

**The
cardiovascular and
immunological impact
of immune suppression
in kidney transplant
recipients**

From calcineurin inhibitors to belatacept

Edwin Bredewold

The cardiovascular and immunological impact of immune suppression in kidney transplant recipients

From calcineurin inhibitors to belatacept

Edwin Bredewold

Copyright: © 2026 Edwin Bredewold

ISBN: 978-94-6534-349-5

Cover: Manon Zuurmond

Layout: ProefschriftMaken - www.proefschriftmaken.nl

Print: Ridderprint - www.ridderprint.nl

All rights reserved. No part of this thesis may be reproduced, stored in a retrieval system or transmitted in any form or by any means without prior permission of the copyright owner.

The cardiovascular and immunological impact of immune suppression in kidney transplant recipients

From calcineurin inhibitors to belatacept

Proefschrift

ter verkrijging van
de graad van doctor aan de Universiteit Leiden,
op gezag van rector magnificus prof. dr. S. de Rijcke,
volgens besluit van het college voor promoties
te verdedigen op dinsdag 26 mei 2026
klokke 13:00 uur

door

Edwin Bredewold

Promotoren

Prof. dr. A.J. van Zonneveld

Prof. dr. J.I. Rotmans

Leden promotiecommissie

Prof. dr. A.P.J. de Vries

Prof. dr. T. van Gelder

Dr. S. Huveneers - Amsterdam Universitair Medisch Centrum

Dr. M. Baas - Radboud Universitair Medisch Centrum

TABLE OF CONTENTS

Chapter 1.	History of Immunosuppressive Therapy in Kidney Transplantation – Rationale for this thesis	9
-------------------	--	---

Chapter 2.	Cardiovascular Risk Following Conversion to Belatacept From a Calcineurin Inhibitor in Kidney Transplant Recipients: A Randomized Clinical Trial	29
-------------------	--	----

OW Bredewold, J Chan, MHS Svensson, A Bruchfeld, JW de Fijter, H Furuland, JM Grinyo, A Hartmann, H Holdaas, O Hellberg, A Jardine, L Mjörnstedt, K Skov, KT. Smerud, I Soveri, SS Sörensen, AJ v Zonneveld, B Fellstrom.

Kidney Medicine, 2023, doi: 10.1016/j.xkme.2022.100574

Chapter 3.	Conversion from calcineurin inhibitors to belatacept-based immunosuppressive therapy skews terminal proliferation of nonclassical monocytes and lowers lymphocyte counts	53
-------------------	--	----

OW Bredewold, AM van Oeveren-Rietdijk, B Florijn, JI Rotmans, JW de Fijter, C v Kooten, AJ v Zonneveld, HC de Boer.

Transplant immunology, 2024, doi: 10.1016/j.trim.2023.101976

Chapter 4.	Attenuation of Torque Teno Viral load over time in kidney transplantation recipients treated with calcineurin inhibitors is mitigated after conversion to belatacept	81
-------------------	--	----

OW Bredewold, WT Moest, JW de Fijter, E Meijers, A Bruchfeld, K Skov, MHS Svensson, J Chan, L Mjornstedt, SS Sorensen, B Fellstrom, MCW. Feltkamp, AJ v Zonneveld, JI Rotmans.

Journal of Medical Virology, 2024, doi: 10.1002/jmv.29905

Chapter 5. An exploration of markers of microvascular dysfunction in 103 kidney transplant recipients randomized to belatacept: no clinical impact of CNI's on endothelial function

OW Bredewold, RJ Postma, J Chan, MHS Svensson, A Bruchfeld, JW de Fijter, H Furuland, A Hartmann, L Mjornstedt, K Skov, I Soveri, SS Sorensen, B Fellström, JM Duijs, R Bijkerk, JI Rotmans, AJ v Zonneveld.

Provisionally accepted for publication in Frontiers in Transplantation

Chapter 6 Summary, general discussion and future perspective for belatacept 131

Appendices	Nederlandse Samenvatting	145
	Curriculum Vitae	151
	Lijst van Publicaties	152
	Dankwoord	154

1

History of Immunosuppressive Therapy in Kidney Transplantation – Rationale for this thesis

The early years of kidney transplantation

The first successful kidney transplantation was performed in 1954, by the team of Joseph E. Murray of Brigham Hospital in Boston, MA, USA [1]. It was a landmark event, and the first successful transfer of a whole organ from one human to another. The first patient transplanted, and also the approximately 20 subsequent patients received a graft from their identical twin-sibling [1-3]. In these cases, immunosuppression was not needed to suppress the immunological response of the recipients.

In 1959, the same team performed a transplantation in which the donor was a heterozygous twin brother of the recipient. In this case, prevention of acute rejection was established by whole body irradiation of the recipient. Bouts of suspected rejection were treated by repeat irradiation, and with a course of prednisone (60 mg) [4]. A similar approach was chosen contemporaneously by two French groups, all three approaches were deemed reasonably successful [5].

In 1962, again the group of Murray and JP Merrill reported on a 24-year-old patient with glomerulonephritis that received a graft from a cadaver donor. The donor was a 30-year old man that had just died after heart surgery in the same hospital [6]. Renal biopsy was performed a total of seven times in the first 6 months, and persistently showed signs of interstitial inflammation, interpreted as allo-graft rejection. Intravenous courses of actinomycin C and various regimens of steroids were prescribed. Fifteen months after transplantation, his glomerular filtration rate was around 9 ml/min/1.73m², but the patient was able to go to work and remained free from dialysis. Though deemed 'successful' at that time such a result would be considered extremely poor from a modern point of view. In 1963, the survival statistics of kidney transplantation in general were extremely discouraging, with less than 5% of 176 transplantations surviving longer than one year [7].

Transplantation in those years, compared to now was hindered by very limited knowledge of donor-recipient immunologic compatibility, a scarcity of medical centers that performed transplantation and lack of potent immunosuppressive drugs.

The discovery of HLA and large-scale organization of transplant programs

In the 1950's, Dausset et al. discovered that serum of poly-transfused patients subjected to cultured leukocytes led to leuko-agglutination in vitro. This serum, injected into patients, resulted in deep leukopenia in the recipient. He con-

cluded that not-natural (acquired) anti-leukocyte antibodies were present in these patients, and that these antibodies resulted from transfusion, and proved to be transferable [8]. He also described four pairs of identical twins, in which no 'leucocidin-reactivity' was found, leading him to speculate about the inheritability of the factors responsible for allo-recognition [9].

Several other researchers, amongst which Jon van Rood from the University of Leiden, investigated the phenomenon of leukocyte agglutination by anti-leukocyte-antibodies. When he and researchers from other parts of the world compared each other's findings, they concluded that their various methods all pointed to the presence of a common antigen presented on white blood cells [10-13]. In a consensus meeting, this antigen was called human leukocyte antigen A2 (the test by one of the researchers with which HLA-A2 was discovered was called 'A2' [13], and this researcher also used A1 for another test. Hence, the antigen was named HLA-A2, though it was the first antigen discovered). In subsequent years, more and more HLA-antigens were discovered. The relevance of HLA-typing for transplantation purposes was immediately clear, and HLA-typing recipients and donors permitted HLA-compatible matching with lower rates of rejection [14]. In later years, these insights on HLA helped transplant centers in Europe (Eurotransplant) to start registering HLA-data of potential organ recipients and (post-mortal) organ donors, and recruit donors from within their combined adherence areas. The Eurotransplant-region started with 3 countries, but later expanded to 5, and to now 8 countries in Europe. Similar initiatives were employed elsewhere in the USA (UNOS), Spain (ONT) and Australia (Donate-Life). Enlarging the donor pool by combining regions and even countries in one large adherence area facilitated matching, and was of particular advantage to immunized patients, that had developed anti-HLA antibodies from previous transplantations or pregnancy [15]. For instance, in the Eurotransplant-region, highly immunized patients that would rarely receive a donor offer in a small region could be transplanted within two years after listing in 85% of cases [16]. A second effect of organization of transplant centers inter-regionally and internationally is the increased volume of organ transplantation. As mentioned previously, organ transplantation was carried out only incidentally in the pioneer years. In 1988, about 10000 patients received either a heart, kidney, liver, lung or other whole organ in the USA. In 2021 (the second year of the worldwide COVID-19 pandemic) 22.917 patients received a kidney transplant alone [17]. In countries like South Korea [18], Japan [19] and Spain [20] the annual rate of transplantation rose considerably in the last 20 to 30 years. **Figure 1** provides information on the rise in volume of transplantations in the Netherlands.

The use of immunosuppressive drugs in renal transplantation: the central role of calcineurin inhibitors

The first kidney recipients did not need immunosuppressive drugs because of their immunologic (twin) kinship to their donor. Patients with incompatible donors received courses of either irradiation, short courses of steroids, high doses of azathioprine (up until 15 mg/kg until leukopenia ensued as a side effect before tapering) and actinomycin C [4-6]. Other methods included splenectomy and thymectomy prior to or concomitantly with graft implantation [21]. ‘Surgical’ immunosuppression and irradiation is not used anymore. No good scientific proof of efficacy has ever been gathered of such procedures.

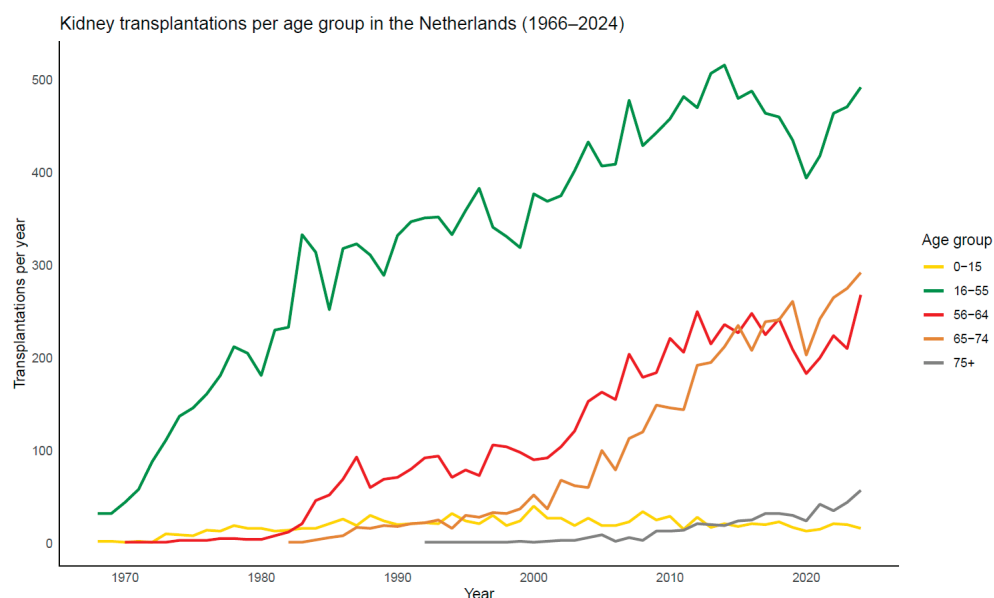


Figure 1

Kidney transplantations (KT) in the Netherlands between 1966 and 2024, showing increasing volume, as well as rising age of recipients. Source: Dutch transplant Society (NTS).

In 1967, T-cell depletion as a means of ‘induction’-therapy was first described. The group of Starzl described a remarkably successful strategy containing of anti-human lymphocyte horse globulin, infused between 5 days before and 14 days after transplantation [22]. Nineteen of 20 patients treated survived, and they all had much better graft function than historical controls and used less azathioprine and less prednisone. Lymphocyte depletion to induce tolerance, in contrast to surgical procedures has stood the test of time and is still used in transplantation nowadays, with an estimated 52% of patients receiving ATG (anti-thymocyte globulin) immediately before transplantation in France [23].

Alemtuzumab, an anti CD52-monoclonal antibody depleting lymphocytes is used for the same purpose [24].

Anxiety over the side-effects of T-cell depleting agents, including the cytokine release syndrome (CRS) and the enhanced risk of opportunistic infections and cancer stimulated the use of other 'induction agents', comprising the interleukin-2 (IL2) pathway. Daclizumab and basiliximab both bind to the IL-2 receptor on activated T-lymphocytes, thereby hampering their proliferative capacity. Clinical trials with both agents resulted in a significant reduction of rejection and steroid-resistant rejection versus placebo [25-28]. Nowadays both strategies are supported in international guidelines, with a slight advantage for T-cell depletion over IL2-R-blockade in preventing rejection in the first year [29, 30]. For highly immunized patients, both the FDA and EMA recently approved the use of imlifidase, an immunoglobulin G-degrading enzyme derived from streptococci. By eliminating donor-specific as well as all other IgG-antibodies, it creates a window of a few days in which a kidney graft can be transplanted without being immediately prone to humoral rejection [31]. It remains to be seen how frequently this drug will be used, as it is extremely expensive and does not negate the need for other highly potent immunosuppressive drugs.

A major breakthrough for maintenance immunosuppression occurred in 1978. Prior successful experiments in rodents, dogs and pigs comparing cyclosporin A (CyA) to azathioprine in various transplantation models convinced Calne et al. to try and use CyA in human renal transplantation with HLA-unmatched donors [32]. This was still a time before evidence-based medicine, and despite the lack of randomized (blinded) studies comparing CyA with the then established drugs, it was swiftly introduced in solid organ transplantation. One of the disadvantages of early CyA was the fact that, due to interaction with food intake, biologic uptake varied from patient to patient, making it hard to find the optimal therapeutic threshold, and making patients prone to experience either rejection through suboptimal dosing, or sometimes to side-effects when uptake was better. The introduction of micro-emulsion Cyclosporin A (CsA) was an improvement [33]. This new formulation led to higher exposure measured either through trough levels or area-under-the-curve (AUC) monitoring, and also to reduced rejection rates in renal transplantation. [34, 35]. Soon, CsA replaced CyA for all transplantation-indications.

At about the same time, tacrolimus (TAC) was introduced, first under the name FK506 [36, 37]. TAC is, like CsA an inhibitor of calcineurin in the cell-nucleus and thus provides a mechanism to counter the proliferation and cytokine-production of T-lymphocytes. From the beginning, it was thought that TAC provided similar or enhanced immunosuppressive potential compared to CsA. This assumption was proven in multiple randomized controlled trials that compared TAC with CsA in kidney transplant recipients (KTR) with various background-immunosuppressive regimens [38-40]. Biopsy-proven rejection was observed significantly less in TAC-treated patients (19.7%) than in those with CsA (33%). The same reduction was seen for steroid-resistant rejection (9.3% vs 19%) [38, 39]. Follow-up reports on these trials also showed that graft-survival, renal function, lipid profile and blood pressure were all better in those on TAC [41-43].

Mycophenolic acid derivatives were introduced in transplantation in the middle of the 1990's. Mycophenolate mofetil (MMF) gradually replaced azathioprine after a number of studies showed improved control of rejection [44, 45]. While this resulted in improved renal function, more patients experienced viral and other opportunistic infections. In the beginning of the twentieth century, yet another class, namely inhibitors of the mammalian target of Rapamune-pathway (mTOR-i) were introduced. In comparison to MMF, their use is particularly associated with a decreased burden of infections without compromising on rejection [46]. On the other hand, everolimus (EVL), the most frequently used of this latest class, frequently leads to proteinuria and carries an enhanced risk of thrombo-embolisms [47, 48].

Figure 2 gives an overview of the timeline of introduction of immunosuppressive drugs in kidney transplantation.

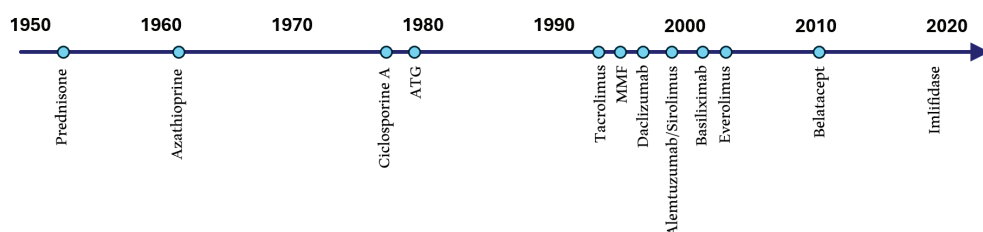


Figure 2.

Timeline of introduction of immunosuppressive drugs in kidney transplantation. ATG = anti-thymocyte globulin. MMF = Mycophenolate Mofetil.

Since introduction of all these drugs, multiple trials have been carried out to find the 'perfect' mix of immunosuppressive drugs. Since most trials are supported for one or two years after transplantation, the rate of (biopsy-proven) rejection, graft survival after 1 year and GFR are all well-studied endpoints studied in those trials. In terms of these endpoints, the publication of the landmark 'symphony'-trial in 2007 proved that the combination of low-dose TAC, MMF and prednisone was superior above combinations with either m-TOR-I and CsA [49]. In 2009, the KDIGO (kidney disease improving global outcome)-network summarized multiple strategies to guide transplant physicians in choosing an immunosuppressive regimen. Its recommendations are; 1) to use a form of induction therapy, 2) to use a calcineurin inhibitor combined with MMF or an m-TOR-I and 3) to use steroid-free regimens only for low-risk patients, and not after having given steroids for more than a week [50]. As such, most centers use a combination of three immunosuppressive drugs (triple-therapy) triple therapy to avoid the side-effects of high doses of either drug, and to suppress multiple pathways of allo-recognition [51]. In fact, patients in over 90% of transplant centers are discharged with TAC, and a few others with CsA as the central immunosuppressive prescription in their regimen [52]. **Figure 3** shows the drug regimen that patients use at discharge after transplantation in American hospitals. The reliance on TAC, and its replacement of CsA since introduction is clearly visible.

Side effects of CNI

The almost universal adherence to TAC and sometimes CsA means that almost all transplant patients are potentially subject to their side effects. These have been known ever since the introduction of CsA. They can be broadly categorized in cardiovascular side effects, the most striking of which is renal toxicity, which is thought to be primarily derived from intrarenal vasoconstriction [53], and non-cardiovascular side effects. These include opportunistic infections, hirsutism, neurotoxicity (posterior reversible encephalopathy syndrome, tremor), gingival hyperplasia and electrolyte disturbances including hyperkalemia.

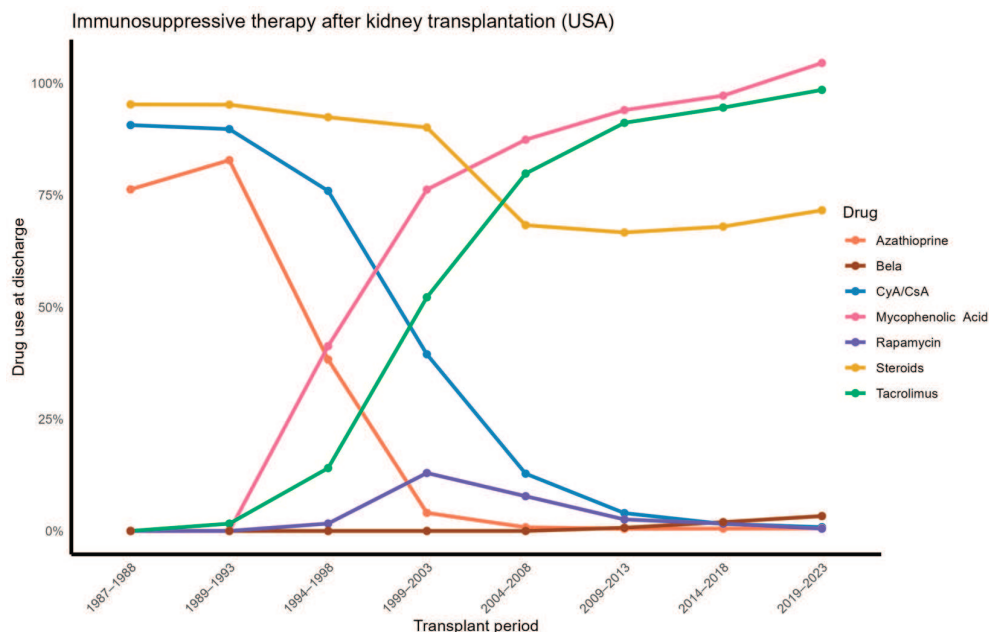


Figure 3.

Variation in discharge-immunosuppressive regimens in American transplant centres (patients use more than one drug at discharge). Source: Chronic Disease Research group (CDRG.org)

On CNI-associated nephrotoxicity Ojo et al. summarized in a landmark paper in 2003 the burden of chronic kidney disease (CKD) in non-renal transplant patients (NKSOT: non-kidney solid-organ-transplantation) [54]. Between 10% (heart) and 21% (intestine) of organ recipients developed renal failure (GFR below 30 ml/min) in five years. Since almost 'all' patients used a calcineurin inhibitor in that study, its influence on the risk of renal failure is impossible to extract. However, the changes seen in renal biopsies of patients developing renal disease after non-renal organ-transplantation resemble those that are associated with CNI-toxicity [55]. More recent studies have confirmed the high prevalence of CKD after NKSOT [56]. Other cardiovascular side-effects of CNI are hypertension, hyperglycemia, disturbed lipid metabolism and rarely thrombotic micro-angiopathy [57-60]. Diabetes after transplantation (PTDM) is a side effect particularly attributed to TAC more than to CsA [61]. Because age contributes vastly to cardiovascular risk, elder patients experiencing these side effects are at particular risk for cardiovascular events and mortality. Over the last thirty years the average age of the transplant recipient has increased, with now more than a third of eligible recipients being over 65 years of age. This, while patients over 50 were not likely to be even listed in 1980. Additional to volume, the rising age of the average KTR in the Netherlands is depicted in **figure**

1. Figure 4 shows the same for the USA, and information on diabetes prevalence is added. In the 50-64 age cohort and the 65+-cohort, diabetes prevalence is 45 and 50% respectively. (data provided by the Chronic Disease Research Group (CDRG) and the Dutch Transplant Society (NTS) in 2025).

With the rising age and comorbidities of the average transplant recipient, and the wide availability of CNI's and T-cell depleting agents, the focus of the transplant physician has broadened, from control of rejection to management of cardiovascular and infectious risk. Paradoxically, superior graft survival through use of CNI paradoxically leads to rise in the fraction of patients prone to develop cardiovascular disease and renal failure. With that in mind, the transplant community has been looking for strategies to minimize, antagonize or eliminate CNI-side effects ever since introduction [62-66]. Despite the relative success of some of these initiatives, the adherence to CNI's has remained strong.

Belatacept as an alternative to CNI

Belatacept was the first co-stimulation blocker used in transplantation. The term co-signaling relates to the process that starts immediately after engaging of the antigen-presenting cell (APC) to a T-cell-receptor (TCR), called 'signal 1' [67]. The signal induced by this engagement, 'signal 2' can either be (co)-stimulatory, or (co)-inhibitory. Without co-signaling, the T-cell is rendered anergic. The Cytotoxic T-lymphocyte antigen number 4 (CTLA4) is genetically homologous to CD28, the most abundant co-stimulatory molecule on T-cells [68]. However, CTLA4-expression actually competes with CD28 for binding to its ligands CD80 and CD86 on APC's, and the resultant binding leads to a (co)inhibitory effect [69]. Belatacept is a drug manufactured by combining the extracellular portion of CTLA4 with the Fc-tail of IgG1. By infusing it, it binds to T-cells and mimics its inhibitory effect, making organ transplantation an ideal application [70].

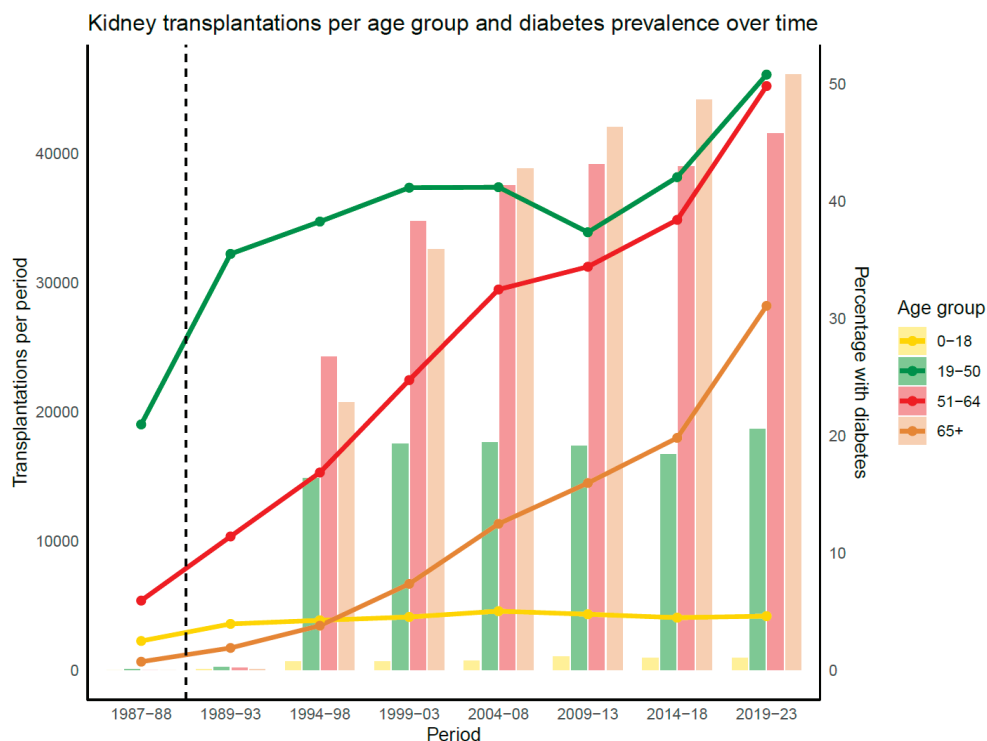


Figure 4

KT in the USA 1987-2024. The number of transplantations, as well as the age at which it is performed is rising, as well as diabetes-prevalence among recipients. The total number of transplantations in the cohort 1987-88 is multiplied by 2.5 (the actual number is 2.5 times lower). A dotted line must make the reader aware of this adjustment, made to provide a fluent trend-line that is more in line with the increase in annual transplantations in that time-period. Source: Chronic Disease Research group (CDRG.org)

Belatacept seemed a major breakthrough when first investigated in the BENE-FIT-studies of the early second decade [71, 72]. It was both successful in providing excellent renal outcome and metabolic outcome compared to CsA, and at the same time disappointingly poor at preventing rejection. Despite a rejection rate of around 33%, GFR of those on belatacept was better than CsA-treated patients. Follow-up analysis of these trials proved that rejection was a problem mainly encountered in the first year after transplantation, while the superiority in terms of renal function was sustained [73, 74]. Other belatacept-trials corroborated the findings of high rejection rates when started early and mostly improved renal function compared to both CsA and TAC [75-77]. The high initial rejection-rates have caused belatacept to not be implemented in guidelines, and not in broad clinical practice. Some centers though, including the Leiden University Medical Center have adopted belatacept as an option in case of side-effects, CNI-intolerability or biopsy proven histologic changes associated

with CNI-use. In such cases the introduction of belatacept, especially after slow waning of CNI has been reported with considerable success [78, 79]. Despite these examples, the necessity of having to administer the drug intravenously, an association with post-transplant-lymphoproliferative disorder, and decreased immunity after COVID-19 vaccination have in all likelihood further hampered broad use of belatacept in renal transplant practice [80-82].

Outline of thesis

Although belatacept has been registered to be used in renal transplantation for nearly fifteen years, its reception in clinical practice was always lukewarm. While nephrologists should be thrilled by the sometimes spectacular advantage that belatacept provides in GFR and graft-half-life [73, 83], most centers still prefer CNI. The work in this thesis aimed to increase clinical knowledge about the use of belatacept in kidney transplant recipients, in comparison to TAC and CsA.

We investigated the change in calculated cardiovascular risk induced by conversion of a CNI-based immunosuppressive regimen to belatacept. For this purpose, we collaborated with researchers at Uppsala University hospital in Sweden, where a clinical calculator was developed that enables physicians to calculate the risk of a cardiovascular event in an individual KTR [84]. Further collaboration was sought with transplant centers in Denmark and Norway. The results of this international randomized trial are described in **chapter 2**.

In **chapter 3**, we performed FACS analysis of circulating monocytes and lymphocytes, to determine if cell populations associated with cardiovascular disease would differ in amount or type after conversion from CNI to belatacept. For this project we used samples derived from 40 Dutch patients that underwent conversion in the context of two trials, both of which had similar conversion protocols.

Belatacept is associated with EBV-driven PTLD, and with CMV-infection in KTR's [85, 86]. Some authors suggest that infection-risk is elevated in belatacept-patients with poor renal function [87]. The increased risk of rejection suggests that belatacept is a relatively weak immunosuppressive drug, while its suppression of allo-antibody induction indicates the opposite [88]. Torque teno virus (TTV) is a virus upregulated in patients with hampered immunity, and is proposed to be a biomarker of immunologic strength. There are indications that its prevalence in blood corresponds to infection and rejection in transplant patients [89]. In **chapter 4**, we measured TTV-levels in patients converted in either of the previously mentioned trials before and after randomization, to compare belatacept's influence on the immune system to that of CNI.

In **chapter 5**, we tried to shed more light on the mechanism of vaso-toxic effects of CNI. Endothelial dysfunction (ED) has long been implied in the pathogenesis of calcineurin-inhibitor-associated nephrotoxicity. A closer look at the literature identifies CNI as sort of a double-edged sword. CNI can indeed be 'nephrotoxic' by inducing vasoconstriction in afferent endothelium through endothelin-1-mediated actions, but CNI's are also the most potent drugs in preventing rejection-induced damage to the microvasculature. Furthermore, the doses at which nephrotoxicity is shown in (animal) literature does not often correlate to modern transplantation medicine. We sought to elucidate some of the mechanisms behind perceived ED, by comparing samples of KTR's before and after conversion from CNI to belatacept and looking for differences in various biomarkers of ED. In this project, we further explored the use of a novel innovative tool that assesses endothelial function dubbed 'cell painting'. This tool consists of a high-throughput high-content image-based assay to assess morphological responses of endothelial cells (EC) to external stimuli [90]. We tested both patient samples of patients randomized from CNI to belatacept, as well as dilution series of concentrations of both TAC and CsA to investigate the effect of CNI on endothelial function.

In **chapter 6**, a summary of the scientific results of this thesis is provided, as well as a discussion on current and future indications for belatacept.

REFERENCES

1. Guild, W.R., et al., Successful homotransplantation of the kidney in an identical twin. *Trans Am Clin Climatol Assoc*, 1955. 67: p. 167–73.
2. Murray, J.E., J.P. Merrill, and J.H. Harrison, Kidney transplantation between seven pairs of identical twins. *Ann Surg*, 1958. 148(3): p. 343–59.
3. Harrison, J.H., J.P. Merrill, and J.E. Murray, Renal homotransplantation in identical twins. *Surg Forum*, 1956. 6: p. 432–6.
4. Merrill, J.P., et al., Successful Homotransplantation of the Kidney between Non-identical Twins. *New England Journal of Medicine*, 1960. 262(25): p. 1251–1260.
5. Hamburger, J., et al., Transplantation Dun Rein Entre Jumeaux Non Monozygotes - Apres Irradiation Du Receveur Bon Fonctionnement Au Quatrieme Mois. *Presse Medicale*, 1959. 67(48): p. 1771–1775.
6. Merrill, J.P., et al., Successful transplantation of kidney from a human cadaver. *JAMA*, 1963. 185: p. 347–53.
7. Goodwin, W.E. and D.C. Martin, Transplantation of the Kidney, Spring 1963. *Urol Surv*, 1963. 13: p. 229–48.
8. Dausset, J., A. Nenna, and H. Brecy, Leukoagglutinins. V. Leukoagglutinins in chronic idiopathic or symptomatic pancytopenia and in paroxysmal nocturnal hemoglobinuria. *Blood*, 1954. 9(7): p. 696–720.
9. Dausset, J. and H. Brecy, Identical nature of the leucocyte antigens detectable in monozygotic twins by means of immune iso-leuco-agglutinins. *Nature*, 1957. 180(4599): p. 1430.
10. Dausset, J., et al., [A new antigen of the system HL-A (Hu-1): the antigen 15, possible allele of the antigens 1, 11, 12]. *Nouv Rev Fr Hematol*, 1968. 8(3): p. 398–406.
11. Van Rood, J.J., et al., RELATIONSHIP OF LEUKOCYTE GROUPS TO TISSUE TRANSPLANTATION COMPATIBILITY. *Ann N Y Acad Sci*, 1964. 120: p. 285–98.
12. Shulman, N.R., et al., Platelet and Leukocyte Isoantigens and Their Antibodies: Serologic Physiologic and Clinical Studies. *Prog Hematol*, 1964. 4: p. 222–304.
13. Payne, R., et al., A New Leukocyte Isoantigen System in Man. *Cold Spring Harb Symp Quant Biol*, 1964. 29: p. 285–95.
14. Patel, R., M.R. Mickey, and P.I. Terasaki, Serotyping for homotransplantation. XVI. Analysis of kidney transplants from unrelated donors. *N Engl J Med*, 1968. 279(10): p. 501–6.
15. Müller, G.A., C. Müller, and P. Wernet, Improved kidney graft survival through HLA-DR-MT matching. *Proc Eur Dial Transplant Assoc*, 1983. 19: p. 464–8.
16. Claas, F.H., et al., The acceptable mismatch program as a fast tool for highly sensitized patients awaiting a cadaveric kidney transplantation: short waiting time and excellent graft outcome. *Transplantation*, 2004. 78(2): p. 190–3.
17. Buchholz, K. Kidney Transplants on the Rise. 2021; Available from: <https://www.statista.com/chart/21110/organ-transplants-timeline-us/>.
18. Yang, C.-W., et al., Global case studies for chronic kidney disease/end-stage kidney disease care. *Kidney International Supplements*, 2020. 10: p. e24–e48.
19. Yagisawa, T., et al., Trends of kidney transplantation in Japan in 2018: data from the kidney transplant registry. *Renal Replacement Therapy*, 2019. 5(1): p. 3.
20. Matesanz, R., et al., How Spain Reached 40 Deceased Organ Donors per Million Population. *American Journal of Transplantation*, 2017. 17(6): p. 1447–1454.

21. Starzl, T.E., et al., SPLENECTOMY AND THYMECTOMY IN HUMAN RENAL HOMO-TRANSPLANTATION. *Proc Soc Exp Biol Med*, 1963. 113: p. 929–32.
22. Starzl, T.E., et al., The clinical use of antilymphocyte globulin in renal homotransplantation. *Transplantation*, 1967. 5(4): p. Suppl:1100–5.
23. Boucquemont, J., et al., Induction therapy in kidney transplant recipients: Description of the practices according to the calendar period from the French multicentric DIVAT cohort. *PLoS One*, 2020. 15(10): p. e0240929.
24. Asderakis, A., et al., Thymoglobulin Versus Alemtuzumab Versus Basiliximab Kidney Transplantation From Donors After Circulatory Death. *Kidney Int Rep*, 2022. 7(4): p. 732–740.
25. Kahan, B.D., P.R. Rajagopalan, and M. Hall, Reduction of the occurrence of acute cellular rejection among renal allograft recipients treated with basiliximab, a chimeric anti-interleukin-2-receptor monoclonal antibody. United States Simulect Renal Study Group. *Transplantation*, 1999. 67(2): p. 276–84.
26. Nashan, B., et al., Reduction of acute renal allograft rejection by daclizumab. Daclizumab Double Therapy Study Group. *Transplantation*, 1999. 67(1): p. 110–5.
27. Nashan, B., et al., Randomised trial of basiliximab versus placebo for control of acute cellular rejection in renal allograft recipients. CHIB 201 International Study Group. *Lancet*, 1997. 350(9086): p. 1193–8.
28. Vincenti, F., et al., Interleukin-2-receptor blockade with daclizumab to prevent acute rejection in renal transplantation. Daclizumab Triple Therapy Study Group. *N Engl J Med*, 1998. 338(3): p. 161–5.
29. Hanaway, M.J., et al., Alemtuzumab induction in renal transplantation. *N Engl J Med*, 2011. 364(20): p. 1909–19.
30. 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: p. S1–155.
31. Kanbay, M., et al., Imlifidase in kidney transplantation. *Clinical Kidney Journal*, 2024. 17(3).
32. Calne, R.Y., et al., Cyclosporin A in patients receiving renal allografts from cadaver donors. *Lancet*, 1978. 2(8104-5): p. 1323–7.
33. Kovarik, J.M., et al., Cyclosporine pharmacokinetics and variability from a microemulsion formulation--a multicenter investigation in kidney transplant patients. *Transplantation*, 1994. 58(6): p. 658–63.
34. Kahan, B.D., et al., The Neoral formulation: improved correlation between cyclosporine trough levels and exposure in stable renal transplant recipients. *Transplant Proc*, 1994. 26(5): p. 2940–3.
35. Keown, P. and D. Niese, Cyclosporine microemulsion increases drug exposure and reduces acute rejection without incremental toxicity in de novo renal transplantation. International Sandimmun Neoral Study Group. *Kidney Int*, 1998. 54(3): p. 938–44.
36. Starzl, T.E., et al., Kidney transplantation under FK 506. *Jama*, 1990. 264(1): p. 63–7.
37. Starzl, T.E., et al., FK 506 for liver, kidney, and pancreas transplantation. *Lancet*, 1989. 2(8670): p. 1000–4.
38. Pirsch, J.D., et al., A comparison of tacrolimus (FK506) and cyclosporine for immunosuppression after cadaveric renal transplantation. FK506 Kidney Transplant Study Group. *Transplantation*, 1997. 63(7): p. 977–83.

39. Margreiter, R., Efficacy and safety of tacrolimus compared with ciclosporin micro-emulsion in renal transplantation: a randomised multicentre study. *Lancet*, 2002. 359(9308): p. 741–6.
40. Johnson, C., et al., Randomized trial of tacrolimus (Prograf) in combination with azathioprine or mycophenolate mofetil versus cyclosporine (Neoral) with mycophenolate mofetil after cadaveric kidney transplantation. *Transplantation*, 2000. 69(5): p. 834–41.
41. Krämer, B.K., et al., Efficacy and safety of tacrolimus compared with cyclosporin A microemulsion in renal transplantation: 2 year follow-up results. *Nephrol Dial Transplant*, 2005. 20(5): p. 968–73.
42. Krämer, B.K., et al., Efficacy and safety of tacrolimus compared with ciclosporin-A in renal transplantation: 7-year observational results. *Transpl Int*, 2016. 29(3): p. 307–14.
43. Ahsan, N., et al., Randomized trial of tacrolimus plus mycophenolate mofetil or azathioprine versus cyclosporine oral solution (modified) plus mycophenolate mofetil after cadaveric kidney transplantation: results at 2 years. *Transplantation*, 2001. 72(2): p. 245–50.
44. Halloran, P., et al., Mycophenolate mofetil in renal allograft recipients: a pooled efficacy analysis of three randomized, double-blind, clinical studies in prevention of rejection. The International Mycophenolate Mofetil Renal Transplant Study Groups. *Transplantation*, 1997. 63(1): p. 39–47.
45. Keown, P.E., A blinded, randomized clinical trial of mycophenolate mofetil for the prevention of acute rejection in cadaveric renal transplantation. The Tricontinental Mycophenolate Mofetil Renal Transplantation Study Group. *Transplantation*, 1996. 61(7): p. 1029–37.
46. Brennan, D.C., et al., Cytomegalovirus incidence between everolimus versus mycophenolate in de novo renal transplants: pooled analysis of three clinical trials. *Am J Transplant*, 2011. 11(11): p. 2453–62.
47. Wiseman, A.C., et al., The effect of everolimus versus mycophenolate upon proteinuria following kidney transplant and relationship to graft outcomes. *Am J Transplant*, 2013. 13(2): p. 442–9.
48. Baas, M.C., et al., Treatment with everolimus is associated with a procoagulant state. *Thromb Res*, 2013. 132(2): p. 307–11.
49. Ekberg, H., et al., Reduced exposure to calcineurin inhibitors in renal transplantation. *N Engl J Med*, 2007. 357(25): p. 2562–75.
50. Kasiske, B.L., et al., KDIGO clinical practice guideline for the care of kidney transplant recipients: a summary. *Kidney Int*, 2010. 77(4): p. 299–311.
51. Slapak, M., et al., The use of low-dose cyclosporine A in combination with azathioprine and steroids in renal transplantation. *Transplant Proc*, 1987. 19(2 Suppl 2): p. 41–5.
52. Lentine, K.L., et al., OPTN/SRTR 2021 Annual Data Report: Kidney. *Am J Transplant*, 2023. 23(2 Suppl 1): p. S21–s120.
53. Nankivell, B.J., et al., The natural history of chronic allograft nephropathy. *N Engl J Med*, 2003. 349(24): p. 2326–33.
54. Ojo, A.O., et al., Chronic renal failure after transplantation of a nonrenal organ. *N Engl J Med*, 2003. 349(10): p. 931–40.
55. de Saint Gilles, D., et al., Kidney Biopsy Findings After Lung Transplantation. *Kidney Int Rep*, 2024. 9(9): p. 2774–2785.

