia: a case report and review of the literature. *Leuk Res.* 2010;34(7):e154-156.

99. Mahadevan U, Wolf DC, Dubinsky M, et al. Placental transfer of anti-tumor necrosis factor agents in pregnant patients with inflammatory bowel disease. *Clin Gastroenterol Hepatol.* 2013;11(3):286-292; quiz e224.

100. Cheent K, Nolan J, Shariq S, et al. Case Report: Fatal case of disseminated BCG infection in an infant born to a mother taking infliximab for Crohn's disease. *J Crohns Colitis.* 2010;4(5):603-605.

101. Hale G, Rye PD, Warford A, Lauder I, Brito-Babapulle A. The glycosylphosphatidylinositol-anchored lymphocyte antigen CDw52 is associated with the epididymal maturation of human spermatozoa. *J Reprod Immunol.* 1993;23(2):189-205.

102. Margolin DH, Rizzo MA, Smith G, et al. Alemtuzumab treatment has no adverse impact on sperm quality, quantity, or motility: A CARE-MS substudy. *J Neurol Sci.* 2013;333:e375-e376.

103. Sageshima J, Ciancio G, Guerra G, et al. Prolonged lymphocyte depletion by single-dose rabbit anti-thymocyte globulin and alemtuzumab in kidney transplantation. *Transplant Immunol.* 2011;25(2-3):104-111.

ELECTRONIC SUPPLEMENTARY MATERIAL:

APPENDIX S1 SYSTEMATIC LITERATURE SEARCH

A systematic literature search was performed with the following search terms.

Embase.com

('alemtuzumab'/mj OR (alemtuzumab OR campath):ab,ti) AND ('pharmacodynamics'/exp OR 'pharmacokinetics'/exp OR 'drug monitoring'/de OR 'toxicity'/exp OR 'immunogenicity'/de OR 'immunosuppressive treatment'/de OR 'immunomodulation'/de OR 'transplantation conditioning'/de OR 'adverse drug reaction'/exp OR (pharmacodynamic* OR pharmacokinetic* OR 'drug monitoring' OR absor* OR distribut* OR metabol* OR excret* OR eliminat* OR induction OR ((reject* OR rescue*) NEAR/6 (therap* OR immunotherap* OR prevent* OR revers*)) OR toxic* OR immunogenicit* OR precondition* OR pretreat* OR pre-condition* OR pre-treat* OR efficac* OR (concentration* NEAR/6 effect*) OR (clinical* NEAR/3 use) OR immunosuppress* OR (immun* NEXT/1 (suppress* OR modulation)) OR immunomodulat* OR ((transplant* OR pretransplant*) NEAR/3 condition*) OR adverse OR side-effect*):ab,ti) AND ('kidney transplantation'/exp OR 'kidney graft rejection'/exp OR 'kidney allograft rejection'/exp OR 'organ transplantation'/de OR 'multiple sclerosis'/exp OR (((kidney* OR renal OR organ*) NEAR/6 (transplant* OR allotransplant* OR graft* OR allograft* OR recipient* OR reject* OR donor* OR donat*)) OR 'multiple sclerosis'):ab,ti) NOT ([animals]/lim NOT [humans]/lim) AND [english]/lim

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("alemtuzumab".mp. OR (alemtuzumab OR campath).ab,ti,kf.) AND (exp "pharmacokinetics"/ OR pharmacokinetics.xs. OR exp "Drug Monitoring"/ OR toxicity.xs. OR "Immunosuppression"/ OR "immunomodulation"/ OR "Transplantation Conditioning"/ OR exp "Drug-Related Side Effects and Adverse Reactions"/ OR (pharmacodynamic* OR pharmacokinetic* OR "drug monitoring" OR absor* OR distribut* OR metabol* OR excret* OR eliminat* OR induction OR ((reject* OR rescue*) ADJ6 (therap* OR immunotherap* OR prevent* OR revers*)) OR toxic* OR immunogenicit* OR precondition* OR pretreat* OR pre-condition* OR pre-treat* OR efficac* OR (concentration* ADJ6 effect*) OR (clinical* ADJ3 "use") OR immunosuppress* OR (immun* ADJ (suppress* OR modulation)) OR immunomodulat* OR ((transplant* OR pretransplant*) ADJ3 condition*) OR adverse OR side-effect*).ab,ti,kf.) AND ("kidney

transplantation"/ OR kidney/tr OR "Organ Transplantation"/ OR exp "multiple sclerosis"/ OR (((kidney* OR renal OR organ*) ADJ6 (transplant* OR allotransplant* OR graft* OR allograft* OR recipient* OR reject* OR donor* OR donat*)) OR "multiple sclerosis"). ab,ti,kf.) NOT (exp animals/ NOT humans/) AND english.la.

Cochrane CENTRAL

((alemtuzumab OR campath):ab,ti) AND ((pharmacodynamic* OR pharmacokinetic* OR 'drug monitoring' OR absor* OR distribut* OR metabol* OR excret* OR eliminat* OR induction OR ((reject* OR rescue*) NEAR/6 (therap* OR immunotherap* OR prevent* OR revers*)) OR toxic* OR immunogenicit* OR precondition* OR pretreat* OR pre-condition* OR pre-treat* OR efficac* OR (concentration* NEAR/6 effect*) OR (clinical* NEAR/3 use) OR immunosuppress* OR (immun* NEXT/1 (suppress* OR modulation)) OR immunomodulat* OR ((transplant* OR pretransplant*) NEAR/3 condition*) OR adverse OR side-effect*):ab,ti) AND ((((kidney* OR renal OR organ*) NEAR/6 (transplant* OR allotransplant* OR graft* OR allograft* OR recipient* OR reject* OR donor* OR donat*)) OR 'multiple sclerosis'):ab,ti)

CHAPTER 4

AIMS OF THE THESIS

Kidney transplant rejection remains a serious complication with long-lasting consequences, including progressive deterioration of renal function, premature transplant failure and death. The objectives of this thesis are to investigate strategies to optimize the prevention, diagnosis and treatment of kidney transplant rejection. In more detail, the following will be investigated:

- To examine the diagnostic performance of gene expression analysis in discriminating aTCMR from no rejection in kidney transplant biopsies (**Chapter 5**).
- To investigate if the pathogenesis of acute transplant rejection is different in transplant biopsies of patients treated with either belatacept or tacrolimus maintenance therapy using gene expression analysis and immunohistochemistry (**Chapter 5**).
- To assess if a proteomic extension assay on sera of kidney transplant recipients is a tool to diagnose aTCMR in a minimally-invasive manner (**Chapter 6**).
- To study the effect of belatacept maintenance therapy on glucose tolerance in a kidney transplant recipient with type 2 diabetes mellitus (**Chapter 7**).
- To analyze the efficacy and long-term outcome of rATG treatment for acute kidney transplant rejection in patients treated with the current standard immunosuppressive regimen consisting of tacrolimus and MPA (**Chapter 8**).
- To investigate patient-, and allograft outcome, in addition to adverse events in kidney transplant recipients treated with alemtuzumab for acute kidney transplant rejection and to compare these to the outcome of patients treated with rATG for the same indication (**Chapter 9**).
- To determine the frequency and outcomes of inflammatory polyneuropathy in kidney transplant recipients treated with either alemtuzumab or rATG (**Chapter 10**).
- To examine the occurrence, treatment and outcome of acquired hemophilia A after treatment with alemtuzumab (**Chapter 11**).

In **Chapters 12 and 13**, the main findings are summarized and placed in a broader context.

PART II

DIAGNOSIS OF ACUTE REJECTION

CHAPTER 5

Immunomics of renal allograft acute T cell-mediated rejection biopsies of tacrolimus- and belatacept-treated patients

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ABSTRACT

Background

Belatacept-based therapy in kidney transplant recipient has been shown to increase long-term renal allograft and patient survival compared with calcineurin inhibitor-based therapy, however with an increased risk of acute T cell-mediated rejection (aTCMR). An improved understanding of costimulation blockade-resistant rejections could lead to a more personalized approach to belatacept therapy. Here, immunomic profiles of aTCMR biopsies of patients treated with either tacrolimus or belatacept were compared.

Methods

Formalin-fixed paraffin-embedded renal transplant biopsies were used for immunohistochemistry and gene expression analysis using the innovative NanoString technique. To validate NanoString, transcriptomic profiles of patients with and without biopsy-proven aTCMR were compared. Biopsies from 31 patients were studied: 14 tacrolimus-treated patients with aTCMR, 11 belatacept-treated patients with aTCMR, and 6 controls without rejection.

Results

A distinct pattern was seen in biopsies with aTCMR compared to negative controls: 78 genes had a higher expression in the aTCMR group (false discovery rate P value < 0.05 to 1.42e-05). The most significant were T cell-associated genes (CD3, CD8, and CD4; $p < 1.98e\text{-}04$), γ-interferon-inducible genes (CCL5, CXCL9, CXCL11, CXCL10, TBX21; $p < 1.33e\text{-}04$) plus effector genes (GNLY, GZMB, ITGAX; $p < 2.82e\text{-}03$). Immuno-phenotypical analysis of the classic immune markers of the innate and adaptive immune system was comparable between patients treated with either tacrolimus or belatacept. In addition, the transcriptome of both groups was not significantly different.

Conclusions

In this small pilot study, no difference was found in immunomics of aTCMR biopsies of tacrolimus- and belatacept-treated patients. This suggests that clinically diagnosed aTCMR reflects a final common pathway of allorecognition which is unaffected by the type of immunosuppressive therapy.

INTRODUCTION

Gene expression analysis of the kidney transplant biopsy has been shown to improve classification and risk stratification of patients when used in combination with current diagnostic standards[1]. The "molecular microscope" has been postulated to give a better insight into the classification of renal transplant pathology[1-4]. With the use of both gene and protein expression analysis, also known as immunomics, more insight can be gained in the pathophysiology of inflammatory reactions in the renal allograft.

The Banff guideline is a pathology-based classification system to diagnose acute renal transplant rejection[5]. However, this classification is vulnerable to misinterpretation and the Banff 2017 guideline states that the combination of conventional histomorphologic examination of a kidney transplant biopsy with molecular diagnostics leads to superior diagnostic classification and has the potential to guide therapy and improve allograft outcomes[5,6]. The novel technique NanoString® allows for multiplex messenger RNA (mRNA) analysis of minute quantities of mRNA from formalin-fixed, paraffin-embedded (FFPE) biopsies without the need of pre-amplification[2]. With this technique, residual material from conventional histopathological diagnosis can be analyzed[7,8]. NanoString® makes it possible to render data on the intragraft gene expression of up to 770 targets of interest within two days and with a comparable sensitivity to quantitative real time-polymerase chain reaction, and a better sensitivity than microarray[8,9].

Long-term outcomes of kidney transplantation are negatively influenced by the nephrotoxicity and metabolic side effects of calcineurin inhibitor (CNI)-based therapy[10,11]. A CNI-free immunosuppressive regimen with the costimulation blocking drug belatacept has been shown to increase long-term renal allograft and patient survival[12-14]. However, belatacept-based immunosuppressive therapy is associated with an increased risk of acute T cell-mediated rejection (aTCMR)[15-18]. Identification of factors that underlie such costimulation blockade-resistant rejection could lead to a more personalized approach to belatacept-based treatment through the identification of patients at "low risk" for acute rejection[15,19-21].

To expand the understanding of the pathogenesis of costimulation blockade-resistant rejections, we have compared the immunomic profiles of aTCMR biopsies of patients treated with maintenance therapy consisting of either tacrolimus or belatacept. Gene expression analysis of 209 genes with the innovative NanoString® technique in combination with

immunohistochemistry (IHC) was used. To validate NanoString® for our research question, transcriptomic profiles of patients with and without biopsy-proven aTCMR were compared.

MATERIALS AND METHODS

Study population and materials

Renal allograft biopsies were obtained from kidney transplant recipients who previously participated in one of two prospective randomized controlled trials (RCT; a belatacept study and a tacrolimus-dosing study) performed at the Erasmus MC, the Netherlands (see below). The design and results of these trials were published previously[20,22]. Both studies were approved by the institutional review board of Erasmus MC (Medical Ethical Review Board numbers 2010-080 and 2012-421). Eleven belatacept-treated patients experienced an aTCMR[20] and the renal allograft biopsies of these patients were analyzed here. These biopsies were compared with 14 biopsies of tacrolimus-treated patients with an aTCMR that were included in one of these two RCTs[20,22]. The biopsies were all for-cause biopsies that were matched for time after transplantation, Banff 2015 category and grade, and age of the recipient (Table 1). All biopsies were scored independently by two pathologists according to the Banff 2015 classification[23]. In case of differences in classification, consensus was met. Six renal transplant biopsies without histomorphologic changes (Banff category 1) were included as negative controls and these were either derived from one of the RCTs[20] or from the archives of the department of pathology of the Erasmus MC (Table 1). The negative controls were matched for age of the recipient and time after transplantation.

The patients who participated in the belatacept study were randomized to a belatacept- or tacrolimus-based immunosuppressive regimen, as described previously[20]. The main objective of the tacrolimus-dosing study was to examine whether a *CYP3A5* genotype-based tacrolimus starting dose leads to earlier achievement of the tacrolimus target predose concentration (C_0)[22]. The target C_0 of tacrolimus and the dosing of mycophenolate mofetil and glucocorticoids were identical in both RCTs[20,22].

Table 1. Patient characteristics.

Biopsy	Primary kidney disease	Age	Sex	Therapy	HLA mm[a]	Type tx	Preemptive[b]	Timing[c]	Pathology diagnosis	DSA[d]	eGFR	Used for IHC	Used for Nanostring®
1	Membranous glomerulopathy	71	M	Tac	2-2-1	LUR	No	152	aTCMR IB	Negative	16	Yes	Yes
2	Hypertension	73	M	Tac	2-1-2	LUR	Yes	82	aTCMR IB	Not tested	40	Yes	Yes
3	Hypertension	46	F	Tac	2-0-1	LUR	No	5	aTCMR IIA	Not tested	41	Yes	No
4	DM2	64	M	Tac	1-2-2	LUR	No	34	aTCMR IIA	Not tested	24	Yes	No
5	ADPKD	73	F	Tac	1-2-1	LUR	Yes	68	aTCMR IIA	Not tested	30	Yes	No
6	DM1	60	M	Tac	1-1-1	LR	Yes	91	aTCMR IIA	Not tested	35	Yes	No
7	Hypertension and DM2	76	M	Tac	2-2-1	LUR	Yes	10	aTCMR IIA	Negative	18	Yes	No
8	Unknown	56	F	Tac	2-2-2	LR	Yes	6	aTCMR IIA and ABMR	Negative	28	Yes	Yes
9	Hypertension	46	F	Tac	1-2-1	LUR	No	9	aTCMR IIA and ABMR	Negative	DGF	Yes	No
10	DM2	65	M	Tac	1-1-1	LUR	Yes	9	aTCMR IIB	Not tested	8	Yes	Yes
11	Urate nephropathy	55	F	Tac	1-1-1	LR	No	6	aTCMR IIB	Not tested	14	Yes	Yes
12	Alport syndrome	34	M	Tac	1-2-1	LUR	No	2	aTCMR IIB	Positive	DGF	Yes	Yes
13	Unknown	50	M	Tac	2-1-2	LUR	No	8	aTCMR IIB	Not tested	34	Yes	Yes
14	Hypertension	55	M	Tac	0-2-2	LUR	No	48	aTCMR IIB	Not tested	12	Yes	No
15	Unknown	26	M	Bela	1-1-1	LR	No	94	aTCMR IIB	Negative	56	Yes	Yes
16	ADPKD	66	F	Bela	1-2-1	LUR	Yes	70	aTCMR IIB	Negative	25	Yes	Yes
17	ATN after sepsis	76	M	Bela	1-2-1	LUR	No	13	aTCMR IIA and ABMR	Negative	18	Yes	Yes
18	Nephrotoxicity chemotherapy	46	M	Bela	0-1-1	LR	Yes	64	aTCMR IIA	Negative	72	Yes	Yes
19	VUR	46	M	Bela	0-1-1	LR	Yes	44	aTCMR IIA	Negative	36	Yes	Yes
20	Hypertension and DM2	69	M	Bela	2-1-1	LUR	Yes	120	aTCMR IIB	Negative	27	Yes	Yes
21	Unknown	46	M	Bela	2-1-2	LUR	Yes	112	aTCMR IIB	Negative	28	Yes	Yes
22	Urologic	45	M	Bela	1-2-1	LUR	Yes	3	aTCMR IIB	Negative	16	Yes	Yes
23	GPA	28	F	Bela	1-1-1	LR	No	5	aTCMR IIB	Negative	34	Yes	No
24	ADPKD	48	F	Bela	1-1-1	LUR	No	4	aTCMR IIB and ABMR	Negative	DGF	Yes	Yes
25	Unknown	62	F	Bela	1-2-2	LUR	Yes	56	aTCMR III	Negative	5	Yes	Yes
26	ADPKD	42	F	Tac	1-1-0	LR	Yes	28	Reactive changes (Banff cat. 1)	Not tested	45	No	Yes

Table 1. (Continued)

Biopsy	Primary kidney disease	Age	Sex	Therapy	HLA mm[a]	Type tx	Preemptive[b]	Timing[c]	Pathology diagnosis	DSA[d]	eGFR	Used for IHC	Used for Nanostring®
27	ADPKD	61	M	Tac	1-1-1	LUR	No	31	Reactive changes (Banff cat. 1)	Not tested	37	No	Yes
28	Unknown	69	M	Tac	0-1-1	LR	No	13	Some infiltration in interstitium with eosinophil granulocytes (Banff cat. 1)	Not tested	58	No	Yes
29	GPA	27	M	Tac	0-0-0	LR	Yes	77	Arteriosclerosis, small area interstitial fibrosis (Banff cat. 1)	Not tested	63	No	Yes
30	Systemic lupus erythematosus	35	F	Tac	0-1-2	LUR	Yes	72	Arteriosclerosis, 10% IF/TA, some glomerular ischemia (Banff cat.1)	Not tested	33	No	Yes
31	Nephrotoxicity chemotherapy	45	M	Bela	0-1-1	LR	Yes	101	Intima fibrosis (Banff cat.1)	Not tested	76	No	Yes

All patients received mycophenolate mofetil with corticosteroids in combination with tacrolimus or belatacept.

[a]HLA mismatch is for HLA-A, HLA-B, and HLA-DR.

[b]Preemptive transplantation before the start of renal replacement therapy.

[c]The time in days between transplantation and the biopsy.

[d]Donor specific antibodies measured with Luminex at time of rejection.

[e]GFR (mL/min per 1.73 m2) at the time of the biopsy.

ABMR, antibody medicated rejection; ADPKD, autosomal dominant polycystic kidney disease; aTCMR, acute T cell mediated rejection; ATN, acute tubular necrosis; DGF, delayed graft function; DM, Diabetes mellitus; DSA, donor specific antibodies; GPA, granulomatous polyangiitis; IF/TA, interstitial fibrosis / tubular atrophy; IHC, immunohistochemistry; LR, living related; LUR, living unrelated; VUR, vesicoureteral reflux.

Histo- and immunohistochemical (IHC) stainings and digital quantification

Two µm sections of FFPE renal allograft biopsies were stained with hematoxylin and eosin, Periodic acid-Schiff-diastase, and Jones' silver stain according to standard diagnostic practice. Subsequently, IHC stainings were performed on 4 µm FFPE cut sections with an automated, validated and accredited staining system (Ventana Benchmark ULTRA, Ventana Medical Systems, Tucson, AZ, USA) using ultraview or optiview universal DAB detection Kit. Antibodies used (CD3, CD4, CD8, CD20, CD56, CD68, PD-1, and granzyme B) and dilutions are summarized in Table S1. FoxP3/CD4 staining was performed at MGH/Harvard, (Boston, MA, USA). All sections were scanned at 40x magnification using Nanozoomer XR digital slide scanner (Hamamatsu, Hamamatsu City, Japan). Digital image analysis was performed using Visiopharm integrator system (version 2017.2.4.3387) with Author™ module (Visiopharm®, Hoersholm, Denmark). For each section, manual selection of only cortical tissue was made, excluding the medulla, artefacts, and the lumen of blood vessels larger than glomeruli. Image analysis Application Protocol Packages were developed to measure the total tissue area (μm^2) and the area percentage of positive staining.

RNA extraction

Three consecutive 20-µm sections cut from each FFPE block were immediately transferred to sterile microcentrifuge tubes and stored at room temperature. Microtome blades were then replaced, and equipment sterilization was performed with RNase AWAY (Life Technologies, Carlsbad, CA) between blocks. Xylene deparaffinization and RNA extraction of the curls were performed with use of the Recover All Total Nucleic Acid Isolation Kit for FFPE (Life Technologies). RNA concentration and purity were measured with the Nano-Drop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

NanoString® nCounter® assay, data normalization and analysis

A custom code set of 216 genes was created: 209 genes that are known to be involved in renal allograft rejection and renal injury, and 7 housekeeping genes. This was based on the panel described in the Banff 2017 report[5]. Probe description and sequences are provided in Table S2. Gene expression was measured on 120 to 200 ng of extracted RNA from FFPE biopsies with NanoString®. NanoString® was previously tested in renal allograft rejection in non-human primates[24,25]. Raw gene expression counts of all samples are provided in Table S3. Background correction, data quality control, normalization of the raw gene expression counts and data analysis was investigated with nSolver™ Analysis Software (Version 4.0.62). The geNorm algorithm was applied to analyze the stability of the reference genes[26]. Seven reference genes (DDX50, HDAC3, GUSB, POLR2A, OAZ1, UBB and SDHA) were used

for normalization. The parameters for quality control flagging as recommended by the manufacturer were used[27].

Statistical analysis

SPSS version 21 (SPSS Inc., Chicago, IL, USA) was used for IHC analysis. Data are summarized as median and interquartile range. For comparisons between groups, the two-tailed Mann-Whitney U test was used. A two-sided *p*-value of less than 0.05 was considered significant. For comparison of the three groups, Kruskal-Wallis test was used. For gene expression analysis, normalized mRNA expression values were evaluated with the R-based advanced nSolver™ Advanced Analysis Software (Version 2.0.115). Differential gene expression data are presented as volcano plots and in a summary table showing the top differentially regulated genes. In addition, the data was subjected to unsupervised hierarchical clustering analysis (HCA). The false discovery rate (FDR; Benjamini-Hochberg) method was used to adjust the *p*-values for multiple *t*-testing.

RESULTS

Patients

The clinical-pathologic characteristics of the patients are presented in Table 1. Of the 25 patients with rejection, 21 patients had an aTCMR (grade IB to III) and 4 patients had a mixed (aTCMR and active antibody mediated rejection [aABMR]) rejection. The timing of the biopsy and patient age at the time of rejection were not significantly different between the patients treated with belatacept, patients treated with tacrolimus and the negative controls ($p = 0.42$, and $p = 0.14$, respectively). The estimated glomerular filtration rate (eGFR) at the time of the acute rejection was similar in patients treated with either tacrolimus or belatacept (median 26; interquartile range [IQR] 15-35 mL/min per 1.73 m^2; and 28; IQR 18-41 mL/min per 1.73 m^2 ($p = 0.57$). The median age of the biopsy used for Nanostring® was 3.2 years (IQR 2.3-4.6).

Quality control of RNA and NanoString®

For the gene expression analysis, 7 samples of patients with tacrolimus maintenance therapy, 10 samples of patients with belatacept maintenance therapy, and 6 negative controls (samples without rejection) were analyzed. The mean A_{260}/A_{280} spectrophotometry ratio was 1.88 (standard deviation 0.17). Two samples (1 negative control and 1 sample of a belatacept-treated patient) did not pass the quality control of the nSolver™ Advanced Analysis Software because of low probe counts and were excluded from further analysis.

Of the 209 probes, 16 did not reach the detection threshold (less than double the counts of the median of the negative control) and were excluded from subsequent analysis (Table S2).

Validation of NanoString®

To validate NanoString®, it was tested whether NanoString® could distinguish between the transcriptome of samples with aTCMR and samples without aTCMR. Unsupervised HCA of the personalized gene panel showed that the samples with aTCMR clustered separately from the negative controls (Figure 1A). However, one sample with aTCMR (patient 18) clustered with the negative controls. The clinical data of this patient revealed that he had a slight deterioration of kidney function at the time of the biopsy (serum creatinine rose from 95 to 107 μmol/L). No rejection was diagnosed in the first examination of the biopsy. However, after revision in the setting of the RCT, the biopsy showed an isolated v-lesion and was therefore classified as an aTCMR grade IIA (Banff 2015 classification[23]). Following this for-cause biopsy, the kidney function of the patient improved to baseline without anti-rejection therapy and without adjusting his maintenance immunosuppressive therapy. At present, 45 months after transplantation, the kidney function of this patient is excellent (serum creatinine concentration 92 μmol/L).

Differential gene expression analysis identified a distinct gene signature in biopsies with aTCMR compared to the negative controls (Figure 1B and Table S4). Comparison of aTCMR and negative controls identified 78 genes with higher expression levels in the aTCMR samples [FDR *p*-value (FDRPV) < 0.05 to 1.42e-05; Table S4], and one gene with significantly higher expression in samples without aTCMR (EEF1A1, FDRPV 0.047). The most differentially expressed genes (DEGs) were T cell-associated transcripts (CD3, CD8, and CD4; $p < 1.98e-04$), γ-interferon-inducible genes (CCL5, CXCL9, CXCL11, CXCL10, TBX21; $p < 1.33e-04$), effector genes (GNLY, GZMB, ITGAX; $p < 2.82e-03$), macrophage-associated transcripts (SLAMF8, CD86, MS4A7, MRC1, ADAMDEC1; $p < 0.04$) and injury-repair response-associated transcripts (LCP2, CTSS, FCGR3A, MYBL1, LCN2 and HAVCR1; $p < 4.63e-02$). The top 15 DEGs were mainly aTCMR-associated transcripts, denoting an aTCMR profile (Table 2). A two-dimensional principal component analysis was performed with the top 15 DEGs and showed separate clustering of the samples with acute rejection compared to the negative controls without aTCMR (Figure 1C).

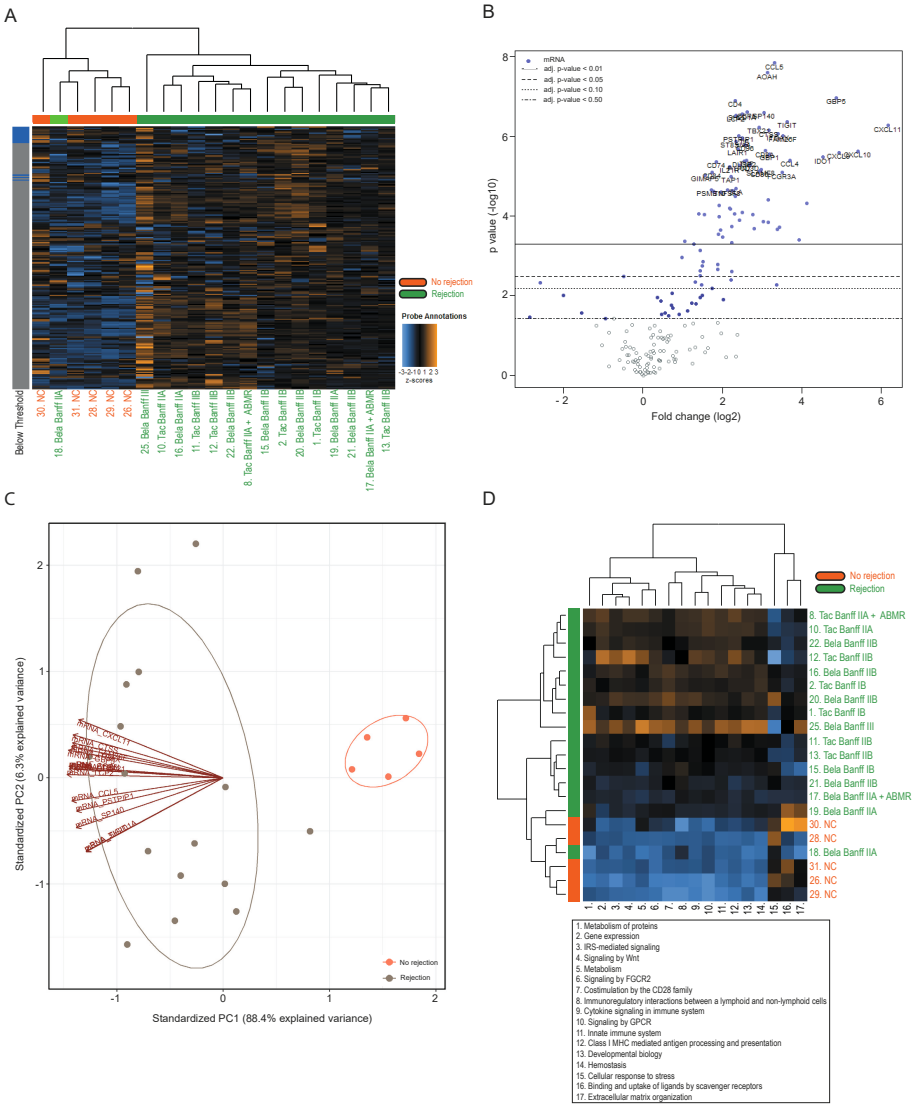

Figure 1. A. Heatmap and unsupervised hierarchical cluster analysis of the 209 normalized genes of samples with aTCMR and samples without aTCMR (negative controls). Each row represents a probe. Each column represents a biopsy sample. The orange samples are the negative controls. The dark green samples are the samples with acute rejection. The color in each cell reflects the level of expression of the mRNA, relative to the geometric mean of all the samples (z-score). Increasing intensities of orange point out higher expression, while increasing intensities of blue indicate lower expression. The degree of relatedness is represented by the dendrogram at the top of the panel. The probe threshold is depicted on the left of the heatmap. Blue cells represent probes that were below the detection threshold (less than double the counts of the median of the negative control). Grey cells represent probes that were above the detection threshold. **B**. Volcano plot of samples with aTCMR *versus* patients without aTCMR. The X-axis represents

fold change (log2). The Y-axis displays each gene's *p*-value (-log10). The horizontal lines indicate various False Discovery Rate *p*-values. The 40 most statistically significant genes are labeled in the plot. Genes with a positive fold change are higher expressed in the samples with an acute rejection. Genes with a negative fold change are higher expressed in the samples without acute rejection. **C**. Principal component analysis (PCA) plot of the top 15 differentially expressed genes in samples with aTCMR and negative controls. PCA samples on the 1st (X-axis) and 2nd PC plane (Y-axis). The samples without acute rejection are displayed in pink. The samples with acute rejection are displayed in grey. **D**. Pathway scores of samples with aTCMR and samples without aTCMR**.** Unsupervised hierarchical cluster analysis and heatmap showing pathway scores. Pathway scores are fit using the first principal component of each gene set's data. Scores are displayed on the same scale via a Z-transformation. Each row represents a sample with patient ID number. Each column represents a pathway. The orange samples are the negative controls. The dark green samples are the samples with rejection. Increasing intensities of orange point out higher pathway scores, while increasing intensities of blue indicate lower pathway scores. The degree of relatedness is represented by the dendrogram at the top of the panel. Each number of the column represents a different pathway: 1. Metabolism of proteins, 2. Gene expression, 3. Insulin receptor substrate signaling mediated signaling, 4. Signaling by Wnt, 5. Metabolism, 6. Signaling by fragment C gamma receptor 2 (FGCR2), 7. Co-stimulation by the CD28 family, 8. Immunoregulatory interactions between lymphoid and non-lymphoid cells, 9. Cytokine signaling in immune system, 10. Signaling by G-protein coupled receptor (GPCR), 11. Innate immune system, 12. MHC mediated Class I antigen processing and presentation, 13. Developmental biology, 14. Hemostasis, 15. Cellular response to stress, 16. Binding and uptake of ligands by scavenger receptors, 17. Extracellular matrix organization.

Table 2. Top 15 of differentially expressed genes in patients with an acute rejection compared with patients without an acute rejection.

mRNA	FC (log2)	SE (log2)	Lower confidence limit (log2)	Upper confidence limit (log2)	FDRPV[a]	Panel[b]
CCL5	3.32	0.354	2.63	4.02	1.42e-05	Rejection
AOAH	3.14	0.347	2.46	3.82	1.42e-05	TCMR
GBP5	4.87	0.592	3.71	6.03	3.59e-05	TCMR
CD4	2.33	0.287	1.77	2.9	3.59e-05	TCMR
CCR5	2.63	0.338	1.97	3.29	4.18e-05	Rejection
SP140	3.05	0.392	2.28	3.81	4.18e-05	TCMR
SH2D1A	2.5	0.322	1.86	3.13	4.18e-05	TCMR
LCP2	2.34	0.304	1.75	2.94	4.18e-05	TCMR
TIGIT	3.63	0.484	2.68	4.58	5.4e-05	TCMR / Exhaustion
CXCL11	6.17	0.835	4.53	7.81	6.01e-05	ABMR
TBX21	2.92	0.397	2.14	3.7	6.01e-05	ABMR / Exhaustion
CTSS	3.17	0.437	2.31	4.02	6.7e-05	AKI
ITGAX	3.41	0.478	2.47	4.35	7.35e-05	TOLs
FAM26F	3.53	0.498	2.55	4.51	7.35e-05	TCMR
PSTPIP1	2.41	0.341	1.75	3.08	7.35e-05	TCMR

Positive ratio means higher expression in samples with rejection.

[a]FDR p-value was obtained from the adjusted *p*-value of FDR correction by Benjamini-Hochberg method

[b]Panel in Banff kidney report 2017[5].

ABMR, antibody mediated rejection; AKI, acute kidney injury; FC, fold change; FDRPV, false discovery rate p-value; SE, standard error; TCMR, T cell-mediated rejection; TOLs, tolerance associated transcripts.

Next, pathway score profiles were compared between samples with aTCMR and negative controls. Seventeen pathways were analyzed (Figure 1D). Each pathway score was a combination of data from 6 to 23 genes (Table S5). Unsupervised HCA of the 17 pathways showed that the samples with aTCMR clustered separately from the samples without rejection (Figure 1D). Almost all pathway scores were higher in patients with aTCMR, for instance co-stimulation by the CD28 family, and cytokine signaling.

Immunomic comparison of aTCMR biopsies under belatacept- or tacrolimus therapy

Immunophenotypical analysis

Twenty-five biopsies were included in the IHC analysis: 11 biopsies of patients treated with belatacept-based maintenance therapy and 14 patients with tacrolimus-based maintenance therapy. The infiltrates in the cortical area of tacrolimus-treated patients with aTCMR mainly consisted of T cells, monocytes and macrophages (Table 3). Representative IHC stainings of the infiltrate in an aTCMR biopsy of a patient with belatacept maintenance therapy are shown in Figure S1. The composition of cells in the cortical area was not significantly different for markers of the adaptive immune response (CD3, CD4, CD8, CD20, FoxP3, PD-1 and granzyme B) and for markers of the innate immune response (CD56 and CD68) in both belatacept- and tacrolimus-treated patients (Table 3). Furthermore, no significant difference was seen in the composition of cells in the cortical area between both groups of patients when only aTCMR grade IIA and IIB were analyzed, or when the mixed AR were compared (data not shown).

Gene expression analysis

In an unsupervised HCA, using the personalized panel, the gene expression profiles of belatacept-maintenance therapy did not cluster separately from the profiles of tacrolimus maintenance therapy (Figure 2A). Differential gene expression analysis demonstrated no significant difference between the aTCMR samples of patients who received maintenance therapy with either belatacept or tacrolimus (Figure 2B and Table S6). The top 15 DEGs (although not statistically different) are summarized in Table 4. In a two-dimensional principal component analysis, no separate clustering was seen between the samples of patients treated with belatacept or tacrolimus maintenance therapy (Figure 2C).

Unsupervised HCA of the pathway scores is depicted in Figure 2D. None of the 17 different pathways distinguished between aTCMR occurring under belatacept or tacrolimus maintenance therapy. Surprisingly, the genes that are involved in the CD28 costimulatory

pathway were similarly expressed in acute rejection samples of patients treated with either tacrolimus or belatacept (Table 5).

Table 3. Immunohistochemistry analysis of CD3, CD4, CD8, FoxP3, CD20, CD56, CD68, PD-1, and granzyme B.

Marker	Treatment	Median[a]	IQR[a]	*p*-value
CD3	Tacrolimus	7.76	4.93-11.35	0.15
	Belatacept	4.18	3.63-8.28	
CD4	Tacrolimus	4.64	1.48-7.84	0.85
	Belatacept	4.86	2.84-6.91	
CD8	Tacrolimus	3.23	1.68-5.73	0.69
	Belatacept	1.96	1.54-4.25	
FoxP3	Tacrolimus	0.05	0.03-0.22	0.58
	Belatacept	0.05	0.02-0.12	
FoxP3/CD4	Tacrolimus	0.009	0.007-0.012	1.00
	Belatacept	0.009	0.006-0.019	
CD20	Tacrolimus	0.43	0.21-2.92	1.00
	Belatacept	1.05	0.29-5.88	
CD56	Tacrolimus	0.05	0.02-0.09	0.12
	Belatacept	0.15	0.05-0.39	
CD68	Tacrolimus	10.6	3.6-19.2	0.37
	Belatacept	5.6	4.2-10.2	
Granzyme B	Tacrolimus	0.21	0.05-0.47	0.81
	Belatacept	0.16	0.08-0.49	
PD-1	Tacrolimus	0.35	0.22-0.70	0.12
	Belatacept	0.05	0.02-0.88	

[a]Median (%) and interquartile range (%) of the ratio of positive stained cortex area divided by the total cortex area of CD3, CD4, CD8, CD20, CD56, CD68, granzyme B and PD-1. The ratio of FoxP3/CD3 is calculated by diving the percentage of FoxP3 staining by the percentage of CD4 staining for each section. IQR, interquartile range; PD-1, programmed cell death protein 1.

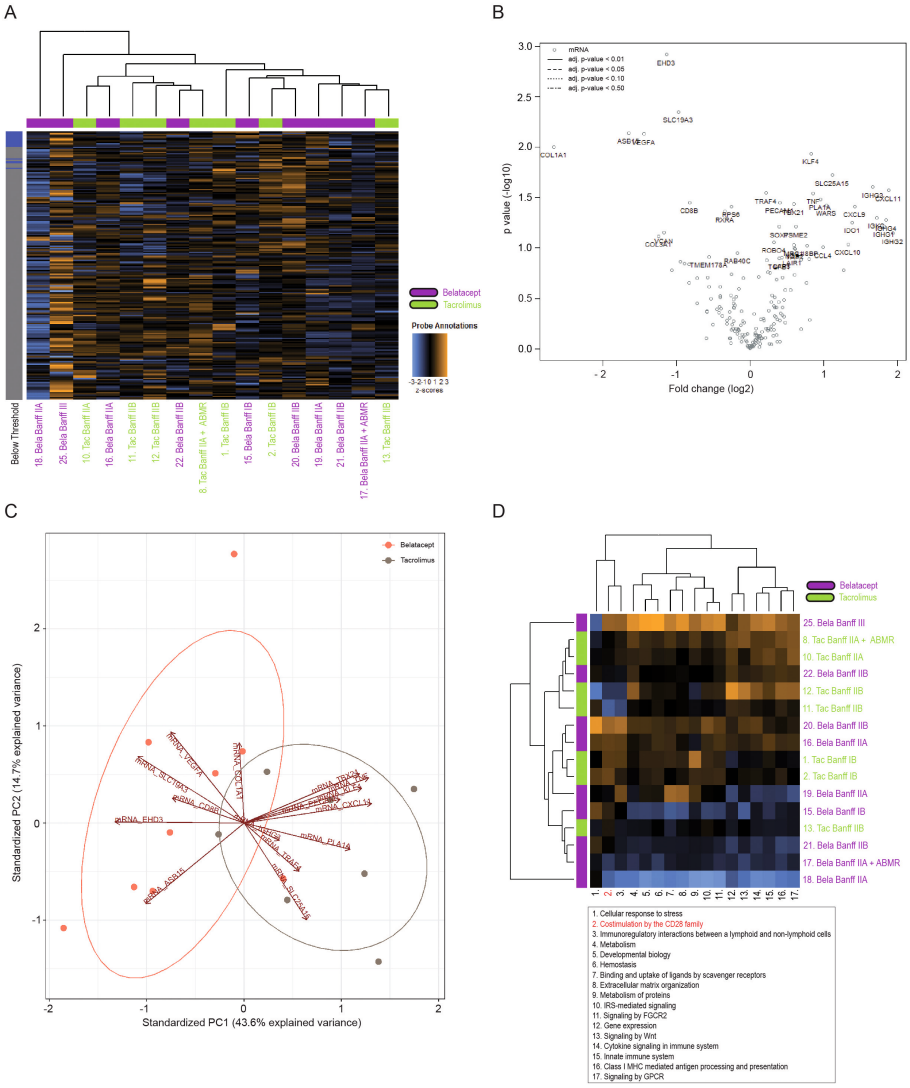

Figure 2. A. Heatmap and unsupervised hierarchical cluster analysis of the 209 normalized genes in samples of patients with aTCMR treated with belatacept or tacrolimus. Each row represents a probe. Each column represents a biopsy sample. The purple samples are the samples from belatacept-treated patients. The light green samples are the samples from tacrolimus-treated patients. The color in each cell reflects the level of expression of the mRNA, relative to the geometric mean of all the samples (z-score). Increasing intensities of orange point out higher expression, while increasing intensities of blue indicate lower expression. The degree of relatedness is represented by the dendrogram at the top of the panel. The probe threshold is depicted on the left of the heatmap. Blue cells represent probes that were below the detection threshold (less than double the counts of the median of the negative control). Grey cells represent probes that were above the detection threshold. **B**. Volcano plot of samples of patients with aTCMR treated with belatacept *versus* patients treated with tacrolimus. The X-axis represents fold change (log2). The Y-axis displays each gene's *p*-value (-log10). None of the genes was significant different between the two groups,

therefore no horizontal lines that indicate various False Discovery Rate *p*-values are visible. The 40 most statistically significant genes are labeled in the plot. Genes with a positive fold change are higher expressed in the samples of patients treated with tacrolimus. Genes with a negative fold change are higher expressed in the samples of patients treated with belatacept. **C**. Principal component analysis (PCA) plot of the top 15 differentially expressed genes in samples of patients with aTCMR treated with belatacept or tacrolimus. PCA samples on the 1st (X-axis) and 2nd PC plane (Y-axis). The samples of patients treated with belatacept are displayed in pink. The samples of patients treated with tacrolimus are displayed in grey. **D**. Pathway scores of samples of patients with aTCMR treated with belatacept or tacrolimus**.** Unsupervised hierarchical cluster analysis and heatmap showing pathway scores. Pathway scores are fit using the first principal component of each gene set's data. Scores are displayed on the same scale via a Z-transformation. Each row represents a sample. Each column represents a pathway. The purple samples are the belatacept-treated patients. The light green samples are the samples of patients treated with tacrolimus. Increasing intensities of orange point out higher pathway scores, while increasing intensities of blue indicate lower pathway scores. The degree of relatedness is represented by the dendrogram at the top of the panel. Each number of the column represents a different pathway: 1. Cellular response to stress, 2. Co-stimulation by the CD28 family, 3. Immunoregulatory interactions between lymphoid and non-lymphoid cells, 4. Metabolism, 5. Developmental biology, 6. Hemostasis, 7. Binding and uptake of ligands by scavenger receptors, 8. Extracellular matrix organization, 9. Metabolism of proteins, 10. Insulin receptor substrate signaling mediated signaling, 11. Signaling by fragment C gamma receptor 2 (FGCR2), 12. Gene expression, 13. Signaling by Wnt, 14. Cytokine signaling in immune system, 15. Innate immune system, 16. Class I MHC mediated antigen processing and presentation, 17. Signaling by G-protein coupled receptor (GPCR).

