New concepts for personalized immunosuppression in the transplanted patient

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Table of contents

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List of papers

I. **RA Klaasen**, S Bergan, S Bremer, L Daleq, AM Andersen, K Midtvedt, MH Skauby, NT Vethe
Longitudinal Study of Tacrolimus in Lymphocytes During the First Year After Kidney Transplantation
Ther Drug Monit 2018; 40: 558-566.

II. **RA Klaasen***, EJ Egeland*, J Chan, K Midtvedt, K, M Svensson, N Bolstad, B Fellstrom, H Haldaas, A Åsberg, S Bergan, NT Vethe, DJ Warren
A Fully Automated Method for the Determination of Serum Belatacept and Its Application in a Pharmacokinetic Investigation in Renal Transplant Recipients.
Ther Drug Monit 2019; 41: 11-18.
(* Contributed equally as first author)

III. **RA Klaasen,** S Bergan, S Bremer, K Hole, C Nordahl, AM Andersen, K Midtvedt, MH Skauby, NT Vethe
Pharmacodynamic assessment of mycophenolic acid in resting and activated target cell population during the first year after renal transplantation
Br J Clin Pharmacol 2019; published online ahead of print, 2020 Jan 11

Abbreviations

6-MP	6-mercaptopurine
AcMPAG	Acyl mycophenolic acid glucuronide
APC	Antigen presenting cell
APRT	Adenine phosphoribosyltransferase
BPAR	Biopsy-proven acute rejection
CD	Cluster of differentiation
CMIA	Chemiluminescent microparticle immunoassay
CMV	Cytomegalovirus
CV	Coefficient of variation
dnDSA	*De novo* donor specific antibodies
FDA	Food and Drug Administration
IL	Interleukin
IMP	Inosine 5'-monophosphate
IMPDH	Inosine-5'-monophosphate dehydrogenase
LLoQ	Lower limit of quantification
MPA	Mycophenolic acid
MPAG	Mycophenolic acid-7-O-glucuronide
MRP2	Multidrug resistance-associated protein 2
NAD	Nicotinamide adenine dinucleotide
NFAT	Nuclear factor of activated T-cells
NODAT	New onset diabetes after transplantation
PBMC	Peripheral blood mononuclear cell
P-gp	P-glycoprotein
TDM	Therapeutic drug monitoring
V_d	Volume of distribution
XMP	Xanthosine 5'-monophosphate

Thesis summary

After renal transplantation, monitoring and adjustment of the immunosuppressive treatment is central. However, rejections and adverse events are still occurring even if drug concentrations are kept within predetermined concentrations. Further development of monitoring practices is therefore warranted. The aim of this thesis was to develop novel assays for therapeutic monitoring of immunosuppressive drugs, and to obtain new knowledge on the pharmacokinetics of tacrolimus and belatacept and the pharmacodynamics of mycophenolic acid (MPA) in renal transplant recipients.

The thesis presents a method for measuring tacrolimus inside a lymphocyte-enriched population (peripheral blood mononuclear cells; PBMC) and the method was applied in a cohort of renal transplant recipients during the first year after transplantation. Intracellular tacrolimus was modestly correlated to whole blood concentrations. Although whole blood concentrations increased from before to 1.5 hours after dose at all study days during the year, the intracellular concentrations had a marked increase after dose early (6- 9 days) after transplantation whilst remaining stable from before to after dose at later time points (>5 weeks after transplantation).

Belatacept is a therapeutic protein and represents a new class of action of immunosuppressive drugs. No assay was available for research or routine monitoring. A ligand-binding assay for measuring belatacept was developed and used to determine pharmacokinetic variables.

MPA limits proliferation of lymphocytes by inhibiting inosine monophosphate dehydrogenase (IMPDH), an enzyme being central in the synthesis of guanine- and deoxyguanine nucleotides. IMPDH-activity, as well as guanine and adenine, was measured in *ex vivo* stimulated and non-stimulated PBMC in renal transplant recipients during the first year after transplantation in a longitudinal setting. MPA showed a stronger inhibitory effect on IMPDH and reduced the guanine and adenine levels in stimulated PBMC, whilst IMPDH was only partially inhibited and the guanine or adenine levels were not altered in non-stimulated PBMC. During the first year after transplantation, PBMC appeared to become less sensitive towards MPA.

1 Background

1.1 History of renal transplantation and early immunosuppression

Although the idea of replacing organs has been proposed for millennia, the first attempts to transfer a kidney into a human was not done until 1906, when Mathieu Jaboulay successfully sutured a pig and a goat kidney into two human recipients [1]. Although initial renal function was observed, neither kidneys nor patients survived for more than a few days. After extensive research done in the first half of the 20th century, it was concluded that the loss of a renal graft was likely due to an immunological response. A genetic match between donor and recipient and inhibition of the recipient's immune system through radiation reduced the risk of rejection. The advantage of matching donor and recipient resulted in Joseph Murray to overcome the immunological challenge by transplanting a kidney between identical twins in 1954 [2]. From 1958 to 1962 several transplantations between non-identical twins and non-twins were shown to be successful utilizing total body irradiation to suppress the immune system [3].

Pharmacological immunosuppression had its first appearance as 6-mercaptopurine (6-MP) in 1959 when it was shown that the drug gave some extension of renal graft survival in humans. Although better than irradiation, the overall survival of patients on 6-MP alone was generally less than 6 months. In 1963, a conference organized by the US National Research Council summarized the current status of renal transplantation. Although the immediate loss of graft function had been overcome to some extent, only 10% of patients that received irradiation were alive at 3 months after transplantation and patients receiving 6-MP had similar outcomes.

Contrasting these grim outcomes was the immunosuppressive protocol presented by Thomas Starzl at the same conference, who showed that combining prednisone and azathioprine (a 6-MP derivate) resulted in one-year survival of >70%. The impact of this discovery can be illustrated in the increase of US transplant programs; within a year after his publication the number of programs had increased from three to fifty [3]. The practice of combining drugs would form the future basis of all immunosuppressive treatment after transplantation.

1.2 Calcineurin inhibitors

The combination of azathioprine and steroids vastly improved outcomes after solid organ transplantation, but there was still a great need for improvement. The next step forward started in 1971, when a fungal extract containing cyclosporine A was identified. Cyclosporin A was the first inhibitor of calcineurin, an enzyme central in the adaptive immune response, and was shown to have considerable immunosuppressive effect without major cytotoxic effects [4]. Clinical studies showed that by adding cyclosporine A to the immunosuppressive regimen after transplantation, the patient survival was considerably improved [5,6]. When compared in a randomized trial, patients on cyclosporine A enjoyed a one year graft survival rate of 72% vs 52% in patients with azathioprine and prednisolone, an improvement of 38% [7].

Cyclosporine A received FDA approval in 1983 as an immunosuppressant for prevention of solid organ allograft rejection in combination with azathioprine and steroids. This triple regimen of immunosuppressants consisting of cyclosporine A (a calcineurin inhibitor), azathioprine (a proliferation inhibitor) and steroids, forms the basis of the protocol for immunosuppressive treatment after solid organ transplantation still in use today [8]. Other pharmaceuticals have largely replaced cyclosporine A and azathioprine, but the principle of suppressing the immune response through three points of actions largely remains.

Cyclosporine A improved short-term survival after transplantation, but it soon became apparent that cyclosporine A had a pronounced nephrotoxic effect resulting in a gradual decrease in renal function. This inspired a search for other immunosuppressive drugs. In 1987 researchers published a paper on the novel macrolide FK506, which they had isolated from a strain of *Streptomyces* [9]. The new compound, later termed tacrolimus, was shown to have a similar mechanism of action as cyclosporine A, but with a 100-fold higher potency [10,11]. In a series of animal studies published in 1987-88, tacrolimus was shown to prevent heart allograft rejection in rats [12,13] , rejection of renal [14,15] and liver [16] allografts in dogs and renal allografts in baboons [17]. Some of the first clinical experiences came from rescue attempts for liver transplant recipients who either had a failing allograft and/or renal dysfunction due to cyclosporine A toxicity. Tacrolimus improved the outcome in 70% of these patients [18,19]. Encouraged by these results, a randomized controlled study comparing tacrolimus to cyclosporine A

in *de novo* liver transplant recipients was initiated. Tacrolimus outperformed cyclosporine A with a rejection free survival after one month of 61% vs. 18% [20]. When tacrolimus was given in combination with glucocorticoids to primary and secondary renal transplant recipients the graft survival rate was 80% [21,22]. Following successful phase III trials, where tacrolimus resulted in lower one year rates of biopsy proven acute rejections (BPAR) compared to cyclosporine A (30.7% vs 46.4%) [23], it gained marketing approval in 1993 (Japan) and 1994 (USA). Large multicenter studies have later confirmed the superiority of tacrolimus over cyclosporine A [24,25].

Although it was hoped that tacrolimus would not have the nephrotoxic effects as cyclosporine A, early clinical experiences showed that this was not the case [26]. When tacrolimus concentrations immediately before next dose, also known as trough concentration, were kept above 8 µg/L, there were no differences in estimated glomerular filtration rate half a year after transplantation compared to cyclosporine A [27]. However, minimization of tacrolimus dose gave an increased renal function compared to cyclosporine A without increasing the risk of rejections [25], indicating that the nephrotoxicity is concentration-dependent. This formed the current immunosuppressive protocol after renal transplantation in Norway, in which tacrolimus is the preferred choice of calcineurin inhibitor for new patients. Tacrolimus has gradually been overtaking cyclosporine A as the most commonly prescribed calcineurin inhibitor (Figure 1).

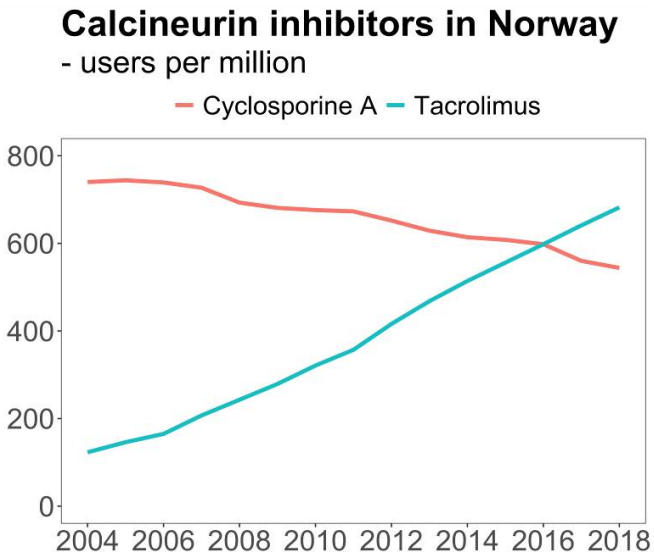

Figure 1: Users of calcineurin inhibitors in Norway between 2004 and 2018. Data from Norwegian prescription registry, accessed February 2020

Besides nephrotoxicity, both calcineurin inhibitors can result in the development of new onset diabetes after transplantation (NODAT). Although tacrolimus is less nephrotoxic than cyclosporine A, it has an increased risk of NODAT. In a direct comparison between tacrolimus and cyclosporine A, the immunosuppressant tacrolimus gave a higher incidence rate of NODAT at six months after transplantation (33.6% vs 26.0%) [27]. Calcineurin inhibitors also have an unfavorable cardiovascular profile. In renal transplant recipients approximately 30% of patients on either cyclosporine A or tacrolimus had cardiovascular complications during Phase III trials [28] and more than 50% of both liver [29] and renal [28] recipients need antihypertensive treatment when receiving calcineurin inhibitors.