56. Mejia, C. and A. Yadav, Kidney Disease After Nonkidney Solid Organ Transplant. *Adv Chronic Kidney Dis*, 2021. 28(6): p. 577–586.
57. Vincenti, F., et al., Results of an international, randomized trial comparing glucose metabolism disorders and outcome with cyclosporine versus tacrolimus. *Am J Transplant*, 2007. 7(6): p. 1506–14.
58. Hoorn, E.J., et al., The calcineurin inhibitor tacrolimus activates the renal sodium chloride cotransporter to cause hypertension. *Nat Med*, 2011. 17(10): p. 1304–9.
59. Heisel, O., et al., New onset diabetes mellitus in patients receiving calcineurin inhibitors: a systematic review and meta-analysis. *Am J Transplant*, 2004. 4(4): p. 583–95.
60. Al-Nouri, Z.L., et al., Drug-induced thrombotic microangiopathy: a systematic review of published reports. *Blood*, 2015. 125(4): p. 616–8.
61. Suilik, H.A., et al., Efficacy of tacrolimus versus cyclosporine after lung transplantation: an updated systematic review, meta-analysis, and trial sequential analysis of randomized controlled trials. *Eur J Clin Pharmacol*, 2024.
62. Mourer, J.S., et al., Late calcineurin inhibitor withdrawal prevents progressive left ventricular diastolic dysfunction in renal transplant recipients. *Transplantation*, 2012. 94(7): p. 721–8.
63. Budde, K., et al., Everolimus with cyclosporine withdrawal or low-exposure cyclosporine in kidney transplantation from Month 3: a multicentre, randomized trial. *Nephrol Dial Transplant*, 2017. 32(6): p. 1060–1070.
64. Morales, J.M., et al., Calcium antagonist treatment of recipients minimizes early cyclosporine nephrotoxicity in renal transplantation: a prospective randomized trial. *Transplant Proc*, 1989. 21(1 Pt 2): p. 1537–9.
65. Devineni, R., et al., Cyclosporine in cardiac transplantation. *Can J Surg*, 1984. 27(3): p. 252–4.
66. Simmons, R.L., et al., Management and prevention of cyclosporine nephrotoxicity after renal transplantation: use of low doses of cyclosporine, azathioprine, and prednisone. *Transplant Proc*, 1985. 17(4 Suppl 1): p. 266–75.
67. Chen, L. and D.B. Flies, Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol*, 2013. 13(4): p. 227–42.
68. Harper, K., et al., CTLA-4 and CD28 activated lymphocyte molecules are closely related in both mouse and human as to sequence, message expression, gene structure, and chromosomal location. *J Immunol*, 1991. 147(3): p. 1037–44.
69. Krummel, M.F. and J.P. Allison, CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med*, 1995. 182(2): p. 459–65.
70. Vincenti, F., et al., Costimulation blockade with belatacept in renal transplantation. *N Engl J Med*, 2005. 353(8): p. 770–81.
71. Vincenti, F., 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): p. 535–46.
72. Durrbach, A., 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): p. 547–57.
73. Vincenti, F., 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): p. 3219–3227.

74. Durrbach, A., et al., Long-Term Outcomes in Belatacept- Versus Cyclosporine-Treated Recipients of Extended Criteria Donor Kidneys: Final Results From BENE-FIT-EXT, a Phase III Randomized Study. *Am J Transplant*, 2016. 16(11): p. 3192–3201.
75. de Graav, G.N., et al., A Randomized Controlled Clinical Trial Comparing Belatacept With Tacrolimus After De Novo Kidney Transplantation. *Transplantation*, 2017. 101(10): p. 2571–2581.
76. Budde, K., et al., Conversion from Calcineurin Inhibitor to Belatacept-based Maintenance Immunosuppression in Renal Transplant Recipients: a Randomized Phase 3b Trial. *J Am Soc Nephrol*, 2021.
77. Rostaing, L., 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): p. 430–9.
78. Adams, A.B., 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): p. 2922–2936.
79. Gupta, G., et al., Safe Conversion From Tacrolimus to Belatacept in High Immunologic Risk Kidney Transplant Recipients With Allograft Dysfunction. *Am J Transplant*, 2015. 15(10): p. 2726–31.
80. Iryaningrum, M.R., et al., Seroconversion rates in kidney transplant recipients following SARS-CoV-2 vaccination and its association with immunosuppressive agents: a systematic review and meta-analysis. *Clin Exp Vaccine Res*, 2023. 12(1): p. 13–24.
81. Chavarot, N., et al., Weak antibody response to three doses of mRNA vaccine in kidney transplant recipients treated with belatacept. *Am J Transplant*, 2021. 21(12): p. 4043–4051.
82. Larsen, C.P., et al., Long-term Safety in Epstein-Barr Virus-Seropositive Kidney-only Transplant Recipients Treated With Belatacept in Clinical Practice: Final Study Results From the ENLIST Registry. *Transplant Direct*, 2024. 10(6): p. e1644.
83. Vincenti, F., et al., Belatacept and Long-Term Outcomes in Kidney Transplantation. *N Engl J Med*, 2016. 374(4): p. 333–43.
84. Soveri, I., et al., A cardiovascular risk calculator for renal transplant recipients. *Transplantation*, 2012. 94(1): p. 57–62.
85. Petrossian, G., et al., Increased CMV disease and “severe” BK viremia with belatacept vs. sirolimus three-drug maintenance immunosuppression. *Transpl Immunol*, 2023. 79: p. 101857.
86. Charpentier, B., et al., Long-term exposure to belatacept in recipients of extended criteria donor kidneys. *Am J Transplant*, 2013. 13(11): p. 2884–91.
87. Bertrand, D., et al., Opportunistic infections after conversion to belatacept in kidney transplantation. *Nephrol Dial Transplant*, 2020. 35(2): p. 336–345.
88. Kim, E.J., et al., Costimulation blockade alters germinal center responses and prevents antibody-mediated rejection. *Am J Transplant*, 2014. 14(1): p. 59–69.
89. van Rijn, A.L., et al., Torque teno virus load as marker of rejection and infection in solid organ transplantation - A systematic review and meta-analysis. *Rev Med Virol*, 2023. 33(1): p. e2393.
90. Postma, R.J., et al., Novel Morphological Profiling Assay Connects ex Vivo Endothelial Cell Responses to Disease Severity in Liver Cirrhosis. *Gastro Hep Adv*, 2024. 3(2): p. 238–249.

Cardiovascular Risk Following Conversion to Belatacept From a Calcineurin Inhibitor in Kidney Transplant Recipients: A Randomized Clinical Trial

OW Bredewold*, J Chan*, MHS Svensson, A Bruchfeld, JW de Fijter, H Furuland, JM Grinyo, A Hartmann, H Holdaas, O Hellberg, A Jardine, L Mjörnstedt, K Skov, KT Smerud, I Soveri, SS Sørensen, AJ v Zonneveld, B Fellstrom.

Published in:

Kidney Medicine 2022 Nov 18;5(1):100574

*OWB and JC have contributed to this manuscript equally

ABSTRACT

Rationale & Objective: In kidney transplant recipients (KTRs), a belatacept-based immunosuppressive regimen is associated with beneficial effects on cardiovascular (CV) risk factors compared with calcineurin inhibitor (CNI)-based regimens. Our objective is to compare calculated CV risk between belatacept and CNI (predominantly tacrolimus) treatments using a validated model developed for KTRs.

Study Design: Prospective, randomized, open label, parallel group, investigator-initiated, international multi-center trial.

Setting & Participants: KTRs aged 18–80 years with a stable graft function (estimated glomerular filtration rate [eGFR] >20 mL/min per 1.73 m²), 3–60 months post-transplantation treated with tacrolimus or Cyclosporine A were eligible for inclusion.

Intervention(s): Continuation with a CNI-based regimen or switch to belatacept for 12 months.

Outcomes: Comparison of the change in estimated 7-year risk of major adverse cardiovascular events (MACE) and all-cause mortality, changes in traditional markers of cardiovascular health, as well as measures of arterial stiffness.

Results: In the 105 KTRs randomized, we found no differences between the treatment groups in predicted risk for MACE or mortality. Diastolic blood pressure, measured both centrally by using a SphygmoCor® device and peripherally, was lower after belatacept treatment compared with CNI. The mean changes in traditional CV risk factors, including renal transplant function, were otherwise similar in both treatment groups. The belatacept group had four acute rejection episodes; two were severe rejections, of which one led to graft loss.

Limitations: heterogeneous baseline eGFR and time from transplantation to trial enrolment in the participants. Limited study duration of 1 year.

Conclusions: We found no effects on calculated CV risk by switching to belatacept treatment. Participants in the belatacept group had lower central and peripheral diastolic blood pressure, but also a higher rejection rate, in the belatacept group.

Funding: The trial has received a financial grant from Bristol-Myers Squibb.

INTRODUCTION

The risk of cardiovascular disease (CVD) in patients with renal failure is much higher than in the general population across all age groups^{1,2}. While a successful transplant reduces this risk significantly, kidney transplant recipients (KTRs) still have an annual cardiovascular (CV) event rate of 3.5–5%³. Accordingly, CVD remains one of the leading causes of death in KTRs^{4,5}. Managing a transplanted patient should therefore include CV risk reduction measures to improve both graft and patient outcomes. Current guidelines for prevention of CVD are based upon data from the general population and from studies specifically targeting CVD in KTRs⁶. In addition to addressing traditional risk factors for CVD, such as lifestyle choices, hypertension, hyperlipidemia, and diabetes, KTRs present two potentially modifiable factors: renal graft function and type of immunosuppressive maintenance regimen.

First, evidence indicates that declining graft function and graft loss are potentially modifiable risk factors for CVD and all-cause mortality in this population, which make strategies for optimizing graft function important^{7,8}. Second, among immunosuppressive drugs used for transplantation, both steroids and calcineurin inhibitors (CNIs) are associated with adverse CV side effects⁹. Therefore, attempts have been made to minimize or eliminate their use. While these have led to reasonably safe steroid-free regimens¹⁰⁻¹², CNIs are still the cornerstone of immunosuppression in modern solid organ transplantation. Early graft survival improved greatly after the introduction of cyclosporine (CsA) in the early 1980s¹³, and tacrolimus (TAC) has been the CNI of choice since the 1990s¹⁴. Despite the benefits of CNIs in the early post-transplant period, they have dose-dependent side effects, including post-transplant diabetes mellitus (PTDM), hypertension, hypercholesterolemia, and nephrotoxicity, leading to progressive decline in renal graft function¹⁵⁻¹⁹. Therefore, there is an ongoing incentive for development of novel immunosuppressive agents without the side effects of CNIs.

Belatacept, a modified form of CTLA4-Ig, binds to CD80 and CD86 on antigen presenting cells, thus blocking CD28 mediated co-stimulation of T-cells. The BENEFIT trials have shown promise for belatacept as an option in designing a more favorable immunosuppressive regimen²⁰⁻²⁴. In brief, despite higher rates of early rejection, the relative risk of death or graft loss after 7 years was reduced by 43% in patients treated with belatacept versus CsA-treated patients, and eGFR in the belatacept-group was on average 22 ml/min/1.73m² higher than in the CsA-group. Furthermore, in a meta-analysis comparing belatacept

with CNIs, treatment with belatacept was associated with lower blood pressure, lower incidence of diabetes and a more favorable lipid profile ²⁵.

However, it is not yet proven whether these findings translate into overall CVD reduction. Soveri et al. have previously developed a risk calculator for CVD and all-cause mortality for use in KTRs ²⁶. The group later used the data of the BENEFIT and BENEFIT-EXT trials to calculate the potential benefit associated with belatacept treatment and found a substantial calculated 7-year risk reduction for major adverse cardiac endpoints (MACE) and mortality by converting from CsA to belatacept ²⁷.

A shortcoming of belatacept that has hindered its implementation in kidney transplantation has been the relatively high rate of early rejection, as well as the lack of studies comparing its efficacy with low-dose TAC, the current standard of care in KTRs. In the present study, our aim was to investigate 1) the effects of conversion from a low-dose CNI-based therapy to belatacept on estimated risk of CVD and all-cause mortality by using the aforementioned risk calculator developed by Soveri et al., validated for use in KTRs ^{26, 27}, and 2) the changes in traditional markers of cardiovascular health, as well as measures of arterial stiffness.

METHODS

Study design

This was a prospective, randomized, open label, parallel group, investigator-initiated, international multi-center trial (EudraCT no. 2013-001178-20). Patients were randomized in 1:1 ratio to either continue treatment with a CNI-based regimen or to switch to belatacept for a study duration of 12 months. An open design was chosen since CNIs were given as tablets daily and belatacept was administered as infusion every four weeks.

Patients were recruited from 9 transplant centers in Denmark, the Netherlands, Norway, and Sweden. KTRs aged 18–80 years with a stable graft function (estimated glomerular filtration rate [eGFR] >20 mL/min per 1.73 m²), 3–60 months post-transplantation treated with TAC or CsA were eligible for inclusion. Patients were excluded if they were Epstein-Barr virus (EBV) IgG seronegative, had severe de novo or recurrent renal disease, had a history of vascular or antibody-mediated rejection in the present transplant or had a history of recent malignancy.

The study was approved by the local ethics committees. Written informed consent was obtained from all patients, and the trial was performed in accordance with the Declaration of Helsinki and the principles of Good Clinical Practice.

Study medication

For patients randomized to the study arm with belatacept, the previous CNI treatment (TAC or CsA) was tapered in the initial period as follows: 100% on day 1, to 70-80% on day 7, to 40-60% on day 15, 20-30% on day 23 and none on day 29 and beyond. Belatacept was dosed 5 mg/kg IV on day 1, 15, 29, 43, 57 and then every month thereafter in the 12-month study period (**Figure 1**). Patients randomized to the control group with continuation of CNI treatment were to maintain trough levels of CsA between 75 and 200 ng/ml and TAC between 5 and 10 ng/ml. Both groups were to continue their underlying immunosuppressive regimen, consisting of mycophenolate mofetil (MMF) or mammalian target of rapamycin inhibitor and corticosteroids. Any other concomitant medication necessary to maintain the patients' baseline condition or to treat a coexisting disease was permitted. Control of blood pressure, glucose and lipid parameters was left to the treating physicians, according to local practice.

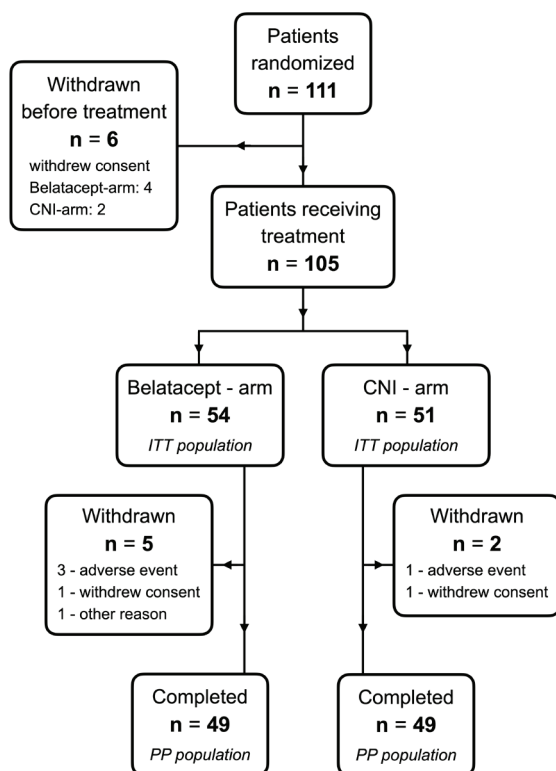


Figure 1. Conversion and dosing scheme

Efficacy assessment and procedures

The primary endpoint of this trial was estimated CV risk using a prediction model developed for KTRs by Soveri et al.^{26, 27}. The prediction model was constructed based on the Assessment of Lescol in Renal Transplantation (ALERT) extension trial data²⁸, and later validated with the Patient Outcomes in Renal Transplantation (PORT) clinical database²⁹.

The estimated 7-year risk of MACE and all-cause mortality in the two treatment groups were calculated as a linear combination of the following variables: age, previous coronary heart disease, previous smoker, current smoker, creatinine, diabetes mellitus, low-density lipoprotein (LDL), number of transplants and total time on renal replacement therapy (**Figure 2**). Secondary endpoints were arterial stiffness, traditional CVD risk factors in KTRs (blood pressure, lipid profiles and eGFR), acute rejections, allograft loss, CV events and patient survival. Blood samples were drawn at a fasting state in the morning at baseline and at end of study visits for measurement of renal function and CV biomarkers: creatinine, high-sensitivity C-reactive protein (hs-CRP), total cholesterol, high-density lipoprotein cholesterol (HDL-cholesterol), LDL cholesterol, triglycerides, apolipoprotein B (ApoB) and apolipoprotein A1 (ApoA1). Arterial stiffness was assessed at the same time points by measuring central pulse pressure, pulse wave velocity and augmentation index using the SphygmoCor® method³⁰.

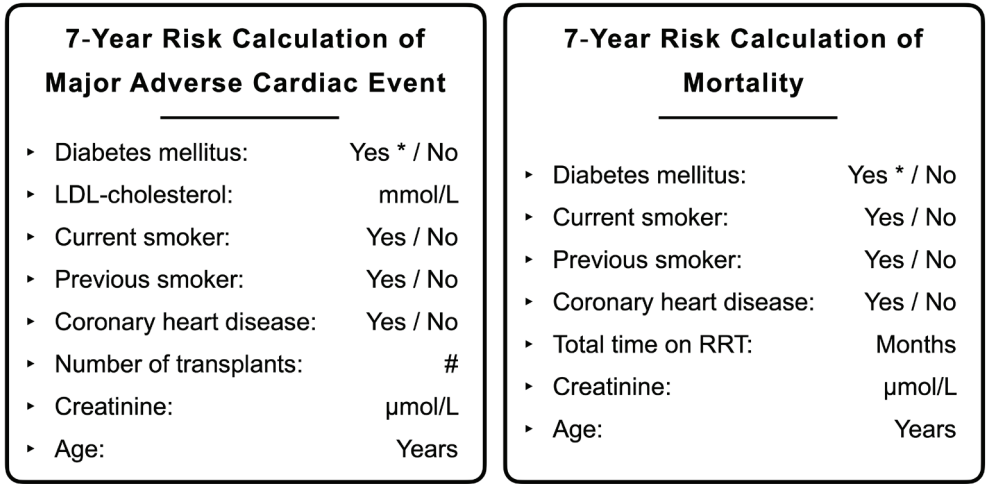


Figure 2. Cardiovascular risk calculator for kidney transplant recipients (Soveri et al., 2012).

List of variables used in the cardiovascular risk calculator. *Includes post-transplant diabetes mellitus. RRT = renal replacement therapy (including dialysis and transplantation)

Sample size and randomization

We performed a power calculation hypothesizing that the intervention arm would decrease the risk of MACE by 30%. We came to that estimate by extrapolation of the reduction in calculated risk in the previously mentioned paper by Soveri et al.²⁷; the calculated risk of MACE for BENEFIT-patients decreased by 31.2% (from 14.3% to 10.9%), and for mortality by 40% (17.5% to 12.5%). The corresponding risk reduction for BENEFIT-EXT-patients was 27.8% (22.5 to 17.6%) and 22.6% (30.9% to 25.2%). For a two-sample t-test on a two-sided significance level of 0.05, assuming a standard deviation of 0.64 (on the natural logarithmic scale), a sample size of 51 per group was required to obtain a power of 0.8 (80%) to detect a 30% calculated risk reduction in MACE. The ANCOVA model was expected to have slightly greater power than the two-sample t-test, and therefore a sample size of 102 patients was seen as sufficient for this study. To account for 8% drop-out, a total of 110 patients, 55 per treatment arm were included in the study. Randomization to treatment arm was performed using a computerized procedure, stratified by center, in a 1:1 ratio.

Statistical analysis

Due to a skewed distribution, the primary variable (estimated risk for MACE and mortality) was log-transformed (natural logarithm). The primary endpoint was the comparison of the log of estimated MACE and mortality risk between treatment groups (CNI- vs. belatacept-based immunosuppression) at one year. For patients who discontinued the study before one year, the last available estimate of CV risk was used in the analysis of the intention-to-treat (ITT) population.

The primary analysis on the primary endpoint was performed using analysis of covariance (ANCOVA) with treatment as a group variable and baseline log risk for MACE and mortality and center as covariates. All other comparisons on primary and secondary endpoints were based on ITT comparisons of treatment groups using two-sample t-test, or ANCOVA with correction for baseline variables and/or center. A two-sided P value of <0.05 was considered statistically significant. Analyses were performed using SAS® version 9.4 (SAS Institute, Cary, NC).

RESULTS

Study participants and characteristics

A total of 112 patients from 9 centres signed the patient informed consent form. Of these, one patient was a screen failure (history of rejection) and was never

randomized. Of the 111 randomized patients, 6 withdrew consent before any study drug was given, 4 in the belatacept arm and 2 in the CNI arm.

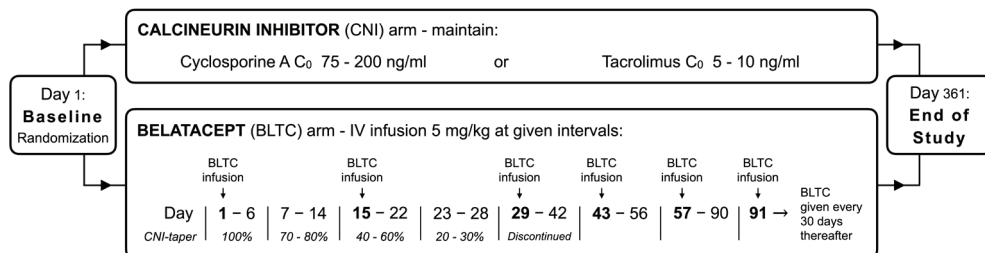


Figure 3. Study flow chart.

Thus, 105 patients were administered study medication: 54 in the belatacept arm and 51 in the CNI arm (defining our ITT population). In the belatacept-arm 5 patients were withdrawn from the study; 3 due to adverse events (AEs), 1 withdrew consent and 1 moved out of the country. Similarly, there were 2 withdrawals in the CNI arm; 1 due to AE and 1 withdrew consent. The remaining 49 patients in each treatment arm were defined as the per protocol (PP) population (**Figure 3**). As the difference between the PP population and the ITT population was quite small, we did not perform PP analyses to avoid the risk of type I error caused by multiple comparisons. The first patient was enrolled September 18th, 2014, and the last patient completed the study on September 13th, 2018. Baseline demographic data and clinical characteristics for each group are presented in Table 1.

Table 1. Baseline demographics and clinical characteristics (ITT population).

	Belatacept arm (n=54)	CNI arm (n=51)
Female	13 (24%)	13 (25%)
Age, years	55.0 (15.2)	54.2 (13.8)
BMI, kg/m ²	26.1 (4.1)	27.1 (4.1)
Renal replacement therapy		
Number of transplantations	1 (1 - 2)	1 (1 - 2)
Time since last transplantation, months	25.3 (3.7 - 59.6)	23.4 (3.1 - 58.8)
Total time on renal replacement therapy, months	35.6 (12.1 - 489.5)	36.8 (5.3 - 220.8)
Prior immunosuppressive therapy		
Tacrolimus	53 (98%)	48 (94%)
Cyclosporine	1 (2%)	3 (6%)
Steroids	50 (93%)	50 (98%)
Mycophenolate	50 (93%)	47 (92%)
mTOR inhibitor	3 (6%)	1 (2%)

Table 1. Baseline demographics and clinical characteristics (ITT population). (*continued*)

	Belatacept arm (n=54)	CNI arm (n=51)
Baseline immunosuppression trough levels		
Tacrolimus	5.8 (1.7)	5.7 (1.7)
Cyclosporine	94 (4.8)	89 (5.1)
Cardiovascular medication		
ACE-inhibitors / angiotensin II antagonists	39 (72%)	29 (57%)
Calcium channel blockers	34 (63%)	39 (77%)
Diuretics	16 (30%)	15 (30%)
α -adrenoceptor antagonists	11 (20%)	7 (14%)
β -adrenoceptor antagonists	29 (54%)	27 (53%)
Nitrate	1 (2%)	3 (6%)
Statins	27 (50%)	32 (63%)
Diabetes mellitus	12 (22%)	7 (14%)
Hypertension	30 (56%)	33 (65%)
Systolic blood pressure, mmHg	137 (17.2)	133 (18.4)
Diastolic blood pressure, mmHg	84 (9.7)	81 (11.2)
Smoking habits		
Non-smoker	29 (54%)	21 (41%)
Current smoker	6 (11%)	8 (16%)
Previous smoker	19 (35%)	22 (43%)
Cardiovascular disease		
Peripheral vascular disease	8 (15%)	7 (14%)
Previous heart failure	2 (4%)	3 (6%)
Previous coronary heart disease	4 (7%)	6 (12%)
Previous cerebrovascular disease	2 (4%)	4 (8%)
Plasma creatinine, $\mu\text{mol/L}$	135 (35.7)	125 (42.6)
eGFR (mL/min/1.73 m^2)	49.4 (14.8)	56.6 (19.1)
hs-CRP, mg/L	3.1 (4.1)	2.8 (2.8)
Plasma glucose, mmol/L	6.2 (1.8)	5.9 (1.7)
Total cholesterol, mmol/L	5.0 (1.0)	5.1 (1.0)
HDL-cholesterol, mmol/L	1.5 (0.5)	1.6 (0.6)
LDL-cholesterol, mmol/L	2.7 (0.9)	2.7 (0.9)
Triglycerides, mmol/L	1.9 (0.9)	1.9 (0.9)
Apolipoprotein B, g/L	1.0 (0.4)	1.0 (0.3)
Apolipoprotein A1, g/L	1.4 (0.3)	1.5 (0.4)

Data are presented as number (percentage) for categorical data, as mean value (standard deviation) for continuous variables and as median (min - max) for renal replacement therapy.

Estimated risk of MACE and mortality

The primary endpoint was the estimated 7-year risk of MACE and all-cause mortality per the risk calculator developed by Soveri et al. (Figure 2). After 12 months of treatment, there was no statistically significant difference between the treatment groups in terms of change in predicted risk, neither for MACE nor for mortality (Table 2).

Table 2. Estimated 7-year risk of MACE and mortality

		Belatacept arm		CNI arm		Difference
		Baseline	End of study	Baseline	End of study	
MACE	Mean (SD)	0.15 (0.13)	0.15 (0.15)	0.14 (0.14)	0.15 (0.15)	
	Log mean risk change [95% CI]	-2.31 [-2.40, -2.23]		-2.25 [-2.33, -2.16]		0.06 [-0.04, 0.16]
Mortality	Mean (SD)	0.21 (0.19)	0.23 (0.20)	0.19 (0.18)	0.21 (0.19)	
	Log mean risk change [95% CI]	-1.94 [-1.96, -1.91]		-1.92 [-1.94, -1.90]		0.02 [-0.01, 0.05]

MACE = major adverse cardiac event. CI = confidence interval

In the belatacept-arm, mean (SD) estimated 7-year risk of MACE at baseline was 0.15 (0.13), and it remained unchanged after 1 year to 0.15 (0.15). Similarly, the risk estimation for the CNI continuation arm was 0.14 (0.14) at baseline and 0.15 (0.15) after 1 year. After applying the ANCOVA models and adjusting for hospital centres, the log mean risk prediction decreased by 2.31 (95% CI: 2.23, 2.40) for the belatacept-group, and 2.25 (95% CI: 2.16, 2.33) for the CNI group. The difference between interventions in log mean risk prediction for MACE was 0.06 (95% CI: -0.04, 0.16).

The estimated 7-year mortality risk in the belatacept-arm at baseline was 0.21 (0.19), which increased non-significantly to 0.23 (0.20) after 1 year. Correspondingly for the CNI continuation arm, the predicted risk of mortality was 0.19 (0.18) at baseline and increased non-significantly to 0.21 (0.19) after 1 year. After applying the ANCOVA models and adjusting for hospital centres, the log mean risk prediction decreased by 1.94 (95% CI: 1.91, 1.96) for the belatacept-group, and 1.92 (95% CI: 1.90, 1.94) for the CNI group. The difference between interventions in log mean risk prediction for mortality was 0.02 (95% CI: -0.01, 0.05). An overview of the variables used in the risk calculation is presented in Table 3.

Table 3. Overview of variables composing estimated cardiovascular risk.

	Variable	Belatacept arm		CNI arm	
		Baseline	End of study	Baseline	End of study
Risk calculator composite					
Common for MACE and mortality	Age, years	54.5 (15.2)	55.5 (15.2)	53.8 (13.7)	54.8 (13.7)
	Creatinine, $\mu\text{mol/L}$	135.1 (35.7)	132.2 (44.1)	124.7 (42.6)	119.1 (38.4)
	Diabetes mellitus	12 (22.2%)	12 (22.2%)	7 (13.7%)	7 (13.7%)
	Coronary HD	4 (7.4%)	4 (7.4%)	6 (11.8%)	6 (11.8%)
	Current smoker	6 (11.1%)	6 (11.1%)	8 (15.7%)	8 (15.7%)
	Previous smoker	19 (35.2%)	19 (35.2%)	22 (43.1%)	22 (43.1%)
MACE only	LDL-cholesterol, mmol/L	2.7 (0.9)	2.6 (1.0)	2.7 (0.9)	2.6 (0.8)
	Number of transplants: 1	51 (94.4%)	51 (94.4%)	48 (94.1%)	48 (94.1%)
	Number of transplants: 2	3 (5.6%)	3 (5.6%)	3 (5.9%)	3 (5.9%)
Mortality only	Total time RRT, months	51.4 (69.5)	62.9 (69.6)	45.1 (37.0)	56.9 (37.0)

Data are presented as number (percentage) for categorical data and mean value (standard deviation) for continuous variables. HD = Heart Disease. MACE = major adverse cardiac event. RRT = renal replacement therapy.

Subgroup analysis was also performed to investigate whether time since transplantation influenced the results in risk calculation. Treatment arms were divided upon the median time after transplantation, thus creating an early and late group (before and after 26 months). There was no difference between belatacept and CNI in calculated risk of MACE ($p = 0.33$) and mortality ($p = 0.56$) in the subgroups.

Traditional CVD risk factors

The changes in traditional CV biomarkers from baseline to end of study are presented in **Table 4**. The mean changes were similar between the treatment groups, except for a significant difference in diastolic blood pressure, with lower levels after belatacept treatment compared with CNI. Systolic blood pressure showed a similar reduction, but the difference was not statistically significant.

Arterial stiffness

Arterial stiffness was measured at baseline and at end of study using the SphygmoCor® method. Compared with the CNI group, central diastolic pressure in patients of the belatacept group decreased by 6.55 mmHg (95%CI: 1.83, 11.27; $p = 0.01$) after one year of treatment. For central systolic pressures, the difference of 6.1 mmHg between study groups (95% CI: -0.11, 12.34; $p = 0.05$) was borderline

significant. There were no differences between the treatment arms in central pulse pressure, pulse wave velocity and augmentation index (**Table 5**).

Cardiovascular events and patient survival

During the one-year study period, there were no cardiovascular events observed (including cardiovascular death, non-fatal myocardial infarction, non-fatal stroke, hospitalization due to congestive heart failure or angina pectoris, or coronary intervention) or deaths in the study population.

Table 4. Change from baseline for traditional CVD risk factors.

Risk factor	Belatacept arm	CNI arm	Difference	p-value
Systolic BP, mmHg	-3.3 (-9.18, 2.68)	2.9 (-1.18, 6.97)	-6.2 (-13.26, 0.97)	0.09
Diastolic BP, mmHg	-2.6 (-5.38, 0.19)	2.8 (-0.24, 5.88)	-5.4 (-9.50, -1.33)	0.01
hs-CRP, mg/L	4.64 (-0.94, 10.23)	0.81 (-0.56, 2.18)	3.83 (-1.90, 9.57)	0.19
Plasma glucose, mmol/L	0.04 (-0.68, 0.77)	-0.06 (-0.65, 0.54)	0.1 (-0.83, 1.03)	0.83
eGFR, mL/min/1.73 m ²	1.40 (-0.81, 3.60)	0.73 (-1.50, 2.95)	0.67 (-2.42, 3.76)	0.67
Total cholesterol, mmol/L	0.22 (-0.55, 0.98)	-0.09 (-0.31, 0.14)	0.3 (-0.49, 1.10)	0.45
HDL-cholesterol, mmol/L	-0.10 (-0.18, -0.03)	-0.02 (-0.09, 0.05)	-0.09 (-0.19, 0.01)	0.08
LDL-cholesterol, mmol/L	-0.10 (-0.31, 0.11)	-0.05 (-0.22, 0.13)	-0.05 (-0.32, 0.22)	0.71
Triglycerides, mmol/L	-0.06 (-0.29, 0.17)	-0.05 (-0.25, 0.14)	-0.01 (-0.31, 0.29)	0.96
Apolipoprotein B, g/L	-0.09 (-0.18, -0.00)	-0.06 (-0.13, 0.01)	-0.03 (-0.14, 0.08)	0.59
Apolipoprotein A1, g/L	0.02 (-0.07, 0.10)	0.01 (-0.08, 0.09)	0.01 (-0.11, 0.13)	0.88

Presented as mean (95% CI). BP = blood pressure. hs-CRP = high-sensitivity C-reactive protein. eGFR = estimated glomerular filtration rate. P-value results from two-sample t-tests.

Table 5. Change from baseline in arterial stiffness variables.

Risk factor	Belatacept arm	CNI arm	Difference	p-value
Augmentation Index, %	-1.26 (-4.51, 1.99)	1.04 (-2.41, 4.48)	-2.30 (-6.96, 2.37)	0.33
Pulse Wave Velocity, cm/sec	-0.44 (-1.02, 0.13)	0.1 (-0.88, 1.08)	-0.54 (-1.67, 0.58)	0.34
Central systolic pressure, mmHg	-4.45 (-9.09, 0.18)	1.65 (-2.63, 5.94)	-6.1 (-12.33, 0.11)	0.05
Central diastolic pressure, mmHg	-3.72 (-7.41, -0.02)	2.83 (-0.17, 5.84)	-6.55 (-11.24, -1.86)	0.01

Table 5. Change from baseline in arterial stiffness variables. (*continued*)

Risk factor	Belatacept arm	CNI arm	Difference	p-value
Central pulse pressure, mmHg	-0.60 (-4.03, 2.83)	-0.37 (-3.51, 2.77)	-0.23 (-4.81, 4.35)	0.92

Presented as mean (95% CI). P-value results from two-sample t-tests.

Safety evaluation

All patients in both study groups reported at least one AE during the duration of the study (**Table 6**). The majority of the events were of mild severity and considered unrelated to study drug. More patients in the belatacept-group (53.7% vs 21.6%) reported AEs that were considered possibly or probably related to the intervention. Three patients in the belatacept-group and one patient in the CNI continuation group reported AEs that led to withdrawal from the study. Serious Adverse Events (SAEs) were reported by 29.6% of the patients in the belatacept-group compared with 15.7% in the CNI group. Patients allocated to the belatacept-group had more infections (**Table 7**). There was 1 case of incident cancer (lung cancer), which occurred in the belatacept-group.

During the study, 8 acute rejection episodes were suspected, and graft biopsies were obtained for further investigation. Acute rejection was confirmed in 4 of the 7 suspected cases in the belatacept-group, and in the single case in the CNI group. Three of the rejection episodes were considered severe (Banff grade IIA or higher): two in the belatacept-group and in the CNI-treated patient. One patient (belatacept) proved refractory despite anti-rejection treatment with methylprednisolone and T-cell depleting antibody. All other rejection episodes recovered upon treatment with corticosteroids or anti-thymocyte globulin as per local practices.

Table 6. Number and proportion of patients with adverse events

	Belatacept arm		CNI arm	
	n	%	n	%
Any adverse event	54	100	51	100
1 adverse event	10	18.5	21	41.2
>1 adverse events	44	81.5	30	58.8
Any possibly or probably intervention-related adverse events	29	53.7	11	21.6
Adverse events leading to withdrawal	3	5.6	1	2.0
Serious adverse events	16	29.6	8	15.7
- suspected acute rejection	7	13.0	1	2.0
- biopsy-proven acute rejection	4	7.4	1	2.0
- graft loss due to acute rejection	1	1.9	0	-
- cancer	1	1.9	0	-

Table 7. Adverse events reported by $\geq 5\%$ of patients in either treatment group given as incidence rates in number (percentage)

Event	Belatacept arm (n=54)	CNI arm (n=51)
Urinary tract infection	19 (35%)	4 (8%)
Pyrexia	17 (32%)	1 (2%)
Abdominal pain/discomfort	10 (19%)	1 (2%)
Nasopharyngitis	10 (19%)	8 (16%)
Respiratory tract infection	8 (15%)	5 (10%)
Coughing	8 (15%)	1 (2%)
Oedema	5 (9%)	3 (6%)
Diarrhoea	5 (9%)	2 (4%)
Anaemia	5 (9%)	1 (2%)
Fatigue	4 (7%)	1 (2%)
Headache	4 (7%)	1 (2%)
Dizziness	4 (7%)	0
Arthralgia	2 (4%)	3 (6%)
Gastroenteritis	1 (2%)	3 (6%)
Nausea	3 (6%)	1 (2%)
Herpes zoster	3 (6%)	1 (2%)
Myalgia	3 (6%)	1 (2%)
Aphthous ulcer	3 (6%)	0

DISCUSSION

In this randomized study, where stable renal transplant patients were converted from a CNI-based maintenance immunosuppressive regimen to belatacept, no difference in calculated 7-year risk of MACE or all-cause mortality could be demonstrated after 1 year of follow-up. We were unable to find a significant effect on any of the three modifiable cardiovascular risk factors which were used as input-variables in the risk calculator (serum LDL-cholesterol, diabetes-prevalence, and serum creatinine). The belatacept arm had significantly lower diastolic blood pressure, measured both centrally (SphygmoCor® method) and peripherally. We found a similar improvement for systolic pressure (Table 4), but this difference was not statistically significant ($p = 0.09$).

Of the three modifiable risk factors in the calculator, we had expected a significant change in eGFR in the intervention arm. Our findings are in contrast to the BENEFIT studies, as well as other belatacept conversion studies reported in the literature^{24, 31-33}. In those studies, there was a consistent improvement in graft function by converting to belatacept. One possible explanation for this was the predominant use of TAC by our study participants with relatively low trough levels (Table 1) at baseline. In the Symphony trial³⁴, the low-dose TAC group had an average trough-level of 6.7 ng/ml one year after transplantation and achieved an eGFR on average 5.7 ml/min/1.73m² higher than the other 3 comparator groups. A belatacept conversion study by Grinyo et al.³⁵ examined 173 patients with a mean time after transplantation to randomization of 19 months, baseline eGFR of 54 ml/min/1.73m², and a low immunologic risk profile, making the population reasonably comparable to ours. Belatacept patients in that study showed an average improvement in eGFR of 4.9 ml/m/1.73m² compared to CNI-patients. At baseline, patients using TAC (56%) had an average trough level of 7.2 ng/ml, while patients on CsA (44%) had an average trough level of 160.2 ng/ml. In our study, the mean trough levels of TAC (5.7 ng/ml) and CsA (91 ng/ml, 4 patients only) at the time of randomization were lower compared to both these studies^{34, 35}. The lower CNI trough levels may have already significantly decreased the nephrotoxic side-effects and explain why our belatacept patients only experienced a non-significant gain in eGFR of 0.7 ml/m/1.73m². However, this hypothesis is not supported by Budde et al. in a recent study that used a very similar design to ours³⁶. Their results showed an average TAC trough level of 6.27 mg/l and 5.82 mg/l respectively at baseline and after one year in the control arm. The average difference in eGFR between belatacept and TAC at that point was already 6.8 ml/min, contrary to our findings. Of note, patients in our study with BPAR had a decrease in eGFR of 2.7 ml/min, compared to a rise of 1.1 ml/min in the others. The low number of events precludes any meaningful interpretation

of these data, and the general lack of improvement in eGFR cannot be explained hereby.

The second element of the calculator is diabetes status. Multiple studies have corroborated the diabetogenicity of TAC in transplantation³⁷⁻³⁹. Furthermore, reversibility of beta cell dysfunction and of PTDM after TAC-withdrawal has been established in both animal studies and in clinical experience⁴⁰⁻⁴³. Thus, we expected to improve glycaemic metabolism in converting from TAC to belatacept. However, no subject in our study reversed diabetes mellitus or developed PTDM in either study arm (Table 3). Also, triglycerides, serum ApoB, and serum ApoA1 did not improve (Table 4), which is of interest, since all three of these parameters are mentioned as risk factors for developing PTDM^{44, 45}.