Table 4. Top 15 of differentially expressed genes in patients with acute rejection treated with belatacept compared with patients treated with tacrolimus.

mRNA	FC (log2)	SE (log2)	Lower confidence limit (log2)	Upper confidence limit (log2)	*p*-value	FDRPV[a]	Panel[b]
EHD3	-1.14	0.28	-1.70	-0.59	0.0012	1.00	Glomerulus
SLC19A3	-0.98	0.29	-1.54	-0.41	0.0045	1.00	eGFR later
ASB15	-1.65	0.53	-2.68	-0.62	0.0073	1.00	GOCAR
VEGFA	-1.44	0.46	-2.35	-0.54	0.0074	1.00	Macrophages
COL1A1	-2.67	0.90	-4.43	-0.91	0.0099	1.00	CADI progression /matrix
KLF4	0.83	0.29	0.27	1.39	0.0116	1.00	ABMR
SLC25A15	1.11	0.42	0.29	1.93	0.0189	1.00	eGFR later
IGHG3	1.66	0.66	0.36	2.95	0.0249	1.00	Plasma cells
CXCL11	1.88	0.76	0.39	3.37	0.0269	1.00	ABMR
TRAF4	0.21	0.09	0.04	0.38	0.0285	1.00	eGFR later
TNF	0.85	0.35	0.17	1.54	0.0288	1.00	ABMR
PLA1A	0.95	0.40	0.16	1.74	0.0328	1.00	ABMR
CD8B	-0.83	0.36	-1.52	-0.13	0.0355	1.00	TCMR
PECAM1	0.40	0.17	0.06	0.74	0.0356	1.00	ABMR
TBX21	0.59	0.26	0.09	1.09	0.0369	1.00	ABMR / Exhaustion

[a]FDR p-value was obtained from the adjusted p value of FDR correction by Benjamini-Hochberg method

[b]Panel in Banff kidney report 2017[5].

ABMR, antibody mediated rejection; CADI, Chronic allograft damage index; FC, fold change; FDRPV, false discovery rate p-value; GOCAR, Genomics of Chronic Allograft Rejection; SE, standard error; TCMR, T cell-mediated rejection.

Table 5. Gene expression ratios of genes involved in the CD28 pathway between patients with an acute rejection treated with belatacept and patients treated with tacrolimus.

mRNA	FC (log2)	SE (log2)	Lower confidence limit (log2)	Upper confidence limit (log2)	*p*-value	FDRPV[a]
BTLA	-0.15	0.52	-1.16	0.87	0.77	1.00
CD274	0.47	0.41	-0.32	1.27	0.24	1.00
CD28	-0.42	0.46	-1.32	0.49	0.37	1.00
CD3D	-0.41	0.38	-1.16	0.35	0.29	1.00
CD4	0.25	0.30	-0.33	0.83	0.40	1.00
CD86	0.05	0.49	-0.91	1.02	0.92	1.00
CTLA4	-0.59	0.73	-2.01	0.84	0.42	1.00
ICOS	-0.17	0.45	-1.05	0.71	0.71	1.00
PDCD1	-0.42	0.53	-1.46	0.62	0.43	1.00
PDCD1LG2	0.03	0.41	-0.77	0.83	0.94	1.00

[a]FDR p-value was obtained from the adjusted p value of FDR correction by Benjamini-Hochberg method.
FC, fold change; FDRPV, false discovery rate p-value; SE, standard error.

Distinct pretransplant subsets of T cells have been described that may be responsible for triggering belatacept-resistant rejections, namely $CD8^+$ $CD28^-$T cells, $CD4^+CD57^+$programmed death 1 (PD-1)$^-$ T cells, and $CD8^+CD28^+T_{EMRA}$[28-31]. However, in our RCT, which included the belatacept-treated patients described here, these three subsets did not predict acute rejection pretransplantation, at least when measured in peripheral blood[20]. In addition, during acute rejection, the three subsets in the blood were not significantly different compared with belatacept-treated patients without acute rejection[20]. In the present study, the intra-graft mRNA concentrations of CD4, CD8, CD28, PD-1, and B3GAT1 (alias CD57) were determined and compared between belatacept- and tacrolimus treated patients with aTCMR Table S6). No difference in the expression of these markers was observed between the two groups.

DISCUSSION

The integration of immunomics with the conventional histomorphologic examination of renal biopsies will lead to improved classification and a deeper understanding of the pathogenesis of acute rejection[5]. This pilot study shows that with the innovative technique NanoString® it is feasible to derive gene expression data from FFPE kidney transplant biopsies and that it was possible to differentiate biopsies with and without aTCMR. These results were used to support our conclusion that the aTCMR immunomic profiles of patients treated with either tacrolimus or belatacept maintenance therapy were not significantly different.

The Banff 2013 working group recommends the use of molecular diagnostics to define ABMR[32]. This includes increased expression analysis of transcripts involved in endothelial injury[32]. The Banff 2017 classification includes more diagnostic and prognostic molecular biomarkers for ABMR[5]. However, the Banff 2017 classification does not contain recommendations on the implementation of molecular diagnostics for the diagnosis of aTCMR. This may be useful in differential diagnostic dilemmas, such as borderline rejection or isolated v-lesions.

NanoString® is a high-throughput gene expression quantification system that delivers direct multiplexed measurements of gene expression through digital readouts of mRNA transcripts. Formalin-fixation can cause cross-linkage of nucleic acids to proteins, which can lead to inhibition of reverse transcriptase. The advantage of the NanoString® over other high-throughput techniques like real time polymerase chain reaction and microarray, is that it does not require a reverse transcriptase step[9,33]. NanoString® is suitable for clinical purposes because it is fast and has minimal hands-on time. Furthermore, the gene expression analysis can be performed in the same formalin fixed paraffin tissue that is used for conventional histopathologic examination. It has been accepted into international treatment guidelines as a prognostic assay for breast cancer[34].

Here, the allograft transcriptome of aTCMR biopsies showed a significantly higher expression of 78 genes compared to the biopsies without aTCMR. The top pathogenesis-based transcripts were mostly T cell-associated, γ-interferon inducible and effector cell, and injury-repair response-associated transcripts denoting an aTCMR profile[35].

This is the first study that compared the immunomics of biopsies with aTCMR of patients treated with either tacrolimus or belatacept. A better understanding of the pathogenesis of costimulation blockade resistant rejections could lead to a more personalized approach of belatacept-based treatment in kidney transplant recipients. Besides, since molecular diagnostics of rejection biopsies are increasingly used in combination with conventional histomorphologic examination[5], it is important to know whether the gene signature of rejection biopsies is dependent of the maintenance immunosuppressive therapy.

In this pilot study with a small sample size, the transcriptome of patients treated with either one of the two immunosuppressive regimens showed no distinct gene signature, including the genes involved in the CD28 costimulatory pathway. In addition, immunophenotypical analysis of the classic immune markers of the innate and adaptive immune system was not

significantly different between the two maintenance therapies. Furthermore, we could not confirm that the previously described T cell subsets ($CD8^+CD28^-$ T cells, $CD4^+CD57^+PD\text{-}1^-$ T cells, and $CD8^+CD28^+T_{EMRA}$[21,28,29]) were associated with belatacept-resistant rejections, neither in the peripheral blood[20] nor in the renal allograft (in this study).

One study analyzed biopsies with aTCMR of patients treated with belatacept or cyclosporine A (CsA) and compared the ratio of $FoxP3^+$ cells among T cells with IHC[36]. This ratio was significantly elevated in acute rejection biopsies of belatacept-treated patients compared to CsA-treated patients (17.99% *versus* 6.45%, respectively, $p = 0.044$)[36]. Here, no difference was found in the ratio of $FoxP3^+$ cells among $CD4^+$ T cells between aTCMR biopsies of belatacept- and tacrolimus-treated patients. Besides, the intra-graft mRNA level of $FoxP3^+$ was similar between the two groups.

No studies have compared the immunomics of biopsies without aTCMR of patients treated with belatacept or tacrolimus. However, several studies compared intra-graft gene expression and IHC of biopsies without rejection of patients treated with belatacept or cyclosporine. Two studies compared the intragraft gene expression of 12-month protocol biopsies without rejection of patients treated with belatacept or CsA[37,38]. Grimbert *et al*. found that the expression of FoxP3 was less in biopsies of patients treated with belatacept compared with CsA. No differences were found in granzyme B expression or the intra-graft expression of genes associated with Th1 (IFNγ, Tbet), Th2 (GATA3) and Th17 (RORγt, IL-17) cells[37]. Vitalone *et al*. compared the intra-graft gene expression of 4451 genes of preimplantation biopsies with 12-month protocol biopsies of patients treated with belatacept or CsA[38]. The biopsies of CsA-treated patients showed higher expression of genes associated with fibrosis, early tubulointerstitial damage and CsA-related toxicity. The biopsies of patients treated with belatacept showed enrichment of genes associated with NK cells and monocytes, progressive immune injury and wound healing[38]. Furuzawa *et al*. analyzed the 1-year protocol biopsies (without rejection) with IHC under belatacept- or CsA-maintenance treatment and observed that biopsies of belatacept-treated patients showed less senescence and a more immunomodulatory phenotype[39].

The explanation for the absence of a difference in the immunomics of aTCMR biopsies of patients treated with tacrolimus or belatacept could be that the aTCMR as seen in biopsies is a shared final common pathway. This phenomenon was previously named the "immunologic constant of rejection"[40,41]. This hypothesis is based on the observation that different immune-mediated tissue destruction processes share the same final molecular

mechanism, like allograft rejection, cancer, autoimmunity and infections and includes activation of γ-interferon-regulated genes, recruitment of cytotoxic cells by chemokine ligands and activation of immune effector function genes[40,41].

We recognize the limitations associated with this pilot study, most notably the small number of patients. This could have influenced the power of this study. However, to date, our RCT is the largest to have compared belatacept to tacrolimus maintenance treatment. Furthermore, we feel that studies on the immunomics of belatacept-resistant rejection with more statistical power are unlikely to become available anytime soon since the treatment of new patients with belatacept is currently very difficult because of a worldwide shortage of the drug[42]. Because of the limited sample size, no correlation between the IHC stainings, Banff grade and gene transcripts could be investigated. Furthermore, the scope of this study was to analyze the immunomics of tacrolimus- and belatacept-treated patients with acute rejections and not to study the gene expression profiles of different types of rejection. Therefore, we studied aTCMR only because ABMR did not occur in our belatacept RCT which compared belatacept and tacrolimus.

Additionally, we have used the NanoString® technique to measure the expression of a limited number of genes instead of using an untargeted approach, thereby excluding other possible differentiating biomarkers. The use of an untargeted genomic approach on the AR biopsies could possibly identify new genes and pathways that we did not analyze with the gene panel used in our study. However, we believe that most genes involved in aTCMR were included as they were derived from the panel presented in the Banff 2017 report[5]. Lastly, more biopsies of the tacrolimus-treated patients used for Nanostring® were taken earlier after transplantation than the biopsies of the belatacept-treated patients and the negative controls. This can be relevant because genes involved in the injury-repair response and inflammation could be affected by the transplant surgery. However, the timing of the acute rejection was not different between the three groups.

In summary, no differences were found in the immunomic profiles of aTCMR biopsies of patients treated with tacrolimus- or belatacept-based maintenance therapy, suggesting that clinically diagnosed rejection is a final common pathway of allo-recognition which is independent of the specific immunosuppressive regimen (tacrolimus or belatacept) under which it occurs. Follow-up studies with larger patient numbers are required to confirm our findings when belatacept is widely available again[42].

REFERENCES

1. Loupy A, Lefaucheur C, Vernerey D, et al. Molecular microscope strategy to improve risk stratification in early antibody-mediated kidney allograft rejection. *J Am Soc Nephrol.* 2014;25(10):2267-2277.
2. Adam B, Afzali B, Dominy KM, et al. Multiplexed color-coded probe-based gene expression assessment for clinical molecular diagnostics in formalin-fixed paraffin-embedded human renal allograft tissue. *Clin Transplant.* 2016;30(3):295-305.
3. Adam B, Mengel M. Molecular nephropathology: ready for prime time? *Am J Physiol Renal Physiol.* 2015;309(3):F185-188.
4. Venner JM, Famulski KS, Badr D, et al. Molecular landscape of T cell-mediated rejection in human kidney transplants: prominence of CTLA4 and PD ligands. *Am J Transplant.* 2014;14(11):2565-2576.
5. Haas M, Loupy A, Lefaucheur C, et al. The Banff 2017 Kidney Meeting Report: Revised diagnostic criteria for chronic active T cell-mediated rejection, antibody-mediated rejection, and prospects for integrative endpoints for next-generation clinical trials. *Am J Transplant.* 2018;18(2):293-307.
6. Schinstock CA, Sapir-Pichhadze R, Naesens M, et al. Banff survey on antibody-mediated rejection clinical practices in kidney transplantation: Diagnostic misinterpretation has potential therapeutic implications. *Am J Transplant.* 2018.
7. Zhang P, Lehmann BD, Shyr Y, Guo Y. The Utilization of Formalin Fixed-Paraffin-Embedded Specimens in High Throughput Genomic Studies. *Int J Genomics.* 2017;2017:1926304.
8. Geiss GK, Bumgarner RE, Birditt B, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat Biotechnol.* 2008;26(3):317-325.
9. Sigdel TK, Nguyen M, Dobi D, et al. Targeted Transcriptional Profiling of Kidney Transplant Biopsies. *Kidney Int Rep.* 2018;3(3):722-731.
10. Nankivell BJ, Borrows RJ, Fung CL, et al. The natural history of chronic allograft nephropathy. *N Engl J Med.* 2003;349(24):2326-2333.
11. Nankivell BJ, P'Ng CH, O'Connell PJ, Chapman JR. Calcineurin Inhibitor Nephrotoxicity Through the Lens of Longitudinal Histology: Comparison of Cyclosporine and Tacrolimus Eras. *Transplantation.* 2016;100(8):1723-1731.
12. Larsen CP, Pearson TC, Adams AB, et al. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. *Am J Transplant.* 2005;5(3):443-453.
13. Archdeacon P, Dixon C, Belen O, Albrecht R, Meyer J. Summary of the US FDA approval of belatacept. *Am J Transplant.* 2012;12(3):554-562.
14. de Graav GN, Bergan S, Baan CC, et al. Therapeutic Drug Monitoring of Belatacept in Kidney Transplantation. *Ther Drug Monit.* 2015;37(5):560-567.
15. Vincenti F, Charpentier B, Vanrenterghem Y, et al. A phase III study of belatacept-based immunosuppression regimens versus cyclosporine in renal transplant recipients (BENEFIT study). *Am J Transplant.* 2010;10(3):535-546.

16. Durrbach A, Pestana JM, Florman S, et al. Long-Term Outcomes in Belatacept- Versus Cyclosporine-Treated Recipients of Extended Criteria Donor Kidneys: Final Results From BENEFIT-EXT, a Phase III Randomized Study. *Am J Transplant.* 2016;16(11):3192-3201.

17. Durrbach A, Pestana JM, Pearson T, et al. A phase III study of belatacept versus cyclosporine in kidney transplants from extended criteria donors (BENEFIT-EXT study). *Am J Transplant.* 2010;10(3):547-557.

18. Vincenti F, Rostaing L, Grinyo J, et al. Belatacept and Long-Term Outcomes in Kidney Transplantation. *N Engl J Med.* 2016;374(4):333-343.

19. Vincenti F, Larsen CP, Alberu J, et al. Three-year outcomes from BENEFIT, a randomized, active-controlled, parallel-group study in adult kidney transplant recipients. *Am J Transplant.* 2012;12(1):210-217.

20. de Graav GN, Baan CC, Clahsen-van Groningen MC, et al. A Randomized Controlled Clinical Trial Comparing Belatacept With Tacrolimus After De Novo Kidney Transplantation. *Transplantation.* 2017;101(10):2571-2581.

21. de Graav GN, Hesselink DA, Dieterich M, et al. An Acute Cellular Rejection With Detrimental Outcome Occurring Under Belatacept-Based Immunosuppressive Therapy: An Immunological Analysis. *Transplantation.* 2016;100(5):1111-1119.

22. Shuker N, Bouamar R, van Schaik RH, et al. A Randomized Controlled Trial Comparing the Efficacy of Cyp3a5 Genotype-Based With Body-Weight-Based Tacrolimus Dosing After Living Donor Kidney Transplantation. *Am J Transplant.* 2016;16(7):2085-2096.

23. Loupy A, Haas M, Solez K, et al. The Banff 2015 Kidney Meeting Report: Current Challenges in Rejection Classification and Prospects for Adopting Molecular Pathology. *Am J Transplant.* 2017;17(1):28-41.

24. Smith RN, Matsunami M, Adam BA, et al. RNA expression profiling of nonhuman primate renal allograft rejection identifies tolerance. *Am J Transplant.* 2018;18(6):1328-1339.

25. Smith RN, Adam BA, Rosales IA, et al. RNA expression profiling of renal allografts in a nonhuman primate identifies variation in NK and endothelial gene expression. *Am J Transplant.* 2018;18(6):1340-1350.

26. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 2002;3(7):RESEARCH0034.

27. Eikmans M, Gielis EM, Ledeganck KJ, et al. Non-invasive Biomarkers of Acute Rejection in Kidney Transplantation: Novel Targets and Strategies. *Front Med (Lausanne).* 2018;5:358.

28. Espinosa J, Herr F, Tharp G, et al. CD57(+) CD4 T Cells Underlie Belatacept-Resistant Allograft Rejection. *Am J Transplant.* 2016;16(4):1102-1112.

29. Mathews DV, Wakwe WC, Kim SC, et al. Belatacept-Resistant Rejection Is Associated With CD28+ Memory CD8 T Cells. *Am J Transplant.* 2017;17(9):2285-2299.

30. Lo DJ, Weaver TA, Stempora L, et al. Selective targeting of human alloresponsive CD8+ effector memory T cells based on CD2 expression. *Am J Transplant.* 2011;11(1):22-33.

31. Xu H, Perez SD, Cheeseman J, Mehta AK, Kirk AD. The allo- and viral-specific immunosuppressive effect of belatacept, but not tacrolimus, attenuates with progressive T cell maturation. *Am J Transplant.* 2014;14(2):319-332.

32. Haas M, Sis B, Racusen LC, et al. Banff 2013 meeting report: inclusion of c4d-negative antibody-mediated rejection and antibody-associated arterial lesions. *Am J Transplant.* 2014;14(2):272-283.

33. Bustin S, Dhillon HS, Kirvell S, et al. Variability of the reverse transcription step: practical implications. *Clin Chem.* 2015;61(1):202-212.

34. Parsons RF, Zahid A, Bumb S, et al. The impact of belatacept on third party HLA alloantibodies in highly sensitized kidney transplant recipients. *Am J Transplant.* 2019.

35. Halloran PF, Famulski K, Reeve J. The molecular phenotypes of rejection in kidney transplant biopsies. *Curr Opin Organ Transplant.* 2015;20(3):359-367.

36. Bluestone JA, Liu W, Yabu JM, et al. The effect of costimulatory and interleukin 2 receptor blockade on regulatory T cells in renal transplantation. *Am J Transplant.* 2008;8(10):2086-2096.

37. Grimbert P, Audard V, Diet C, et al. T-cell phenotype in protocol renal biopsy from transplant recipients treated with belatacept-mediated co-stimulatory blockade. *Nephrol Dial Transplant.* 2011;26(3):1087-1093.

38. Vitalone MJ, Ganguly B, Hsieh S, et al. Transcriptional profiling of belatacept and calcineurin inhibitor therapy in renal allograft recipients. *Am J Transplant.* 2014;14(8):1912-1921.

39. Furuzawa-Carballeda J, Lima G, Alberu J, et al. Infiltrating cellular pattern in kidney graft biopsies translates into forkhead box protein 3 up-regulation and p16INK4alpha senescence protein down-regulation in patients treated with belatacept compared to cyclosporin A. *Clin Exp Immunol.* 2012;167(2):330-337.

40. Wang E, Worschech A, Marincola FM. The immunologic constant of rejection. *Trends Immunol.* 2008;29(6):256-262.

41. Spivey TL, Uccellini L, Ascierto ML, et al. Gene expression profiling in acute allograft rejection: challenging the immunologic constant of rejection hypothesis. *J Transl Med.* 2011;9:174.

42. Gabardi S, van Gelder T. Causes and Consequences of the Worldwide Belatacept Shortage. *Transplantation.* 2017;101(7):1520-1521.

SUPPLEMENTALS

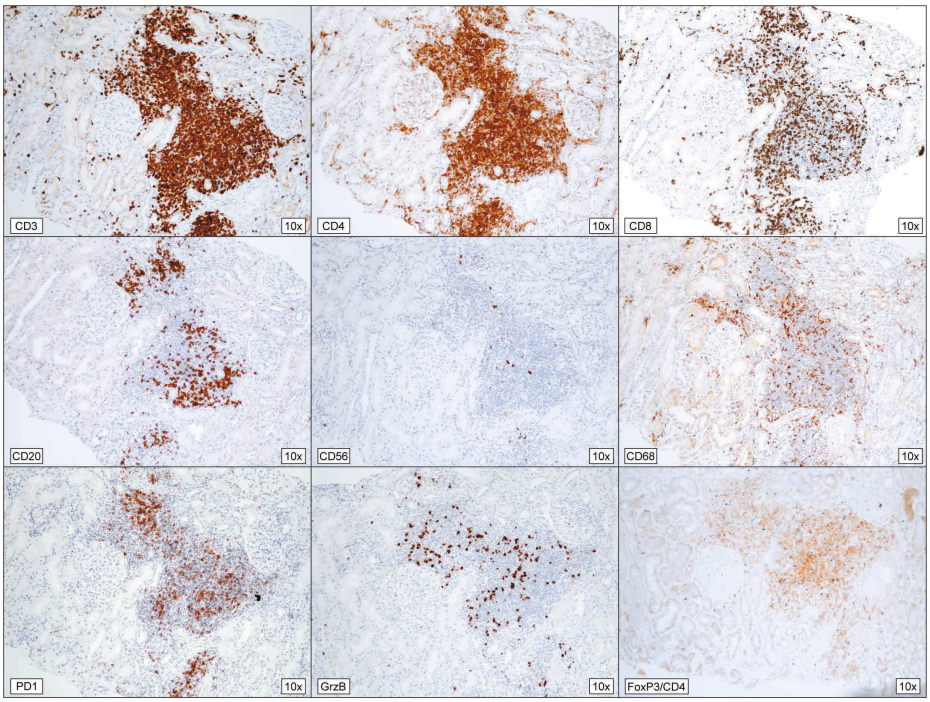

Figure S1. Representative immunohistochemical staining of an infiltrate in the kidney allograft biopsy of a belatacept-treated patient with an acute rejection. Stainings of CD3, CD4, CD8, CD20, CD56, CD68, PD1, granzyme B (GrzB) and FoxP3/CD4 were assessed on subsequent slides. Magnification of all slides is 10x.

Tables:

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CHAPTER 6

Targeted proteomic analysis detects acute T cell-mediated kidney allograft rejection in belatacept-treated patients

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Therapeutic Drug Monitoring 2019;41(2):243-8

ABSTRACT

Background

There is an unmet need for reliable minimally invasive diagnostic biomarkers for immunological allograft monitoring and for the detection of acute kidney transplant rejection. Here, targeted proteomic analysis was applied to compare 92 proteins in sera of belatacept-treated patients who had biopsy-proven, acute T-cell-mediated rejection (aTCMR) with patients without aTCMR.

Methods

Proximity extension immunoassay was used to measure 92 inflammation-related protein concentrations in the pre-rejection and rejection sera of 11 patients with aTCMR and 9 patients without aTCMR. This assay uses two matched oligonucleotide-labeled antibody probes for each protein and polymerase chain reaction to measure normalized protein expression values.

Results

Five proteins (CD5, CD8A, NCR1, TNFRSF4 and TNFRSF9) were expressed significantly higher in samples with aTCMR compared with samples without aTCMR (adjusted *p*-value<0.014) and had a good predictive capacity for aTCMR (area under the curve in a receiver operator curve ranged from 0.83 to 0.91 [*p*<0.014]). These proteins are associated with $CD8^+$ cytotoxic T cell and NK cell functions. Nonhierarchical clustering analysis showed distinct clustering of samples with aTCMR and samples without aTCMR. This clustering was not found in pre-rejection samples (one month after transplantation). In pre-rejection samples, IFN-γ was expressed at a significantly lower level (normalized protein expression value median -0.15, interquartile range: -0.27 - 0.04) than in samples of patients without rejection (median 0.13, interquartile range: -0.07 - 0.15, adjusted *p*-value=0.00367).

Conclusions

Targeted proteomic analysis with proximity extension immunoassay is a promising minimally invasive technique to diagnose aTCMR in kidney transplant recipients.

INTRODUCTION

Histopathological examination of a kidney allograft biopsy is the gold standard for diagnosing acute rejection (AR)[1]. However, sampling errors, limited reproducibility, and differential diagnostic dilemma's remain a problem when examining a renal biopsy[2]. Furthermore, a percutaneous needle biopsy of the kidney is a costly and invasive procedure with considerable risk for complications, most notably bleeding[3]. A biopsy is not always possible in patients with bleeding diathesis, or uncontrolled hypertension. In addition, obtaining a biopsy from children is challenging. Therefore, there is an unmet need for reliable and minimally invasive biomarkers for the diagnosis of kidney transplant rejection[4]. Such a biomarker should have a short turnaround time, be cost-effective, and must have a high positive and negative predictive value.

Proteomic screening of blood or urine of kidney transplant recipients may be an alternative mean to diagnose AR without the need for a biopsy. Rejection may be identified before the onset of clinical symptoms or the occurrence of histomorphological damage[5-8]. AR is a heterogeneous time-dependent process that requires the activation of the immune system; it causes injury to the kidney and leads to scarring during the healing process. It is unlikely that a single biomarker can capture the multitude of these biological events. The use of a panel of proteins, also called targeted proteomics, may be more suitable[9]. For AR, a targeted proteomic analysis should include markers of the immune response, acute kidney injury, tissue regeneration and fibrosis.

Since 2011, belatacept (a costimulatory blocking drug) is registered for the prevention of AR in kidney transplant patients as part of a calcineurin inhibitor (CNI)-free immunosuppressive regimen[10]. CNI-free immunosuppressive regimen with belatacept results in a comparable patient- and kidney allograft survival as achieved with CNI therapy, but with a superior metabolic profile[11-16]. However, belatacept-treated patients have more acute T cell-mediated rejections (aTCMR) compared with CNI-treated patients[17,18].

We performed a proteomic screening of 92 inflammation-related proteins in serum of belatacept-treated kidney transplant recipients with and without aTCMR with the multiplex Proximity Extension Immunoassay (PEA; Olink Bioscience, Uppsala, Sweden)[19]. This assay is widely used in several disciplines of medicine, including cardiovascular and inflammatory diseases. PEA has also been studied in the field of nephrology. In a cohort of 1047 senior adults, using a panel of 92 proteins with PEA, 20 proteins were independently associated

with the estimated glomerular filtration rate (eGFR) decline per year. These proteins are involved in phosphate homeostasis, inflammation, angiogenesis, apoptosis, extracellular matrix remodeling and endothelial dysfunction[20].

The objectives of this pilot study were: 1) to compare the proteomic profiles with PEA between belatacept-treated patients with and without a biopsy-proven aTCMR, and 2) to screen pre-rejection serum samples of belatacept-treated patients for proteins that can potentially predict AR.

MATERIALS AND METHODS

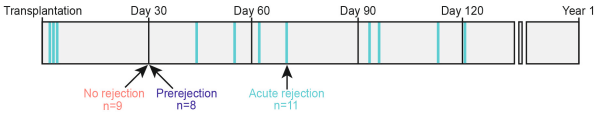
Study population and materials

Serum samples were collected from kidney transplant recipients who participated in a prospective randomized controlled trial at Erasmus University Medical Center, Rotterdam, the Netherlands[17]. In this trial, kidney transplant recipients were randomized to receive belatacept- and tacrolimus-based immunosuppressive regimens. Patient characteristics, inclusion criteria and the immunosuppressive regimens have been described before[17]. In brief, all patients received induction therapy with basiliximab (Simulect®, Novartis Pharma, Basel, Switzerland), followed by maintenance therapy consisting of mycophenolate mofetil (MMF, Cellcept®, Roche Pharmaceuticals, Basel, Switzerland), glucocorticoids and either belatacept (Nulojix®, Bristol Myers-Squibb, New York City, NY) or tacrolimus (Prograft®, Astellas Pharma, Leiden, the Netherlands)[17].

As reported by de Graav *et al.*, 11 of the 20 belatacept-treated patients experienced AR during the 1-year study period: 10 patients had aTCMR and 1 patient had a mixed rejection (aTCMR and acute/active antibody mediated rejection)[17,21]. All AR occurred within the first 4 months after transplantation, three even occurred in the first week after transplantation (Figure 1A)[17]. The median time elapsed between transplantation and AR was 56 days (IQR 5-94).

The serum samples were centrifuged (3000 rpm, 10 minutes) within 4 hours after blood collection and the supernatant was stored at -80°C. The samples of two time points (day 30 after transplantation, and the day of kidney biopsy, Figure 1A) were used for this study. All biopsies with AR were scored independently by two pathologists according to the Banff 2015 classification[22].

A



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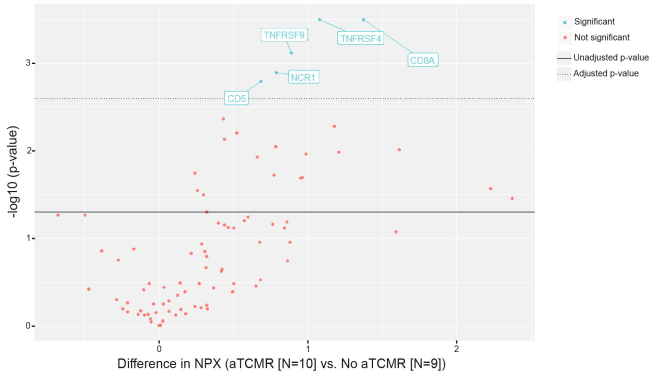

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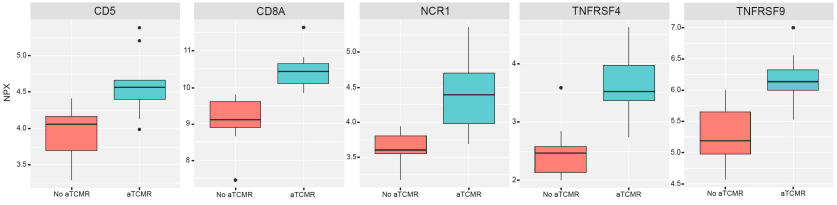

D

Protein	Description	Cell type	Pathway
CD5	T cell surface glycoprotein CD5	B-1 cells and T cells	B cell development T cell development and activation
CD8A	T cell surface glycoprotein CD8 α chain	Cytotoxic T cells	Immunoregulatory interactions between lymphoid and a non-lymphoid cell T cell receptor signaling pathway
NCR1 (CD355)	Natural cytotoxicity triggering receptor 1	Activated natural killer cells	Immunoregulatory interactions between lymphoid and a non-lymphoid cell
TNFRSF4 (OX40 receptor)	TNF receptor superfamily member 4	T cells	Costimulatory molecule involved in NF-κβ pathway TNFs bind their physiological receptors Cytokine signaling
TNFRSF9 (CD137)	TNF receptor superfamily member 9	T cells	Costimulatory molecule involved in NF-κβ pathway TNFs bind their physiological receptors Cytokine signaling

E

Protein	AUC (95%-CI)	SE	p-value	NPX Cutoff	Sensitivity	Specificity	PPV	NPV
CD5	0.83 (0.65-1.00)	0.094	0.014	4.28	0.78	0.80	0.81	0.77
CD8A	0.90 (0.71-1.00)	0.095	0.003	9.81	1.00	0.90	0.92	1.00
NCR1	0.91 (0.78-1.00)	0.065	0.003	3.96	0.78	0.90	0.89	0.79
TNFRSF4	0.84 (0.65-1.00)	0.098	0.011	2.85	0.89	0.80	0.83	0.87
TNFRSF9	0.84 (0.66-1.00)	0.093	0.011	5.90	0.78	0.80	0.81	0.77

F

		CD5	CD8A	NCR1	TNFRSF4	TNFRSF9
CD5	Correlation coefficient	1.0	0.76	0.78	0.86	0.92
	p-value		<0.001	<0.001	<0.001	<0.001
CD8A	Correlation coefficient	0.76	1.0	0.63	0.79	0.79
	p-value	<0.001		0.005	<0.001	<0.001
NCR1	Correlation coefficient	0.78	0.63	1.0	0.68	0.73
	p-value	<0.001	0.005		0.002	0.001
TNFRSF4	Correlation coefficient	0.86	0.79	0.68	1.0	0.88
	p-value	<0.001	<0.001	0.002		<0.001
TNFRSF9	Correlation coefficient	0.92	0.79	0.73	0.88	1.0
	p-value	<0.001	<0.001	0.001	<0.001	

Figure 1. A. Timeline of included serum samples. The green bars represent the patients with an acute rejection (n=11). The pink samples represent patients without acute rejection throughout the total follow-up period of 1 year (n=9). The blue samples represent the pre-rejection samples (day 30) of patients with aTCMR more than 30 days after transplantation (n=8). **B**. Volcano plot of 89 proteins of serum samples with and without aTCMR. The X-axis displays the difference in NPX of 89 proteins of the samples with aTCMR and samples without aTCMR. Proteins with a positive NPX value are higher in samples with aTCMR. The Y-axis represents each protein *p*-value (-log 10). The two horizontal lines depicts adjusted (dotted line) and unadjusted (solid line) *p*-values. **C**. Boxplot of the significantly different proteins of the comparison of samples with and without aTCMR. The pink samples are of 9 patients without aTCMR, and the green samples are of 10 patients with aTCMR. The Y-axis represents the NPX expression value and interquartile range. **D**. Table of the five significant proteins. The aliases, description, cell types and involved pathways are described. **E**. Area under the curve (AUC) of the significant proteins. A receiver operator characteristic curve analysis was performed of the 5 significant proteins and the AUC, NPX cutoff value with the sensitivity, specificity, positive predictive value and negative predictive value were calculated. **F**. Pearson's correlation coefficient analysis. The correlation between the 5 significant proteins (CD5, CD8A, NCR1, TNFRSF4 and TNFRSF9) was tested with Pearson's correlation coefficient analysis. 95%-CI, 95%-confidence interval; NPV, negative predictive value; NPX, normalized protein expression; PPV, positive predictive value; SE, standard error.

Proximity Extension Immunoassay

Ninety-two proteins were simultaneously measured in 1 µL serum by using the Immuno-Oncology panel of Olink (Uppsala, Sweden). Each of the 92 proteins is recognized by two matched oligonucleotide-labelled antibody probes. Upon simultaneous binding to the correct target protein, a real-time polymerase chain reaction (PCR) amplicon is created by a proximity-dependent DNA polymerization event. The resulting sequences were amplified and quantified by real time PCR (Biomark™ HD system, Fluidigm, San Francisco, CA, USA). Quality control of the samples was performed using 2 incubation controls (2 different non-human antigens), and a detection control (a complete double stranded DNA amplicon which does not require any proximity binding or extension step). A negative control was included in triplicate on each plate to monitor background levels of each protein assay and were used to calculate the limit of detection. To minimize variation between samples, raw measurements were normalized. An extension control (an antibody linked to 2 matched unique olinonucleotides for immediate proximity independent of antigen binding) was used for normalization. For each sample and data point, the corresponding quantitation cycle (Cq)-value for the extension control was subtracted. Three inter-plate controls (pool of 92 matching oligonucleotide pairs) are added to each 96 wells-plate to normalize the measurements between plates. In our study, only one plate was used. Finally, the data were transformed using a pre-determined correction factor (estimated during validation of the panel) of Olink. The generated Normalized Protein eXpression (NPX) value is on a log2 scale where higher NPX values correspond to higher protein concentrations. Biomarkers with values below the limit of detection (less than 2.5 SDs above the negative controls)

were excluded from the analysis. The intra-assay and inter-assay coefficients of variation of PEA were 8% and 12% when tested in a validation study[19].

Statistical analysis

All analyses were performed using R (R Foundation for Statistical Computing, Vienna, Austria, version 3.4.4), RStudio version 1.1.447 (RStudio Inc., Boston, MA, USA) and SPSS version 21 (SPSS Inc., Chicago, IL, USA). Continuous variables are expressed as median and interquartile range (first and third quartile, IQR). The unpaired t-test with false discovery rate (FDR) correction was used for the comparison between 2 groups. Results were considered statistically significant if $p < 0.05$. *P*-values were corrected for multiple testing using Benjamini-Hochberg's approach. Receiver-operator characteristic curve analysis was performed on the significant proteins and the area under the curve (AUC) was calculated as a measure of discriminatory ability. Pearson's correlation coefficient analysis was used to calculate correlations between the significantly different proteins.

RESULTS

The NPX values of the 92 proteins of the Immuno-Oncology panel were compared between the 11 samples with aTCMR (day of rejection, green samples in Figure 1A) and 9 samples without rejection (day 30 after transplantation, pink samples in Figure 1A). One sample (aTCMR on day 3) did not pass the quality control after PEA and was excluded from further analysis. Three proteins (IL-1α, TNF, and IL-35) did not pass the limit of detection and were excluded.

Five proteins (T cell surface glycoprotein CD5 [CD5], T cell surface glycoprotein CD8 [CD8a], Natural cytotoxicity triggering receptor 1 [NCR1], TNF receptor superfamily member 4 [TNFRSF4] and TNF receptor superfamily member 9 [TNFRSF9]) were expressed significantly higher in samples with aTCMR compared with samples without aTCMR (adjusted *p*-value<1.14E-02; Figure 1B and C). The pathways most enriched among these 5 proteins are related to T cell activation, T cell proliferation, and NK cell-mediated immune responses (Figure 1D). Receiver-operator characteristic curve analysis of these proteins revealed a good predictive capacity for aTCMR (Figure 1D). The AUC ranged from 0.83 for CD5 to 0.91 for NCR1 (p<0.014; Figure 1E). The 5 proteins (CD5, CD8A, NCR1, TNFRSF4 and TNFRSF9) were all significantly correlated with each other (Figure 1F).

A non-hierarchical clustering analysis and heatmap showed clustering of samples with aTCMR and samples without aTCMR (Figure 2A). Two samples with aTCMR clustered with the samples without aTCMR. One sample was from a patient with an eGFR deterioration from 48 to 36 mL/min per 1.73 m^2, 6 weeks after transplantation. The renal biopsy showed an isolated vascular lesion and was therefore classified as Banff category aTCMRIIA. This patient was treated with methylprednisolone. The other sample was obtained from a patient with an eGFR deterioration from 52 to 30 mL/min per 1.73 m^2 4 months after transplantation. The renal biopsy showed aTCMRIIB. This patient was treated with methylprednisolone and T cell-depleting therapy (alemtuzumab). One sample without aTCMR clustered with the aTCMR group. This patient was 1 month after transplantation and showed no clinical signs of AR. The kidney function (eGFR of 50 mL/min per 1.73m^2) was stable at the time of serum collection and thereafter.

Identification of patients with a higher probability for AR could potentially improve the long-term allograft outcome by changing the immunosuppressive regimen. Therefore, the pre-rejection serum (day 30 after transplantation, blue samples in Figure 1A) of 8 patients were compared with samples of 9 patients without aTCMR (pink samples in Figure 1A). Three proteins (IL-1α, IFN-β, and IL-35) were excluded due to low detection. No clustering was seen of pre-rejection samples (blue samples in Figure 1A) and no rejection samples (pink samples in Figure 1A) in a hierarchical cluster analysis (Figure 2B). One protein, IFN-γ, was expressed significantly higher in samples of patients without rejection (NPX value median 0.13, IQR -0.07 - 0.15) compared with pre-rejection samples (median -0.15, IQR -0.27 - 0.04, adjusted p-value=0.00367). The NPX values of the 5 proteins (CD5, CD8A, NCR1, TNFRSF4 and TNFRSF9) that were significantly higher in rejection samples when compared with those without rejection, were not higher in pre-rejection samples.

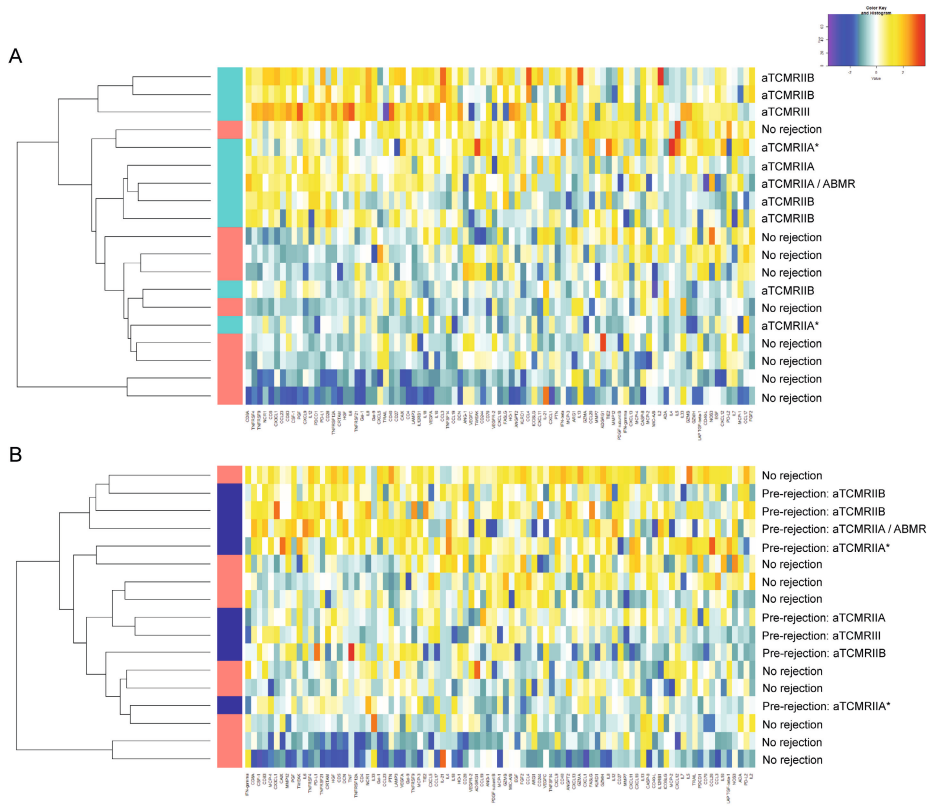

Figure 2. A. Unsupervised hierarchical cluster analysis and heatmap of the 89 proteins of samples with and without aTCMR. Each row represents a serum sample. The green samples are the samples with aTCMR and the pink samples are without aTCMR. Each column represents a NPX value of one of the 89 proteins. The columns are ordered by *p*-value, with the most significant proteins on the left and the least significant proteins on the right. The color in each cell reflects the level of relative expression of the protein (red is high and blue is low). *Sample with isolated vascular lesion. **B**. Unsupervised hierarchical cluster analysis and heatmap of the 89 proteins of pre-rejection samples (day 30) and samples without aTCMR (day 30). Each row represents a serum sample. The blue samples are the pre-rejection samples of patients with aTCMR in the follow up period. The pink samples are of patients without aTCMR during the follow-up period. Each column represents a NPX value of one of the 89 proteins. The columns are ordered by *p*-value, with the most significant proteins on the left and the least significant proteins on the right. *Sample with isolated vascular lesion.

DISCUSSION

In the present study, targeted proteomic analysis with PEA was performed for the first time in kidney transplant recipients. Sera of belatacept-treated patients were used in an attempt to diagnose aTCMR in a minimally invasive way. Five proteins (CD5, CD8A, NCR1, TNFRSF4 and TNFRSF9) were associated with aTCMR and had good positive and negative predictive values. These proteins are strongly associated with effector lymphocytes of adaptive and innate immune systems, *i.e.* the classical $CD8^+$ cytotoxic T cells and NK cells. These cells have important roles in AR[23]. After activation, these cells release cytotoxic molecules like perforin and granzyme, which can result in donor cell lysis and allograft damage. The observations of our study in serum of patients with AR are in accordance with the biology of AR found in kidney allografts[24]. The top molecular transcripts found in AR biopsies reflected the presence and actions of effector T cells, antigen presenting cells, NK cells, and γ-interferon-inducible genes[25].