1.3 Co-stimulatory pathway inhibitors

It was hoped that tacrolimus would solve the problem of gradual loss of renal function seen with cyclosporine A. Although the renal function was improved with minimized tacrolimus dose, nephrotoxicity is still a major hurdle for both calcineurin inhibitors indicating that this is a class-specific adverse effect [30,31]. To solve the issue of nephrotoxicity, immunosuppression through other mechanisms than calcineurin inhibition have therefore been explored.

Abatacept, a biological drug approved for treatment of rheumatoid arthritis [32], was known to inhibit T-cell activation through a different mechanism of action than calcineurin inhibitors. Although abatacept blocked rejection of cells or organs transplanted into mice [33], it had limited efficacy in rhesus monkeys receiving allografts [34,35]. This was possibly due to abatacept only partially binds to its target on the T-cell surface and only partially inhibiting the T-cell response [32,36].

To improve the immunosuppressive effect of abatacept, mutagenesis was used to create a mutant that inhibited the T-cell activation more completely. This resulted in the development of belatacept, which had the same mechanism of action but performed better than abatacept in preventing organ rejection in animal models [37]. Belatacept was compared against cyclosporine A in clinical trials and although the incidence of BPAR was higher during the first year after transplantation [38], belatacept gave better [39] or similar [40] long term patient survival of recipients with a living and deceased donor, respectively. Moreover, the renal function was considerably better in patients

receiving belatacept. Unfortunately, few studies compare belatacept to tacrolimus, the most commonly used calcineurin inhibitor today [41].

1.4 Purine synthesis inhibition

Mycophenolic acid (MPA), a naturally occurring molecule first found in a *Penicillum* strain [42,43] was initially of interest due to its apparent antibacterial [44-47], antiviral [48,49] and antitumor [48-52] capabilities. *In vitro*, MPA has been shown to delay or inhibit growth of *S.aureus* [44,45] and a number of viruses [49]. However, in 1969, when MPA was injected into mice infected with mouse sarcoma virus, no anti-viral activity could be demonstrated, but it was observed that MPA acted as an immunosuppressant [50].

Further *in vivo* studies showed that MPA indeed functioned as an immunosuppressant, and that it had effect when administered at and after the antigen challenge, most likely by inhibiting proliferation of lymphocytes [53]. Due to its ability to inhibit cell division, a pharmacological capability advantageous in the treatment of cancer or psoriasis, these were some of the first conditions subjected to clinical trials. MPA showed limited effect in cancer patients [54], but showed potential as a treatment for psoriasis [55-59]. Although substantial amount of studies on the anti-tumor aspect was done in the following decades [60], few studies transitioned from *in vitro* studies to *in vivo* or clinical trials.

In 1990, the compound mycophenolate mofetil (MMF), a morpholinoethylester derivate of MPA, was synthesized to improve the bioavailability of MPA [61]. This prodrug showed promising applicability for anti-rejection treatment after solid organ transplantation in animal models [62,63], especially in combination with cyclosporine A and methyl-prednisolone. Based on these encouraging results a Phase I clinical trial was initiated where MMF was given in combination with cyclosporine A and glucocorticoids to *de novo* renal transplant recipients. It was shown that MMF was generally well tolerated and gave a dose-dependent reduction of risk of rejection. In addition, reversal of refractory renal allograft rejection was demonstrated [64,65]. Further experimental studies strengthened the hypothesis that MMF was efficacious in preventing allograft rejection [66-71] and this was confirmed in Phase III blinded randomized controlled trials comparing MMF to placebo [72] or azathioprine [73,74] in combination with cyclosporine A and corticosteroids. In both settings, MMF proved superior in reducing

the rate of renal allograft rejection episodes and it received FDA approval for maintenance immunosuppression in 1995. MMF has largely replaced azathioprine in prevention of renal allograft rejection and is currently prescribed to 90% of renal transplant recipients in the US [75]. In addition, MMF has proven comparable to high dose intravenous steroids in the treatment of ongoing rejections [76].

Adverse events associated with MMF are often gastrointestinal (22 – 48%), hematological/lymphatic (25 – 64%) or infectious (36 – 75%) [72,77]. Adverse effects lead to 50-60% of patients reducing the MMF dose, and this increases the risk of experiencing BPAR 2-fold (halving of dose) or 3-fold (complete discontinuation) [78,79]. There are concerns that MMF treatment is associated with increase in cytomegalovirus (CMV) incidence or severity. Although MMF does not increase the incidence of CMV infections compared to azathioprine [74,80] it can cause more severe outcome in cases of CMV reactivation [81,82]

1.5 Pharmacodynamics

Pharmacodynamics can be defined as what a drug does to the body, *i.e.* how it asserts its pharmacological effect. A figure summarizing the different mechanisms of action of cyclosporine A, tacrolimus, MPA and belatacept is shown in Figure 2.

1.5.1 Calcineurin inhibition – tacrolimus and cyclosporine A

When the T-cell receptor is presented to an antigen by an antigen presenting cell, there is increase in intracellular Ca^{2+}, both from intracellular stores and from extracellular space through calcium release activated calcium channels. This increase in intracellular Ca^{2+} results in calmodulin binding to and activating the serine/threonine protein phosphatase calcineurin. In turn calcineurin dephosphorylates nuclear factor for activated T-cells (NFAT). NFAT then translocates to the nucleus [83] and promotes transcription of several pro-inflammatory cytokines, including interleukin (IL) 2, IL-3, IL-4, IL-5, interferon gamma, tumor necrosis factor alpha and granulocyte colony-stimulating factor [84]. IL-2 is secreted from the T-cell and stimulates other T-cells to differentiate into effector T-cells, promotes T-cells growth and augments natural killer cell cytolytic activity [85].

Both cyclosporine A and tacrolimus are assumed to be passively taken up by lymphocytes. Intracellularly, they bind to different immunophilins (tacrolimus binds to

FKBP-12 and cyclosporine A to cyclophilin) [86], but both drug-immunophilin complexes inhibit calcineurin, resulting in decreased NFAT activity and thereby reducing the T-cells activity [87]. Both cyclosporine A and tacrolimus inhibit calcineurin, however tacrolimus appears to only partially inhibit calcineurin whilst cyclosporine A inhibits the enzyme almost completely [88]. FKBP-12 is also a regulator of the cell-cycle and FKBP-12 deficient cells are arrested in the G_1 phase [89]. Tacrolimus may therefore disrupt T-cell proliferation independently from NFAT, but there is currently little knowledge about this.

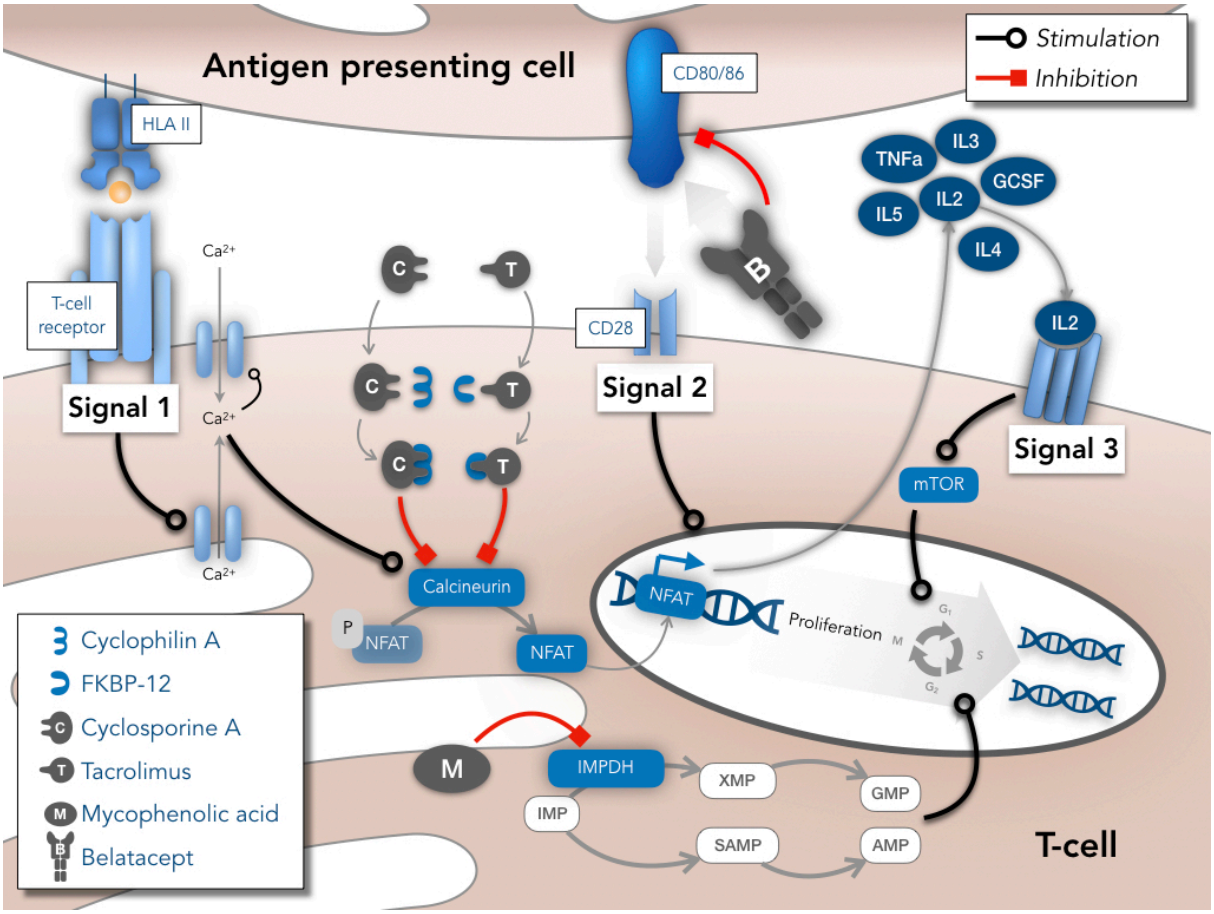

Figure 2: Mechanism of action for calcineurin inhibitors, mycophenolic acid and belatacept. (NFAT: Nuclear factor of activation of T-cells. IMPDH: Inosine monophosphate dehydrogenase. IMP: Inosine monophosphate. XMP: Xanthosine monophosphate. SAMP: Adenylosuccinate. GMP: Guanosine monophosphate. AMP: Adenosine monophosphate)

1.5.2 Proliferation inhibition – mycophenolic acid

Purines are synthesized through two pathways; the salvage pathway and the *de novo* synthesis. In the salvage pathway, guanine is resynthesized to GMP and adenine to AMP, through hypoxanthine-guanine phosphoribosyltransferase and adenine

phosphoribosyltransferase (APRT), respectively. Purine bases can therefore be recycled into purine nucleotides, limiting the energy intensive synthesis of new nucleotides.