Expectations regarding the effect on lipid profile, the third element in the calculator, were limited. While CsA has been implicated in dyslipidaemia⁴⁶, TAC seems to be less detrimental to lipid status. In our study, 94% of the participants were on TAC before randomization. Ferguson et al.⁴⁷ compared three steroid-avoiding regimens of immunosuppression: belatacept with MMF vs belatacept with sirolimus vs TAC with MMF. Both belatacept-arms had lower LDL (23.9 mg/ml and 25.0 mg/ml vs 34.0 mg/ml for TAC with MMF) after one year, but the difference was non-significant, possibly related to the limited sample-size of the study. Another observational study focusing on the metabolic effects of conversion from TAC to belatacept found improvement in GFR and acid-base status, but not in blood lipids⁴⁸. Our findings are in line with these reports, as we found no effect on LDL-cholesterol (Table 3).

In trying to explain the lack of significant improvement of CV-risk, we need to consider another bias besides the low CNi trough levels. Patients were already treated with CNi for a median of 26 months since transplantation. Serious negative side effects of CNi-treatment could be less likely found in the control group, as patients suffering from these side effects could have been converted to alternative immunosuppression earlier on and thus not be eligible for this study. The only positive effect that we found for belatacept was a significant improvement in diastolic blood pressure, measured both centrally (Sphygmo-Cor® method) and peripherally. For systolic pressure, a similar improvement was found (Table 4), but it was not statistically significant ($p = 0.09$), most likely due to the relatively small sample size of this study. Calcineurin inhibitors (CNi) are known as potent vasoconstrictors and contribute to post-transplant hypertension⁴⁹. A previous study comparing belatacept and CsA on central aortic blood pressure and arterial stiffness after renal transplantation has shown no differences in blood pressure, both centrally and peripherally, in the two groups

⁵⁰. However, the study was also hampered by a low sample size and may have lacked the power to detect significant differences. Although not included in the calculator, blood pressure is of course an established risk factor for cardiovascular disease. Moreover, high blood pressure is strongly associated with risk of graft failure and finding an improvement in this parameter could still indicate an advantage for belatacept-treatment ⁵¹.

Regarding safety, AEs occurred in both groups, but SAEs were reported almost twice as often in belatacept-treated patients (29.6% vs 15.7%), and these were more likely to discontinue their study treatment than patients treated with CNI (5.6% vs 2.0%). Rejection was seen more often in the belatacept-patients. Four episodes of biopsy-proven acute rejections (BPAR) occurred in the belatacept-group vs one single episode in the CNI-group (7.4% vs 2.0%). Three patients showed signs of vascular inflammation in the biopsy, corresponding to Banff grade II, two of which were in the belatacept-group. All three patients were treated according to local protocol with high-dose steroids and T-cell depleting antibodies, despite which one belatacept-patient suffered graft loss and re-initiated dialysis treatment. The other two recovered with anti-rejection treatment. The median number of days between transplantation and randomization was 225 in those on belatacept with BPAR, while no rejection was seen in any patient after 1 year after transplantation (mean 818 days after transplantation for those without BPAR). This trend of rejection after early conversion corroborates the findings by Budde et al., in which all belatacept-rejections was seen within 1 year of transplantation ³⁶.

The rate of rejection in this study is in line with earlier reports. For example, in the trial by Grinyo et al. ³⁵, 7.1% of belatacept-patients experienced rejection versus none in the CNI-group. In another trial by Adams et al. ⁵², 1-year rejection rates were around 50% when belatacept was used right after transplantation, declining to 33% when TAC was tapered off 3-5 months after transplantation. When TAC was tapered after 11 months, the rejection rates between TAC- and belatacept-treated patients were similar, around 16%. Other reports have described varying (0-11%) rates of rejection, but these are data from non-randomized 'rescue'-settings after even longer time post-transplantation and are therefore comparable with our results ^{53, 54}.

Beside rejection, urinary tract infections (UTIs), nasopharyngitis and other respiratory tract infections (RTIs) were more often seen in the belatacept arm (Table 7). The present study's planned visits could have led to a bias in the reporting of uncomplicated infections, since a study visit was planned every month for belatacept-patients, instead of every 3 months for the CNI-continuation group.

Not a single case of pneumocystis-jirovecii pneumonia, cytomegalovirus- (CMV), polyoma- or EBV-associated disease was seen in the belatacept-patients. Three cases of CMV-infection were seen in CNI-patients. Previous reports have been inconclusive on opportunistic infections (OPIs) in belatacept-treatment. The follow-up study to the first belatacept-conversion trial noted a slightly higher incidence of viral infection (11% vs 14%)³⁵. In a recent study by Bertrand et al., 50 OPI's were noted in 453 patients treated with belatacept (9.8%)⁵⁵. In a multivariate analysis of that study, the authors concluded that patients with low GFR (<25 ml/min) and patients converted early after transplantation (within six months) were more likely to develop OPIs.

There was one case of lung cancer in the belatacept group in the present study. Previous studies have not indicated a higher risk of malignancy in belatacept beyond post-transplant-lymphoproliferative disorder^{24,35}.

A major strength of the current study is the international multicentre approach, making it representative for European transplantation practice. However, this study also has important limitations which must be considered. The study duration of 1 year was most likely too short to reveal a significant difference in renal function between the two study groups. We have overestimated the potential reduction in MACE and mortality for patients that use low-dose TAC instead of CsA. Another limitation was the heterogeneous time from transplantation to trial enrolment, and the small number of patients on CsA and the relatively large span of eGFR at baseline also contributed to the heterogeneity. Patients with severely diminished graft function were less likely to benefit from conversion.

In conclusion, we have shown no effect on calculated CV risk or renal function in this study comparing conversion to belatacept with continuation of CNI-based immunosuppression. We did show a significant difference in diastolic blood pressure. We re-confirmed the increased chance of rejection when converting to belatacept. After more than 10 years of clinical experience, the place of belatacept in kidney transplantation is still not fully established, but it may be an attractive option when patients suffer from significant side effects of CNI, like nephrotoxicity or PTDM. However, it is hard to define a significant benefit of belatacept for patients that are doing well on low-dose TAC-based therapy without severe CNI-related side-effects. Further studies are needed to define the place of belatacept in kidney transplantation.

REFERENCES:

1. Baigent C, Burbury K, Wheeler D. Premature cardiovascular disease in chronic renal failure. *Lancet*. 2000;356(9224): 147-152.
2. Jardine AG, Gaston RS, Fellstrom BC, Holdaas H. Prevention of cardiovascular disease in adult recipients of kidney transplants. *Lancet*. 2011;378(9800): 1419-1427.
3. 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.
4. Methven S, Steenkamp R, Fraser S. UK Renal Registry 19th Annual Report: Chapter 5 Survival and Causes of Death in UK Adult Patients on Renal Replacement Therapy in 2015: National and Centre-specific Analyses. *Nephron*. 2017;137 Suppl 1: 117-150.
5. Saran R, Robinson B, Abbott KC, et al. US Renal Data System 2017 Annual Data Report: Epidemiology of Kidney Disease in the United States. *Am J Kidney Dis*. 2018;71(3S1): A7.
6. Holdaas H, Fellstrom B, Jardine AG, et al. Effect of fluvastatin on cardiac outcomes in renal transplant recipients: a multicentre, randomised, placebo-controlled trial. *Lancet*. 2003;361(9374): 2024-2031.
7. Fellstrom B, Jardine AG, Soveri I, et al. Renal dysfunction as a risk factor for mortality and cardiovascular disease in renal transplantation: experience from the Assessment of Lescol in Renal Transplantation trial. *Transplantation*. 2005;79(9): 1160-1163.
8. Svensson M, Jardine A, Fellstrom B, Holdaas H. Prevention of cardiovascular disease after renal transplantation. *Curr Opin Organ Transplant*. 2012;17(4): 393-400.
9. Jardine AG. Assessing the relative risk of cardiovascular disease among renal transplant patients receiving tacrolimus or cyclosporine. *Transpl Int*. 2005;18(4): 379-384.
10. Birkeland SA. Steroid-free immunosuppression in renal transplantation. *Lancet*. 1996;348(9034): 1105-1106.
11. Pathak V, Madhavan D, Narayanasamy K, et al. Low-dose Rituximab and Thyroglobulin Induction With Steroid-free Maintenance Immunosuppression and Protocol Biopsies Improves Long-term Patient and Graft Survival After Kidney Transplantation: Survival and Safety Outcomes in More Than 1100 Patients From a Single Center. *Transplant Direct*. 2019;5(8): e475.
12. Woodle ES, First MR, Pirsch J, et al. A prospective, randomized, double-blind, placebo-controlled multicenter trial comparing early (7 day) corticosteroid cessation versus long-term, low-dose corticosteroid therapy. *Ann Surg*. 2008;248(4): 564-577.
13. Hariharan S, Johnson CP, Bresnahan BA, Taranto SE, McIntosh MJ, Stablein D. Improved graft survival after renal transplantation in the United States, 1988 to 1996. *N Engl J Med*. 2000;342(9): 605-612.
14. Ong SC, Gaston RS. Thirty Years of Tacrolimus in Clinical Practice. *Transplantation*. 2020;Publish Ahead of Print.
15. 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.

16. Hoorn EJ, Walsh SB, McCormick JA, et al. The calcineurin inhibitor tacrolimus activates the renal sodium chloride cotransporter to cause hypertension. *Nat Med*. 2011;17(10): 1304-1309.
17. Heisel O, Heisel R, Balshaw R, Keown P. New onset diabetes mellitus in patients receiving calcineurin inhibitors: a systematic review and meta-analysis. *Am J Transplant*. 2004;4(4): 583-595.
18. Nankivell BJ, Chapman JR, Bonovas G, Gruenewald SM. Oral cyclosporine but not tacrolimus reduces renal transplant blood flow. *Transplantation*. 2004;77(9): 1457-1459.
19. Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Allen RD, Chapman JR. The natural history of chronic allograft nephropathy. *N Engl J Med*. 2003;349(24): 2326-2333.
20. 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.
21. 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.
22. Larsen CP, Grinyo J, Medina-Pestana J, et al. Belatacept-based regimens versus a cyclosporine A-based regimen in kidney transplant recipients: 2-year results from the BENEFIT and BENEFIT-EXT studies. *Transplantation*. 2010;90(12): 1528-1535.
23. 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.
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. Masson P, Henderson L, Chapman JR, Craig JC, Webster AC. Belatacept for kidney transplant recipients. *The Cochrane database of systematic reviews*. 2014(11): Cd010699.
26. Soveri I, Holme I, Holdaas H, Budde K, Jardine AG, Fellstrom B. A cardiovascular risk calculator for renal transplant recipients. *Transplantation*. 2012;94(1): 57-62.
27. Soveri I, Snyder J, Holdaas H, et al. The external validation of the cardiovascular risk equation for renal transplant recipients: applications to BENEFIT and BENEFIT-EXT trials. *Transplantation*. 2013;95(1): 142-147.
28. Jardine AG, Fellstrom B, Logan JO, et al. Cardiovascular risk and renal transplantation: post hoc analyses of the Assessment of Lescol in Renal Transplantation (ALERT) Study. *Am J Kidney Dis*. 2005;46(3): 529-536.
29. Israni AK, Snyder JJ, Skeans MA, et al. Predicting coronary heart disease after kidney transplantation: Patient Outcomes in Renal Transplantation (PORT) Study. *Am J Transplant*. 2010;10(2): 338-353.
30. Butlin M, Qasem A. Large Artery Stiffness Assessment Using SphygmoCor Technology. *Pulse (Basel)*. 2017;4(4): 180-192.
31. 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.

32. Florman S, Becker T, Bresnahan B, et al. Efficacy and Safety Outcomes of Extended Criteria Donor Kidneys by Subtype: Subgroup Analysis of BENEFIT-EXT at 7 Years After Transplant. *Am J Transplant.* 2017;17(1): 180-190.
33. Poster Abstracts. 2020;20(S3): 539-1166.
34. 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.
35. Grinyó 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.
36. Budde K, Prashar R, Haller H, et al. Conversion from Calcineurin Inhibitor to Belatacept-based Maintenance Immunosuppression in Renal Transplant Recipients: a Randomized Phase 3b Trial. *J Am Soc Nephrol.* 2021.
37. Fan Y, Xiao YB, Weng YG. Tacrolimus versus cyclosporine for adult lung transplant recipients: a meta-analysis. *Transplant Proc.* 2009;41(5): 1821-1824.
38. Knoll GA, Bell RC. Tacrolimus versus cyclosporin for immunosuppression in renal transplantation: meta-analysis of randomised trials. *BMJ.* 1999;318(7191): 1104-1107.
39. Penninga L, Penninga EI, Moller CH, Iversen M, Steinbruchel DA, Gluud C. Tacrolimus versus cyclosporin as primary immunosuppression for lung transplant recipients. *The Cochrane database of systematic reviews.* 2013(5): CD008817.
40. Dai C, Walker JT, Shostak A, et al. Tacrolimus- and sirolimus-induced human beta cell dysfunction is reversible and preventable. *JCI Insight.* 2020;5(1).
41. 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.
42. Terrec F, Jouve T, Naciri-Bennani H, et al. Late Conversion From Calcineurin Inhibitors to Belatacept in Kidney-Transplant Recipients Has a Significant Beneficial Impact on Glycemic Parameters. *Transplant Direct.* 2020;6(1): e517.
43. Ghisdal L, Bouchta NB, Broeders N, et al. Conversion from tacrolimus to cyclosporine A for new-onset diabetes after transplantation: a single-centre experience in renal transplanted patients and review of the literature. *Transpl Int.* 2008;21(2): 146-151.
44. Malyala R, Rapi L, Nash MM, Prasad GVR. Serum Apolipoprotein B and A1 Concentrations Predict Late-Onset Posttransplant Diabetes Mellitus in Prevalent Adult Kidney Transplant Recipients. *Can J Kidney Health Dis.* 2019;6: 2054358119850536.
45. Guzman GE, Victoria AM, Ramos I, et al. Risk Factors Related to New-Onset Diabetes after Renal Transplantation in Patients of a High Complexity University Hospital in Colombia, 20 Years of Experience. *Int J Endocrinol.* 2020;2020: 8297192.
46. Mathis AS, Dave N, Knipp GT, Friedman GS. Drug-related dyslipidemia after renal transplantation. *Am J Health Syst Pharm.* 2004;61(6): 565-585; quiz 586-567.
47. 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.
48. Schulte K, Vollmer C, Klasen V, et al. Late conversion from tacrolimus to a belatacept-based immuno-suppression regime in kidney transplant recipients improves

- renal function, acid-base derangement and mineral-bone metabolism. *J Nephrol.* 2017;30(4): 607-615.
- 49.** Calo LA, Ravarotto V, Simioni F, et al. Pathophysiology of Post Transplant Hypertension in Kidney Transplant: Focus on Calcineurin Inhibitors Induced Oxidative Stress and Renal Sodium Retention and Implications with RhoA/Rho Kinase Pathway. *Kidney Blood Press Res.* 2017;42(4): 676-685.
- 50.** 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.
- 51.** Mangray M, Vella JP. Hypertension after kidney transplant. *Am J Kidney Dis.* 2011;57(2): 331-341.
- 52.** 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.
- 53.** Brakemeier S, Kannenkeril D, Dürr M, et al. Experience with belatacept rescue therapy in kidney transplant recipients. *Transpl Int.* 2016;29(11): 1184-1195.
- 54.** 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.
- 55.** Bertrand D, Terrec F, Etienne I, et al. Opportunistic Infections and Efficacy Following Conversion to Belatacept-Based Therapy after Kidney Transplantation: A French Multicenter Cohort. *J Clin Med.* 2020;9(11).

3

Conversion from calcineurin inhibitors to belatacept-based immunosuppressive therapy skews terminal proliferation of non-classical monocytes and lowers lymphocyte counts

OW Bredewold, AM van Oeveren-Rietdijk, B Florijn, JI Rotmans, JW de Fijter, C v Kooten, AJ v Zonneveld, HC de Boer.

Published in:

Transplant Immunology 2024 Feb;82:101976

ABSTRACT:

Belatacept, a modified form of CTLA-Ig that blocks CD28-mediated co-stimulation of T cells, is an immune-suppressant that can be used as an alternative to calcineurin inhibitors (CNIs). In kidney transplant recipients, belatacept has been associated with improved renal function and reduced cardiovascular toxicity. Monocytes as well as T-lymphocytes play causal roles in the pathophysiology of atherosclerotic disease. We hypothesized that the beneficial impact of the use of belatacept over CNIs on cardiovascular risk could be partly explained by the impact of belatacept therapy on these circulating leukocytes. Hence, we phenotyped circulating leukocytes in transplanted patients with a stable renal function that were randomized between either continuation of CNI or conversion to belatacept in two international studies in which we participated. In 41 patients, we found that belatacept-treated patients consistently showed lower numbers of B-lymphocytes, T-lymphocytes as well as CD14-negative monocytes (CD14NM), especially in non-diabetic patients. Our observation that this decrease was associated to plasma concentrations of $\text{TNF}\alpha$ is consistent with a model where CD14NM-production of $\text{TNF}\alpha$ is diminished by belatacept-treatment, due to effects on the antigen-presenting cell compartment.

INTRODUCTION

Kidney transplantation is the treatment of choice for end-stage kidney disease (ESKD). Since the introduction of calcineurin inhibitors (CNI) and especially tacrolimus, early graft survival has spectacularly risen [1]. Belatacept was introduced in the beginning of the previous decade and was shown to be a safe alternative to CNI [2-4]. While, compared to CNI, it is quite consistently linked to higher rates of rejection, the use of belatacept was associated with improvement of renal function and favorable effects on blood pressure, glucose metabolism and bone mineralization [5-7]. Belatacept seems less likely to result in formation of donor specific antibodies (DSA) compared to CNI illustrating its impact on humoral immunity [8].

Cardiovascular disease (CVD) remains the most important cause of death after transplantation [9, 10]. While multiple clinical studies on the immunological aspects of atherosclerosis were reported in the last 10 years, not much is known about the influence of belatacept on circulating cells of the immune system, which may directly impact the progression of CVD [11, 12]. Although monocytes are a main component of the innate immune system, they are also able to present antigens to cognate T-lymphocytes, thereby playing a role in adaptive immunity [13]. Traditionally, monocytes have been divided into three subsets, classical- (CM), intermediate- (IM) and non-classical monocytes (NCM), based on the expression of the LPS-receptor CD14 and Fcγ receptor III (FcγRIII)/CD16 with CMs being CD14⁺⁺/CD16^{neg}, IMs are CD14⁺⁺/CD16⁺ and NCMs CD14⁺/CD16⁺⁺. CD14-expression on monocytes varies accordingly from 'low' or 'dim' to 'high' in NCM versus CM. Most authors require at least some amount of CD14-expression in their gating strategy for monocytes [14]. However, in our laboratory we have found a population of CD14-negative monocytes in patients with diabetic nephropathy (de Boer, manuscript in progress). In our interpretation, loss of expression of CD14 is a sign of terminal differentiation of NCM in inflammatory conditions. For that reason we included monocytes that were negative for CD14 in our analysis.

Marked variations in the relative abundance of various monocytic subsets has been observed in numerous disease states including CVD and chronic kidney disease (CKD). For instance, levels of classical monocytes predict cardiovascular events in patients with coronary heart disease [15]. In pre-transplantation-patients, CD16⁺ monocytes (both IM and NCM) exert an upregulation of pro-atherosclerotic genes in human endothelial cells, underlining their mechanistic role in CVD [16]. In patients with CKD, uremic toxins shifts monocytes to a CD16-positive phenotype [17] leading to significantly higher numbers of IMs,

independently predicting cardiovascular outcomes in patients with CKD [18]. Even after resolution of CKD by kidney transplantation, elevated numbers of circulating IMs are associated with subclinical atherosclerosis [19].

Immunosuppression used in transplantation mostly exerts effects on T-lymphocytes, either by depletion or interference with its function [20]. Naïve T-lymphocytes are activated by antigen-presenting cells (APC) in a 'direct' or indirect fashion. In the first, donor-derived APC's present molecules of the major histocompatibility complex (MHC) to recipient T-cells. In indirect allograft recognition, mostly dendritic cells but also other host-APC's like monocytes, macrophages and B-lymphocytes phagocytose a donor-pathogen and present it in small peptides loaded onto molecules of the MHC II-class. These peptides can be presented on the surface of APC's to engage the T-cell-receptor. In transplantation, donor-antigen of the HLA-class can present itself this way to T-cells, and elicit T-cell mediated (cellular) rejection [21]. In atherosclerosis, macromolecules like oxidized LDL, present in atherosclerotic plaque are taken up by APC's, fragmented into peptides that are loaded up on MHC-molecules and also presented to CD4+ T-cells[22]. Depending on whether the APC will be simultaneously activated through Toll-like-receptors, the subsequent T-cell action will either be a pro-, or anti-atherogenic response [23]. Production of antibodies (donor-specific antibodies, DSA) requires specific cytokine production and the recruitment of Follicular helper-T-cells. This process is frequently seen in transplantation, but not in atherosclerosis.

Belatacept is a co-stimulation blocker, existing of the cytotoxic T-lymphocyte antigen 4 (CTLA4) coupled to the Fc-tail of Immunoglobulin G. It acts by binding to the co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2) expressed by antigen presenting cells (APCs) [24]. In so doing, it prevents that these ligands bind to CD28, a stimulus that plays a key role in cell remodelling, cytokine production and proliferation [25, 26]. Multiple trials have investigated the effect of conversion of immune-suppressive regimens based on CNI to regimens including belatacept. Our institution has recently participated in two of those studies [27, 28]. Data from the participating patients have presented us with an opportunity to explore whether belatacept impacts the phenotype of circulating leukocytes. We performed a thorough fluorescent assorting cell sorting (FACS) analysis on selected leucocytes of 44 of those patients, revealing a remarkable effect on CD14-negative monocytes, a cell relatively scarcely described. We also found that this effect clusters with TNF-alfa and lymphocytes, only in belatacept-treated patients.

We hypothesized that belatacept influences the phenotype or reduces the circulating numbers of leucocytes relative to the effect of calcineurin inhibitors. While phenotypic changes can happen early after conversion, the clinical effects of reduced inflammation could impact patients and grafts on the long term. In order to investigate this hypothesis we compared monocytes and lymphocytes in kidney transplant recipients (KTR) that were randomized for conversion to belatacept [27, 28] to those of the control group (continuation of CNI-therapy) during the first year after conversion.

METHODS

Description of the study

At the time of the study, two international randomized trials were recruiting KTR at the Leiden University Medical Center [27, 28]. Both trials randomized patients to convert from CNI-based immunosuppressive therapy to belatacept, or to continue on CNI. The goal of these original studies was to compare kidney function and cardiovascular outcomes after conversion to belatacept, after a minimum of 3 months after transplantation (to limit rejections that are typically associated with its use directly after transplantation). We designed a local amendment that permitted us to include measurements of circulating cells in all patients from Leiden during the first year of both studies. Our medical ethical committee approved all procedures prior to initiation of the study, in June 2015. All participants provided written informed consent.

For the original studies, patients between 18 and 80 years old were eligible when they had undergone kidney transplantation at least 3 months, and at the latest 5 years before, and had stable renal function. Patients with rejection rated BANFF 2a or greater were not eligible for inclusion. Of the included patients, three patients had received alemtuzumab, a T-cell depleting agent, as a means of 'induction'-therapy. Alemtuzumab was given respectively 30, 50, and 56 months before randomization to this study, meaning that repopulation of lymphocytes was completed long before participation [29].

The precise criteria for inclusion and exclusion are published elsewhere [27, 28]. Our amendment study aimed to include 40 of these patients and perform measurements of circulating cells at three time-points, at randomization (T0), 6 months (T6) and 12 months (T12) after randomization.

The randomization itself was performed within the main studies that overlapped our study. Belatacept was initiated on the day of randomization, immediately

after obtaining blood samples for the measurements. Five mg/kg of belatacept was infused on day 1, 15, 29, 43, 57 and every 29th day after day 57, for a total of 1 year after randomization. CNI's were discontinued in 4 weeks after initiation of belatacept. Patients randomized to the control group continued CNI after randomization in the same dose as they used before (trough level of tacrolimus between 4-7 mg/L, cyclosporine 100-150 mg/L). All patients received maintenance immunosuppression consisting of mycophenolate mofetil (MMF) (dosed twice daily to obtain an area under the concentration over 12 hour-curve (AUC-12) of 30-60 mg/L*h, or the mammalian target of sirolimus-inhibitor (mTOR-i) everolimus (trough levels 5-8 mg/L) and low-dose prednisone (5-7,5 mg per day). All patients were routinely tested for clinical parameters including renal function every 3 months, or monthly when in the belatacept group in the first 3 months after randomization.

Blood sampling

For measurement of circulating cells, 5 mL of EDTA-anticoagulated blood was drawn through venous sampling during routine visits at randomization and at 6 and 12 months after randomization. EDTA-blood was centrifuged for 10 minutes at 3000 rpm (room temperature) after which plasma was stored in microcentrifuge tubes at -80°C until required for analysis.

Fluorescent Assisted Cell Sorting (FACS).

From all antibodies in the cocktails saturating concentrations were determined. Conjugated antibodies were added within 2 hours of blood withdrawal in the appropriate dilutions and incubated for 30 minutes on ice in the dark. For monocyte subset measurements one tube with 100 µL of whole blood was incubated with a monocyte cocktail containing conjugated antibodies CD45-BUV395, CD14-V500, CD31-FITC, and CD16-PE-CF594. In a second tube, 100 µL of whole blood was incubated with a lymphocyte cocktail containing CD45-BUV395, CD3-PerCP, CD19-eFluor700 and CD27-PE. All antibodies were obtained from BD Biosciences (Etten-Leur, The Netherlands). Red blood cells (RBCs) were lysed with shock buffer (0.155 M NH₄Cl, 0.01 M KHCO₃, 0.1 mM EDTA, pH 7.4) for 10 minutes on ice and the samples were measured immediately using the LSRII (Becton Dickinson) after appropriate maintenance- and compensation protocols. This multicolour lyse-no-wash, whole blood protocol guarantees minimal sample manipulation and thus minimal cell activation. Per tube 955.843±248.673 total events (average ± SD of 41 blood samples) were recorded. Absolute cell-numbers were calculated by measuring the percentage of cells in the "no debris" gate and relating this cell population to the total WBC-count measured by a routine haematological laboratory, yielding absolute numbers of cells expressed as nx10E6/mL, unless stated otherwise. The gating

strategies for monocytes and lymphocytes are illustrated and described in **Supplemental Figure 1** and a list of all antibodies used, including clone-identifier is depicted in the **Supplemental Table 1**.

Additional FACS for markers of antigen-presentation.

After interpretation of the first results, in which we found the predominant effect on CD14NM, we hypothesized that these cells would contain high expression of markers associated with antigen-presentation. Therefore, plasma of 4 additional KTRs was collected, since the original samples were not suited for a new FACS-experiment. All were treated with tacrolimus, mycophenolate and prednisone. The same FACS-procedure was carried out, with additional markers for CD80, CD86 and HLA-DR to estimate antigen-presenting capacity.

Measurement of TNF α -levels.

Circulating TNF α antigen levels were measured for all patients in EDTA-plasma samples using a Human Magnetic Luminex Assay (Bio-Techne, Abingdon, UK).

Statistical analysis

Clinical characteristics were expressed as either categorical variables or continuous variables. Comparison between the intervention groups was done using Pearson Chi Square test, with p-value of < 0.05 as discriminant for significant difference. All cell counts are continuous variables, expressed in whole numbers as mean cell counts $\pm$ standard error of mean (SEM), or median cell counts with IQR when populations were non-parametrically distributed. Comparison of the initial values at baseline was done using independent T-testing. Comparing of mean differences at the longitudinal time points was done using either a two-way mixed ANOVA, with one-way ANOVA to follow up on significant simple main effects, or by paired samples T-testing. Differences between continuous variables were considered significant when p-values were < 0.05 . For determination of the proportion of variance the R-squared value (R^2) was calculated. The analysis was performed using SPSS version 27 (SPSS Inc, Chicago, IL) and GraphPad Prism, version 8 (GraphPad Prism Software Inc, San Diego, CA).

RESULTS

Inclusion and patient characteristics

From September 2015 until October 2018, 44 KTRs were recruited for this study. Twenty-two patients were converted from CNI to belatacept (**Figure 1**; flow chart). All but one patient finished one year of belatacept-treatment after conversion (one patient emigrated to another continent 4 months after random-

ization). Twenty-two patients were randomized to the control group. Of these, one withdrew consent and refused participation after randomization for CNl. One patient unfortunately developed postrenal failure due to ureterovesical stenosis of the anastomosis and presented with irreversible renal failure shortly after randomization. No patient in the belatacept-group developed rejection during the course of the study, 1 patient on TAC developed BANFF-2a rejection, for which he was successfully treated with a course of methylprednisolone (3 consecutive days of 1000 mg intravenously).

Baseline characteristics are presented in **Table 1**. No significant differences were seen initially for any of the categories, though slightly more diabetics were in the belatacept-group (8 of 21= 38% versus 3 of 20=15% for CNl).

Table 1. Baseline-characteristics. Continuous variables with normal distribution are presented as means with standard deviation, those with non-Gaussian distribution are presented by IQR (P25, median, P75). Categorical variables are presented with count-numbers with corresponding percentages. * Others used tacrolimus, ** Including ADPKD and urological conditions, *** Others used Mycophenolate Mofetil.

Baseline characteristics				Calcineurin inhibitor	Belatacept
Patients				20	21
Gender	(%male)			13 (65%)	12 (57%)
Donor age	(y, mean, SD)			59 (11)	58 (12)
CsA before Ktx*	(n,%)			4 (20%)	2 (9,5%)
Cause of ESRD	Diabetes or hypertension	(n,%)		5 (25%)	5 (23,8%)
	Structural CKD**	(n,%)		3 (15%)	7 (33,3%)
	Glomerulonephritis	(n,%)		8 (40%)	6 (28,6%)
	Other/unknown	(n,%)		4 (20%)	3 (14,3%)
Dialysis-duration before KTX	(months, IQR)			(0,5,28)	(0,7,30)
Glomerular filtration rate	(CKD-EPI (ml/min/1.73m2) mean, SD)			56 (17)	47 (14)
Living donor	(%)			11 (55%)	11 (52,4%)
Time since Ktx	(months, IQR)			(15,26,54)	(26,32,44)
Mtor-i ***				2 (10%)	3 (14,3%)
Smoking	Previous	(n,%)		10 (50%)	8 (38,1%)
	Current	(n,%)		2 (10%)	2 (9,5%)
Diabetes	(n,%)			3 (15%)	8 (38,1%)
LDL	mmol/L (mean, SD)			2,89 (1,12)	2,99 (1,03)

Table 1. Baseline-characteristics. (continued)

Baseline characteristics				
HDL		mmol/L (mean, SD)	1,51 (0,52)	1,62 (0,45)
Previous cardiovascular disease	Coronary artery disease	(n,%)	5 (25%)	5 (23,8%)
	Peripheral vascular disease	(n,%)	4 (20%)	3 (14,3%)
	Congestive heart failure	(n,%)	2 (10%)	2 (9,5%)
	Cerebrovascular disease	(n,%)	1 (5%)	1 (4,8%)
Any previous transplants		(n,%)	2 (10%)	1 (4,8%)
Number of anti-hypertensive drugs	one (n,%)		11 (55%)	10 (47,6%)
	two, (n,%)		3 (15%)	5 (23,8%)
	three or more (n,%)		3 (15%)	4 (19,1%)
Use of ACE-i or ARB			7 (35%)	10 (47,6%)
Statin user			11 (55%)	10 (47,6%)
HLA-mismatch	A (mean n of mismatches, SD)		0,95 (0,7)	1,19 (0,7)
	B (mean n of mismatches, SD)		1,2 (0,5)	1,05 (0,6)
	DR (mean n of mismatches, SD)		0,8 (0,7)	1,1 (0,5)

FACS analysis and definition of 4 monocyte subsets

Using a minimal phenotypic cocktail containing the “backbone” markers CD45, CD31, CD14 and CD16, monocyte conversion into 4 stages was monitored in both patient groups. CD31 was included to discriminate between CD14NM and natural killer cells (NKs), since both CD14NM and NKs are highly positive for CD16 and negative for CD14, but monocyte are highly positive for CD31, while NKs are intermediately positive. In the fourth subset, monocytes were as negative for CD14 as a negative reference population (NRP) and thus denoted CD14-negative monocyte (CD14NM).

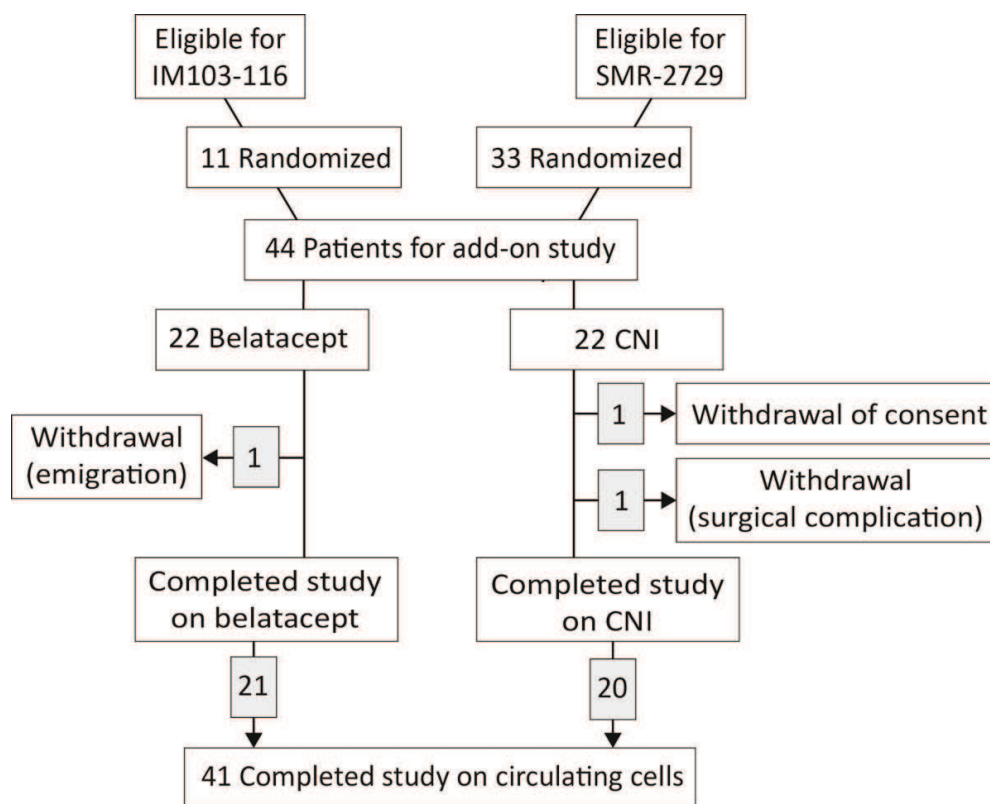


Figure 1. Flowchart of patient inclusion. Forty-four patients from two clinical trials were included, of which 41 completed the add-on study.

The FACS gating strategy for both monocytes and lymphocytes is described and illustrated in **Supplemental Figure 1**. An illustration of extended monocyte conversion into four monocyte subsets and accompanying lymphocytes is depicted in **Figure 2A and B**.

WBC and monocyte- and lymphocyte subsets after CNI-and belatacept-treatment

At baseline (BL), absolute counts for white blood cells (WBC), monocyte subsets, T- and B-lymphocytes and granulocytes were not significantly different between the CNI- and belatacept groups. After 1 year of treatment, WBC count increased in the CNI-group (Figure 3A), but not in the belatacept-group. This increase in total numbers of WBC in the CNI-group was reflected in absolute numbers of granulocytes, the highest abundant circulating cell type (Figure 3B), but not in the monocyte- or lymphocyte subsets (figure 3C-H). Interestingly, belatacept treatment decreased the numbers of CD14NM at both 6 months (Figure 3F, $P < 0.05$) and 1 year (also $p < 0.05$), while the numbers of the other monocyte subsets were not significantly changed (Figures 3C, D and E). In addition, numbers

of T-cells were significantly decreased after 1 year of belatacept treatment (Figure 3G, $p<0.01$), but not in the CNI-group. Numbers of B-cells were also lower after a year on belatacept, although the change was not significant. (Figure 3H)

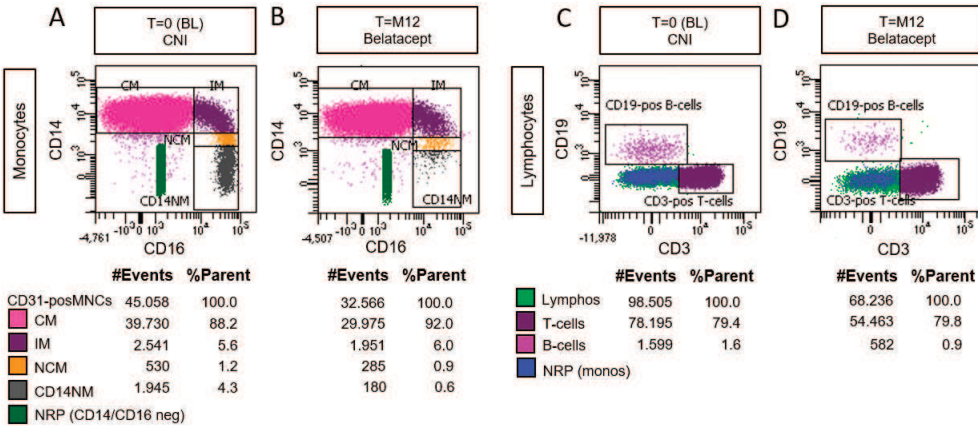


Figure 2. Illustration of impact of belatacept conversion on monocyte subset and lymphocyte levels in one patient. A) CD14-negative monocytes represents 4.3% of all monocytes at randomization, under CNI-treatment (T=0). **B)** After 1 year of belatacept treatment this decreased to 0.6%, limiting monocyte conversion to mainly 3 subsets. From the same patient, lymphocyte subsets showed a small decrease of B-cells from 1.9% of all lymphocytes at baseline to 0.9% 1 year after belatacept treatment, while the percentage of T-cells was not different (**C and D**). CNI = calcineurin inhibitor. BL = baseline. M12 = month 12. CM/IM/NCM = classical/intermediate/non-classical monocyte. NRP = Negative Reference Population.

Diabetes mellitus determines effect of belatacept on circulating leucocytes

Diabetes mellitus (DM) has been shown to affect conversion of CM towards NCM [30-34]. The finding of a significant decline in CD14NM in our study, together with our own (unpublished) previous finding of extended conversion into CD14NM in DN led us to investigate if DM affected the skewing of monocytes after conversion. In our cohort, 11 patients had DM of which 3 in the control group. We saw that at randomization, CM were slightly higher in patients with diabetes, while IM and NCM were lower. No difference was seen in CD14NM (supplemental figure 2).

When stratifying for DM, the longitudinal effects of treatment on CD14NM and T-cells as shown in Figure 3 appeared to be restricted to non-diabetic patients, since exclusion of the diabetic patients (**Figure 4**) resulted in consistent and more pronounced statistical differences. For patients without DM on belatacept, CD14NMs decreased (Figure 4B: Bela-BL versus Bela-M6, $P<0.01$; Bela-BL

versus Bela-M12, $P < 0.05$), as well as T-cells (Figure 4C: Bela-BL versus Bela-M12, $P < 0.01$) and B-cells (Figure 4D: Bela-BL versus Bela-M12, $P < 0.05$). These effects were not observed in diabetic patients. Interestingly, kidney function parameters improved for non-diabetics on belatacept (Figure 4E, creatinine: Bela-BL versus Bela-M12, $P < 0.05$ and Figure 4F, eGFR: Bela-BL versus Bela-M12, $P = 0.057$), while this was not the case for diabetic subjects.

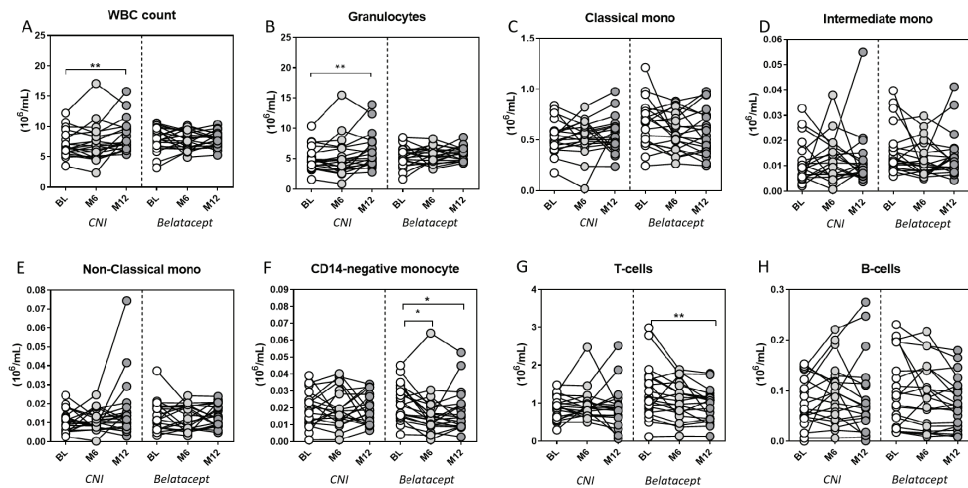


Figure 3. Quantitative differences in monocyte subset counts and lymphocyte counts upon conversion from CNI to belatacept. We observed an increase in whole blood cells (A) and (B) granulocytes in controls on CNI treatment. In the belatacept patients, CD14-negative monocytes (E) and T-cells (F) were significantly lower after conversion while the decline in B-cells (G) trended towards significance ($p = 0.0521$). Paired repeated measures 1-way ANOVA with Tukey's multiple comparisons test. * indicates significant difference, p -value < 0.05 . ** indicates $p < 0.01$. WBC: white blood cell. CNI = calcineurin inhibitor. BL = baseline. M6/M12 = month 6/12. Patients randomized to belatacept used CNI at randomization but are represented in the squares named 'belatacept'.