The protein panel tested in this study also contained several other biomarkers that have been associated with AR. For instance, IFN-γ, CXCL9, CXCL10, CXCL11, granzyme B, PD ligand 1, FASLG and NK cell marker KLRD1[24,26]. Here, these markers were not significantly different between the belatacept-treated patients with and without aTCMR. However, AR is a process of time-dependent events involving different combinations of proteins. It is, therefore, better not to look at a single protein but at a panel of proteins. In this study, cluster analysis of all proteins showed a distinct clustering of sera with and without aTCMR.

Prediction of AR might help to stratifying immunosuppressive therapy of kidney transplant recipients. Therefore, pre-rejection samples (after the first month of transplantation) were compared with samples of patients without rejection in the first year. No protein profile could be identified that predicted aTCMR, and the 5 proteins that were significantly higher in rejection samples were not higher in pre-rejection samples compared with samples without rejection. A possible explanation for this is the timing of measurements: expression of these proteins might increase shortly before AR. One protein, IFN-γ, was found to be lower in pre-rejection samples compared with samples of patients without rejection. Although IFN-γ is a proinflammatory cytokine, it can also exert immunoregulatory activities[27]. Xu *et al.* also reported decreased IFN-γ levels in serum of kidney transplant patients with aTCMR compared with samples of patients without rejection. They postulated that IFN-γ is secreted from Th1-like regulatory T cells and thereby negatively modulates aTCMR[28].

Only one study in solid organ transplant recipients was performed with PEA before. This study shows promising results to discriminate AR from no rejection in serum of heart transplant recipients[29]. Ten proteins, mainly associated with T cell and NK cells (CCL19, CD244, CSF1, CXCL9, CXCL10, CXCL11, IL-6, IL-12B, LTA, SLAMF1) were expressed significantly higher in heart transplant recipients with AR compared to patients without AR[29]. An explanation for the discrepancy between our results and theirs may be that AR of a heart allograft is most often recognized at an earlier stage compared with a kidney allograft. In heart transplantation, diagnosing AR is mainly based on the histopathologic examination of protocol endomyocardial biopsies. In contrast, in kidney transplantation, AR is usually diagnosed in a for-cause biopsy. This can delay the diagnosis of AR in kidney transplant recipients and making the diagnosis at a later stage of rejection and subsequently differences in the proteome.

The advantages of PEA are that it is a fast method (results within 24 hours and 3 hours hands-on time), with high sensitivity and specificity, and only a very low sample volume (1 µL) is required. Furthermore, different types of samples (plasma, serum, urine, saliva, dried blood spot *etc.*) can be tested and low-abundant (below pg/mL) proteins can be measured[19]. We, therefore, believe that PEA is a candidate screening tool for acute kidney transplant rejection. This assay can potentially reduce the burden of kidney biopsies, especially in patients with a high risk for biopsy-related complications and in children.

We acknowledge the limitations of this pilot study. First, the number of included patients was limited and therefore, no validation study was performed. However, expansion of the sample size is currently impossible, because of a manufacturing delay of belatacept[30]. Despite the limited sample size, a clear distinction in the protein profile was seen between serum samples from patients with aTCMR and those without aTCMR. Second, a selection of proteins was analyzed, thereby excluding other possibly relevant proteins. Third, with PEA, no absolute levels of the proteins are measured and therefore comparison with other studies is difficult. Fourth, in this study only patients treated with a belatacept-based regimen were analyzed. The low number of patients with an AR (n=2) in the tacrolimus group of our RCT precluded a meaningful analysis. Currently, we are collecting samples of tacrolimus-treated patients with an AR for a proteomic analysis. This study will include n = 225 patients and is expected to be completed at the end of 2019. Lastly, it would be of interest to analyze PEA in serum of patients with other types of rejection (*e.g.* antibody-mediated rejection), rejections occurring longer after transplantation (as opposed to early AR), and other common kidney

pathology in kidney transplant recipients (like delayed graft function, acute tubular necrosis, pyelonephritis, BK nephropathy, and recurrent glomerulonephritis).

CONCLUSION

Targeted proteomic analysis with PEA of serum of kidney transplant recipients appears to be a promising minimally invasive technique to diagnose aTCMR additionally to histopathological examination. The encouraging results from this pilot study warrant further study with the inclusion of a larger group of patients and different types of kidney diseases.

REFERENCES

1. Hart A, Smith JM, Skeans MA, et al. OPTN/SRTR 2016 Annual Data Report: Kidney. *Am J Transplant.* 2018;18 Suppl 1:18-113.
2. Broecker V, Mengel M. The significance of histological diagnosis in renal allograft biopsies in 2014. *Transpl Int.* 2015;28(2):136-143.
3. Morgan TA, Chandran S, Burger IM, Zhang CA, Goldstein RB. Complications of Ultrasound-Guided Renal Transplant Biopsies. *Am J Transplant.* 2016;16(4):1298-1305.
4. Verhoeven J, Boer K, Van Schaik RHN, et al. Liquid Biopsies to Monitor Solid Organ Transplant Function: A Review of New Biomarkers. *Ther Drug Monit.* 2018.
5. Clarke W. Proteomic research in renal transplantation. *Ther Drug Monit.* 2006;28(1):19-22.
6. Christians U, Klawitter J, Klawitter J. Biomarkers in Transplantation--Proteomics and Metabolomics. *Ther Drug Monit.* 2016;38 Suppl 1:S70-74.
7. Sigdel TK, Sarwal MM. Assessment of Circulating Protein Signatures for Kidney Transplantation in Pediatric Recipients. *Front Med (Lausanne).* 2017;4:80.
8. Perez JD, Sakata MM, Colucci JA, et al. Plasma proteomics for the assessment of acute renal transplant rejection. *Life Sci.* 2016;158:111-120.
9. Brunet M, Shipkova M, van Gelder T, et al. Barcelona Consensus on Biomarker-Based Immunosuppressive Drugs Management in Solid Organ Transplantation. *Ther Drug Monit.* 2016;38 Suppl 1:S1-20.
10. Archdeacon P, Dixon C, Belen O, Albrecht R, Meyer J. Summary of the US FDA approval of belatacept. *Am J Transplant.* 2012;12(3):554-562.
11. Vincenti F, Charpentier B, Vanrenterghem Y, et al. A phase III study of belatacept-based immunosuppression regimens versus cyclosporine in renal transplant recipients (BENEFIT study). *Am J Transplant.* 2010;10(3):535-546.
12. Durrbach A, Pestana JM, Pearson T, et al. A phase III study of belatacept versus cyclosporine in kidney transplants from extended criteria donors (BENEFIT-EXT study). *Am J Transplant.* 2010;10(3):547-557.
13. Durrbach A, Pestana JM, Florman S, et al. Long-Term Outcomes in Belatacept- Versus Cyclosporine-Treated Recipients of Extended Criteria Donor Kidneys: Final Results From BENEFIT-EXT, a Phase III Randomized Study. *Am J Transplant.* 2016;16(11):3192-3201.
14. Vincenti F, Rostaing L, Grinyo J, et al. Belatacept and Long-Term Outcomes in Kidney Transplantation. *N Engl J Med.* 2016;374(4):333-343.
15. de Graav GN, van der Zwan M, Baan CC, Janssen JAMJL, Hesselink DA. Improved Glucose Tolerance in a Kidney Transplant Recipient With Type 2 Diabetes Mellitus After Switching From Tacrolimus To Belatacept: A Case Report and Review of Potential Mechanisms. *Transplantation Direct.* 2018;4(3):e350.
16. de Graav GN, Bergan S, Baan CC, et al. Therapeutic Drug Monitoring of Belatacept in Kidney Transplantation. *Ther Drug Monit.* 2015;37(5):560-567.
17. de Graav GN, Baan CC, Clahsen-van Groningen MC, et al. A Randomized Controlled Clinical Trial Comparing Belatacept With Tacrolimus After De Novo Kidney Transplantation. *Transplantation.* 2017;101(10):2571-2581.

18. Newell KA, Mehta AK, Larsen CP, et al. Lessons Learned: Early Termination of a Randomized Trial of Calcineurin Inhibitor and Corticosteroid Avoidance Using Belatacept. *Am J Transplant.* 2017;17(10):2712-2719.

19. Assarsson E, Lundberg M, Holmquist G, et al. Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. *PLoS One.* 2014;9(4):e95192.

20. Carlsson AC, Ingelsson E, Sundstrom J, et al. Use of Proteomics To Investigate Kidney Function Decline over 5 Years. *Clin J Am Soc Nephrol.* 2017;12(8):1226-1235.

21. de Graav GN, Hesselink DA, Dieterich M, et al. An Acute Cellular Rejection With Detrimental Outcome Occurring Under Belatacept-Based Immunosuppressive Therapy: An Immunological Analysis. *Transplantation.* 2016;100(5):1111-1119.

22. Loupy A, Haas M, Solez K, et al. The Banff 2015 Kidney Meeting Report: Current Challenges in Rejection Classification and Prospects for Adopting Molecular Pathology. *Am J Transplant.* 2017;17(1):28-41.

23. Nankivell BJ, Alexander SI. Rejection of the kidney allograft. *N Engl J Med.* 2010;363(15):1451-1462.

24. Halloran PF, Venner JM, Madill-Thomsen KS, et al. Review: The transcripts associated with organ allograft rejection. *Am J Transplant.* 2018;18(4):785-795.

25. Halloran PF, Famulski K, Reeve J. The molecular phenotypes of rejection in kidney transplant biopsies. *Curr Opin Organ Transplant.* 2015;20(3):359-367.

26. Jamshaid F, Froghi S, Di Cocco P, Dor FJ. Novel non-invasive biomarkers diagnostic of acute rejection in renal transplant recipients: A systematic review. *Int J Clin Pract.* 2018:e13220.

27. Smigiel KS, Srivastava S, Stolley JM, Campbell DJ. Regulatory T-cell homeostasis: steady-state maintenance and modulation during inflammation. *Immunol Rev.* 2014;259(1):40-59.

28. Xu X, Huang H, Wang Q, et al. IFN-gamma-producing Th1-like regulatory T cells may limit acute cellular renal allograft rejection: Paradoxical post-transplantation effects of IFN-gamma. *Immunobiology.* 2017;222(2):280-290.

29. Sukma Dewi I, Gidlof O, Hollander Z, et al. Immunological Serum Protein Profiles for Noninvasive Detection of Acute Cellular Rejection After Heart Transplantation. *J Am Coll Cardiol.* 2017;70(23):2946-2947.

30. Gabardi S, van Gelder T. Causes and Consequences of the Worldwide Belatacept Shortage. *Transplantation.* 2017;101(7):1520-1521.

PART III

PREVENTION AND TREATMENT OF ACUTE REJECTION AND ITS COMPLICATIONS

CHAPTER 7

Improved glucose tolerance in a kidney transplant recipient with type 2 diabetes mellitus after switching from tacrolimus to belatacept: A case report and review of potential mechanisms

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ABSTRACT

The introduction of immunosuppressant belatacept, an inhibitor of the CD28-80/86 pathway, has improved 1-year outcomes in kidney transplant recipients with pre-existent diabetes mellitus and has also reduced the risk of posttransplant diabetes mellitus. So far, no studies have compared a tacrolimus-based to a belatacept-based immunosuppressive regimen with regard to improving glucose tolerance after kidney transplantation. Here, we present the case of a 54-year-old male with type 2 diabetes mellitus who was converted from belatacept to tacrolimus 1 year after a successful kidney transplantation. Thereafter, he quickly developed severe hyperglycemia, and administration of insulin was needed to improve metabolic control. Six months after this episode, he was converted back to belatacept because of nausea, diarrhea, and hyperglycemia. After switching back to belatacept and within 4 days after stopping tacrolimus glucose tolerance improved and insulin therapy could be discontinued. Although belatacept is considered less diabetogenic than tacrolimus, the rapid improvement of glucose tolerance after switching to belatacept is remarkable. In this article, the potential mechanisms of this observation are discussed.

INTRODUCTION

Kidney transplant recipients who have pre-existent diabetes mellitus (DM) or who develop DM after transplantation (posttransplant DM [PTDM]) have a worse survival and suffer from more cardiovascular morbidity than those without[1-3]. The calcineurin-inhibitors (CNI), cyclosporine A (CsA) and tacrolimus, may decrease insulin secretion and increase insulin resistance[4]. The latter is characterized by a decreased insulin sensitivity, that is, more insulin is needed to maintain serum glucose within the reference range[5].

Belatacept, an inhibitor of the CD28-CD80/86 pathway[6], does not induce hyperglycemia nor PTDM. Despite the higher acute rejection risk observed in belatacept-treated patients[7-9], it improves 1-year allograft survival and renal function in kidney transplant recipients with pre-existent DM compared with CsA-treated patients[3].

In addition, belatacept-based therapy decreases the risk of developing PTDM. A meta-analysis which included 729 belatacept-treated and 320 CsA-treated patients showed a relative risk of 0.61 (95%-confidence interval, 0.40 - 0.93) to develop PTDM with belatacept compared to CsA[10]. Tacrolimus is, nowadays, the most-widely used CNI, and treatment with tacrolimus carries a higher risk of developing PTDM than CsA[11,12].

Belatacept may be a therapeutic option for kidney transplant recipients that develop PTDM or for those with pre-existent DM who develop a worsening of metabolic control after starting a CNI-based regimen. Glucose metabolism has not been compared between belatacept- and tacrolimus-treated patients. In addition, no data on the safety and efficacy regarding insulin sensitivity of conversion from CNIs to belatacept after kidney transplantation have been reported.

Here, a kidney transplant recipient with DM is described, who after conversion from belatacept to tacrolimus developed severe hyperglycemia. Glucose control was difficult and did not improve despite high doses of insulin. Within 4 days after stopping tacrolimus and reintroducing belatacept, a marked improvement of glucose tolerance was observed. The purpose of this case report is to discuss the possible pathophysiologic mechanisms explaining this observation and the role of immunosuppressive therapy therein.

CASE DESCRIPTION

A 54-year-old Caucasian male received a preemptive, living-unrelated donor kidney transplant in October 2013 because of hypertensive and diabetic nephropathy. His medical history included hypertension since 1992; type 2 DM since 2002; and since 2008, histologically confirmed diabetic and hypertensive nephropathies. The transplant was 2-2-1 mismatched (for HLA-A, HLA-B and HLA-DR, respectively). Peak and actual panel reactive antibodies were 0%.

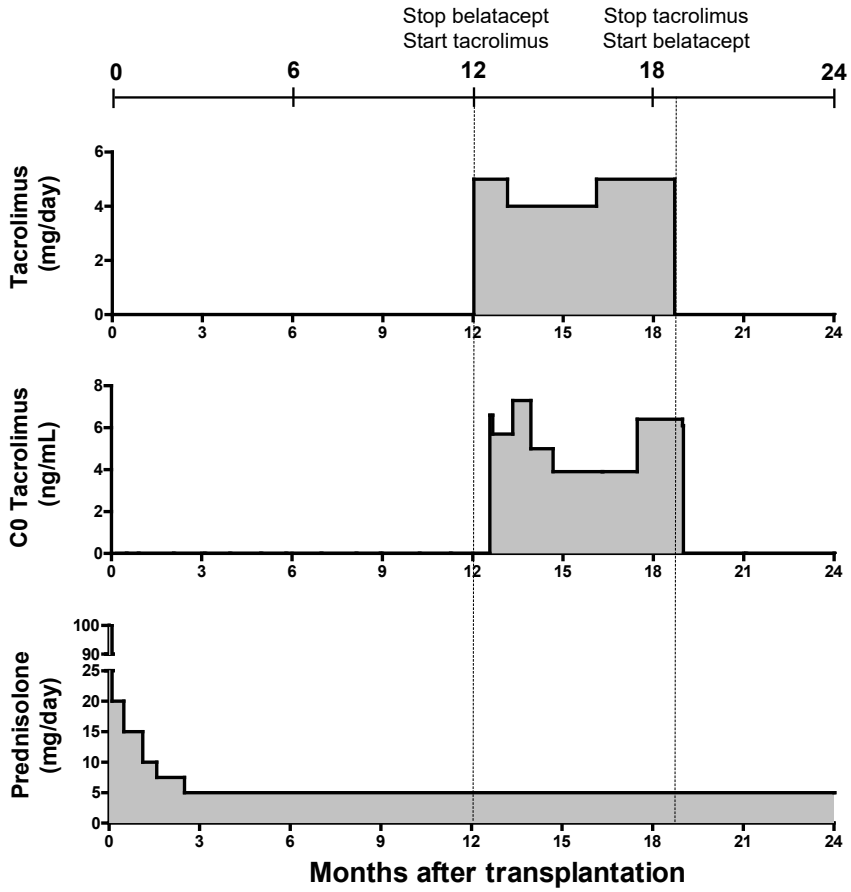

Figure 1. Overview of the dose of tacrolimus and prednisolone. The depicted doses of tacrolimus and prednisolone are oral daily doses per time period. Tacrolimus was adjusted to whole blood predose concentrations (C_0). Prednisolone was given as an intravenous dose of 100 mg on days 0-3. From day 4 until day 18 the prednisolone dose was 20 mg/d; in weeks 3-4 the prednisolone dose was 15 mg/d; in weeks 5-6 the prednisolone dose was 10 mg/d; in weeks 7-10 the prednisolone dose was 7.5 mg/d; thereafter prednisolone dose was 5 mg/d. De dashed vertical lines indicate the time points when belatacept was discontinued and restarted.

The patient was treated with belatacept according to the less-intensive regimen of the Belatacept Evaluation of Nephroprotection and Efficacy as First-line Immunosuppression (BENEFIT) trials during the first posttransplantation year as part of a randomized-controlled trial[13,14]. This trial compared belatacept to tacrolimus and its main findings were described previously[14,15]. In addition to belatacept, he also received induction therapy with 20 mg/kg basiliximab (day 0 and 4) and maintenance therapy with mycophenolate mofetil (targeted to pre-dose concentrations of 1.5-3.0 mg/mL), and prednisolone, tapered to 5 mg/d by month 3 and maintained at 5 mg/d thereafter (Figure 1). The clinical course of the first posttransplant year was uneventful. Belatacept was not reimbursed by the health insurance companies in the Netherlands at the time the patient was 1 year after transplantation (October 2014). Belatacept was discontinued, and he was switched to tacrolimus (Advagraf®; Astellas Pharma, Tokyo, Japan) targeted to predose concentrations of 5-7 ng/mL (Figure 1).

At the time of transplantation, his DM was well-controlled: glycated hemoglobin (HbA1c) was 44 mmol/mol with 24 International Units (IU) of long-acting insulin-glargine (Lantus®; Sanofi, Paris, France) daily. Before transplantation, he was taken care of by a nephrologist in a local hospital. Initially, his diabetes was managed with metformin only. When his renal function deteriorated, metformin was stopped, and he was started on long-acting insulin. After transplantation and in an attempt to take patient off insulin, he was started on metformin and glimepiride. Insulin-glargine was initially continued (mean dose, 28 IU/d). In addition, during the first month after transplantation, he received short-acting insulin-aspart (NovoRapid®; Novo Nordisk, Bagsværd, Denmark, mean dose 18 IU/day). Four months after his transplantation and when prednisolone had been tapered to 5 mg daily, insulin could be discontinued completely. Figure 2 gives an overview of diabetes-related events, glucose and HbA1c concentrations, and glucose lowering medication. We believe that the combination of improved kidney function, possibly increased physical activity, the introduction of metformin and glimepiride and the tapering of prednisolone to 5 mg daily (Figure 2) allowed for the complete withdrawal of insulin therapy.

Within 14 days after conversion to tacrolimus, the patient developed severe hyperglycemic episodes (Figure 2). Glimepiride and metformin were increased to 6 mg and 3000 mg daily, respectively, without improvement of glucose control (Figure 2). Insulin therapy was restarted (Figure 2). The patient needed up to 50 IU of short- and long-acting insulin on a daily basis to improve metabolic control. Despite the high insulin dose, hyperglycemia persisted. During these hyperglycemic episodes, no infections or changes in bodyweight were observed.

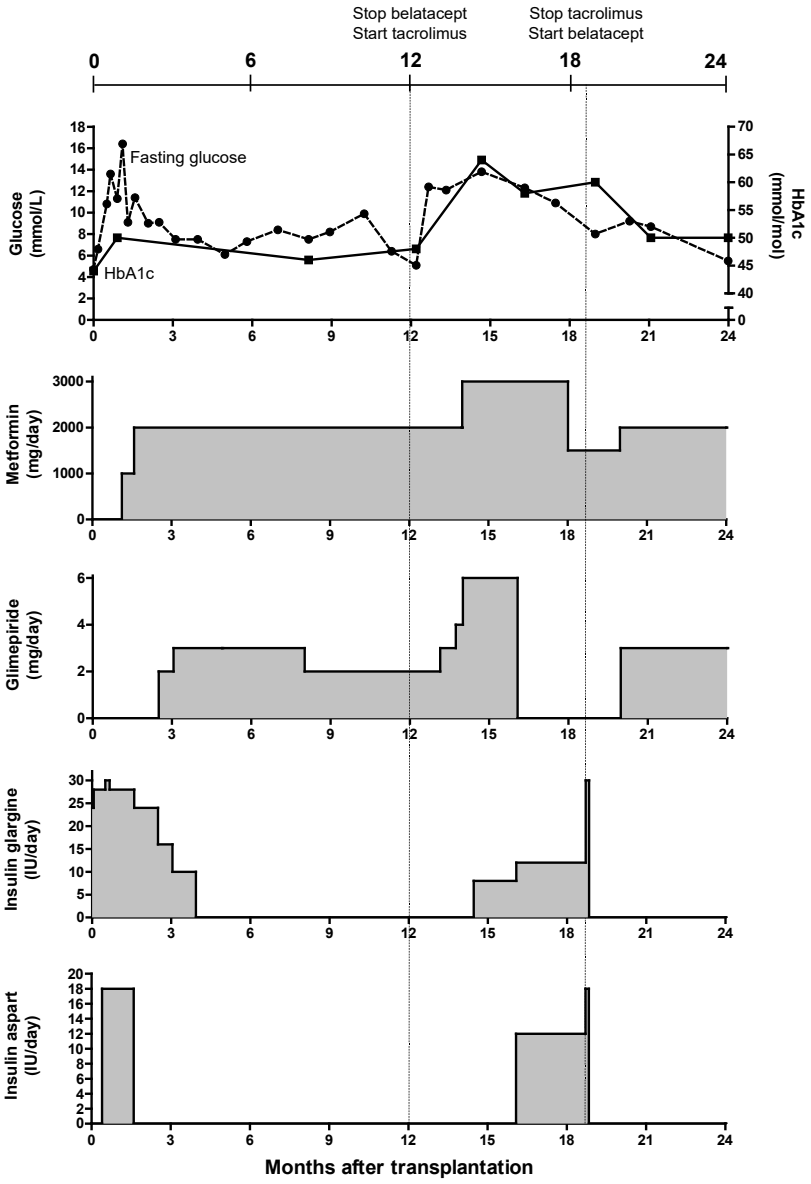

Figure 2. Overview of diabetes-related events, measurements and glucose-lowering medication. A timeline is depicted indicating important events related to changes in glucose concentrations. The presented glucose and HbA1c concentrations were measured in hospital at the outpatient clinic. The maximum target concentration of HbA1c was 53 mmol/mol. Glucose concentrations measured at home are not included. The daily doses per time period are given for metformin, glimepiride, insulin-glargine and insulin-aspart. From day 12 to 48 after transplantation, doses of insulin-aspart were adjusted to target a premeal glucoses concentration of <10 mmol/L (average dose was 18 IU/d). De dashed vertical lines indicate the time points when belatacept was discontinued and restarted.

In addition, the patient developed tremors of the hands, nausea, and diarrhea that were all considered as side effects of tacrolimus. Clinically, no signs of tacrolimus-associated nephrotoxicity were observed. No (protocol) kidney biopsy was performed after conversion to tacrolimus. Because of these side effects, 6 months after conversion, belatacept (5 mg/kg bodyweight monthly) was restarted. Tacrolimus was discontinued overnight. Four days after discontinuing tacrolimus, the patient's blood glucose concentrations improved tremendously and insulin therapy could be stopped. During the 2-year follow up period, the patient has remained in good clinical condition. No acute rejection occurred and his eGFR has remained stable (55 mL/min per 1.73 m^2; Figure S1). His current glucose-lowering treatment consists of metformin and glimepiride.

DISCUSSION

In this patient, a marked improvement in glucose tolerance was observed after switching from belatacept to tacrolimus. Improvement of glucose control occurred immediately after withdrawal of tacrolimus and exogenous insulin could be stopped within 4 days. The improvement in glucose was not related to changes in BMI (Figure S1). Although it is well known that belatacept is less diabetogenic than CNIs[10], the rapid improvement of glucose tolerance was remarkable and unexpected.

Tacrolimus may induce DM by several mechanisms. First, tacrolimus reduces insulin secretion by the pancreatic β cells via decreasing insulin mRNA expression through inhibition of 2 pathways (nuclear factor of activated T cells and cAMP response element-binding protein)[16-19]. Second, the insulin content of the β cell is diminished by tacrolimus[17]. Third, the glucose-induced insulin release is inhibited by tacrolimus through reduced glucokinase activity[17,20]. Fourth, tacrolimus directly induces β cell apoptosis and reduces islet cell proliferation[19,21]. Altogether, these effects lead to a reduction of insulin secretion. Furthermore, CNIs may induce insulin resistance by stimulating endocytic removal of glucose transporter type 4 (Glut-4) from the cell membrane of adipocytes and muscle cells[22].

The induction of hypomagnesemia is another mechanism by which tacrolimus may directly influence glucose tolerance[23]. Tacrolimus can downregulate the magnesium absorbing channel transient receptor of potential melastatin (TRPM6) in the distal collecting tubule and thereby induce hypomagnesemia via renal magnesium wasting[24]. Hypomagnesemia may contribute to insulin resistance by decreasing autophosphorylation of the β subunits of the insulin receptor[25]. Besides, hypomagnesemia may reduce insulin secretion[25]. Unfortunately,

it is unknown if hypomagnesemia played a role in our case, since magnesium concentrations were not measured.

We think that the fast improvement of glucose control after switching to belatacept in our patient was mainly related to (1) the discontinuation of tacrolimus and/or (2) a direct effect on insulin resistance by CD80-86 blockade by belatacept. Several animal studies have described the effects of discontinuation of tacrolimus on glucose metabolism. Redmon *et al*[17] observed reversibility of insulin secretion 72 hours after discontinuation of tacrolimus in hamster β cells (HIT-T15). Another *in vitro* study using rat β cells found similar results[26]. In an *in vivo* study, reversibility of rat β cells insulin gene expression, insulin content, and insulin secretion was observed 7 days after discontinuing tacrolimus[27]. Another study in rats showed that the insulin resistance improved 5 days after the last dose of tacrolimus[28].

All these studies show reversibility of impaired glucose tolerance after discontinuation of tacrolimus. However, these studies are limited by the short duration of tacrolimus treatment. Prolonged administration could possibly lead to a more severe reduction of functional β cell mass and irreversibility of impaired glucose control. Furthermore, to the best of our knowledge, no studies in humans have analyzed directly the effects of insulin secretion and resistance after discontinuation of tacrolimus. Boots *et al*[29] described the effect of tacrolimus dose reduction on insulin secretion. In 15 kidney transplant recipients without DM, a 33% reduction in the tacrolimus predose concentrations resulted in a 36% increase in β cell secretion capacity[29].

The fast improvement of glucose tolerance in this case may also have been caused by the introduction of belatacept (rather than the withdrawal of tacrolimus).One study has suggested that CD86 may play a role in insulin resistance via interaction with the adiponectin axis[30].

Interestingly, a case report of a patient with rheumatoid arthritis reported an improvement of insulin resistance 4 weeks after treatment with abatacept, which is considered a lower-affinity version of belatacept[31]. In another study, improved insulin sensitivity was observed in 15 patients 6 months after the start of abatacept treatment[32].

In contrast to the findings of the studies described above, Zhong *et al*[33] found in mice and humans that a higher CD80/86 expression was negatively correlated with insulin resistance.

No effect of adiponectin on CD80/86 expression was noted on human macrophages in another study[34].

The main limitation of this case report is that the mechanistic evidence of the effect of belatacept and tacrolimus on glucose tolerance is lacking. We did not examine endogenous insulin secretion and insulin resistance.

In conclusion, a kidney transplant recipient with pre-existing type 2 DM is described who showed a rapid improvement of glucose tolerance after switching from tacrolimus to belatacept. Such a strategy may be beneficial in comparable cases although high-quality evidence of the safety of this intervention in terms of rejection is currently lacking.

REFERENCES

1. Cosio FG, Hickson LJ, Griffin MD, Stegall MD, Kudva Y. Patient survival and cardiovascular risk after kidney transplantation: the challenge of diabetes. *Am J Transplant.* 2008;8(3):593-599.
2. Revanur VK, Jardine AG, Kingsmore DB, et al. Influence of diabetes mellitus on patient and graft survival in recipients of kidney transplantation. *Clin Transplant.* 2001;15(2):89-94.
3. Rostaing L, Neumayer HH, Reyes-Acevedo R, et al. Belatacept-versus cyclosporine-based immunosuppression in renal transplant recipients with pre-existing diabetes. *Clin J Am Soc Nephrol.* 2011;6(11):2696-2704.
4. Bamgbola O. Metabolic consequences of modern immunosuppressive agents in solid organ transplantation. *Ther Adv Endocrinol Metab.* 2016;7(3):110-127.
5. American Diabetes Association. Standards of medical care in diabetes. *Diabetes Care.* 2017;40 (Suppl. 1):S1-S135.
6. de Graav GN, Bergan S, Baan CC, et al. Therapeutic Drug Monitoring of Belatacept in Kidney Transplantation. *Ther Drug Monit.* 2015;37(5):560-567.
7. Grinyo JM, Del Carmen Rial M, Alberu J, et al. Safety and Efficacy Outcomes 3 Years After Switching to Belatacept From a Calcineurin Inhibitor in Kidney Transplant Recipients: Results From a Phase 2 Randomized Trial. *Am J Kidney Dis.* 2017;69(5):587-594.
8. Cohen JB, Eddinger KC, Forde KA, Abt PL, Sawinski D. Belatacept Compared With Tacrolimus for Kidney Transplantation: A Propensity Score Matched Cohort Study. *Transplantation.* 2017;101(10):2582-2589.
9. Keith DS, Vranic G, Nishio-Lucar A. Graft Function and Intermediate-Term Outcomes of Kidney Transplants Improved in the Last Decade: Analysis of the United States Kidney Transplant Database. *Transplant Direct.* 2017;3(6):e166.
10. Masson P, Henderson L, Chapman JR, Craig JC, Webster AC. Belatacept for kidney transplant recipients. *Cochrane Database Syst Rev.* 2014(11):CD010699.
11. Luan FL, Steffick DE, Ojo AO. New-onset diabetes mellitus in kidney transplant recipients discharged on steroid-free immunosuppression. *Transplantation.* 2011;91(3):334-341.
12. Vincenti F, Friman S, Scheuermann E, et al. Results of an international, randomized trial comparing glucose metabolism disorders and outcome with cyclosporine versus tacrolimus. *Am J Transplant.* 2007;7(6):1506-1514.
13. Vincenti F, Charpentier B, Vanrenterghem Y, et al. A phase III study of belatacept-based immunosuppression regimens versus cyclosporine in renal transplant recipients (BENEFIT study). *Am J Transplant.* 2010;10(3):535-546.
14. de Graav GN, Baan CC, Clahsen-van Groningen MC, et al. A Randomized Controlled Clinical Trial Comparing Belatacept With Tacrolimus After De Novo Kidney Transplantation. *Transplantation.* 2017;101(10):2571-2581.
15. de Graav GN, Hesselink DA, Dieterich M, et al. An Acute Cellular Rejection With Detrimental Outcome Occurring Under Belatacept-Based Immunosuppressive Therapy: An Immunological Analysis. *Transplantation.* 2016;100(5):1111-1119.
16. Ozbay LA, Smidt K, Mortensen DM, et al. Cyclosporin and tacrolimus impair insulin secretion and transcriptional regulation in INS-1E beta-cells. *Br J Pharmacol.* 2011;162(1):136-146.

17. Redmon JB, Olson LK, Armstrong MB, Greene MJ, Robertson RP. Effects of tacrolimus (FK506) on human insulin gene expression, insulin mRNA levels, and insulin secretion in HIT-T15 cells. *J Clin Invest.* 1996;98(12):2786-2793.

18. Kruger M, Schwaninger M, Blume R, Oetjen E, Knepel W. Inhibition of CREB- and cAMP response element-mediated gene transcription by the immunosuppressive drugs cyclosporin A and FK506 in T cells. *Naunyn Schmiedebergs Arch Pharmacol.* 1997;356(4):433-440.

19. Heit JJ, Apelqvist AA, Gu X, et al. Calcineurin/NFAT signalling regulates pancreatic beta-cell growth and function. *Nature.* 2006;443(7109):345-349.

20. Radu RG, Fujimoto S, Mukai E, et al. Tacrolimus suppresses glucose-induced insulin release from pancreatic islets by reducing glucokinase activity. *Am J Physiol Endocrinol Metab.* 2005;288(2):E365-371.

21. Drachenberg CB, Klassen DK, Weir MR, et al. Islet cell damage associated with tacrolimus and cyclosporine: morphological features in pancreas allograft biopsies and clinical correlation. *Transplantation.* 1999;68(3):396-402.

22. Pereira MJ, Palming J, Rizell M, et al. Cyclosporine A and tacrolimus reduce the amount of GLUT4 at the cell surface in human adipocytes: increased endocytosis as a potential mechanism for the diabetogenic effects of immunosuppressive agents. *J Clin Endocrinol Metab.* 2014;99(10):E1885-1894.

23. Augusto JF, Subra JF, Duveau A, et al. Relation between pretransplant magnesemia and the risk of new onset diabetes after transplantation within the first year of kidney transplantation. *Transplantation.* 2014;97(11):1155-1160.

24. Nijenhuis T, Hoenderop JG, Bindels RJ. Downregulation of Ca(2+) and Mg(2+) transport proteins in the kidney explains tacrolimus (FK506)-induced hypercalciuria and hypomagnesemia. *J Am Soc Nephrol.* 2004;15(3):549-557.

25. Gommers LM, Hoenderop JG, Bindels RJ, de Baaij JH. Hypomagnesemia in Type 2 Diabetes: A Vicious Circle? *Diabetes.* 2016;65(1):3-13.

26. Uchizono Y, Iwase M, Nakamura U, et al. Tacrolimus impairment of insulin secretion in isolated rat islets occurs at multiple distal sites in stimulus-secretion coupling. *Endocrinology.* 2004;145(5):2264-2272.

27. Hernandez-Fisac I, Pizarro-Delgado J, Calle C, et al. Tacrolimus-induced diabetes in rats courses with suppressed insulin gene expression in pancreatic islets. *Am J Transplant.* 2007;7(11):2455-2462.

28. Rodriguez-Rodriguez AE, Trinanes J, Velazquez-Garcia S, et al. The higher diabetogenic risk of tacrolimus depends on pre-existing insulin resistance. A study in obese and lean Zucker rats. *Am J Transplant.* 2013;13(7):1665-1675.

29. Boots JM, van Duijnhoven EM, Christiaans MH, Wolffenbuttel BH, van Hooff JP. Glucose metabolism in renal transplant recipients on tacrolimus: the effect of steroid withdrawal and tacrolimus trough level reduction. *J Am Soc Nephrol.* 2002;13(1):221-227.

30. Pang TT, Chimen M, Goble E, et al. Inhibition of islet immunoreactivity by adiponectin is attenuated in human type 1 diabetes. *J Clin Endocrinol Metab.* 2013;98(3):E418-428.

31. Fedele AL, Alivernini S, Gremese E, Ferraccioli G. CTLA-4 Ig as an effective treatment in a patient with type I diabetes mellitus and seropositive rheumatoid arthritis. *Clin Exp Rheumatol.* 2016;34(2):315-317.

32. Ursini F, Russo E, Letizia Hribal M, et al. Abatacept improves whole-body insulin sensitivity in rheumatoid arthritis: an observational study. *Medicine (Baltimore).* 2015;94(21):e888.

33. Zhong J, Rao X, Braunstein Z, et al. T-cell costimulation protects obesity-induced adipose inflammation and insulin resistance. *Diabetes.* 2014;63(4):1289-1302.

34. Wolf AM, Wolf D, Rumpold H, Enrich B, Tilg H. Adiponectin induces the anti-inflammatory cytokines IL-10 and IL-1RA in human leukocytes. *Biochem Biophys Res Commun.* 2004;323(2):630-635.

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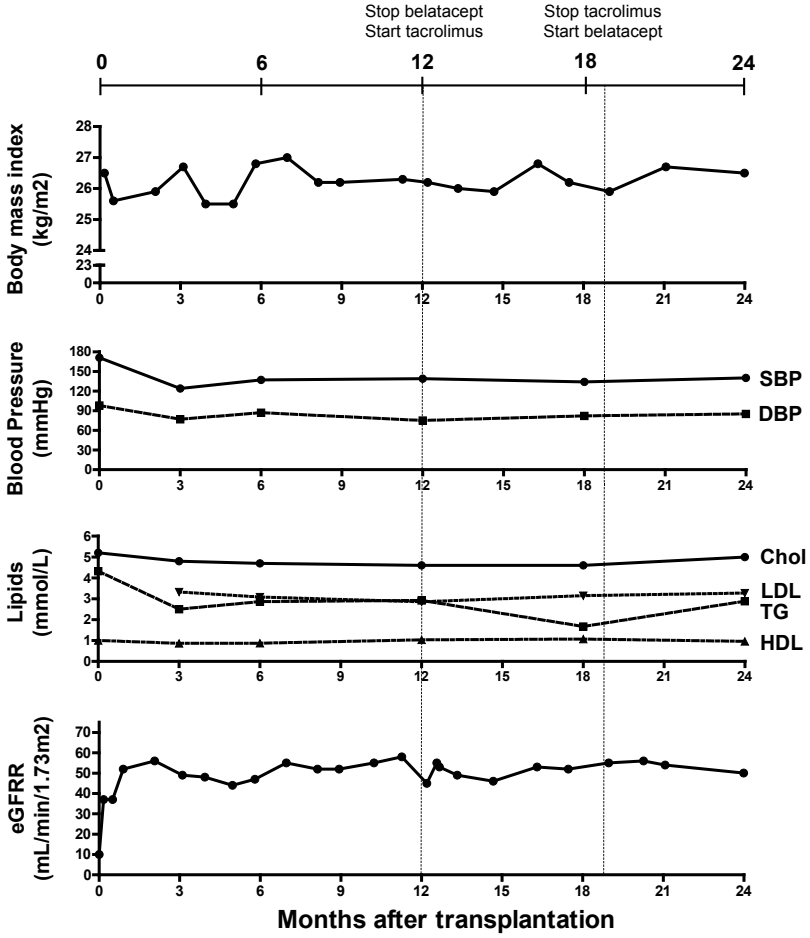

Figure S1. Body mass index, blood pressure, lipids and graft function during 2-year follow-up

CHAPTER 8

The efficacy of rabbit anti-thymocyte globulin for acute kidney transplant rejection in patients using calcineurin inhibitor and mycophenolate mofetil-based immunosuppressive therapy

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ABSTRACT

Background

T cell depleting antibody therapy with rabbit anti-thymocyte globulin (rATG) is the treatment of choice for glucocorticoid-resistant acute kidney allograft rejection (AR) and is used as first-line therapy in severe AR. Almost all studies investigating the effectiveness of rATG for this indication were conducted at the time when cyclosporine A and azathioprine were the standard of care. Here, the long-term outcome of rATG for AR in patients using the current standard immunosuppressive therapy (*i.e.* tacrolimus and mycophenolate mofetil), is described.

Methods

Between 2002 to 2012, 108 patients were treated with rATG for AR. Data on kidney function in the year following rATG and long-term outcomes were collected.

Results

Overall survival after rATG was comparable to overall survival of all kidney transplant patients ($p = 0.10$). Serum creatinine 1 year after rATG was 179 μmol/L (interquartile range (IQR) 136-234 μmol/L) and was comparable to baseline serum creatinine ($p = 0.22$). Early AR showed better allograft survival than late AR ($p = 0.0007$). In addition, 1 year after AR, serum creatinine was lower in early AR (157 mol/L; IQR 131-203) compared to late AR (216 mol/L; IQR 165-269; $p < 0.05$). The Banff grade of rejection, kidney function at the moment of rejection, and reason for rATG (severe or glucocorticoid resistant AR) did not influence the allograft survival.

Conclusion

Treatment of AR with rATG is effective in patients using current standard immunosuppressive therapy, even in patients with poor allograft function. Early identification of AR followed by T cell depleting treatment leads to better allograft outcomes.

INTRODUCTION

T cell depleting antibody therapy with rabbit anti-thymocyte globulin (rATG) is the treatment of choice for glucocorticoid-resistant or recurrent acute rejection (AR)[1]. In addition, many physicians use rATG as first-line therapy for severe acute T cell-mediated rejection (aTCMR, Banff grade IIA or higher) or as a component of treatment directed against acute antibody-mediated rejection (aABMR)[2].

The evidence of the efficacy of rATG therapy for AR dates from the era when azathioprine and cyclosporine A (CsA) were the standard of care. However, there is little evidence to guide the type of anti-rejection therapy in kidney transplant recipients treated with the current standard maintenance immunosuppressive therapy, consisting of tacrolimus (TAC) and mycophenolate mofetil (MMF)[3]. The advice on anti-rejection therapy in the KDIGO guideline[1] is based on a systematic review with meta-analysis[4], which was recently updated[5]. This review compared T cell depleting therapy (ATG, both horse and rabbit, anti-lymphocyte globulin and muromonab-CD3) to pulse glucocorticoids as treatment of the first episode of kidney transplant AR. ATG therapy showed less failure of reversal of AR compared with pulse glucocorticoids (relative risk (RR) 0.40; 95%-confidence interval (CI) 0.22-0.74). Furthermore, allograft loss 18 months after AR was significantly less in the ATG group compared with patients treated with pulse glucocorticoids (RR 0.63, 95%-CI 0.44-0.89)[5].

The studies included in this systematic review had a wide variation in definition of outcomes. Not all studies reported the immunization status of the patients, details about trial methodology were often incompletely reported and most studies included only small numbers of patients[5]. Most importantly, because the studies were performed more than 2 decades ago, all patients received maintenance immunosuppressive treatment consisting of CsA and/or azathioprine. No patients received TAC plus MMF-based therapy.

The lack of data on the effectiveness of rATG in kidney transplant patients using TAC plus MMF-based immunosuppressive therapy and the availability of several new options to treat aTCMR, such as anti-CD52 therapy (alemtuzumab) and anti-CD20 therapy[2,6,7], prompted us to analyze the results of rATG for AR after kidney transplantation at our center. The objectives of this study were to: 1) investigate the long-term outcomes (patient survival, allograft survival and adverse events) and 2) characterize which patients were at greater risk for adverse outcome after rATG therapy for AR. The outcomes presented here could serve

as a basis for future studies that describe other anti-rejection therapies in patients treated with TAC and MMF.

MATERIALS AND METHODS

Study design and inclusion criteria

A retrospective analysis was conducted of all kidney transplant recipients who received rATG (Thymoglobulin®, Sanofi Genzyme, United States) because of AR between 2002 and 2012 in the Erasmus Medical Center. This specific period was chosen because from 2000 onwards, patients received TAC as the standard maintenance immunosuppression. After 2012, the anti-rejection protocol was changed and ever since patients have been treated with alemtuzumab in case of glucocorticoid-resistant or severe aTCMR. According to Dutch law, the present study did not require formal approval of the local medical ethical review board[8]. All AR episodes were proven by biopsy except for one. In this patient, no biopsy was performed. This case was included in all analyses except the analyses with the Banff classification. For the present study, all kidney allograft biopsies were revised by an experienced renal-pathologist (M.C.C-v.G) and categorized according to the Banff 2015 classification[9].