While most cells can maintain purine nucleotide levels through salvage alone, proliferating lymphocytes have such an increased need that *de novo* synthesis is necessary. A key step in the *de novo* synthesis is the oxidation of Inosine-5'-monophosphate dehydrogenase (IMP) to xanthosine 5'-monophosphate (XMP) by inosine monophosphate dehydrogenase (IMPDH) as illustrated in Figure 2. XMP is converted further to guanosine (mono, di and tri-) phosphates and deoxy guanosine (di and tri-) phosphate, collectively termed guanine nucleotides. These are vital in DNA-replication and therefore cellular proliferation. The conversion of IMP to XMP via IMPDH is dependent on oxidized nicotinamide adenine dinucleotide (NAD^+) as a co-factor. Binding and releasing of substrates, cofactor and product is strictly sequential; first IMP binds to IMPDH followed by NAD^+ binding to the IMP-IMPDH complex. Hydrogen is transferred from IMP to NAD^+, resulting in the formation of NADH and an IMP thiomidate intermediate-IMPDH complex (XMP*-IMPDH). NADH must be released before water catalyzes the oxidation of XMP* to XMP and XMP is thereafter released from IMPDH [90].

It has been shown that MPA inhibits IMPDH by stabilizing the XMP*-IMPDH intermediate in an uncompetitive, reversible matter [91]. The inhibition of IMPDH results in depletion of guanine nucleotides, arresting the cell in S-phase resulting in apoptosis [92] and limits therefore the clonal expansion of lymphocytes [53]. Two isoforms of IMPDH have been identified (IMPDH 1 and 2). In resting lymphocytes IMPDH is mostly expressed as IMPDH 1, but following activation a larger proportion is present as IMPDH 2, although expression of both isoenzymes is markedly increased. Both isotypes have similar enzyme kinetics regarding synthesis of XMP, but MPA inhibits IMPDH 2 approximately 5-fold more than IMPDH 1 [90]. Experiments show that MPA does not reduce the production of cytokines in lymphocytes early (<24 hours) after mitogen stimulation compared to untreated cells, but did reduce cytokine production after 72 hours compared to untreated cells, presumably when the untreated cells had undergone proliferation [93]. This indicated that inhibition of proliferation is the major immunosuppressive effect. In addition, supplementing cells with guanosine and deoxyguanosine reverses the effect of MPA to a large degree [94], indicating that no other downstream enzymes vital for cell function is inhibited.

1.5.3 Co-signal inhibition – belatacept

A naïve T-cell requires both a stimulatory signal from the T-cell receptor, mediated through calcineurin/NFAT, and a stimulatory co-signal which is mediated through stimulation of cluster of differentiation (CD) 28, a receptor located on the T-cell membrane [95]. Naïve T-cells receiving signal through the T-cell receptor, but not through CD28, enters a state of anergy [96] leaving the T-cell without ability to attack the graft [95]. In order to initiate the co-stimulatory signal, CD28 must bind to CD80 and CD86, ligands located on antigen presenting cells such as dendritic cells and B-cells [97]. In order to regulate the immune response, activated T-cells express another receptor, called CTLA4, with much stronger affinity to CD80 and CD86, outcompeting CD28 [98]. Contrasting CD28, CTLA4 sends a tempering signal to the T-cell when bound to CD80 and CD86 and thereby limits the immune response.

Belatacept consists of a mutated CTLA4 and IgG Fc and can block the interaction between CD80/86 and CD28 and thereby inhibits the co-stimulatory signal, resulting in unresponsive T-cells [99]. The mutated CTLA4-domain on belatacept binds quicker and stays on longer than unmodified CTLA4 [37], contributing to a stronger immunosuppressive effect. CD86 saturation measurements in renal transplant recipients show that using the currently approved dosing regimens, 80-92% of CD86 on CD14+ monocytes are saturated.

Some T-cells do not require CD80/86 – CD28 interaction to be activated [100,101]. These are assumed to be T-memory cells and may be the reason why patients receiving belatacept (that only inhibit activation of naïve T-cells) have higher incidence of BPAR than patients receiving calcineurin inhibitors (that block stimulation of both types of T-cells). Memory T-cells are dependent on stimulation by IL15 and blocking of CD122, a subunit of the IL15 receptors, has shown reduced rates of rejection in mice and non-human primates when combined with belatacept compared to belatacept alone [102].

CD86 expressed on B-cells does not only stimulate T-cells through CD28, but also stimulates the B-cells itself to produce IgG and IgE [103]. A secondary mechanism of action could therefore be B-cell inhibition in addition to T-cell inhibition, although this has not been explored in detail.

1.6 Pharmacokinetics

The pharmacodynamics (what the drugs do to the body) of immunosuppressive drugs are outlined above, but equally important is what the body does to the drug (pharmacokinetics, or the movement of drugs). Pharmacokinetics can be divided into absorption, distribution, metabolism and excretion. Pharmacokinetic parameters of tacrolimus, MPA and are outlined below.

1.6.1 Tacrolimus

The average bioavailability of tacrolimus is 25% and ranges from 5- 93% [104]. Tacrolimus is a substrate of several members of the CYP3A-family and variation in genotype or expression of these enzymes in gut and liver can alter the first-pass metabolism and contributes to a large extent to the range in bioavailability [105]. In immediate-release formulation the time to maximum concentration is generally 0.5-1 hour, but can be prolonged by simultaneous intake of food rich in fat. This is probably due to tacrolimus being highly lipophilic [106].

The bioavailability may also be influenced by expression of P-glycoprotein (P-gp). P-gp is a transporter capable of expelling xenobiotics from cells and tacrolimus has been shown to be a substrate for this transporter [107]. In gut, P-gp expels substrates back into lumen and reduces the amount of drug reaching the circulation [108]. The tacrolimus rate of efflux varies with P-gp polymorphism [107,109], and may be a source of variability of tacrolimus absorption.

After entering the blood, tacrolimus distributes to erythrocytes to a large extent and the concentration in whole blood is 14 times that in plasma, although with a large variation (4-114 times) [110]. This high affiliation to erythrocytes means that the whole blood concentration is dependent on hematocrit [111]. The ratio between total and plasma concentration is lower at a higher total concentration of tacrolimus indicating that the erythrocyte compartment is saturable. The volume of distribution (V_d) is high (98-140 L [112]), further underlining the lipophilic character of tacrolimus. There is also a large variation in the V_d, with an inter-individual coefficient of variation of 110% [113]. In plasma, 99% of tacrolimus is bound to proteins [114], leaving only a small fraction free to enter the site of action inside the lymphocyte. P-gp is also expressed on lymphocytes and P-gp polymorphisms influence the amount of tacrolimus that enters

the lymphocyte; in patients with P-gp polymorphisms that alters substrate affinity (ABCB1 1199G>A) or reduces P-gp expression (ABCB1 3435C>T) the ratio between lymphocyte and whole blood concentration are 1.3 – 1.8 times higher compared to wild-type [107].

Tacrolimus is removed from the circulation mainly through metabolism and < 0.5% is removed directly through renal or biliary elimination [115]. Metabolism occurs in the liver with CYP3A4 and CYP3A5 being the main metabolizing enzymes. Not everyone carries functional CYP3A5 (CYP3A5*1) and there are large differences between racial groups (<20% of Whites, > 80% Blacks). Patients with functional CYP3A5 have a 2-fold higher tacrolimus clearance compared to those with non-functional CYP3A5 (CYP3A5*3) [116]. Since CYP3A5 also exists in the intestine as well as liver, patients with functional CYP3A5 may also have reduced bioavailability of tacrolimus. The current Norwegian immunosuppressive treatment protocol after renal transplantation requires patient with functional CYP3A5 to have a higher initial dose to compensate for the increased clearance and reduced bioavailability.

A large variation in tacrolimus clearance has been reported [110] and a part of this variation may be due to CYP3A5-polymorphism. However, there is a large variation within patients with the same CYP3A5 genotype [110] showing that CYP3A5-polymorphism only partly explains the variation and individual pharmacokinetic parameters are difficult to predict [117]. The inter-individual coefficient of variation in tacrolimus clearance is 54% [113] and combined with a large variation in V_d results in terminal half-life ranging from 4 – 41 hours [118].

Tacrolimus forms several demethylated and hydroxylated metabolites [119], with main metabolites being 13-O-desmethyl tacrolimus and 15-O-desmethyl tacrolimus. These metabolites have limited immunosuppressive activity (<9% compared to tacrolimus) and is also found in much lower concentration than tacrolimus (<10%) [120], indicating that the immunosuppressive effect is mediated largely by tacrolimus itself. Similarly to tacrolimus, its metabolites are almost completely eliminated by biliary excretion, with 93% of radiolabeled tacrolimus being recovered from feces and 2.3% in urine [115].

1.6.2 Mycophenolic acid

MPA is usually given orally either in the form of MMF (CellCept®, Roche) or as a sodium salt (Myfortic®, Novartis). Both forms are rapidly and completely hydrolyzed to MPA by carboxylesterase 1 and 2 [121] in the gastrointestinal system and liver. Bioavailability is 94% in healthy volunteers [122], but is lower (81%) in renal transplant recipients in the immediate postoperative period [123], possibly due to reduced gastrointestinal motility occurring after surgery [124]. After oral administration of MMF, peak plasma concentration of MPA is reached within 1 – 2 hours [125].