Association of $\text{TNF}\alpha$ with skewing of cell populations

$\text{TNF}\alpha$ is a central proinflammatory cytokine that is produced by monocytes [35]. Given the impact of belatacept on the skewing of the monocyte subpopulations, we decided to associate the results to plasma levels of $\text{TNF}\alpha$. In patients continuing CNI-treatment, $\text{TNF}\alpha$ increased significantly over time especially in the last half year (Figure 5A and 5B), while no effect was visible after belatacept treatment. In fact, the increase in plasma $\text{TNF}\alpha$ (delta $\text{TNF}\alpha$) in the last half year on CNI was significantly higher when compared to patients that switched to belatacept (Figure 5B; M12-M6 for CNI versus M12-M6 for belatacept; $P < 0.01$). These results were not dependent on diabetes status. (Figure 5C).

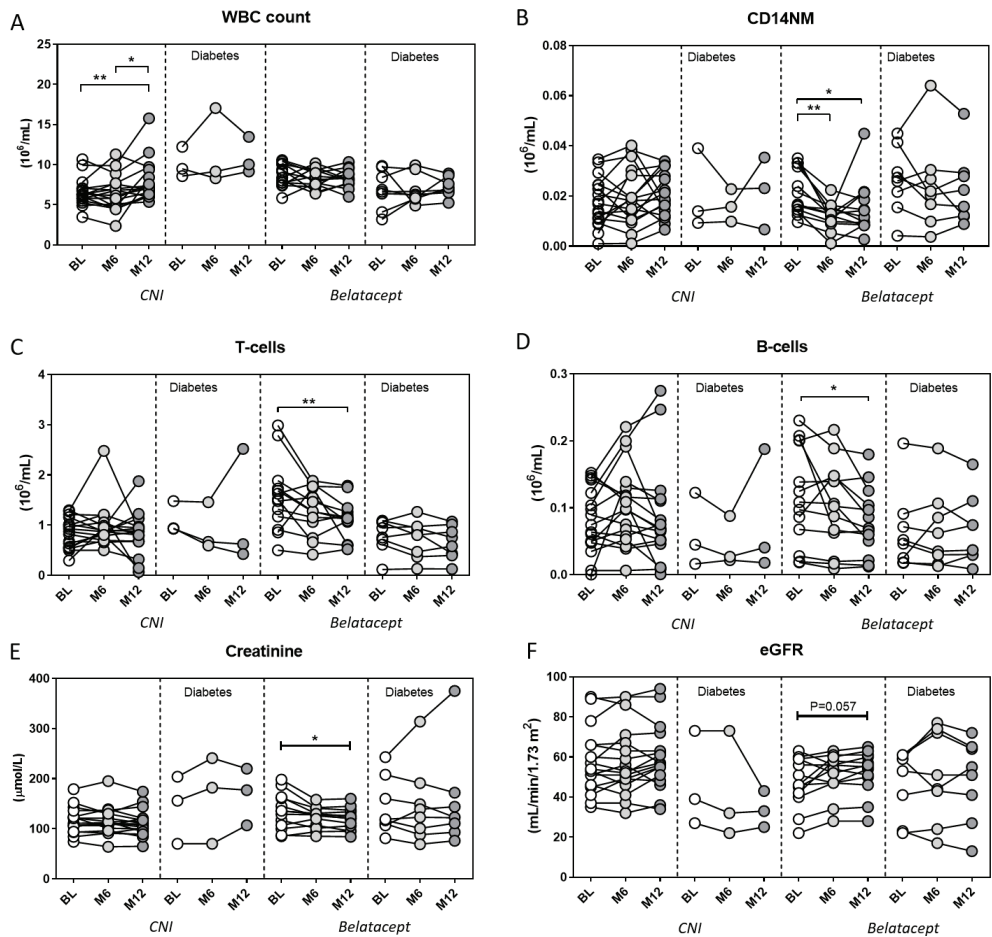


Figure 4. Effect of conversion stratified for diabetes. In the control group, a change upward is observed for total white blood cells in the control group (A). The observed changes in CD14NM (B), T-cells (C) and B-cells (D) after conversion to belatacept is more pronounced in non-diabetic patients (left squares are non-diabetics), as is the improved renal function (E) and (F). Paired repeated measures 1-way ANOVA with Tukey's multiple comparisons test. * indicates significant difference, p-value < 0,05. ** indicates p < 0,01. Patients randomized to belatacept used CNI at randomization but are represented in the squares named 'belatacept'.

To explore if the impact of treatment on the observed longitudinal changes in cell numbers were interrelated, we calculated delta values (M12 minus BL) for each individual patient and performed multivariate linear regression analysis to correlate changes in granulocytes and monocyte- and lymphocyte subsets. **Supplemental figure 3** shows correlation matrices for significance (2-way ANOVA P-values, matrices A and C) and covariance (Pearson R values; matrices B and D). Under continuous CNI regime (CNI, n=20; matrices A and B), only changes in CM versus T-cells and changes in NCM versus IM were correlated

significantly. In patients converted from CNI to belatacept however (Bela, n=21; matrices C and D), a cluster of interrelated changes for CM, IM and NCM (cluster 1) and for CD14NM, T-cells and B-cells (cluster 2) exists. Interestingly, although CD14NMs are considered to be descendants from the monocyte lineage, changes in CD14NM did not correlate with changes of its ancestors, but was rather interrelated with changes in T- and B-cells.

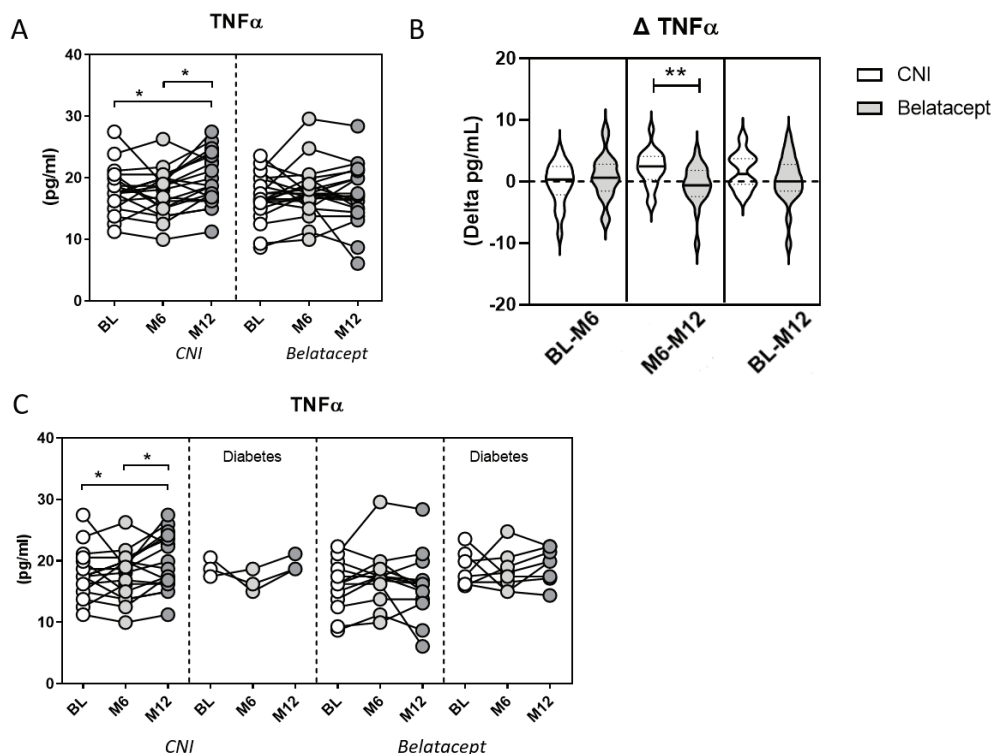


Figure 5. Conversion to belatacept leaves TNFα levels unaffected while a significant increase is seen in those on CNI. **A)** TNF-alfa concentrations at baseline (BL), 6 months and 12 months (M6/M12). **B)** the change in concentration for patients on CNI (white) and belatacept (grey). **C)** same as A) stratified for diabetes. Paired repeated measures 1-way ANOVA with Tukey's multiple comparisons test. * indicates significant difference, p-value < 0,05. ** indicates p < 0,01.

To give a more detailed insight in the direction of changes, the linear regression curves for patients remaining on CNI (n=20; graphs A, B and C) and for all patients converted to belatacept (Bela, n=21; graphs D, E and F) are shown in **Figure 6**, depicting changes in cell numbers of cluster 2. As already shown in the correlation matrices, there were no significant relations between delta values of circulating cells or TNFα after continuation on CNI-treatment (Figure 6 A-D), but they were highly significant in those converted to belatacept. With delta (Δ)

CD14NM as variable, significant correlations were found with Δ T-cells (Figure 6E) and Δ B-cells (Figure 6F). As could be expected, Δ T-cells also showed a highly significant correlation with Δ B-cells (Figure 6G). Finally, changes in CD14NM also correlated with Δ TNF α (Figure 6H). The magnitude of the variance ranges greatly, from a R^2 -value for variance of just 0,32 (R 0,569, Supplemental fig 3) for the correlation between Δ T-cells and Δ CD14NM to 0,59 (R 0,769) for Δ T-cells and Δ B-cells. When assessing the direction of changes, cell populations decreased consistently under belatacept-treatment, while such a general trend was not seen in CNI. (Figure 6 I, J, K)

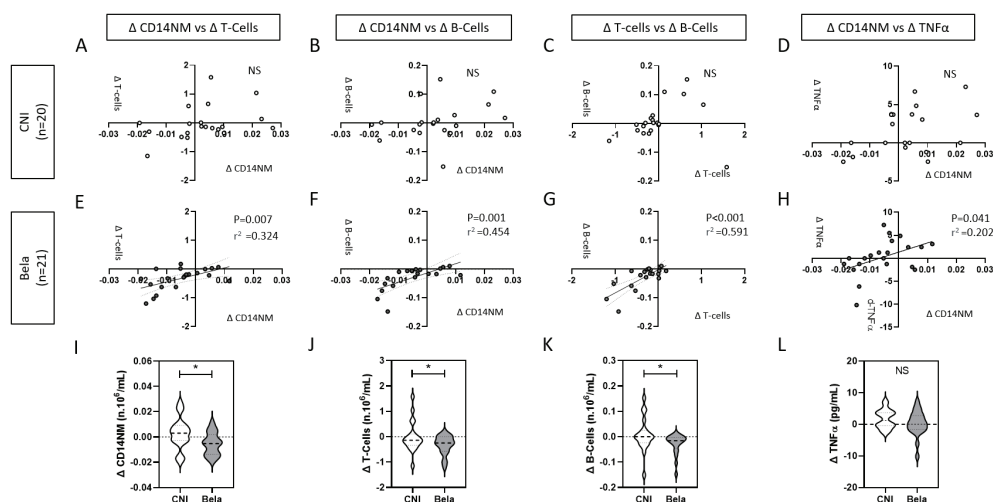


Figure 6. Simple Linear regression analysis of changes in cell populations after 1 year of treatment. R^2 indicates proportion of variance explained by the independent variable. No association between Δ CD14NM and Δ T-cells (A) and Δ B-cells (B), nor for Δ T-cells and Δ B-cells (C) or Δ CD14NM and Δ TNF- α (D) is seen in CNI-treated-patients. In Belatacept-treated-patients, significant associations are observed for these changes: Δ CD14NM with Δ T-cells (E) and B-cells (F) respectively, for Δ T-cells and Δ B-cells, and for Δ CD14NM and TNF- α (H). For CD14NM (I), T-cells (J) and B-cells (K) belatacept-treatment exerts a consistent decreasing significant effect in these populations, contrary to CNI. TNF- α levels are not significantly changed (L, also 5B). Univariate linear regression. $P < 0,05$ indicates significant difference.

CD14NM express large amounts of CD80 and CD86.

Since belatacept acts on co-stimulation through CD80/CD86, we investigated whether these markers were present on CD14NM. For that purpose we collected new blood samples from four KTR's on standard therapy consisting of tacrolimus, mycophenolate and prednisone. We compared the expression of CD80 and CD86 on CD14NM to other monocytes and to professional APC's, plasmacytoid and conventional dendritic cells (pDC's and cDC's). **Figure 7** shows the

mean fluorescent intensity (MFI) of all APC-subsets correlated to a reference population (RefPop; CD45-negative cells). It illustrates that expression of CD80 (**Figure 7A**) in these immunosuppressed conditions is very low and that IM, NCM and CD14NM exhibit expression of CD86 (**Figure 7B**) to an extent even higher than the DCs (**Figure 7D**). For comparison HLA-DR expression was also determined (**Figure 7C**). Furthermore, in peripheral blood of these KTRs, monocyte subsets outnumbered the DCs of which CD14NM showed a significant increase (**Figure 7E**; CD14NM vs pDC and cDC $P < 0.05$).

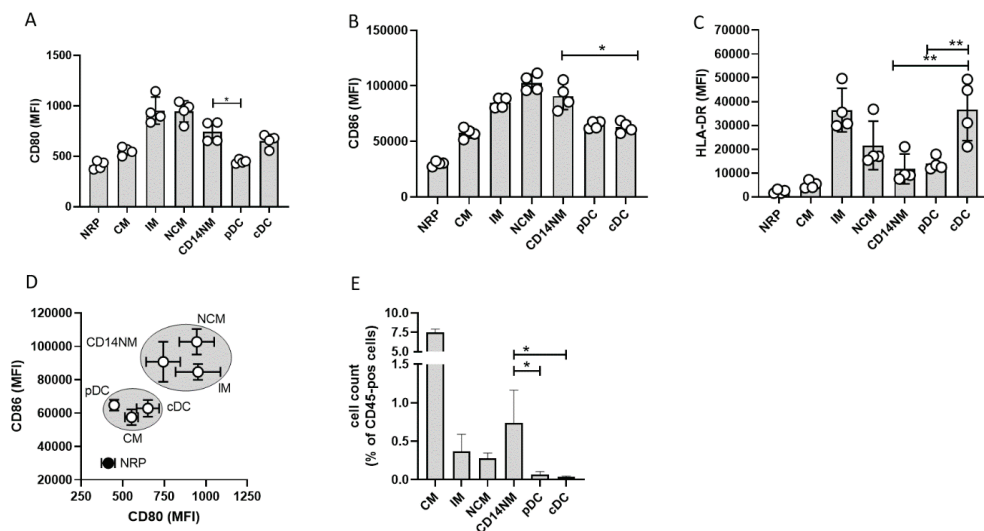


Figure 7. Mean Fluorescence Index of CD80 (**A**), CD86 (**B**), HLA-DR (**C**) on CD45-positive cells of kidney transplant recipients on tacrolimus, prednisone and MMF). Note that CD14NM presents abundant CD80/86, even more than dendritic cells (**A, B, D**). (**E**) In KTR's, monocytes outnumber dendritic cells. PDC and cDC plasmacytoid and conventional dendritic cells. Note: to enable readability, multiple significant differences between populations in these graphs are not highlighted with asterisks. * p-value < 0,05 in standard t-test for comparison of groups.

DISCUSSION

In this study, we correlated phenotypical and quantitative changes in white blood cells to conversion from 'standard CNI-based-therapy' to belatacept. While prednisone is known to influence skewing of monocytes, intake of MMF, mTOR-inhibitors and CNIs has no effect on monocyte subset numbers in kidney transplant recipients [36]. Since the intake of 'background' therapy was identical for all KTRs in our study, the observed results are attributable to the conversion of therapy.

The main result is that we found a fourth subset of monocytes, phenotypically highly positive for CD16 and CD31 and as negative for CD14 (CD14NM) as a negative reference population (CD14⁻/CD16⁺ lymphoid cells) and we consider this subset as an end-stage entity of monocyte conversion (manuscript in preparation). Interestingly, this CD14NM subset is equipped with the essential stimulatory markers for belatacept to act, namely CD80 and CD86, and decreases in abundance in patients converted to belatacept compared to patients that continued CNI-based therapy. Other observations were a significant decline in B-cells, T-cells and TNF α in belatacept, and a rise in overall white blood cells in patients continuing CNI.

Most existing literature has not reported on CD14-negative monocytes because of two reasons: firstly, in peripheral blood samples from healthy volunteers this CD14NM was hardly detectable [37]. Secondly, the definition of monocytes in most studies requires CD14 to be at least slightly (dim) positive, and thus CD14NM is excluded by default [38, 39]. Or, since CD14NM are slightly smaller than other monocytes (supplemental figure 1B) they were excluded by cell size selection in studies using FSC/SSC characteristics [40-42]. In some papers, CD14-negative monocytes were shared among the CD14-expressing/low NCM population leading to phenotypic definition of NCMs with CD14 expression varying from CD14⁻ to CD14⁺ [43-45].

However, few studies have reported a CD14-negative NCM. For instance, 6-Sulfo-Lac-Nac (SLAN) has been described as an additional identifying marker for CD14-low or negative NCM (SlanMo) [44, 46]. The CD14NM in only 1 in the 4 subjects retested to evaluate APC-potential were positive for SLAN, all but ruling out the possibility that our CD14NM was the SlanMo.

In the patients in the CNI-arm, TNF α in the circulation increased. This effect may be explained by the fact that 90% of the patient's received tacrolimus, which has been shown to increase the potential production of TNF α , IL1 β , IL-6 and IL-10 by monocytes after stimulation in comparison to CsA [47]. Furthermore, it has been shown that monocytes are the dominant TNF α producing cells in peripheral blood of KTRs [48]. In our cohort, this increase in TNF α was not seen during belatacept treatment, indicating either an anti-inflammatory effect of belatacept, or the elimination of the influence of tacrolimus. There was a linear correlation between the changes in CD14NM numbers and TNF α after 1 year of belatacept, which suggests that these cells may contribute to TNF α secretion into the circulation. Interestingly, the decline of CD14NM numbers and TNF α also coincided with a reduction in both T- and B-cells in belatacept-treated

patients but not in CNI. This observation is most convincing in non-diabetic patients, but significant for the whole cohort of belatacept-patients.

We think our findings are best explained by proposing a model in which belatacept first and foremost exerts an effect on the 'upstream' monocyte-population. In that model, the terminal differentiation of monocytes to CD14NM is influenced by the conversion to belatacept through either apoptosis of the latter, or by hampering of differentiation of NCM to CD14NM. By diminishing this cell population, less presentation of antigen to naïve T-cells sequentially causes less T-cells to proliferate and thus lower numbers of B-cells to be stimulated.

Previous studies provide some support for this hypothesis. First, Tono et al. tested the influence of abatacept, a different CTLA4-Ig-compound on human monocytes. He indeed found that cultured monocytes went into apoptosis after stimulation with enterotoxin when abatacept was added. Furthermore, the expression of CD80 and CD86 decreased on the remaining monocytes [49]. We cannot elaborate on that last finding from our results, since we only tested CD80/86 on patients 'on' tacrolimus. Monocyte conversion from CM to NCM has been shown in disease states associated with systemic inflammation, like rheumatoid arthritis and sepsis [50, 51]. Furthermore, in a cohort of patients with diabetic nephropathy (DN) we observed extended conversion of monocytes beyond the NCM stage to CD14NM (De Boer et al. manuscript in preparation).

The second proposed step of CD14NM-depletion leading to less proliferation of T-cells was prompted by the striking clustering of CD14NM, T-cells, B-cells (and $\text{TNF}\alpha$) in patients treated with belatacept, but not in those on CNI. Other monocytes have previously been recognized for their involvement in allo-recognition. For instance, increased monocyte count was associated with rejection, and with low GFR in a cohort of KTR's. [52]. Also, monocytes are capable of distinguishing between self- and non-self tissues [53] and allogeneic non-self acts as an immune adjuvant causing persistent maturation of monocytes into APCs [54]. Furthermore, depletion of monocytes blunted T cell-mediated allograft rejection [54]. Since we did not include the activation markers CD25 and CD71 in our phenotypic cocktail however, we cannot definitively proof but only speculate that the lower numbers of T-cells in belatacept patients could be explained by downregulation of the CD14NM. Not including these and other markers is a limitation to our study.

We also did not assess the exact phenotype of the B-lymphoid subsets, but the simultaneous decline in CD14NM and B-cell-numbers in belatacept-patients could also help understand why belatacept- treatment is associated with a reduced tendency for formation of donor-specific antibodies [55].

Surprisingly, KTR that were already diabetic before transplantation did not show belatacept-induced reduction of CD14NM, T- and B-cells, nor an improvement in kidney function. While CNI treatment is associated with an increased risk of developing post-transplant diabetes mellitus (PTDM) [56], apparently diabetic patients switching CNI treatment to belatacept show less of the mentioned effects on cell-populations. A possible explanation may be that monocytes of patients with DM2 have shown to express less CD86, thereby impairing the effect of belatacept on APCs [30]. However, caution against overinterpretation of this observation is warranted since it only involves a small number of patients. Small group size also limited further subgroup analysis. For example, we observed that two among three patients in the intervention-group using everolimus (EVL) were among the patients experiencing the largest 'drop' in CD14NM. However, since the total number of EVL-patients were only three in the belatacept-group and two in the CNI-group, we cannot build on that observation any further.

An interesting possibility we consider is that the CD14NM is related to the cells described in a sub-study of the BENEFIT-trial [57]. There, in blood of patients treated with belatacept, qualitative analysis showed that a subgroup of circulating CD16^{pos} leukocytes expressed indoleamine 2,3-dioxygenase 1 (IDO) after 12 months of treatment, while these cells were not CD14^{pos} monocytes, and were not observed in patients treated with CsA. From that same group, 16 patients on belatacept and 10 on CNI had repeat investigation of graft histology and peripheral blood [58]. CD16+TNF α -positive cells were found significantly less frequently in circulation, while higher frequencies of CD16+IDO+ cells were found in the graft. Besides the presence of CD16^{pos}/IDO^{pos} cells in the circulation, kidney grafts after 10 years of treatment with belatacept in the BENEFIT study contained more CD16^{pos}/IDO^{pos} cells, which points to trafficking of these cells from the circulation into the transplanted kidney. These IDO-positive cells may actually represent the fourth CD14NM subset.

In conclusion, our study indicates enhanced proliferation of monocytes into a CD14NM, and correlates this finding with conversion to belatacept in KTR's. Belatacept-treated patients show significantly less of these terminally differentiated monocytes, and this decline is associated with a comparable lowering of TNF α , B-cells and T-cells, most notably in non-diabetics. This effect was not seen in CNI-treated patients. We propose a model to explain these findings.

Further studies are needed to evaluate the exact role this cell has in trans-endothelial migration, cell-cell interaction of APC with lymphocytes, inflammation and possibly fibrosis in transplant recipients.

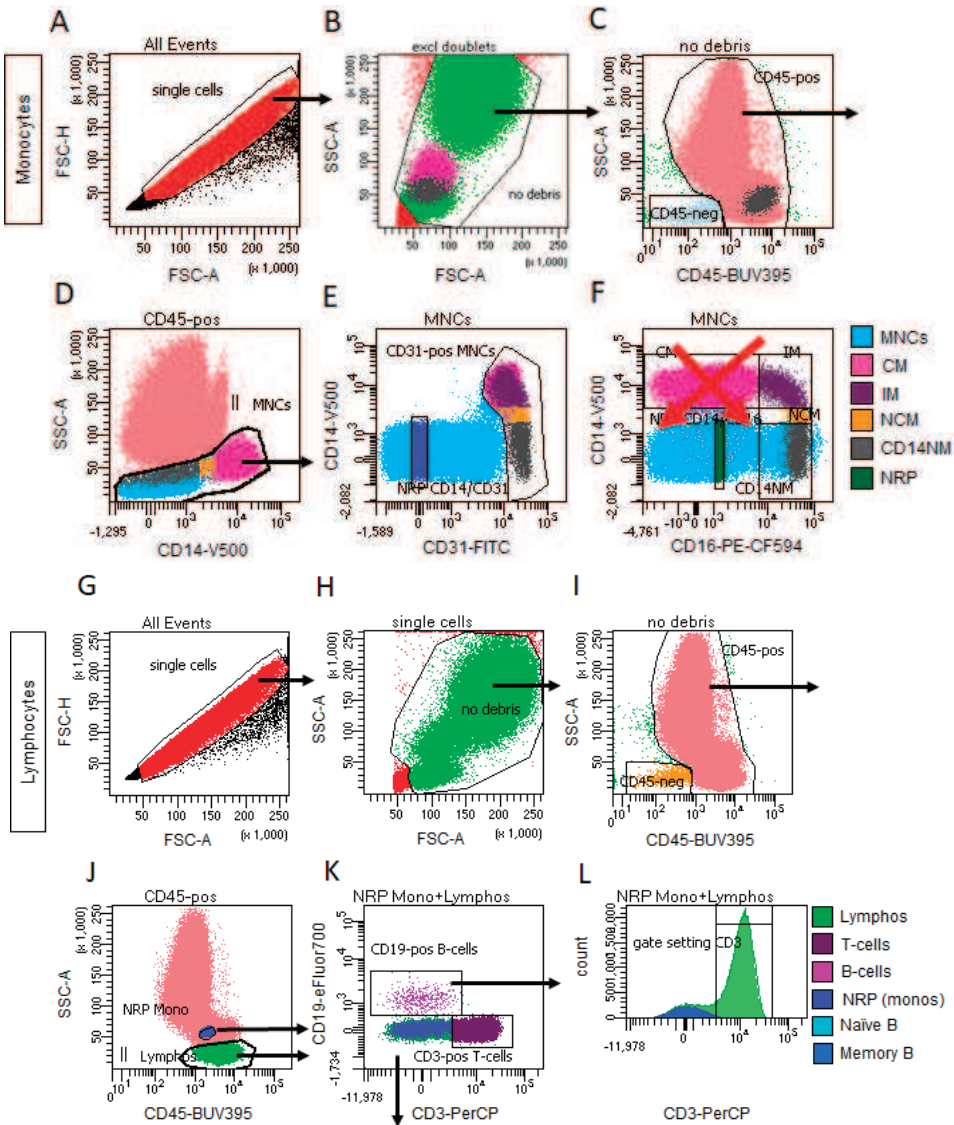
REFERENCES

1. Ong, S.C. and R.S. Gaston, *Thirty Years of Tacrolimus in Clinical Practice*. Transplantation, 2020. Publish Ahead of Print.
2. Vincenti, F., 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): p. 535-46.
3. Vanrenterghem, Y., 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): p. 976-83.
4. Rostaing, L., 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): p. 430-9.
5. Schulte, K., et al., *Late conversion from tacrolimus to a belatacept-based immunosuppression regime in kidney transplant recipients improves renal function, acid-base derangement and mineral-bone metabolism*. J Nephrol, 2017. 30(4): p. 607-615.
6. de Graav, G.N., et al., *A Randomized Controlled Clinical Trial Comparing Belatacept With Tacrolimus After De Novo Kidney Transplantation*. Transplantation, 2017. 101(10): p. 2571-2581.
7. Adams, A.B., 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): p. 2922-2936.
8. Bray, R.A., 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): p. 1783-1789.
9. Ojo, A.O., *Cardiovascular complications after renal transplantation and their prevention*. Transplantation, 2006. 82(5): p. 603-11.
10. Jardine, A.G., et al., *Prevention of cardiovascular disease in adult recipients of kidney transplants*. Lancet, 2011. 378(9800): p. 1419-27.
11. Gistera, A. and G.K. Hansson, *The immunology of atherosclerosis*. Nat Rev Nephrol, 2017. 13(6): p. 368-380.
12. Ewing, M.M., et al., *T-cell co-stimulation by CD28-CD80/86 and its negative regulator CTLA-4 strongly influence accelerated atherosclerosis development*. Int J Cardiol, 2013. 168(3): p. 1965-74.
13. Jakubczik, C.V., G.J. Randolph, and P.M. Henson, *Monocyte differentiation and antigen-presenting functions*. Nat Rev Immunol, 2017. 17(6): p. 349-362.
14. Merah-Mourah, F., et al., *Identification of Novel Human Monocyte Subsets and Evidence for Phenotypic Groups Defined by Interindividual Variations of Expression of Adhesion Molecules*. Sci Rep, 2020. 10(1): p. 4397.
15. Hopfner, F., et al., *Subgroups of monocytes predict cardiovascular events in patients with coronary heart disease. The PHAMOS trial (Prospective Halle Monocytes Study)*. Hellenic J Cardiol, 2019. 60(5): p. 311-321.
16. Roy-Chowdhury, E., et al., *Human CD16+ monocytes promote a pro-atherosclerotic endothelial cell phenotype via CX3CR1-CX3CL1 interaction*. Cardiovasc Res, 2021. 117(6): p. 1510-1522.

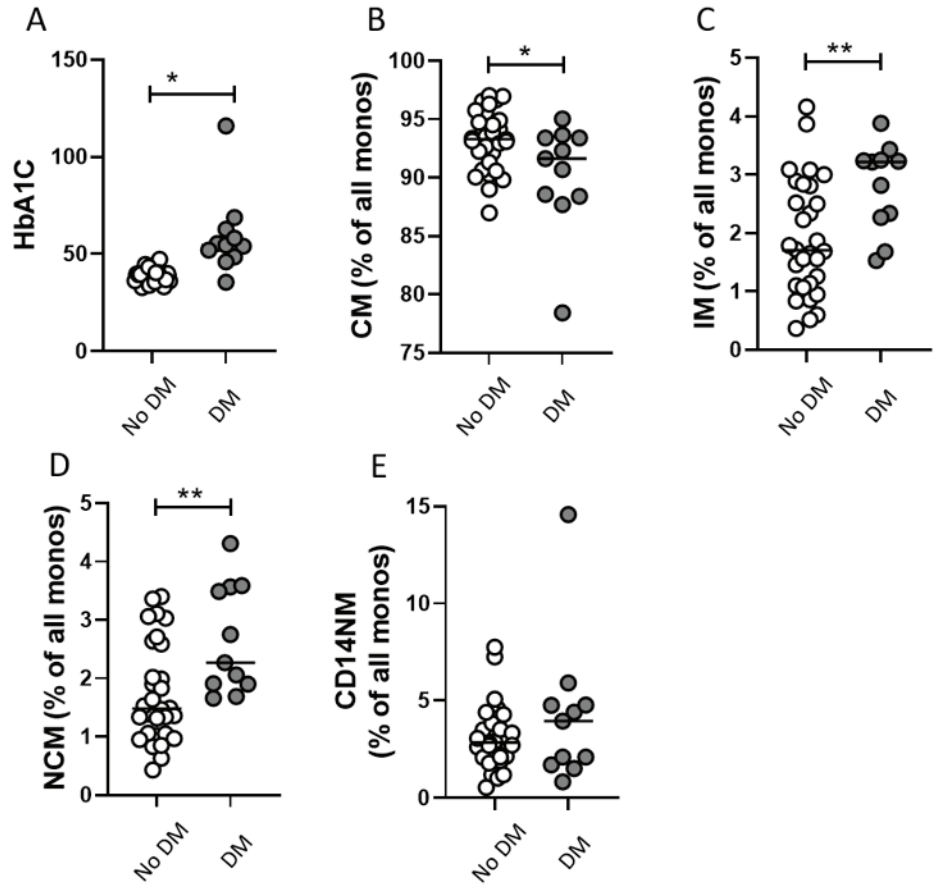
17. Borges Bonan, N., et al., *Contribution of the uremic milieu to an increased pro-inflammatory monocytic phenotype in chronic kidney disease*. Sci Rep, 2019. 9(1): p. 10236.
18. Rogacev, K.S., et al., *CD14⁺⁺CD16⁺ monocytes and cardiovascular outcome in patients with chronic kidney disease*. Eur Heart J, 2011. 32(1): p. 84-92.
19. Ulrich, C., et al., *Proinflammatory CD14⁺CD16⁺ monocytes are associated with subclinical atherosclerosis in renal transplant patients*. Am J Transplant, 2008. 8(1): p. 103-10.
20. Halloran, P.F., *Immunosuppressive drugs for kidney transplantation*. N Engl J Med, 2004. 351(26): p. 2715-29.
21. Nankivell, B.J. and S.I. Alexander, *Rejection of the kidney allograft*. N Engl J Med, 2010. 363(15): p. 1451-62.
22. Stemme, S., et al., *T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein*. Proc Natl Acad Sci U S A, 1995. 92(9): p. 3893-7.
23. Wigren, M., J. Nilsson, and D. Kolbus, *Lymphocytes in atherosclerosis*. Clin Chim Acta, 2012. 413(19-20): p. 1562-8.
24. Herrero-Beaumont, G., M.J. Martinez Calatrava, and S. Castaneda, *Abatacept mechanism of action: concordance with its clinical profile*. Reumatol Clin, 2012. 8(2): p. 78-83.
25. Esensten, J.H., et al., *CD28 Costimulation: From Mechanism to Therapy*. Immunity, 2016. 44(5): p. 973-88.
26. Rochman, Y., et al., *Functional characterization of human T cell hyporesponsiveness induced by CTLA4-Ig*. PLoS One, 2015. 10(4): p. e0122198.
27. Budde, K., et al., *Conversion from Calcineurin Inhibitor to Belatacept-based Maintenance Immunosuppression in Renal Transplant Recipients: a Randomized Phase 3b Trial*. J Am Soc Nephrol, 2021.
28. Bredewold, O.W., et al., *Cardiovascular Risk Following Conversion to Belatacept From a Calcineurin Inhibitor in Kidney Transplant Recipients: A Randomized Clinical Trial*. Kidney Med, 2023. 5(1): p. 100574.
29. Bouvy, A.P., et al., *Alemtuzumab as Antirejection Therapy: T Cell Repopulation and Cytokine Responsiveness*. Transplant Direct, 2016. 2(6): p. e83.
30. Valtierra-Alvarado, M.A., et al., *Type 2 diabetes mellitus metabolic control correlates with the phenotype of human monocytes and monocyte-derived macrophages*. J Diabetes Complications, 2020. 34(11): p. 107708.
31. Terasawa, T., et al., *Bezafibrate, a peroxisome proliferator-activated receptor α agonist, decreases circulating CD14⁽⁺⁾CD16⁽⁺⁾ monocytes in patients with type 2 diabetes*. Transl Res, 2015. 165(2): p. 336-45.
32. Ryba-Stanisławowska, M., et al., *Elevated levels of peripheral blood CD14(bright) CD16⁺ and CD14(dim) CD16⁺ monocytes may contribute to the development of retinopathy in patients with juvenile onset type 1 diabetes*. APMIS, 2015. 123(9): p. 793-9.
33. Ren, X., et al., *Increase in Peripheral Blood Intermediate Monocytes is Associated with the Development of Recent-Onset Type 1 Diabetes Mellitus in Children*. Int J Biol Sci, 2017. 13(2): p. 209-218.
34. Myśliwska, J., et al., *Expansion of CD14⁺CD16⁺ monocytes producing TNF- α in complication-free diabetes type 1 juvenile onset patients*. Cytokine, 2012. 60(1): p. 309-17.

35. Belge, K.U., et al., *The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF*. J Immunol, 2002. 168(7): p. 3536-42.
36. Rogacev, K.S., et al., *Immunosuppression and monocyte subsets*. Nephrol Dial Transplant, 2015. 30(1): p. 143-53.
37. Schmidl, C., et al., *Transcription and enhancer profiling in human monocyte subsets*. Blood, 2014. 123(17): p. e90-9.
38. Schepers, E., et al., *Transcriptome analysis in patients with chronic kidney disease on hemodialysis disclosing a key role for CD16+CX3CR1+ monocytes*. PLoS One, 2015. 10(4): p. e0121750.
39. Merino, A., et al., *Senescent CD14+CD16+ monocytes exhibit proinflammatory and proatherosclerotic activity*. J Immunol, 2011. 186(3): p. 1809-15.
40. Komura, T., et al., *CD14+ monocytes are vulnerable and functionally impaired under endoplasmic reticulum stress in patients with type 2 diabetes*. Diabetes, 2010. 59(3): p. 634-43.
41. Dopheide, J.F., et al., *Phenotypic characterisation of pro-inflammatory monocytes and dendritic cells in peripheral arterial disease*. Thromb Haemost, 2012. 108(6): p. 1198-207.
42. Tak, T., et al., *Circulatory and maturation kinetics of human monocyte subsets in vivo*. Blood, 2017. 130(12): p. 1474-1477.
43. Dutertre, C.A., et al., *Pivotal role of M-DC8+ monocytes from viremic HIV-infected patients in TNF α overproduction in response to microbial products*. Blood, 2012. 120(11): p. 2259-68.
44. Hofer, T.P., et al., *6-Sulfo LacNAc (Slan) as a Marker for Non-classical Monocytes*. Front Immunol, 2019. 10: p. 2052.
45. Zawada, A.M., et al., *SuperSAGE evidence for CD14++CD16+ monocytes as a third monocyte subset*. Blood, 2011. 118(12): p. e50-61.
46. Ahmad, F., et al., *Current Concepts on 6-sulfo LacNAc Expressing Monocytes (slanMo)*. Front Immunol, 2019. 10: p. 948.
47. Weimer, R., et al., *Switch from cyclosporine A to tacrolimus in renal transplant recipients: impact on Th1, Th2, and monokine responses*. Hum Immunol, 2000. 61(9): p. 884-97.
48. De Serres, S.A., et al., *Monocyte-secreted inflammatory cytokines are associated with transplant glomerulopathy in renal allograft recipients*. Transplantation, 2011. 91(5): p. 552-9.
49. Tono, T., et al., *Effects of CTLA4-Ig on human monocytes*. Inflamm Regen, 2017. 37: p. 24.
50. Poehlmann, H., et al., *Phenotype changes and impaired function of dendritic cell subsets in patients with sepsis: a prospective observational analysis*. Crit Care, 2009. 13(4): p. R119.
51. Rossol, M., et al., *The CD14(bright) CD16+ monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17 cell population*. Arthritis Rheum, 2012. 64(3): p. 671-7.
52. Buberçi, R. and S. Paydas, *Elevated Monocyte Count and Loss of Renal Function in Renal Transplant Patients*. Transplant Proc, 2020. 52(10): p. 3080-3084.
53. Zecher, D., et al., *An innate response to allogeneic nonself mediated by monocytes*. J Immunol, 2009. 183(12): p. 7810-6.
54. Oberbarnscheidt, M.H., et al., *Non-self recognition by monocytes initiates allograft rejection*. J Clin Invest, 2014. 124(8): p. 3579-89.

55. Vincenti, F., et al., *Belatacept and Long-Term Outcomes in Kidney Transplantation*. N Engl J Med, 2016. 374(4): p. 333-43.
56. Wen, X., et al., *Comparison of Utilization and Clinical Outcomes for Belatacept- and Tacrolimus-Based Immunosuppression in Renal Transplant Recipients*. Am J Transplant, 2016. 16(11): p. 3202-3211.
57. Furuzawa-Carballeda, J., et al., *High levels of IDO-expressing CD16+ peripheral cells, and Tregs in graft biopsies from kidney transplant recipients under belatacept treatment*. Transplant Proc, 2010. 42(9): p. 3489-96.
58. Furuzawa-Carballeda, J., 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. 197(2): p. 250-261.



Supplemental figure 1. FACS gating strategy for monocytes and lymphocytes. The FACS gating strategy for monocytes. After exclusion of doublets (A) and debris (B), mononuclear cells (MNCs) are gated (D) in preselected CD45-positive leukocytes (C). Next, by selecting CD31-positive MNCs, NKs were excluded (E) and CM, IM, NCM and CD14NM are gated based on their expression of CD16 and CD14 (F). Please note that the CD14NM population was as negative as the negative reference population (NRP CD14/CD16), located in the lymphocyte population (F) and that they were smaller in size than CMs (SSC in plot B). Next to monocyte subsets, also lymphocyte subsets were measured using a separate FACS cocktail containing antibodies directed against CD45, CD3, CD19 and CD27. After exclusion of doublets (G) and debris (H) and preselection of CD45-pos cells, lymphocytes were gated based on CD45- expression and SSC (J). Next, B-cells were selected utilizing CD19-expression and T-cells utilizing their CD3-expression (K, L).



Supplemental figure 2. All patients at baseline according to diabetes status **(A)**. Classical monocytes **(B)** are lower in diabetics while intermediate **(C)** and non-classical monocytes **(D)** are higher in diabetics. For CD14NM **(E)** no difference is seen. Standard T-test comparison for group differences, * indicates $p < 0,05$, ** indicates $p < 0,01$.