In the period 2002-2012, 1463 patients received a kidney transplantation at our center. Patients treated with rATG were identified by means of our kidney transplant registry and the electronic medication prescription system of our hospital pharmacy. Sixteen patients with blood group ABO-incompatible kidney transplantations who received rATG were excluded from the analysis.

Immunosuppressive protocol

The standard immunosuppressive regimen after 2009 included induction therapy with basiliximab (Simulect®, Novartis Pharma, Basel, Switzerland) 20 mg intravenously on days 0 and 4 after transplantation. Before 2009, induction therapy was not given in our center on a routine basis except to recipients of a deceased-after-circulatory-death donor kidney. The standard maintenance immunosuppressive regimen consisted of TAC (Prograf®, Astellas Pharma, Leiden, the Netherlands), MMF (Cellcept®, Roche Pharmaceuticals, Basel, Switzerland) and glucocorticoids.

Dosing of TAC was based on pre-dose concentrations (C_0). Target C_0 for tacrolimus were 10-15 µg/L (week 1-2), 8-12 µg/L (week 3-4), 5-10 µg/L (week 4-12), and 4-8 µg/L from

month 4 onwards. MMF was started at 1000 mg twice daily. Before 2010, the dose of MMF was adjusted if the patient experienced side effects, like gastro-intestinal complaints and leucopenia. From 2010, dosing of MMF was based on C_0, aiming for target C_0 of 1.5 – 3.0 mg/L. Glucocorticoids were given as an intravenous dose of 100 mg on days 0-3 and thereafter were started in a dose of 20 mg/day (days 4 – 20). Thereafter, glucocorticoids were tapered and completely withdrawn around month 4. Patients using other experimental immunosuppressive drugs as part of clinical studies were included in the current analysis.

Treatment of AR

Patients with AR were initially treated with intravenous methylprednisolone 1000 mg (Solu-Medrol®, Pfizer, New York, United States) daily for 3 consecutive days. Treatment with rATG was left at the discretion of the attending physician and was based on the effect of pulse glucocorticoids, severity of the AR (Banff category), previous transplant rejection, medical history, and patient immunization status. For a subgroup analysis, two reasons for rATG therapy were distinguished: 1) glucocorticoid-resistant and (2) severe AR. In glucocorticoid-resistant AR, patients were initially treated with pulse glucocorticoids. If the effect was not satisfactory, rATG therapy was administered subsequently. In patients with severe AR (based on Banff category and kidney function) rATG was given as first-line therapy or shortly after pulse glucocorticoids (without awaiting the full effect of glucocorticoids).

Rabbit ATG was administered in a high flow vein or central venous catheter as a single bolus (4 mg/kg [actual bodyweight, no maximum dose limit]) during 6 hours. The aim was an absolute whole blood CD3+ T cell count below 200 x 10^6/L for a duration of 2 weeks. If CD3+ T cell counts increased during this period, a repeat dose of rATG (4 mg/kg) was administered. Patients with an aABMR or a mixed type AR might be treated additionally with intravenous immunoglobulins, rituximab, or plasma-exchange, according to the KDIGO guideline and local protocol [1].

All patients received pre-medication prior to rATG administration: prednisolone 50 mg intravenously, 4 mg clemastine and 1000 mg acetaminophen. For 3-6 months, patients received *Pneumocystis jirovecii* prophylaxis (sulfamethoxazole/trimethoprim) and cytomegalovirus (CMV) prophylaxis ([val]ganciclovir or CMV immunoglobulins).

Outcomes

The following data were collected: baseline characteristics, anti-rejection therapy, rejection type and severity according to the Banff 2015 classification[9], allograft function (serum creatinine and estimated glomerular filtration rate (eGFR; Chronic Kidney Disease Epidemiology Collaboration [CKD-EPI][10] and proteinuria), allograft survival (censored for death) and serious adverse events. Baseline serum creatinine was defined as the lowest serum creatinine in the 3 months before AR. Baseline eGFR was defined as the highest eGFR in the 3 months before AR. Data on serum creatinine and eGFR were included in the analysis when the patient had a functioning allograft. The follow-up period for infection was from rATG administration until death, loss to follow-up, or re-transplantation. Malignancies and mortality were evaluated until last follow-up, which could be after a subsequent kidney transplantation. Allograft loss was defined as the need for dialysis or re-transplantation. In all patients who received a kidney transplant between 2002 and 2012 in our center, allograft- and patient survival were analyzed and compared with that of patients suffering from AR and requiring rATG therapy. The hospital information system, NKR (Netherlands Cancer Registry, https://www.cijfersoverkanker.nl/) and NOTR (Netherlands Organ Transplant Registry, https://www.transplantatiestichting.nl/) were used for data retrieval regarding the occurrence of malignancies. Infections were considered serious if the infection necessitated hospitalization or occurred during hospital admission for another reason. Viral infections were recorded. BK viremia was tested on indication. Unexplained fever, chills, hypotension, rash, dyspnea, lymphadenopathy, arthralgia or myalgia were considered serum sickness.

Statistical methods

Categorical variables are presented as number (percentage). Continuous variables are presented as mean with standard deviation for parametric variables or median with interquartile range (IQR) for non-parametric variables. For differences between paired samples the paired two-sample *t*-test or Wilcoxon signed rank test were used. For unpaired non-parametric continuous data, the Kruskal-Wallis test and Mann-Whitney U test were used. Allograft survival between groups was analyzed by means of Kaplan-Meier survival analysis. The influence of independent variables was analyzed with univariate Cox proportional hazard regression analysis. Because of the number of events (49 allograft losses), it was only possible to test a maximum number of variables mounting up to 5 degrees of freedom per analysis in the multivariate Cox proportional hazard regression analysis. The influence of the most significant variable was tested in the presence of all the other variables one by one in order of increasing *p*-values. Variables were eliminated from the model by backward elimination. They were replaced by other variables so that at last all variables

had been present in the model. A two-sided p-value < 0.05 was considered statistically significant. For statistical analysis, GraphPad Prism, version 5 (San Diego, CA, USA) and SPSS version 21 (SPSS Inc., Chicago, IL, USA) were used.

RESULTS

Patient demographics

A total of n = 108 episodes of AR requiring rATG therapy were identified in 103 patients (Table 1). Five patients were diagnosed with a second episode of AR in the same kidney transplant, which also required rATG treatment. Most rejections were aTCMR (Table 1).

Forty-five patients received induction therapy (Table 1). At the time of AR, the majority of patients were treated with combination therapy consisting of TAC, MMF, with or without glucocorticoids (72.2%). Six patients (5.6%) used a TAC-based immunosuppressive regimen and 20 patients (18.5%) were treated with a MMF-based immunosuppressive regimen (without TAC) at the time of rejection. TAC and glucocorticoid dosing was stable during the 2002-2012 study period. In contrast, MMF dosing, was significantly lower in the period 2010-2012 compared with 2002-2006: 1000 mg (IQR 1000-2000 mg) *versus* 2000 mg (IQR 1250-2000 mg; p = 0.04).

Efficacy

Allograft survival

Allograft survival and event-free survival (survival free from allograft loss or death) in rATG treated patients was significantly worse than in the total group of kidney transplant recipients without rATG treatment (p < 0.0001, HR 3.9, 95%-CI 2.6-5.8 and p < 0.0001, HR 15.9, 95%-CI 9.2-27.4, respectively; Figure 1A). In the year after rATG treatment, 28 patients (25.9%) experienced allograft loss, 5 of whom had primary non-functioning allografts (Figure 1A). In the full observation period (median 6.8 years, IQR 4.9-9.1) 49 patients lost their allograft. Median allograft survival of the total group was 7.0 years (Figure 1A).

Table 1. Baseline characteristics of patients requiring rATG because of AR

Characteristic	
Patients - no.	103
Kidney transplantations - no.	107
Recipient age - yr.	46 (35-56)
Donor age - yr.	54 (46-61)
Female sex - no. (%)	64 (62.1)
Cause of ESRD - no.	
DM/HTN/GN/PKD/reflux/other/unknown	23/10/18/16/17/16/3
Ethnic distribution - no.	
Caucasian/Black/Asian/Arab/other	70/16/5/5/7
Transplant number - no.	
1/2/3/4	76/25/5/1
Preemptive kidney transplantation - no. (%)	25 (23.4)
Donor type - no.	
LR/LUR/DBD/DCD	35/47/15/10
HLA mismatch	
HLA A: 0/1/2	21/60/23
HLA B: 0/1/2	11/55/38
HLA DR: 0/1/2	13/50/41
PRA actual - no. (%)	
0-5%	81 (77.1)
6-83%	21 (20)
84-100%	3 (2.9)
PRA peak - no. (%)	
0-5%	62 (59)
6-83%	32 (30.5)
84-100%	11 (9.5)
CMV IgG serostatus recipient	
Positive / negative	75/31
EBV IgG serostatus recipient	
Positive / negative	90/7
Induction therapy - no.	
None	62
Basiliximab/ ATG/ Daclizumab	33/10/2
Maintenance immunosuppression - no. (%)	
TAC/MMF/glucocorticoids	58 (53.7)
TAC/MMF	20 (18.5)
TAC + other (non-MMF)	6 (5.6)
MMF + other (non-TAC)	20 (18.5)
Other combinations	4 (3.7)
rATG administration - no.	108
DGF during rejection episode - no. (%)	19 (17.6)
Primary non-function - no. (%)	5 (4.6)
Time to rejection - days	24 (8-339)
Early rejection (<1 month) - no. (%)	56 (51.9)
Intermediate rejection (1-3 months) - no. (%)	8 (7.4)
Late rejection (>3 months) - no. (%)	44 (40.7)

Table 1. (Continued)

Characteristic	
Banff 2015 classification - no.*	
aTCMR	
aTCMR IA	6
aTCMR IB	8
aTCMR IIA	21
aTCMR IIB	20
aTCMR III	1
ABMR	
a/aABMR	12
c/aABMR	3
Mixed aTCMR with a/aABMR	
aTCMR IA	1
aTCMR IB	7
aTCMR IIA	2
aTCMR IIB	8
Mixed aTCMR with c/aABMR	
aTCMRIIA	1
C4d positive ABMR	18
C4d negative ABMR	10
No diagnosis after reclassification*	18
Anti-rejection therapy	
Methylprednisolone prior to rATG - no. (%)	93 (86.1)
Cumulative dose of methylprednisolone, mg	
1000/2000/3000/6000	2/9/79/3
Cumulative dose of rATG per patient, mg	555 (250-715)
Cumulative dose of rATG per patient, mg/kg	7.4
rATG number of gifts - no.	
1/2/3/4	30/62/15/1
Necessity for additional anti-rejection therapy < 3 months - no.	
Methylprednisolone	10
Intravenous immunoglobulins	6
Rituximab	3
Plasma exchange	3
Muromonab-CD3	1

Data are numbers (%) or median (interquartile range). Other kidney diseases included focal segmental glomerulosclerosis, hemolytic uremic syndrome, nephronophtisis, tuberous sclerosis or tubulo-interstitial nephritis. TAC + other regime contained combinations of TAC, glucocorticoids, sirolimus, everolimus, AEB071, or FTY720. MMF + other regime contained combinations of MMF, glucocorticoids, sirolimus, cyclosporine A, everolimus, or CP-690550. Other combinations existed of a combination of azathioprine, glucocorticoids, everolimus, cyclosporine A, AEB071, or FTY720.* Banff 2015 re-classification in 18 biopsies was not possible. Fifteen patients' biopsies were either missing from archives or there was insufficient material to allow for reclassification. In 3 patients, no histologic diagnosis of AR was made (although the clinical picture was strongly suspect for AR). ABMR antibody mediated rejection; a/aABMR acute/active antibody mediated rejection; aTCMR acute T cell mediated rejection; c/aABMR chronic/active antibody mediated rejection; CMV cytomegalovirus; DBD donation after brain death; DCD donation after circulatory death; DGF Delayed graft function (need for dialysis in the first week after transplantation); DM diabetes mellitus; EBV Epstein-Barr virus; ESRD end stage renal disease; GN glomerulonephritis; HTN hypertensive nephropathy; LR living related; LUR living unrelated; MMF mycophenolate mofetil; PKD polycystic kidney disease; PRA panel reactive antibody; rATG rabbit anti-thymocyte globulin; TAC tacrolimus.

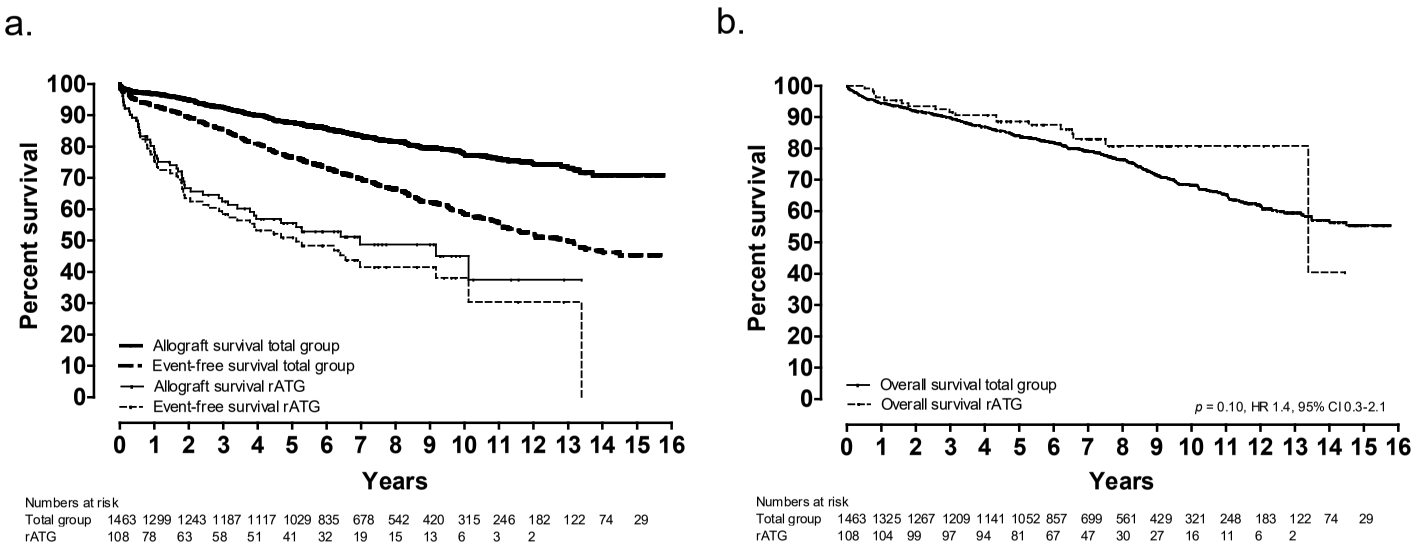


Figure 1. Event-free survival, overall survival and allograft survival curves of patients treated with rATG because of AR. (A) Event-free survival and allograft survival censored for death of patients treated with rATG for AR and all patients transplanted with a kidney and not treated with rATG between 2002 and 2012. Event-free survival is time from transplantation (in case of the total group of patients) or AR (in case of rATG treated patients) to death or allograft loss. Event-free survival of the total group of patients *versus* patients treated with rATG: $p < 0.0001$, HR 3.9, 95%-CI 2.6-5.8. Allograft survival of the total group *versus* patients treated with rATG; $p < 0.0001$, HR 15.9, 95%-CI 9.2-27.4. **(B)** Overall survival of all patients transplanted with a kidney between 2002 and 2012 and patients treated with rATG for AR; $p = 0.10$, HR 1.4, 95%-CI 0.3-2.1.

In univariate Cox proportional hazard analysis, two variates had a significant influence on death-censored allograft survival: timing of AR and glucocorticoid use during AR (Table 2). Allograft survival was significantly better in the patients with early AR (<1 month) than in patients with late AR (>3 months; $p < 0.0001$, HR 3.64, 95%-CI 1.97-6.72) (Table 2 and Figure 2A). Allograft survival was not significantly different between intermediate (1-3 months after transplantation) and late AR ($p = 0.50$; data not shown). Allograft survival was better in patients using glucocorticoids as part of the maintenance immunosuppressive therapy during AR ($p < 0.0001$, HR 0.40, 95%-CI 0.22-0.72; Table 2). Glucocorticoids were significantly more often used during AR in patients with early rejections (98%) compared to late rejections (42%; $p < 0.001$).

Table 2. Results of the univariate cox proportional hazards analysis

Variable (reference category)	Exp (B)	95% CI for Exp (B)	*p*-value
Patient characteristics			
Recipient age at transplantation (per yr)	0.99	0.97-1.01	0.32
Recipient age at acute rejection (per yr)	0.99	0.97-1.01	0.47
Donor age (per yr)	0.99	0.97-1.02	0.54
Gender (female)	0.95	0.54-1.68	0.86
Race (Caucasian)	0.86	0.46-1.59	0.63
Transplant number (1)	0.84	0.45-1.57	0.59
PRA current (<6%)	1.13	0.58-2.20	0.73
PRA (per %)	1.00	0.99-1.01	1.00
Transplant characteristics			
Type donor (living donor)	1.36	0.69-2.68	0.37
HLA mismatch (per HLA mismatch)	0.94	0.78-1.14	0.54
Therapy characteristics			
Induction therapy (no)	0.90	0.50-1.63	0.74
Maintenance therapy (TAC+MMF)	0.70	0.38-1.27	0.23
Glucocorticoid maintenance (no)	0.40	0.22-0.72	<0.0001
Rejection characteristics			
Timing rejection (< 1 month)			<0.0001
1-3 months	1.54	0.45-5.22	0.49
>3 months	3.64	1.97-6.72	<0.0001
Type rejection (aTCMR I)			0.55
CKD at time rejection (CKD 3b)			0.64
Reason rATG (GC resistant rejection)	0.88	0.50-1.54	0.65

Univariate analysis of the risk of allograft loss with hazard ratio (Exp(B), 95% confidence interval and p-value. Race is caucasian or non-caucasian. Transplant number is 1 or >1. PRA current is < 6% or ≥6%. Type donor is living or postmortal. Maintenance therapy is TAC+MMF or TAC+other and MMF+other. Glucocorticoid use at the time of rejection. Type rejection is aTCMR I, aTCMR II+III, ABMR, or mixed. CDK at time rejection is CKD3b, CKD4, CKD5, or delayed graft function. Reason rATG is glucocorticoid (GC) resistant or severe rejection.

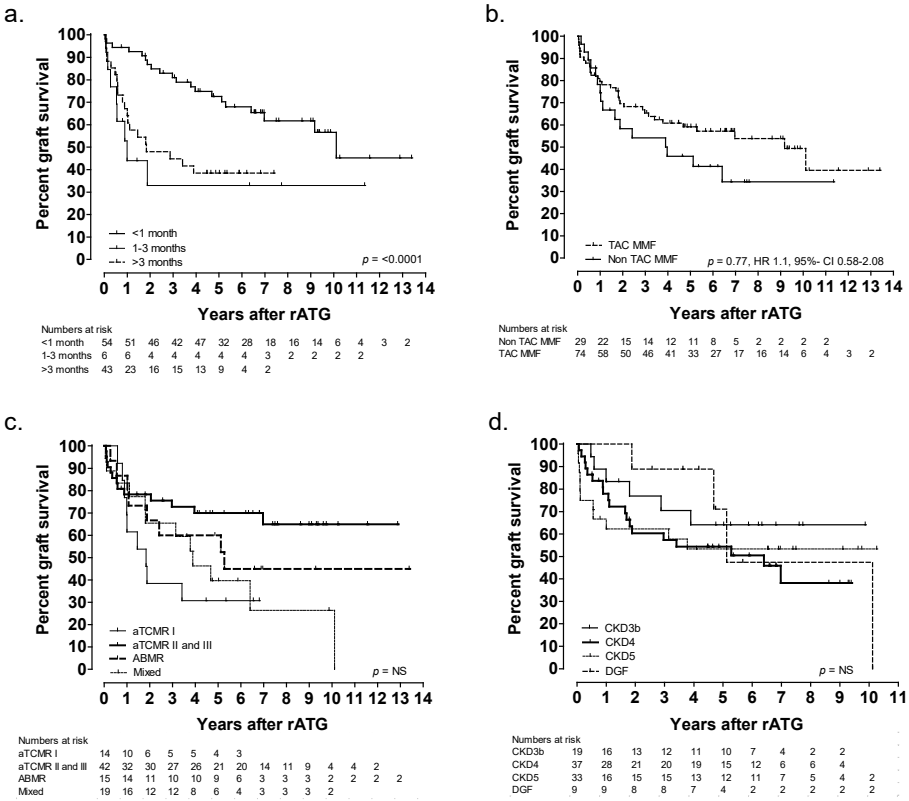


Figure 2. Allograft survival curves of different subgroups of patients treated with rATG for AR. (A) Death-censored allograft survival in early (< 1 month after transplantation), intermediate (1-3 months) and late AR (> 3 months). **(B)** Death-censored allograft survival of patients using the combination of maintenance immunosuppression TAC/MMF *versus* patients using other combinations of immunosuppression. **(C)** Death-censored allograft survival of patients after rATG therapy grouped by the categories of the Banff 2015 classification. aTCMR I = acute T cell mediated rejection grade IA+IB, aTCMR II and III = acute T cell mediated rejection grade II and III. ABMR = acute and chronic active antibody mediated rejection, mixed = patients with a mixed AR (aTCMR and aABMR). **(D)** Death-censored allograft survival grouped by chronic kidney disease (CKD) stage. CKD3b = 30-45 mL/min, CKD4 = 15-30 mL/min, CKD5 = < 15 mL/min. *DGF* - Delayed graft function (need for dialysis in the first week after transplantation) NS - not significant

Death-censored allograft survival of patients using TAC/MMF (± glucocorticoids) and patients using other combinations of immunosuppressive drugs was comparable (p = 0.23, HR 0.70, 95%-CI 0.38-1.27; Table 2 and Figure 2B). Furthermore, no difference in allograft survival was seen between aTCMR grade I, aTCMR grade II+III, aABMR and mixed-type AR (Figure 2C; p = 0.55; Table 2). Remarkably, death-censored allograft survival was comparable between all CKD stages (CKD 3b, CKD 4, CKD 5) and delayed graft function (DGF; Figure 2D and Table 2, p = 0.64).

Sixty-two patients (57.4%) received rATG because of glucocorticoid-resistant AR, whereas 46 patients (42.6%) were treated with rATG because of severe AR. Death-censored allograft survival were comparable between the 2 groups (p = 0.65, HR 0.88, 95%-CI 0.50-1.54; Table 2 and Supplementary Figure 1).

In a multivariate analysis, the influence of timing of AR on allograft survival in presence of each variable was tested. Apart from timing of AR, no other variables showed a significant influence on allograft survival. This means that the final result of multivariate analysis is the same as that of univariate analysis of the influence of timing of AR (Table 2).

Allograft function

Rabbit ATG efficacy was also reflected in kidney function. One year after rATG therapy, kidney function (median serum creatinine) in the patients without allograft loss in the first year was comparable to baseline kidney function (p = 0.22; Figure 3). Twelve months after rATG, 33.9% of patients had a serum creatinine comparable to baseline level (± 25%), 43.5% of patients showed an increase of serum creatinine of more than 25%, and 22.6% of patients showed a decrease in serum creatinine of more than 25% compared to baseline (data not shown). The urine protein/creatinine ratio (UPCR) was significantly higher at the moment of rejection compared to baseline level (p < 0.001, Figure 3C). However, the UPCR had normalized to baseline level at 3 months after transplantation (Figure 3C).

Besides better allograft survival, the patients with early AR also had a significantly lower serum creatinine 12 months after AR compared to the patients with late AR (157 µmol/L [IQR 131-203] *versus* 216 µmol/L [IQR 165-269], respectively, p < 0.05; Table 3). In patients with late AR, serum creatinine 12 months after AR did not return to baseline: 216 µmol/L *versus* 148 µmol/L, respectively (p < 0.001; Table 3).

Table 3. Serum creatinine of the subgroups

	Baseline		**rATG**		**M12**	
Subgroups	Median (IQR)	Number	Median (IQR)	Number	Median (IQR)	Number
Banff classification						
aTCMR IA and IB	148 (208-299)	13	299 (229,5-382)[a]	13	248 (182-372)[b]	8
aTCMR IIA, IIB and III	165 (132-223)	39	270 (212-412)[a]	39	144 (132-190)	31
ABMR	153 (114-182)	11	224 (186-274)[a]	10	203 (140-320)	13
Mixed	143 (111-163)	19	211 (166-245)[a]	13	205 (140-239)	15
Reason for rATG therapy						
GC-resistant rejection	152 (111-176)	55	247 (198-367)[a]	55	180 (142-237)[a]	42
Severe rejection	163 (132-223)	35	285 (222-445)[a]	33	171 (129-223)	33
CKD stage						
CKD 3b	148 (116-162)	18	182 (165-200)[acd]	18	182 (131-208)	15
CKD 4	164 (124-207)	37	247 (224-276)[ad]	37	186 (140-252)[a]	25
CKD 5	157 (132-255)	19	428 (367-626)[a]	19	157 (122-203)	15
DGF	DGF	19	DGF	19	170 (137-212)	14
Timing of rejection						
<1 month	165 (141-220)[ef]	37	270 (223-420)[a]	37	157 (131-203)[f]	49
1-3 months	130 (98-152)	7	210 (193-412)[a]	7	182 (135-234)	4
>3 months	148 (109-174)	42	247 (200-340)[a]	40	216 (165-269)[a]	22

Data are median (interquartile range) and number of patients with available serum creatinine (µmol/l) at that specific time point. [a] Significantly different ($p < 0.05$) compared with baseline, [b] Significantly different ($p < 0.05$) compared with aTCMR II and III at M12, [c] Significantly different ($p < 0.05$) compared with CKD4, [d] Significantly different ($p < 0.05$) compared with CKD5, [e] Significantly different ($p < 0.05$) compared with 1-3 months, [f] Significantly different ($p < 0.05$) compared with > 3 months. aTCMR I= Acute T cell mediated rejection grade IA+IB; aTCMR II and III= Acute T cell mediated rejection grade IIA, IIIB and III; ABMR = acute and chronic active antibody mediated rejection; Mixed = Patients with a mixed rejection (aTCMR and aABMR); M12 - 12 months (±8 weeks) after rATG therapy; GC Glucocorticoid-resistant

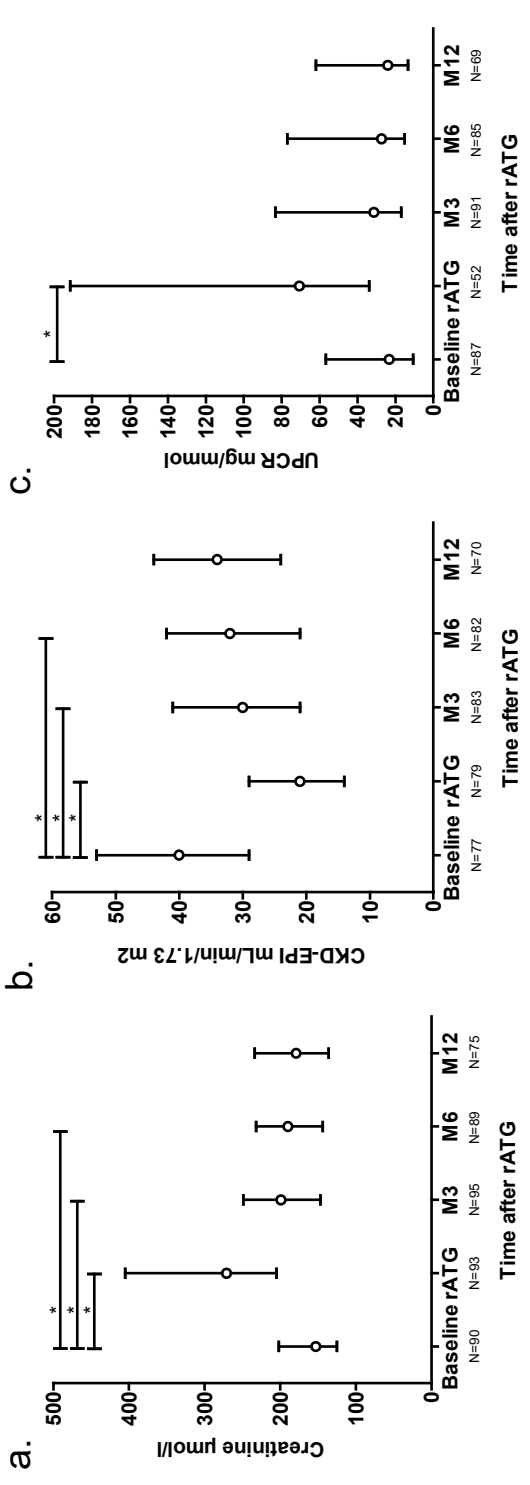


Figure 3. Serum creatinine (µmol/L), creatinine clearance (CKD-EPI, mL/min/1.73 m^2) and urine protein to creatinine ratio (UPCR) before and after AR and rATG therapy. Data are median and IQR. M3- 3 months after rATG ± 4 weeks. M6- 6 months after rATG ± 6 weeks. M12 - 12 months after rATG ± 8 weeks. *Significantly different compared with baseline ($p < 0.05$). N - number of patients with laboratory parameters at that specific time point.

Serum creatinine was comparable between patients with various types of AR at baseline and at time of diagnosis (Table 3). Twelve months after rATG therapy, serum creatinine in patients aTCMR grade I was significantly higher than in patients with aTCMR grade II and III: 248 µmol/L *versus* 144 µmol/L, respectively ($p < 0.05$; Table 3) in the patients without allograft loss in the first year. Allograft function at 12 months was comparable between the other groups (Table 3). The interval between pulse glucocorticoids and rATG was significantly longer in patients with aTCMR grade I than in patients with aTCMR grade II and III: 31 days (IQR 23-92) *versus* 8 days (IQR 3-15; $p < 0.05$).

In patients with glucocorticoid-resistant AR, serum creatinine after 12 months did not return to baseline (180 µmol/L *versus* 152 µmol/L, $p = 0.04$) in the patients without allograft loss in the first year after rejection (Table 3). The interval between pulse glucocorticoids and rATG was significantly longer in patients with glucocorticoid-resistant AR than in patients with severe AR (15 days (IQR 5-27) *versus* 4 days (1-9); $p = 0.0003$).

Serum creatinine in the patients with a functioning allograft 1 year after rATG was comparable between all CKD stages (CKD 3b, CKD 4, CKD 5) and DGF (Table 3). In the DGF group, all patients had a functioning allograft after 1 year and median serum creatinine was 170 µmol/L.

Complications

Adverse events and mortality

The overall survival of the patients treated with rATG for AR was similar to the overall survival of all patients who received a kidney transplantation between 2002 and 2012 in our center after exclusion of those treated with rATG ($p = 0.10$, HR 1.4, 95%-CI 0.3-2.1; Figure 1B).

Median length of hospital stay after rATG infusion was 15 days (IQR 13-19). Five (7.4%) patients were transferred to the intensive care unit because of hemodynamic instability (Table 4). None of these patients died during the intensive care unit stay. Six (5.6%) experienced serum sickness after rATG treatment and one patient cytokine release syndrome.

A significant drop in hemoglobin, thrombocytes and leukocytes was seen after rATG therapy (Supplementary Figure 2). T cells dropped from 0.54 x 10^9/L to a minimum of 0.01 x 10^9/L in the first week ($p = 0.01$). After 4 weeks, T cell count was still significantly lower than before rATG therapy (0.11 x 10^9/L, $p = 0.001$; data not shown).

Table 4. Adverse events

Adverse events	
Serum sickness - no. (%)	6 (5.6)
Cytokine release syndrome - no. (%)	1 (0.9)
Fever - no. (%)	42 (61.8)
Interventions - no. (%)	
Transfer to ICU	5 (4.6)
Infection in the first year after rATG - no.	124
Viral	19
Fungal	8
Bacterial	97
CMV infections	
CMV reactivation - no. (%)	27 (25)
Primary CMV infection - no. (%)	0 (0)
EBV infections	
EBV reactivation - no. (%)	4 (3.7)
Primary EBV infection - no. (%)	1 (0.9)
BK infections	
BK viremia - no. (%)	6 (5.6)
Malignancy	
Number	14
Time after rATG therapy - months	63 (45)

Data are numbers (percentage), median (IQR), or mean (standard deviation) *Clinical data of the first 24 hours after rATG administration was retrieved from 67 patients. Fever was defined as temperature above 38.5°C. CMV cytomegalovirus; EBV Epstein-Barr virus. The types of malignancies were endometrial carcinoma, adenocarcinoma of the lung, non-seminoma testis, colon carcinoma, renal carcinoma, meningeoma, prostatic cancer, non-Hodgkin lymphoma and EBV related lymphoma. The EBV-related lymphoma was in an IgG seropositive patient and occurred fourteen months after treatment with rATG and was treated with irradiation.

Infections

A total of 124 serious infections were recorded in the first year following rATG therapy (median time after rATG 44 days, IQR 10-157). The most common infections were urinary tract infections and pneumonia (Table 4). In 4 of 15 patients with pneumonia, *Pneumocystis jiroveci* was the causative pathogen. One patient died of *Pneumocystis jiroveci* pneumonia and one patient died of *Candida* sepsis 6 months after rATG therapy.

Median duration of follow-up for viral infections was 4.7 years (IQR 2-6.9). CMV reactivation occurred in 25% of patients (Table 4). One patient was diagnosed with CMV colitis and another with CMV retinitis. Four reactivations and 1 primo infection of Epstein-Barr virus (EBV) occurred (Table 4).

Malignancy

Median duration of follow-up for malignancies was 6.8 years (IQR 4.9-9.1). Twelve primary solid tumors occurred in 11 patients and two patients developed a lymphoma after a mean follow up of 63 months (standard deviation 45; Table 4). In addition, 11 basal cell carcinomas and 4 squamous cell carcinomas were diagnosed in 6 patients after a median of 107 months (IQR 60-117).

DISCUSSION

Rabbit ATG is a purified polyclonal immunoglobulin fraction obtained from sera of rabbits immunized with human thymocytes[11]. Administration of rATG leads to a fast and profound depletion of T cells and to a lesser extent, B cells which lasts for several months[11,12]. Rabbit ATG also modulates T cell activation by downregulation of molecules that control T cell activation[12]. Repopulation of lymphocytes occurs through homeostatic proliferation of CD4+ and CD8+ memory cells with a senescent and exhausted functional profile[13,14].

Here, the long-term outcomes and adverse events are described of treatment of AR with rATG in patients using the current standard immunosuppressive therapy. In this cohort, overall 5-year patient survival after rATG treatment for AR was 89% and was similar to the overall survival of all kidney transplant patients transplanted in our center between 2002-2012 and who did not receive treatment with rATG. In comparison, literature reported a 5-year patient survival (with and without AR) after deceased donor kidney transplantation and living donor kidney transplantation of 91.8% and 95.6%, respectively[15]. In a systematic review, ATG therapy for AR was not associated with increased mortality after one year compared to therapy with pulse glucocorticoids[5]. Our findings support the notion that survival is not affected by rATG in everyday clinical practice.

One year after rATG therapy, 78.2% of all patients had a functioning allograft and 5-year allograft survival was 55.6%. The allograft survival reported here is inferior to that described in 4 other studies where rATG was used as anti-rejection therapy. Two studies describing patients who received rATG as first-line anti-rejection therapy (cumulative dose of rATG 10.5-21 mg/kg)[16] and for glucocorticoid-resistant AR (cumulative dose of rATG 7.5 mg/kg)[17] showed one-year allograft survival rates of 83% and 89%, respectively. Two other studies demonstrated 5-year allograft survival rates of 78% (cumulative dose rATG 40 mg/kg)[18] and 74% (cumulative dose of rATG not reported)[19] in patients treated with ATG as first-line treatment for AR. These studies are not entirely comparable to ours because

in these studies, patients were mainly treated with azathioprine and CsA, rATG was used as first-line therapy in three of the four studies, and the cumulative dose of rATG was not similar (being lower in the present study with a cumulative dose of 7.4 mg/kg). Because the efficacy of rATG is dose-dependent[20,21], the differences in the cumulative dose of rATG between the other studies and the present study can influence allograft survival, Given the favorable outcomes of these older studies, perhaps we should have used rATG sooner and in higher dose.

Various parameters of AR determined the risk for allograft loss and recovery of kidney function 1 year after rATG treatment, including timing, Banff grade of rejection and the reason for rATG therapy (severe or glucocorticoid-resistant AR). Allograft survival and serum creatinine at 12 months were superior in patients with early AR *versus* late AR. This has also been described by others[22-25]. Late AR is different from early AR for two reasons. First, late AR occurs in patients who visit the outpatient clinic less frequently and with intervals of 1-4 months, likely leading to a delay in diagnosis. Second, a major proportion of late AR may have been related to non-adherence to immunosuppressive drugs. Furthermore, late AR is associated with the formation of *de novo* donor-anti-HLA antibodies and the development of aABMR for which no proven therapy exits[26-28].

Death-censored allograft survival was comparable for aTCMR I, aTCMR II+III, ABMR and mixed-type rejection. Surprisingly, kidney function after 12 months in patients with aTCMR grade II+III was superior to those with aTCMR grade I. Allograft survival rates according to Banff grade rejection have been described by others[29,30] and showed better allograft survival in patients with aTCMR I than in patients with aTCMR II and III. Our surprising finding may have resulted from a longer interval between pulse glucocorticoids and rATG in patients with aTCMR grade I. This may have been caused by reluctance of nephrologists to treat with rATG because of fear for complications. This delay may have resulted in more irreversible damage due to ongoing AR. Other possible explanations are that the attending physician did not intensify the maintenance immunosuppression after rATG treatment in the group with aTCMR grade I leading to ongoing and subclinical AR. Based on these results, we suggest treatment of patients with aTCMR grade I in whom kidney function does not improve after pulse glucocorticoids should be more aggressive and the administration of rATG not to be delayed too long. The choice to prescribe rATG may be guided by a repeat kidney transplant biopsy. However, in this study, no data were collected of patients with aTCMR grade I treated with pulse glucocorticoids only, so we may have excluded the population with the most favorable prognosis.

The allograft survival of patients with ABMR is worse compared with patients experiencing TCMR[31]. In this study, no significant difference was seen in the allograft survival of patients with aTCMR or ABMR. A possible explanation for this surprising finding may be the fact that the number of patients with ABMR was small which may have resulted in limited statistical power to detect any difference in allograft survival.

Serum creatinine was significantly higher in patients with glucocorticoid-resistant AR compared to patients with severe AR. The interval between pulse glucocorticoids and rATG in patients with a glucocorticoid-resistant AR was 15 days. Possibly, kidney function may have been better if rATG had been given sooner. A multivariate prediction model, using intra-graft mRNA expression of immune and non-immune biomarkers, designed to predict which patients will not respond to pulse glucocorticoid therapy may serve as a tool to guide the type of anti-rejection therapy[32].

Despite the efficacy of rATG for AR, treatment with rATG is associated with considerable toxicity and morbidity. In this study, 5 patients were transferred to the intensive care unit and 6.5% of patients experienced serum sickness. Other studies described an incidence of serum sickness between 1.7 and 28%[17,33-36]. These infusion-related side-effects are the reason that certain patients cannot be treated with rATG (*e.g.*, those with cardiac failure or fluid overload). Alternative treatment, such as alemtuzumab, is indicated in these patients[37,38]. Besides the infusion-related events, the rATG-treated patients experienced 124 serious infections in the first year after rATG treatment and 25% of patients suffered from CMV-related complications.

Although this is the largest cohort of patients treated with rATG for AR in the era of current standard immunosuppressive medicine, we realize that this study is heterogeneous and single-center. However, and unlike in clinical trials, this study illustrates the long-term outcomes in real life and not in highly selected subpopulations. We think this study provides more insight in the long-term outcomes of rATG therapy for AR in de modern era of immunosuppressive therapy. Future prospective studies should include a comparator and focus on the optimal dosing of rATG to better weigh the benefits and risks[3].

This study has limitations. Due to its retrospective character, not all patients treated with rATG may have been included. Since patients were identified by means of our institution's transplant database and the hospital pharmacy records; we believe not many patients were missed. Second, some clinical parameters could not be retrieved and data on long-term

outcomes may have been missed if patients were admitted to other hospitals. Third, in the first period of the study, DSA (donor-specific antibodies) were not routinely tested in patients with AR in our hospital. Because of the incomplete data on the presence or absence of DSA, no meaningful analysis into the role of DSA could be performed. Fourth, follow-up biopsies after rATG therapy to evaluate for post-AR treatment changes, like ongoing inflammation or interstitial fibrosis, were not routinely performed. Finally, although all patients received the current gold standard immunosuppression (TAC plus MMF), subtle changes may have occurred over the study period. At the beginning of the 21st century, many of our patients received immunosuppression-minimizing treatment[39-41], whereas in the more recent era and with the recognition of aABMR as an important cause of allograft loss, we may have aimed for higher TAC exposure and have become more careful when considering glucocorticoid minimization. The median dose of MMF was lower in the period 2010-2012 compared with the period 2002-2006. Possibly, the introduction of therapeutic drug monitoring for mycophenolic acid in our clinic led to the difference in MMF dosing[42]. The retrospective design of the present study precluded a meaningful analysis of any such trend.

CONCLUSION

Rabbit ATG is an effective anti-rejection treatment in patients using current standard immunosuppressive therapy, even in patients with poor allograft function. Treatment with rATG for AR does not seem to be associated with increased mortality although it is associated with considerable toxicity, especially CMV-related complications. Timing of rATG therapy is important. Early recognition of severe and /or glucocorticoid-resistant AR followed by aggressive treatment leads to better allograft function and allograft survival. When this window of opportunity is used, the benefits may outweigh the risks.

REFERENCES

1. Tai E, Chapman JR. The KDIGO review of the care of renal transplant recipient. *Pol Arch Med Wewn.* 2010;120(6):237-242.
2. van den Hoogen MW, Hilbrands LB. Use of monoclonal antibodies in renal transplantation. *Immunotherapy.* 2011;3(7):871-880.
3. Bamoulid J, Staeck O, Crepin T, et al. Anti-thymocyte globulins in kidney transplantation: focus on current indications and long-term immunological side effects. *Nephrol Dial Transplant.* 2017;32(10):1601-1608.
4. Webster AC, Pankhurst T, Rinaldi F, Chapman JR, Craig JC. Monoclonal and polyclonal antibody therapy for treating acute rejection in kidney transplant recipients: a systematic review of randomized trial data. *Transplantation.* 2006;81(7):953-965.
5. Webster AC, Wu S, Tallapragada K, et al. Polyclonal and monoclonal antibodies for treating acute rejection episodes in kidney transplant recipients. *Cochrane Database Syst Rev.* 2017;7:CD004756.
6. Sautenet B, Blancho G, Buchler M, et al. One-year Results of the Effects of Rituximab on Acute Antibody-Mediated Rejection in Renal Transplantation: RITUX ERAH, a Multicenter Double-blind Randomized Placebo-controlled Trial. *Transplantation.* 2016;100(2):391-399.
7. Zarkhin V, Li L, Kambham N, et al. A randomized, prospective trial of rituximab for acute rejection in pediatric renal transplantation. *Am J Transplant.* 2008;8(12):2607-2617.
8. Halloran PF, Famulski K, Reeve J. The molecular phenotypes of rejection in kidney transplant biopsies. *Curr Opin Organ Transplant.* 2015;20(3):359-367.
9. Loupy A, Haas M, Solez K, et al. The Banff 2015 Kidney Meeting Report: Current Challenges in Rejection Classification and Prospects for Adopting Molecular Pathology. *Am J Transplant.* 2017;17(1):28-41.
10. Levey AS, Stevens LA, Schmid CH, et al. A new equation to estimate glomerular filtration rate. *Ann Intern Med.* 2009;150(9):604-612.
11. Admiraal R, Jol-van der Zijde CM, Furtado Silva JM, et al. Population Pharmacokinetics of Alemtuzumab (Campath) in Pediatric Hematopoietic Cell Transplantation: Towards Individualized Dosing to Improve Outcome. *Clin Pharmacokinet.* 2019.
12. Preville X, Flacher M, LeMauff B, et al. Mechanisms involved in antithymocyte globulin immunosuppressive activity in a nonhuman primate model. *Transplantation.* 2001;71(3):460-468.
13. Bouvy AP, Klepper M, Kho MM, et al. T cells Exhibit Reduced Signal Transducer and Activator of Transcription 5 Phosphorylation and Upregulated Coinhibitory Molecule Expression After Kidney Transplantation. *Transplantation.* 2015;99(9):1995-2003.
14. Bouvy AP, Kho MM, Klepper M, et al. Kinetics of homeostatic proliferation and thymopoiesis after rATG induction therapy in kidney transplant patients. *Transplantation.* 2013;96(10):904-913.
15. Neuberger JM, Bechstein WO, Kuypers DR, et al. Practical Recommendations for Long-term Management of Modifiable Risks in Kidney and Liver Transplant Recipients: A Guidance Report and Clinical Checklist by the Consensus on Managing Modifiable Risk in Transplantation (COMMIT) Group. *Transplantation.* 2017;101(4S Suppl 2):S1-S56.