In the systemic circulation, MPA is almost exclusively distributed to plasma with only 0.01% found in the cellular compartment. Pharmacokinetics is best described in a two-compartment pharmacokinetic model, with a central V_d of 91L and peripheral V_d of 237 L [126]. MPA is glucuronidated through uridine diphosphate glucuronosyltransferases located in the liver, mainly UGT1A9, 1A7, 1A8, 1A10 and 2B7 [127] and is either glucuronidated or glucosidated at one of two positions, forming four metabolites. The main metabolite is MPA-7-O-glucuronide (MPAG), with plasma concentrations more than 20 fold higher than MPA [128-130], but with no pharmacological activity [131]. Almost all (96%) of MPA is excreted into urine, almost exclusively as MPAG [132].

MPAG also binds to serum proteins, similar to MPA. In patient with reduced renal function, the excretion of MPAG is decreased resulting in higher a MPAG concentration in plasma. At higher concentrations, MPAG displaces MPA from albumin, resulting in a higher MPA free-fraction in patients with decreased renal function [133] or hypoalbuminemia [134]. When the free fraction is increased a larger proportion of MPA is subjected to metabolism, resulting in higher clearance [135], and possibly also an increased amount that can distribute to lymphocytes and exert IMPDH inhibition.

On a pharmacokinetic curve of MPA, a secondary peak can often be seen 6-12 hours after a dose has been given. This peak is due to enterohepatic circulation, where MPAG is excreted into bile via the multidrug resistance-associated protein 2 (MRP2), then degraded to MPA and reabsorbed into circulation [136,137]. When bile reabsorption is inhibited using cholestyramine, the MPA $AUC_{0\text{-}12h}$ is markedly lower. It is suggested that enterohepatic circulation contributes to approximately 40% of the MPA AUC, but with a wide range (10 to 61%) [132]. Due to the considerable contribution the enterohepatic circulation has on the AUC, traditional estimation of terminal half-life may

not reflect true elimination half-life. The apparent half-life (by including the enterohepatic recirculation) is 16 – 18 hours [132]. Cyclosporine A inhibits MRP2 and thereby the enterohepatic circulation of MPAG [138], but tacrolimus does not. This leads to patients receiving tacrolimus having 25 – 35% higher MPA exposure than patients receiving cyclosporine A [139].

Another metabolite, the acyl glucuronide of MPA (AcMPAG), is formed at a much lower rate than MPAG. It has an AUC that is approximately 10% of MPA and <0.5% of MPAG. AcMPAG is considered an active metabolite, although the degree of IMPDH inhibition is uncertain, being reported from 10% [140] to 100% [141] of MPA.

1.6.3 Belatacept

Pharmacokinetic studies on belatacept are limited [142-144], and are mostly based on data from the clinical studies performed by the manufacturer [143,144]. Belatacept is a large chimeric protein and falls into the category of biological pharmaceuticals. Such drugs are readily digested after oral administration and little is absorbed from the gastrointestinal tract, resulting in a low oral bioavailability. Biological pharmaceuticals must therefore be given parenterally [145]. Belatacept is only approved for infusion, although abatacept has an almost identical chemical structure and can be given sub-cutaneous [146]. Data from unpublished studies suggest that sub-cutaneous belatacept can give AUC similar to intravenous administration, although with lower and prolonged peak concentrations (Clinical Trial.gov, NCT00569803, accessed 16.july 2019). After entering the circulation, belatacept distributes mainly to the extravascular space, as little is able to distribute into cellular compartments. This results in a low V_d of 0.09 - 0.12 L/kg [142,143]. No distribution studies using radiolabeled belatacept have been reported, so details regarding specific distribution are not available.

Contrasting low molecular drugs like tacrolimus and MPA, belatacept is not metabolized through metabolizing enzymes in the liver or kidneys. Since belatacept has an IgG-Fc domain, it is expected that it is metabolized in a similar manner as IgG. This occurs through internalization and lysosomal degradation in endothelial cells and monocytes, and a large fraction is recycled via the neonatal Fc receptor [147]. Another possible route of elimination is through target-mediated elimination. This is a process where binding to the target on cell surface results in intracellular compartmentalization

and degradation [148]. Since belatacept binds to antigen presenting cells (APC), target-mediated elimination is a possibility. In adolescent and adult renal transplant recipients, as well as healthy adults, the belatacept half-life is 7 – 9 days [142,143].

1.7 Therapeutic drug monitoring

All pharmacological agents used to prevent allograft rejection exert their effect through inhibition of the immune system. An inherent adverse effect is therefore immunological failure. This is reflected in the increased risk of infections [149] and cancer [150] in immunosuppressed patients. Since both the desired and adverse effect are closely related it follows that efficacious and toxic concentrations are overlapping. This means that immunosuppressants often have a small therapeutic index, meaning that the ratio between efficacious and toxic concentrations is small. In addition, several immunosuppressants display a high pharmacokinetic variability as described above. To avoid both over- and underexposure, both detrimental for a transplant recipient, it is evident that adjusting the dose according to exposure is necessary. The concept of adjusting the dose according to drug measurement forms the basis of therapeutic drug monitoring (TDM).

Classically, there are five requirements for TDM to be of value; a) narrow therapeutic window with severe consequences of over- or underexposure, b) an unpredictable and variable dose-exposure relationship between individuals, c) a predictable and less varying dose-exposure relationship within one individual, d) an exposure-outcome relationship, and e) there are assays available for measuring exposure.

1.7.1 Tacrolimus

Tacrolimus is almost always monitored in current immunosuppressive protocols. Although AUC is considered to be the golden standard for measuring exposure, few studies have examined the relationship between AUC and risk of BPAR after renal transplantation. In addition, AUC measurement requires intensive sampling over a dosing interval and is not suited in most routine settings. Trough measurements (C_0) are therefore used instead. However, studies show that C_0 is [151,152] and is not [153,154] related to the risk of BPAR and may [155-157] and may not be correlated with AUC [158-160]. Co-medication, like the use of MPA, steroid usage and induction therapy can

vary between studies and makes it a challenge to determine the effect of tacrolimus exposure on its own. Toxicity distinctive for tacrolimus it often not influenced by co-medication and a more concise association between tacrolimus and toxicity is usually stronger [161,162]. Early protocols aimed for high tacrolimus C_0 in whole blood; 7-20 µg/L during month 1-3 and 5-15 µg/L thereafter [163]. A large study showed that target whole blood tacrolimus C_0 of 10-20 µg/L from 0-3 months (no induction treatment) and 5-15 thereafter resulted in a BPAR incidence of <20% within the first year, with the large majority occurring within the first six months [164]. To reduce the nephrotoxic severity of tacrolimus, lower levels of whole blood tacrolimus C_0 from day 1 (3 – 7 µg/L) after transplantation combined with induction treatment (daclizumab) was studied in the ELiTE Symphony-trial. Tacrolimus whole blood concentrations were above target (4-11 µg/L the first month, 4-10 µg/L month 2-5 and 4 – 9 µg/L month 6-12), but resulted in better renal function without increasing the risk for BPAR [25]. This study formed the basis of the current immunosuppressant protocol used after renal transplantation in Norway for standard risk patients.

Whilst traditional tacrolimus monitoring aims to achieve target concentration, there is increasing evidence that alternative interpretation of tacrolimus concentration other than absolute concentration has merit. Several papers have shown that higher intra-individual variation in tacrolimus trough concentration, rather than absolute concentration, is associated with increased risk for BPAR [165-169] and development of donor specific antibodies [168]. Although the mechanism of this association is currently unknown, it is possible that a high inter-individual variation is associated with periods of low tacrolimus concentrations and subsequent increased risk of rejection. In addition, a high intra-individual variation may be due to non-adherence, also a risk factor for rejection.

It has also been shown that whole blood tacrolimus C_0 relative to dose, and not concentration alone, is also associated with both BPAR and nephrotoxicity. Patients requiring higher dose to achieve the target concentration are assumed to have a higher clearance, with the ratio (concentration/dose) as a pseudo clearance value. Patients with higher ratios have increased risk of both BPAR [154] and nephrotoxicity [170], presumably because they reach lower concentrations when doses are delayed or forgotten and higher peak concentrations due to higher absolute doses.

While whole blood C_0 monitoring is the current method of monitoring tacrolimus, the lack of strong correlation with clinical outcome has led to efforts to find more suitable alternatives. One alternative that has been explored is pharmacodynamic, rather than pharmacokinetic, monitoring. Since tacrolimus inhibits the dephosphorylation and activation of NFAT, the downstream effect of NFAT has been suggested as a pharmacodynamic marker of tacrolimus. One approach to quantify NFAT inhibition is to measure gene expression of three NFAT specific cytokines (IL2, IFN-γ and GM-CSF) [171]. One broadly used method for NFAT monitoring is to calculate the expression of these genes at $C_{1.5}$ (at assumed maximum concentration of tacrolimus) relative to C_0 (minimum tacrolimus concentration), a measurement termed NFAT-remaining gene expression (NFAT-RGE). This was measured in 262 stable renal transplant recipients and showed that patients with >30% NFAT-RGE (n=119) had a larger risk of BPAR (25%) than those (n=143) with <30% NFAT-RGE (1.3%) [172]. Conversely, patients with higher NFAT-RGE have a decreased risk of viral infections [172,173], making it difficult to set a target for NFAT-RGE. In a recent prospective trial, the cyclosporine A dose was adjusted to get NFAT-RGE within 15-30%. Standard treatment in the control group resulted in NFAT-RGE between 9.4 and 11.4%, indicating over immunosuppression. Compared to controls, patients subjected to NFAT-RGE monitoring had lower concentrations of cyclosporine A, lower cardiovascular adverse events, better renal function and no increase in incidence of BPAR [174]. This study showed value in NFAT-RGE monitoring in patients receiving cyclosporine A, but if this can be translated to tacrolimus is not known.

Currently, tacrolimus concentration monitoring is performed in whole blood, with the reason being practicality rather than pharmacological. Tacrolimus is largely distributed to erythrocytes resulting in much higher concentrations in whole blood than in plasma, which is attractive from an analytical perspective. In addition, whole blood is readily attainable through venipuncture or dried blood spot sampling [175,176], making it suitable in a routine setting. However, tacrolimus exerts its pharmacological effect inside lymphocytes, cells that only represents <0.1% of the cells in whole blood. Since whole blood concentration only has a limited correlation with efficacy, interest in concentrations inside peripheral blood mononuclear cells (PBMC), a lymphocyte enriched cell-population, has been explored [107,177-183]. All studies show none or weak correlation between whole blood and intracellular tacrolimus concentrations,

indicating that whole blood concentration does not reflect the concentration at the site of action. Tacrolimus concentration in PBMC correlated negatively with severity of rejection in liver transplant recipients on tacrolimus monotherapy [178], demonstrating that intracellular measurements have merit in these patients. Most studies have either been conducted in immediate post-operative phase [178] or at a single time-point [179-183], meaning that longitudinal data on intracellular tacrolimus concentration are lacking. Whole blood measurement is always reported as mass per volume, since whole blood is a liquid. However, the unit of measurements for intracellular drugs has been more inconsistent, with some papers reporting mass per number of cells [183,184], per cellular volume [180] or amount of cellular protein [185]. No studies have reported correlations between the units of measurements, so it is hard to compare results between studies with different units.