Supplemental Table 1.

All antibodies used in FACS protocols

	CD number	Fluorochrome	Clone	Firm
Mono tube	CD45	BUV395	HI10	BD Biosciences
	CD14	V500	MφP9	BD Biosciences
	CD31	FITC	WM59	BD Biosciences
	CD16	PE-CF594	3G8	BD Biosciences
Lympho tube	CD45	BUV395	HI10	BD Biosciences
	CD3	PerCP	SK7	BD Biosciences
	CD19	eFluor700	HIB19	BD Biosciences
Validation tubes	CD45	Pac.Blue	F10-98-4	Serotec
	CD14	V500	MφP9	BD Biosciences
	CD16	AF700	3G8	Biolegend
	CD31	PE	WM59	BD Biosciences
	CD3	PerCP-Cy5	HIT3A	Biolegend
	CD19	PerCP-Cy5	SJ25C1	Biolegend
	CD20	PerCP-Cy5	2H7	Biolegend
	CD56	PerCP-Cy5	5.1H11	Biolegend
	CD80	FITC	L307	BD Biosciences
	CD86	BV605	BU63	Biolegend
	HLA-DR	PE-CF594	G46-6	BD Biosciences
	CD11c	PE-Cy7	3,9	eBioscience
	CD123	BV650	6H6	Biolegend
	SLAN	APC	M-DC8	Miltenyi

Attenuation of Torque Teno Viral load over time in kidney transplantation recipients treated with calcineurin inhibitors is mitigated after conversion to belatacept

OW Bredewold, WT Moest, JW de Fijter, E Meijers, A Bruchfeld, K Skov, MHS Svensson, J Chan, L Mjornstedt, SS Sorensen, B Fellstrom, MCW Feltkamp, AJ v Zonneveld, JI Rotmans

Published in:

Journal of Medical Virology 2024 Sep;96(9):e29905

ABSTRACT

Torque Teno Virus (TTV) is a non-pathogenic anellovirus, highly prevalent in healthy populations. Variations in its viral load have been associated with states of diminished immunity, as occurs after organ transplantation. It is hypothesized that TTV-load might be used as a diagnostic tool to guide prescription and dosing of immunosuppressive drugs.

Not much is known about the effects of combined immunosuppressive drugs on TTV replication in renal transplantation. Belatacept was introduced to counter side-effects of calcineurin inhibitors (CNI). It was never widely adopted, mainly because its association with increased risk of rejection. To investigate the differential effects of a regimen based on calcineurin inhibitors versus belatacept on TTV-loads, we measured TTV-levels in 105 patients from two randomized controlled trials in kidney transplant recipients (KTRs).

We observed that time after transplantation was inversely related to TTV-levels of patients that remained on a CNI-containing regime, whereas this decline over time was diminished after conversion to belatacept. In addition, a correlation with tacrolimus-trough levels and age were found. Our study is the first report on the impact of conversion from CNI to belatacept on TTV-levels in KTR. In conclusion, the time-related decline in TTV-levels is mitigated after conversion from CNI to belatacept.

INTRODUCTION

The introduction of effective immunosuppressive drugs in kidney transplantation (KT), including calcineurin inhibitors (CNI) and induction therapy with T-lymphocyte depleting agents and interleukin-2 receptor inhibitors (IL2R-i) have lowered rejection rates from an initial 50% to 12-% in the first year on current standard regimens (1, 2). The downside of the increased efficacy is the high prevalence of opportunistic infections and malignancies. For instance, viral infection with cytomegalovirus (CMV) or BK-polyoma-virus (BKPyV) is prevalent in 19% and 10-15% of KTR's respectively, and can lead to substantial morbidity and graft loss (3-6). Furthermore, kidney transplant recipients (KTRs) are at risk for post-transplant lymphoproliferative disease (PTLD) (7). Besides the frequency, the severity of common viral infections is also enhanced in KTRs. This was recently exemplified during the COVID-19 era, where mortality and hospitalisation was consistently higher in KTRs, both prior to and after the availability of vaccines (8, 9).

Currently, the transplant physician can adjust the immunosuppressive drug used and its dose by therapeutic drug monitoring, by screening for opportunistic viral infections and antibodies to donor-HLA (donor-specific antibodies, DSA) and by performing protocol biopsies. A new development is the measuring of levels of torque teno virus (TTV) in KTR. TTV is a non-enveloped DNA-virus belonging to the anelloviridae family. It is a non-pathogenic and highly prevalent virus that is generally considered to be the major constituent of the blood virome, in healthy subjects and in transplant patients (10, 11). A recent study from our group revealed that low TTV-levels are associated with rejection in KTRs (12). Other studies showed associations of high TTV-levels with age, with mortality, and with diminished response to COVID-19-vaccination in patients with chronic kidney disease (CKD) (13-15), while a lower TTV level was associated with rejection in lung transplant recipients (16). These and other results suggest that TTV-levels are broadly reflecting the strength of the immune system. Thus, TTV-monitoring could serve as an additional tool to be used to optimize dosing of immunosuppressive medication. To that purpose, an international consortium is currently exploring TTV-based-decision-making in KTRs in an ongoing clinical trial (17).

Thus far, knowledge regarding the influence of different immunosuppressive drugs on the blood levels of TTV is scarce. Standard immunosuppression includes a CNI, mostly tacrolimus (TAC) in the vast majority of patients (18). Belatacept, a co-stimulation blocker provides physicians with an option to prevent CNI-related renal toxicity, but its use has been minimal, since several studies

show increased rates of acute rejection despite the beneficial effect on renal function and metabolic parameters (19-22).

We recently published the results of an international multi-centre trial in which KTRs treated with CNi (96% on TAC) were randomly assigned to belatacept or CNi-continuation (SMR-2729 trial) (19). Our centre also participated in the comparable, but larger trial performed by Budde et al (20). In these studies, more allograft rejections were observed in belatacept-treated patients during the first year after transplantation. To explore the potential use of TTV-load measurement in decision-making in KTR-management, we determined levels of TTV in 105 patients in these trials, hypothesizing the TTV load would decrease after conversion to belatacept, reflecting stronger immunity due to a less effective immunosuppressive effect.

MATERIALS AND METHODS

Patient selection

Samples of patients in two recent studies were used. In both SMR-2729 (Eu-draCT no. 2013-001178-20) (19) and IM103-116 (NCT01820572) (20), KTR's were randomized between conversion to belatacept and continuation of CNi in a 1:1 fashion. The background immune-suppression had been identical, consisting of either mycophenolate mofetil (MMF) or the mammalian target of rapamune inhibitor (m-TOR-i) everolimus, and low-dose prednisone. Study visits and sample collection had occurred at the same time-points, including baseline (T0) and visits after 6 and 12 months after randomization (T6, T12). Patients in the first trial had been included after a minimum of three months after transplantation, while the second trial had included patients after 6 months. All patients had stable kidney function and no proteinuria or any other clinical reason to be subjected to kidney biopsy at the time of randomization. Any patients that had experienced rejection classified as at least BANFF2a (or greater) or with cancer or any chronic viral infection had been excluded from the original trials. Beside the time after randomization, there had been small differences in both studies, most notably the primary and secondary outcome measures as well as the follow-up. The first trial had investigated an innovative tool to calculate cardiovascular risk one year after conversion, while Budde's trial had followed-up renal endpoints during two years. However, since both studies had included patients that were essentially identical from a clinical perspective, samples from all participating patients in either trial were eligible to be used for TTV-determination. Stored plasma from ninety-five patients from SMR-2729 and 10 additional pa-

tients from IM103-116 were used. All patients had provided extended informed consent through the original studies that they participated in.

TTV measurement

Plasma samples of patients collected at baseline (immediately before randomization) and after 6 and 12 months of randomization were used for this study. For measurement of TTV load, we used the TTV R-gene[®] kit manufactured by Biomerieux Clinical Diagnostics (Marcy-l'Etoile, France). Plasma was stored at -80° Celsius. After defrosting, 200 µL of plasma was collected from each sample for DNA isolation. Nucleic acids were extracted and eluted in a 100-µL volume using the MagNA Pure 96 DNA and Viral NA small volume kit (2.0) with the Pathogen Universal 200 protocol on a MagNA Pure 96 instrument (Roche, Mannheim, Germany). Ten µL of the elution volume was added to 15 µL of TTV-R-Gene amplification premix (TTV R-GENE[®] kit). Amplification was performed on the CFX96 real-time detection system (Bio-Rad, Veenendaal, The Netherlands). Samples were extracted in combination with internal control (TTV R-GENE[®] kit). All measurements were performed twice to ensure reproducibility. TTV load was measured in viral DNA copies per mL and log₁₀-transformed, as is universally done to express viral loads. The TTV DNA limit of detection (LoD) was determined by probit analysis and was set at 250 copies/ml (Log₁₀ 2.4).

Statistical analysis

One-way ANOVA was used to compare TTV-levels between belatacept-patients and CNI-patients after randomization, and to compare subgroups based on age, renal function, tacrolimus-trough-level, dialysis vintage and time after transplantation. Standard student's T-test was performed to compare baseline values and TTV-load values between different sexes and diabetes-status. Paired sample-testing was performed to detect statistical difference in means of groups between two time-points after transplantation. Regression analysis was performed to determine association between TTV-levels at the end of study with various independent variables. Besides sex and age, we chose other potentially confounding parameters for their possible influence on strength of the immune system and hence their possible effect on TTV-replication. As a proxy for renal function the CKD-EPI-2021 formula was used. As TTV-load is assumed to follow a Gaussian distribution, we had to correct for samples of which the TTV-level fell between 0 (zero) and the lower limit of detection (LOD). To correct for that underestimation, values of 0 were converted to Log LoD/ $\sqrt{2}$ (1.70) (23). The analyses were performed using SPSS version 29 and Graphpad Prism, version 9.3.1.

RESULTS

Patients and sample collection

Plasma samples representing three time points (T0: randomization, T6: 6 months after randomization and T12: 1 year after randomization) were collected of 105 patients. Samples of 39 patients from the Netherlands, 31 patients from Sweden, 20 patients from Denmark and 15 from Norway were collected. Of the 39 patients from the Netherlands, 29 were from the SMR-2729-study and 10 patients were from the IM103-106-study. Patients were predominantly male. Fifty-five patients (54 on TAC, 1 on Cyclosporine A [CsA]) were converted to belatacept, while 50 patients continued using CNI (47 on TAC, 3 on CsA). All patients used 5 mg of prednisone and MMF, except four patients that used Everolimus (two in both groups). Patients with diabetes were overrepresented in the belatacept-group (13 vs 8). Renal function at baseline was significantly worse in patients converting to belatacept (49.9 ml/min/1.73m²) compared to patients in which CNI was continued (58.4 ml/min/1.73m², $p = 0.006$). Baseline patient characteristics are shown in **table 1**.

Table 1.

Baseline Characteristics	CNI (50)		Belatacept (55)*	
Sex (female), count, %	11	22	16	29.1
Age (y), (mean, range)	57.5	23-82	56.8	28-81
Time from Tx (from tx to baseline) (months), mean, range	26.6	3-60	27.7	4-60
Dialysis vintage (months), (median, P25-P75)	11	0-23	10	0-25
GFR (ml/m/1.73m ²), (SD)	61.4	18.3	52.4	14.2
Tacrolimus-trough-level (ng/mL, SD**)	5.7	1.7	5.7	1.8
Diabetes (count, %)	8	16	13	23.6

Table 1. Baseline-characteristics. Patients converted to belatacept had significantly lower GFR compared to control group. * At baseline, all patients used CNI. ** For those on tacrolimus. CsA = 1 in belatacept, 3 in CNI. Tx = transplantation

TTV-levels decline over time after randomization, but this change is reduced in patients converted to belatacept

TTV-loads were measured at three time points in all patients (**Figure 1A**). The average TTV-level at T0 is 4.23 log, with patients ranging from a level of 1.7 log (limit of detection) to almost 9 log. The average TTV-log then declined to 3.81 and further to 3.76. One-way ANOVA was performed, showing statistical significance for the decline between T0 and T12 ($p = 0.04$).

Next, we evaluated whether conversion to belatacept influenced this decline. **Figure 1B** shows TTV-levels at the same three time-points, for CNI and belatacept-converted patients respectively. The change in TTV-log in the CNI group proves to be the largest, with an average decline between T0 and T12 of 4.15 log to 3.53 log ($p = 0.03$), while for those randomized to belatacept it is only decreased from 4.30 log to 3.96 log ($p = 0.21$). It is of note to realize that before randomization, all patients were still on CNI at T0. In both groups, there was a decline in the first interval after randomization, from 4.15 log to 3.77 log in the CNI-group, and from 4.30 log to 3.84 log in the belatacept-group. In the second interval, only the CNI-group patients declined further (from 3.77 log to 3.53 log), while such a further decline was not observed and even reversed in belatacept-patients (3.84 log to 3.96 log). The difference between the average decline in TTV-log between both study groups was itself not significant ($p = 0.33$).

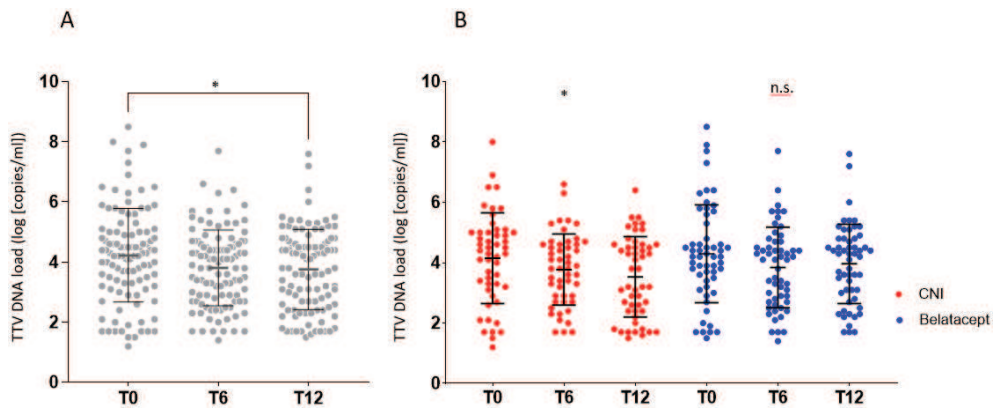


Figure 1.

A. Scatter-plots of measurements at three time points, with error bars for mean with SD. ANOVA shows a significant decline of the average TTV-level over time. **B.** Continuation of CNI shows sustained lowering of TTV-levels, not seen after conversion to belatacept. * $P < 0.05$, n.s. = non-significant.

Baseline TTV-levels associate with time after transplantation in both groups

Next, we considered the influence of time after transplantation on TTV-levels at baseline (T0). We ranked the patients according to time after transplantation and calculated the P25, P50 (median) and P75, being 13 months, 22 months and 44 months after transplantation, respectively. **Figure 2A** shows the TTV-levels at baseline of those randomized before and after the median time after transplantation. The average TTV-load of the earlier period is 4.67 log, while those in the later period had an average TTV-level of 3.79 log ($p = 0.003$). **Figure 2B** shows the same comparisons for the earliest quartile (p25) vs the latest (P75),

also at baseline: 4.82 log vs 3.45 log ($p = 0.004$). In **figure 2C**, we depicted the average TTV-log at baseline and after randomization at T6 and T12. In patients converted early and randomized to continue using CNI, we saw that the average TTV log declined from 4.68 log to 3.68 log after 12 months ($p = 0.009$), while the equivalent change for patients that were randomized to belatacept was 4.66 log to 4.42 log ($p = 0.51$). For patients converted late, we saw non-significant changes in TTV-Log for both CNI (3.62 log to 3.38 log, $p = 0.54$) and belatacept (3.94 log to 3.52 log, $p = 0.24$).

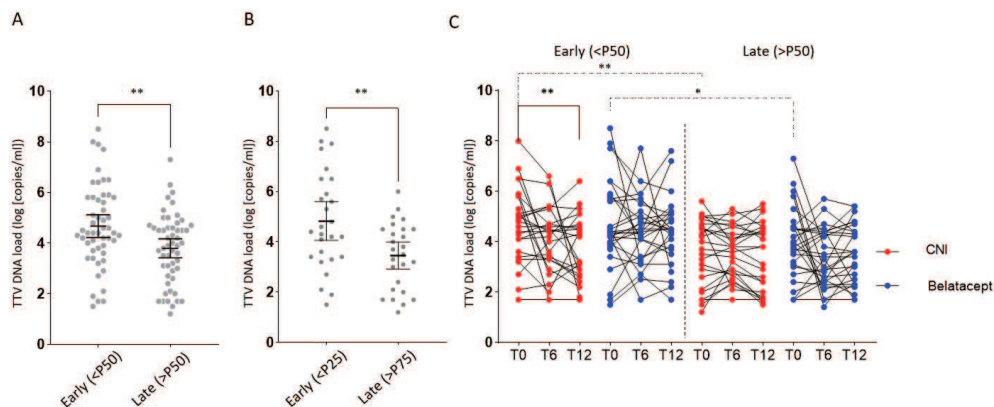


Figure 2.

A, B. Standard T-tests show significantly higher mean TTV-levels for those converted early after transplantation. **C.** Significantly higher TTV-levels at baseline compared to T12 for both study groups, but TTV declines after randomization only in control group. One way ANOVA, * $P < 0.05$, ** $P < 0.01$.

TTV-decline is associated with tacrolimus-trough-level and age in the control group, but not with renal function or dialysis vintage, and not in those converted to Belatacept.

Next, we investigated whether other variables such as age, trough level of tacrolimus, renal function and dialysis vintage correlate with TTV-log. We ranked all patients and determined P25, median and P75 of the above-mentioned parameters. The results are illustrated in **figures 3 and 4**. **Figure 3A** shows a comparison of patients of which trough levels at baseline were under (Low TAC) or above (High TAC) the median trough level (5.4 ng/mL). A standard T-test shows that there is a slight non-significant difference in TTV-level. However, the patients on a high TAC-trough-level in the CNI-group had their TTV-levels regress, while those that converted to belatacept did not. (**Figure 4A**, right panel. CNI-group from 4.43 log to 3.38 log, $p = 0.01$; Belatacept-group from 4.28 log to 4.14 log, $p = 0.69$). The equivalent non-significant changes in those with low TAC-levels are seen in the left panel (CNI-group 3.97 log to 3.73 log; Belatacept-group 4.21 log to 3.79 log).

The median age of patients at randomization was 57 years old. To assess the impact of age, we analysed TTV-levels of those older and younger than the median. For those in the older age group, the baseline TTV-level is roughly the same as for younger patients (**figure 3B**). As in patients with low tacrolimus-trough-level, the left panel in **figure 4B** shows that younger patients have a non-significant change in TTV-level, whether they were converted to belatacept or continue using CNI. However, the decline in TTV-log was significant in older patients in the CNI-group, but not for those converted to belatacept (CNI: 4.14 log to 3.17 log, $p = 0.01$. Belatacept: 4.33 log to 3.91 log, $p = 0.27$) (right panel in **figure 4B**).

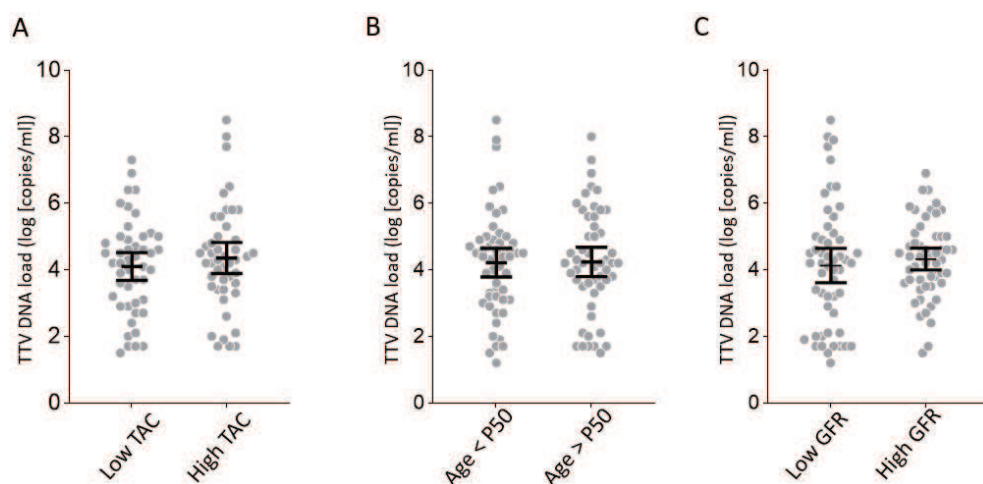


Figure 3.

A. Standard t-tests show no difference in TTV-log between those under or over the median tacrolimus-trough-level, or **(B)** age, or **(C)** GFR.

Figure 3C shows that those with an eGFR above the median eGFR (52 ml/min/1.73m²) have a slightly higher TTV-level than those with lower eGFR, though the difference is not significant. In **4C**, no difference was observed for the effects of conversion versus continuation of CNI in either those with higher or lower eGFR. Neither effect was seen in comparison of those with eGFR under P25 with those with an eGFR above P75 (data not shown).

Furthermore, we looked at dialysis vintage (DV). Thirteen patients in the CNI-group and 15 patients in the belatacept-group were transplanted pre-emptively. The median dialysis exposure for all patients was 11 months. The fourth inter-quartile range for dialysis vintage (P75) was 24 months and longer. **Figure 5A** and **5B** show the TTV-levels at baseline for those with either short DV (< median) vs long DV (>median), and those transplanted pre-emptively versus those with DV above the P75. None of those comparisons show any significant difference

to the comparator. **Figure 6A** and **6B** show the TTV-levels over time after randomization. The course after conversion shows no discrepancy with that of the CNI-group.

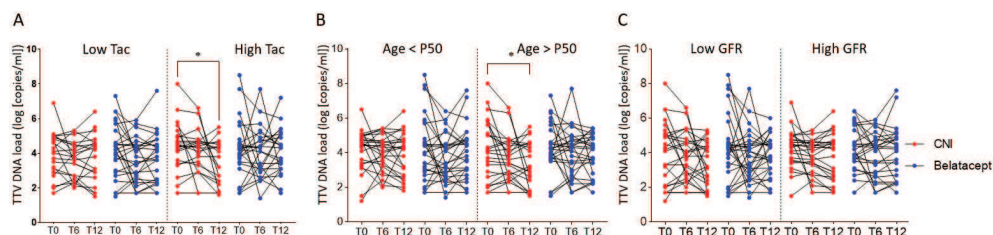


Figure 4.

A. One-way ANOVA shows that the 'natural' decline in TTV-levels as seen in control group is mitigated by conversion to belatacept in those with higher tacrolimus trough levels, and in **(B)** older patients. **C.** No influence of conversion is seen in those with higher or lower GFR. * $P < 0,05$

Lastly, we performed multiple regression analysis to predict change in TTV-load, from age, TAC-trough-level, use of belatacept, time after transplantation and initial TTV load. The model was statistically significant ($p < 0.01$) but none other than BL-load contributed significantly to the model (Confidence interval running through zero for all other coefficients 'B' in **supplemental table 1**).

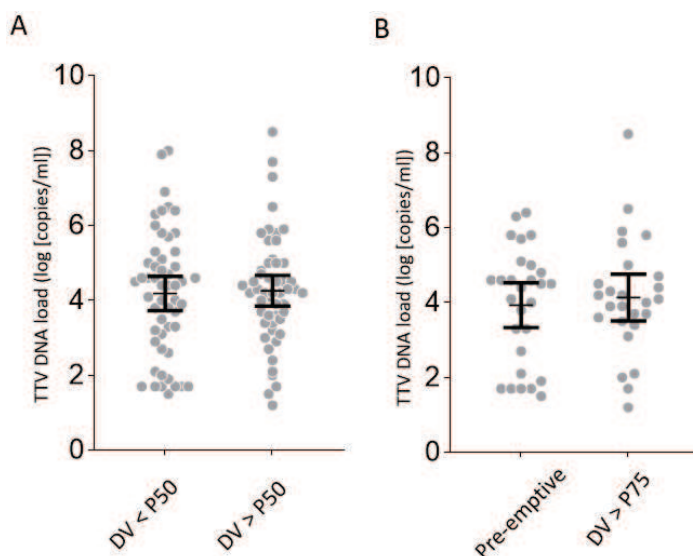


Figure 5.

Dialysis Vintage (DV). **A.** T-tests show no difference in TTV-level between those with DV greater or smaller than the median, nor in those **(B)** with very long DV compared to those that were transplanted pre-emptively.

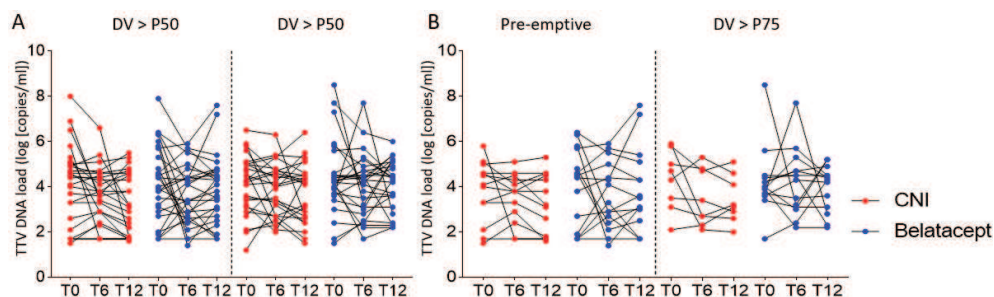


Figure 6

A) Change in TTV-levels is equal among patients with shorter, or longer **(B)** dialysis-longevity.

Conversion to belatacept and risk of rejection

Four participants in this study experienced an episode of biopsy proven acute rejection (BPAP) of their kidney allograft during the course of the study. Three of these patients were in the belatacept-group. They were randomized after 4, 9 and 12 months after transplantation. One patient was in the CNI-group, randomized 7 months after transplantation. All patients were successfully treated with pulse-courses of steroids. Unfortunately, no data were collected on the timing of BPAP or the steroid course relative to the measurement of TTV-levels.

Supplemental figure 1 shows the TTV-levels of those with BPAP.

DISCUSSION

In this study, we measured TTV levels to explore its potential use as a tool to facilitate prescribing immunosuppressive drugs in KTR. Our paper is the first report on TTV-levels in patients that were randomized from calcineurin inhibition to belatacept. It offers a straight comparison between a CNI-based regimen (mostly on TAC) and belatacept-based therapy of this evolving biomarker of immunity.

Regular course of TTV-levels after transplantation

Before discussing our results, it is imperative to consider that previous literature shows that the immediate TTV-response after induction-therapy is in fact a drop to levels below those before transplantation. This is true for patients treated with both T-cell-depleting agents as with IL2R-i. After thirty days, TTV-levels rise to pre-transplant levels (24), and subsequently increase before reaching their peak after more than two months after transplantation (25). The dynamic of TTV-levels in the very early phase is explained by the assumption that lymphocytes are the most important reservoir for the virus, and the fall and

rise in TTV-levels are paralleled by total lymphocyte count (24). Only after this early period consideration of the use of TTV-levels as a marker of immunity is warranted.

We have measured an average decline of TTV-levels in our patients from 4.23 log to 3.76 log. In comparison to other studies, the absolute values seem quite low. Other studies in transplantation have focused on the capabilities of TTV to predict infection and/or rejection. These studies compared pre-transplant-levels with TTV-levels after transplantation. For example, Solis et al. showed that TTV rose by 2.0 log three months after kidney transplantation (26). The average TTV-level in plasma (66 patients) at that time was around 6.6 log. After 24 months, it was still above 4.5 log. Van Rijn et al. tested a much larger cohort, in which the highest mean TTV-level was 7.4 log after three months after transplantation (12). An average of 5.2 log was measured at the end of their follow-up of 12 months. Of note, both these studies used the same lab-test as we did here (TTV R-gene[®] kit manufactured by Biomerieux Clinical Diagnostics (Marcy-l'Etoile, France)). In our study, only 11 patients were randomized within 6 months after transplantation. The average TTV-level of these patients was 6.1 log at baseline, which is comparable to the other studies. The large difference in the rest of the cohort is most likely attributable to the inclusion of more patients long after transplantation, and indicates that the 'down-trend' of TTV-levels continues for a much longer time after transplantation, as reported by Roberto et al. (27).

Conversion to belatacept counteracts the fall of TTV-levels

The main finding of our study is that the fall of TTV-levels observed in the CNI-group over time is not seen in patients converted to belatacept. While TTV load went down at every interval in the CNI-group, reflecting the dynamics of TTV in the aforementioned studies, it went slightly up in the second interval in the belatacept-group, while the change in the first interval is already less pronounced. Our results seem comparable to those in a recent cohort-study by Cabezas et al.; it showed TTV-load after conversion from CNI to Belatacept in a cohort of KTR, after roughly 4 years after transplantation (28). In that study, despite the rise 12 months after conversion being insignificant, all TTV-levels after conversion (4.4 Log, 4.0 Log, 4.2 Log after 3, 6 and 12 months) were higher than those at baseline (3.8 Log). The uptrend that we report here in randomized patients is contrary to our expectation that belatacept, as a possibly less potent immunosuppressant, would indirectly suppress TTV-replication. Because of the randomization, the difference is most likely to pertain to the conversion itself. While it could also be due to an indirect effect of the conversion, like rejection, the total incidence of BPAR is too low to explain the mitigation.

Although belatacept has long been associated with viral disease, not much is known about the exact mechanism by which belatacept suppresses anti-viral host responses. In 19 patients converted from CNI to belatacept because of CNI-intolerance, antiviral response was measured by analysis of EBV- and CMV-specific cytokine-secreting T-cells. No difference with the CNI-group was found (29). In another experiment, Kühne et al. stimulated PBMCs from healthy donors and KTRs with CMV, EBV and influenza (CEF)-derived peptides and measured the CD8⁺-T-cell response. Production of neither IFN- γ , interleukin (IL)-2 or IL-10 were influenced by belatacept when compared to healthy donors (30). In contrast, CNI did suppress production of these cytokines. Both observations do not explain the increased burden due to viral disease recognized in patient care. For instance, enhanced risk of post-transplant lymphoma (PTLD) in patients seronegative for the Epstein Barr virus (EBV) was noted, leading to a warning by the manufacturer to use belatacept in EBV-positive patients only (31). Severity of BK-virus-nephropathy and CMV-disease was higher in belatacept-treated KTRs than in those treated with sirolimus (32). It is possible that CD28-positivity of T-cells plays a role in the anti-viral response of belatacept. Older patients gradually lose CD28-positive CD8⁺-T-cells (33). The result is a CD8⁺-T-cell-repertoire that is relatively CMV-competent, and less responsive to belatacept-therapy (also proposed as a partial explanation for the enhanced risk of rejection due to belatacept (34, 35)). To our knowledge, no studies have specifically investigated the antiviral immune response against TTV.

Data on co-stimulation blockers and TTV-levels in other settings are scarce. Martin-Lopez et al. investigated TTV-levels in patients with rheumatoid arthritis (36). They showed that patients on abatacept, a subcutaneous variant inhibitor of the CD80/86-CD28-costimulatory pathway have higher TTV loads than patients on methotrexate and leflunomide. Another report compared the TTV-response after various immunosuppressive interventions to TTV-level at baseline, and found the effect of abatacept to be significantly greater compared to tocilizumab and infliximab, but much less than that of rituximab, a B-cell depleting agent (37).

Other findings on TTV-levels after conversion

Another interesting finding in our study was the difference in effect of conversion on TTV-levels between those with higher and lower TAC-trough levels (**figure 4A, D**). The finding that the fall of TTV levels in the control group is only seen in those with higher trough-levels and not in others could suggest that the induced change in strength of immunity is larger in those on high trough-levels. Unfortunately, we did not collect trough-levels at later intervals. But, assuming that tacrolimus-dosage is generally reduced longer after transplantation, this

finding could mean that tacrolimus-trough-levels influence TTV-replication. This hypothesis is corroborated by a recent report by Regele et al., which shows that a reduction in TTV-levels is seen approximately 60 days after a reduction of tacrolimus (38).

We also found that TTV-levels in the CNI-group mitigated over time in older patients, but not in the young, and not in those converted to belatacept. This indicates a differential effect of belatacept on older patients than younger, possibly related to T-cell-variability induced by belatacept. We were not able to find a difference between older and younger patients in our study at baseline, contrary to an earlier study that did find TTV-levels to be higher in the elderly (39).

Notable difference in GFR

Baseline eGFR was lower in belatacept-converted patients than in the CNI-group (table 1). Block-randomization, as used in the SMR-2729-study (19), is associated with enhanced predictability in un-blinded studies (40). It is hard to imagine that this explanation caused the quite large difference we observed. However, since all our patients were included from two randomized studies we have not been able to provide any satisfactory other reason. Hemodialysis (HD)-patients in India and Italy were tested for TTV-DNA-presence and compared to healthy blood donors (41, 42). The results showed higher prevalence in HD patients (83% resp 41.7%) compared to controls (43% resp 10.7%) Conversely, in a small cohort of peritoneal dialysis patients from Turkey, no difference in TTV-levels were seen compared to healthy controls (43). In 136 liver transplant recipients, no correlation was seen between renal function and TTV-presence (44). Furthermore, our own analysis revealed no influence of dialysis vintage on TTV. On this basis we think that the small, but significant difference in eGFR in our study is unlikely to have had any impact on our results.

Limitations to our study are the slight variation in background immunosuppressive regimens of patients in our study, and the lack of specific data around episodes of rejection. The CNI used was mostly TAC, but 4 patients used CsA. Four patients used m-TOR-i instead of MMF. M-TOR-i use is associated with enhanced anti-viral immunity against BK compared to MMF (45) and CNI (46). Because of that, we would have had to make separate analyses if the m-TOR-i-cohort had been larger. However, since our main interest was comparison of CNI to belatacept we decided to include samples of all randomized patients in this study.

In conclusion, this analysis adds data on belatacept to the body of literature concerning the use of TTV as a biomarker of immunocompetence. Contrary to

what we had expected, conversion to belatacept did not result in a decrease of TTV-levels over time compared to CNi. This could point to an enhanced immunosuppressive effect of belatacept over CNi. However, larger studies, with more homogeneity in background immunosuppressive therapy and detailed follow-up of rejections is needed to fully corroborate that effect. Also, although we think the difference in GFR would have had little effect on TTV levels, most belatacept-studies show improved renal function, and it would be interesting to see if our findings can be confirmed in other randomized patients, either retrospectively or in a new trial. We affirmed earlier observations that found an important decline in TTV-levels over time after transplantation. In further studies on TTV as a biomarker of immunity after transplantation, it is essential that the effect of time after transplantation on TTV-levels is appreciated in the design.

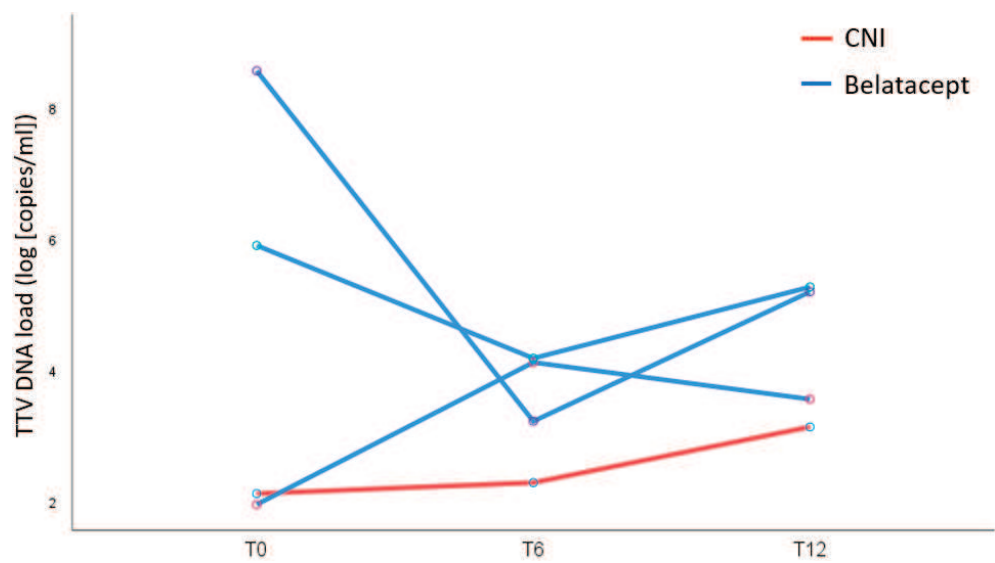
REFERENCES

1. Baron PW, Ojogho ON, Yorgin P, Sahney S, Cutler D, Ben-Youssef R, et al. Comparison of outcomes with low-dose anti-thymocyte globulin, basiliximab or no induction therapy in pediatric kidney transplant recipients: a retrospective study. *Pediatr Transplant*. 2008;12(1):32-9.
2. Ekberg H, Tedesco-Silva H, Demirbas A, Vitko S, Nashan B, Gürkan A, et al. Reduced exposure to calcineurin inhibitors in renal transplantation. *N Engl J Med*. 2007;357(25):2562-75.
3. Hellemans R, Abramowicz D. Cytomegalovirus after kidney transplantation in 2020: moving towards personalized prevention. *Nephrology Dialysis Transplantation*. 2020;37(5):810-6.
4. Hirsch HH, Knowles W, Dickenmann M, Passweg J, Klimkait T, Mihatsch MJ, et al. Prospective study of polyomavirus type BK replication and nephropathy in renal-transplant recipients. *N Engl J Med*. 2002;347(7):488-96.
5. Manzano Sánchez D, Jimeno García L, Manzano Sánchez D, López Jiménez I, Saura Luján IM, González Soriano MJ, et al. Renal Function Impairment in Kidney Transplantation: Importance of Early BK Virus Detection. *Transplant Proc*. 2019;51(2):350-2.
6. Tang Y, Guo J, Li J, Zhou J, Mao X, Qiu T. Risk factors for cytomegalovirus infection and disease after kidney transplantation: A meta-analysis. *Transplant Immunology*. 2022;74:101677.
7. Opelz G, Naujokat C, Daniel V, Terness P, Dohler B. Disassociation between risk of graft loss and risk of non-Hodgkin lymphoma with induction agents in renal transplant recipients. *Transplantation*. 2006;81(9):1227-33.
8. Nimmo A, Gardiner D, Ushiro-Lumb I, Ravanan R, Forsythe JLR. The Global Impact of COVID-19 on Solid Organ Transplantation: Two Years Into a Pandemic. *Transplantation*. 2022;106(7):1312-29.
9. Barnes E, Goodyear CS, Willicombe M, Gaskell C, Siebert S, T IdS, et al. SARS-CoV-2-specific immune responses and clinical outcomes after COVID-19 vaccination in patients with immune-suppressive disease. *Nat Med*. 2023;29(7):1760-74.
10. Vasilyev EV, Trofimov DY, Tonevitsky AG, Ilinsky VV, Korostin DO, Rebrikov DV. Torque Teno Virus (TTV) distribution in healthy Russian population. *Virology*. 2009;6:134.
11. van Rijn AL, Roos R, Dekker FW, Rotmans JI, Feltkamp M. Torque teno virus load as marker of rejection and infection in solid organ transplantation - A systematic review and meta-analysis. *Reviews in medical virology*. 2023;33(1):e2393.
12. van Rijn AL, Wunderink HF, Sidorov IA, de Brouwer CS, Kroes AC, Putter H, et al. Torque teno virus loads after kidney transplantation predict allograft rejection but not viral infection. *J Clin Virol*. 2021;140:104871.
13. Brassard J, Gagne MJ, Leblanc D, Poitras E, Houde A, Boras VF, et al. Association of age and gender with Torque teno virus detection in stools from diarrheic and non-diarrheic people. *J Clin Virol*. 2015;72:55-9.
14. Giacconi R, Maggi F, Macera L, Pistello M, Provinciali M, Gianecchini S, et al. Torquetenovirus (TTV) load is associated with mortality in Italian elderly subjects. *Experimental gerontology*. 2018;112:103-11.
15. Graninger M, Stumpf J, Bond G, Görzer I, Springer DN, Kessel F, et al. Prediction of humoral and cellular immune response to COVID-19 mRNA vaccination by

- TTV load in kidney transplant recipients and hemodialysis patients. *J Clin Virol.* 2023;162:105428.
16. Frye BC, Bierbaum S, Falcone V, Köhler TC, Gasplmayr M, Hettich I, et al. Kinetics of Torque Teno Virus-DNA Plasma Load Predict Rejection in Lung Transplant Recipients. *Transplantation.* 2019;103(4):815-22.
17. Hauptenthal F, Rahn J, Maggi F, Gelas F, Bourgeois P, Hugo C, et al. A multicentre, patient- and assessor-blinded, non-inferiority, randomised and controlled phase II trial to compare standard and torque teno virus-guided immunosuppression in kidney transplant recipients in the first year after transplantation: TTVguideIT. *Trials.* 2023;24(1):213.
18. Kasiske BL, Zeier MG, Chapman JR, Craig JC, Ekberg H, Garvey CA, et al. KDIGO clinical practice guideline for the care of kidney transplant recipients: a summary. *Kidney Int.* 2010;77(4):299-311.
19. Bredewold OW, Chan J, Svensson M, Bruchfeld A, de Fijter JW, Furuland H, et al. Cardiovascular Risk Following Conversion to Belatacept From a Calcineurin Inhibitor in Kidney Transplant Recipients: A Randomized Clinical Trial. *Kidney Med.* 2023;5(1):100574.
20. Budde K, Prashar R, Haller H, Rial M, Kamar N, Agarwal A, et al. Conversion from Calcineurin Inhibitor to Belatacept-based Maintenance Immunosuppression in Renal Transplant Recipients: a Randomized Phase 3b Trial. *J Am Soc Nephrol.* 2021.
21. Larsen CP, Grinyo J, Medina-Pestana J, Vanrenterghem Y, Vincenti F, Breshahan B, et al. Belatacept-based regimens versus a cyclosporine A-based regimen in kidney transplant recipients: 2-year results from the BENEFIT and BENEFIT-EXT studies. *Transplantation.* 2010;90(12):1528-35.
22. Ippoliti G, D'Armini AM, Lucioni M, Marjeh M, Viganò M. Introduction to the use of belatacept: a fusion protein for the prevention of posttransplant kidney rejection. *Biologics.* 2012;6:355-62.
23. Hornung RW, Reed LD. Estimation of Average Concentration in the Presence of Nondetectable Values. *Applied Occupational and Environmental Hygiene.* 1990;5(1):46-51.
24. Focosi D, Macera L, Boggi U, Nelli LC, Maggi F. Short-term kinetics of torque teno virus viraemia after induction immunosuppression confirm T lymphocytes as the main replication-competent cells. *J Gen Virol.* 2015;96(Pt 1):115-7.
25. Görzer I, Jaksch P, Kundi M, Seitz T, Klepetko W, Puchhammer-Stöckl E. Pre-transplant plasma Torque Teno virus load and increase dynamics after lung transplantation. *PLoS One.* 2015;10(3):e0122975.
26. Solis M, Velay A, Gantner P, Bausson J, Filipputti A, Freitag R, et al. Torqueteno-virus viremia for early prediction of graft rejection after kidney transplantation. *J Infect.* 2019;79(1):56-60.
27. Roberto P, Cinti L, Napoli A, Paesani D, Riveros Cabral RJ, Maggi F, et al. Torque teno virus (TTV): A gentle spy virus of immune status, predictive marker of sero-conversion to COVID-19 vaccine in kidney and lung transplant recipients. *J Med Virol.* 2023;95(2):e28512.
28. Cabezas L, Truffot A, Germi R, Bugnazet M, Malvezzi P, Gnesotto M, et al. Evaluation of Torque Teno Virus DNA Load as a Predictive Biomarker in Kidney Transplant Recipients Converted from Calcineurin Inhibitors to Belatacept. *Kidney International Reports.* 2024.