16. Gaber AO, First MR, Tesi RJ, et al. Results of the double-blind, randomized, multicenter, phase III clinical trial of Thymoglobulin versus Atgam in the treatment of acute graft rejection episodes after renal transplantation. *Transplantation.* 1998;66(1):29-37.

17. Mariat C, Alamartine E, Diab N, et al. A randomized prospective study comparing low-dose OKT3 to low-dose ATG for the treatment of acute steroid-resistant rejection episodes in kidney transplant recipients. *Transpl Int.* 1998;11(3):231-236.

18. Baldi A, Malaise J, Mourad M, Squifflet JP. A prospective randomized study comparing poly-ATG to mono-OKT3 clonal antibodies for the first rejection therapy after kidney transplantation: long-term results. *Transplant Proc.* 2000;32(2):429-431.

19. Kainz A, Korbely R, Soleiman A, Mayer B, Oberbauer R. Antithymocyte globulin use for treatment of biopsy confirmed acute rejection is associated with prolonged renal allograft survival. *Transpl Int.* 2010;23(1):64-70.

20. Mohty M, Bacigalupo A, Saliba F, et al. New directions for rabbit antithymocyte globulin (Thymoglobulin((R))) in solid organ transplants, stem cell transplants and autoimmunity. *Drugs.* 2014;74(14):1605-1634.

21. Kho MM, Bouvy AP, Cadogan M, et al. The effect of low and ultra-low dosages Thymoglobulin on peripheral T, B and NK cells in kidney transplant recipients. *Transpl Immunol.* 2012;26(4):186-190.

22. Basadonna GP, Matas AJ, Gillingham KJ, et al. Early versus late acute renal allograft rejection: impact on chronic rejection. *Transplantation.* 1993;55(5):993-995.

23. Joseph JT, Kingsmore DB, Junor BJ, et al. The impact of late acute rejection after cadaveric kidney transplantation. *Clin Transplant.* 2001;15(4):221-227.

24. Sijpkens YW, Doxiadis, II, Mallat MJ, et al. Early versus late acute rejection episodes in renal transplantation. *Transplantation.* 2003;75(2):204-208.

25. Krisl JC, Alloway RR, Shield AR, et al. Acute Rejection Clinically Defined Phenotypes Correlate With Long-term Renal Allograft Survival. *Transplantation.* 2015;99(10):2167-2173.

26. Vlaminck H, Maes B, Evers G, et al. Prospective study on late consequences of subclinical non-compliance with immunosuppressive therapy in renal transplant patients. *Am J Transplant.* 2004;4(9):1509-1513.

27. Denhaerynck K, Dobbels F, Cleemput I, et al. Prevalence, consequences, and determinants of nonadherence in adult renal transplant patients: a literature review. *Transpl Int.* 2005;18(10):1121-1133.

28. Hidalgo LG, Campbell PM, Sis B, et al. De novo donor-specific antibody at the time of kidney transplant biopsy associates with microvascular pathology and late graft failure. *Am J Transplant.* 2009;9(11):2532-2541.

29. Wu K, Budde K, Lu H, et al. The severity of acute cellular rejection defined by Banff classification is associated with kidney allograft outcomes. *Transplantation.* 2014;97(11):1146-1154.

30. Lamarche C, Cote JM, Senecal L, Cardinal H. Efficacy of Acute Cellular Rejection Treatment According to Banff Score in Kidney Transplant Recipients: A Systematic Review. *Transplant Direct.* 2016;2(12):e115.

31. Halloran PF, Chang J, Famulski K, et al. Disappearance of T Cell-Mediated Rejection Despite Continued Antibody-Mediated Rejection in Late Kidney Transplant Recipients. *J Am Soc Nephrol.* 2015;26(7):1711-1720.

32. Rekers NV, de Fijter JW, Claas FH, Eikmans M. Mechanisms and risk assessment of steroid resistance in acute kidney transplant rejection. *Transpl Immunol.* 2016;38:3-14.

33. Lebranchu Y, Bridoux F, Buchler M, et al. Immunoprophylaxis with basiliximab compared with antithymocyte globulin in renal transplant patients receiving MMF-containing triple therapy. *Am J Transplant.* 2002;2(1):48-56.

34. Soulillou JP, Cantarovich D, Le Mauff B, et al. Randomized controlled trial of a monoclonal antibody against the interleukin-2 receptor (33B3.1) as compared with rabbit antithymocyte globulin for prophylaxis against rejection of renal allografts. *N Engl J Med.* 1990;322(17):1175-1182.

35. Buchler M, Hurault de Ligny B, Madec C, Lebranchu Y, French Thymoglobuline Pharmacovigilance Study G. Induction therapy by anti-thymocyte globulin (rabbit) in renal transplantation: a 1-yr follow-up of safety and efficacy. *Clin Transplant.* 2003;17(6):539-545.

36. Tanriover B, Chuang P, Fishbach B, et al. Polyclonal antibody-induced serum sickness in renal transplant recipients: treatment with therapeutic plasma exchange. *Transplantation.* 2005;80(2):279-281.

37. van den Hoogen MW, Hesselink DA, van Son WJ, Weimar W, Hilbrands LB. Treatment of steroid-resistant acute renal allograft rejection with alemtuzumab. *Am J Transplant.* 2013;13(1):192-196.

38. van der Zwan M, Baan CC, van Gelder T, Hesselink DA. Review of the Clinical Pharmacokinetics and Pharmacodynamics of Alemtuzumab and Its Use in Kidney Transplantation. *Clin Pharmacokinet.* 2017.

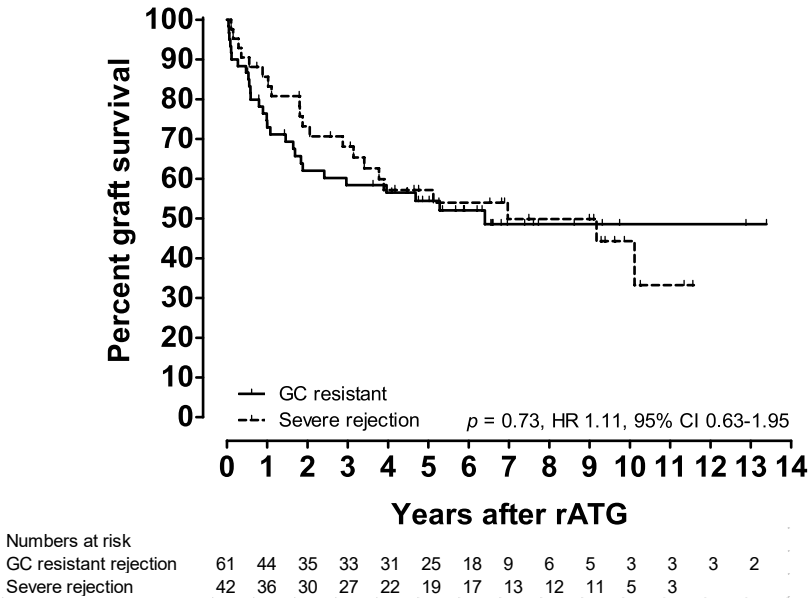
39. Roodnat JI, Hilbrands LB, Hene RJ, et al. 15-year follow-up of a multicenter, randomized, calcineurin inhibitor withdrawal study in kidney transplantation. *Transplantation.* 2014;98(1):47-53.

40. Smak Gregoor PJ, de Sevaux RG, Ligtenberg G, et al. Withdrawal of cyclosporine or prednisone six months after kidney transplantation in patients on triple drug therapy: a randomized, prospective, multicenter study. *J Am Soc Nephrol.* 2002;13(5):1365-1373.

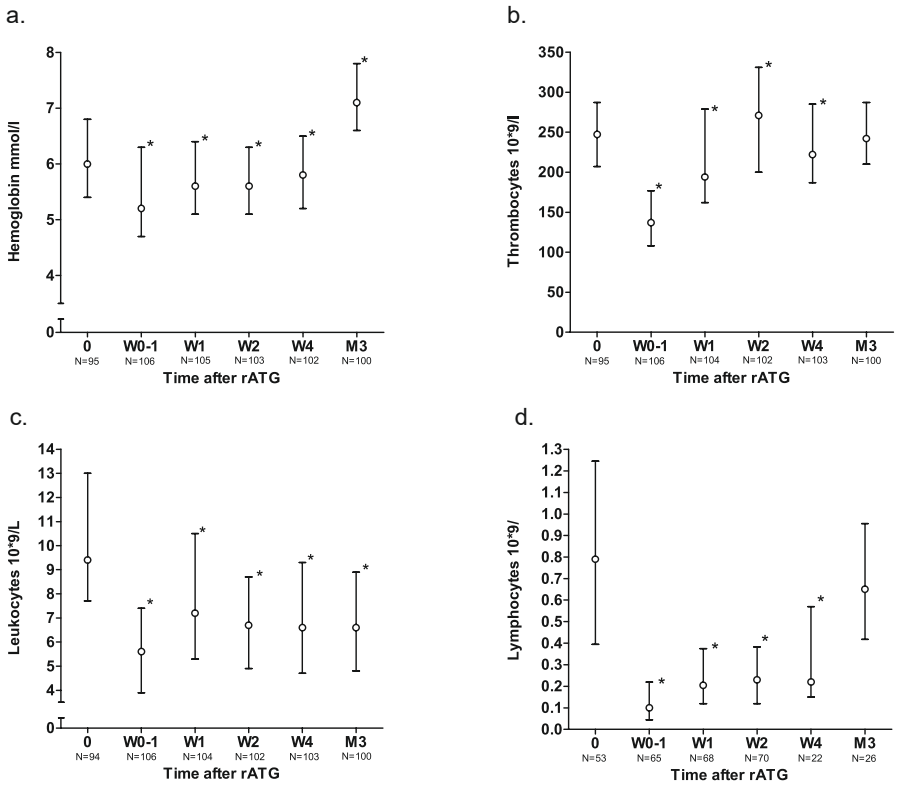
41. de Sevaux RG, Gregoor PJ, Hene RJ, et al. A controlled trial comparing two doses of cyclosporine in conjunction with mycophenolate mofetil and corticosteroids. *J Am Soc Nephrol.* 2001;12(8):1750-1757.

42. van Gelder T, Hesselink DA. Mycophenolate revisited. *Transpl Int.* 2015;28(5):508-515.

SUPPLEMENTALS



SDC Figure 1. Death-censored allograft survival of glucocorticoid-resistant AR versus severe AR. GC Glucocorticoid



SDC Figure 2. Laboratory parameters of hemoglobin (**a.**), thrombocytes (**b.**), leukocytes (**c.**) and lymphocytes (**d.**). Data are median and interquartile range. 0 = the day of rATG. W0-1 = the lowest measured value in the first week after rATG. W1 = the value 1 week after ATG ± 3 days. W2 = the value of 2 weeks after rATG ± 4 days. W4 = 4 weeks after rATG ± 7 days. M3 = 3 months after rATG ± 4 weeks. *Significant different compared with T=0 ($p < 0.05$). N = number of patients with laboratory parameters at that specific time point.

CHAPTER 9

Comparison of alemtuzumab and anti-thymocyte globulin treatment for acute kidney allograft rejection

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ABSTRACT

Rabbit anti-thymocyte globulin (rATG) is currently the treatment of choice for glucocorticoid-resistant, recurrent or severe acute allograft rejection (AR). However, rATG is associated with severe infusion-related side effects. Alemtuzumab is incidentally given to kidney transplant recipients as treatment for AR. In the current study, the outcomes of patients treated with alemtuzumab for AR were compared with that of patients treated with rATG for AR. The patient-, allograft-, and infection-free survival and adverse events of 116 alemtuzumab-treated patients were compared with those of 108 patients treated with rATG for AR Propensity scores were used to control for differences between the two groups. Patient- and allograft survival of patients treated with either alemtuzumab or rATG were not different (hazard ratio [HR] 1.14, 95%-confidence interval [CI] 0.48-2-69, p=0.77, and HR 0.82, 95%-CI 0.45-1.5, p=0.52, respectively). Infection-free survival after alemtuzumab treatment was superior compared with that of rATG-treated patients (HR 0.41, 95%-CI 0.25-0.68, p<0.002). Infusion-related adverse events occurred less frequently after alemtuzumab treatment. Alemtuzumab therapy may therefore be an alternative therapy for glucocorticoid-resistant, recurrent or severe acute kidney transplant rejection.

INTRODUCTION

Alemtuzumab is incidentally used to treat acute kidney allograft rejection (AR)[1-5]. Alemtuzumab is a humanized monoclonal rat antibody directed against the cell surface glycoprotein CD52[6]. Treatment with alemtuzumab causes a long-lasting depletion of various cells of the adaptive (T- and B cells) and innate immune system (NK cells, dendritic cells, monocytes and granulocytes)[6]. The drug is registered for the treatment of relapsing-remitting multiple sclerosis[7]. The Campath® Distribution Program offers off-label treatment with alemtuzumab for other indications, including therapy for kidney transplant recipients and patients with chronic lymphocytic leukemia[8].

Currently, rabbit anti-thymocyte globulin (rATG) is the treatment of choice for glucocorticoid-resistant, recurrent or severe (Banff grade IIA or higher) acute T cell-mediated rejection (aTCMR)[9]. Although effective, rATG has several limitations, for instance infusion-related side effects[10-12]. Alemtuzumab might be an alternative T cell-depleting therapy for AR with fewer infusion-related side effects[1-5].

The outcomes of alemtuzumab therapy for AR in kidney transplant recipients have only been reported in five small case series (with a cumulative number of 88 patients), concluding that patients with AR responded well to therapy with alemtuzumab[1-5]. However, in only one of these reports, alemtuzumab was compared to rATG therapy and none of them were randomized controlled trials[1]. Our center participated in one of these case series[1]. In this case series, 11 patients with AR and a contra-indication for rATG were treated with alemtuzumab. The incidence of the composite endpoint 'treatment failure' was comparable between both groups (alemtuzumab 27% *versus* rATG 40%, p = 0.89) and treatment with alemtuzumab was associated with fewer infusion related side effects and reduced costs[1].

Since 2012 and after our initial positive experience with alemtuzumab, it became the treatment of choice for all patients with glucocorticoid-resistant, severe or recurrent AR in the Erasmus MC[1]. Here, we present further data on patient- and allograft outcome on subsequent patients treated with alemtuzumab for AR in our center. Factors that influenced allograft survival were investigated, and we focused on the occurrence of infections, malignancies and autoimmune diseases. Patient-, allograft-, and infection-free survival of alemtuzumab-treated patients were compared with those of patients treated with rATG for AR[10].

MATERIALS AND METHODS

Study design

A retrospective analysis was performed on data of kidney transplant recipients who were treated in the Erasmus MC, University Medical Center Rotterdam, with alemtuzumab (Campath®, Sanofi Genzyme, United States) because of AR between January 2012 and January 2018. The study was approved by the medical ethical review board of the Erasmus MC (number 2018-1430). The patients were identified by the electronic medication prescription system of our hospital pharmacy. Patients with blood group AB0-incompatible kidney transplantations were excluded from the analysis, because they receive alemtuzumab as induction therapy[13].

The outcomes were compared to those of a cohort of patients treated with rATG (Thymoglobulin®, Sanofi Genzyme, United States) for AR between January 2002 and January 2012. The characteristics and outcomes of this cohort were described in detail previously[10].

All AR episodes (including recurrent AR) were biopsy-proven and biopsies were re-evaluated according to the Banff 2015 (for rATG-treated patients) and Banff 2017 classification (for alemtuzumab-treated patients) by one dedicated renal-pathologist (M.C.C-v.G.)[14-16]. The presence of donor-specific anti-HLA antibodies (DSA) and non-donor-specific HLA antibodies against HLA-A, HLA-B, HLA-DR, and HLA-DQ were examined in alemtuzumab-treated patients using the single-antigen bead Luminex assay on serum samples collected at the time of AR. DSA directed against Cw and DP HLA molecules were not tested. The presence of DSA was not routinely tested in the period 2002-2012 when rATG still was the therapy of choice[10]. Therefore, the biopsies of the rATG-treated patients could not be reclassified according to the Banff 2017 criteria[14].

Of patients treated with alemtuzumab, patient survival, allograft function (estimated glomerular filtration rate (eGFR; Chronic Kidney Disease Epidemiology Collaboration [CKD-EPI][17]), allograft survival (censored for death), variables that could influence allograft survival (patient and donor characteristics, type of immunosuppressive therapy, and type and grade of rejection), and adverse events were assessed. Baseline eGFR was defined as the highest eGFR in the three months prior to AR. Delayed graft function (DGF) was defined as the need for dialysis in the first week after transplantation. Allograft loss was defined as the need for dialysis or retransplantation. The follow-up period for allograft loss and

infection was from the day of T cell-depleting therapy until death, retransplantation, or loss to follow-up. Malignancies and mortality were evaluated until the last follow-up visit, which could be after subsequent kidney transplantation. The Dutch national pathology archive PALGA (Pathologisch-Anatomisch Landelijk Geautomatiseerd Archief, https://www.palga.nl/) was used for collecting of data relating to the occurrence of malignancy. Infections were considered serious if the infection necessitated hospitalization or occurred during hospital admission for another reason.

The allograft- and patient survival data of patients who had received a kidney transplant in the same time periods in our center and were not treated with T cell-depleting therapy was also compared to the patients treated with T cell-depleting therapy for AR.

Maintenance immunosuppressive therapy

The standard immunosuppressive regimen included induction therapy with basiliximab (Simulect®, Novartis Pharma, Basel, Switzerland) 20 mg intravenously on days 0 and 4 after transplantation, followed by maintenance therapy with tacrolimus (Prograf®, Astellas Pharma, Leiden, the Netherlands), mycophenolate mofetil (MMF; Cellcept®, Roche Pharmaceuticals, Basel, Switzerland) and glucocorticoids.

Basiliximab became part of our standard immunosuppressive regimen in 2009. Dosing of tacrolimus and MMF was based on pre-dose concentrations (C_0). Target C_0 for tacrolimus was respectively 10-15 μg/L (week 1-2), 8-12 μg/L (week 3-4), 5-10 μg/L (week 5-12), and 4-8 μg/L from month 4 onwards. MMF was started at 1,000 mg twice daily and subsequent dosing was based on C_0 (target C_0 was 1.5-3.0 mg/L). Glucocorticoids were given as an intravenous dose of 100 mg on days 0-3 and followed by a dose of 20 mg/day (days 4-14). Thereafter, glucocorticoids were tapered off and completely withdrawn around month 4.

Treatment of AR

The first-line treatment of aTCMR was methylprednisolone 1,000 mg (Solu-Medrol®, Pfizer, New York, the United States) intravenously daily for three consecutive days, followed by a second-line treatment with alemtuzumab or rATG in case of a glucocorticoid-resistant, recurrent or severe aTCMR (Banff grade IIA or higher). rATG was administered as a single bolus (4 mg/kg [actual bodyweight, no maximum dose limit]) intravenously[10]. Alemtuzumab was administered subcutaneously[18]. The first 14 patients were treated with alemtuzumab (30 mg) daily for two consecutive days. Since T cell-depletion already occurred after one dose of alemtuzumab, the next patients received a single dose (30 mg).

To prevent infusion-related side effects patients were premedicated with glucocorticoids (50 mg intravenously), acetaminophen (4 times daily 1,000 mg) and clemastine (4 mg intravenously). The alemtuzumab-treated patients were discharged the same day if no severe side-effects were noted. T- and B cell counts were measured with BD FACSCanto™ software every three months until the T cell count was >200 x 10^6/L. In the patients treated with rATG, a $CD3^+$ T cell count <200×10^6/L was aimed for a duration of two weeks during which patients were hospitalised[10]. If $CD3^+$ T cell counts increased during this period, a repeat dose of rATG was administered. All patients received prophylaxis for *Pneumocystis jirovecii* (sulfamethoxazole/trimethoprim) and cytomegalovirus (CMV; valganciclovir, except for CMV seronegative recipients with CMV seronegative donors) until the T cell count was >200 x 10^6/L. Patients with aABMR or mixed type AR could additionally be treated with intravenous immunoglobulins (IVIg), plasma-exchange, or both according to the Kidney Disease: Improving Global Outcomes (KDIGO) guideline[9].

Statistical methods

Categorical variables are presented as number (percentage). Continuous variables are presented as mean with standard deviation for normally distributed variables or median with interquartile range (IQR) for non-normally distributed variables. For differences between unpaired non-normally distributed continuous data or unpaired categorical data, the Kruskal-Wallis and Mann-Whitney U tests, and the Chi-squared and Fisher's exact tests were used. Kaplan-Meier survival analysis was used to examine subgroups (*e.g.* age categories and rejection types) within the alemtuzumab group and to compare allograft- and patient survival between alemtuzumab-treated patients and patients transplanted in the same period and who were not treated with alemtuzumab.

The influence of predictor variables on allograft survival in alemtuzumab-treated patients was analyzed with multivariable Cox proportional hazard regression analysis. Due to the number of events (41 allograft losses), the number of variables that could be included per analysis was limited. The influence of the most significant variable was tested in the presence of all the other variables one by one, and the non-significant variables were eliminated from the model by backward elimination.

Propensity scores were used to control for baseline differences between the patients treated with rATG and alemtuzumab[19]. They were acquired by performing a logistic regression with therapy type as the outcome variable. Covariates included in the logistic model were: age of the patient at time of AR, gender, primary kidney disease, donor type (living/deceased),

induction therapy (43% of patients treated with rATG received induction therapy, *versus* 97.3% of alemtuzumab treated patients), maintenance therapy, time to AR, and type of AR. The resulting propensity score was used as a covariate in Cox proportional hazards regression models (for calculation of the patient-, allograft-, and infection-free survival), in linear regression models (for continuous outcomes), and in logistic regression models (for categorical outcomes).

A two-sided p-value <0.05 was considered statistically significant. GraphPad Prism, version 5 (San Diego, CA, USA), SPSS version 21 (SPSS Inc., Chicago, IL, USA), and R (R Foundation for Statistical Computing, Vienna, Austria, version 3.5.1) were used for the statistical analysis.

RESULTS

Patient demographics

Between January 2012 and January 2018, 1,214 patients received a kidney transplant at our center. Of these, 113 patients (9.3%) were treated with alemtuzumab for AR. Three patients were treated with alemtuzumab twice because of two separate rejection episodes of the same kidney transplant. Between January 2002 and January 2012, 1,107 patients were transplanted with a kidney and 108 patients of these (9.8%) were treated with rATG for AR[10]. The median cumulative dose of rATG per patient was 7.4 mg/kg. Baseline characteristics of patients treated with either alemtuzumab or rATG for AR are presented in Table 1.

Induction therapy with basiliximab was given to 303 (27.4%) of all patients transplanted between 2002 and 2012 (the rATG period) and to 1065 (87.8%) of all patients between 2012 and 2018 (the alemtuzumab period). As a result, significantly more patients treated with alemtuzumab (93.8%) had previously received basiliximab induction therapy compared to rATG-treated patients (29.2%; p<0.0001 [Table 2]). A tacrolimus- and MMF-based maintenance therapy was given to 81% of alemtuzumab-treated patients and to 72.2% of rATG-treated patients (p=0.08; Table 2). First line therapy for AR was methylprednisolone in 94.8% of alemtuzumab-treated patients, and to 86.1% or rATG-treated patients (Table 2).

Table 1. Baseline characteristics of patients treated with either alemtuzumab or rATG

Characteristic	Alemtuzumab (n=113)	rATG (n=108)	p-value
Recipient age at transplantation- yr.	56 (39-63)	45 (34-55)	0.0001
Recipient age at rejection- yr.	56 (40-63)	46 (35-56)	0.0002
Donor age - yr.	54 (43-63)	54 (46-61)	0.82
Female sex - no. (%)	47 (40.5)	40 (37.0)	0.69
Cause of ESRD - no.			
DM/HTN/GN/PKD/reflux/other*/unknown	26/22/21/9/7/27/4	23/10/18/16/17/16/3	0.04
Ethnic distribution - no.			
Caucasian/Black/Asian/Arab/other	79/17/8/11/1	70/16/5/5/7	0.12
Transplant number - no.			
1/2/3	88/22/6	76/25/5	0.71
Preemptive kidney transplantation - no. (%)	41 (35.3)	25 (23.4)	0.06
Donor type - no.			
LR/LUR/DBD/DCD	27/55/12/22	35/47/15/10	0.12
HLA mismatch			
HLA A: 0/1/2	26/61/29	21/60/23	0.75
HLA B: 0/1/2	10/53/53	11/55/38	0.39
HLA DR: 0/1/2	21/55/40	13/50/41	0.48
PRA actual - no. (%)			0.52
0-5%	93 (80.2)	81 (77.1)	
6-83%	22 (19.0)	21 (20)	
84-100%	1 (0.8)	3 (2.9)	
PRA peak - no. (%)			0.15
0-5%	69 (59.5)	62 (59)	
6-83%	31 (26.7)	32 (30.5)	
84-100%	16 (13.8)	11 (9.5)	
CMV IgG serostatus recipient - no. (%)			
Positive	83 (73.6)	75 (70.8)	0.76
EBV IgG serostatus recipient - no. (%)			
Positive	106 (93.8)	90 (92.8)	0.78

Data are numbers (%) or median (interquartile range). *Other kidney diseases included focal segmental glomerulosclerosis, vascular disease, septic shock, kidney dysplasia /nephrectomy, congenital nephrotic syndrome, Alport syndrome, nephronophtisis, drug intoxication, RCAD syndrome, or tubulointerstitial nephritis. Data of rATG-treated patients are prescribed previously[10]. CMV cytomegalovirus; DBD donation after brain death; DCD donation after circulatory death; DM diabetes mellitus; EBV Epstein-Barr virus; ESRD end stage renal disease; GN glomerulonephritis; HLA human leucocyte antigen; HTN hypertensive nephropathy; LR living related; LUR living unrelated; PKD polycystic kidney disease; PRA panel reactive antibody; rATG rabbit anti-thymocyte globulin.

Table 2. Immunosuppressive therapy in patients treated with alemtuzumab or rATG

Immunosuppressive therapy	Alemtuzumab (n=113)	rATG (n=108)	p-value
Induction therapy - no. (%)			<0.0001
None	3 (2.7)*	62 (57.4)	
Basiliximab	106 (93.8)	33 (29.2)	
rATG	2 (1.8)	10 (8.8)	
Rituximab	2 (1.8)	0 (0)	
Daclizumab	0 (0)	2 (1.9)	
Maintenance immunosuppression - no. (%)			0.08
TAC/MMF/glucocorticoids	78 (67.2)	58 (53.7)	
TAC/MMF	16 (13.8)	20 (18.5)	
TAC + other (non-MMF)	11 (9.5)	6 (5.6)	
MMF + other (non-TAC)	11 (9.5)	20 (18.5)	
Anti-rejection therapy - no. (%)			
Methylprednisolone prior to T cell-depleting therapy	110 (94.8)	93 (86.1)	0.04
Cumulative dose of methylprednisolone, mg			0.004
1000	0 (0)	2 (2.2)	
2000	1 (0.9)	9 (8.2)	
3000	96 (87.3)	79 (71.8)	
4000	1 (0.9)	0 (0)	
6000	12 (10.9)	3 (2.7)	
Additional anti-rejection therapy in patients with ABMR			
Intravenous immunoglobulins	10	1	
Plasma-exchange + intravenous immunoglobulins	3	2	
Additional anti-rejection therapy in patients with mixed AR			
Intravenous immunoglobulins	8	4	
Plasma-exchange + intravenous immunoglobulins	0	4	

Data are numbers (%).*In three patients no induction therapy was administered because of an HLA-identical donor. TAC + other regime contained combinations of TAC, glucocorticoids, everolimus, or azathioprine. Other combinations existed of a combination of azathioprine, glucocorticoids, everolimus, cyclosporine A, AEB071, or FTY720. MMF + other regime contained combinations of MMF, glucocorticoids, cyclosporine A, everolimus, or belatacept. Data of rATG-treated patients are prescribed previously[10]. MMF mycophenolate mofetil; TAC tacrolimus.

Sixty-four alemtuzumab-treated patients (55.2%) and 64 (59.3%) rATG-treated patients had an early AR (within three months after transplantation; Table 3). The distribution of the Banff grade of AR was not different between the patients treated with alemtuzumab or rATG (p=0.89; Table 3). In 18 patients (15.5%), a second kidney allograft biopsy was performed after the initial treatment with methylprednisolone and immediately before alemtuzumab treatment to confirm ongoing AR (Table S1).

Table 3. Rejection characteristics

Rejection characteristic	Alemtuzumab (n=116)[$]	rATG (n=108)	p-value
Time to rejection - days	32 (2-1644)	24 (8-339)	0.83
Early rejection (<3 months) - no. (%)	64 (55.2)	64 (59.3)	0.59
Late rejection (>3 months) - no. (%)	52 (44.8)	44 (40.7)	0.59
Delayed graft function during AR	33 (28)	19 (17.6)	0.06
Banff classification - no.*			0.89
aTCMR			
aTCMR IA/IB	1/9	6/8	
aTCMR IIA/IIB/III	29/23/2	21/20/1	
Borderline aTCMR	3	0	
ABMR			
aABMR	17	12[@]	
DSA+ and C4d+	7		
DSA+ and C4d-	0		
DSA- and C4d+	7		
C4d+, no DSA tested	2		
Histologic features of ABMR, no DSA/C4d	1[¥]		
c/aABMR	1[§]	3	
Mixed aTCMR with aABMR			
aTCMR I/II/III	9/7/2	8/10/0[@]	
DSA+ and C4d+	5		
DSA+ and C4d-	6		
DSA- and C4d+	4		
C4d+, no DSA tested	3		
Mixed aTCMR with c/aABMR	1[$]	1	

Data are numbers (%) or median (interquartile range). [$]A total of 113 patients were treated with alemtuzumab, however three patients were treated with alemtuzumab twice because of two separate rejection episodes of the same kidney transplant *Banff classification of aTCMR, ABMR and mixed AR were compared. Re-classification in 12 biopsies of alemtuzumab-treated patients and 18 biopsies of rATG-treated patients was not possible because the biopsies were missing from archives. The primary pathological diagnosis of these biopsies was aTCMR in five patients, ABMR in two patients, and mixed AR in five patients. Data of rATG-treated patients are prescribed previously[10]. [¥]Histologic features of ABMR with glomerulitis and peritubular capillaritis, but C4d staining was negative and no DSAs were present. [§]The patients with c/aABMR had no DSAs and C4d staining was positive in the peritubular capillaries. [$]The patient with mixed c/aABMR had DSAs and C4d staining was negative. [@]DSAs were not routinely measured in the rATG-treated patients. ABMR antibody mediated rejection; aABMR active antibody mediated rejection; aTCMR acute T cell mediated rejection; c/aABMR chronic/active antibody mediated rejection; DGF Delayed graft function (need for dialysis in the first week after transplantation); DSA de novo donor specific antibodies.

Patient survival

Patient survival of patients treated with either alemtuzumab or rATG for AR is depicted in Figure 1A. Compared with the historical rATG cohort, the patient survival of the alemtuzumab group was not different (hazard ratio [HR] 1.14, 95%-confidence interval [CI] 0.48-2.69, p=0.77; Figure 1A), also when only those patients who were treated with basiliximab induction therapy were included (HR 1.74, 95%-CI 0.68-4.46, p=0.25; Figure 2A).

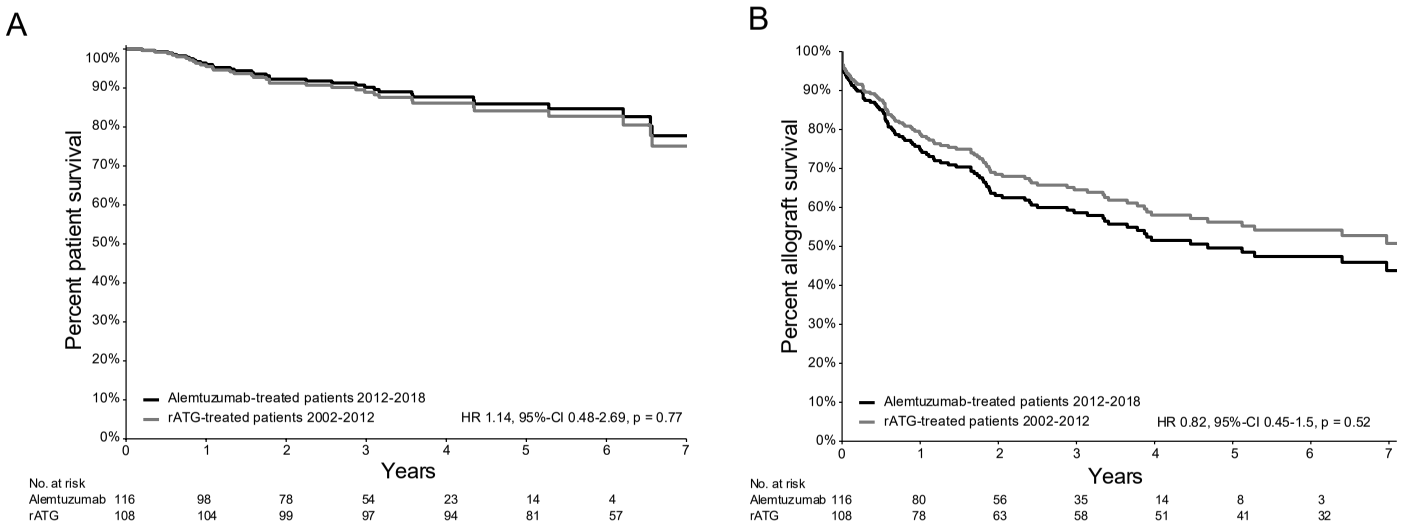


Figure 1. Survival plots of patient- and allograft survival of patients with acute rejection and treated with either rATG or alemtuzumab. **a.** Patient survival curve (from the time of treatment) based on the propensity score Cox regression model of patients treated with either alemtuzumab (2012-2018) or rATG (2002-2012) for acute kidney allograft rejection. **b.** Allograft survival curve (from the time of treatment) based on the propensity score Cox regression model (event = allograft loss, censored for death) of patients treated with either alemtuzumab (2012-2018) or rATG (2002-2012) for acute kidney allograft rejection.

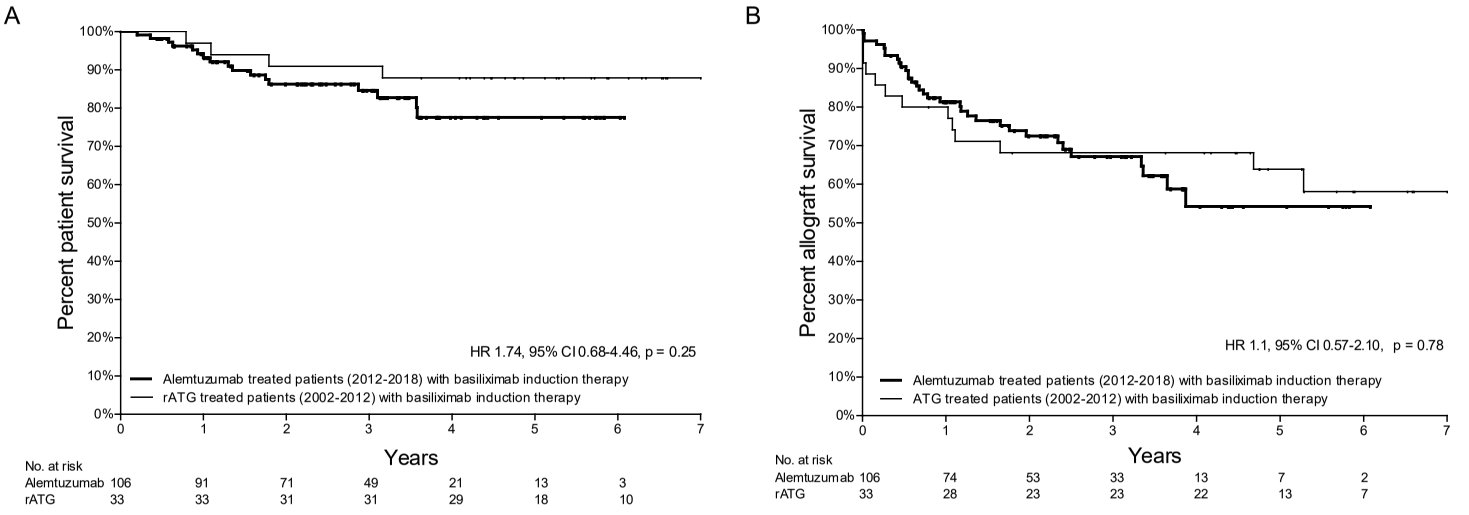


Figure 2. Kaplan-Meier survival curves of the patient- and allograft survival of patients treated with basiliximab induction therapy. **a.** Kaplan-Meier curve of the patient survival curve (from the time of treatment) of patients treated with either alemtuzumab (2012-2018) or rATG (2002-2012) for AR and who received induction therapy with basiliximab. **b.** Kaplan-Meier curve of the allograft survival curve (from the time of treatment) of patients treated with either alemtuzumab (2012-2018) or rATG (2002-2012) for AR and who received induction therapy with basiliximab.

The patient survival of alemtuzumab-treated patients was significantly lower compared with the patients transplanted in the same period and who were not treated with alemtuzumab (HR 2.38, 95%-CI 1.25-4.54, p=0.0036, Figure S1A). In the total follow-up period (median 2.8 years [IQR 1.3-3.8 years]), 18 patients died after a median of 1.45 years (IQR 0.92-2.93; Table S2). A univariable Cox proportional hazard regression analysis was performed to investigate which variables influenced the risk of death in patients treated with alemtuzumab. The only variable that was associated with the risk of death was age of the recipient (HR per year 1.09, 95%-CI 1.04-1.14, p<0.0001; Table S3). This increased risk of death was seen in alemtuzumab-treated patients older than 50 years at the time of transplantation (Figure S2).

A comparison between the patient survival of rATG-treated and patients transplanted in the same period and who were not treated with rATG is shown in Figure S1C and was described previously[10].

Kidney allograft survival

Death-censored kidney allograft survival of alemtuzumab-treated patients was not different compared to that of patients who received rATG for AR (HR 0.82, 95%-CI 0.45-1.50, p=0.52; Figure 1B). A similar survival was also observed when only those patients who were treated with basiliximab induction therapy were included (HR 1.10, 95%-CI 0.57-2.10, p=0.78; Figure 2B). Additional information about the kidney allograft function after alemtuzumab or rATG therapy for AR is provided in Figure S3.

The allograft survival of alemtuzumab-treated patients was significantly worse compared to the allograft survival of patients that were transplanted in the same period and who were not treated with alemtuzumab (HR 258.0, 95%-CI 112.0-591.3, p<0.0001; Figure S1B). During the follow-up (median 2.2 years, IQR 1-3.5), 41 (35.3%) patients lost their kidney allograft after alemtuzumab therapy for AR, of which six never had a functioning graft (primary non-function [PNF]). To investigate which variables influenced allograft survival in alemtuzumab-treated patients, a Cox proportional hazard regression analysis was performed. In the univariable model, age of the recipient, number of HLA mismatches, glucocorticoid maintenance treatment, timing of AR, and the Δ eGFR (percentage change between baseline eGFR and eGFR at the moment of AR) significantly influenced the risk for death-censored allograft loss (p<0.05, Table S4) in alemtuzumab-treated patients. The variables glucocorticoid use and timing of rejection were related because all patients with an early acute rejection used glucocorticoids as maintenance therapy, while only 56.6%

of patients with a late acute rejection used glucocorticoids (p<0.0001). The Banff grade of rejection did not influence allograft survival (p=0.19). Allograft survival of alemtuzumab-treated patients suffering from either aTCMR or aABMR is shown in Figure S4.

The final multivariable model showed that patients with actual panel reactive antibodies (PRA) >6%, and patients with a *Δ* eGFR of more than 50% had an inferior allograft survival after alemtuzumab therapy (Figure 3). Patients using glucocorticoids at time of AR, and patients with more HLA mismatches, showed a superior allograft survival after alemtuzumab therapy (Figure 3). Several variables were compared between patients with an HLA mismatch of 0-3 and an HLA mismatch of 4-6 (Table S5). One variable was significantly different: 42 (72%) recipients with 4-6 HLA mismatches received a living unrelated donor kidney (Table S5), while 13 (23%) recipients with 0-3 HLA received a living unrelated donor kidney (p<0.001; Table S5).

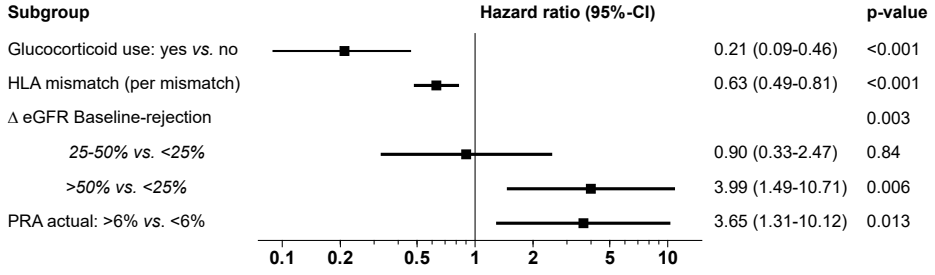


Figure 3. Multivariable Cox proportional hazard regression analysis of risk for allograft loss in alemtuzumab-treated patients. Multivariable analysis of the risk of allograft loss with hazard ratio (Exp(B), 95%-confidence interval and p-value). Delta (*Δ*) eGFR baseline-moment of rejection is the percentage change between the baseline eGFR and eGFR at the moment of rejection. Glucocorticoid use means maintenance therapy with glucocorticoids during the rejection. PRA Panel reactive antibodies.

The allograft survival of patients treated with survival of rATG was worse compared to that of patients who were not treated with rATG and transplanted in the same time period (HR 15.9, 95%-CI 9.2-27.4, p < 0.0001; Figure S1D). A multivariable Cox proportional hazard regression analysis was performed for patients treated with rATG for AR and reported previously[10]. This analysis demonstrated that allograft survival was superior in patients with an early AR compared with a late AR[10].

Adverse events

Infusion-related side effects also occurred less frequently in alemtuzumab-treated patients compared with the patients treated with rATG (Table 4). No alemtuzumab-treated patients experienced serum sickness *versus* five patients in the rATG group (p=0.02). No patients experienced cytokine release syndrome or pulmonary edema after alemtuzumab. The median duration of hospitalization was three days (IQR 1-6) in patients treated with alemtuzumab and 15 days (IQR 13-19) in rATG-treated patients.