1.7.2 Mycophenolic acid

MPA is approved for the prevention of rejection using a fixed dose (1 gram twice daily for MMF and 720 mg twice daily for the sodium salt of MPA) and without monitoring. However, questions have been raised whether the treatment can be optimized with TDM [186,187]. A requirement for TDM is an association between exposure and efficacy. This has been shown to be valid for MPA. In a study on 72 renal transplant recipients given basiliximab, prednisolone, cyclosporine A and 2 g/day fixed dose MMF, 15% of the patients experienced BPAR within 3 months. An MPA AUC < 22 $mg \cdot h \cdot L^{-1}$ measured within the first week after transplantation was associated with BPAR, although with limited strength (positive predictive value = 30% and negative predictive value= 89%) [188]. Measurement of AUC is cumbersome and requires intense sampling throughout the dose interval. Limited sampling strategies (LSS) have therefore been developed. LSS is an approach where only a few samples are needed to calculate the whole MPA AUC. One study has shown good correlation between full AUC and a LSS estimated AUC based on three samples taken 20 min, 1 hour and 3 hours after MMF administration [189]. In stable patients (> 3 months post-transplantation) the model predicted an AUC within +/- 20% of the true AUC in 90% of cases. AUC prediction in patients recently transplanted (7 days) was poorer (60% of cases within +/- 20%), but still considered usable in MPA TDM [77].

Several prospective trials utilizing MPA TDM have been performed [77,139,190-192]. No clear recommendation on MPA TDM could be drawn, with results both in favor [77,190,192] and disfavor [139,191] of TDM. The majority of evidence for or against MPA monitoring comes from three large studies; APOMYGRE [77], FDCC [191] and OptiCept [139] with a total of 1758 renal transplant recipients. In the APOMYGRE-trial, all patients were given induction therapy (basiliximab and methylprednisolone) followed by maintenance treatment consisting of cyclosporine A, prednisolone and MMF. The control-group received fixed dose MMF, only adjusted according to clinical experience blinded to concentration measurements. The MMF dose in the intervention group was adjusted according to an LSS-strategy aiming for an AUC of 40 $mg \cdot h \cdot L^{-1}$. In the intervention group 84% needed dose adjustment, resulting in a higher MMF dose 14 days after transplantation (mean 2.70 vs 2.00 g/day), but similar dose at 12 months (mean 1.82 vs 1.96 g/day). Consequently, 70% of patients in the intervention group reached the target AUC at 14 days, compared to only 30% in the control group. AUC-monitored patients also had lower incidence of BPAR (7.7% vs 24.6%), with the largest differences seen within 4 months after transplantation. Adverse effects were similar between the groups. Based on these results, MPA TDM seemed useful, particularly in the early period after transplantation.

All patients received cyclosporine A in the APOMYGRE-trial, and it is uncertain whether patients treated with tacrolimus would have the same benefit of MPA TDM. The value of MPA TDM in patients receiving tacrolimus was assessed in the OptiCept-trail [139], where both patients receiving standard or reduced dose of tacrolimus or cyclosporine A were included. In the intervention group, MMF dose was adjusted to get a MPA C_0 > 1.3 mg/L if patients received cyclosporine A and >1.9 mg/L if they received tacrolimus, whilst patients in the control group received fixed dose MMF. MPA TDM did not improve the clinical outcome, although pooled analysis showed that patients with a MPA C_0 > 1.6 mg/L had reduced risk for BPAR within 12 months (5 vs 15%).

The FDCC-trial showed similar results as OptiCept. In FDCC, AUC-based TDM of MPA in patients receiving either tacrolimus or cyclosporine A was assessed. Patients were either given 2 g/day fixed dose or had MMF dose adjusted to get an AUC of 45 $mg \cdot h \cdot L^{-1}$ based on LSS measurements. Although the rate of BPAR the first year after transplantation was similar between the groups (15 vs 16%), indicating that TDM does not improve outcome, the study showed that a low AUC at day 3 after transplantation

was predictive of BPAR within the first year. All three studies mentioned above demonstrate that the MPA exposure is associated with clinical outcome, but highlights that a protocol to achieve target exposure is difficult to determine.

Pharmacodynamic monitoring of MPA was already suggested in 1995 [193]. Since MPA inhibits IMPDH, the activity of this enzyme is a reasonable marker on the pharmacodynamic response. IMPDH measurements in both healthy individuals and renal transplant recipients receiving MMF show a large inter-individual variation [193-196]. Prospective trials adjusting the MMF dose according to IMPDH measurements have not been reported, but it has been shown that pre-transplantation IMPDH-activity can identify patients who later would need reduced dose due to toxicity (positive predictive value: 65%, negative predictive value: 80%) and that a high pre-transplant IMPDH-activity results in 3.6-fold (p=0.009) increase in risk of BPAR when adjusted for other factors [197].

Since proliferation of lymphocytes is dependent on the guanosine nucleotide pool, another approach of pharmacodynamic monitoring of MPA may be to measure the alteration of this pool. This approach has only been explored to a small degree [198-200], and data from patients receiving MMF is limited [200]. *In vitro* studies suggest that mitogen-stimulated PBMC is much more susceptible to inhibition by MPA than non-stimulated cells [198,199], but only the effect on non-stimulated PBMC has been studied in transplant recipients [200].

1.7.3 Belatacept

Whilst TDM for tacrolimus and MPA has been explored, and in the case of tacrolimus, universally implemented in routine treatment, the utility of belatacept monitoring has not been tested. Two dosing regimens were compared in phase III clinical trials [39,40]; more intensive (MI) and less intensive (LI). Patients in the MI group received an induction treatment of 10 mg/kg at day 1 and day 5, thereafter at week 2, 4, 6, 8, 10, 12, 16, 20 and 24. Patients in the LI group received 10 mg/kg at day 1 and day 5, and then at week 2, 4, 8, 12. After this, both groups received 5 mg/kg every 4 weeks. Patients in both groups had similar rates of BPAR, but patients in the MI group had more adverse effects (post-transplant lymphoproliferative disorder, infections and malignancies). This indicated that the LI and MI regimens represent the upper range of the therapeutic window, but information on the lower limit is still lacking. An assay

capable of measuring belatacept concentrations is required in order to find a lower limit of the therapeutic concentrations, and although the drug manufacturer has described an assay [201], none are commercially available.

Since belatacept blocks CD80/86 on APC, the saturation of these ligands has been suggested as a potential pharmacodynamic measurement. A relevant assay utilizes anti-CD86 and anti-CD80 antibodies labeled for flow cytometry measurements and measures the CD80/CD86 occupancy in a competitive assay format. In samples from renal transplant recipients treated with belatacept, approximately 82% of CD86 on circulating cells were occupied at trough concentration, while approximately 94% was occupied at peak concentration [202]. However, in a trial on 20 belatacept-treated renal recipients, of which 11 (55%) experienced BPAR, the occupancy of CD86 was similar between the groups [203], indicating that the pharmacodynamic response may be diverse between individuals although the drug exposure appears to be sufficient.

2 Rationale and objectives of the thesis

Since the introduction of cyclosporine A in 1983, the protocols for immunosuppression after renal transplantation have been continuously improved with the introduction of new pharmaceuticals or optimization by TDM. This has resulted in improved graft survival in the first year after transplantation. However, the rate of graft loss beyond the first year is similar for patients receiving their transplant in 1989-1995 and 2007-2017 [204], inciting the need for further improvements. Tacrolimus nephrotoxicity is still a frequently seen challenge [170,205], even when TDM via whole blood concentration is applied, prompting the question whether alternative methods of monitoring could improve the treatment outcome. Patients receiving belatacept have an increased risk of BPAR, but no TDM of belatacept has been examined so it is unknown whether such monitoring could reduce BPAR incidences. TDM of MPA is still a controversial topic and although pharmacokinetic and pharmacodynamic measurements correlate with outcomes, more knowledge of pharmacodynamics may be needed in order to identify an efficient therapeutic drug monitoring protocol for this drug.

Overall, the main objective of this thesis was to develop novel methods of monitoring for different immunosuppressants and apply these in an exploratory setting. Specific objectives for the individual projects were as follows:

2.1 Paper I – Alternative matrices for tacrolimus

The aim of the first project was to develop, validate and apply an assay capable of measuring tacrolimus inside PBMC. Since tacrolimus is assumed to assert its immunosuppressive effect inside lymphocytes, it is implicit that concentration inside a population largely consisting of such cells would better reflects the concentration at the site of action than the current standard practice of measuring in whole blood. Since studies on intracellular tacrolimus uses different methods of normalizing the amount of tacrolimus to amount of cell, it would be valuable to determine how different methods of normalization correlates. For that reason, a secondary goal was therefore to simultaneously measure tacrolimus per number of cells and per amount of cellular protein, and determine whether these produced comparable results.

Intracellular measurement of tacrolimus has been described before, but little is known about the longitudinal behavior of this drug. The assay developed in this project would therefore be used in a cohort of renal transplant recipients to describe intracellular tacrolimus concentration over time, by measuring at several time points during the first year after renal transplantation. Measurements of intracellular concentration of tacrolimus would be performed both at trough and at assumed peak concentrations at each sampling day to gain additional information about intracellular pharmacokinetics during the dose interval.

2.2 Paper II – Serum monitoring of belatacept

The clinical usefulness of belatacept monitoring has not been investigated. To be able to perform studies on both pharmacokinetics and monitoring of belatacept, a reliable and convenient assay for concentration measurement of this drug had to be developed.

The aim of this project was to develop and validate a method for determining belatacept levels in serum using a ligand binding assay utilizing the target molecule (CD80/86) to capture belatacept, and to apply this assay in a research setting to show applicability and describe basic pharmacokinetics. The intention was also to make the assay as automated as possible, making it suitable for both clinical studies on a larger scale and for routine drug monitoring.