29. Schaenman J, Rossetti M, Pickering H, Sunga G, Wilhalme H, Elashoff D, et al. Preservation of Antiviral Immunologic Efficacy Without Alloimmunity After Switch to Belatacept in Calcineurin Inhibitor-Intolerant Patients. *Kidney Int Rep.* 2023;8(1):126-40.
30. Kühne JF, Neudörfl C, Beushausen K, Keil J, Malysheva S, Wandrer F, et al. Differential effects of Belatacept on virus-specific memory versus de novo allo-specific T cell responses of kidney transplant recipients and healthy donors. *Transpl Immunol.* 2020;61:101291.
31. Charpentier B, Medina Pestana JO, Del CRM, Rostaing L, Grinyó J, Vanrenterghem Y, et al. Long-term exposure to belatacept in recipients of extended criteria donor kidneys. *Am J Transplant.* 2013;13(11):2884-91.
32. Petrossian G, Ortiz J, Ortiz AC, Addonizio K, Hsiao A, James R, et al. Increased CMV disease and "severe" BK viremia with belatacept vs. sirolimus three-drug maintenance immunosuppression. *Transpl Immunol.* 2023;79:101857.
33. Fagnoni FF, Vescovini R, Mazzola M, Bologna G, Nigro E, Lavagetto G, et al. Expansion of cytotoxic CD8⁺ CD28⁻ T cells in healthy ageing people, including centenarians. *Immunology.* 1996;88(4):501-7.
34. Sansoni P, Vescovini R, Fagnoni F, Biasini C, Zanni F, Zanlari L, et al. The immune system in extreme longevity. *Experimental gerontology.* 2008;43(2):61-5.
35. de Graaf GN, Hesselink DA, Dieterich M, Kraaijeveld R, Weimar W, Baan CC. Down-Regulation of Surface CD28 under Belatacept Treatment: An Escape Mechanism for Antigen-Reactive T-Cells. *PLoS One.* 2016;11(2):e0148604.
36. Martín-López M, Albert E, Fernández-Ruiz M, González-Álvaro I, Rodríguez E, Aguado JM, et al. Torque teno virus viremia in patients with chronic arthritis: Influence of biologic therapies. *Semin Arthritis Rheum.* 2020;50(1):166-71.
37. Studenic P, Bond G, Kerschbaumer A, Bécède M, Pavelka K, Karateev D, et al. Torque Teno Virus quantification for monitoring of immunomodulation with biologic compounds in the treatment of rheumatoid arthritis. *Rheumatology (Oxford).* 2022;61(7):2815-25.
38. Regele F, Haupenthal F, Doberer K, Gorzer I, Kapps S, Strassl R, et al. The kinetics of Torque Teno virus plasma load following calcineurin inhibitor dose change in kidney transplant recipients. *J Med Virol.* 2024;96(3):e29554.
39. Haloschan M, Bettesch R, Görzer I, Weseslindtner L, Kundi M, Puchhammer-Stöckl E. TTV DNA plasma load and its association with age, gender, and HCMV IgG serostatus in healthy adults. *Age (Dordr).* 2014;36(5):9716.
40. Efird J. Blocked randomization with randomly selected block sizes. *Int J Environ Res Public Health.* 2011;8(1):15-20.
41. Chattopadhyay S, Rao S, Das BC, Singh NP, Kar P. Prevalence of transfusion-transmitted virus infection in patients on maintenance hemodialysis from New Delhi, India. *Hemodial Int.* 2005;9(4):362-6.
42. Rivanera D, Lozzi MA, Idili C, Lilli D. Prevalence of TT virus infection in Italian dialysis patients. *Pathol Biol (Paris).* 2009;57(1):97-100.
43. Ozener IC, Ture F, Koc M, Avsar E. Prevalence of TT virus in a CAPD population. *Nephron.* 2002;91(2):341-3.
44. Herrmann A, Sandmann L, Adams O, Herrmann D, Dirks M, Widera M, et al. Role of BK polyomavirus (BKV) and Torque teno virus (TTV) in liver transplant recipients with renal impairment. *J Med Microbiol.* 2018;67(10):1496-508.

45. van Doesum WB, Gard L, Bemelman FJ, de Fijter JW, Homan van der Heide JJ, Niesters HG, et al. Incidence and outcome of BK polyomavirus infection in a multicenter randomized controlled trial with renal transplant patients receiving cyclosporine-, mycophenolate sodium-, or everolimus-based low-dose immunosuppressive therapy. *Transpl Infect Dis.* 2017;19(3).
46. de Fijter JW, Holdaas H, Øyen O, Sanders JS, Sundar S, Bemelman FJ, et al. Early Conversion From Calcineurin Inhibitor- to Everolimus-Based Therapy Following Kidney Transplantation: Results of the Randomized ELEVATE Trial. *Am J Transplant.* 2017;17(7):1853-67.



Supplemental figure 1. Spaghetti-plot of TTV-levels of patients with rejection.

**An exploration of markers of
microvascular dysfunction in kidney
transplant recipients randomized to
belatacept: no clinical impact of CNI's on
endothelial function.**

OW Bredewold, RJ Postma, J Chan, MHS Svensson, A Bruchfeld, JW de Fijter, H Furuland, A Hartmann, L Mjornstedt, K Skov, I Soveri, SS Sorensen, B Fellström, JM Duijs, R Bijkerk, JI Rotmans, AJ v Zonneveld

ABSTRACT

Calcineurin inhibitors (CNI) have been the drug of choice in kidney transplantation since their introduction. However, their side-effects comprise hypertension, diabetes and nephrotoxicity. Endothelial dysfunction (ED) has been proposed as a key mechanism underlying these adverse effects. Belatacept, a costimulation blocker, may offer a more favorable vascular profile.

In the present study, we investigated markers of endothelial function in blood samples from kidney transplant recipients (KTR) that participated in two randomized clinical conversion trials from CNI (control group) to belatacept (intervention group). In a broad range of experiments, we assessed established markers of ED including soluble thrombomodulin, P-selectin and angiopoietin 1 and 2 in plasma samples of patients before and after conversion. In addition, we assessed expression levels of micro-RNAs, which have been identified as discriminative markers for ED, rejection and renal fibrosis in the setting of transplantation. Lastly, we used patient samples and CNI-dilution series in a validated model of ED in which changes in a large set of morphological characteristics of endothelial cells were evaluated.

In contrast to our hypothesis, we did not find any significant differences in our assays between the intervention-and control-groups. We did observe dose-related effects of CNI on endothelial cell (EC) morphology upon direct exposure of EC to tacrolimus and ciclosporin A, but a similar effect was not seen when plasma samples of KTR who were converted to belatacept were compared with samples from KTR on CNI's. In conclusion, we found no significant effect of conversion on established biomarkers of endothelial toxicity in patients using CNI or belatacept. These data suggest that CNI-side-effects bundled under the term 'CNI-toxicity' are not derived from a significant clinical effect of CNI on endothelial function.

INTRODUCTION

Regimens containing a calcineurin inhibitor (CNI) are used by most recipients of solid organ transplantation [1]. Their introduction in the late seventies has led to significant decrease in rejection rates and improved graft survival. Since their introduction, standard regimens have been periodically changed, and now typically consist of tacrolimus (TAC) or sometimes ciclosporin A (CsA), together with Mycophenolic acid (MMF) or a mammalian target of Rapamune inhibitor (mTORi) for patients with low immunological risk. For those with higher risk, steroids are advised [2].

An alternative to CNI-based regimens is available in the form of belatacept, which is a co-stimulation blocker, designed by coupling the extracellular part of cytotoxic T-lymphocyte antigen 4 (CTLA4) to the Fc-chain of IgG. In the past fifteen years, various clinical studies with belatacept have shown that it carries a relatively high risk of rejection in the first year after transplantation, but a clear benefit for renal function, with glomerular filtration rate (GFR) consistently higher when compared to patients on CNI's in the majority of conversion-trials [3-5]. Because of this, most centers that use belatacept introduce it later after transplantation, or only prescribe it to patients that have severe CNI-related toxicity [6, 7].

Notoriously, CNI affect GFR, as well as a number of long-term risk factors for kidney function decline such as diabetes, dyslipidemia and hypertension [8-10]. The nephrotoxic effects are mostly easily recognizable for transplant physicians as a negative effect that introduction or dose-increment of CNI's exerts on GFR. More broadly, patients experiencing CNI-toxicity have a clinical presentation that includes declining GFR, hypertension and hyperkalemia. However recognizable, not much is known about the actual mechanisms through which CNI's exert their toxicity. Endothelial dysfunction (ED) is often implied, based on data from preclinical studies. Specifically, CsA induces acute efferent arteriolar vasoconstriction due to disturbance of the balancing effects of endothelium-relaxing factor (NOS) and prostaglandin E2 on the vasodilating side and endothelin-1 (ET-1) and thromboxane on the other [11-13]. Similar effects on ET-1 production and release by CsA have been described for TAC [14]. The finding of consistently lower blood pressures and reduced arterial stiffness are clinical indications of ED being favorably influenced by conversion to belatacept [15-18]. Wang e.a. have shown an improvement of several measures of ED and renal hemodynamics in a rat model of diabetic nephropathy, indicating a direct effect of CTLA-Ig on renal parenchyma [19]. Besides a direct effect it is assumed that part of any conversion effect is due to discontinuation of CNI and its associated

vaso-constriction [20]. Histologic features of the effect of CNI's on the micro-vasculature can be observed after prolonged use and include arterial hyalinosis, interstitial fibrosis and tubular atrophy [21]. However, those features are not at all unique for CNI-exposure. Specifically, chronic rejection, caused by anti-HLA-antibodies (ABMR) can cause similar features, and face the physician with a clinical dilemma: does the graft suffer more from CNI-exposure, or from ABMR, for which CNI still is the cornerstone of therapy, despite its toxicity [22].

At the Leiden University Medical Center (LUMC), we have participated in two randomized controlled trials (RCT) exploring the effect on cardiovascular risk factors and renal function after conversion from CNI to belatacept [5, 15]. Both studies had similar in- and exclusion criteria and follow up, providing a chance to investigate features of ED in kidney transplant recipients (KTR) converted to belatacept and weaned off CNI. We hypothesized that patients converted to belatacept would exhibit changes indicating an improvement in ED associated with CNI, and we expected those changes to be early reflections of the clinical benefit that belatacept-treated patients have shown in previous trials in terms of renal function and metabolic health. To that purpose, we used blood samples of patients participating in either of the two conversion trials, and compared samples before and after conversion. Samples of these patients were used to perform a broad investigation of markers of endothelial function, ranging from established biomarkers as angiotensin I and II (Ang-1/Ang-2), soluble thrombomodulin (s-TM) and P-selectin (P-sel), to an exploratory investigation of micro-RNA's previously associated with ED, renal fibrosis and restoration of kidney function after kidney transplantation. Furthermore, we explored the effects of different concentrations of calcineurin inhibitors on cultured endothelial cells, as well as the effects of plasma samples from participants on an innovative endothelial cell morphological profiling assay. This assay was previously validated in our hospital in a cohort of patients with cirrhosis, and in other conditions associated with microvascular disease [23]. This study could provide insight into the reversibility of early ED, and contribute to our knowledge of mechanistic differences between belatacept and CNI.

MATERIALS AND METHODS

Plasma samples of KTR's of both RCT's (EudraCT no. 2013-001178-20 (SMR-2729) and NCT01820572 (IM103-116)) stored at the Leiden University Medical Centre were used for this study. . Both studies involved KTR's that were randomized to convert from standard immune suppression with CNI (93% had TAC, 7% CsA) to belatacept, or to continue with CNI. The inclusion- and exclusion cri-

teria, follow-up and background immunosuppression were very similar in both studies [5, 15]. The standard regimens in all participating centers consisted of prednisone (5 mg standard-dose) and MMF. Blood samples were collected immediately before and at 12 months after randomization, and stored at 80° Celsius. Informed consent was provided upon participation in the respective trials.

Measurement of biomarkers of vascular function

Plasma was isolated from 5 mL of patient-derived venous blood after being freshly drawn into EDTA-coated vacutainers and mixed carefully head-to-head to ensure proper exposure of the blood to the EDTA. Subsequently, samples were centrifuged at room temperature at 150 g for 15 minutes. Platelet-rich plasma (PRP) was isolated and centrifuged again at 2000 g for 10 minutes. Aliquots of 500 µL plasma were transferred into freezer tubes and stored at -80°C. Serum was collected by allowing full blood to coagulate for 30 minutes. The supernatant was then centrifuged at 4 °C at 2000 g for 10 minutes. After that, samples were similarly stored in aliquots at -80°C.

For this study, plasma was defrosted and levels of Ang-2, P-sel and s-TM were determined using Enzyme-Linked Immunosorbent Assay (ELISA) according to manufacturers' protocol (R&D Systems, Minneapolis, MN, USA; catalog number DANG20, DPSE00, DTHBD0). For measurement of Ang-1, serum was used (R&D Systems, Minneapolis, MN, USA; catalog number DANG10).

Extraction, profiling and measurement of micro-RNA's.

A total of 200 µL of plasma was spiked with 5 fmol of synthetic, exogenous *C. elegans* miR-238 as a technical control (Biolegio, Nijmegen, The Netherlands) and mixed with 800 µL of Trizol LS reagent (Invitrogen, Breda, The Netherlands). Total RNA was then isolated using the RNeasy Micro Kit (Qiagen, Venlo, The Netherlands) with a modified protocol. Chloroform was added to the plasma/ Trizol LS mixture, followed by centrifugation for 15 min at 12000 rpm. For extraction of RNA, further centrifugation and washing steps were performed.

After extraction, reverse transcription of total RNA was performed using the micro-RNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). C-DNA was subsequently pre-amplified using Megaplex PreAmp primer pools A V2.1 (Applied Biosystems) according to the manufacturer's instructions. The resulting DNA was then loaded onto custom-made Megaplex cards to determine the expression profile of a selected set of micro-RNA's in serum, with RNU44, RNU48 and U6 serving as controls. These Megaplex arrays were run and analyzed on a 7900HT Fast Real-Time PCR System (Applied Biosystems).

Ct values for designated micro-RNA's were normalized to the median expression of Micro-RNA of each megaplex-plate. Expression of individual micro-RNA-levels was presented as fold changes compared to one index case at baseline, using the standard $\Delta\Delta\text{Ct}$ method. The methods for extraction, profiling and measurement of micro-RNA's were similar to the protocol used by Bijkerk e.a. [24].

Selection of micro-RNA's

Table 1 gives an overview of the selected Micro-RNA, as well as the biological function with which it is implicated, as well as a literature reference if available. We chose micro-RNA's associated with AD, allograft dysfunction or CNI-toxicity, with a preference for those we found to be discriminative for diabetes and restoration of renal function after transplantation. For capacity reasons, we selected 20 micro-RNA's.

Table 1. Selected micro-RNA's

Micro-RNA	Biological process	Reference
MiR92a, MiR210, MiR30c, MiR27a, MiR126, MiR130b, MiR132, MiR200c, MiR320, MiR340, MiR660	Endothelial dysfunction, renal fibrosis, restoration of renal function.	[25-31]
MiR199a-3p, MiR214, MiR21, MiR150, MiR155, MiR192, MiR200b, MiR142-5p	CNI-toxicity, allograft dysfunction	[32-35]
MiR16	Renal fibrosis	[36]

Endothelial Cell Morphology Assay

The endothelial cell morphological profiling assay has been described previously [23, 37]. In short, human umbilical vein endothelial cells (HUVEC) were obtained and cultured as described previously [23]. Low-passage HUVECs were thawed and cultured in endothelial cell growth medium 2 (EGM-2) (PromoCell C-22111) supplemented with 1% Penicillin/Streptomycin (Gibco 15070-063) in a gelatin (1% in PBS, Merck; 104078) coated T75 flask for 3 days. Cells (20.000/well) were then seeded in a gelatin coated 96-wells plate (PerkinElmer Pheno-Plate, 6055300) 4 days prior to the experiment.

Two experiments were performed using this assay; 1: CNI titration series, and 2: patient-derived plasma exposure.

Morphological responses to different concentrations of CNI were investigated for CsA (Neoral®, Novartis Pharma by, Switzerland) and TAC (Prograft®, Astellas

Pharma bv, Japan). A titration series of 0 (zero), 5, 10, 20, 40, 80, 160, 320, 640, 1280, 2560 and 5120 mcg/L (CsA) and 0, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256 and 512 ng/mL (TAC) was performed in endothelial cell basal medium (PromoCell C-2211), supplemented with 5% healthy human serum. Both dilution series are compatible with blood-concentrations that represent subclinical, clinical, and toxic levels. The cultured endothelial cells were incubated with the different mixes for 18 hours, followed by immunofluorescent staining of the cells.

Morphological responses to patient-derived plasma were investigated by exposing cultured endothelial cells to plasma collected from patients treated in the LUMC in either of the two randomized trials. K₂EDTA plasma was stabilized and recalcified by adding 0.5 μ M recombinant Hirudin (ABCAM ab201396), 25 μ g/ml Corn trypsin inhibitor (HTI CTI-01), and 1.85 mM calcium-dichloride (CaCl₂) (Merck 1.02381) to a K₂EDTA plasma volume equaling 25% of the total volume. Endothelial cell basal medium (EBM) (PromoCell C-22211) containing 1:100 insulin transferrin selenium supplement (ITS) (Gibco 41400) was added to obtain the final volume. The cultured endothelial cells were incubated with the different plasma mixes for 18 hours, followed by immunofluorescent staining of the cells.

Cells were stained according to the previously published protocol, modified to include a staining for von Willebrand factor (vWF). Cells were incubated with Mi-toTracker Deep Red FM (Invitrogen M22426) 1:2000 for 30 minutes, followed by incubation with 4% paraformaldehyde (Alfa Aesar J61899) for 10 minutes, and 30 minute incubation with blocking buffer (2% (w/v) BSA + 0.5% Glycine (Merck 1.04201) + 1% (v/v) Triton X-100 in PBS). Afterwards, wells were incubated with primary antibodies against VE-Cadherin (BD 555661, 2 μ g/ml) and vWF (DAKO a0082, 1:1000) in PBS with 2% BSA for one hour, followed by incubation with 488 Alexa-fluorophore labeled Donkey anti-mouse antibody (Invitrogen A-11001, 2 μ g/ml), Qdot 625 labeled Goat anti-rabbit antibody (Invitrogen A-10194, 1:500), 1:500 Rhodamine Phalloidin (Invitrogen R415), and 1:1000 HOECHST 33258 (Molecular Probes) in 50mM Borate Buffer, pH=8.3 with 2% BSA + 0.05% glycine for one hour. Wells were washed with borate buffer, and stored under borate buffer for imaging. Max-projections of 9 z-steps with 0.7 μ m step size were acquired using a high content confocal microscope (Molecular Devices, ImageXpress™ Micro Confocal) at 20x magnification (Nikon Plan Apo Lambda; NA = 0.75), using a 60 μ m pinhole. Six sites without overlap were imaged per well.

For each image, a focus-score metric, intensity sum, median, standard deviation, and the 0.01, 0.25, 0.75, 0.99 quantiles were computed. Results were plotted in histograms comprised of all images for a given channel to assess

outliers. Visual inspection of outliers was performed to assess image quality before discarding. Images that contained artifacts (eg out-of-focus images, debris, clipping/saturation artifacts) were discarded. Sites that contained failed images in a single channel were discarded altogether. Following image quality control, images were corrected for uneven illumination and vignetting. For a description of the IF staining, imaging, image control, we refer to a previous publication [23].

Image Analysis

Single-cell morphological profiles were extracted from the images using R (version 4.1.1)[38], the R-package *EImage* (version 4.29.2) [39], and in-house scripts. In short, individual nuclei were identified from Dapi-channel images and used as seeds to identify the cell borders using the VE-Cadherin signal. To filter out debris, upper and lower thresholds were applied for the nuclei size. R-package *EImage* “compute” functions were used to compute morphological features. Functions to extract distribution and colocalization were based on the procedure by Verbeek et al. [40].

Data Analysis

Data analysis concerning patient data was performed using R(V 4.1.1.) Qualitative data are described using frequencies and percentages. Quantitative variables are described using mean (standard deviation) or median (IQR) when appropriate. Comparisons between treatment groups were performed using ANOVA and student’s t-test for continuous variables. A p-value < 0,05 was considered to be statistically significant. R-packages *haven* (V2.5.4), *ggplot2* (V3.5.1) and *factoextra* (V1.0.7) were used to perform principal component analysis (PCA) of micro-RNA’s.

Data analysis of the morphological data was performed in Python version 3.7.6, using functions from the Scikit-learn package [41]. The approach was as follows: Z-score normalization was applied to the single-cell data of each plate dataset separately [37]. Afterwards, datasets were combined. Single-cell profiles were reduced in dimension by factor analysis [42], capturing about 80% of total variance, resulting in 18 factors. Mean profiles were created by averaging the cells for each patient/time point in the reduced subset. Resulting profiles were scaled to 0-mean and unit variance. These profiles were used for modelling the endothelial morphological response, either by principle component analysis, linear discriminant analysis, or 5-fold cross-validated support vector machine (SVM) with rbf-kernel.

Statistics for vascular markers and micro-RNA's

For analysis of markers of vascular function (concentrations in ng/ml, continuous variables), we used Graphpad prism 10.2.3. We used a mixed-effects model for longitudinal analysis (baseline vs T12). Fisher's LSD (least significant difference) was used for post hoc analysis. For between-group comparisons we performed unpaired t-tests. A p-value of $< 0,05$ was considered statistically significant.

For micro-RNA's, we calculated fold changes (FC) for each selected micro-RNA. We compared average FC per micro-RNA between baseline and T12 using unpaired two-sample t-tests when data were normally distributed. If not we performed Wilcoxon rank-sum-test. P-values were corrected for multiple testing using the FDR-method. We made volcano plots to visualize effect size and significance. We performed principal component analysis to explore clustering patterns within micro-RNA-profiles. All analyses were done using R version 4.3.2 (*ggplot2*, *enhancedvolcano*-packages).

RESULTS

Data collection and patient characteristics.

Samples of 100 patients from 4 different countries were used. From the Netherlands, there were 29 patients from SMR-2729, and 10 from IM-103-116 [5, 15]. All other patients were recruited from SMR-2729 (20 from Denmark, 14 from Norway and 27 from Sweden). Patients were eligible for both studies after 3 and 6 months respectively. Median time after transplantation at recruitment was 26 months. Patients in both studies were either randomized to convert to belatacept, at a dose of 5 mg/kg at day 1, 15, 29, 43, 57 and every 28th day thereafter, or to continue on CNI according to their local protocol. Data on traditional vascular markers Ang 1/2, STM and P-selectin were obtained from all patients, at baseline (T0) and 12 months later (T12). The measurements were performed shortly after completion of the study in 2019. To perform the micro-RNA-measurements, we used samples that had been stored for more than six years, and defrosted only after definite resolution of the COVID-19 pandemic. Unfortunately, at that time we found that some samples had lost their identification label. Therefore, we used only those samples of which the labels were readable, and of which one was available at baseline (before randomization) and one year after randomization (T12). After normalization of the micro-RNA-data, only samples with a cycle threshold of lower than 35 were used. In total, samples of 70 patients with measurements from at least two time-points were included.

For the cell-painting assays, we used only patient-samples from local (Leiden) patients that had participated in the RCT's. From that group, we possessed samples collected at baseline, at 6 months after randomization and after one year (T0, T6, T12). Data from all 100 patients are depicted in table 2. Data from the smaller group of 70 (micro-RNA) and 39 (cell-painting) patients are available as supplemental tables S1 and S2. Of note, the patients in the control group of all cohorts had slightly better GFR despite randomization, whereas all other clinical characteristics were evenly distributed. 93,1% of patients used TAC before randomization (89,6% in control group, 96,3% in belatacept).

Table 2.

Baseline-characteristics for all patients

Variable	CNI (48)	Belatacept (52)
Age (mean, SEM)	57 (1,9)	57 (2,0)
Female sex (n,%)	11 (22%)	16 (29%)
LDL (mean, mmol/L, SEM)	2,7 (0,1)	2,7 (0,1)
Months since transplantation	26,6 (3)	27,7 (2)
Dialysis vintage in months	15,6 (3)	15,6 (2)
History of smoking	30 (60%)	24 (45%)
Diabetes	8 (16%)	13 (23%)
Systolic BP (mmHg)	134	136
Diastolic BP (mmHg)	82	83
eGFR (ml/min/1.73m ²)	61 (2,8)	52 (2,0)
CsA before randomization	5 (10,4%)	2 (3,8%)

No significant change in traditional biomarkers of vascular health after conversion from tacrolimus to belatacept.

Figure 1a shows measurements for Ang-1 and Ang-2. The mean level of Ang-1 at baseline was 7,4 ng/mL (7,9 ng/mL for CNI, 6,9 ng /mL for belatacept). After 12 months after randomization, it was 7,9 ng/mL for CNI again, while it had increased to 8,2 ng/mL in belatacept (p-value 0,18). For Ang-2, the average was 2,7 ng/mL (in both groups). Twelve months later it remained at 2,9 ng/mL for both groups. Because Ang-1 and Ang-2 have opposing endothelial effects, the ratio of Ang-2 over Ang-1 is

often used to indicate whether a change in concentration has a net activating (rise) or stabilizing (decline) effect on endothelial cells. In CNI-patients, the ratio of Ang-2 over Ang-1 (Ang-2/1) increased from 0,29 to 0,31 (p-value 0,63), while in belatacept it decreased from 0,32 to 0,29 (p 0.38) The p-value for the difference in Ang-2/1 ratio between CNI and belatacept was non-significant (Mann-Whitney-test, p = 0,10).

In **figure 1b**, the values for s-TM are depicted. Average s-TM-concentration was 9,2 ng/mL at baseline and decreased to 8,7 ng/L after 12 months in CNI-patients. For belatacept, the average concentration was 9,3 ng/mL, unchanged. For P-sel, the average concentration decreased from 32,0 to 29,4 ng/mL in CNI, and was stable in belatacept-patients (27,6 ng/mL to 27,9 ng/mL). When comparing the average change between groups, no difference for either s-TM or P-sel was observed after conversion.

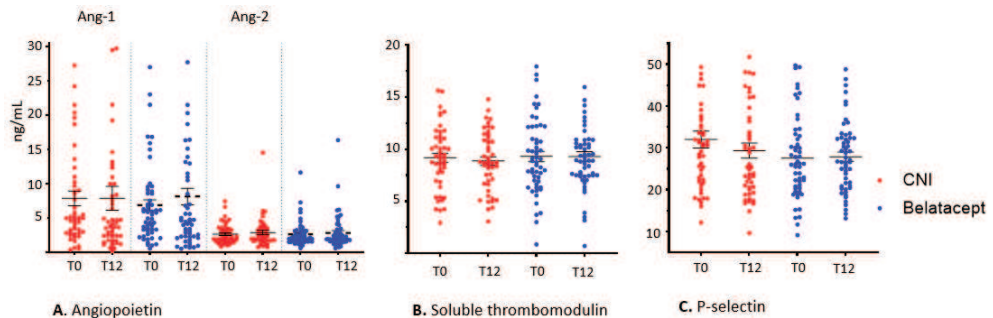


Figure 1.

A. Levels of angiopoietin 1 and 2 **B.** Levels of soluble thrombomodulin **C.** Levels of P-selectin. None of the markers show significant difference after conversion to belatacept. All values in ng/mL. Error bars signify mean $\pm$ SEM.

Micro-RNA's

To test if the conversion from tacrolimus to belatacept exerted relevant change on the expression of micro-RNA's specifically selected for their discriminative properties regarding ED and association with (transplant)-renal function, we performed effect-size analysis of the fold change (FC) of micro-RNA's. The results are visualized in a Volcano plot (**figure 2a**). The upper striped blue line represents a p-value of 0.05 after correction for multiple testing. It becomes clear from the picture that most micro-RNA's are expressed in lower quantities after conversion to belatacept than when patients continued using CNI. Only three micro-RNA's (MiR130b, MiR200c, and MiR200b) increase after conversion while the rest is expressed in lower quantities. However, the changers are small, and none of them are significant.

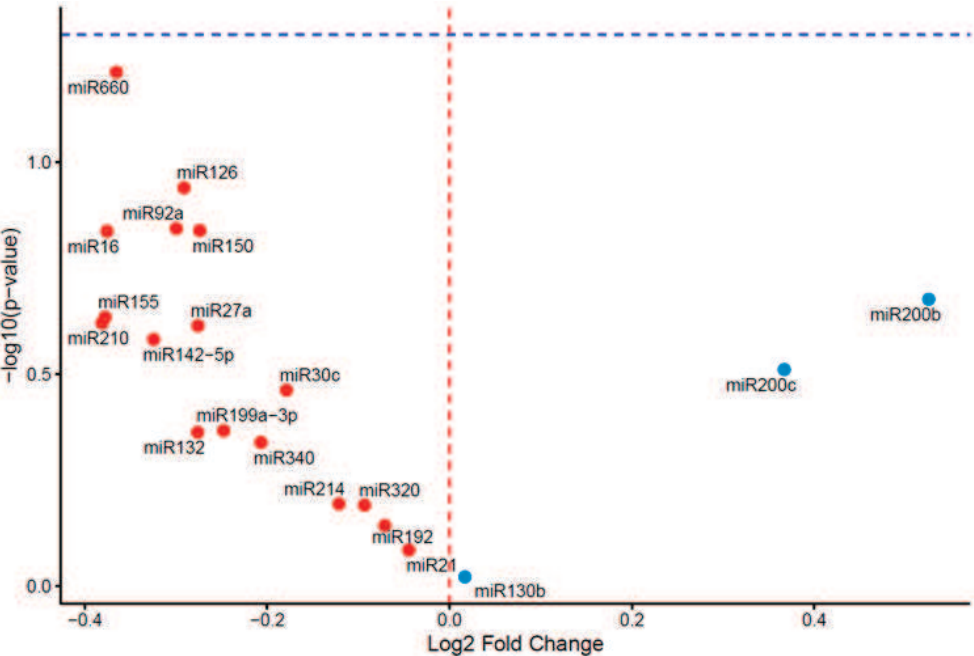


Figure 2
Volcano-plot, showing Micro-RNA-fold-change after conversion. The blue line indicates significance (p-value corrected for multiple testing < 0.05). Blue dots represents micro-RNA's that rise after conversion to belatacept. Red dots represents a decrease in expression. MiR = micro-RNA.

To visualize whether any clustering-patterns exist between inter-related micro-RNA's, we performed principal component (PC) analysis (PCA). This methods transforms multiple variables (the micro-RNA's) into PC's, thereby reducing complexity and enabling the discovery of associated variables. In **supplemental figure 1**, the result of the PCA is shown. No apparent clustering of micro-RNA's is seen. Also, it confirms the lack of significant change after conversion, indicated by the larger red (CNI) and blue (belatacept) centroid-dots in the middle of the ellipses indicating the averages of all micro-RNA's, as well as by the low percentage of total variance explained by the main PC's 1 (42.4%) and 2 (11.7%).

Cell-painting assay: the impact of ciclosporin A and tacrolimus on endothelial phenotype in vitro

We used linear discriminant analysis (LDA) to assess whether we were able to find a difference in the morphological response of endothelial monolayers on exposure to dose responses of CNI and TAC. It is essential to understand that LDA is a supervised analysis technique, where-as PCA is unsupervised. This means that in LDA-plots, the analysis uses knowledge of group levels (e.g. what CNI-experiment (TAC or CsA), what concentration) to find the combination of variables that best separates the groups. The larger the distance between two samples, the greater the accumulated difference in variables. **Figure 3a** shows the LDA-plot. On the right side of the picture, images from the experiment are shown. In the actual plot, blue dots represent the control-samples (no CNI). Those samples show endothelial cells in an quiescent state. The cell membranes (green) are broad, and have an intense fluorescence pattern. Stress fibers (yellow) are sparsely seen, and mitochondria (red) are interconnected and located perinuclearly.

The middle of the plot shows a large cloud of samples representing different dilution samples in which higher concentration is visualized by larger dots. A clear gradual change is seen along the horizontal axis, where samples seem to follow a dose-related shift to the left side of the picture. Furthermore, the highest concentrations of both TAC and CsA are represented on the extreme lower and higher margin of the plot respectively. Both groups are clearly discernible from controls and samples. In the images from those patients, we clearly see changes in the cell membrane (less broad, loss of reticular 'fishnet' pattern). Besides, the mitochondria show fragmentation, where the interconnection is lost, and the mitochondria are less closely located to the nuclei. Stress fibers (F-actin) are seen more abundantly. In comparison to each other, the intensity of F-actin staining is more pronounced in the higher CsA-concentrations relative to TAC. All the mentioned changes are indicative of an activated state of endothelial cells.

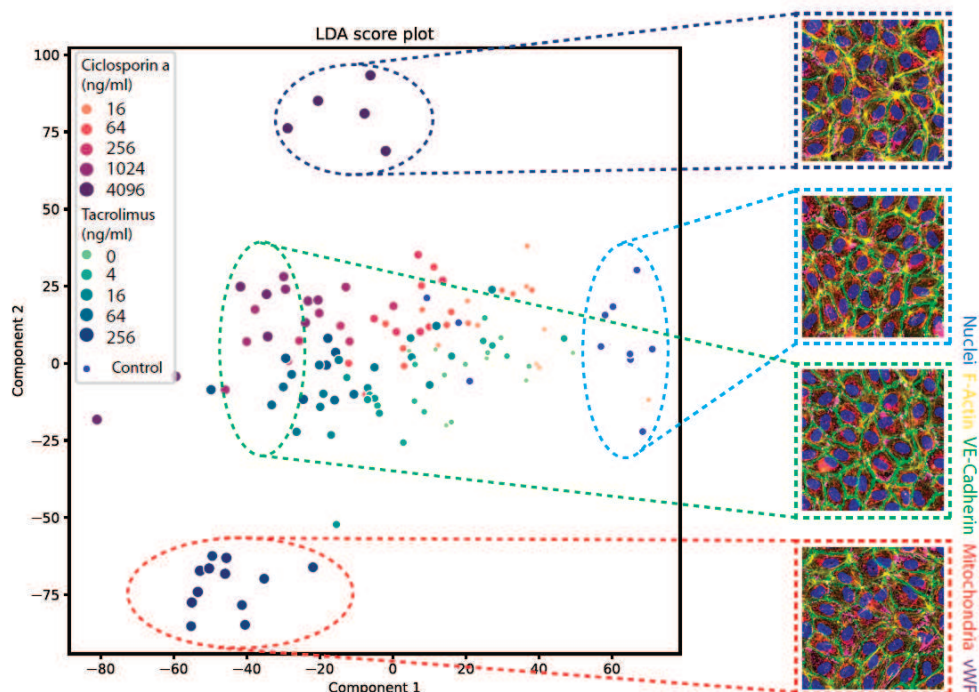


Figure 3.

A. Linear discriminant analysis of cell painting essays under different concentrations of CNI. Clear distinction of controls (light-blue oval) from high-dose (HD) CsA (dark-purple oval) and HD TAC (dark-blue oval). Green oval contains intermediate doses of both TAC and CsA. **B.** Regression-fit-plot for estimated vs true CsA-concentration.

Next, we performed regression-fit-plotting to estimate CNI-concentration based on endothelial morphology. Predicted concentrations were derived from analysing endothelial cell features using leave-one-out cross validation. The model performed reasonably well for CsA ($R^2 = 0.72$, $MAE = 1.19 \pm 0.45$). In **figure 3b**, the results of regression-fit-plotting for CsA are shown. For TAC, we performed the same analysis, which underperformed compared to CsA. (**supplemental figure 2**).

Patient samples

Cell-painting assay reveals endothelial changes not related to treatment

For the patient samples, we also used PCA to visualize patterns in the variables extracted from the cell-images. The results are portrayed in **figure 4a**. It shows all the samples, 4b and 4c show the groups. In all patients, we see a

migration of the cluster of samples in time. Note that, in contrast to LDA, PCA is non-supervised, meaning it is blind to the group to which the patient-sample belongs. Despite this unsupervised approach, clustering of patients at baseline, at T6 and at T12 is clearly visible, both for belatacept-patients as for CNI (**figure 4b, c**). In belatacept-patients, the separation between baseline (when CNI was still used by all patients) and T6 is more pronounced than between T6 and T12. In CNI-patients, the separation is maximal between T6 and T12.

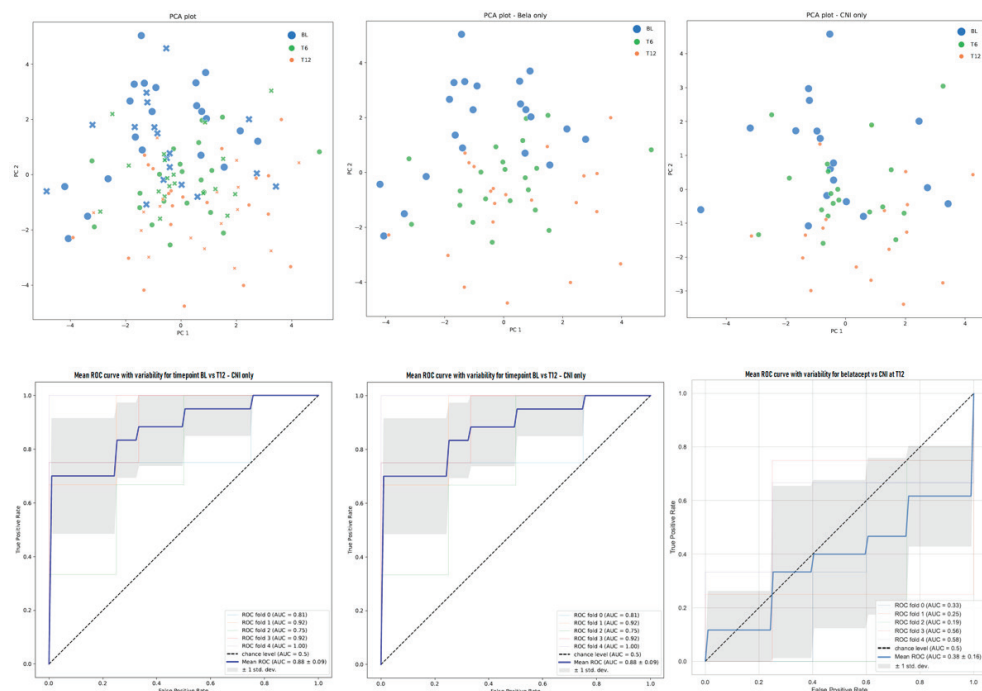


Figure 4

A. PCA for patient samples. Rounds represent belatacept, crosses are controls (CNI). Symbol-size negatively correlates to time after randomization. **B.** Same, but only belatacept-patients are represented **C.** Same, but only CNI-patients are represented **D.** ROC curves for cell-painting essays separate BL from T12 for belatacept. **E.** Same for CNI. **F.** ROC curves cannot distinguish endothelial cell morphology of belatacept vs CNI at month 12.