Table 4. Adverse events after therapy with alemtuzumab or rATG

Adverse events	Alemtuzumab	rATG	p-value
Fever* - no. (%)	10 (8%)	42 (61.8%)	<0.001
Systolic blood pressure <90 mmHg - no. (%)	1 (0.8%	7 (10.4%)	0.003
Tachycardia >100/minute - no. (%)	18 (15.5%)	44 (69.8%)	<0.001
Interventions - no.			
Transfer to ICU	1 (0.9%)	5 (4.6%)	0.11
Supplemental oxygen	1 (0.9%)	9 (13.4%)	0.03
Volume resuscitation	0	6 (9.0%)	0.06

Data are numbers (percentage) and median (interquartile range). Fever, blood pressure, tachycardia and interventions were registered in the 24 hours after therapy. Under-reporting of the incidence of infusion-related adverse events is possible in 37 patients who were dismissed on the day of alemtuzumab therapy. Data of rATG-treated patients are prescribed previously[10]. *Fever was defined as temperature above 38.5°C.

The infection-free survival (excluding CMV, EBV and BK virus infections) in the first year after alemtuzumab treatment was significantly better compared with the infection-free survival of the rATG-treated patients (HR 0.41, 95%-CI 0.25-0.68, p<0.002; Figure 4). CMV reactivation occurred in 25 patients (21.6%) treated with alemtuzumab (Table S6), compared to 27 patients (25%) in the rATG group (p=0.10). In both the alemtuzumab- and rATG-treated groups, two patients experienced a primary CMV infection (p=0.50). Additional information on the occurrence of infections is presented in Table S6.

Secondary autoimmune events have been described after administration of alemtuzumab[6]. In the current study, two patients developed inflammatory polyneuropathy (one case of Guillain-Barre syndrome and one case of chronic inflammatory demyelinating polyneuropathy) after alemtuzumab treatment[20]. No patients were diagnosed with autoimmune thyroid disorders, idiopathic thrombocytopenic purpura or autoimmune nephropathy.

Repopulation of T cells >200x10^6/L occurred in 55.7% of alemtuzumab-treated patients in the first year after administration (Figure S5A and B). In 40.2% of the patients, repopulation of B cells >100x10^6/L (Figure S5C and D) was seen at 1 year.

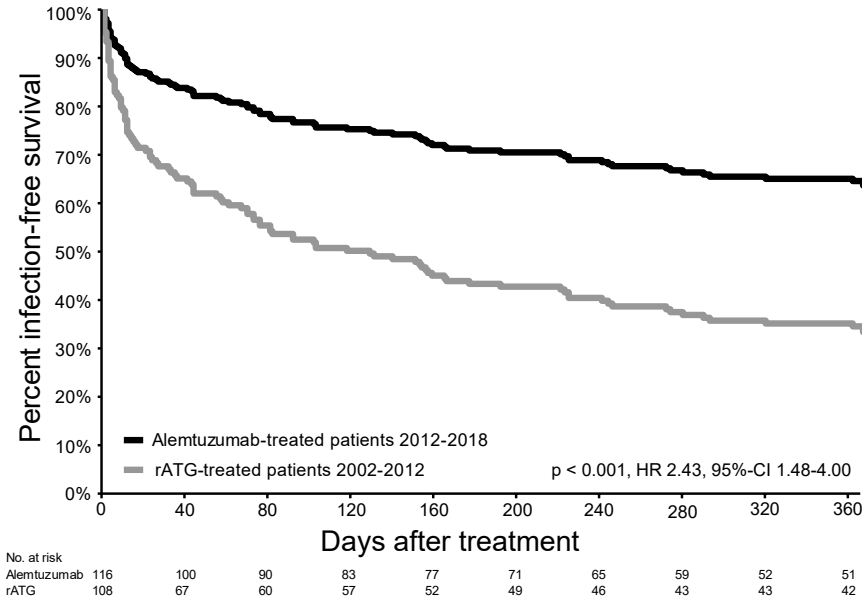


Figure 4. Infection-free survival in the first year after treatment for acute rejection. Infection-free survival (excluding CMV, EBV and BK virus infections) of patients with AR and treated with alemtuzumab (2012-2018) and patients treated with rATG for AR between 2002-2012.

Solid tumors were diagnosed in seven alemtuzumab-treated patients during the total follow-up (median 2.8 years [IQR 1.3-3.8]; Table S7). The incidence of solid tumors was 2.3 per 100 person-years with a median time after alemtuzumab therapy of 28 months (IQR 9-38), and the age at the time of diagnosis was 65 years (IQR 60-76). Seven patients were diagnosed with skin cancer: 21 basal cell carcinomas and 12 squamous cell carcinomas. In the rATG treated patients, 14 malignancies were diagnosed after a mean time of 63 months (standard deviation 45 months) during the follow-up of 6.8 years (IQR 4.9–9.1; Table S8)[10].

DISCUSSION

In the current study, the largest cohort of patients treated with alemtuzumab for AR is described. The results of this study suggest that alemtuzumab could be an alternative to rATG for the treatment of glucocorticoid-resistant, severe or recurrent AR. Compared to rATG, allograft- and patient survival of patients treated with alemtuzumab was not different. Moreover, adverse events and infections seemed to occur less frequently in patients treated with alemtuzumab-compared with rATG-treated patients.

Alemtuzumab seems as effective as rATG for the prevention of allograft loss after AR. Five case series have described allograft outcome in patients treated with alemtuzumab for AR[1-5]. The results of these case series are difficult to compare with our study. Four of the five studies were performed more than 15 years ago and patients in these studies were not treated with the current gold standard therapy (induction therapy in combination with tacrolimus and MMF)[2-5]. Furthermore, these case series were of heterogeneous design. First, alemtuzumab was prescribed as first line treatment for AR in two studies[4,5], and in one study alemtuzumab was prescribed to patients with AR resistant to ATG or OKT3[2]. Second, the dose of alemtuzumab ranged from 15 mg[1] to 93 mg[2]. Third, the follow-up period of these studies ranged from three months[1] to ten years[4]. Compared with treatment with methylprednisolone or rATG, allograft survival in alemtuzumab-treated patients was similar[1,4]. The result of our study supports this conclusion.

Treatment with alemtuzumab is associated with serious side effects and therefore the assessment of the benefit-risk balance in the individual patient before initiation of treatment is necessary. We investigated which clinical factors influenced allograft survival. Factors that were associated with a good response were a low Δ eGFR between baseline and the moment of AR, glucocorticoid maintenance therapy at the time or AR, and an actual PRA below 6%. The use of glucocorticoid maintenance therapy and timing of rejection were related, because all patients with an early acute rejection used glucocorticoids as maintenance therapy. Therefore, we are not sure if glucocorticoid use is a protective factor, or that an early rejection is associated with a better allograft outcome compared with a late rejection. Late rejections occur in patients who visit the outpatient clinic less frequently and with intervals of 1–4 months, likely leading to a delay in diagnosis.

Surprisingly, patients with more HLA mismatches had a better response to alemtuzumab therapy compared with those with less HLA mismatches. Analysis of all factors showed that patients with more HLA mismatches (4-6) more often received a kidney from a living unrelated donor. How this is related to a better response to alemtuzumab treatment is unclear. It is known that results of living donor kidney transplantation are better compared to deceased donor transplantation, even with higher numbers of HLA mismatches[21]. Taken together, based on these results, we treat patients with an early AR aggressively with alemtuzumab and are more reluctant to administer alemtuzumab in patients with a late AR who also have a considerable loss of renal function.

Seventeen patients with aABMR were treated with alemtuzumab. Treatment options for aABMR are limited and no specific drugs have received US Food and Drug Administration approval. Currently, the therapy for aABMR consists of glucocorticoids, IVIg and/or plasma-exchange, although the evidence for this treatment is not strong[22,23]. Since alemtuzumab causes lysis of T- and B cells, as well as antigen presenting cells, alemtuzumab may be considered in patients with aABMR. In our study, patients with aABMR showed a good response to alemtuzumab therapy. However, larger studies are necessary to confirm our results and analyze the best therapeutic strategy.

Although T cell-depletion after alemtuzumab therapy lasts longer than after rATG[24], the infection-free survival was better after therapy with alemtuzumab compared with rATG. The biggest difference in the number of infections in patients treated with rATG or alemtuzumab occurred in the first few weeks after therapy (Table S6). A possible explanation for this is the longer duration of hospitalization after therapy for AR in rATG-treated patients compared with alemtuzumab-treated patients. A longer hospitalization is associated with a higher risk for health care-associated infections[25]. The occurrence of CMV disease or reactivation was similar between patients treated with alemtuzumab or rATG. In literature, similar results (lower frequency of infections and no difference in CMV infections) are seen when alemtuzumab or rATG are used as induction therapy[26].

In contrast to reports investigating the occurrence of autoimmune disorders in patients suffering from multiple sclerosis and treated with alemtuzumab, we observed no clinically apparent autoimmune thyroid disorders or idiopathic thrombocytopenic purpura in the present cohort[27]. Possibly, the follow-up period of the present study was too short for these autoimmune events to occur. Another reason could be that patients with multiple sclerosis are susceptible to autoimmune disorders because of their genetic constitution.

The administration of rATG is associated with serious infusion-related side effects and the drug is relatively contra-indicated in patients with cardiac failure or fluid overload[11,12]. Infusion-related side effects in our study were less prevalent in patients treated with alemtuzumab compared with rATG therapy and subcutaneous administration of alemtuzumab therefore appears to be safe in frail patients and patients with cardiac morbidity. In this study, rATG was given as a bolus of 4 mg/kg. We cannot exclude the possibility that another dosing regimen such as a repeated, standard dose of rATG may have resulted in fewer infusion-related side effects[28].

We acknowledge the limitations of the current study. First, this was a retrospective single-center study. Second, several variables (including time period, the use of induction therapy and others) were different between the patients treated with alemtuzumab and the patients treated with rATG. A propensity score analysis was performed to correct for potential differences between the alemtuzumab and rATG group, but we cannot exclude the possibility that other (unmeasured) confounding factors influenced the outcomes of this analysis. Currently, these data offer the best available evidence for the treatment of AR with alemtuzumab as it is unlikely that a randomized controlled trial comparing alemtuzumab with other anti-rejection therapies will be performed anytime soon. Third, the allograft survival of patients who were treated with alemtuzumab seemed (although not significant) to be worse compared with rATG-treated patients. Again, we cannot exclude the possibility that inclusion of more patients may have resulted in a significant difference between the two groups. Fourth, in our study 93.8% of alemtuzumab-treated patients were treated with basiliximab induction therapy. In the United States, only 33.8% of kidney transplant recipients are treated with basiliximab, whereas 65.9% of patients receive induction therapy with T cell-depleting antibodies[29]. We don't know the influence of this difference on the outcomes after alemtuzumab therapy for AR. Fifth, due the unavailability of data on DSAs in the rATG-treated patients, it was not possible to apply the Banff 2017 classification on biopsies of these patients which may have biased the diagnosis of ABMR.

To conclude, alemtuzumab therapy could be an alternative therapy to rATG for glucocorticoid-resistant or severe AR. This may be especially relevant for patients with a relative contraindication for rATG, including patients suffering from fluid overload or previous rATG treatment. Further studies, preferably multicenter randomized controlled trials, are necessary to explore the potential advantages of alemtuzumab for severe rejection.

REFERENCES

1. van den Hoogen MW, Hesselink DA, van Son WJ, Weimar W, Hilbrands LB. Treatment of steroid-resistant acute renal allograft rejection with alemtuzumab. *Am J Transplant.* 2013;13(1):192-196.
2. Csapo Z, Benavides-Viveros C, Podder H, Pollard V, Kahan BD. Campath-1H as rescue therapy for the treatment of acute rejection in kidney transplant patients. *Transplant Proc.* 2005;37(5):2032-2036.
3. Basu A, Ramkumar M, Tan HP, et al. Reversal of acute cellular rejection after renal transplantation with Campath-1H. *Transplant Proc.* 2005;37(2):923-926.
4. Clatworthy MR, Friend PJ, Calne RY, et al. Alemtuzumab (CAMPATH-1H) for the treatment of acute rejection in kidney transplant recipients: long-term follow-up. *Transplantation.* 2009;87(7):1092-1095.
5. Rebello PR, Hale G, Friend PJ, Cobbold SP, Waldmann H. Anti-globulin responses to rat and humanized CAMPATH-1 monoclonal antibody used to treat transplant rejection. *Transplantation.* 1999;68(9):1417-1420.
6. van der Zwan M, Baan CC, van Gelder T, Hesselink DA. Review of the Clinical Pharmacokinetics and Pharmacodynamics of Alemtuzumab and Its Use in Kidney Transplantation. *Clin Pharmacokinet.* 2018;57(2):191-207.
7. Genzyme Submits Applications to FDA and EMA for Approval of LEMTRADA™ (alemtuzumab) for Multiple Sclerosis, http://news.genzyme.com/press-release/genzyme-submits-applications-fda-and-ema-approval-lemtrada-alemtuzumab-multiple-sclero. Published on June 12, 2012.
8. Campath Distribution Program, http://www.campath.com/. Assessed on 12 april 2019.
9. Tai E, Chapman JR. The KDIGO review of the care of renal transplant recipient. *Pol Arch Med Wewn.* 2010;120(6):237-242.
10. van der Zwan M, Clahsen-Van Groningen MC, Roodnat JI, et al. The Efficacy of Rabbit Anti-Thymocyte Globulin for Acute Kidney Transplant Rejection in Patients Using Calcineurin Inhibitor and Mycophenolate Mofetil-Based Immunosuppressive Therapy. *Ann Transplant.* 2018;23:577-590.
11. Buchler M, Hurault de Ligny B, Madec C, Lebranchu Y, French Thymoglobuline Pharmacovigilance Study G. Induction therapy by anti-thymocyte globulin (rabbit) in renal transplantation: a 1-yr follow-up of safety and efficacy. *Clin Transplant.* 2003;17(6):539-545.
12. Tanriover B, Chuang P, Fishbach B, et al. Polyclonal antibody-induced serum sickness in renal transplant recipients: treatment with therapeutic plasma exchange. *Transplantation.* 2005;80(2):279-281.
13. van Agteren M, Weimar W, de Weerd AE, et al. The First Fifty ABO Blood Group Incompatible Kidney Transplantations: The Rotterdam Experience. *J Transplant.* 2014;2014:913902.
14. Haas M, Loupy A, Lefaucheur C, et al. The Banff 2017 Kidney Meeting Report: Revised diagnostic criteria for chronic active T cell-mediated rejection, antibody-mediated rejection, and prospects for integrative endpoints for next-generation clinical trials. *Am J Transplant.* 2018;18(2):293-307.
15. Roufosse C, Simmonds N, Clahsen-van Groningen M, et al. A 2018 Reference Guide to the Banff Classification of Renal Allograft Pathology. *Transplantation.* 2018;102(11):1795-1814.

16. Loupy A, Haas M, Solez K, et al. The Banff 2015 Kidney Meeting Report: Current Challenges in Rejection Classification and Prospects for Adopting Molecular Pathology. *Am J Transplant.* 2017;17(1):28-41.

17. Levey AS, Stevens LA, Schmid CH, et al. A new equation to estimate glomerular filtration rate. *Ann Intern Med.* 2009;150(9):604-612.

18. Clatworthy MR, Sivaprakasam R, Butler AJ, Watson CJE. Subcutaneous administration of alemtuzumab in simultaneous pancreas-kidney transplantation. *Transplantation.* 2007;84(12):1563-1567.

19. Using propensity scores with small samples, http://www.faculty.umb.edu/william_holmes/usingpropensityscoreswithsmallsamples.pdf, assessed on 3 June 2019.

20. Van der Zwan M HD, Brusse E, et al. . GBS and CIDP after alemtuzumab therapy in kidney transplant recipients. *Neurology: Neuroimmunology and Neuroinflammation.*2020; april. In press.

21. Laging M, Kal-van Gestel JA, Haasnoot GW, et al. Transplantation results of completely HLA-mismatched living and completely HLA-matched deceased-donor kidneys are comparable. *Transplantation.* 2014;97(3):330-336.

22. Archdeacon P, Chan M, Neuland C, et al. Summary of FDA antibody-mediated rejection workshop. *Am J Transplant.* 2011;11(5):896-906.

23. Sablik KA, Clahsen-van Groningen MC, Looman CWN, et al. Treatment with intravenous immunoglobulins and methylprednisolone may significantly decrease loss of renal function in chronic-active antibody-mediated rejection. *BMC Nephrol.* 2019;20(1):218.

24. Product monograph of Thymoglobulin, http://products.sanofi.ca/en/thymoglobulin.pdf, assessed on 24 May 2019.

25. Leape LL, Brennan TA, Laird N, et al. The nature of adverse events in hospitalized patients. Results of the Harvard Medical Practice Study II. *N Engl J Med.* 1991;324(6):377-384.

26. Hanaway MJ, Woodle ES, Mulgaonkar S, et al. Alemtuzumab induction in renal transplantation. *N Engl J Med.* 2011;364(20):1909-1919.

27. Ziemssen T, Thomas K. Alemtuzumab in the long-term treatment of relapsing-remitting multiple sclerosis: an update on the clinical trial evidence and data from the real world. *Ther Adv Neurol Disord.* 2017;10(10):343-359.

28. Kho MM, Bouvy AP, Cadogan M, et al. The effect of low and ultra-low dosages Thymoglobulin on peripheral T, B and NK cells in kidney transplant recipients. *Transpl Immunol.* 2012;26(4):186-190.

29. Hart A, Smith JM, Skeans MA, et al. OPTN/SRTR 2017 Annual Data Report: Kidney. *Am J Transplant.* 2019;19 Suppl 2:19-123.

SUPPLEMENTAL DIGITAL CONTENT

Figures

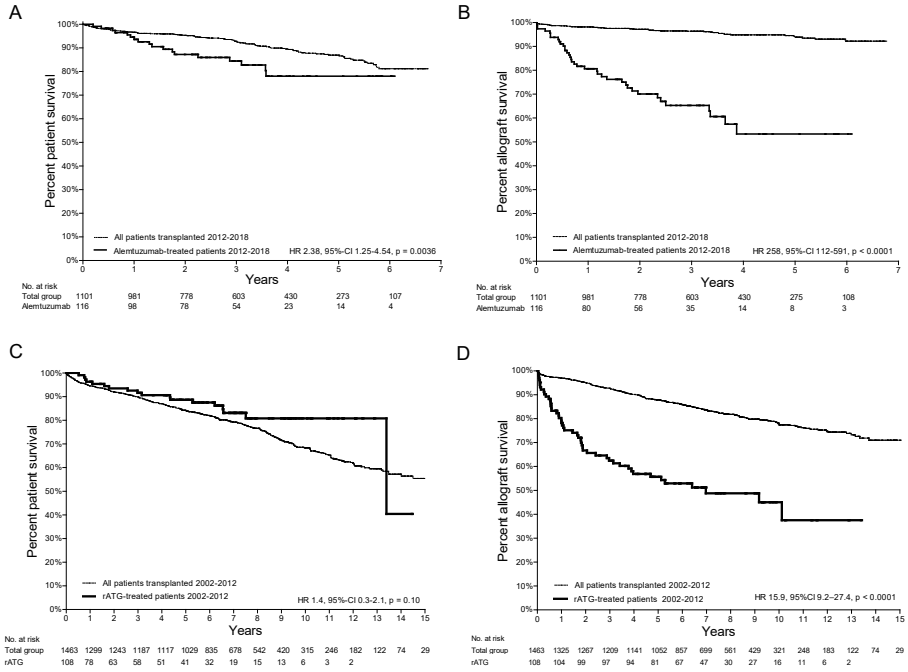
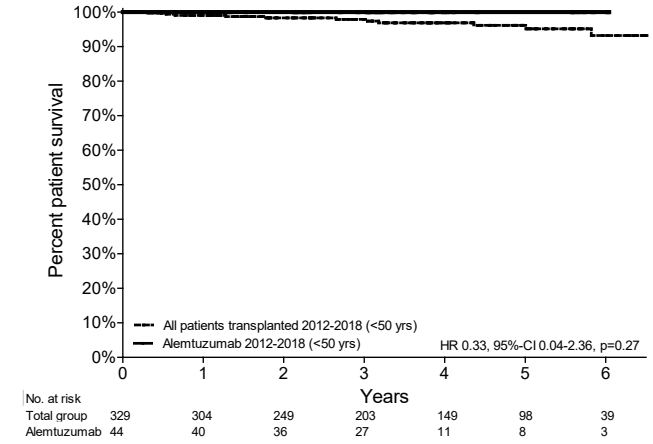


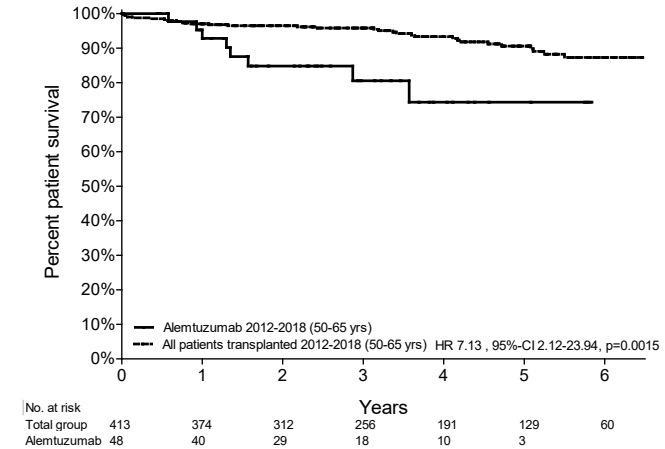
Figure S1. Survival plots of allograft- and patient survival in the period between 2002-2012 and 2012 -2018. a. Kaplan-Meier patient survival curve of patients treated with alemtuzumab for AR (2012-2018) and patients transplanted in the same period and not treated with alemtuzumab. From time point of acute rejection (alemtuzumab group) and time point of kidney transplantation (patients not treated with alemtuzumab). **b.** Kaplan-Meier allograft survival curve (event = allograft loss, censored for death) of patients treated with alemtuzumab for AR (2012-2018) and patients transplanted in the same period and not treated with alemtuzumab. From time point of acute rejection (alemtuzumab group) and time point of kidney transplantation (patients not treated with alemtuzumab). **c.** Kaplan-Meier patient survival curve of patients treated with rATG for AR (2002-2012) and patients transplanted in the same period and not treated with rATG. From time point of acute rejection (rATG group) and time point of kidney transplantation (patients not treated with rATG). **d.** Kaplan-Meier allograft survival curve (event = allograft loss, censored for death) of patients treated with rATG for AR (2002-2012) and patients transplanted in the same period and not treated with rATG. From time point of acute rejection (rATG group) and time point of kidney transplantation (patients not treated with rATG).

A



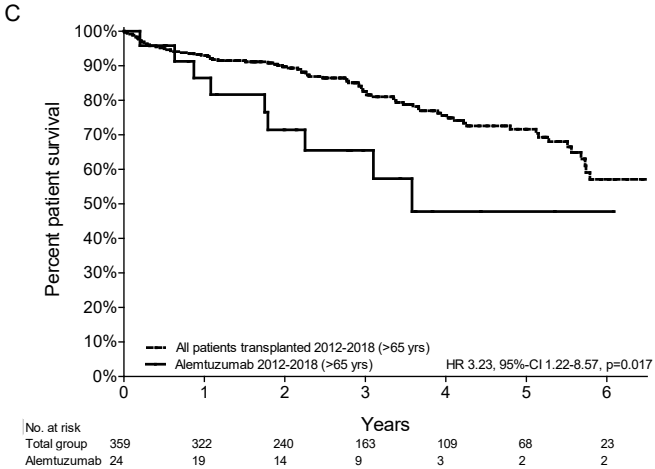
No. at risk							
Total group	329	304	249	203	149	98	39
Alemtuzumab	44	40	36	27	11	8	3

B



No. at risk							
Total group	413	374	312	256	191	129	60
Alemtuzumab	48	40	29	18	10	3	

C



No. at risk							
Total group	359	322	240	163	109	68	23
Alemtuzumab	24	19	14	9	3	2	2

Figure S2. Kaplan-Meier survival curves of patient survival of different age categories. a. Patient survival of patients (<50 years at time of transplantation) treated with alemtuzumab for AR (2012-2018) and patients (<50 years at time of transplantation) transplanted in the same period and not treated with alemtuzumab. **b.** Patient survival of patients (50-65 years at time of transplantation) treated with alemtuzumab for AR (2012-2018) and patients (50-65 years at time of transplantation) transplanted in the same period and not treated with alemtuzumab. **c.** Patient survival of patients (>65 years at time of transplantation) treated with alemtuzumab for AR (2012-2018) and patients (>65 years at time of transplantation) transplanted in the same period and not treated with alemtuzumab.

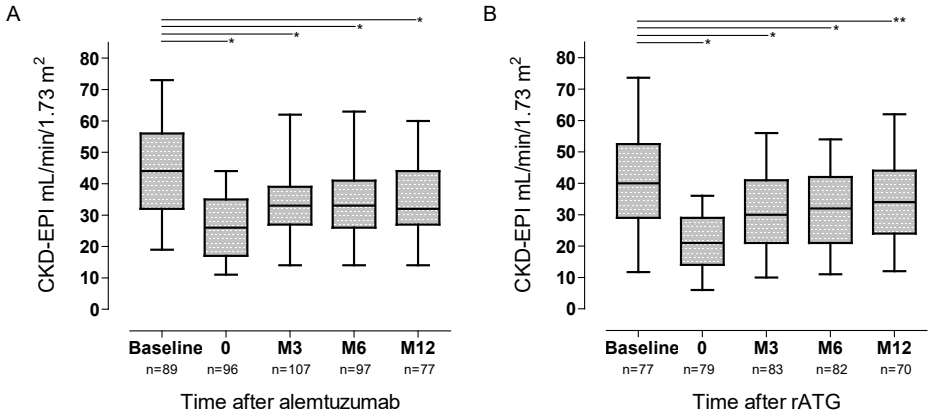


Figure S3. The creatinine clearance (mL/min/1.73m²) of patients treated with alemtuzumab or rATG for AR. The boxes represent median and IQR and the whiskers 5th and 95th percentile. n=number of patients with an eGFR. Baseline = best serum creatinine or eGFR in three months before AR, 0 = serum creatinine or eGFR on day of AR, M3 = 3 months after alemtuzumab (±4 weeks), M6 = 6 months (±6 weeks) after alemtuzumab, M12 = 12 months after alemtuzumab (±8 weeks). *p<0.05, **p=not significant

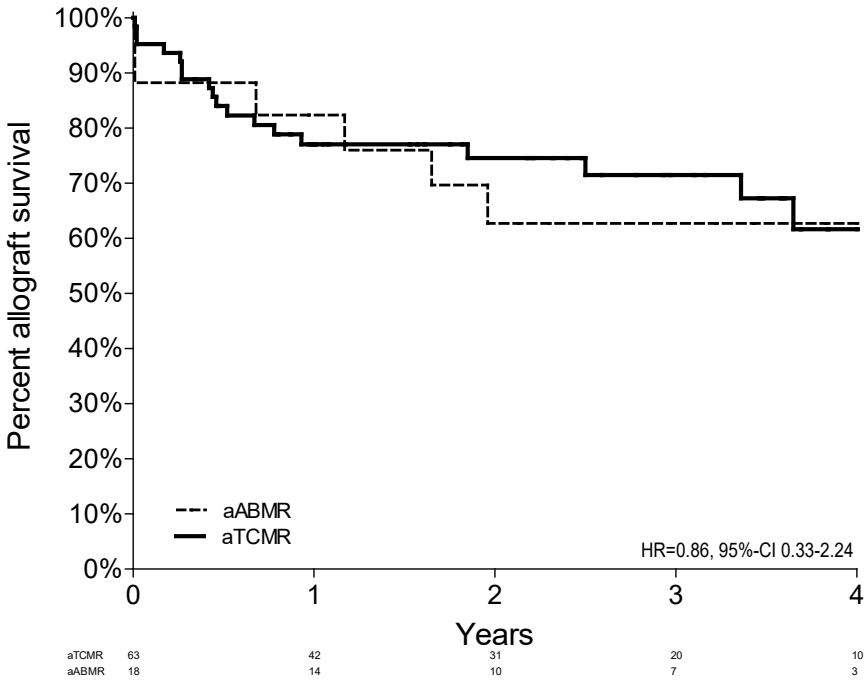


Figure S4. Kaplan-Meier survival curve of allograft survival of alemtuzumab-treated patients with aTCMR or aABMR

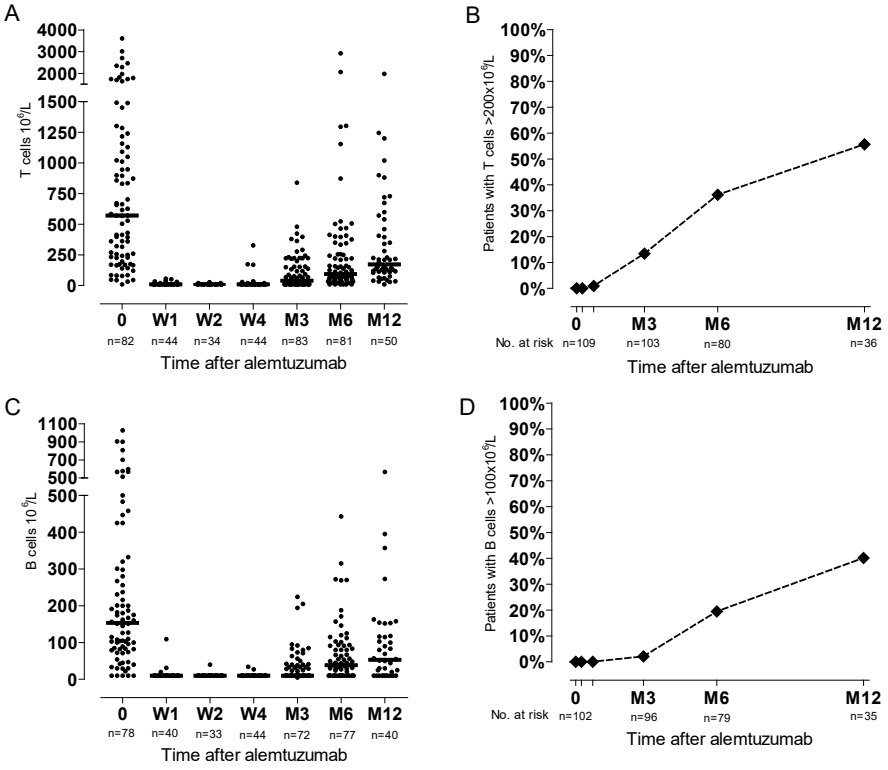


Figure S5. T- and B cells after alemtuzumab therapy. T- and B cells were measured every three months, until T cells were >200 x 10^6/L. **a**. Scatter dot plot of all measured T cells on different time points after alemtuzumab therapy. The horizontal line depicts the median. **b**. Percent of patients with repopulation of T cells >200 x 10^6/L in the year after alemtuzumab therapy. **c**. Scatter dot plot of all measured B cells on different time points after alemtuzumab therapy. The horizontal line depicts the median. **d.** Percent of patients with repopulation of B cells >100 x 106/L in the year after alemtuzumab therapy.

SUPPLEMENTAL DIGITAL CONTENT

Tables

SDC Table 1. Patients with a second biopsy between methylprednisolone and alemtuzumab to confirm ongoing rejection.

Patient no.	1st Banff diagnosis	Treatment	Days between 1st and 2nd biopsy	2nd Banff diagnosis
1	aABMR	Methylprednisolon 3 x 1000 mg	8	aTCMRIIA
2	aABMR	Methylprednisolon 3 x 1000 mg + IVIg	50	aABMR
3	aABMR	Methylprednisolon 6 x 1000 mg	7	aTCMRIIB
4	aTCMRIB	Methylprednisolon 3 x 1000 mg	85	aTCMRIB
5	aTCMRIIA	Methylprednisolon 6 x 1000 + IVIg	16	aTCMRIIA
6	aABMR	Methylprednisolon 3 x 1000 mg + IVIg	61	aABMR
7	aTCMRIIB	Methylprednisolon 3 x 1000 mg + IVIg	111	chronic active TCMR
8	aTCMR (biopsy missing)	Methylprednisolon 3 x 1000 mg	77	aTCMRIB
9	aTCMRIA	Methylprednisolon 3 x 1000 mg + IVIg	52	c/aABMR, borderline TCMR
10	aTCMRIB	Methylprednisolon 3 x 1000 mg	80	aTCMRIIA, aABMR
11	aTCMRIB	Methylprednisolon 6 x 1000 mg	17	borderline TCMR
12	aTCMRIB	Methylprednisolon 3 x 1000 mg	54	aTCMRIB
13	borderline aTCMR	Methylprednisolon 3 x 1000 mg	86	Acute tubular necrosis
14	aABMR	Methylprednisolon 3 x 1000 mg + IVIg	33	aABMR
15	aTCMRIA	Methylprednisolon 3 x 1000 mg	126	chronic active TCMR
16	aTCMRIIA	Methylprednisolon 3 x 1000 mg	9	aTCMRIIA
17	aTCMRIB	Methylprednisolon 3 x 1000 mg	111	chronic active TCMR
18	aTCMRIIB	Methylprednisolon 3 x 1000 mg + IVIg	7	ABMR (biopsy missing)

aABMR active antibody mediated rejection; aTCMR acute T cell mediated rejection; IVIg intravenous immunoglobulins

SDC Table 2. Cause of death after therapy with alemtuzumab or rATG

Adverse events	Alemtuzumab	rATG
Patient death - no (%)	18 (15.5)	17 (16.5)
Time after therapy - yr.	1.45 (0.92-2.93)	3.1 (1-6.3)
Cause of death - no.		
Infectious	7	5
Carcinoma	4	2
Cardiovascular	2	3
Hepatic failure	1	1
Allograft failure	2	0
Unknown	2	6

Data are numbers (percentage) and median (interquartile range).

SDC Table 3. Univariable Cox proportional hazard regression analysis for risk of death within patients treated with alemtuzumab

Variable (reference category)	Exp (B)	95% CI for Exp (B)	p-value
Patient characteristics			
Recipient age at transplantation (per yr)	1.09	1.04-1.14	<0.0001
Recipient age at acute rejection (per yr)	1.09	1.04-1.14	<0.0001
Donor age (per yr)	1.00	0.97-1.03	1.00
Gender (female)	0.44	0.29-1.73	0.44
Ethnicity (Caucasian)	0.40	0.12-1.37	0.14
Transplant number (1)	0.55	0.16-1.88	0.34
PRA current (<6%)	1.07	0.36-3.23	0.90
Transplant characteristics			
Type donor (living)	1.20	0.46-3.16	0.71
HLA mismatch (per HLA mismatch)	0.88	0.64-1.19	0.40
Therapy characteristics			
Maintenance therapy (TAC+MMF±glucocorticoids)	5.70	0.76-42.83	0.09
Glucocorticoid maintenance (no)	1.63	0.47-5.60	0.44
Rejection characteristics			
Timing rejection (< 3 months)	0.52	0.20-1.32	0.17
Type rejection			0.41
DSA vs no DSA	0.40	0.11-1.44	0.16
CKD at time rejection (CKD 3)			0.30
Δ eGFR Baseline- moment of rejection			0.99
Interval methylprednisolon-alemtuzumab	1.00	0.99-1.02	0.83
Allograft loss	1.18	0.47-2.94	0.72
T cells after 3 months	1.00	0.99-1.01	0.85

Increasing age at time of transplantation or AR resulted in increased risk of death. Ethnicity is caucasian or non-caucasian. Transplant number is 1 or >1. PRA current is < 6% or ≥6%. Type donor is living or deceased. Maintenance therapy is TAC+MMF± glucocorticoids or the other combinations of drugs. Glucocorticoid maintenance is use at the time of rejection. Timing of the rejection is <3 or > 3months after transplantation. Type rejection is aTCMR, ABMR, or mixed. CDK at time rejection is CKD1+2+3, CKD4, CKD5, and delayed graft function+ primary non-function. Interval between methylprednisolon and alemtuzumab is days. T cells three months after alemtuzumab is a continue variable. Data of rATG-treated patients were described previously[10]. CKD chronic kidney disease; MMF mycophenolate mofetil; PRA panel reactive antigen; TAC tacrolimus

SDC Table 4. Univariable Cox proportional hazard regression analysis for allograft loss in patients treated with alemtuzumab

Variable (reference category)	Exp (B)	95%-CI for Exp (B)	p-value
Patient characteristics			
Recipient age at transplantation (per yr)	0.97	0.95-0.99	0.002
Recipient age at acute rejection (per yr)	0.97	0.95-0.99	0.003
Donor age (per yr)	0.99	0.97-1.01	0.27
Gender (female)	0.99	0.53-1.86	0.97
Ethnicity (Caucasian)	1.11	0.57-2.15	0.77
Transplant number (1)	1.1	0.53-2.23	0.81
PRA actual (<6%)	1.35	0.64-2.87	0.43
Transplant characteristics			
Type donor (living)	1.48	0.77-2.85	0.24
HLA mismatch (per HLA mismatch)	0.72	0.58-0.89	0.002
HLA mismatch (0-3)	0.34	0.17-0.65	0.001
Therapy characteristics			
Maintenance therapy (TAC+MMF± glucocorticoids)	1.19	0.55-2.60	0.66
Glucocorticoid maintenance (no)	0.32	0.17-0.60	<0.0001
Frequency alemtuzumab (1)	1.19	0.56-2.54	0.65
Rejection characteristics			
Timing rejection (< 3 months)	1.39	1.01-1.91	0.04
Type rejection (aTCMR)			0.19
DSA vs no DSA	1.36	0.66-2.81	0.40
CKD at time rejection (CKD1-3)			0.20
Δ eGFR baseline-moment of rejection			0.004
25 till 50% drop versus <25% drop	0.91	0.33-2.53	0.81
>50% drop versus <25% drop	3.36	1.29-8.75	0.008
Interval methylprednisolone-alemtuzumab	1.00	0.99-1.01	0.49

Transplant number is 1 or >1. PRA current is < 6% or ≥6%. Type donor is living or deceased. Maintenance therapy is TAC+MMF± glucocorticoids or the other combinations of drugs. Glucocorticoid maintenance is use at the time of rejection. Frequency of alemtuzumab is 1 or >1. Timing of the rejection is <3 or > 3 months after transplantation. Type rejection is aTCMR, ABMR, or mixed. CDK at time rejection is CKD1+2+3, CKD4, CKD5+ delayed graft function+ primary non-function. Interval between methylprednisolone and alemtuzumab is days. Data of rATG-treated patients were described previously[10]. CKD chronic kidney disease; DSA de novo donor specific antibodies; MMF mycophenolate mofetil; PRA panel reactive antibodies; TAC tacrolimus

SDC Table 5. Characteristics and statistical analysis of alemtuzumab-treated patients with HLA mismatch of 0-3, and patients with HLA mismatch of 4-6

Characteristic	HLA MM 0-3 (n=57)	HLA MM 4-6 (n=58)	p-value
Recipient age at transplantation- yr.	54 (38-63)	57 (40-63)	0.31
Recipient age at rejection- yr.	54 (38-63)	58 (42-64)	0.28
Donor age - yr.	54 (40-63)	54 (40-63)	0.59
Female sex - no. (%)	25 (43)	22 (38)	0.71
Cause of ESRD - no.			
DM/HTN/GN/PKD/reflux/other/unknown	12/9/12/4/5/16/0	14/13/9/5/2/11/4	0.28
Ethnic distribution - no.			
Caucasian/Black/Asian/Arab/other	40/8/4/6/0	39/9/4/5/1	1.00
Transplant number - no.			
1/2/3	41/12/5	47/10/1	0.22
Preemptive kidney transplantation - no. (%)	22 (38)	19 (32.8)	0.70
Donor type - no.			
LR/LUR/DBD/DCD	24/13/6/15	3/42/6/7	<0.001
Living/deceased	37/21	45/13	0.15
HLA mismatch - no.			
HLA A: 0/1/2	22/33/3	4/28/26	<0.001
HLA B: 0/1/2	10/43/5	0/10/48	<0.001
HLA DR: 0/1/2	19/36/3	2/19/37	<0.001
PRA actual - no. (%)			0.35
0-5%	44 (75.9)	49 (84.4)	
6-83%	13 (22.4)	9 (15.5)	
84-100%	1 (1.7)	0 (0)	
PRA peak - no. (%)			0.29
0-5%	33 (56.9)	36 (62.1)	
6-83%	14 (24.1)	17 (29.3)	
84-100%	11 (19.0)	5 (8.6)	
De novo DSA (ABMR and mixed type rejections)			
DSA+/non-donor HLA antibodies/no DSA/not tested	6/4/2/2	14/1/3/3	0.24
Class I/II/I+II	1/3/2	3/9/2	0.62
CMV IgG serostatus recipient - no. (%)			
Positive	43 (74)	41 (71.9)	0.84
EBV IgG serostatus recipient - no. (%)			
Positive	52 (91.2)	55 (96.5)	0.44
Maintenance therapy (TAC+MMF± glucocorticoids) - no. (%)	49 (84.4)	47 (79.3)	0.63
Glucocorticoid maintenance - no. (%)	44 (75.9)	49 (84.5)	0.35
Timing rejection (< 3 months) - no. (%)	31 (53.4)	32 (55.2)	1.00
Type rejection	34/6/8	32/10/11	0.54
CKD at time rejection	17/21/20	20/18/19	0.80
Interval methylprednisolone-alemtuzumab	6 (4-24)	7 (4-22)	0.80

Data are numbers (%) or median (interquartile range). Type rejection is aTCMR, ABMR, or mixed. CDK at time rejection is CKD1+2+3, CKD4, CKD5+ delayed graft function+ primary non-function. Interval between methylprednisolone and alemtuzumab is days. CMV cytomegalovirus; DBD donation after brain death; DCD donation after circulatory death; DM diabetes mellitus; DSA de novo donor specific antibodies; EBV Epstein-Barr virus; ESRD end stage renal disease; GN glomerulonephritis; HLA human leucocyte antigen; HTN hypertensive nephropathy; LR living related; LUR living unrelated; MMF mycophenolate mofetil; PKD polycystic kidney disease; PRA panel reactive antibody; rATG rabbit anti-thymocyte globulin; TAC tacrolimus

SDC Table 6. Infections during the total follow-up after alemtuzumab and rATG treatment

Infections	Alemtuzumab	rATG
Infection in the first year - no.	96	124
Viral	14	19
Fungal	3	8
Bacterial	79	97
Blood	3	8
Urinary tract/urosepsis	47	51
Skin and soft tissue	5	9
Lung	16	15
Tuberculosis	2	0
Other/unknown	6	14
Infection in the first 15 days	9	29
CMV infections		
CMV reactivation - no. (%)	25 (21.6)	27 (25)
CMV disease - no. (%)	2 (1.7)	0
Primary CMV infection - no. (%)	2 (1.7)	2 (1.9)
CMV, time after therapy	76 (52-167)	32 (19-74)
EBV infections - no. (%)	0	1 (9)
BK infections		
BK viremia - no. (%)	20 (17.2)	6 (5.6)
BK viremia, time after therapy	106 (59-186)	458 (322-844)

Data are numbers (%) or median (interquartile range). CMV, EBV and BK virus infections were scored apart from the infections during the total follow-up, in the first year, and after the first year.

SDC Table 7. Malignancies after alemtuzumab treatment

Malignancy	Months after alemtuzumab	Months after transplantation	Age of patient
Lung cancer	9	10	57
Lung cancer	27	38	61
Pancreatic cancer	37	43	73
Breast cancer	28	32	65
Prostate carcinoma	43	44	80
Adenocarcinoma of unknown primary*	38	46	60
Colon cancer**	8	58	76

Age of patient is at the time of malignancy. *No biopsy of the metastasis was taken. **This patient had pre-transplantation colon cancer and developed metastasis of colon cancer after alemtuzumab.