2.3 Paper III – Pharmacodynamic assessment of mycophenolic acid

The overall aim of the third project was to apply a previously developed assay to assess pharmacodynamic effects of MPA in the target cell population.

Pharmacodynamic variables to be measured were both IMPDH-capacity – a direct effect of MPA – and alterations in the adenine- and guanine nucleotide pool – an indirect effect of MPA. Since the main mechanism of action of MPA is to inhibit the proliferation of lymphocytes through IMPDH-inhibition inside these cells, PBMC (a cell population enriched in lymphocytes) was chosen as the sample material in order to measure pharmacodynamics at the site of action. There are large differences in the intracellular environment in resting and stimulated cells *e.g.* the differences in expression of IMPDH 1

and IMPDH 2 [206-208] . To evaluate whether MPA pharmacodynamics differed between activated and non-activated cells, IMPDH-capacity and adenine- and guanine nucleotide concentrations was measured in both *ex vivo* stimulated and non-stimulated PBMC.

In order to gain longitudinal understanding of MPA pharmacodynamics in transplant recipients, these pharmacodynamic markers were measured immediately before and at several time points during the first year after renal transplantation. To assess the variation in pharmacodynamics during a dosing interval, all pharmacodynamic markers were measured at trough (before dose) and peak (1.5 hours after dose) concentration of MPA in plasma. A secondary objective was to relate the pharmacodynamic measurements to the occurrence of BPAR or the need to reduce the dose.

3 Summary of papers

3.1 Paper I

Longitudinal study of tacrolimus in lymphocytes during the first year after kidney transplantation

Paper I consists of two parts. The first part is development and validation of a method for measuring tacrolimus inside lymphocytes. The second part is the application of the assay in a cohort of renal transplant recipients during the first year after transplantation. PBMC was chosen as the sample matrix and isolated using Ficoll to create cell pellets. Both number of cells and amount of protein was measured to be able to normalize the amount of intracellular tacrolimus in two alternative ways. Lysis of PBMC was done by freeze-thaw, methanol extraction and sonication, and tacrolimus was measured in the extract using HPLC coupled to tandem mass spectrometry. Validation showed that the assay was accurate (100 – 102%) and precise (Coefficient of variation (CV) < 7.4%). Variation due to the cell isolation, counting and tacrolimus extraction was assessed by preparing five samples from a single patient taken simultaneously and showed a CV of 7.4%. An assessment of matrix effects showed that the sample matrix did not alter retention time or signal intensity, and calibrators and controls could therefore be prepared in neat methanol instead of blank PBMC. Initially, the tacrolimus extract was evaporated and reconstituted during sample preparation, but a high signal to noise ratio allowed for evaporation to be omitted. The omission of evaporation did not alter the reported concentration (mean deviation 3.0%, range 0.18 to 10.5%). There was only a modest correlation between results reported as mass per cell number and mass per amount of cellular protein.

The assay was used in a cohort of renal transplant patients (n=29) to measure intracellular tacrolimus at three different time points during the first year after transplantation; 6-9 days, 5-7 weeks and 1 year. At all three days samples were collected at trough and assumed peak whole blood concentration (1.5 hours). In order to compare intracellular to whole blood concentration, whole blood tacrolimus concentration was measured using a routine chemiluminescent microparticle immunoassay (CMIA) method approved for *in vitro* diagnostic use. The concentrations are summarized in *Table 1*. There was only a weak correlation between whole blood and intracellular

tacrolimus concentrations at trough (rho -0.32 to 0.59) and modest correlation at 1.5 hours after dose (rho 0.40 to 0.82). Although the whole blood tacrolimus concentration increased from t_0 to $t_{1.5}$ at all sampling days, the intracellular tacrolimus concentrations only increased from t_0 to $t_{1.5}$ in the immediate post-transplant phase (6-9 days post transplantation). The intra-individual variation across the year was also higher for intracellular tacrolimus concentrations (CV 42 to 43%) than for whole blood concentrations (23 – 36%).

Table 1: Tacrolimus concentration in PBMC and whole blood from renal transplant recipient during the first year after transplantation (n=29)

Time after transplantation	6-9 days		5-7 weeks		1 year	
	t_0	$t_{1.5}$	t_0	$t_{1.5}$	t_0	$t_{1.5}$
PBMC (pg/10^6 cells) *median, quartiles*	22.5 (21.0–35.2)	43.9 (36.3–61.8)	33.0 (22.9–41.3)	29.9 (25.3–55.2)	27.4 (25.1–36.3)	27.2 (21.0–45.1)
Whole blood (µg/L) *median, quartiles*	5.0 (4.5–6.2)	10.5 (7.1–16.4)	6.0 (5.7–6.8)	8.3 (7.0–10.7)	5.4 (4.0–6.6)	9.1 (7.10–11.5)

t_0; before dose. $t_{1.5}$; 1.5 hours after dose. PBMC; peripheral mononuclear blood cells.

3.2 Paper II

A fully automated method for the determination of serum belatacept and its application in a pharmacokinetic investigation in renal transplant recipients

In order to perform pharmacokinetic studies of belatacept, a suitable assay was developed in Paper II. A ligand binding format was chosen, utilizing the target molecule (CD80) of belatacept to capture the drug. The final method consisted of trapping CD80 in microtiter plates via biotin-streptavidin interaction followed by adding diluted serum to the wells. Unbound components in the sample was then washed out, leaving belatacept, bound to CD80, remaining. To quantify the amount of belatacept in the well, Protein A labelled with europium was added and time resolved fluorometry used to create a signal proportional to amount of belatacept. The signal was linear from 0.003 to 0.3 mg/L in diluted samples, meaning that belatacept could be measured in concentration 0.3 to 30 mg/L without further dilution. Variation and accuracy allowed for a lower limit of

quantification (LLoQ) to be set to 1.0 mg/L and pre-dilution extended upper limit of quantification to 130 mg/L. Accuracy was 91 - 99% and imprecision (CV) <10%. The predilution in either serum or assay buffer did not affect the results. The whole assay, with exception of pre-dilution, was automated on a pipetting robot capable of running 126 samples within 3 hours.

The applicability of the assay was then used to measure belatacept concentrations from five patients over a total of 26 dosing intervals (shown in Figure 3). Data was collected from both the induction phase (2 week dose interval) and maintenance phase (4 week interval). A model with 3-compartment (one central and two peripheral) fitted the observed data and gave an estimated (mean +/- standard deviation) V_d of 3.5 +/- 0.6 L for the central compartment and elimination constant of 0.013 +/- 0.002 h^{-1}. Trough concentrations were within 12.6 – 14.4 mg/L (induction phase) and 1.8 – 4.9 mg/L (maintenance phase)

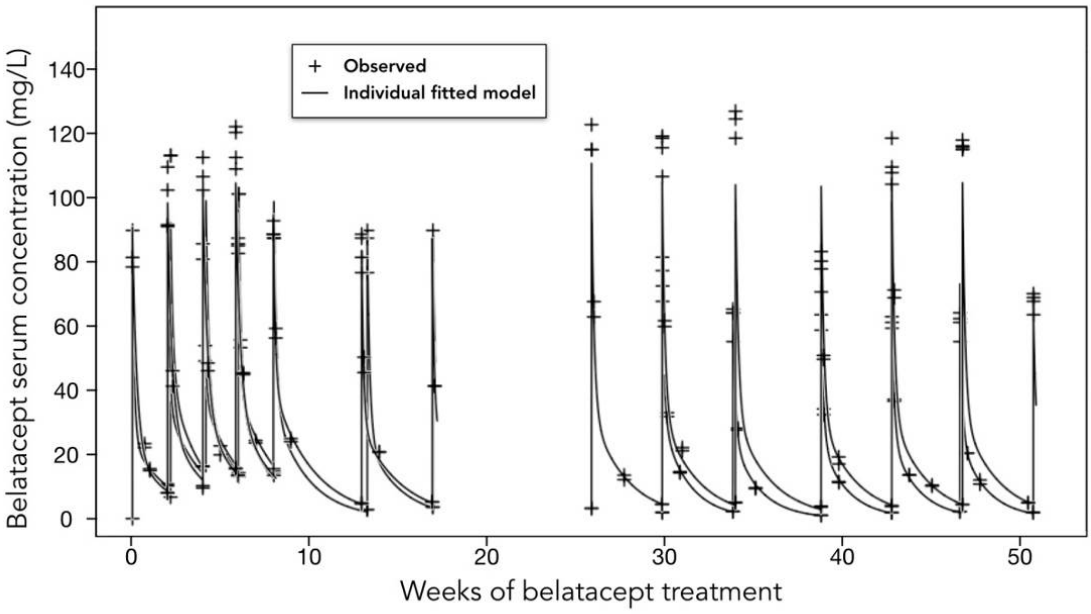

Figure 3: Belatacept pharmacokinetic profiles from stable renal transplant recipients (n=5).

3.3 Paper III

Pharmacodynamic assessment of mycophenolic acid in resting and activated target cell population during the first year after renal transplantation

To explore the pharmacodynamic effects of MPA, IMPDH-capacity, guanine nucleotides, and adenine nucleotides were monitored in the patients (n=24) that received MPA in the cohort of Paper I. In addition to the samples collected before and 1.5 hour after dose at 6-9 days, 5-7 weeks and 1 year after transplantation, measurements were also performed 0-4 days prior transplantation *i.e*. before immunosuppression was initiated. Since activation of lymphocytes alters the ratio between IMPDH 1 and IMPDH 2, and MPA inhibits IMPDH 2 more effectively than IMPDH 1, the IMPDH-capacity and nucleotides were measured in both *ex vivo* mitogen stimulated PBMC and unstimulated PBMC. The sampling schedule allowed us to explore how the pharmacodynamic biomarkers changes within a dose interval (from before to 1.5 hours after dose) and across the first year after transplantation.

All MPA pharmacodynamic and -kinetic measurements are summarized in Table 2. This study revealed several novel pharmacodynamic aspects of MPA during immunosuppressive treatment. First, stimulated PBMC were more sensitive towards MPA compared with unstimulated cells. In the stimulated cells, the IMPDH-capacity after dose was between 21% and 34% of the pre-dose capacity, whilst between 57% – 75% of the capacity in the non-stimulated PBMC remained. Second, whilst alteration in the purine pools in non-stimulated PBMC remained largely unchanged from before to after transplantation and before to after dose, there was a more substantial reduction of purines in the stimulated PBMC. Thirdly, the pre-dose IMPDH-capacity increased from early (6-9 days) to late (1 year) after transplantation, both in stimulated and non-stimulated PBMC.

The six patients that needed dose-reduction at some stage during the first year after transplantation tended to have lower IMPDH-capacities both in stimulated and non-stimulated cells before and just after transplantation.