We assembled receiver-operator curves (ROC) to evaluate if samples could be reliably classified to belatacept or control and to what time-point, on the basis of endothelial cell morphology (**figure 4d, e, f**). ROC's correctly classify baseline-patients from T12 patients for both belatacept and CNI. However, they do not reliably differentiate belatacept-patients from CNI-patients at month 12 (**figure 4f**). This indicates that the distinction in endothelial cell morphology patterns

that is recognized is associated with the factor 'time after randomization, and not with the result of the randomization itself.

To provide more information on what features are important to separate the samples, we used unsupervised multivariate analysis (factor analysis, FA). **Supplemental figure 3** shows a ranking of the factors identified, and the importance of their contribution to the separation of samples. In factor analysis, the factors have no inherent meaning, but they are constructed from combinations of measured variables that may or may not carry relevance. In our analysis, fifteen factors are identified, of which factor 6, containing information on vWF and mitochondria. Since none of the factors were significantly associated to the conversion in drug-regimen we did not further explore this finding.

DISCUSSION

In this study, we investigated the effects of a conversion from CNI to belatacept on endothelial dysfunction in KTR's. Our most striking conclusion is that we found no significant change in ED in any of the biomarkers we explored. Neither traditional biomarkers of vascular function, nor micro-RNA's, nor our novel 'cell-painting' assay showed any marked change in patients on belatacept when compared to patients that continued to use CNI.

We had expected our current study to show a decrease in Ang-2/1 ratio, indicative of improved endothelial function. The angiopoietin system involves two antagonizing components: Ang-1 is secreted by pericytes underlying the endothelium, binding to it through its Tie-2-receptor (Tie-2R). Ang-2 is released from Weibel-Palade bodies in conditions like sepsis. Ang-1 promotes vessel integrity, while Ang-2 expels Ang-1 from Tie-2R, stimulating detachment of endothelial cells from pericytes and promoting vascular leakage. In transplantation, early skewing of the angiopoietin system towards Ang-2 after ischemia-reperfusion injury (IR/I) is a predictor of pericyte loss and capillary rarefaction [43, 44]. Previous work from our own lab proved Ang-2/1 disturbances after simultaneous pancreas-after-kidney transplantation (SPK) and kidney-rejection [24, 45]. Furthermore, TAC is associated with suppression of Ang-1 in vitro, while Ang-2-upregulation is also implied in TAC-associated hypertension [20, 46]. However, we found no change in Ang-1, nor Ang-2, and only a small non-significant decrease in Ang-2/1.

We also investigated the effect of conversion on s-TM and P-sel. S-TM is shed from endothelial cells after inflammatory injury. Previous work from our lab

identified s-TM to be increased in kidney failure. However, it was reversed after transplantation and restoration of renal function, only to rise again in those patients developing interstitial fibrosis and tubular atrophy [47]. Since IF/TA is one of the features of CNI-toxicity in renal transplant-biopsies we had hypothesized that s-TM would possibly be decreased in belatacept-patients. However, we did not see such difference. The same result, no difference was found for P-sel, a thrombocyte-derived marker of endothelial activation [48] and ED, and the third traditional biomarker studied [24, 43, 49-51]. Besides the obvious conclusion that belatacept does nothing to change the concentration of these agents, it is also possible that any effect that would have occurred was missed due to suboptimal timing of sampling, as changes in concentrations are thought to be dynamic, and may not be preserved after 6 or 12 months [24, 45]. However, the ratio of Ang-2/1 did remain elevated in another experiment by our lab, one year after kidney-graft-rejection [24]. Of note, in that study, the mean eGFR in those that had rejection was 11 ml/m/1.73m² lower than in those with stable GFR. In our study, baseline-eGFR of those on belatacept was significantly lower than in controls, and no difference in eGFR was observed after a year of follow-up in the SMR-2729, from which the majority of our samples were obtained [15].

We found no effect of conversion on micro-RNA-expression either. We had selected 20 micro-RNA's that were previously associated with ED, kidney fibrosis and restoration of kidney function after kidney transplantation [25-36]. We had expected several of those to be differentially expressed after conversion to belatacept. Previous literature, including our own showed excellent biomarker-potential of micro-RNA's like miR126 and miR132 in transplantation-settings of IR/I, rejection and graft dysfunction [24, 28, 34]. Others, like miR21, miR214 and miR199a have been suggested to play a role in CNI-related toxicity [32]. However, our results lack any discriminative effect of conversion on a single micro-RNA selected. Neal e.a. previously showed the total concentration of micro-RNA's to be lower in patients with severe CKD. In this regard, we have to consider again that the belatacept-group had lower GFR, and a lower total amount of RNA may have made it harder to detect a change in concentration, despite the fact that most of our patients had renal function in the CKD-3-range, where Neal showed the most relevant differences in CKD 4 and 5 [36].

We saw that almost all micro-RNA's except three were downregulated after belatacept-conversion. Although the lower expression itself did not reach significance from any individual micro-RNA, we must consider some explanations for overall-lower expression of genetic material. One is subclinical viral infection, as some viral infections are known to interfere with mRNA-transcription through inhibition and degradation of RNA-polymerase II [52, 53]. We have

previously shown Torque Teno Virus (TTV) concentrations to be higher in belatacept-treated patients [54]. Secondly, since micro-RNA's are shed from vesicles that originate from circulating cells, lower numbers of circulating cells may also be implicated. We have previously shown that conversion to belatacept is associated with lower numbers of T-cells, B-cells and some subclasses of monocytes [55].

In our dilution-series cell-painting experiments, we showed dose-dependent effects of CNI on endothelial cell morphology. Gradual loss of VE-cadherin-staining and the appearance of F-actin stress fibers under influence of CsA have been shown before in cultured pulmonary endothelial cells, albeit in concentrations 200-20000 times the highest concentration in our study [56]. Effects on EC-mitochondria have also been described, but are somehow two-pronged. Calcineurin is essential for the translocation of dynamin-related protein-1 (DRP1) to mitochondria and its activation by dephosphorylation. When activated, DRP-1 causes mitochondrial fragmentation and ultimately cell death. Both CsA and TAC revert this process, protecting against mitochondrial dysfunction [57]. On the other hand, calcineurin inhibition causes mitochondrial accumulation of calcium, and the ensuing result is the production of toxic reactive oxygen species [58, 59]. In the literature, more examples of this protective effect of CNI can be found. For instance, CNI have an attenuating effect on ED after brain death in a pig model, and earlier studies have shown protection against IR/I-induced ED in multiple animal models (although clinical studies did not corroborate the results in humans) [60-63].

In our last experiment, we used the cell painting model to test the effect of patient plasma samples on endothelial cell morphology. As such, we could compare the differential effects of belatacept, - and CNI-treatment, incorporating the effects of comorbidities such as age, diabetes and CKD. Although we did see endothelial morphology change after six and twelve months, we did not see a treatment effect, as the cell changes were observed in both groups, and the belatacept-samples did not show reversal of toxicity. Of note, the dose-response curve of CNI with endothelial changes seems to be stronger for CsA than for TAC in the dilution-series experiment (fig 3b). Most patients (over 90%) represented in the samples used TAC, in 'low' (trough-level 3-7) doses. Not finding a treatment effect might reflect the fact that such a dose is below the toxic threshold for endothelial (renal) cells. So, although our results suggest a clear toxic effect of increasing doses of both CsA and TAC on endothelial cell structure in vitro, it is clear that this model of ED was not significantly influenced by the presence of serum derived from patients on belatacept or CNI, indicating that clinically-relevant ED depends on other comorbidities and possibly dose.

For more than a decade now, belatacept has provided an alternative immunosuppressive option for KTR's that experience CNI-related toxicity, associated with improvement in eGFR and cardiovascular risk. A possible mechanistic explanation is that belatacept results in less ED when compared to CNI. Processes in which ED plays a role are known to show their clinical detrimental effects years after early ED is established, and that is why we had hypothesized to find early changes in markers of ED [64]. To our knowledge, this study is the first attempt at comparing features of ED in KTR's randomized to belatacept. A major strength of our approach is the fact that our samples are taken from international RCT's, and that the patients reflect current international standards of using 'low-dose' CNI, (more than 90% TAC). A limitation is the unfortunate loss of samples we had to endure. Also, in interpreting the results, one has to remember that almost 90% of the samples were derived from patients from the SMR-2729-study [15]. This study, besides a small trial by de Graav e.a. is the only 'negative' (conversion had no significant beneficial effect on eGFR) study on belatacept-conversion ever published [65]. Although the patients in this trial were randomized, the intervention group had worse renal function before randomization. We can only speculate whether these features have influenced the results of our study, i.e. whether belatacept truly has a negligible effect on CNI-associated ED after kidney transplantation.

In conclusion, we have investigated whether a conversion of immunosuppressive regimen resulted in early changes in features of ED in KTR, and saw surprisingly little difference between patients on CNI and those converted to belatacept. Thus, we have not been able to find support for the hypothesis that conversion to belatacept to alleviate CNI-toxicity in KTR's acts can be explained by a mitigation of endothelial dysfunction.

REFERENCES.

1. Lentine, K.L., et al., *OPTN/SRTR 2021 Annual Data Report: Kidney*. Am J Transplant, 2023. 23(2 Suppl 1): p. S21–s120.
2. Kasiske, B.L., et al., *KDIGO clinical practice guideline for the care of kidney transplant recipients: a summary*. Kidney Int, 2010. 77(4): p. 299–311.
3. Vincenti, F., 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): p. 535–46.
4. Durrbach, A., 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): p. 547–57.
5. Budde, K., et al., *Conversion from Calcineurin Inhibitor to Belatacept-based Maintenance Immunosuppression in Renal Transplant Recipients: a Randomized Phase 3b Trial*. J Am Soc Nephrol, 2021.
6. Gupta, G., et al., *Safe Conversion From Tacrolimus to Belatacept in High Immunologic Risk Kidney Transplant Recipients With Allograft Dysfunction*. Am J Transplant, 2015. 15(10): p. 2726–31.
7. Adams, A.B., 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): p. 2922–2936.
8. Suilik, H.A., et al., *Efficacy of tacrolimus versus cyclosporine after lung transplantation: an updated systematic review, meta-analysis, and trial sequential analysis of randomized controlled trials*. Eur J Clin Pharmacol, 2024.
9. Heisel, O., et al., *New onset diabetes mellitus in patients receiving calcineurin inhibitors: a systematic review and meta-analysis*. Am J Transplant, 2004. 4(4): p. 583–95.
10. van Schaik, M., et al., *Long-term renal and cardiovascular risks of tacrolimus in patients with lupus nephritis*. Nephrol Dial Transplant, 2024. 39(12): p. 2048–2057.
11. Farouk, S.S. and J.L. Rein, *The Many Faces of Calcineurin Inhibitor Toxicity-What the FK? Adv Chronic Kidney Dis*, 2020. 27(1): p. 56–66.
12. De Nicola, L., et al., *Arginine feeding modifies cyclosporine nephrotoxicity in rats*. J Clin Invest, 1993. 92(4): p. 1859–65.
13. Lanese, D.M. and J.D. Conger, *Effects of endothelin receptor antagonist on cyclosporine-induced vasoconstriction in isolated rat renal arterioles*. J Clin Invest, 1993. 91(5): p. 2144–9.
14. Haug, C., et al., *Endothelin release by rabbit proximal tubule cells: modulatory effects of cyclosporine A, tacrolimus, HGF and EGF*. Kidney Int, 1998. 54(5): p. 1626–36.
15. Bredewold, O.W., et al., *Cardiovascular Risk Following Conversion to Belatacept From a Calcineurin Inhibitor in Kidney Transplant Recipients: A Randomized Clinical Trial*. Kidney Med, 2023. 5(1): p. 100574.
16. Melilli, E., et al., *Impact of immunosuppressive therapy on arterial stiffness in kidney transplantation: are all treatments the same?* Clin Kidney J, 2018. 11(3): p. 413–421.
17. Vanrenterghem, Y., 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): p. 976–83.
18. Melilli, E., 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): p. 58–65.
 19. Wang, L., et al., *Effects of Cytotoxic T Lymphocyte-Associated Antigen 4 Immuno-globulin Combined with Microbubble-Mediated Irradiation on Hemodynamics of the Renal Artery in Rats with Diabetic Nephropathy*. *Ultrasound Med Biol*, 2020. 46(3): p. 703–711.
 20. Wang, X., et al., *Tacrolimus Causes Hypertension by Increasing Vascular Contractility via RhoA (Ras Homolog Family Member A)/ROCK (Rho-Associated Protein Kinase) Pathway in Mice*. *Hypertension*, 2022. 79(10): p. 2228–2238.
 21. Liptak, P. and B. Ivanyi, *Primer: Histopathology of calcineurin-inhibitor toxicity in renal allografts*. *Nat Clin Pract Nephrol*, 2006. 2(7): p. 398–404; quiz following 404.
 22. Udomkarnjananun, S., M.R. Schagen, and D.A. Hesselink, *A review of landmark studies on maintenance immunosuppressive regimens in kidney transplantation*. *Asian Biomed (Res Rev News)*, 2024. 18(3): p. 92–108.
 23. Postma, R.J., et al., *Novel Morphological Profiling Assay Connects ex Vivo Endothelial Cell Responses to Disease Severity in Liver Cirrhosis*. *Gastro Hep Adv*, 2024. 3(2): p. 238–249.
 24. Bijkerk, R., et al., *Acute Rejection After Kidney Transplantation Associates With Circulating MicroRNAs and Vascular Injury*. *Transplant Direct*, 2017. 3(7): p. e174.
 25. Shang, F., et al., *MicroRNA-92a Mediates Endothelial Dysfunction in CKD*. *J Am Soc Nephrol*, 2017. 28(11): p. 3251–3261.
 26. Shang, F., et al., *Obstructive Sleep Apnea-induced Endothelial Dysfunction Is Mediated by miR-210*. *Am J Respir Crit Care Med*, 2023. 207(3): p. 323–335.
 27. Demolli, S., et al., *MicroRNA-30 mediates anti-inflammatory effects of shear stress and KLF2 via repression of angiopoietin 2*. *J Mol Cell Cardiol*, 2015. 88: p. 111–9.
 28. Bijkerk, R., et al., *Circulating microRNAs associate with diabetic nephropathy and systemic microvascular damage and normalize after simultaneous pancreas-kidney transplantation*. *Am J Transplant*, 2015. 15(4): p. 1081–90.
 29. Zhang, H., et al., *Inhibition of miR-200c Restores Endothelial Function in Diabetic Mice Through Suppression of COX-2*. *Diabetes*, 2016. 65(5): p. 1196–207.
 30. Jiang, Y., et al., *Upregulation of miR-200c-3p induced by NaF promotes endothelial apoptosis by activating Fas pathway*. *Environ Pollut*, 2020. 266(Pt 1): p. 115089.
 31. Bijkerk, R., et al., *Silencing of microRNA-132 reduces renal fibrosis by selectively inhibiting myofibroblast proliferation*. *Kidney Int*, 2016. 89(6): p. 1268–80.
 32. Vandebussche, C., et al., *Tacrolimus-induced nephrotoxicity in mice is associated with microRNA deregulation*. *Arch Toxicol*, 2018. 92(4): p. 1539–1550.
 33. Liang, J.Y., et al., *Increased expression of miR-155 correlates with abnormal allograft status in solid organ transplant patients and rat kidney transplantation model*. *Life Sciences*, 2019. 227: p. 51–57.
 34. Vahed, S.Z., et al., *Circulating miR-150, miR-192, miR-200b, and miR-423-3p as Non-invasive Biomarkers of Chronic Allograft Dysfunction*. *Archives of Medical Research*, 2017. 48(1): p. 96–104.
 35. Lan, H.Y., *Smads as therapeutic targets for chronic kidney disease*. *Kidney Res Clin Pract*, 2012. 31(1): p. 4–11.

36. Neal, C.S., et al., *Circulating microRNA expression is reduced in chronic kidney disease*. *Nephrol Dial Transplant*, 2011. 26(11): p. 3794–802.
37. Bray, M.A., et al., *Cell Painting, a high-content image-based assay for morphological profiling using multiplexed fluorescent dyes*. *Nat Protoc*, 2016. 11(9): p. 1757–74.
38. Team, R.C., *R: A Language and Environment for Statistical Computing*. 2021, R Foundation for Statistical Computing.
39. Pau, G., et al., *EImage--an R package for image processing with applications to cellular phenotypes*. *Bioinformatics*, 2010. 26(7): p. 979–81.
40. Manders, E.M.M., F.J. Verbeek, and J.A. Aten, *Measurement of co-localization of objects in dual-colour confocal images*. *J Microsc*, 1993. 169(3): p. 375–382.
41. Pedregosa, F., et al., *Scikit-learn: Machine Learning in Python*. *Journal of Machine Learning Research*, 2011. 12(85): p. 2825–2830.
42. Young, D.W., et al., *Integrating high-content screening and ligand-target prediction to identify mechanism of action*. *Nat Chem Biol*, 2008. 4(1): p. 59–68.
43. de Vries, D.K., et al., *Renal ischemia-reperfusion induces release of angiopoietin-2 from human grafts of living and deceased donors*. *Transplantation*, 2013. 96(3): p. 282–9.
44. Khairoun, M., et al., *Renal ischemia-reperfusion induces a dysbalance of angiopoietins, accompanied by proliferation of pericytes and fibrosis*. *Am J Physiol Renal Physiol*, 2013. 305(6): p. F901–10.
45. Khairoun, M., et al., *Microvascular damage in type 1 diabetic patients is reversed in the first year after simultaneous pancreas-kidney transplantation*. *Am J Transplant*, 2013. 13(5): p. 1272–81.
46. Choe, J.Y., et al., *Tacrolimus (FK506) inhibits interleukin-1 β -induced angiopoietin-1, Tie-2 receptor, and vascular endothelial growth factor through down-regulation of JNK and p38 pathway in human rheumatoid fibroblast-like synoviocytes*. *Joint Bone Spine*, 2012. 79(2): p. 137–43.
47. Dane, M.J., et al., *Association of kidney function with changes in the endothelial surface layer*. *Clin J Am Soc Nephrol*, 2014. 9(4): p. 698–704.
48. Blann, A.D., S.K. Nadar, and G.Y. Lip, *The adhesion molecule P-selectin and cardiovascular disease*. *Eur Heart J*, 2003. 24(24): p. 2166–79.
49. de Boer, H.C., et al., *Aspirin treatment hampers the use of plasma microRNA-126 as a biomarker for the progression of vascular disease*. *Eur Heart J*, 2013. 34(44): p. 3451–7.
50. Groeneweg, K.E., et al., *Circulating Long Noncoding RNA LNC-EPHA6 Associates with Acute Rejection after Kidney Transplantation*. *Int J Mol Sci*, 2020. 21(16).
51. Bijkerk, R., et al., *Circulating angiopoietin-2 and angiogenic microRNAs associate with cerebral small vessel disease and cognitive decline in older patients reaching end-stage renal disease*. *Nephrol Dial Transplant*, 2022. 37(3): p. 498–506.
52. Akhrymuk, I., S.V. Kulemzin, and E.I. Frolova, *Evasion of the innate immune response: the Old World alphavirus nsP2 protein induces rapid degradation of Rpb1, a catalytic subunit of RNA polymerase II*. *J Virol*, 2012. 86(13): p. 7180–91.
53. Vreede, F.T. and E. Fodor, *The role of the influenza virus RNA polymerase in host shut-off*. *Virulence*, 2010. 1(5): p. 436–9.
54. Bredewold, O.W., et al., *Attenuation of Torque teno viral load over time in kidney transplantation recipients treated with calcineurin inhibitors is mitigated after conversion to belatacept*. *J Med Virol*, 2024. 96(9): p. e29905.

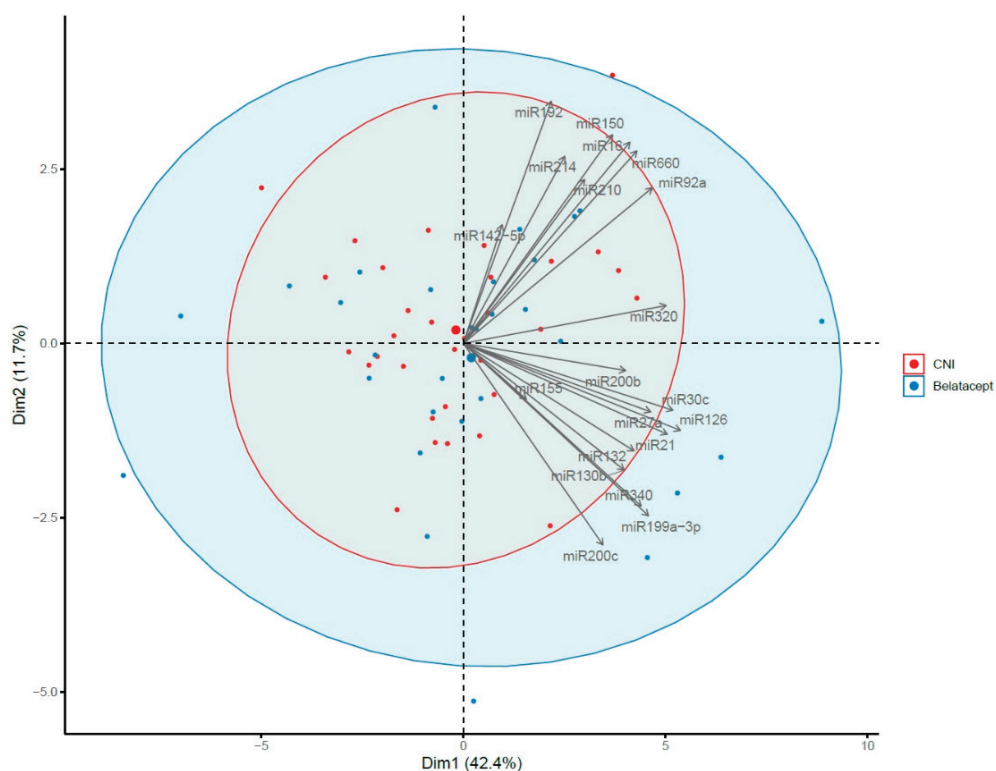
55. Bredewold, O.W., et al., *Conversion from calcineurin inhibitors to belatacept-based immunosuppressive therapy skews terminal proliferation of non-classical monocytes and lowers lymphocyte counts*. Transpl Immunol, 2024. 82: p. 101976.
56. Boittin, F.X., et al., *The immunosuppressant drug Cyclosporin A aggravates irradiation effects in endothelial cells*. Biochem Biophys Res Commun, 2022. 602: p. 127–134.
57. Cho, S.G., et al., *Drp1 dephosphorylation in ATP depletion-induced mitochondrial injury and tubular cell apoptosis*. Am J Physiol Renal Physiol, 2010. 299(1): p. F199–206.
58. Nsengimana, B., et al., *Involvement of oxidative species in cyclosporine-mediated cholestasis*. Front Pharmacol, 2022. 13: p. 1004844.
59. J. Perez de Hornedo, G.d.A., M. Calvino Fernandez, S. benito, T. Parra Cid, *Cyclosporin a causes oxidative stress and mitochondrial dysfunction in tubular renal cells*. Nefrologia, 2007. 27(5): p. 565–73.
60. Idouz, K., et al., *Cascading renal injury after brain death: Unveiling glycocalyx alteration and the potential protective role of tacrolimus*. Front Cell Dev Biol, 2024. 12: p. 1449209.
61. Travis, D.L., et al., *Protection by cyclosporine A against normothermic liver ischemia-reperfusion in pigs*. J Surg Res, 1998. 75(2): p. 116–26.
62. Squadrito, F., et al., *Tacrolimus limits polymorphonuclear leucocyte accumulation and protects against myocardial ischaemia- reperfusion injury*. J Mol Cell Cardiol, 2000. 32(3): p. 429–40.
63. Krishnadasan, B., et al., *Decreased lung ischemia-reperfusion injury in rats after preoperative administration of cyclosporine and tacrolimus*. J Thorac Cardiovasc Surg, 2002. 123(4): p. 756–67.
64. Gimbrone, M.A., Jr. and G. Garcia-Cardena, *Endothelial Cell Dysfunction and the Pathobiology of Atherosclerosis*. Circ Res, 2016. 118(4): p. 620–36.
65. de Graav, G.N., et al., *A Randomized Controlled Clinical Trial Comparing Belatacept With Tacrolimus After De Novo Kidney Transplantation*. Transplantation, 2017. 101(10): p. 2571–2581.

Supplemental table 1. Baseline characteristics of patients in micro-RNA-experiments

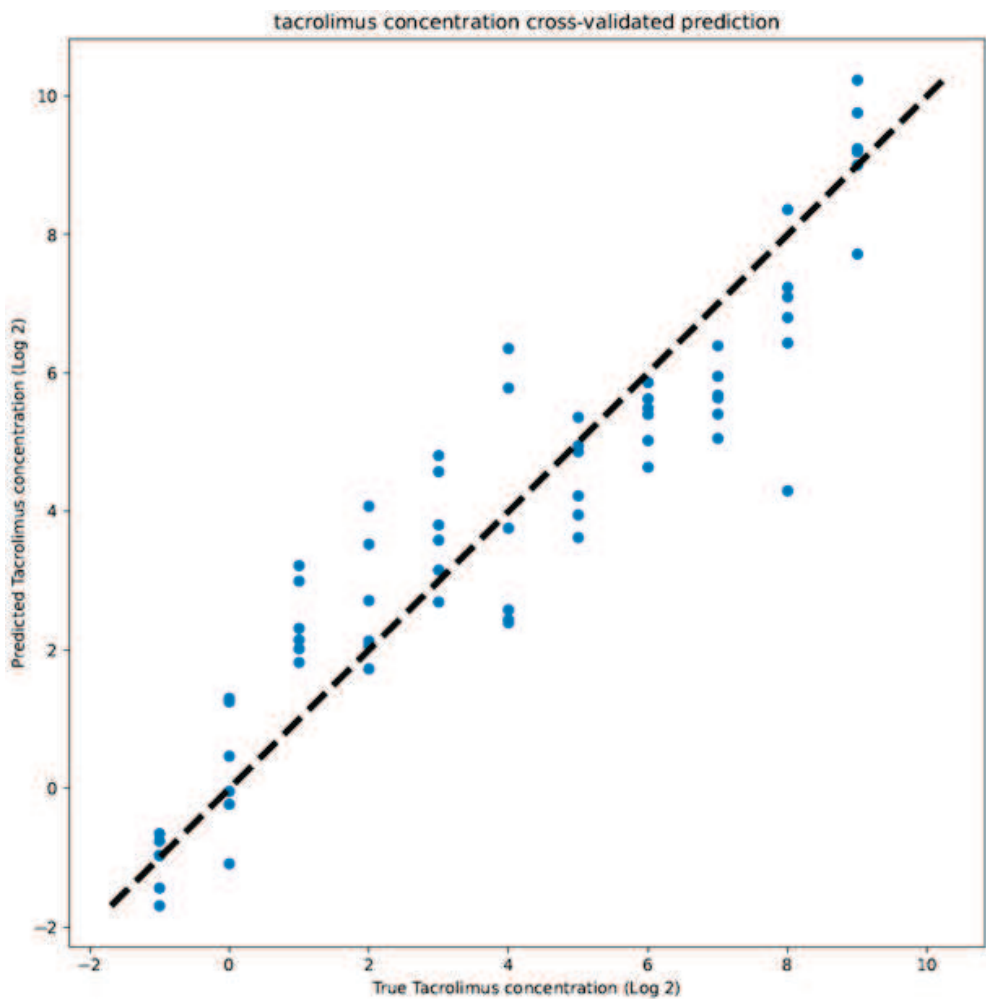
Variable	CNI (39)	Belatacept (31)
Age (mean, SEM)	57 (2,1)	55 (2,8)
Female sex (n,%)	10 (26%)	10 (32%)
LDL (mean, mmol/L, SEM)	2,7 (0,2)	2,7 (0,2)
Months since transplantation	29 (3)	33 (3)
Dialysis vintage in months	14 (3)	14 (3)
History of smoking	24 (62%)	14 (45%)
Diabetes	6 (15%)	5 (16%)
Systolic BP (mmHg)	135 (3)	134 (2)
Diastolic BP (mmHg)	82 (2)	81 (2)
eGFR (ml/min/1.73m2)	58 (3,2)	52 (2,7)

Supplemental table 2. Baseline characteristics of patients participating in cell-painting essay.

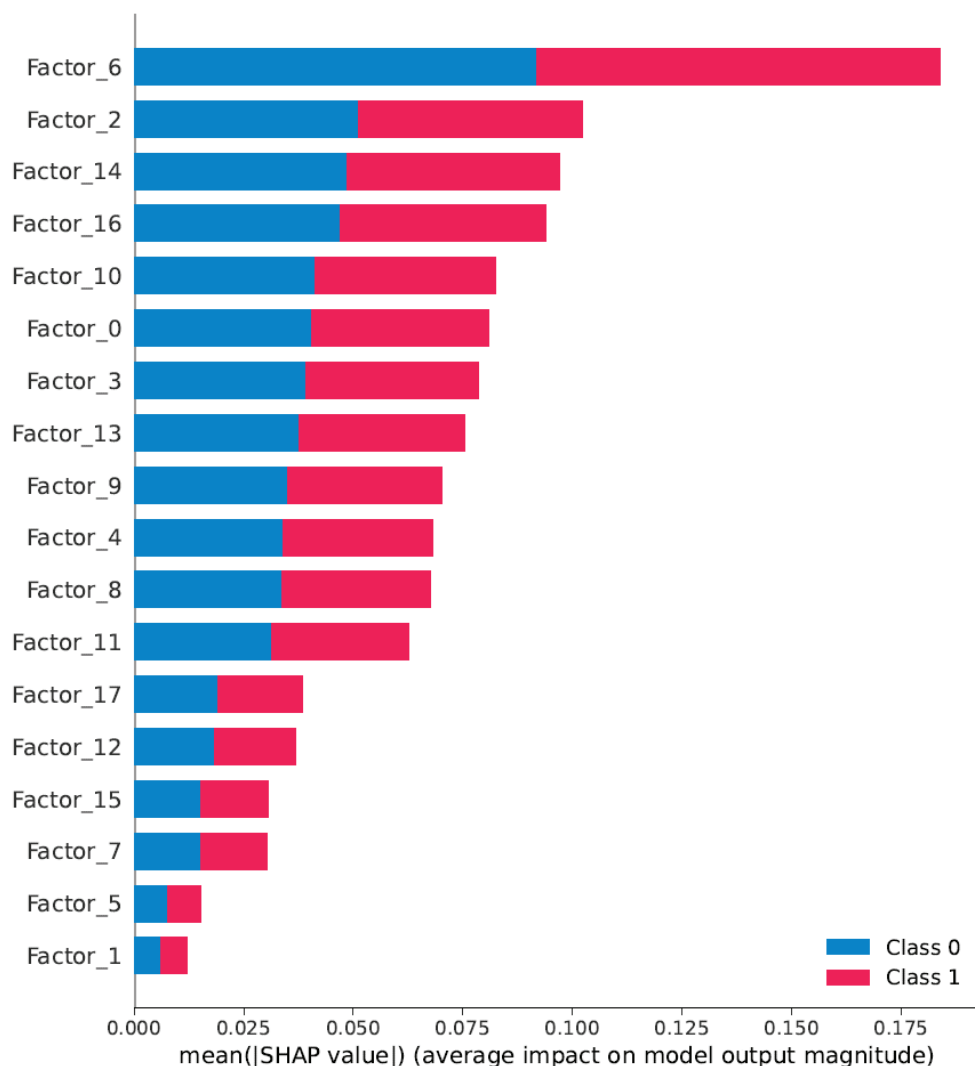
Variable	CNI (18)	Belatacept (21)
Age (mean, SEM)	59 (2,6)	58 (3,6)
Female sex (n,%)	6 (33%)	9 (43%)
LDL (mean, mmol/L, SEM)	2,8 (0,3)	3,0 (0,2)
Months since transplantation	31 (5)	35 (3)
Dialysis vintage in months	19 (6)	18 (6)
History of smoking	11 (61%)	10 (48%)
Diabetes	3 (17%)	8 (38%)
Systolic BP (mmHg)	142 (5)	133 (4)
Diastolic BP (mmHg)	84 (2)	78 (2)
eGFR (ml/min/1.73m2)	56 (4)	47 (3)



Supplemental figure 1. Principal component analysis. The average change in micro-RNA-expression after conversion to belatacept (blue centroid) does not differ from controls (red centroid).



Supplemental figure 2. Regression-fit-plot for estimated vs true TAC-concentration.



Supplemental Figure 3. Factor analysis reveals that factor 6 (containing information on vWf and mitochondriae) shows the greatest variation in endothelial cell patterns of KTR.

6

Summary, discussion and future perspective for belatacept

SUMMARY

In the present thesis, differences between belatacept and calcineurin inhibitors (CNI) in kidney transplantation regarding their effect on cardiovascular and immunologic parameters were explored, to help define the place of belatacept in current practice. To achieve this, data derived from two randomized controlled trials (RCT's) that compared kidney-transplant recipients (KTR) before and after conversion from CNI to belatacept were used. Our findings aimed to provide much-needed information about the clinical utility of belatacept implementing clinical, immunological and vascular effects.

In **chapter 2**, we describe the results of an RCT on conversion from CNI to belatacept. In a collaboration with researchers from hospitals in Sweden, Norway and Denmark we compared the 7-year risk of developing an endpoint defined within 'MACE' (Major Adverse Cardiovascular Endpoint) or all-cause mortality, using a cardiovascular risk-calculator [1]. In 105 patients that were followed-up for one year, we found no difference in the risk-calculation between patients that were converted to belatacept, when compared to patients that remained on a CNI-based regimen. Likewise, we also failed to demonstrate a significant effect of conversion on any of three individual components of the calculator: LDL-cholesterol, renal function and diabetes-risk. A small improvement in diastolic blood pressure was observed in belatacept patients. These data provided some contrast with results of previous trials, in which an improvement in renal function was invariably found [2-4]. Possible explanations discussed in the article are underpowering, a somewhat skewed distribution of GFR between the two randomized groups and a selection bias favouring patients not suffering from CNI-side-effects. However, our findings indirectly support existing guidelines regarding the dosing of tacrolimus (TAC) in transplantation, as these modern doses are probably less toxic than previous CNI-based regimens.

At the time patients were randomized and treated in the trial described in **chapter 2**, the LUMC was involved in a second international RCT with a comparable design [4]. In-and exclusion-criteria were largely the same, although the endpoints were different. However, the follow-up in the first year was so similar, that it facilitated us to create a larger, homogeneous patient cohort of which the samples could be used for the other research in this thesis.

With the expanded data, we explored whether conversion from CNI to belatacept resulted in alteration of the presence and phenotypic profile of circulating monocytes and lymphocytes, to assess differences in cardiovascular risk as well as immunologic action. We hypothesized that belatacept would result in a decrease and a phenotypic change in circulating cells, indicative of a less inflammatory phenotype. We identified a significant change in a monocyte-subset that is only scarcely described in other literature [5]. In patients converted to belatacept, this cell-type, which is negative for cell-marker CD14 decreased in the circulation while its concentration stayed constant in the CNI-group. In belatacept-treated patients, we saw a parallel decrease in T-lymphocytes, B-lymphocytes and TNF-alfa, with all these changes showing significant correlation in a regression model. On the basis of our findings, we propose a model in which CD14NM, this subset of monocytes behave as an antigen-presenting cell (APC) facilitating proliferation of T-cells and B-cells. In this model, belatacept negatively influences the terminal conversion of CD14-positive monocytes into CD14NM. This effect would then lead to downstream suppression of T-cell-differentiation and proliferation, as well as T-cell mediated stimulation of B-cells. These results are described in detail in **chapter 3** [6].

Although belatacept is associated with rejection, especially in the first year after transplantation, multiple studies and pre-clinical research have also indicated that belatacept-treated patients have a decreased likelihood to form donor-specific anti-HLA-antibodies (DSA) compared to both ciclosporin A (CsA) as TAC [4, 7, 8]. Also, since the introduction of belatacept, a caution for EBV-associated lymphoproliferative disease is given to physicians prescribing belatacept to EBV-seronegative transplant recipients. These observations point to a somewhat paradoxical effect of belatacept regarding its immunosuppressive strength. There is an urgent need of biomarkers that reliably indicate if immunosuppression in an individual patient is either suboptimal, with the risk of rejection, or conversely dosed too high, with a risk of infections and cancer.

Torque Teno Virus (TTV) is a non-pathogenic anellovirus that can serve as a marker of immunogenic strength: present in high concentrations in plasma when the host is immunocompromised, and lower when immunity is stronger [9]. We measured the copy number of this virus in samples taken before and after conversion from CNI to belatacept. We found that TTV continually decreases in concentration after transplantation, but that this decline is negated in patients converted to belatacept. It indicates that belatacept, though associated with more rejections in the first year after transplantation, is not necessarily weaker in its immunosuppressive effect. These findings can be found in **chapter 4** [10].

In **chapter 5**, we set out to compare parameters of endothelial dysfunction (ED) in patients converted to belatacept with patients that maintained on a CNI-based immunosuppressive regimen. For that purpose, traditional and novel biomarkers to explore ED in KTR's after conversion were measured. We used several different markers, to explore the hypothesis that belatacept improved ED compared to CNI. Traditional markers, like angiopoietins, P-selectin and Soluble thrombomodulin were investigated. Twenty micro-RNAs associated with ED, renal fibrosis, CNI-toxicity and allograft dysfunction, including miR21, miR126, miR155 and miR132 were selected, again hypothesizing that these markers would be influenced by the conversion in immunosuppression [11-14]. Lastly, we employed a novel method, called 'cell-painting' to study ED. This assay uses human umbilical vein endothelial cells (HUVEC) that are cultured, and in which stainings are performed to visualize cellular components, like cell wall, mitochondria, nuclei and stress fibres. Images of these cells are then acquired using a high content confocal microscope, and after that they are subjected to computational analysis of features [15]. In our study, we subjected these cells to patient sera, and to dilution series of CNI. To our surprise, we found no effect of the conversion from CNI to belatacept in either the traditional methods or the micro-RNAs, nor with the cell-painting-assay. The essay did corroborate earlier findings on the effect of CNI on ED, particularly in high doses, and more outspoken for CsA than for TAC. These observations suggest that CNI-toxicity as encountered in clinical practice is not a problem singularly caused by an influence of CNI on endothelial cells, although the design of the study might have been suboptimal to include the patients most heavily affected by it. Secondly, our dilution-series experiments emphasize that assumed knowledge on toxicities of CNI is quite often derived from studies that investigate CNI in doses incomparably higher than used in practice [16]. In addition to the results of chapter 2, the lack of a clear effect of CNI on ED in these broad experiments indirectly lends credence to the contemporary practice of subscribing low-dose TAC in renal transplantation.

DISCUSSION

In this thesis, we investigated the effect of conversion from CNI to belatacept on a multitude of different aspects; ranging from kidney function and risk factors for cardiovascular disease, to quantitative effects on cell populations involved in inflammation, effects on viral replication of TTV as marker of immune status and effects on biomarkers of microvascular health. When the trial underlying the results in this thesis was designed, the hypothesis was that belatacept would exert a significant effect on parameters of cardiovascular health as well on the risk factors associated with it such as renal function and ED. Instead, we found an effect on the phenotypic composition of monocyte subsets leading us to propose a potential carry-over effect to lymphocytes (Chapter 3). The effect of conversion on vascular biomarkers was limited to a subtle influence on blood pressure (chapters 2 & 5). The effects of belatacept on TTV-replication were contrary to our expectations (chapter 4). The question is how to interpret these results.

The most striking observation in the studies presented in this thesis was the absence of significant improvements in any of the parameters of vascular health in patients that were converted from a CNI to a belatacept-based immune suppressive regime. It is important to note that our study-design, with the limited follow-up of 1 year and no comparison of biopsies does not allow us to draw conclusions regarding chronic CNI-toxicity to endothelial cells. However, the results of chapter 5 make it doubtful whether an acute endothelial toxicity of TAC is of any clinical importance, contrary to the hypotheses in this thesis, and possibly contrary to long-held beliefs in the transplant community. A couple of limitations need to be considered at this point.