SDC Table 8. Malignancies after rATG treatment

Malignancy	Months after rATG	Months after transplantation	Age of patient
Endometrial carcinoma	67	69	57
Adenocarcinoma lung	68	157	61
Non seminoma testis	17	23	44
Colon carcinoma	21	21	45
Rectal carcinoma	17	62	52
Meningioma	28	44	62
Renal carcinoma	107	107	34
Renal carcinoma	77	77	69
Renal carcinoma	140	140	55
Renal carcinoma	144	144	56
Prostatic carcinoma	10	35	54
Prostatic carcinoma	77	77	69
Non-Hodgkin lymphoma	78	79	56
EBV related B-lymphoma	14	14	65

Age of patient is at the time of malignancy. The Epstein-Barr virus (EBV)-related lymphoma was in an IgG seropositive patient and occurred fourteen months after treatment with rATG and was treated with irradiation.

CHAPTER 10

Guillain-Barré syndrome and chronic inflammatory demyelinating polyradiculoneuropathy after alemtuzumab therapy in kidney transplant recipients

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INTRODUCTION

Alemtuzumab is approved for the treatment of relapsing-remitting multiple sclerosis and is used off-label for patients with chronic lymphocytic leukemia and as induction- and anti-rejection therapy in kidney transplant recipients[1]. Guillain-Barré syndrome (GBS) or chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) complicating alemtuzumab treatment was reported in nine patients with hematologic malignancy or multiple sclerosis[1-3]. The risk of GBS or CIDP in solid organ transplant recipients treated with alemtuzumab is unknown.

Rabbit anti-thymocyte globulin (rATG) is another T cell-depleting drug used to treat acute kidney allograft rejection. Only one patient was reported who developed GBS after rATG treatment for aplastic anemia[4]. We found no reports of GBS or CIDP complicating rATG treatment in kidney transplant recipients. Here, we investigated the frequency, type, and outcome of GBS and CIDP in a single-center cohort of kidney transplant recipients treated with either alemtuzumab or rATG.

PATIENTS AND METHODS

Study design

A retrospective analysis was performed of a cohort of kidney transplant recipients who received either alemtuzumab (Campath, Sanofi-Genzyme, Cambridge, Massachusetts, USA) or rATG (Thymoglobulin, Sanofi-Genzyme) between 2002 and 2018 in the Erasmus MC, Rotterdam. Alemtuzumab was administered as a single dose of 30 mg subcutaneously and rATG in a dose of 4 mg/kg intravenously.

Statistical methods

Continuous variables are presented as median and interquartile ranges (IQRs). The 95%-confidence intervals (CI) were calculated with the modified Wald method. For statistical analysis, SPSS version 21 (SPSS Inc., Chicago, IL, USA) was used.

Standard protocol approval, registrations and patient consents

The study was approved by the Erasmus MC Medical Ethical Review Board (number 2018-1430).

RESULTS

Between 2002 and 2018, 2,788 patients received a kidney transplant at our center. Alemtuzumab was administered to 143 (5.1%) patients and rATG to 108 (3.9%) patients. The total follow-up period of patients treated with alemtuzumab was 3.0 years (IQR 1.7-4.1) for a total of 444.3 person-years. A tacrolimus-based immunosuppressive regimen was given to 92% of patients. Three patients (2.1%, 95%-CI 0.4%-6.3%) developed GBS or CIDP after alemtuzumab. Two patients fulfilled the diagnostic criteria for GBS and one fulfilled the diagnostic criteria for CIDP. The clinical presentation and diagnostic findings of these patients are presented in Table 1. Laboratory tests, including clinical chemistry, serology and virology demonstrated no alternative diagnoses, and there was no recent *Campylobacter jejuni* or cytomegalovirus infection (PCR negative for cytomegalovirus). The total follow-up period for rATG-treated patients was 8.2 (IQR 6.3-11) years for a total of 829.4 person-years. Seventy-eight percent of patients received a tacrolimus-based immunosuppressive regimen. None of the patients treated with rATG (0%, 97.5%-CI 0-4.1%) developed GBS or CIDP.

Table 1. Clinical characteristics, diagnosis and outcome of patients with GBS and CIDP after alemtuzumab

Case	1 (GBS)	2 (GBS)	3 (CIDP)
Gender	Male	Female	Male
Age at onset symptoms (years)	54	57	63
Primary kidney disease	Polycystic kidney disease	Reflux nephropathy	Polycystic kidney disease
CMV status at transplantation	Seropositive	Seronegative	Seropositive
Induction therapy	Alemtuzumab (30 mg, 30 days before transplantation, IVIg (0.4g/kg on day of transplantation), immunoabsorption	Basiliximab	Basiliximab
Anti-rejection therapy	None	Alemtuzumab (30 mg)	Alemtuzumab (30 mg)
Immunosuppressive treatment at onset symptoms	Tacrolimus/ MMF/ prednisolone 5 mg/day	Tacrolimus/ MMF/ prednisolone 2.5 mg/day	Tacrolimus/ MMF/ prednisolone 5 mg/day
Diagnosis	GBS, level 2 of Brighton classification (No electrophysiological studies available)	GBS (AIDP), level 1 of Brighton classification	CIDP (fulfillment of clinical criteria and definite electrophysiological criteria EFNS/PNS 2010)
Interval between alemtuzumab treatment and symptoms (months)	4	8	42
Onset to maximum severity (days)	21	10	90
Maximum mRS (range 0-6)	4	5	4
Maximum GBS disability score (range 0-6)	4	5	-
EGOS (range 0-7)	3.5	6.5	-
Treatment	IVIg (0.4 g/kg) for 5 days	IVIg (0.4 g/kg) for 5 days	2x IVIg (0.4 g/kg) for 5 days, 4 sessions of plasma-exchange, methylprednisolone (3x1000mg), prednisone 5mg daily (maintenance)
mRS after treatment	1	6	1
GBS disability score after treatment for GBS	1	6	-
Neurologic outcome at the last follow-up	Partial recovery (follow up 1 year)	Death (6 months later, due to malignancy)	Full recovery (follow-up 3 years)

AIDP, acute inflammatory demyelinating polyradiculoneuropathy; CIDP, chronic inflammatory demyelinating polyradiculoneuropathy; EGOS, Erasmus GBS outcome score; GBS, Guillain-Barre syndrome; IVIg, intravenous immunoglobulins; MMF mycophenolate mofetil; mRS modified Rankin Scale

DISCUSSION

This study shows that 2.1% of patients treated with alemtuzumab developed GBS or CIDP. This is higher than the incidence rate of these neuropathies in the general population and of kidney transplant recipients not treated with alemtuzumab[5-7]. Secondary autoimmunity after alemtuzumab appears to be mainly B cell-driven. A mismatched reconstitution of T and B cells after alemtuzumab can lead to an expansion of B cells in the absence of appropriate T cell regulation. This may enable the escape of autoreactive B cells and production of pathogenic autoantibodies to self-antigens which can lead to secondary autoimmunity, such as thyroiditis, idiopathic thrombocytopenic purpura, GBS or CIDP[1].

None of the patients treated with rATG developed GBS or CIDP. A possible explanation for the difference in the risk of developing these neuropathies with alemtuzumab is that the depletion of immune cells lasts longer after alemtuzumab[1]. Alternatively, rATG may protect from GBS and CIDP.

Limitations of the current study are that we were unable to define the frequency of GBS and CIDP in kidney transplant recipients not treated with T cell-depleting therapy. Second, no causality between alemtuzumab and the risk of GBS or CIDP was demonstrated, and our findings may therefore relate to chance. Third, cytomegalovirus could have played a role in the development of GBS or CIDP because patients 1 and 3 were seropositive for cytomegalovirus at the time of transplantation. However, no signs of a reactivation were observed at the time the patients were diagnosed with GBS and CIDP. Fourth, we cannot exclude that the increased incidence of GBS and CIDP among alemtuzumab-treated patients may relate to the fact that in this group, more patients used tacrolimus as maintenance immunosuppression compared with the rATG cohort. Fifth, this observation is based on kidney transplant recipients who have several reasons to have an underlying neuropathy (*i.e.,* renal insufficiency and diabetes mellitus) and it is uncertain whether it is also applicable to patients with multiple sclerosis.

In conclusion, alemtuzumab therapy in kidney transplant recipients may be associated with the development of GBS and CIDP. Clinicians should be alert for these neurological complications in kidney transplant recipients treated with alemtuzumab.

REFERENCES

1. van der Zwan M, Baan CC, van Gelder T, Hesselink DA. Review of the Clinical Pharmacokinetics and Pharmacodynamics of Alemtuzumab and Its Use in Kidney Transplantation. *Clin Pharmacokinet.* 2018;57(2):191-207.
2. Avivi I, Chakrabarti S, Kottaridis P, et al. Neurological complications following alemtuzumab-based reduced-intensity allogeneic transplantation. *Bone Marrow Transplant.* 2004;34(2):137-142.
3. Hradilek P, Woznicova I, Slonkova J, Lochmanova A, Zeman D. Atypical acute motor axonal neuropathy following alemtuzumab treatment in multiple sclerosis patient. *Acta Neurol Belg.* 2017;117(4):965-967.
4. Kaya B, Davies CE, Oakervee HE, et al. Guillain Barre syndrome precipitated by the use of antilymphocyte globulin in the treatment of severe aplastic anaemia. *J Clin Pathol.* 2005;58(9):994-995.
5. Willison HJ, Jacobs BC, van Doorn PA. Guillain-Barre syndrome. *Lancet.* 2016;388(10045):717-727.
6. Ostman C, Chacko B. Guillain-Barre syndrome post renal transplant: A systematic review. *Transpl Infect Dis.* 2019;21(1):e13021.
7. Echaniz-Laguna A, de Seze J, Chanson JB. Chronic inflammatory demyelinating polyradiculoneuropathy in solid organ transplant recipients: a prospective study. *J Neurol Neurosurg Psychiatry.* 2012;83(7):699-705.

CHAPTER 11

Acquired hemophilia A after alemtuzumab therapy

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Haemophilia 2020; Jul

To the editor:

Treatment with alemtuzumab leads to a prolonged depletion of T- and B cells, natural killer cells, dendritic cells and monocytes[1]. Alemtuzumab therapy is associated with secondary auto-immune disorders, including auto-immune thyroid disease, immune thrombocytopenia and inflammatory neuropathy[1]. Here, two patients with acquired hemophilia A (AHA) are described who were previously treated with alemtuzumab.

Patient 1 was a 24-year-old male who received a kidney transplant because of end-stage kidney disease caused by Alport syndrome. One year after transplantation he was treated for a mixed-type kidney transplant rejection. He received methylprednisolone (total dose 3,000 mg) and intravenous immunoglobulins (2 g/kg) followed by alemtuzumab (30 mg, subcutaneously on two consecutive days). Four years after treatment for rejection, he presented with spontaneous bruising. He did not experience unintentional weight loss, fever, infection or night sweats. His immunosuppressive regimen at that time consisted of tacrolimus and prednisolone (5 mg/day). Physical examination at presentation revealed multiple large hematomas on all extremities and on his thorax. Laboratory testing demonstrated an isolated prolonged activated partial thromboplastin time (aPTT) of 80 seconds (Table 1). The aPTT did not correct after mixing with normal pooled plasma (46 seconds). Factor VIII coagulation activity (FVIII:C) was strongly reduced (0.02 U/mL; normal 0.60-1.40 U/mL). A Factor VIII inhibitor was confirmed and quantified with the Nijmegen modification of the Bethesda assay (10 Bethesda Units [BU/ml]; Table 1 and Figure 1A). Additional work-up showed repopulation of T- and B-lymphocytes (Table 1). The patient was diagnosed with AHA and was treated with a single administration of activated prothrombin complex concentrate (factor eight inhibitor bypassing activity [FEIBA®] 50 U/kg) to stop bleeding of a severe bleeding in his arm resulting in pain and impairment of joint mobility of the elbow (Figure 1A). Immunosuppressive therapy was immediately started with prednisolone (1 mg/kg/day, *i.e.* 80 mg/day) and cyclophosphamide (100 mg/day). The aPTT shortened and FVIII:C increased within two weeks whereupon cyclophosphamide was discontinued (Figure 1A). The patient achieved complete remission of the AHA after six weeks with normalization of FVIII:C and no detectable Factor VIII inhibitor (Figure 1A). Prednisolone was tapered to 5 mg over a period of six months. Thereafter, prednisolone was continued (5 mg/day) as immunosuppressive therapy for the kidney transplantation.

Table 1.

Parameter	Patient 1	Patient 2	Reference range
Hemoglobin (g/dL)	10.3	10.8	13.7-17.7
Platelets (x10^9/L)	391	217	150-400
Leukocytes (x10^9/L)	15.6	5.8	4-10
Lymphocytes (x109/L)	0.84	0.80	0.50-5.00
B-lymphocytes (x109/L)	0.21	-	-
T-lymphocytes (x109/L)	0.63	-	-
CD4/CD8 ratio	3.9	-	-
aPTT (seconds)	80	62	25-31
PT (seconds)	11.3	13.1	9.5-13-5
aPTT mix (seconds)	46	54	25-31
Factor VIII (U/mL)	0.02	0.02	0.60-1.40
vWF antigen (U/mL)	1.79	2.31	0.60-1.40
vWF activity (U/mL)	1.67	2.94	0.60-1.40
Bethesda VIII (BU/ml)	10	24.6	<0.3

Laboratory testing of the hemostasis parameters at presentation. aPTT, activated partial thromboplastin time; PT, prothrombin time; vWF, von Willebrand Factor

Patient 2 was a 42-year-old male who received alemtuzumab (intravenously, 12 mg/day for five days and one year later 12 mg/day for three days) for relapsing-remitting multiple sclerosis. Fourteen months after the last dose, he presented with a spontaneous large hematoma on his back without other complaints. He did not use any medications at the time of presentation. Physical examination revealed a large hematoma in the lumbar region which extended to his right upper leg, which was illustrated earlier[2]. Laboratory testing revealed an isolated prolonged aPTT of 62 seconds which did not normalize after mixing with normal pooled plasma (54 seconds; Table 1). FVIII:C was strongly reduced (0.02 U/mL) and a Factor VIII inhibitor was present (24.6 BU/ml; Table 1). The lymphocyte count was normal (Table 1). A diagnosis of AHA was made and he was treated because of severe bleeding with a hemoglobin drop to 10.8 g/dL with FEIBA® 100 U/kg on the first day followed by 50 U/kg two times daily for six days. He was also treated with prednisolone (1 mg/kg/day, *i.e.* 90 mg/day) and cyclophosphamide (200 mg/day) as immunosuppressive therapy (Figure 1B). Complete remission of AHA was achieved after six weeks (Figure 1B). Cyclophosphamide was stopped ten weeks after presentation and prednisolone was tapered over a period of seven months.

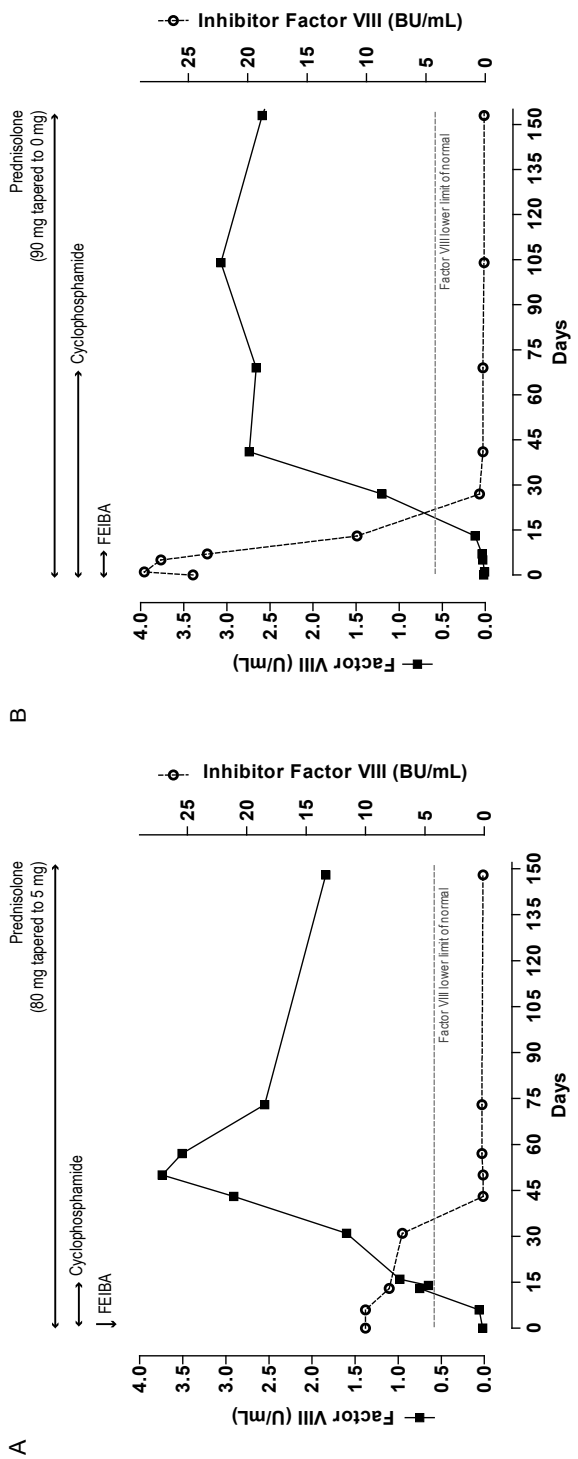

Figure 1. The concentrations of Factor VIII and inhibitor of Factor VIII in the first six months of patient 1 (**A**) and patient 2 (**B**). The dashed line depicts the lower limit of normal of Factor VIII. BU, Bethesda Units.

AHA is a rare auto-immune disorder caused by autoantibodies directed against Factor VIII. It occurs mainly at (very) high age, or in young women after pregnancy. It is usually idiopathic (50% of cases) but AHA may also be associated with malignancies, auto-immune diseases, infections, pregnancy and drugs[3-5]. In our patients, additional investigations, including auto-immune serology (for systemic lupus erythematosus and rheumatoid arthritis), virology (of hepatitis B and C, and Epstein Barr virus) and a CT scan of the thorax and abdomen, did not reveal any underlying cause of AHA in our patients. There was no monoclonal gammopathy, white blood cell and platelet counts were normal, nor were there other signs of a hematological malignancy. Currently, both patients (three and one year after presentation) are still in remission of AHA after treatment with glucocorticoids and cyclophosphamide and no other auto-immune disorders or malignancies have occurred[6].

In literature, an association between alemtuzumab and AHA has been described in four patients[7-11]. The indication for alemtuzumab was multiple sclerosis in three cases[7-10] and anti-neutrophil cytoplasmic antibody-associated vasculitis in one case[11]. The interval between alemtuzumab therapy and the diagnosis of AHA ranged from 11 months[9,10] to 5 years[8,11]. Depletion of regulatory T cells, natural killer and dendritic cells and escape of auto-reactive B cells after alemtuzumab therapy have been proposed to contribute to the increased susceptibility of secondary autoimmunity[1]. Because of the lack of another explanation and the remarkable young age of our patients, we believe that AHA in our patients was related to the administration of alemtuzumab. Therefore, AHA should be considered in the differential diagnosis of an alemtuzumab-treated patients presenting with an acquired bleeding disorder.

REFERENCES

1. van der Zwan M, Baan CC, van Gelder T, Hesselink DA. Review of the Clinical Pharmacokinetics and Pharmacodynamics of Alemtuzumab and Its Use in Kidney Transplantation. *Clin Pharmacokinet*. 2018;57(2):191-207.
2. Brink HS, Moll W, Sandberg Y. Acquired haemophilia A after alemtuzumab treatment of multiple sclerosis [published online ahead of print, 2020 Apr 16]. *Br J Haematol* 2020.
3. Kruse-Jarres R, Kempton CL, Baudo F, et al. Acquired hemophilia A: Updated review of evidence and treatment guidance. *Am J Hematol*. 2017;92(7):695-705.
4. Knoebl P, Marco P, Baudo F, et al. Demographic and clinical data in acquired hemophilia A: results from the European Acquired Haemophilia Registry (EACH2). *J Thromb Haemost.* 2012;10(4):622-631.
5. Tengborn L, Baudo F, Huth-Kuhne A, et al. Pregnancy-associated acquired haemophilia A: results from the European Acquired Haemophilia (EACH2) registry. *BJOG.* 2012;119(12):1529-1537.
6. Collins PW. Therapeutic challenges in acquired factor VIII deficiency. *Hematology Am Soc Hematol Educ Program*. 2012;2012:369-374.
7. Pisa M, Della Valle P, Coluccia A, et al. Acquired haemophilia A as a secondary autoimmune disease after alemtuzumab treatment in multiple sclerosis: A case report. *Mult Scler Relat Disord*. 2019;27:403-405.
8. Madeley J, Hodges G, Birchley A. Development of acquired haemophilia A in a patient treated with alemtuzumab for multiple sclerosis. *BMJ Case Rep*. 2018;2018.
9. McCaughan G, Massey J, Sutton I, Curnow J. Acquired haemophilia A complicating alemtuzumab therapy for multiple sclerosis. *BMJ Case Rep*. 2017;2017.
10. Massey J, Barnett Y, Curnow J, Sutton I. B cell depletion therapy resulting in sustained remission of severe autoimmune complications following Alemtuzumab treatment of Multiple Sclerosis. *Mult Scler Relat Disord.* 2019;35:100-103.
11. Clatworthy MR, Jayne DR. Acquired hemophilia in association with ANCA-associated vasculitis: response to rituximab. *Am J Kidney Dis.* 2006;47(4):680-682.

PART IV

SUMMARY AND DISCUSSION

CHAPTER 12

SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Based on:

Snijders MLH, Varol H, **van der Zwan M**, Becker JU, Hesselink DA, Baan CC, von der Thüsen JH, Clahsen-van Groningen MC. *Molecular analysis of renal allograft biopsies: where do we stand and where are we going.* Transplantation 2020; Mar 6

Van der Zwan M, Hesselink DA, Baan CC, Clahsen-van Groningen MC**.** *Chronic active antibody mediated rejection: to belatacept or not, that is the HOT question.* Transplantation 2020; Jun

SUMMARY

Kidney transplantation is a lifesaving procedure. However, transplantation of a foreign organ leads to transplant rejection if the kidney transplant recipient is not treated with immunosuppressive drugs. Kidney transplant rejection still occurs in approximately a quarter of kidney transplant recipients and is associated with a high burden of morbidity and mortality[1]. The quote:

"An ounce of prevention is worth a pound of cure."
Benjamin Franklin (1705-1790)

therefore can be applied to the care of kidney transplant recipients. Minimizing or preventing adverse events related to the current standard immunosuppressive maintenance therapy will aid in improvement of patient- and allograft kidney transplant outcomes. In addition, early detection and optimal treatment of acute rejection are important to prevent irreversible allograft injury. This thesis explores ways to optimize maintenance immunosuppressive treatment, and diagnosing and treating kidney transplant rejection.

Prevention of acute rejection

Current immunosuppressive regimens consist of induction therapy and maintenance therapy. Induction therapy is administered during transplant surgery. Maintenance therapy is initiated directly after surgery and must be continued life-long[2]. The current standard of care maintenance therapy in most kidney transplant centers includes tacrolimus (a calcineurin inhibitor [CNI]) and mycophenolic acid (MPA, an anti-proliferative agent). With this regimen, the short-term kidney allograft survival is good[3]. However, this immunosuppressive regimen has limitations that impact long-term patient- and allograft survival: i) harmful adverse events, including nephrotoxicity, infection, malignancy, diabetes mellitus, hypertension and dyslipidemia, and ii) incomplete prevention of acute kidney transplant rejection still occurs in a significant proportion of patients[4].

The current standard immunosuppressive maintenance therapy also is a "one size fits all" protocol. Almost all kidney transplant recipients are currently treated with a combination of CNI, MPA and/or glucocorticoids[3]. This disregards the fact that each patient has individual needs. Patient- or donor-related factors such as co-morbidity, organ quality and immunological risk are in general not considered when choosing a specific immunosuppressive regimen[5]. The last few years only a few of novel immunosuppressive

drugs have been tested in kidney transplant recipients[3,6-11]. One group of drugs that has the potential to be part of the immunosuppressive regimen in the future are biologicals that intervene with the costimulatory pathway between T cells and antigen-presenting cells (APCs).

In **Chapter 2**, we provide an overview of costimulation blockade therapies that are currently in use or being developed for kidney transplantation. Belatacept, a biological that inhibits the interaction between the antigens CD28 and CD80/86, is currently the only costimulation blockade drug that is approved by the U.S. Food and Drug Administration for the prevention of acute kidney transplant rejection[12]. Belatacept therapy is associated with a superior kidney function and reduced incidence of *de novo* donor-specific anti-HLA antibodies (DSA; **Chapter 2**) in comparison with CNI-based immunosuppression. However, the use of this drug in the clinic is not widespread because of the associated increased risk of acute rejection (**Chapter 2**). A better understanding of the pathogenesis of belatacept-resistant acute rejection and identification of patients with a low risk of belatacept-resistant acute rejection will likely expand the use of belatacept. This was investigated in **Chapter 5**. We compared kidney transplant biopsies with acute T cell-mediated rejection (aTCMR) of patients treated with belatacept maintenance immunosuppressive therapy to that of patients treated with tacrolimus. We could not detect a difference in the expression of 209 genes known to be involved in acute kidney transplant rejection. There was also no difference in immunophenotypic analysis by immunohistochemistry between patients treated with belatacept or tacrolimus. These findings suggest that patients treated with either belatacept- or tacrolimus-based immunosuppressive therapy share a common pathway of aTCMR.

Therefore, we questioned if it was possible to identify belatacept-treated patients with a low risk of acute rejection (**Chapter 6**)**.** We analyzed the concentrations of 92 inflammation-related proteins in prerejection serum samples of belatacept-treated patients and compared them with serum samples of belatacept-treated patients without rejection. No difference was observed between the two groups, which implies that this proteomic analysis does not predict acute rejection. To conclude, no clinical tests are currently available to reliably predict the risk of belatacept-resistant rejection besides the conventional pre-transplant assessment of DSAs and HLA mismatch.

In **Chapter 2** we discussed the current position of belatacept as maintenance immunosuppressive therapy in kidney transplant recipients. We concluded that belatacept

still is a promising drug. Kidney transplant recipients that appear to benefit the most of belatacept therapy are those with a low risk of acute rejection and/or have contraindications to CNI, for instance CNI-induced nephrotoxicity, impaired allograft function, delayed graft function, CNI-mediated thrombotic microangiopathy, atypical hemolytic uremic syndrome and poorly controlled type 2 diabetes mellitus. In **Chapter 7** we describe an example of such a patient. This kidney transplant recipient had severe hyperglycemia and neurotoxicity (tremor) during tacrolimus maintenance therapy, both well-known side effects of tacrolimus. After conversion to belatacept a rapid and profound improvement of glucose tolerance was noted. In addition, the patient reported that the tremor disappeared after withdrawal of tacrolimus.

Diagnosis of acute rejection

Despite improvement in the maintenance therapy of kidney transplant recipients, acute rejection still occurs in a significant proportion of patients. Currently, the gold standard test to diagnose acute kidney transplant rejection is the histopathologic examination of a kidney biopsy. However, this is an invasive procedure and interobserver variability and sampling error occur frequently[13,14]. At present, multiple approaches are being developed to improve the diagnosis of acute kidney transplant rejection, for example integration of molecular analysis into the conventional histomorphologic examination of a kidney biopsy, and minimally invasive screening of acute rejection biomarkers in blood or urine. The Banff (2017) report introduced the molecular analysis of genes associated with kidney transplant rejection as a criterion for antibody-mediated rejection (ABMR)[15]. Interestingly, only limited recommendations were made regarding the implementation of molecular analysis for the diagnosis of aTCMR.

In **Chapter 5** we analyzed the expression of the genes presented in the Banff 2017 report in biopsies with and without aTCMR. The gene signature of biopsies with aTCMR was different compared to those without aTCMR. Moreover, a significant increased expression of 78 genes was seen in biopsies with aTCMR. These genes were primarily associated with T cell-associated genes, γ-interferon-inducible genes and effector genes. Importantly, one patient with an isolated vascular lesion was diagnosed with aTCMR grade IIA. However the gene profile was comparable to that of biopsies without aTCMR and the patient did not have a clinical picture of acute rejection. Therefore, we conclude from these preliminary data that implementation of molecular analysis to the conventional examination of a kidney transplant biopsy could help in improving diagnostics of aTCMR in challenging cases. For instance, an isolated vascular lesion may not always be a sign of aTCMR.

As described in **Chapter 6** we tested a minimally-invasive assay to diagnose aTCMR in the serum of kidney transplant recipients. In this pilot study the concentrations of 92 inflammation-related proteins were tested in sera of patients with and without aTCMR. A significant difference was noted between the two groups: Five proteins (T-cell surface glycoprotein CD5 [CD5], T cell surface glycoprotein CD8 [CD8a], Natural cytotoxicity triggering receptor 1 [NCR1], TNF receptor superfamily member 4 [TNFRSF4], and TNF receptor superfamily member 9 [TNFRSF9]) were significantly higher in sera at the time of aTCMR. Moreover, the protein profile of patients with aTCMR was also different from that of patients without aTCMR. These findings implicate that this proteomic analysis is a promising minimally-invasive tool to screen for aTCMR in the blood of kidney transplant recipients.

Treatment of acute rejection

The treatment of choice for glucocorticoid-resistant, severe or recurrent acute kidney transplant rejection is, according to the international guideline, the T cell-depleting drug rabbit anti-thymocyte globulin (rATG)[2]. However, the guideline is based on studies performed more than 25 years ago when induction therapy was not routinely administered and kidney transplant recipients were mainly treated with azathioprine and ciclosporin. None of these patients were treated with current standard of care therapy consisting of tacrolimus and MPA. Therefore, we investigated the long-term patient- and allograft outcomes of rATG treatment for acute rejection in 108 patients treated with tacrolimus- and MPA-based immunosuppressive therapy (**Chapter 8**). We observed that rATG is an effective therapy for glucocorticoid-resistant, severe or recurrent acute kidney transplant rejection, especially in patients with an early acute rejection (in the first month post-transplantation). In addition, patients with tubulointerstitial rejection (aTCMR grade I) had an inferior kidney function one year after therapy compared with patients with a vascular rejection (aTCMR grade II or III). The interval between methylprednisolone treatment and rATG administration was the longest in the aTCMR grade I group suggesting that the clinicians were reluctant to prescribe rATG to these patients, which seems to have resulted in more irreversible damage to the kidney transplant. rATG therapy was not associated with increased mortality compared with patients not treated with rATG. However, severe infusion-related adverse events frequently occurred.

Alemtuzumab is another T cell-depleting agent and is sometimes used off-label in kidney transplant recipients as induction therapy (mostly in highly sensitized kidney transplant recipients) or as anti-rejection therapy. **Chapter 3** reviews the current literature on the

application of alemtuzumab in kidney transplant recipients. Since 2012, in the Erasmus MC, patients are treated with alemtuzumab for glucocorticoid-resistant, severe or recurrent acute kidney transplant rejection. In **Chapter 9** the patient- and allograft outcomes and adverse events of patients treated with either alemtuzumab (n = 113) or rATG (n = 108) in the Erasmus MC were compared. The main conclusions of this study were that the patient- and allograft survival was similar between the two therapies. However, infusion-related side effects and infection occurred less frequently after alemtuzumab therapy and the length of hospital stay was 12 days shorter in the group of patients treated with alemtuzumab.

Alemtuzumab is associated with secondary auto-immune events, such as autoimmune thyroid disorders, immune thrombocytopenia and anti-glomerular basement membrane disease. Alemtuzumab-associated auto-immunity can occur even years after treatment (**Chapter 3**)[16]. We analyzed the kidney transplant recipients treated with alemtuzumab in the Erasmus MC for the occurrence of such auto-immune events. Three kidney transplant recipients treated with alemtuzumab (2.1% of all patients treated with alemtuzumab) developed an inflammatory polyneuropathy (**Chapter 10**). Other causes of inflammatory polyneuropathy were excluded and alemtuzumab may therefore have played a crucial role in the development of inflammatory polyneuropathy. In **Chapter 11**, we describe two patients who developed acquired hemophilia A after therapy with alemtuzumab. Alemtuzumab may have played a role in the formation of auto-antibodies to factor VIII in these patients.

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Prevention of acute rejection

Eight years after the drug was first approved for the prevention of kidney transplant rejection, belatacept remains a promising immunosuppressive drug. Because of the increased rejection risk associated with belatacept compared to tacrolimus-based immunosuppression, many transplant physicians are reluctant to prescribe this drug. Unfortunately, at this moment, no biomarker(s) exists that can predict the risk of belatacept-resistant acute kidney transplant rejection. In **Chapter 6**, we described that identification of belatacept-treated patients with a low risk of kidney transplant rejection was not possible. In that study, we compared (prerejection) serum samples taken on day 30 after transplantation with serum samples on the time of rejection. However, the time between the prerejection samples and the time of rejection ranged between 10 and 90 days and this may have influenced the results. A proteomic study with serial monitoring in these patients, for instance every two weeks, may show protein alterations in the blood before the rejection becomes clinically overt and may provide further information on the pathogenesis of rejections occurring under belatacept-based immunosuppression.

Because no clinical test is available that can predict the risk of belatacept-resistant acute kidney transplant rejection, belatacept is currently reserved for subgroups of patients. In **Chapter 2** we suggest an approach to the selection of patients who may benefit from belatacept and discuss possible treatment protocols and strategies to monitor belatacept treatment. As shown in **Chapter 7**, patients with post-transplantation diabetes mellitus can benefit from conversion from tacrolimus to belatacept. One of the explanations for this improvement in diabetes control is that tacrolimus can induce hypomagnesemia via renal magnesium wasting, which can cause insulin resistance and decreased insulin secretion[17-19]. In addition to a higher risk of diabetes mellitus, hypomagnesemia is one of the factors that contribute to progression of arterial stiffness and increased cardiovascular risk in kidney transplant recipients treated with CNIs[20]. There is some evidence that CNI sparing immunosuppressive regimens based on belatacept are associated with a positive effect on cardiovascular risk[20]. In two small retrospective studies, belatacept therapy resulted in a small improvement in arterial stiffness compared with CNIs[21,22]. In the BENEFIT trials, belatacept therapy was associated with a superior cardiovascular and metabolic profile, including lower blood pressure, improved serum lipids concentrations and a decreased incidence of post-transplantation diabetes mellitus one year after transplantation compared with ciclosporin[23]. However, the extension BENEFIT study (seven-year evaluation) did not

report these outcomes[24]. Furthermore, no studies compared the cardiovascular risk between patients treated with belatacept or tacrolimus. Whether belatacept therapy leads to superior long-term outcomes compared with tacrolimus therapy with respect to cardiovascular and metabolic risk remains to be seen and warrants further investigation.

Although the risk of T cell-mediated rejection is increased in belatacept-treated patients, the formation of *de novo* DSAs is reduced compared with ciclosporin-treated patients[25]. Two reasons for the reduced formation of DSAs may be that 1) costimulation blockade with belatacept leads to more effective prevention of DSA formation by B cells and 2) intravenous administration of belatacept ensures better drug adherence and lower day to day variability in immunosuppressive drug exposure compared with CNI-based regimens[26]. DSAs are a risk factor for the development of chronic active antibody-mediated rejection (c-aABMR) which is an important cause of late kidney transplant loss[27]. However, whether belatacept therapy leads to a decreased rate of c-aABMR is not known and is an interesting research question.

Diagnosis of acute rejection

Molecular analysis of acute rejection biopsies

The combination of molecular diagnostics with the conventional histomorphologic examination of a kidney biopsy improves the accuracy of transplant rejection diagnosis[15]. In **Chapter 5** we used the Nanostring® assay to evaluate gene expression in kidney biopsies. An increasing number of studies use Nanostring® in solid organ transplant recipients, and recently Nanostring® released a commercially-available 770-gene expression panel in collaboration with the Banff community named the Banff-Human Organ Transplant (B-HOT) panel[28]. We showed that the gene signature of biopsies with aTCMR was different compared with those without aTCMR with the Nanostring® assay (**Chapter 5**). These findings suggest that a logical next step is to investigate if gene expression differs between the different grades of aTCMR (including borderline aTCMR, isolated vascular lesions [aTCMR IIA] and mixed type rejections) and if these differ in frequently occurring disorders after kidney transplantation, such as CNI-related nephrotoxicity, BK virus nephropathy, pyelonephritis or recurrence of the primary kidney disease. This could aid in tailored therapeutic regimens resulting in improved renal allograft outcome.

A possible next step in the application of molecular diagnostics to kidney transplant biopsies could be to evaluate whether gene expression profiles can predict if a patient with aTCMR will respond to anti-rejection treatment and in this way guide therapy. This could lead to improved outcomes after aTCMR and prevent (unnecessary) side effects of the anti-

rejection therapy. These gene profiles could then for example be integrated with clinical factors of patients who respond to alemtuzumab and result in a risk prediction score to evaluate which patients with a glucocorticoid-resistant, recurrent or severe acute rejection may benefit from alemtuzumab.

Minimally-invasive biomarkers of acute rejection

In our proof-of-concept study we identified a specific protein profile in serum of patients with aTCMR by the use of a targeted proteomic assay (**Chapter 6**). Several steps must be performed before this assay can be used as a reliable tool to screen for acute kidney transplant rejection. As described in the introduction of this thesis (**Chapter 1**), the standard-of-care therapy is a tacrolimus-based immunosuppressive regimen, and in our study we investigated serum taken form belatacept-treated patients. It is not expected that large differences between the two treatment groups exist, because the gene expression in the aTCMR biopsies was also similar between tacrolimus- and belatacept-treated patients (**Chapter 5**). Additionally, a larger number of patients need to be tested, and it would be of additional value to include other types of rejection. Our study analyzed a small patient cohort (n = 20) and only patients with aTCMR were included in our study. Influence of factors such as age, the severity of chronic kidney disease, the type of induction therapy and disorders with immune activation (auto-immune disease and infection) also need to be taken into consideration and investigated[29-32].

The timing of the rejection is also an important aspect to consider. In our study, only patients with a rejection between three days and four months after transplantation were analyzed. Due to a limited number of samples, we could not investigate if the time after transplantation influenced the protein expression in the serum. Of interest, in a pilot study that we presented at a the Banff 2019 conference, the gene expression of three interferon-γ-inducible chemokines (chemokine [C-X-C motif] ligand 9 [CXCL9], CXCL10 and CXCL11) in aTCMR biopsies at different time points after transplantation were compared[33]. These three chemokines are important for the recruitment of T lymphocytes during kidney transplant rejection and are promising biomarkers to diagnose kidney transplant rejection. A significant difference was seen in the gene counts of these chemokines between early aTCMR (rejection in the first two weeks after transplantation) and late aTCMR (rejection after two weeks; Table 1)[33]. Additionally, these findings were strengthened through urine analysis on a different patient population, in which we also found a difference in these genes when comparing them in early and late rejection, These results seem to indicate a

different immunologic mechanism of rejection early after transplantation and warrants further investigation.

Table 1. Gene counts (median and interquartile range) of CXCL9, CXCL10 and CXCL11.

	Early aTCMR (n=6)	Late aTCMR (n=9)	No rejection (n=5)
CXCL9	4017 (2440-9822)	968 (638-2180)*	52 (11-194)**
CXCL10	6027 (2560-9181)	680 (439-1758)*	23 (13-147)**
CXCL11	3581 (2120-6802)	453 (204-1152)*	19 (13-55)**

*Significantly different (P<0.003) compared with early aTCMR. **Significantly different compared with early aTCMR (P<0.004) and late aTCMR (P<0.002)

We also found that certain proteins that are associated with acute rejection in other studies, such as IFN- g, CXCL9, CXCL10, CXCL11, granzyme B, and PD ligand 1, were not different between the sera of patients with and without aTCMR in our study (**Chapter 6**)[34-36]. Two possible explanations for this are: i) rejection is a time-dependent process that involves immune activation, tissue injury and tissue repair and the protein expression may differ during these events and ii) rejections located in the tubulointerstitial space may show a different protein profile in the blood compared with rejections involving the arteries (aTCMR with vasculitis or ABMR). Perhaps this may also be the case for protein profiles in the urine of kidney transplant recipients.

A concluding remark is that thorough validation is necessary, for instance in a multi-center setting, before this assay can be implemented into the routine clinical care of kidney transplant recipients.

Treatment of acute rejection

Alemtuzumab and rATG are both effective drugs to treat glucocorticoid-resistant, severe or recurrent acute kidney transplant rejections (both aTCMR and ABMR). The advantage of alemtuzumab above rATG is that alemtuzumab is associated with less severe infusion-related side effects (**Chapter 8 and 9**). In addition, rATG must in general be administered through a central venous catheter, while alemtuzumab is injected subcutaneously. Another advantage is that the length of hospitalization was 12 days shorter in patients treated with alemtuzumab. In the Erasmus MC, the costs for a day of hospitalization on an internal medicine ward are € 700,-, and therefore € 8.400,- can be saved with alemtuzumab. Alemtuzumab is used off-label in kidney transplant recipients and is free of charge via the Campath Access program[37]. The administration of alemtuzumab is therefore more cost-effective than rATG therapy.

The outcomes (infusion-related side effects and cost-effectiveness) may be different when a low-dose rATG regimen is administered.

A disadvantage of alemtuzumab treatment is that the depletion of T and B cells is much longer than after rATG therapy (**Chapter 8 and 9**). We found, rather surprisingly, that this does not cause an increased incidence of infections in the first year after T cell-depleting therapy. The infection-free survival in the first year after alemtuzumab was lower compared with rATG therapy (**Chapter 9**). The largest difference between the two groups was seen in the first few weeks after T cell-depletion therapy. Because the duration of hospitalization after rATG was longer than after alemtuzumab therapy, these patients were exposed to a higher risk of nosocomial infection. The influence of alemtuzumab on the immune system persists much longer than after rATG therapy. We do not know the consequence of this on the incidence of infections occurring more than one year after T cell-depleting therapy. The longer duration of T and B cell depletion after alemtuzumab therapy may cause an increased rate of secondary auto-immune diseases (**Chapter 2, 10 and 11**). Another disadvantage of alemtuzumab is that it is not indicated, approved or marketed for kidney transplant recipients. As described in **Chapter 3**, alemtuzumab is registered for the treatment of multiple sclerosis and through the Campath Access Program available for off-label use in other indications[37]. Therefore, the future of alemtuzumab in kidney transplantation is uncertain. Funding agencies are reluctant to fund studies that includes off-label use of drugs and this will limit further development of alemtuzumab as anti-rejection therapy. It is therefore unlikely that additional large studies, such as a randomized, controlled trial comparing alemtuzumab to rATG as anti-rejection therapy, will be performed anytime soon.

The depletion of T and B cells can be long-lasting (up to 36 months). In our study with alemtuzumab (**Chapter 9**), in only 55.7% of alemtuzumab-treated patients T cells repopulated above $200x10^6$/L one year after therapy. Such a long and profound depletion may not be required to successfully treat acute rejection and may unnecessarily increase the risk of infection, malignancy and auto-immunity. The dose of alemtuzumab (a single dose of 30 mg) in kidney transplant recipients was based on the maximum dose used in patients with chronic lymphocytic leukemia. No dose-finding studies have been performed in kidney transplant recipients[38]. A lower dose of alemtuzumab for acute rejection could potentially lead to a shorter T- and B cell-depletion and fewer complications. In our study, the first 14 patients were treated with alemtuzumab (30 mg) daily for two consecutive days (for a total of 60 mg). Since T cell-depletion already occurred after one dose of alemtuzumab,

the next patients received a single dose (30 mg). No difference in the allograft survival was seen between patients treated with either one or two injections with alemtuzumab (Hazard ratio 1.19, 95% confidence-interval 0.56-2.54, p = 0.65; **Chapter 9**). One study compared different dosages of alemtuzumab in kidney transplant patients[39]. Willicombe *et al.* compared induction therapy with a fixed dose alemtuzumab (30 mg) to a weight-adjusted dose (0.4 mg/kg) [39]. One year after therapy, a significant difference was observed in lymphocyte count (fixed dose $1.1x10^9$/L [1.03-1.14] and weight-adjusted dose $1.27x10^9$/L [1.17-1.38], p < 0.0001). Fewer episodes of urosepsis occurred in the weight-adjusted dose group (HR 1.38, 95%-CI 1.03-1.85, p = 0.037). However, the infection-free survival after one year was similar (fixed dose 63.8% and weight-adjusted dose 67.4%, p = 0.14)[39]. Although this study shows that an adjusted dose is associated with earlier lymphocyte repopulation, alemtuzumab dosing based on the bodyweight may still be suboptimal. In children undergoing hematopoietic stem cell transplantation, bodyweight-adjusted dosing of alemtuzumab leads to a highly variable exposure[40]. Recently, a pharmacokinetic model was published for individualized dosing in pediatric patients[41]. We believe that such a pharmacokinetic model also needs to be developed for kidney transplant recipients treated with alemtuzumab to determine the optimal dose and improve the clinical outcomes after alemtuzumab.