Table 2: Mycophenolic acid concentrations and pharmacodynamics in renal transplant recipients during the first year after transplantation

Time after transplantation	*0-4 days before*	*6-9 days*		*5-7 weeks*		*1 year*	
		t0	*t1.5*	*t0*	*t1.5*	*t0*	*t1.5*
Number of patients	**19**	**22**	**22**	**24**	**24**	**23**	**24**
Plasma MPA (median, IQR) mg/L	-	**1.47** *(1.14-2.39)*	**5.27** *(3.84-7.59)*	**2.75** *(1.75-3.26)*	**7.02** *(4.56-9.95)*	**1.98** *(1.77-4.09)*	**6.47** *(4.77-7.98)*
***Ex vivo* mitogen stimulated PBMC**							
IMPDH capacity pmol 10^{-6} cells min^{-1} (median, quartiles)	**127** *(95.8-147)*	**33.1** *(13.4-50.7)*	**5.11** *(2.30-25.1)*	**35.3** *(16.1-83.4)*	**10.7** *(5.72-19.5)*	**84.8** *(43.6-99.3)*	**18.4** *(7.34-31.2)*
Guanine nmol 10^{-6} cells (median, quartiles)	**4.06** *(2.68-5.69)*	**1.14** *(0.61-2.14)*	**0.45** *(0.33-0.88)*	**1.06** *(0.59-2.41)*	**0.56** *(0.34-1.25)*	**1.83** *(1.26-2.51)*	**0.99** *(0.47-1.33)*
Adenine nmol 10^{-6} cells (median, quartiles)	**6.41** *(4.63-7.19)*	**2.63** *(1.47-3.63)*	**1.44** *(0.75-2.48)*	**2.59** *(1.67-4.44)*	**1.37** *(1.12-2.97)*	**4.69** *(3.71-5.53)*	**2.41** *(1.79-2.92)*
Non-stimulated PBMC							
IMPDH capacity pmol 10^{-6} cells min^{-1} (median, quartiles)	**5.71** *(3.79-6.93)*	**2.55** *(1.78-4.83)*	**1.98** *(1.27-3.66)*	**3.17** *(2.41-4.37)*	**2.97** *(1.73-3.90)*	**4.67** *(3.06-8.32)*	**2.77** *(1.89-4.42)*
Guanine nmol 10^{-6} cells (median, quartiles)	**0.89** *(0.74-1.16)*	**0.65** *(0.49-0.88)*	**0.58** *(0.48-0.96)*	**0.91** *(0.44-1.18)*	**0.69** *(0.46-1.29)*	**0.96** *(0.55-1.10)*	**0.61** *(0.52-0.99)*
Adenine nmol 10^{-6} cells (median, quartiles)	**2.00** *(1.67-2.51)*	**1.33** *(1.21-1.97)*	**1.46** *(1.09-1.89)*	**1.62** *(1.33-1.97)*	**1.90** *(1.50-2.70)*	**1.89** *(1.28-2.66)*	**1.75** *(1.19-2.55)*

Median (quartiles). t_0; before dose. $t_{1.5}$; 1.5 hours after dose. PBMC; peripheral mononuclear blood cells. MPA; mycophenolic acid. IMPDH; Inosine monophosphate dehydrogenase.

4 Discussion

Therapeutic drug monitoring has been used to improve the immunosuppressive treatment after renal transplantation ever since the introduction of cyclosporine A. For an assay to be of value, several aspects need to be addressed. First, the choice of sample material needs consideration. Concentration in blood, either serum/plasma or whole blood, has been the universal method used to date mainly because of convenience regarding sample collecting and the relatively low sensitivity needed to measure in these sample matrices. However, concerns are being raised about the validity of these matrices [8]. The papers in this thesis highlight how the choice of matrix is not arbitrary, both as to which cells are used and the immunological state of these cells. Second, the analytical technique needs to be considered. Until the introduction of belatacept, all immunosuppressant drugs used in maintenance treatment were relatively small molecules. These are efficiently measured using high performance liquid chromatography with or without mass spectrometry. A larger proportion of new pharmaceuticals entering the marked today are biologicals and alternate techniques may be required to be able to monitor these drugs. Thirdly, when pharmaceuticals are measured by novel principles we need to reassess what target to aim for and how the new assays can be applied.

4.1 Sample matrices

For both Paper I and Paper III PBMC was chosen as sample material. In Paper I, tacrolimus was measured inside this cell population. Tacrolimus inhibits signaling from the T-cells receptor and its site of action is therefore T-cells specific. PBMC is a heterogeneous population consisting of approximately 60% CD3+ T-cells, 5-10% CD20+ B-cells, 5-10% CD14+ monocytes and 5-15% CD56+ Natural killer cells [182,209] in addition to small amounts of dendritic cells. Concentration within the PBMC population could for that reason be expected to reflect the concentration within the T-cells. However, is has been shown that the percentage of T-cells may vary from 27 – 91% and that tacrolimus concentration within the T-cell sub-population does not correlate with the concentration within PBMC in general [182]. Tacrolimus is a substrate for P-gp [107], an efflux protein capable of expelling tacrolimus from the intracellular space. Association between low P-gp activity and tacrolimus nephrotoxicity has been

demonstrated [109], indicating that accumulation of tacrolimus inside the cells is dependent on P-gp. If the T-cells express different P-gp activity than other PBMC sub-populations, this may explain the lack of correlation between concentrations.

PBMC represents an attractive matrix since isolation from whole-blood is easily attained by using Ficoll-based techniques and this may be the reason why several assays measuring intra-cellular levels of immunosuppressant use this population [179,180,185,210-213]. Since the PBMC presents only a small percentage of all cells in peripheral blood many assays for intracellular measurements require some amount of blood, generally between 7-8 mL [107,179-181,183,184]. If sub-populations within PBMC are to be studied, the required amount of whole blood may be unacceptable high in many settings; one method measuring tacrolimus in CD4+ and CD18+ sub-populations requires 40 mL whole blood [182]. In the assay described in Paper I, evaporation of tacrolimus extract from PBMC was evaluated. The signal-to-noise ratio at LLoQ, a common measurement of sensitivity, was 10-fold without evaporation and 33-fold with evaporation. In addition, five PBMC aliquots were isolated from 3 mL whole blood and only one aliquot was needed for intracellular tacrolimus measurement. By using a single aliquot from 3 mL whole blood and including evaporation in the sample preparation, the sample preparation could be scaled to sensitive enough for analysis in sub-populations using an acceptable amount of whole blood.

PBMC was also used in Paper III, where IMPDH-capacity and levels of purines were measured in response to MPA. MPA exerts immunosuppressive effect through inhibition of proliferation; a central step in both T-cell and B-cell mediated immune response. MPA inhibits lymphocytes in general, contrasting tacrolimus which is T-cell specific, and PBMC may represent the target cells of MPA better than as the target of tacrolimus. As shown in Paper III, however, there is a distinct difference between the non-stimulated resting lymphocyte and the activated lymphocyte. MPA had a stronger inhibitory effect on the later than the former. Proliferation, and thereby the stage when MPA exerts its effect, happens after stimulation and stimulated PBMC could be a more relevant sample matrix for IMPDH-capacity measurements that non-stimulated.

However, measuring in stimulated lymphocytes is mostly relevant for assessing the immunosuppressive effect. It is not necessarily a valid sample matrix to evaluate the relationship between IMPDH-capacity and adverse events, since to mechanism for adverse events may be mediated through other pathways than stimulated lymphocytes.

Paper III indicated that IMPDH-measurements in both stimulated and non-stimulated PBMC has potential to identify patients needing reduced doses later on. Measurements in non-stimulated PBMC performed better than measurements in stimulated-PBMC and IMPDH-measurements in non-stimulated PBMC is considerably less technically challenging than in stimulated. For identifying patients at risk for needing reduced doses, IMPDH-measurements should therefore be performed in non-stimulated PBMC.

In Paper II, serum was chosen as the sample matrix for the measurement of belatacept. Since belatacept has a large protein structure, it is expected to mainly be contained within plasma, similarly to other therapeutic proteins [148]. This is confirmed in the pharmacokinetic evaluation in Paper II, where the estimated V_d of 3.5 L reflects the extracellular volume in the circulation. The mechanism of action of belatacept is to block the costimulatory signal during APC and T-cell interaction by binding to CD80 and CD86 on the APC (see Figure 2). The interaction between these cells is assumed to happen in lymph nodes. The lymph node is therefore the site of action of belatacept. Target site concentration monitoring of belatacept could therefore be done by measuring inside lymph nodes, although accessing this compartment is cumbersome and presumably not suitable in a routine analysis. Although lymph nodes can be considered to be the site of action, APC expressing CD80/86 circulates and initial contact between belatacept and CD80/86 is likely to happen in plasma, making this also a site of action. In patients treated with belatacept, >80% of CD80/86 on circulating monocytes are saturated [202] and combined with the slow off-kinetics of belatacept [37] this may also reflect the saturation in lymph nodes.

4.2 Analytical techniques

Whole blood tacrolimus in Paper I was assessed using CMIA. This method generally reports higher tacrolimus concentration compared to HPLC-MS/MS-based methods, although with considerable variation; deviations ranges from 0.0 to 36.7% [214]. In the routine laboratory at Rikshospitalet (where the whole blood tacrolimus concentration in Paper I was measured) the CMIA method was later compared with HPLC-MS/MS. The CMIA based assay reported on average 8.7% higher value than the HPLC-MS/MS assay, but ranged from 8.3% lower to 33% higher (n=113 samples).

This bias is most likely due to the CMIA kit not being specific for tacrolimus alone, but also binds to metabolites. Interference from metabolites was not assessed in the

HPLC-MS method developed in Paper I, but HPLC with tandem MS is less prone to interference from metabolites compared to immunoassay-based techniques [215]. Only a modest correlation between tacrolimus concentration in whole blood and PBMC was seen in Paper I, and a varying degree of interference from the metabolites in the whole blood assay could partly explain this lack of correlation. This is further supported by the observation that the correlation seemed to be stronger at assumed peak concentration compared to trough. At peak concentration, a larger fraction of tacrolimus is in its unmetabolized form and therefore less interference from metabolites is expected. However, studies utilizing HPLC-MS/MS for determination of both whole blood and intracellular tacrolimus concentration show similar weak correlation when measured at trough [180] indicating that weak correlation between whole blood and PBMC concentration is not mainly due to interfering metabolites.