First of all, a selection bias is probably present in any conversion trial that investigates toxicity of the comparator drug. Namely, in any conversion trial (in contrast to trials where the study drug is introduced immediately after transplantation), patients experiencing severe side effects of CNI might already have been excluded from participation, while any patient converting to belatacept would have their first exposure to belatacept in the trial. As such, the full extent of side effects of CNI is likely to have been diluted in a conversion trial. In choosing this design, we have probably missed patients with the most pronounced manifestations of acute CNI-toxicity. It is well known that a creatinine-rise is frequently observed after introduction of calcineurin inhibitors. The reversibility of it after dose-adjustment, as well as the directness of both the effect and its recovery after dose-adjustment or discontinuation, together with the known effects of CNI on a factor like ET-1 all suggested to us that the vascular endothe-

lium is affected by acute CNI-toxicity. Possibly, patients most targeted by such proposed 'acute endothelial toxicity' were left out of our trials by design. We did not investigate other cells implicated in microvascular function, like vascular smooth muscle cells (VSMC's), or pericytes. Effects of CsA on pericytes (brain pericytes) have been studied, but involved supra-pharmacological concentrations amounting to 6 mg/L, fifty times the trough level used in patients [17]. One of the explanations for a lack of effect on endothelial cell parameters was that many studies on CNI-toxicity have used doses that do not relate to those used in clinical medicine [16]. Studies involving CNI-effect on VSMC's have to our knowledge never been performed, either in vitro or clinically.

Secondly, the method of randomization used in the trial in chapter 2 might have contributed to the uneven distribution of renal function over the intervention group and the controls. That imbalance in renal function might have influenced the results of our trial, as discussed in the respective chapters. To explain that we have to emphasize that the trial design in chapter 2 was one of single-blind randomization, as were all belatacept-trials (no trial was ever done with sham infusions in the CNI arm). However, ours additionally used block-randomization, of 10-patient-blocks of 5 slots for each arm. Although many centres were participating in the trial, not all centres were constantly recruiting patients in the period between 2015 and 2018 (partly due to drug-scarcity in certain periods). In fact, in some periods, just one or two centres were actively recruiting, and that information was known to the researchers. For that reason, it is conceivable that the block-design might have contributed to uneven distribution of renal function between study arms. For example, a local researcher, knowing that three slots for belatacept had already been filled in the current block, might have been influenced to not include a patient with low GFR in the study, for fear of his patient to have to continue on CNI. Although it remains speculative whether block-randomization was indeed the underlying cause of the uneven distribution of GFR in our study, some literature exists to support the concept of uneven distribution by block-randomization [18].

Lastly, the magnitude of the effect of conversion to belatacept on kidney function deserves some discussion. First, we should emphasize at this point that the power calculation underlying the inclusion of approximately 110 patients into the trial in chapter 2 was based upon the primary endpoint in that study, the CV-risk-calculator. The cardiovascular risk was expected to decline by 30% through conversion, and that expectation was based on a calculated risk reduction for MACE of 31.2% in the BENEFIT, and 40% in the BENEFIT-EXT-study [19]. However, the corresponding changes in eGFR in assuming that reduction were never realized in conversion from TAC to belatacept. Recent studies have re-

vealed that the improvement of eGFR upon conversion from TAC to belatacept was smaller than in the earlier conversion from CsA to belatacept. The largest such study included 447 patients, of which 80% used TAC. The difference in eGFR two years after conversion was 5.7 ml/m/1.73m² after one year, 7 ml/min/1.73m² after two [4]. Another conversion study randomized 173 patients including 56% on TAC. There, an improvement in eGFR of 5.7 ml/m/1.73m² was noted after three years [3]. These studies were not all available at the time we designed the trial in chapter 2, where we based our expectations mainly on the CsA-conversions in the BENEFIT-trials. Those showed improvements of approximately 13 (1 year) to 20 ml/min/1.73m² (after 7 years) for the conversion of CsA to belatacept, in spite of the initially increased chances of rejection [8]. In conclusion, an improvement in renal function is also proven for conversion from TAC, but the magnitude of the effect was much lower than for CsA. Despite the mentioned explanations, we were still surprised to find no improvement in GFR in our study, since it is the first study on conversion from CNI to belatacept ever published that did not show that. As explained above, since the power calculation was based on an overestimation of the decline in cardiovascular risk, an element of underpowering regarding the effect on renal function might also be responsible. Other findings in this thesis merit mentioning as well. First of all, we again corroborated the higher incidence of rejection for belatacept compared to TAC in chapter 2. However, all rejections fell into the group of patients converted within one year after transplantation. This finding matches the reports by Adams, Budde et al., indicating that rejection under belatacept-based triple therapy mainly occurs in the first year after transplantation [4, 20]. Possibly, the strength of the immune system of patients after kidney transplantation is still recovering after that period, consistent with our findings on TTV-titers described in chapter 4. There, we found that time after transplantation negatively correlates with TTV-load, and that this load is decreasing even after years after transplantation. In that same chapter, we also found that belatacept seems to mitigate the downward trend of the TTV-load. In the future, a low TTV-load might become one of the parameters on which to base decisions regarding immunosuppressive drug prescription.

Finally, in chapter 2 we found that conversion to belatacept led to slightly more side-effects, including UTI's and upper respiratory infections. We did not see any opportunistic infections, or cancer. There is no consensus in the transplant community regarding the risk of getting opportunistic infections after conversion to belatacept. Recently, a French study described the outcomes after conversion from TAC to belatacept of 311 patients, and compared these to a matched cohort where CNI was continued [21]. CMV was noted slightly more often, but the difference was not significant. No difference was seen for other opportunistic

infections. Another French study found that the risk of opportunistic infections after conversion was inversely related to eGFR [22]. In chapter 3, we reported that treatment with belatacept leads to a decrease in numbers of circulating T-cells, B-cells and CD14NM. Although this effect should be corroborated in larger trials, it sounds reasonable to hypothesize that a partial depletion of immune cells can increase the chance of opportunistic infection in KTR's.

CURRENT AND FUTURE INDICATIONS FOR BELATACEPT.

Considering the multitude of factors discussed above, the question arises if and when belatacept should be used as an immune-suppressive drug in kidney transplant recipients. In our opinion, TAC should still be considered the backbone of standard immunosuppression in kidney transplantation, as it has been for almost two decades now. For those patients that experience side effects, including acute nephrotoxicity, thrombotic micro-angiopathy, post-transplant diabetes or biopsy-proven signs of toxicity, we believe belatacept is a viable option, but with consideration of some criteria. Most importantly, we do not advocate conversion to belatacept within the first year of transplantation when side effects of TAC are not clearly present. From the first year after transplantation onwards, the higher chance of rejection is most probably mitigated efficiently, whereupon conversion can be considered. A gradual introduction such as the scheme described by researchers from Emory University in Atlanta is a useful consideration [20]. Second of all, we do not consider belatacept to be a less potent immunosuppressive option. For patients particularly at risk for opportunistic infections or cancer, conversion should only be considered in circumstances where continuation of TAC is reasonably contra-indicated. EBV-seropositivity is a requirement for all potential conversion candidates. Importantly, belatacept is always to be combined with other immunosuppressive drugs. By and large, the other drugs in the regimen are low-dose prednisone and MMF, as in this thesis. When considering a conversion, the physician should consider whether his patient can start to use or continue to use MMF. For that reason, there should be some hesitation in giving belatacept to patients with a GFR under 25 ml/min/1.73m², as metabolites of MMF can accumulate with declining renal function and possibly increased risk of adverse effects [23].

At this point, roughly 15 years after the introduction of belatacept in clinical transplantation it is also worthwhile to consider current developments in transplantation, especially in the field of co-stimulatory drug development. Belatacept continues to be an interesting option because of its capacity to block

development of DSA's. However, its intravenous use is an issue that complicates its broad uptake. Despite efforts to develop a subcutaneous method of administration, that development seems to be halted for belatacept (clinicaltrials.gov NCT00569803). Other drugs possibly provide more promising options in the near future. Interestingly, abatacept, a CTLA4-pathway blocker similar to belatacept, was used instead of belatacept in 176 patients during the COVID-19 pandemic, to make sure patients would not have to visit the hospital in that period. In contrast to belatacept, abatacept can be used subcutaneously, facilitating patients to self-isolate during the pandemic. In this pragmatic real-life experiment, 171 patients (97.2%) survived with a functioning graft for three months. Two patients died, and three patients lost their graft. Those three already had severe graft dysfunction at the time of conversion to belatacept. Since there was no control group, no hard conclusion can be made, but the authors themselves interpreted these results to generally indicate safety and feasibility of using abatacept in KTR's [24]. Despite the fact that abatacept is as old as belatacept with a much lower affinity for CD86 and thus a less potent CD28-blockade, other small reports are available that could favour future use of this subcutaneous CTLA4-Ig-agonist [25]. The field of xeno-transplantation has accelerated the development of newer co-stimulatory options. Tegoprubart, an antagonist to CD40-ligand in the CD40-CD40L-pathway was used in the early reports on porcine kidney transplantation into a human recipient, and it was also used in porcine heart transplantation [26, 27]. Of note, the heart-transplantation case was complicated by humoral rejection despite T-cell depletion, complement blockade and tegoprubart. A phase II study that compares its use to tacrolimus is currently underway (Clinicaltrials.gov ID NCT05983770) and results are expected in the near future. Frexalimab is an anti-CD40L antibody also blocking the CD40-CD40L-costimulatory pathway. It is currently being investigated in auto-immune diseases like multiple sclerosis and SLE [28]. Since its mechanism is similar to tegoprubart, trials in kidney transplant recipients could be on the horizon, whereas its subcutaneous administration is an important upside, facilitating the future clinical implementation of frexalimab. VEL-101 targets CD28 directly, without suppressing signals down-stream from CTLA4. This potentially leaves the proliferation of regulatory T-cells intact, providing a promising mix of potent suppression of immunity against donor-derived cells and antigens, while maintaining anti-infectious immunity more or less intact. A study in non-human primates showed prevention of allo-immunization in combination with tacrolimus. However, without it, half the animal subjects developed graft rejection [29]. Other drugs involving costimulatory pathways are reviewed by Yakubu et al [30].

In diagnostics, the last decade has seen increased interest in recognizing early manifestations of allo-recognition of the graft by the recipient immune system. It is now commonplace to periodically screen for the presence of DSA's. A growing number of transplant centres screen additionally for donor-derived cell-free DNA (dd-cf-DNA), a marker of graft injury which provides additional value in assessing potential antibody-mediated rejection on top of DSA-detection [31]. Possibly, combining data derived from these parameters can guide indications for biopsy or a switch in immunosuppression. A couple of years ago, the iBox-score was developed, which combines clinical, histological and immunological data to help predict graft longevity [32]. Furthermore, evaluation of graft biopsies will increasingly be supplemented with modern diagnostic techniques. More specifically, classic histology and immune-fluorescence will be supplemented by molecular genetic methods and single cell proteomics that can help recognize early patterns of rejection and possibly drug-toxicity.

The next generation of co-stimulation-blockers, including tegoprubart, frexalimab and VEL-101 will hopefully find its way to clinical use through high-level randomized controlled trials, against the current gold-standard, which is still tacrolimus. The approach we followed in this thesis to elucidate mechanistic differences between belatacept and CNIs can be expanded in future studies to acquire more detailed knowledge into the mechanisms behind co-stimulation blockade. However, that research, though urgently needed to understand why belatacept affects cell differentiation and the formation of DSA, will probably not all be performed with belatacept, but with these newer agents as the intervention. Such research should be combined with novel markers of allo-immunity, such as dd-cf-DNA and composite prognostic models like the iBox-score. When biopsies are performed, differences in long-term nephrotoxicity between co-stimulation blockers and tacrolimus can also be revealed. A focus on the differential effect of both drugs on other cells like VSMC's and pericytes can give insight into the contribution of these cells to endothelial dysfunction. These trials are warranted, because a significant proportion of patients treated with TAC still face side-effects for which co-stimulation can be a viable alternative. By comparing co-stimulators to TAC, using the approach in this thesis, we can not only learn more about the newest generation of costimulatory drugs, but also expand our knowledge on a key drug we have been using for over two decades. That way, we can use the expanding spectrum of immunosuppressive drugs in renal transplantation wisely, and maximize both patient perspectives, and the utilization of health care resources.

REFERENCES

1. Bredewold, O.W., et al., *Cardiovascular Risk Following Conversion to Belatacept From a Calcineurin Inhibitor in Kidney Transplant Recipients: A Randomized Clinical Trial*. *Kidney Med*, 2023. 5(1): p. 100574.
2. Vincenti, F., 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): p. 535–46.
3. Grinyó, J.M., 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): p. 587–594.
4. Budde, K., et al., *Conversion from Calcineurin Inhibitor to Belatacept-based Maintenance Immunosuppression in Renal Transplant Recipients: a Randomized Phase 3b Trial*. *J Am Soc Nephrol*, 2021.
5. Ahmad, F., et al., *Current Concepts on 6-sulfo LacNAc Expressing Monocytes (slanMo)*. *Front Immunol*, 2019. 10: p. 948.
6. Bredewold, O.W., et al., *Conversion from calcineurin inhibitors to belatacept-based immunosuppressive therapy skews terminal proliferation of non-classical monocytes and lowers lymphocyte counts*. *Transpl Immunol*, 2024. 82: p. 101976.
7. Kim, E.J., et al., *Costimulation blockade alters germinal center responses and prevents antibody-mediated rejection*. *Am J Transplant*, 2014. 14(1): p. 59–69.
8. Vincenti, F., et al., *Belatacept and Long-Term Outcomes in Kidney Transplantation*. *N Engl J Med*, 2016. 374(4): p. 333–43.
9. van Rijn, A.L., et al., *Torque teno virus load as marker of rejection and infection in solid organ transplantation - A systematic review and meta-analysis*. *Rev Med Virol*, 2023. 33(1): p. e2393.
10. Bredewold, O.W., et al., *Attenuation of Torque teno viral load over time in kidney transplantation recipients treated with calcineurin inhibitors is mitigated after conversion to belatacept*. *J Med Virol*, 2024. 96(9): p. e29905.
11. Dane, M.J., et al., *Association of kidney function with changes in the endothelial surface layer*. *Clin J Am Soc Nephrol*, 2014. 9(4): p. 698–704.
12. Bijkerk, R., et al., *Acute Rejection After Kidney Transplantation Associates With Circulating MicroRNAs and Vascular Injury*. *Transplant Direct*, 2017. 3(7): p. e174.
13. Vandenbussche, C., et al., *Tacrolimus-induced nephrotoxicity in mice is associated with microRNA deregulation*. *Arch Toxicol*, 2018. 92(4): p. 1539–1550.
14. Lorenzen, J.M., et al., *MicroRNA-24 Antagonism Prevents Renal Ischemia Reperfusion Injury*. *Journal of the American Society of Nephrology*, 2014. 25(12): p. 2717–2729.
15. Postma, R.J., et al., *Novel Morphological Profiling Assay Connects ex Vivo Endothelial Cell Responses to Disease Severity in Liver Cirrhosis*. *Gastro Hep Adv*, 2024. 3(2): p. 238–249.
16. Boittin, F.X., et al., *The immunosuppressant drug Cyclosporin A aggravates irradiation effects in endothelial cells*. *Biochem Biophys Res Commun*, 2022. 602: p. 127–134.
17. Takata, F., et al., *Inhibition of transforming growth factor-beta production in brain pericytes contributes to cyclosporin A-induced dysfunction of the blood-brain barrier*. *Cell Mol Neurobiol*, 2007. 27(3): p. 317–28.

18. Efird, J., *Blocked randomization with randomly selected block sizes*. Int J Environ Res Public Health, 2011. 8(1): p. 15–20.
19. Soveri, I., et al., *The external validation of the cardiovascular risk equation for renal transplant recipients: applications to BENEFIT and BENEFIT-EXT trials*. Transplantation, 2013. 95(1): p. 142–7.
20. Adams, A.B., 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): p. 2922–2936.
21. Divard, G., et al., *Long-Term Outcomes after Conversion to a Belatacept-Based Immunosuppression in Kidney Transplant Recipients*. Clin J Am Soc Nephrol, 2024. 19(5): p. 628–637.
22. Bertrand, D., et al., *Opportunistic Infections and Efficacy Following Conversion to Belatacept-Based Therapy after Kidney Transplantation: A French Multicenter Cohort*. J Clin Med, 2020. 9(11).
23. MacPhee, I.A., et al., *Pharmacokinetics of mycophenolate mofetil in patients with end-stage renal failure*. Kidney Int, 2000. 57(3): p. 1164–8.
24. Bertrand, D., et al., *Conversion From Intravenous In-Hospital Belatacept Injection to Subcutaneous Abatacept Injection in Kidney Transplant Recipients During the First COVID-19 Stay-at-Home Order in France*. Transpl Int, 2023. 36: p. 11328.
25. Larsen, C.P., et al., *Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties*. Am J Transplant, 2005. 5(3): p. 443–53.
26. Kawai, T., et al., *Xenotransplantation of a Porcine Kidney for End-Stage Kidney Disease*. N Engl J Med, 2025. 392(19): p. 1933–1940.
27. Griffith, B.P., et al., *Transplantation of a genetically modified porcine heart into a live human*. Nat Med, 2025. 31(2): p. 589–598.
28. Fatima, T., et al., *Frexalimab (SAR441344) as a potential multiautoimmune disorder tackling mAB targeting the CD40-CD40L pathway undergoing clinical trials: a review*. Ann Med Surg (Lond), 2024. 86(12): p. 7305–7313.
29. Poirier, N., 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): p. 88–100.
30. Yakubu, I., et al., *Costimulation blockade: the next generation*. Curr Opin Organ Transplant, 2025. 30(2): p. 96–102.
31. Budde, K., et al., *Clinical utility of donor-derived cell-free DNA testing for kidney transplant monitoring in selected patients*. Kidney Int, 2025. 107(5): p. 792–796.
32. Loupy, A., et al., *Prediction system for risk of allograft loss in patients receiving kidney transplants: international derivation and validation study*. Bmj, 2019. 366: p. 14923.

A large, bold, black capital letter 'A' is positioned on the left side of a light gray square. The square is partially visible, with its right edge cut off by the frame. The letter 'A' is centered vertically within the square's height.

Nederlandse Samenvatting
Curriculum Vitae
Publicatielijst
Dankwoord

NEDERLANDSE SAMENVATTING

In de wereld van orgaantransplantatie wordt voortdurend gezocht naar nieuwe geneesmiddelen (immuunsuppressiva), met betere werking tegen afstoting en minder bijwerkingen. Die bijwerkingen zijn gevoeligheid voor infecties, en kanker, maar daar komt nog bij dat de standaardbehandeling met calcineurineremmers de kans op hoge bloeddruk, nierfalen en diabetes verhoogt. Die aandoeningen vormen nu juist de reden dat veel patiënten überhaupt een nieuwe nier nodig hebben. Daardoor ontstaat de paradox dat de sterkste medicijnen tegen afstoting zelf ook nierschade geven. Deze middelen hebben de uitkomsten van niertransplantatie gunstig beïnvloed, maar kunnen tegelijkertijd de nierfunctie op de lange termijn verslechteren en bijdragen aan cardiovasculaire complicaties. Een illustratie van deze toxiciteit is dat ontvangers van een transplantaat anders dan de nier, zoals een long, hart of lever, 20% kans hebben om nierfalen te ontwikkelen. Tegen deze achtergrond wordt er voortdurend gezocht naar minder toxische alternatieven. Belatacept is zo'n alternatief: een immuunsuppressivum zonder directe nefrotoxische effecten dat in vroege studies werd geassocieerd met een betere nierfunctie en een gunstiger cardiovasculair risicoprofiel. Kanttekening is het feit dat er wel een fors verhoogde kans op afstoting lijkt te bestaan, zeker vroeg na transplantatie. In deze context ontstond het idee voor het wetenschappelijk onderzoek dat ten grondslag ligt aan dit proefschrift: het vergelijken van de effecten van klassieke calcineurine-remmers met het 'nieuwe' belatacept op een aantal domeinen waarop immuunsuppressie invloed heeft: de immunologie, hart- en bloedvaten, en de gevoeligheid voor infectie. En dat in een vertraagde setting: pas minimaal 3 maanden na transplantatie. De hypothese was dat belatacept positief uit die vergelijking zou komen.

In hoofdstuk 2 wordt een grote internationale studie beschreven waarin niertransplantatie-patiënten werden vervolgd voor wie door middel van loting (randomiseren) werd bepaald of ze hun calcineurineremmer bleven gebruiken, of werden omgezet naar belatacept. De uitkomstmaat van deze studie was een toen net ontwikkelde risico-calculator die voor transplantatie-patiënten specifiek het risico op hart- en vaatziekten kon schatten. De hypothese achter die studie was dat overstappen op belatacept dat risico zou verlagen. Echter, na een jaar follow-up bleek er geen verschil in het berekende risico tussen beide groepen. Ook afzonderlijke componenten van deze risicoschatting, zoals hoogte van het cholesterol, nierfunctie en het risico op diabetes, verschilden niet wezenlijk. Wel werd bij patiënten die belatacept gebruikten een lichte verbetering van de bloeddruk waargenomen. Deze uitkomsten stonden haaks op eerdere studies, waarin steeds een verbetering van de nierfunctie werd gevonden, alsmede een duidelijke verbetering van risicofactoren voor hart- en vaatziekten.

In het hoofdstuk worden verschillende mogelijke verklaringen aangedragen om te verklaren waarom dit verschil nu zo klein was. Een van die verklaringen komt voort uit het feit dat je een klein verschil in risico statistisch alleen kan aantonen in een grote groep patiënten. Omdat het verschil in risico in dit experiment onverwachts klein bleek, was de onderzoeksgroep in retrospect te klein gekozen. Een andere verklaring ligt mogelijk in het feit dat de patiënten die belatacept kregen een slechtere nierfunctie hadden voor de omzetting, dan de mensen die doorgingen met calcineurineremmer. Tenslotte zou er sprake kunnen zijn van het 'uitselecteren' van patiënten die de grootste last hebben van bijwerkingen van calcineurineremmers, mogelijk al vóór deelname. De conclusie van dit hoofdstuk is dus verrassend, maar vormt wel ondersteuning voor de huidige klinische praktijk, waarin lagere doseringen tacrolimus worden voorgeschreven dan in het verleden.

Het LUMC participeerde in dezelfde periode ook in een tweede internationale studie met een vergelijkbaar ontwerp. Het was daarom mogelijk om het patiëntencohort uit hoofdstuk 2 uit te breiden met een aantal patiënten die volgens dezelfde manier waren gerandomiseerd. Dit maakte het mogelijk om in de daaropvolgende hoofdstukken met een grotere groep patiënten onderzoek te doen naar secundaire effecten van het veranderen van immuunsuppressie. In hoofdstuk 3 werd onderzocht of belatacept leidt tot veranderingen in aantallen en kenmerken van in het bloed circulerende ontstekingscellen, met name monocyten, T-lymfocyten en B-lymfocyten. De hypothese was hier dat belatacept zou leiden tot een minder inflammatoir profiel van deze circulerende witte bloedcellen. In deze studie werd een specifieke subpopulatie van cellen (CD14NM, monocyten zonder het oppervlakte-eiwit CD14) geïdentificeerd, die niet of nauwelijks eerder werd beschreven. Deze CD14NM namen af bij patiënten die belatacept gebruikten, terwijl ze stabiel bleven bij patiënten op die calcineurineremmers doorgebruikten. Tegelijkertijd daalden de aantallen T- en B-lymfocyten, evenals de concentratie van de ontstekingsmarker TNF-alfa. Op basis van deze bevindingen wordt een theoretisch model voorgesteld waarin belatacept ingrijpt op de ontwikkeling van monocyten en daarmee indirect de activatie van het immuunsysteem remt. Dit model biedt een kader om de immunologische effecten van belatacept beter te begrijpen.

Van belatacept is een ogenschijnlijk paradoxaal effect bekend: het wordt geassocieerd met een hogere kans op afstoting in het eerste jaar na transplantatie, terwijl transplantatie-patiënten die ermee worden behandeld minder vaak antistoffen tegen hun donor vormen (DSA's: donor-specifieke antistoffen) dan patiënten die worden behandeld met CNI. Belatacept zou dus tegelijkertijd als 'zwakker' danwel 'sterker' immuunsuppressivum kunnen worden beschouwd. In

hoofdstuk 4 wordt onderzocht of middels meting van TTV (Torque Teno Virus) inzicht kan worden verkregen in deze paradox. TTV is een niet-ziekmakend virus waarvan de hoeveelheid in het bloed een weerspiegeling kan zijn voor de mate van immuunsuppressie. Er zijn verschillende studies gedaan die suggereren dat TTV meebeweegt op de kracht van ons immuunsysteem: het zit in hoge concentraties in het bloed van ouderen, en mensen die bijvoorbeeld een long-transplantatie hebben gehad, en het is relatief laag als het immuunsysteem (te) sterk is, zoals bij afstoting van een orgaan. In hoofdstuk 4 blijkt dat TTV-niveaus na transplantatie geleidelijk afnemen, maar dat deze daling wordt afgeremd bij patiënten die worden behandeld met belatacept. Dit suggereert dat belatacept niet per definitie een zwakkere vorm van immuunsuppressie is, ondanks het hogere risico op vroege afstoting.

In hoofdstuk 5 wordt onderzocht of belatacept minder toxisch is voor endotheelcellen als dat aangenomen wordt van calcineurineremmers. Endotheelcellen vormen de binnenste belijning van (microscopisch kleine) bloedvaatjes. Endotheeldysfunctie (ED) is een belangrijke vroege stap in het ontstaan van cardiovasculaire ziekte. ED werd op verschillende manieren gemeten, middels 'klassieke' biomarkers, alsook door het vergelijken van micro-RNA-profielen en een innovatieve setting waarbij endotheelcellen in het laboratorium worden blootgesteld aan verschillende stimuli, en waarbij de morfologische reactie van deze cellen kan worden geobserveerd. Deze techniek wordt 'cell-painting' genoemd. In geen van de genoemde experimenten werd een duidelijk voordeel van belatacept ten opzichte van calcineurineremmers gevonden. Wel bevestigde het onderzoek dat hoge doseringen calcineurineremmers schadelijk zijn voor endotheelcellen, vooral in het geval van ciclosporine. Deze bevindingen suggereren dat de endotheeltoxiciteit van calcineurineremmers bij de lage doseringen die tegenwoordig worden gebruikt, klinisch minder relevant is dan vaak wordt aangenomen, danwel dat de toxiciteit niet optreedt via een effect op deze cellen, maar op andere componenten van de bloedbaan. Die conclusie sluit aan op de bevindingen van hoofdstuk 2.

Samenvattend lijkt belatacept ten opzichte van CNI weinig duidelijke voordelen te laten zien op het gebied van cardiovasculaire gezondheid of endotheelfunctie. De meest opvallende effecten liggen op immunologisch niveau, met veranderingen in specifieke celpopulaties en virale markers. Verschillende methodologische beperkingen, zoals selectie van relatief stabiele patiënten en de korte follow-up, kunnen hebben bijgedragen aan het uitblijven van de verwachte gunstige effecten. Tegelijkertijd kunnen de resultaten ook positief worden geïnterpreteerd: de huidige klinische praktijk waarin lage doseringen tacrolimus worden gebruikt, lijkt de nadelige effecten van calcineurineremmers in belang-

rijke mate te hebben beperkt. Dit uiteraard met opnieuw de kanttekening, dat het onderzoek alleen is uitgevoerd bij die patiënten die calcineurineremmers goed verdragen.

In de toekomst zal het gebruik van belatacept waarschijnlijk vooral worden overwogen bij patiënten die evidente bijwerkingen hebben van calcineurineremmers, bij voorkeur na het eerste jaar na transplantatie. Er is een nieuwe generatie co-stimulatie-blokkers (de klasse medicijnen waartoe belatacept behoort) in ontwikkeling, en in de komende jaren zullen die worden geïntroduceerd in de markt. Diagnostische mogelijkheden naar transplantaat-dysfunctie nemen toe. Met moleculaire technieken kan steeds eerder worden vastgesteld dat het immuunsysteem een reactie maakt tegen onderdelen van het getransplanteerde orgaan. Deze vroege signalen, zoals het ontdekken van donor-DNA of DSA's in de bloedsomloop worden al gebruikt in voorspellingsmodellen, die kunnen worden verfijnd door gebruik van kunstmatige intelligentie. Dergelijke modellen zullen beter dan nu kunnen identificeren welke patiënten optimaal profiteren van (nieuwe) co-stimulatie-blokkers of juist van de oude standaard calcineurineremmers. De aanpak in dit proefschrift, waarin klinische uitkomsten worden gecombineerd met mechanistische analyses, kan aangevuld met moderne diagnostiek opnieuw worden toegepast in toekomstig onderzoek naar nieuwe immuunsuppressiva.

CURRICULUM VITAE

Obbo Winne Bredewold (roepnaam Edwin) werd geboren op 10 augustus 1976 te Deventer. Na zijn jeugd in Indonesië en Tanzania te hebben doorgebracht, keerde hij terug naar Epse, bij Deventer, waar hij zijn VWO-examen afrondde in 1995. Hij studeerde geneeskunde aan het Academisch Medisch Centrum/ Universiteit van Amsterdam. Hij combineerde zijn studie met wedstrijdroeien, en had zitting in het bestuur van studenten-roeivereniging Nereus alvorens zijn co-schappen te lopen. In 2004 behaalde hij zijn arts-examen in het AMC.

Na een periode te hebben gewerkt als arts-niet-in-opleiding in het Onze Lieve Vrouwe Gasthuis in Amsterdam startte hij met de opleiding tot internist in 2006, in het Rode Kruis-ziekenhuis te den Haag met dr. Rob Valentijn als bezielend opleider. In 2008 vervolgde hij zijn opleiding in het LUMC. Hij begon in 2009 met de differentiatie tot internist-nefroloog. In 2011 behaalde hij het C-formulier ten bewijze van het afronden van de opleiding tot internist, onder begeleiding van professor dr. J.H.W. Smit. Nadien bleef hij werken in het LUMC op de afdeling Interne Geneeskunde als internist-nefroloog.

In 2015 begon hij formeel aan een promotie-traject onder begeleiding van prof dr. A.J. van Zonneveld, initieel met als doel om biomarkers van cardiovasculaire ziektes te onderscheiden in een grote groep mensen met kransslagaderverkalking. Echter, al snel kreeg hij de kans om binnen de transplantatie-nefrologie onderzoek te doen naar het toen vrij nieuwe medicijn belatacept, middels het begeleiden van een internationaal wetenschappelijk onderzoek in samenwerking met wetenschappers uit Zweden, Denemarken en Noorwegen. Naast professor van Zonneveld begeleidde achtereenvolgens prof dr. J.H.W. de Fijter en later prof dr. J.I. Rotmans hem in het voltooien van dit proefschrift.

LIJST VAN PUBLICATIES

1. van Leeuwen, J.R., et al., Obinutuzumab in Rituximab-Intolerant Antineutrophil Cytoplasmic Antibody-Associated Vasculitis Patients. *Kidney Int Rep*, 2025. 10(4): p. 1288–1291.
2. van Schaik, M., et al., Long-term renal and cardiovascular risks of tacrolimus in patients with lupus nephritis. *Nephrol Dial Transplant*, 2024. 39(12): p. 2048–2057.
3. Bredewold, O.W., et al., Conversion from calcineurin inhibitors to belatacept-based immunosuppressive therapy skews terminal proliferation of non-classical monocytes and lowers lymphocyte counts. *Transpl Immunol*, 2024. 82: p. 101976.
4. Bredewold, O.W., et al., Attenuation of Torque teno viral load over time in kidney transplantation recipients treated with calcineurin inhibitors is mitigated after conversion to belatacept. *J Med Virol*, 2024. 96(9): p. e29905.
5. Bredewold, O.W., et al., Cardiovascular Risk Following Conversion to Belatacept From a Calcineurin Inhibitor in Kidney Transplant Recipients: A Randomized Clinical Trial. *Kidney Med*, 2023. 5(1): p. 100574.
6. van Leeuwen, J.R., et al., Compassionate Use of Avacopan in Difficult-to-Treat Antineutrophil Cytoplasmic Antibody-Associated Vasculitis. *Kidney Int Rep*, 2022. 7(3): p. 624–628.
7. Dirikgil, E., et al., ExploriNg DUrable Remission with Rituximab in ANCA-associatEd vasculitis (ENDURRANCE trial): protocol for a randomised controlled trial. *BMJ Open*, 2022. 12(9): p. e061339.
8. van Dam, L.S., et al., PR3-ANCA's predict relapses in ANCA-associated vasculitis patients after rituximab. *Nephrol Dial Transplant*, 2021. 36(8): p. 1408–1417.
9. Kraaij, T., et al., Long-term effects of combined B-cell immunomodulation with rituximab and belimumab in severe, refractory systemic lupus erythematosus: 2-year results. *Nephrol Dial Transplant*, 2021. 36(8): p. 1474–1483.
10. Zhang, H., et al., Prediction Power on Cardiovascular Disease of Neuroimmune Guidance Cues Expression by Peripheral Blood Monocytes Determined by Machine-Learning Methods. *Int J Mol Sci*, 2020. 21(17).
11. van Dam, L.S., et al., Highly Sensitive Flow Cytometric Detection of Residual B-Cells After Rituximab in Anti-Neutrophil Cytoplasmic Antibodies-Associated Vasculitis Patients. *Front Immunol*, 2020. 11: p. 566732.
12. Teng, Y.K.O., et al., An evidence-based approach to pre-pregnancy counseling for patients with systemic lupus erythematosus. *Rheumatology (Oxford)*, 2018. 57(10): p. 1707–1720.
13. Kraaij, T., et al., The NET-effect of combining rituximab with belimumab in severe systemic lupus erythematosus. *J Autoimmun*, 2018. 91: p. 45–54.

14. Bijkerk, R., et al., MicroRNA-132 controls water homeostasis through regulating MECP2-mediated vasopressin synthesis. *Am J Physiol Renal Physiol*, 2018. 315(4): p. F1129–F1138.
15. Kraaij, T., et al. Poster session: Synergetic B-Cell Immunomodulation with Rituximab and Belimumab Is Clinically Effective in Severe and Refractory Systemic Lupus Erythematosus - the Synbiose Proof-of-Concept Study. in *Annals of the Rheumatic Diseases*. 2017.
16. Kraaij, T., et al., TAC-TIC use of tacrolimus-based regimens in lupus nephritis. *Lupus Sci Med*, 2016. 3(1): p. e000169.
17. Trappenburg, M.C., et al., Elevated numbers and altered subsets of procoagulant microparticles in breast cancer patients using endocrine therapy. *Thromb Res*, 2011. 127(4): p. 363–9.
18. Bredewold, O.W., J.W. de Fijter, and T. Rabelink, A case of mononucleosis infectiosa presenting with cholemic nephrosis. *NDT Plus*, 2011. 4(3): p. 170–2.
19. Bauer, M.P., et al., [Recurrence of *Clostridium difficile*-associated diarrhoea prevented by the administration of a whey concentrate from specifically immunised cows; prospective study]. *Ned Tijdschr Geneesk*, 2008. 152(35): p. 1919–26.

DANKWOORD

Het zal niemand ontgaan zijn dat er erg veel tijd is besteed aan de totstandkoming van dit proefschrift. Hieronder wil ik eenieder bedanken die tijd, toewijding, doorzettingsvermogen, wijsheid en expertise heeft ingezet om me te helpen dit project tot een goed einde te brengen.

Allereerst de mensen die daadwerkelijk arbeid hebben verricht, zonder wie ik hier echt nooit zou hebben gestaan. Jacques, met jou ben ik destijds begonnen, met toen nog het CTMM-project als onderwerp. Duizenden micro-RNA's geanalyseerd, die maar zeer ten dele hierin terecht zijn gekomen. Hans, jij vroeg me op een dinsdagmiddag of ik woensdagochtend mee wilde vliegen naar Zweden, en dat was het startpunt van de belatacept-studie. Van die vroege woensdagochtend op Arlanda pluk ik vandaag de vruchten. Mijn uiteindelijke reis duurde langer dan dat jij in Leiden bleef zitten, maar ik ben je zeer dankbaar voor je initiatief en je begeleiding. Annemarie, samen met Hetty alle cellen afgedraaid en tot in de late uurtjes ge-FACS-ed. Jasmin, Christy, Sandra, Inger, met de speciaal hiervoor in huis gehaalde micro-scan alle wangslimvliezen ge-echood. Tevens alle internationale samples verzameld en koud bewaard. Erin, dankzij jou werd hoofdstuk 4 over TTV's een supersnel succes. Rudmer, je cell-painting essay was de kers op de taart, dank voor je tijd, ook voor analyse en advies. Barend en Hanneke, jullie hulp bij het rekruteren van patiënten was goud waard, blij dat jullie hier nog rondlopen nu het af is. Hetty, jij hebt lange tijd gefungeerd als degene die het circulating-cells project blijvend stuwde. Je voltooide de FACS-experimenten, en hielp enorm bij het manuscript. En vooral: je bleef me motiveren om het af te schrijven. Ik hoop dat je van je zeilboot geniet. Elke, we spendeerden vele uren aan een experiment dat we uiteindelijk nooit beschreven, maar je liet me wel zien dat samenwerken heel motiverend werkt.

Thanks to Joe, who I still hope to meet somewhere for a beer sometime. Also to Annette, Mariet, Hans, Josip, Anders, Olof, Halvard, Alan, Lars, Wouter, Karin, Roel, Knut, Soeren, Inga and My, for patiently and critically reading and re-formulating my manuscripts. My special regards go out to Bengt, as I have you to thank for re-igniting the spark to write chapter 2, that I had lost after COVID19. You really have been of crucial importance in that process.

Aan alle fijne collega's van de sectie nierziekten: dank voor de blijvende motivatie om af te maken waar ik ooit aan begonnen ben. Samenwerken in de kliniek met jullie is echt een dagelijks plezier waar ik nooit, of in ieder geval nog lang niet, zonder hoop te hoeven. In geheel willekeurige volgorde: (weer) Hanneke, Ton, Soufian, Aiko, Paul, Andre, Wieneke, Marije, Siebe, Wouter, Tineke, Ramzi,

Raju, Matthijs, Eline, Koen, Esther, Stephanie, Mieke, Tessa, Martine, Judith, Gelisa, Shakeel, you rock! Laten we niet vergeten af en toe hard te spelen.

Anton-Jan, je bent 11 jaar lang onafgebroken enthousiast, toekomstgericht, positief en inspirerend geweest, en zonder die eigenschappen was ik afgehaakt. 'Je kan elke dag opnieuw beginnen' was je motto, en dat heeft zich uiteindelijk uitbetaald: je hebt van deze dokter een doctor weten te maken. Joris, jij hebt me uiteindelijk de spiegel voorgehouden die ik nodig had: doorgaan op hetzelfde pad, of het radicaal omgooien: concrete doelen stellen, met tussenevaluaties, deadlines, nieuwe projecten en mezelf committeren aan het leven van een pHd-student, met als uiteindelijk resultaat dat ik hier vandaag sta. Thanks vriend: laten we onze andere hobby's nu ook weer oppakken, samen , Lowlands?

Aan mijn paranimfen; Argho, klinische inzichten, lastige dilemma's en creatieve oplossingen in de nefrologie: ik haal ze altijd het liefst op bij jou. Superscherpe spitsvondige en vasthoudende dokter alleskunner, dank dat je vandaag ook op wetenschappelijk vlak naast me wil staan. Michel, levenslange boezemvriend: dank voor je vriendschap. Quando se...?

Lieve papa, mama, Francine, Leffert, Margriet en Harro. In onze familie werd aan tafel geleerd scherp te discussiëren, kritisch om te gaan met alle informatie die we ontvingen uit welke bron dan ook. Oeverloos, tot in de late uurtjes. Tot irritatie aan toe. Maar altijd met liefde, waardering en respect voor elkaars argumenten. Ik heb dat oeverloze wel eens vervloekt, doe dat nog steeds wel eens, maar stiekem heb ik er ook veel gebruik van gemaakt. Dank daarvoor, en ik hoop dat we als familie nog vaak samenkomen en de discussie voortzetten.

Lieve Simon en Fenne, jullie zijn echt het allerbelangrijkste in mijn leven. Met jullie samen balspelletjes te doen, met jullie op vakantie te gaan, rugby te kijken, hockey te kijken, te skiën, te sporten, vakanties te vieren en te praten over de wereld, over later, over relaties, schoolleven en thuis, ik hoop er altijd van te mogen genieten. Jullie maken het leven mooi. Lieve Jes, ik kan me niet voorstellen dit, of wat dan ook ooit alleen te moeten hebben gedaan. We delen al 20 jaar alles samen. Onze liefde voor elkaar, die voor de kinderen, P&J, sportieve vakanties, strand, en onze hernieuwde liefde voor uitgaan en short-trips naar overal. Ooit zat ik gebroken op de voorste rij bij jouw promotie. Nu jij bij de mijne. Ik leef nog lang en gelukkig met je.