As described in **Chapter 2, 10 and 11** alemtuzumab is associated with rare and sometimes fatal secondary auto-immune diseases, such as inflammatory polyneuropathy (Guillain-Barré syndrome and chronic inflammatory demyelinating polyneuroradiculopathy) and acquired hemophilia A) that can occur even years after therapy and despite the repopulation of lymphocytes. Besides, alemtuzumab therapy exposes patients to a higher risk of (opportunistic) infections and malignancy. Because of these adverse events, alemtuzumab therapy for patient with multiple sclerosis is only available via the Risk Evaluation and Mitigation Strategy program in the United States[42]. This program informs health care professionals about the risks of alemtuzumab and an advice is included to monitor auto-immune events until four years after treatment (Table 2). Besides, several patient support tools, including smartphone applications, are developed to educate patients and send reminders for doctor's appointments and blood tests[43].

Table 2. Data derived from Lemtrada Risk Education and mitigation website[42]

Condition	Activity	Timing
Immune thrombocytopenia	Complete blood count and differential	- Prior to treatment - Monthly until 48 months after last infusion
Glomerular nephropathies, including anti-GBM disease	Urine protein to creatinine ratio	Prior to treatment
	Serum creatinine	- Prior to treatment - Monthly until 48 months after last infusion
	Urinalysis	- Prior to treatment - Monthly until 48 months after last infusion
Thyroid disorders	Thyroid function tests	- Prior to treatment - Monthly until 48 months after last infusion
Autoimmune hepatitis	Serum transaminases and total bilirubin	- Prior to treatment - Monthly until 48 months after last infusion
Melanoma	Skin examinations	- Prior to treatment - Yearly

As alemtuzumab is used off-label in kidney transplant recipients a safety monitoring program is not mandatory for this population. After the promising results of alemtuzumab as anti-rejection therapy, the nephrologists in Erasmus MC continue to administer alemtuzumab in patients with glucocorticoid-resistant, severe or recurrent acute rejection. Since 2012 a total of 197 kidney transplant recipients have been treated with alemtuzumab for these types of acute rejection. Furthermore, alemtuzumab is also administered in the Erasmus MC as induction therapy in highly sensitized kidney transplant recipients and in patients with a blood group incompatible kidney transplantation. Although most of these above-mentioned tests are already implemented into the clinical care of kidney transplant recipients, the introduction of a prospective registry with a safety monitoring program in the Erasmus MC (to be expanded to a national scale) will lead to increased alertness for the occurrence of rare adverse events even long after therapy. An improved understanding of the risks of alemtuzumab in kidney transplant recipients is important to inform patients and balance the risks and benefits of this therapy in the future.

Conclusions and future perspectives

Kidney transplant rejection is complex clinical problem with long-lasting consequences for the patient. The focus of this thesis is optimization of the maintenance immunosuppressive therapy, diagnosis and treatment of kidney transplant rejection. The conclusions of this thesis are: i) belatacept remains a promising immunosuppressive drug for subgroups of kidney transplant recipients, for instance in patients with post-transplantation diabetes mellitus, but a more tailored strategy in the selection of kidney transplant recipients, treatment regimen

and post-conversion monitoring is necessary to expand the use, ii) novel applications, such as gene expression profiling of kidney transplant biopsies and a proteomic assay in the blood have the ability to improve the diagnosis of acute kidney transplant rejection, iii) alemtuzumab and rATG are both effective drugs to treat glucocorticoid-resistant, severe or recurrent acute kidney transplant rejections (both aTCMR and ABMR). The advantage of alemtuzumab above rATG is that alemtuzumab is associated with less severe infusion-related side effects, a shorter hospital stay and lower costs, and iv) the use of alemtuzumab is associated with secondary auto-immune disorders, such as inflammatory polyneuropathy and acquired hemophilia A, and this risk may be higher compared with the use of rATG. The outstanding recommendations for future research are summarized in Table 3.

Table 3. Recommendations for future research

To identify kidney transplant recipients with a high risk for belatacept-resistant transplant rejection, for instance through serial testing of molecular or protein markers in peripheral blood and urine of belatacept-treated patients
To investigate if gene expression differs between the different grades of aTCMR and of frequently occurring kidney transplant diseases after kidney transplantation, in order to improve the accuracy of transplant rejection diagnosis and subsequent tailored treatment options
To evaluate if an integrated risk prediction score consisting of clinical variables and gene expression profiles of transplant biopsies can predict if a patient with aTCMR will respond to anti-rejection treatment
To investigate the following aspects of a targeted proteomic assay to diagnose kidney transplant rejection minimally-invasive:
• *Inclusion of a larger number of kidney transplant recipients*
• *Patients treated with the current standard-of-care immunosuppressive therapy*
• *Influence of age, the severity of chronic kidney disease, type of induction therapy and disorders with immune activation*
• *The effect of time between transplantation and rejection on the protein expression of CXCL9, CXCL10 and CXCL11*
• *Test peripheral blood and urine*
• *Validation of the data in an independent cohort of patients and thereafter a prospective study*
To conduct a randomized controlled trial comparing rATG to alemtuzumab for acute kidney transplant rejection
To implement a prospective registry with a safety monitoring program for kidney transplant recipients treated with alemtuzumab to screen for the occurrence of adverse events
To investigate the incidence, pathogenesis and risk factors of secondary auto-immune diseases after alemtuzumab therapy in kidney transplant recipients
To develop a pharmacokinetic model to determine the optimal dose of alemtuzumab in kidney transplant recipients

REFERENCES

1. Clayton PA, McDonald SP, Russ GR, Chadban SJ. Long-Term Outcomes after Acute Rejection in Kidney Transplant Recipients: An ANZDATA Analysis. *J Am Soc Nephrol.* 2019;30(9):1697-1707.
2. Kidney Disease: Improving Global Outcomes Transplant Work G. KDIGO clinical practice guideline for the care of kidney transplant recipients. *Am J Transplant.* 2009;9 Suppl 3:S1-155.
3. Hart A, Smith JM, Skeans MA, et al. OPTN/SRTR 2017 Annual Data Report: Kidney. *Am J Transplant.* 2019;19 Suppl 2:19-123.
4. Neuberger JM, Bechstein WO, Kuypers DR, et al. Practical Recommendations for Long-term Management of Modifiable Risks in Kidney and Liver Transplant Recipients: A Guidance Report and Clinical Checklist by the Consensus on Managing Modifiable Risk in Transplantation (COMMIT) Group. *Transplantation.* 2017;101(4S Suppl 2):S1-S56.
5. Peeters LEJ, Andrews LM, Hesselink DA, de Winter BCM, van Gelder T. Personalized immunosuppression in elderly renal transplant recipients. *Pharmacol Res.* 2018;130:303-307.
6. Tedesco-Silva H, Kho MM, Hartmann A, et al. Sotrastaurin in calcineurin inhibitor-free regimen using everolimus in de novo kidney transplant recipients. *Am J Transplant.* 2013;13(7):1757-1768.
7. Baan CC, Kannegieter NM, Felipe CR, Tedesco Silva H, Jr. Targeting JAK/STAT Signaling to Prevent Rejection After Kidney Transplantation: A Reappraisal. *Transplantation.* 2016;100(9):1833-1839.
8. Vincenti F, Silva HT, Busque S, et al. Evaluation of the effect of tofacitinib exposure on outcomes in kidney transplant patients. *Am J Transplant.* 2015;15(6):1644-1653.
9. Bouamar R, Shuker N, Osinga JAJ, et al. Conversion from tacrolimus to everolimus with complete and early glucocorticoid withdrawal after kidney transplantation: a randomised trial. *Neth J Med.* 2018;76(1):14-26.
10. Shipkova M, Hesselink DA, Holt DW, et al. Therapeutic Drug Monitoring of Everolimus: A Consensus Report. *Ther Drug Monit.* 2016;38(2):143-169.
11. Pascual J, Berger SP, Witzke O, et al. Everolimus with Reduced Calcineurin Inhibitor Exposure in Renal Transplantation. *J Am Soc Nephrol.* 2018;29(7):1979-1991.
12. Archdeacon P, Dixon C, Belen O, Albrecht R, Meyer J. Summary of the US FDA approval of belatacept. *Am J Transplant.* 2012;12(3):554-562.
13. Morgan TA, Chandran S, Burger IM, Zhang CA, Goldstein RB. Complications of Ultrasound-Guided Renal Transplant Biopsies. *Am J Transplant.* 2016;16(4):1298-1305.
14. Broecker V, Mengel M. The significance of histological diagnosis in renal allograft biopsies in 2014. *Transpl Int.* 2015;28(2):136-143.
15. Haas M, Loupy A, Lefaucheur C, et al. The Banff 2017 Kidney Meeting Report: Revised diagnostic criteria for chronic active T cell-mediated rejection, antibody-mediated rejection, and prospects for integrative endpoints for next-generation clinical trials. *Am J Transplant.* 2018;18(2):293-307.
16. Coles AJ, Cohen JA, Fox EJ, et al. Alemtuzumab CARE-MS II 5-year follow-up: Efficacy and safety findings. *Neurology.* 2017;89(11):1117-1126.
17. Nijenhuis T, Hoenderop JG, Bindels RJ. Downregulation of Ca(2+) and Mg(2+) transport proteins in the kidney explains tacrolimus (FK506)-induced hypercalciuria and hypomagnesemia. *J Am Soc Nephrol.* 2004;15(3):549-557.

18. Gommers LM, Hoenderop JG, Bindels RJ, de Baaij JH. Hypomagnesemia in Type 2 Diabetes: A Vicious Circle? *Diabetes.* 2016;65(1):3-13.

19. van der Burgh AC, Moes A, Kieboom BCT, et al. Serum magnesium, hepatocyte nuclear factor 1beta genotype and post-transplant diabetes mellitus: a prospective study. *Nephrol Dial Transplant.* 2019.

20. Melilli E, Manonelles A, Montero N, et al. Impact of immunosuppressive therapy on arterial stiffness in kidney transplantation: are all treatments the same? *Clin Kidney J.* 2018;11(3):413-421.

21. Melilli E, Bestard-Matamoros O, Manonelles-Montero A, et al. Arterial stiffness in kidney transplantation: a single center case-control study comparing belatacept versus calcineurin inhibitor immunosuppressive based regimen. *Nefrologia.* 2015;35(1):58-65.

22. Seibert FS, Steltzer J, Melilli E, et al. Differential impact of belatacept and cyclosporine A on central aortic blood pressure and arterial stiffness after renal transplantation. *Clin Transplant.* 2014;28(9):1004-1009.

23. Vanrenterghem Y, Bresnahan B, Campistol J, et al. Belatacept-based regimens are associated with improved cardiovascular and metabolic risk factors compared with cyclosporine in kidney transplant recipients (BENEFIT and BENEFIT-EXT studies). *Transplantation.* 2011;91(9):976-983.

24. Vincenti F, Rostaing L, Grinyo J, et al. Belatacept and Long-Term Outcomes in Kidney Transplantation. *N Engl J Med.* 2016;374(4):333-343.

25. Bray RA, Gebel HM, Townsend R, et al. De novo donor-specific antibodies in belatacept-treated vs cyclosporine-treated kidney-transplant recipients: Post hoc analyses of the randomized phase III BENEFIT and BENEFIT-EXT studies. *Am J Transplant.* 2018;18(7):1783-1789.

26. Leibler C, Thiolat A, Henique C, et al. Control of Humoral Response in Renal Transplantation by Belatacept Depends on a Direct Effect on B Cells and Impaired T Follicular Helper-B Cell Crosstalk. *J Am Soc Nephrol.* 2018;29(3):1049-1062.

27. Zhang R. Donor-Specific Antibodies in Kidney Transplant Recipients. *Clin J Am Soc Nephrol.* 2018;13(1):182-192.

28. https://www.nanostring.com/products/gene-expression-panels/gene-expression-panels-overview/human-organ-transplant-panel, assessed on 2 October 2019.

29. Andersson E, Bergemalm D, Kruse R, et al. Subphenotypes of inflammatory bowel disease are characterized by specific serum protein profiles. *PLoS One.* 2017;12(10):e0186142.

30. Lind L, Sundstrom J, Larsson A, et al. Longitudinal effects of aging on plasma proteins levels in older adults - associations with kidney function and hemoglobin levels. *PLoS One.* 2019;14(2):e0212060.

31. Molin CJ, Westerberg E, Punga AR. Profile of upregulated inflammatory proteins in sera of Myasthenia Gravis patients. *Sci Rep.* 2017;7:39716.

32. Carlsson AC, Ingelsson E, Sundstrom J, et al. Use of Proteomics To Investigate Kidney Function Decline over 5 Years. *Clin J Am Soc Nephrol.* 2017;12(8):1226-1235.

33. van der Zwan M, Baan CC, Colvin R, et al., P121 Increased gene expression levels of CXCL9, CXCL10 and CXCL11 in renal allograft biopsies with an early acute T cell-mediated rejection. *Human Immunology*.Volume 80, Supplement, 2019, page 145, https://doi.org/10.1016/j.humimm.2019.07.174.

34. Halloran PF, Famulski K, Reeve J. The molecular phenotypes of rejection in kidney transplant biopsies. *Curr Opin Organ Transplant.* 2015;20(3):359-367.

35. Halloran PF, Venner JM, Madill-Thomsen KS, et al. Review: The transcripts associated with organ allograft rejection. *Am J Transplant.* 2018;18(4):785-795.

36. Jamshaid F, Froghi S, Di Cocco P, Dor FJ. Novel non-invasive biomarkers diagnostic of acute rejection in renal transplant recipients: A systematic review. *Int J Clin Pract.* 2018:e13220.

37. Campath Access Program: https://www.campathproviderportal.com/Home.aspx?ReturnUrl=%2fSecure%2fWelcome.aspx, assessed on 26 september 2019.

38. van der Zwan M, Baan CC, van Gelder T, Hesselink DA. Review of the Clinical Pharmacokinetics and Pharmacodynamics of Alemtuzumab and Its Use in Kidney Transplantation. *Clin Pharmacokinet.* 2018;57(2):191-207.

39. Willicombe M, Goodall D, McLean AG, Taube D. Alemtuzumab dose adjusted for body weight is associated with earlier lymphocyte repletion and less infective episodes in the first year post renal transplantation - a retrospective study. *Transpl Int.* 2017;30(11):1110-1118.

40. Willemsen L, Jol-van der Zijde CM, Admiraal R, et al. Impact of serotherapy on immune reconstitution and survival outcomes after stem cell transplantations in children: thymoglobulin versus alemtuzumab. *Biol Blood Marrow Transplant.* 2015;21(3):473-482.

41. Admiraal R, Jol-van der Zijde CM, Furtado Silva JM, et al. Population Pharmacokinetics of Alemtuzumab (Campath) in Pediatric Hematopoietic Cell Transplantation: Towards Individualized Dosing to Improve Outcome. *Clin Pharmacokinet.* 2019.

42. LEMTRADA (Alemtuzumab): REMS (Risk Evaluation and Mitigation Strategy) Program. (2019). https://www.lemtradarems.com/, assessed on 27 September 2019.

43. Barclay K, Carruthers R, Traboulsee A, et al. Best Practices for Long-Term Monitoring and Follow-Up of Alemtuzumab-Treated MS Patients in Real-World Clinical Settings. *Front Neurol.* 2019;10:253.

CHAPTER 13

NEDERLANDSE SAMENVATTING

De nieren zijn onmisbare organen en hebben veel functies, zoals het filteren van afvalstoffen uit het bloed, het regelen van de bloeddruk, het op peil houden van de vocht- en zouthuishouding, de productie van het hormoon erytropoëtine wat belangrijk is voor de aanmaak van rode bloedcellen, en ze spelen een belangrijke rol in de calcium en fosfaat stofwisseling. Indien de nieren zeer ernstig beschadigd zijn en hierdoor onvoldoende werken heet dit eindstadium nierfalen. Bij eindstadium nierfalen moet de nierfunctie kunstmatig worden overgenomen door een kunstnier en dit wordt ook wel dialyse genoemd. Dialyse heeft grote gevolgen voor het dagelijkse leven en veel patiënten ervaren een lage kwaliteit van leven; zij moeten vaak een streng dieet volgen, mogen niet te veel drinken, moeten vaak naar het ziekenhuis en hebben een lagere levensverwachting. Voor veel patiënten met eindstadium nierfalen is daarom een niertransplantatie de beste behandeling. Bij een niertransplantatie wordt een nier van een levende of overleden donor in het lichaam van de patiënt geplaatst. De donornier neemt dan alle functies van de oude nieren over.

Een nadeel van niertransplantatie is dat het immuunsysteem van de patiënt de nier als lichaamsvreemd herkent en de nier kan afstoten. Hierbij spelen humane anti-leukocyten antigenen (HLA) een belangrijke rol. Deze HLA-eiwitten zijn aanwezig op het oppervlak van elke kernhoudende cel van het menselijk lichaam. Elk individu heeft een unieke “barcode” van deze HLA-eiwitten, die genetisch is vastgelegd in het DNA. Het immuunsysteem is in de normale situatie belangrijk voor de bestrijding van infecties met micro-organismen (bijvoorbeeld virussen, bacteriën of parasieten) en bestaat uit verschillende typen cellen zoals antigeen-presenterende cellen, T- cellen, B cellen, macrofagen, monocyten, natural killer (NK) cellen en neutrofiele granulocyten. Antigeen-presenterende cellen nemen delen van het micro-organisme op en presenteren het HLA van het micro-organisme aan T en B cellen. Deze immuuncellen bevinden zich in de bloedbaan, organen, weefsels en lymfeklieren van de patiënt en na activatie gaan deze cellen naar de plaats van de infectie, bijvoorbeeld naar de longen, darmen, huid of urinewegen, of worden lokaal geactiveerd om aldaar de infectie te bestrijden. Na niertransplantatie herkennen de immuuncellen de HLA-eiwitten van de donornier als lichaamsvreemd. Dit leidt tot een activatie van de immuuncellen en vervolgens tot beschadiging van het transplantaat. Een afstoting van een niertransplantaat lijkt dus erg op de normale reactie van het immuunsysteem op een infectie.

Er bestaan twee typen afstoting van een niertransplantaat, namelijk T cel-gemedieerde afstoting en antilichaam-gemedieerde afstoting. Deze twee typen kunnen acuut of chronisch zijn en afzonderlijk maar ook tegelijk voorkomen. Bij een T cel-gemedieerde afstoting worden T cellen geactiveerd na herkenning van lichaamsvreemd HLA van de donornier.

Hierdoor wordt het niertransplantaat direct (via het uitscheiden van cytokines en enzymen) en indirect (via activatie van monocyten, NK cellen en macrofagen) aangevallen door de T cellen waardoor de cellen van de donornier beschadigen. Bij een antilichaam-gemedieerde afstoting ontwikkelen geactiveerde B cellen zich tot plasmacellen. Deze plasmacellen kunnen antilichamen maken die gericht zijn tegen de cellen van het niertransplantaat (zogenoemde donor-specifieke antilichamen). Binding van deze antilichamen aan de cellen zet een cascade van ontstekingseiwitten in gang die leidt tot schade van de donornier.

Om afstoting van een niertransplantaat te voorkomen is het noodzakelijk om levenslang medicatie (immunosuppressiva) te gebruiken die het immuunsysteem onderdrukken. Het gebruik van deze immunosuppressiva gaat gepaard met bijwerkingen. Ondanks het gebruik van deze combinatie van immunosuppressiva komt afstoting van een donornier nog voor in ruim een kwart van de niertransplantatiepatiënten. Afstoting kan een kortere overleving van de donornier en een lagere levensverwachting van de patiënt tot gevolg hebben. In dit proefschrift wordt onderzocht hoe de preventie, diagnostiek en behandeling van acute afstoting van het niertransplantaat geoptimaliseerd kunnen worden.

Preventie van afstoting van het niertransplantaat

De immunosuppressieve behandeling na een niertransplantaat bestaat uit inductietherapie en onderhoudstherapie. Inductietherapie wordt rondom de operatie toegediend. Onderhoudstherapie wordt direct na de operatie gestart en moet levenslang gecontinueerd worden. De huidige onderhoudstherapie bestaat uit een combinatie van de geneesmiddelen tacrolimus, mycofenolzuur en prednisolon. Met deze combinatie is de overleving van een niertransplantaat op korte termijn goed, maar op de lange termijn komen afstotingreacties nog altijd voor. Daarnaast ervaren patiënten veel bijwerkingen van deze geneesmiddelen zoals infecties, kanker, diabetes mellitus (“suikerziekte”), hypertensie (“hoge bloeddruk”) en paradoxaal genoeg nierschade. Daarom is er behoefte naar nieuwe immunosuppressieve geneesmiddelen met minder bijwerkingen en een betere (langetermijn) effectiviteit. Een veelbelovende groep medicijnen die in de toekomst een rol kan gaan spelen als immunosuppressiva zijn middelen die de interactie tussen T cellen en antigeen-presenterende cellen blokkeren, ook wel costimulatie blokkers genoemd.

In **hoofdstuk 2** wordt een overzicht gegeven van de costimulatie blokkers die nu gebruikt worden in de kliniek of nog in ontwikkeling zijn. Belatacept is de costimulatie blokker die aan transplantatie patiënten wordt voorgeschreven. Dit medicijn blokkeert de interactie tussen de CD28 en CD80/86 (eiwitten op de celwand van respectievelijk T cellen en

antigeen-presenterende cellen). Het gebruik van belatacept leidt tot een betere nierfunctie en minder aanmaak van donor-specifieke antilichamen. Het nadeel is dat er meer acute afstotingen van de donornier voorkomen in vergelijking met tacrolimus. Een beter begrip van het achterliggende mechanisme van de verhoogde kans op afstoting is belangrijk om het gebruik van belatacept gerichter te kunnen voorschrijven. Optimaal zou zijn om patiënten met een laag risico op afstoting te behandelen met belatacept en patiënten met een hoog risico met tacrolimus. In **hoofdstuk 5** is dit mechanisme onderzocht. Niertransplantaatbiopten met acute afstoting van patiënten die zijn behandeld met belatacept of tacrolimus werden vergeleken. We hebben de expressie van 209 genen gemeten en de depositie van 9 eiwitten beoordeeld in die biopten. Er was geen verschil tussen de beide groepen te zien. Dit impliceert dat het afstotingsproces na belatacept behandeling via een vergelijkbaar mechanisme verloopt als na tacrolimus behandeling.

Vervolgens hebben we onderzocht of het mogelijk was om te voorspellen welke patiënten een laag risico hebben op afstoting tijdens belatacept behandeling (**hoofdstuk 6**). We hebben daarvoor de aanwezigheid van 92 eiwitten onderzocht in het bloed van patiënten die geen afstoting ontwikkelden en patiënten die wél een afstoting ontwikkelden. Er werd geen verschil gevonden tussen de beide groepen. Concluderend is met deze analyse niet mogelijk om in te schatten wat het risico op afstoting is tijdens een behandeling met belatacept.

In **hoofdstuk 2** wordt de huidige positie van belatacept als onderhoudsbehandeling na niertransplantatie beschreven. De conclusie van dit hoofdstuk is dat belatacept een veelbelovende therapie is voor een subgroep van patiënten, namelijk patiënten met een laag risico op afstoting en patiënten met een contra-indicatie voor tacrolimus (bijvoorbeeld een slecht ingestelde diabetes mellitus). In **hoofdstuk 7** beschrijven we een patiënt die een ernstige ontregeling van diabetes mellitus en neurotoxiciteit (trillen van de handen) had na start van tacrolimus therapie. Na het stoppen van tacrolimus en starten van belatacept had de patiënt een forse verbetering van de regulatie van de diabetes mellitus en het trillen verdween.

Diagnostiek van acute afstoting

Een afstoting van een niertransplantaat kan momenteel alleen met een microscopische beoordeling van een nierbiopsie worden vastgesteld. Het nemen van een nierbiopsie heeft een aantal nadelen, namelijk i) het afnemen van een nierbiopt is een invasieve belastende procedure waarbij een patiënt moet worden opgenomen in het ziekenhuis en complicaties, zoals een bloeding, kunnen ontstaan, ii) het is een kostbare procedure en iii) interobserver

variabiliteit tussen pathologen die nierbiopten beoordelen komt voor. Momenteel worden er meerdere studies gedaan om de diagnostiek van afstoting te verbeteren, zoals moleculaire analyse van nierbiopten en minimaal invasieve testen in het bloed en urine van patiënten. Dit hebben we ook onderzocht in **hoofdstuk 5 en 6**.

We hebben een genexpressieanalyse verricht van nierbiopten met en zonder een acute T cel-gemedieerde afstoting (**hoofdstuk 5)**. Van de 209 geteste genen kwamen 78 genen meer tot expressie in biopten met een acute T cel-gemedieerde afstoting. Ook werd gezien dat de genexpressieanalyse een toevoeging kan zijn aan de microscopische beoordeling van een nierbiopt bij ingewikkelde casuïstiek. Bijvoorbeeld, bij één patiënt liet het nierbiopt een afstoting zien, maar er waren klinisch geen aanwijzingen voor een afstoting (stabiele nierfunctie). Het genexpressieprofiel toonde hetzelfde profiel als van de patiënten zonder een afstoting. Deze patiënt werd niet behandeld voor afstoting en de nierfunctie bleef hetzelfde. De genexpressieanalyse kan in zulke casus dus bijdragen aan een verbeterde diagnostiek van acute T cel-gemedieerde afstoting.

In **hoofdstuk 6** beschrijven we een minimaal-invasieve test om acute T cel-gemedieerde afstoting in het bloed van niertransplantatiepatiënten die behandeld zijn met belatacept vast te stellen. Analyse van 92 eiwitten toonde dat de concentratie van 5 eiwitten hoger was in het bloed van patiënten op het moment van een acute T cel-gemedieerde afstoting. Dit resultaat laat zien dat deze zogenaamde proteomic extensie analyse techniek een veelbelovende methode is om patiënten te screenen op het ontstaan van een acute afstoting middels onderzoek van het bloed. Een belangrijke kanttekening bij dit onderzoek is dat het een studie betrof met een beperkt aantal patiënten. De waarde van deze minimaal-invasieve test wordt momenteel verder onderzocht in een grotere groep patiënten die behandeld worden met tacrolimus.

Behandeling van een acute afstoting

Indien een acute afstoting van een niertransplantaat plaatsvindt worden patiënten veelal als eerste behandeld met een hoge dosis prednison. Bij een ernstige afstoting of een afstoting die niet verbetert na prednison wordt een behandeling met anti-thymocyten globuline (ATG) geadviseerd in de internationale richtlijn. ATG is een medicijn dat zorgt voor afbraak van T- en B cellen gedurende enkele maanden. De richtlijn is gebaseerd op studies van meer dan 25 jaar geleden toen het immunosuppressieve schema na niertransplantatie nog niet bestond uit tacrolimus en mycofenolzuur. Het doel van de studie beschreven in **hoofdstuk 8** was om de effectiviteit van ATG te onderzoeken in patiënten met een acute afstoting die behandeld

worden met tacrolimus en mycofenolzuur. ATG blijkt een effectieve therapie te zijn bij een ernstige afstoting of een acute afstoting die niet verbetert na hoge dosering prednison in patiënten behandeld met tacrolimus en mycofenolzuur. Het was voornamelijk effectief in patiënten met een acute afstoting die binnen 1 maand na niertransplantatie plaatsvindt. Er waren wel frequente (ernstige) infusie-gerelateerde bijwerkingen na het gebruik van ATG.

Alemtuzumab is een ander medicijn wat zorgt voor afbraak van T- cellen en B cellen. Deze therapie is geregistreerd voor patiënten met multiple sclerose (een ziekte van het centraal zenuwstelsel) en wordt buiten deze indicatie gebruikt in niertransplantatiepatiënten als inductie- en anti-afstotingstherapie. In **hoofdstuk 3** wordt een overzicht gegeven van de literatuur over het gebruik van alemtuzumab in niertransplantatiepatiënten. In het Erasmus MC worden sinds 2012 patiënten met een ernstige acute afstoting of een afstoting die niet verbetert na hoge dosering prednison behandeld met alemtuzumab. We hebben de uitkomsten van deze patiënten geanalyseerd en vergeleken met de hierboven beschreven uitkomsten van patiënten die met ATG werden behandeld. De patiënten- en niertransplantaat overleving bleek hetzelfde in beiden groepen. Het gebruik van alemtuzumab lijkt echter tot minder infusie-gerelateerde bijwerkingen en minder infecties te leiden in vergelijking met ATG. Bovendien was de duur van opname in het ziekenhuis van de met alemtuzumab behandelde patiënten 12 dagen korter dan die van de patiënten die ATG kregen voorgeschreven.

Het gebruik van alemtuzumab heeft dus voordelen ten opzichte van het gebruik van ATG. Een belangrijk nadeel is dat de toediening van alemtuzumab auto-immuunziekten kan veroorzaken, zoals schildklierziektes en immuun trombocytopenische purpura (ITP, een pathologische afbraak van bloedplaatjes). Dit hebben we ook gezien in de patiënten behandeld met alemtuzumab in het Erasmus MC. Drie patiënten ontwikkelden een zeldzame vorm van een inflammatoire polyneuropathie (Guillain-Barré syndroom en chronische inflammatoire demyeliniserende polyneuroradiculopathie) na alemtuzumab (**hoofdstuk 10**). Verder kregen twee patiënten een verworven hemofilie A (bloedstollingsziekte) na toediening van alemtuzumab (**hoofdstuk 11**). Alemtuzumab heeft mogelijk een rol gespeeld in het ontstaan van deze auto-immuunziekten. Het is daarom belangrijk om alert te zijn op zeldzame bijwerkingen bij patiënten die behandeld zijn met alemtuzumab.

CONCLUSIE

Acute niertransplantaat afstoting is een complex probleem met nadelige gevolgen voor de patiënt. Verbetering van de preventie, diagnostiek en behandeling van deze complicatie leidt tot een verbetering van de niertransplantaat- en patiënt overleving. De belangrijkste conclusies van dit proefschrift zijn: i) belatacept is een veelbelovend immunosuppressief geneesmiddel voor subgroepen van niertransplantaat patiënten, bijvoorbeeld patiënten met een contra-indicatie voor tacrolimus, ii) nieuwe technieken zoals genexpressie analyse van nierbiopten of eiwit analyse in het bloed, kunnen leiden tot een verbeterde diagnostiek van acute niertransplantaat afstoting, en iii) alemtuzumab en ATG zijn beiden effectieve geneesmiddelen om een ernstige of glucocorticoïd-resistente acute afstoting van een niertransplantaat te behandelen. Behandeling met alemtuzumab leidt tot minder infusie-gerelateerde bijwerkingen, een kortere opname in het ziekenhuis en lagere kosten in vergelijking met ATG maar tot meer auto-immuunziekten zoals inflammatoire polyneuropathie en verworven hemofilie. Mijn aanbevelingen voor toekomstig onderzoek zijn:

1) Het optimaliseren van de genexpressie en eiwit analyse zoals onderzocht in dit proefschrift. De resultaten zoals beschreven in dit proefschrift dienen te worden gevalideerd in grotere groepen niertransplantatiepatiënten met verschillende types van afstoting. Validatie in andere groepen van orgaan transplantaatontvangers (anders dan een nier) is m.i. eveneens een vereiste alvorens deze technieken klinisch kunnen worden ingezet.

2) Het bijhouden van de uitkomsten en bijwerkingen na behandeling met alemtuzumab in een patiëntenregistratie. De ernstige, auto-immuun bijwerkingen van alemtuzumab zijn zeldzaam en het is niet precies duidelijk hoe vaak deze voorkomen en welke patiënten het meeste risico lopen. Een (landelijke) registratie van deze bijwerkingen zou hier meer inzicht in kunnen geven.

3) Het vaststellen van de meest optimale dosering van alemtuzumab. De huidige dosering van 30 mg subcutaan is gebaseerd op data uit de hematologie. Gezien de langdurige leukopenie is het denkbaar dat een lagere dosis wellicht net zo effectief is maar met minder bijwerkingen gepaard gaat. Een dose-finding studie zou hier antwoord op kunnen geven.

PART V

APPENDICES

LIST OF ABBREVIATIONS

3C study	calcineurin, campath and chronic allograft nephropathy study
aABMR	active ABMR
ABMR	antibody-mediated rejection
ACR	acute cellular rejection
aIRR	adjusted incidence rate ratio
AHA	acquired hemophilia A
Akt	AKT8 virus oncogene cellular homolog
APCs	antigen-presenting cells
aPTT	activated partial thromboplastin time
AR	acute kidney transplant rejection
aTCMR	acute T cell-mediated rejection
AUC	area under the curve
BAFF	B- lymphocyte activating factor
b-aTCMR	borderline aTCMR
BENEFIT	Belatacept Evaluation of Nephroprotection and Efficacy as First-line Immunosuppression
BENEFIT-EXT	BENEFIT-extended criteria donors
BPAR	biopsy proven acute rejection
BU	Bethesda Units
C0	pre-dose concentration
C4d	complement 4d
c-aABMR	chronic-active ABMR
CARE-MS	comparison of alemtuzumab and Rebif® efficacy
c-aTMCR	chronic-active TCMR
CI	confidence interval
CIDP	chronic inflammatory demyelinating polyradiculoneuropathy
CKD	chronic kidney disease
CKD–EPI	chronic kidney disease Epidemiology Collaboration
CLL	chronic lymphocytic leukemia
C_{max}	maximum concentration
CMV	cytomegalovirus
CNI	calcineurin inhibitor
CXCL	chemokine (C-X-C motif) ligand
CsA	cyclosporin A
CTLA	cytotoxic T lymphocyte antigen
DEG	differentially expressed gene
DGF	delayed graft function
DNA	deoxyribonucleic acid
DM	diabetes mellitus
DSA	donor-specific anti-HLA antibodies
EBV	Epstein-Barr virus
eGFR	estimated glomerular filtration rate

EMA	European Medicines Agency
ESRD	end-stage renal disease
ERK1/2	extracellular signal-Regulated Kinases 1 and 2
FVIII:C	factor VIII coagulation activity
FDR	false Discovery Rate
FDRPV	false Discovery Rate p-value
Fc	crystallizable fragment
FCγR	IgG fragment C receptor
FDA	United States Food and Drug Administration
FFPE	formalin-fixed paraffin-embedded
FoxP3	forkhead box P3
GBS	Guillain-Barré syndrome
HbA1c	glycated hemoglobin
HCA	hierarchical clustering analysis
HLA	human leucocyte antigens
HR	hazard ratio
ICOS	inducible T cell costimulator
IFNγ	interferon gamma
i-IF/TA	inflammation in areas with interstitial fibrosis and tubular atrophy
Ig	immunoglobulin
IgG1	immunoglobulin G1
IHC	immunohistochemistry
IL	interleukin
IQR	interquartile range
IU	international units
IVIg	intravenous immunoglobulins
JAK	Janus kinase
KDIGO	Kidney Disease: Improving Global Outcomes
KTR	kidney transplant recipients
LFA-3	lymphocyte function-associated antigen 3
MAPK	mitogen-activated protein kinase
MMF	mycophenolate mofetil
MPA	mycophenolic acid
mRNA	messenger ribosomal nucleic acid
MS	multiple sclerosis
mTOR	mammalian target of rapamycin inhibitors
NCR1	natural cytotoxicity triggering receptor 1
NK	natural killer
NPX	normalized protein expression
PCR	polymerase chain reaction
PD-1	programmed death-1
PEA	proximity extension immunoassay
PNF	primary non-function

PRA	panel reactive antibodies
PTDM	post-transplant diabetes mellitus
PTLD	post-transplant lymphoproliferative disease
SLAM	signaling lymphocytic activation molecule;
rATG	rabbit anti-thymocyte globulin
RCT	randomized controlled trial
RR	relative risk
RRMS	relapsing remitting multiple sclerosis
SPKT	simultaneous pancreas-kidney transplantation
SOT	solid organ transplantation
TAC	tacrolimus
TCR	T cell receptor
TIM	T cell/transmembrane, immunoglobulin, and mucin
TNF	tumor necrosis factor
TNFRSF	TNF receptor superfamily
Treg	regulatory T cells

LIST OF PUBLICATIONS

van der Zwan M, Baan CC, van Gelder T, Hesselink DA. *Review of the clinical pharmacokinetics and pharmacodynamics of alemtuzumab and its use in kidney transplantation.* Clinical Pharmacokinetics. 2018 Feb;57(2):191-207

van der Zwan M*, de Graav GN*, Baan CC, Janssen JAMJL, Hesselink DA. *Improved glucose tolerance in a kidney transplant recipient with type 2 diabetes mellitus after switching from tacrolimus to belatacept: A case report and review of potential mechanisms.* Transplantation Direct. 2018 Feb 20;4(3):e350. *Authors contributed equally

van der Zwan M, Clahsen-Van Groningen MC, Roodnat JI, Bouvy AP, Slachmuylders CL, Weimar W, Baan CC, Hesselink DA, Kho MML. *The efficacy of rabbit anti-thymocyte globulin for acute kidney transplant rejection in patients using calcineurin inhibitor and mycophenolate-based immunosuppressive therapy.* Ann Transplant. 2018 Aug 17;23:577-590.

van der Zwan M, Hesselink DA, Clahsen-van Groningen MC, Baan CC. *Targeted proteomic analysis detects acute T cell-mediated kidney allograft rejection in belatacept-treated patients.* Ther Drug Monit. 2018 Dec 4.

van der Zwan M, Baan CC, Colvin RB, Smith RN, White RA, Ndishabandi D, Nigg AL, van den Bosch TPP, de Graav GN, Clahsen-van Groningen MC, Hesselink DA. *Immunomics of renal allograft acute T cell-mediated rejections biopsies of tacrolimus- and belatacept-treated patients.* Transplantation Direct 2018 Dec 20;5(1):e418.

van der Zwan M, Hesselink DA, van den Hoogen MWF, Baan CC. *Costimulatory blockade in kidney transplant recipients.* Drug 2020;80(1):33-46

van der Zwan M, Hesselink DA, Brusse E, van Doorn PA, van den Hoogen MWF, de Weerd AE, Jacobs BC. *Guillain-Barré syndrome and chronic inflammatory demyelinating polyradiculoneuropathy after alemtuzumab in kidney transplant recipients.* Neurology: Neurimmunology & Neuroinflammation, 2020 Apr 16;7(4): e721

van der Zwan M, Clahsen-van Groningen MC, van den Hoogen MWF, Kho MML, Roodnat JI, Mauff KAL, Roelen DL, van Agteren M, Baan CC, Hesselink DA. *Comparison of alemtuzumab and anti-thymocyte globulin treatment for acute kidney allograft rejection*. Frontiers in immunology 2020; Jun

Van der Zwan M, Leebeek FWG, Kruip MJHA, Hesselink DA. *Acquired hemophilia A after alemtuzumab therapy*. Haemophilia 2020; Jul

Snijders MLH, Varol H, **van der Zwan M**, Becker JU, Hesselink DA, Baan CC, von der Thüsen JH, Clahsen-van Groningen MC. *Molecular analysis of renal allograft biopsies: where do we stand and where are we going.* Transplantation 2020; Mar 6

Van der Zwan M, Hesselink DA, Baan CC, Clahsen-van Groningen MC**.** *Chronic active antibody mediated rejection: to belatacept or not, that is the HOT question.* Transplantation 2020; Jun

PHD PORTFOLIO

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PhD period:	January 2017- October 2019
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Total workload:	33.5 ECTS

Courses and workshops	**Year**	**Workload (ECTS)**
Masterclass kidney transplantation, Barcelona	2015	0.6
HESPERIS course (ESOT), Rome	2017	1.45
SPSS*	2017	1.0
Scientific integrity*	2017	0.3
Graphpad prism*	2017	0.3
Young professionals day (NTV), Utrecht	2017	0.3
Klinisch Review Symposium (NTV), Utrecht	2017	0.3
Nephrology Winterschool (Duch kidney foundation), Driebergen	2018	1.45
ACADEMIA (ESOT), Vienna	2018	1.45
BROK (Basiscursus Regelgeving Klinisch Onderzoek)*	2018	1.5
Advanced immunology*	2018	4.5
Biomedical English writing*	2018	2.0
Young professionals day (NTV), Utrecht	2018	0.3
Nephropathology course, New York	2019	1.45
ERKNET CERTAIN meeting*	2019	0.5
* Erasmus MC, Rotterdam		

Conferences		
ESOT, Barcelona (Poster)	2017	1.45
Dutch Transplantation Society, Rotterdam (Oral)	2018	0.6
Dutch nephrology federation, Veldhoven (Poster)	2018	0.6
The transplantation society, Madrid (2 Orals)	2018	2.15
Basic science meeting ESOT, Rotterdam (Poster)	2018	1.1
Dutch Transplantation Society, Amsterdam (2 Orals)	2019	0.6
ESOT, Copenhagen (3 Orals)	2019	1.25
Dutch Transplantation Society, Roermond	2020	0.6

Grants		
Bootbeurs NTV (annual meeting NTV)	2018	
NTV scholingsbeurs	2018	
STAR grant (ESOT)	2018	

Awards		
Young investigator Science award The Transplantation Society	2018	

Lecturing		
Teaching medical students (minor kidney transplantation, patient demonstration, and writing a systematic review)	2017-2020	2.0
Chairman regional nephropathology meeting	2017	1.0

Other		
Associate editor of the Netherlands Journal of Medicine	2017-2020	5.0
Lab meetings internal medicine department, transplantation lab, Erasmus MC	2017-2019	
Journal Club internal medicine department, Erasmus MC	2017-2020	

Memberships
Nederlandse Transplantatie Vereniging (NTV)
The European Society of Organ Transplantation (ESOT)
The Transplantation Society (TTS)
Nederlandse Internisten Vereniging
Nederlandse Federatie voor Nefrologie

ABOUT THE AUTHOR

Marieke van der Zwan was born on September 21th, 1984 in The Hague, the Netherlands. After completing her secondary education (VWO) at the Interconfessionele Scholengroep Westland (location Gasthuislaan) in 2002, she started her medical training in Erasmus University Rotterdam, the Netherlands. During the medical training, she obtained a Master of Science degree in Molecular Medicine in Erasmus MC (2007). Her graduation research focused on the XY chromosome inactivation in primary spermatocytes. She received her Medical Doctor degree cum laude in 2009, after which she worked as a resident at the department of Internal Medicine in the Albert Schweitzer hospital (Dordrecht). In 2011, she started her medical specialization in internal medicine under the supervision of Dr. E.F.H. van Bommel (Albert Schweitzer hospital), Prof. Dr. J.L.C.M van Saase (Erasmus MC) and dr. S.C.E. Klein Nagelvoort-Schuit (Erasmus MC). Four years later, she started a fellowship nephrology in Erasmus MC under supervision of Prof. dr. R. Zietse including an internship of six months in Maasstad hospital in Rotterdam. During her training in nephrology, she started part of her work described in this thesis. In 2017, she started as a PhD candidate under the supervision of Prof. Dr. Carla Baan, dr. D.A. Hesselink and dr. M.C. Clahsen-van Groningen at the department of Internal Medicine, Division of Nephrology and Transplantation, at the Erasmus MC. The results of her research are described in this thesis. From 2017 onwards, she is an associate editor for the Netherlands Journal of Medicine. In 2019, she continued to work as resident nephrology and she finished her training in April 2020.

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