In Paper II, belatacept in serum was measured using its pharmacodynamic target (CD80) and Protein A labeled with europium (see Figure 1 in Paper II for illustration). This format eliminates the need for analyte capturing antibodies used in traditional immunoassays. In normal immunoassays, a pair of antibodies with affinity towards the analyte is used to capture (capture antibody) and measure the amount of captured analyte (tracer antibody, see Figure 4A). Some patients express antibodies which can cross-bind these assay antibodies, resulting in major interference [216,217] (see Figure 4B). Many analytical antibodies are derived from animals (often mice) and exposure to these animals may be a source of biological production of interfering antibodies [218]. By applying the CD80 as the capture-phase and Protein A as a tracer molecule, we avoid using antibodies for this purpose and reduce the susceptibility of interfering antibodies.

Belatacept binds to both CD80 and CD86, but only CD80 was used as a capture reagent in Paper II. Belatacept binds to CD80 with 10-fold higher affinity than CD86 [37] and analytical sensitivity is expected to increase with CD80. Conversely, since more belatacept is needed to saturate CD86, this receptor is used in saturation monitoring [202,203] as it is assumed that CD80 will always be more saturated than CD86.

A possible bias in immunoassay or ligand binding assay used to measure drug concentrations, such as described in Paper II, may arise from reagents cross-reacting with metabolites. This was the case for the tacrolimus CMIA assay described above. Interfering metabolites presents a limited problem for belatacept measurements, as it

does not form metabolites in the traditional sense but is degraded extensively by lysosomal degradation. Other structures with similar Fc-domain and CD80 binding capabilities, currently only abatacept, may cause interference (falsely elevated levels). This was seen during assay validation in Paper II, but may not pose a real issue since these two drugs rarely are co-administered.

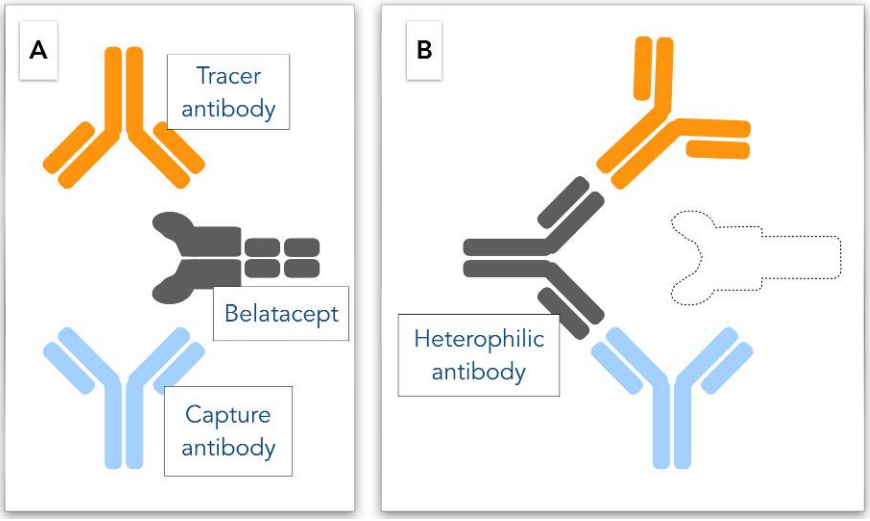

Figure 4: Principle of heterophilic antibody interference in immunoassays.
Panel A (normal situation): A pair of antibodies form a "sandwich" in presence of analyte.
Panel B (interference): Antibody capable of binding both analytical antibodies and forming sandwich without presence of analyte.

By using CD80 to capture belatacept in the sample, only belatacept free to bind to the CD80 would be quantified. The presence of neutralizing antibodies blocking the antigen-biding site of belatacept can therefore be expected to result in low measured levels of belatacept, making it possible to identify patients with neutralizing antibodies. Between 12 and 23% of patients receiving belatacept have been reported to develop anti-belatacept antibodies [219] and 1/5 of these are assumed to be neutralizing [143].

However, proof of this concept was not part of the validation procedure in Paper II. Since samples were diluted 100-fold in non-serum buffer and the assay was performed at room-temperature, it is possible that antibodies bound to belatacept *in vivo* were not bound during analysis due to altered environment. A similar setup for infliximab analysis, using TNF-alfa instead of CD80, has been shown to efficiently identify patients with neutralizing anti-infliximab antibodies [220].

The assay in Paper III was based on quantifying the rate of XMP production after addition of excess amount of substrate (IMP) and cofactor (NAD^+). This format measures

the total capacity of IMPDH, and not the *in vivo* activity that could be limited by available substrate. The term IMPDH-capacity is therefore applied in this thesis.

In both Paper I and Paper III, measurements were normalized to the number of cells. In addition, the amount of intracellular tacrolimus in Paper I was normalized to the amount of protein in the sample. There was only a modest correlation between these two methods of normalization, showing that these are not interchangeable. This is an important consideration since different studies apply different normalization strategies. The most common method for normalizing measurements in PBMC is per million cells [178,179,181-185], although some are per volume [180,221], some per amount of cellular protein [185,194,196,197] and some per concentration of AMP [195]. Studies normalizing intracellular tacrolimus concentration to number of cells [178] and IMPDH capacity to amount of protein [197] have shown the strongest correlation to clinical outcome, although larger studies on the other methods are still lacking.

4.3 Pharmacokinetics

In Paper I, the mean pre-dose concentrations of tacrolimus in PBMC was 31 – 43 pg/10^6 cells, whilst tacrolimus in whole blood was mean 5.3 – 6.3 µg/L. These are comparable concentrations to studies where similar methods have been used [178,179,181], where mean PBMC tacrolimus ranged from 29 to 75 pg/10^6 cells. The correlation between concentration of tacrolimus in whole blood and PBMC at trough was only modest and there was a large inter-individual variation, which is in concordance with other studies [178,179,181]. The modest correlation could partly explain why there is no clear relationship between whole blood monitoring and outcome, since whole blood not necessarily reflects the concentration at the target site. The longitudinal setting in Paper I allowed an intra-individual variation in tacrolimus concentrations to be calculated. The intra-individual coefficient of variation (CV) at t_0 was larger in intracellular concentrations (mean 43%) than in whole blood (mean 23%). This indicates that patients may have a stable whole blood concentration but still experience varying intracellular concentration, and may be an additional reason why tacrolimus whole blood TDM not always predicts outcomes.

Hematocrit during the follow-up in Paper I was increasing, as is expected after renal transplantation. Tacrolimus is highly bound to erythrocytes and its free fraction in plasma is increased at higher whole blood concentrations, indicating that the

erythrocyte compartment is saturable. This could explain the absence of increase in intracellular tacrolimus from t_0 to $t_{1.5}$ in the intermediate (5-7 weeks) and stable post-transplantation phase (1 year), as compared to the immediate post-transplantation phase (6-9 days); at the later days, the erythrocyte compartment is "larger", leading to more tacrolimus being bound here and delaying the distribution to PBMC. Another explanation could be that P-gp activity in lymphocytes may increase after transplantation [222]. P-gp effluxes tacrolimus from the lymphocytes and an increased P-gp expression could therefor delay or reduce the distribution of tacrolimus from whole blood to PBMC.

It is important to note that patients in Paper I were given tacrolimus with immediate release twice daily initially after transplantation, but were given the option to switch to tacrolimus with prolonged release given once per day after stabilizing. Most patients switched formulation, but mainly after the sampling day at 5-7 weeks. A switch to prolonged formulation intuitively explains a lower increase in concentration from t_0 to $t_{1.5}$, but only one patient had switched formulation at 5-7 weeks, when the lack of increase was also evident. Differences between immediate and prolonged release formulation therefore does not explain the lack of increase in intracellular concentration from t_0 to $t_{1.5}$, also supported by the observed increase in whole blood concentration from t_0 to $t_{1.5}$. Since samples were only collected at two time points during each dose interval it could not be determined in Paper I if the time-concentration curve of intracellular remained constant (flat) between doses, or if the concentration peak was merely delayed. If the intracellular concentration remains constant over the dose interval, then intracellular concentrations could be measured at other time points than trough and still reflect the intracellular exposure overall. However, Paper I indicated that this only occurs at 5-7 weeks and later after transplantation, and studies measuring complete intracellular AUC at 5-7 weeks and later should be performed to confirm that a constant intracellular concentration is the case.

Basic pharmacokinetic parameters of belatacept were calculated in Paper II. Although only five patients were included and patients were not on the same dose interval (two in the maintenance phase, two in the induction phase, and one transiting between the phases) and one patient did not participate on all sampling days, the model was able to propose a 3-compartment model with a V_d of the central compartment being 3.5 +/- 0.6 L and an elimination constant of 0.013 +/- 0.002 h^{-1}. There are few published

pharmacokinetic models on belatacept and the sample size in Paper II is limited. There are therefore limitations to the assessment of pharmacokinetic values in Paper II. However, abatacept is expected to have similar pharmacokinetics since the chemical structure is almost identical as belatacept. The central compartment V_d of abatacept has been calculated to be 3.3 L, although with a lower elimination constant (0.006 h^{-1}) [223], compared to the findings in Paper II.

Belatacept dosing is adjusted according to bodyweight to ensure a more homogenous exposure between patients. However, the results in Paper II suggest that heavier patients may have a higher systemic exposure. Two patients had vastly different body weights (63 kg versus 109 kg) and therefore received different doses (315 mg vs 550 mg). Both peak and trough-concentrations were almost double for the heavier patient, indicating that adjusting on body weight alone may not result in equal drug exposure. Heavy or light patients may therefore be subject to over- or underexposure, respectively. Since belatacept is largely distributed to the blood plasma, adjusting dose to lean body mass instead of total body weight may potentially result in a more homogenous exposure between individuals.

4.4 Pharmacodynamics

Although pharmacodynamic measurement of tacrolimus was not presented in Paper I, Bremer *et al* measured NFAT-RGE in the same patient cohort [173]. NFAT-RGE (*i.e.* NFAT regulated cytokine expression 1.5 hours after dose as a percentage of pre-dose expression) was lower at 6-9 days after transplantation compared to 5-7 weeks and 1 year. This is in concordance with the lesser increase in intracellular tacrolimus concentration from t_0 to $t_{1.5}$ seen at the later days in Paper I. However, NFAT-RGE at 5-7 weeks and 1 year were approximately 40% and 50%. This means that NFAT regulated cytokine expression was more than halved without a noticeable increase in intracellular tacrolimus concentration. This discrepancy may be related to the choice of PBMC as sample matrix. Since intracellular concentration of tacrolimus in PBMC not necessarily correlates with specific subpopulations such as CD4+ T-cells [182] there could still be an increase in tacrolimus concentrations in CD8+ and CD4+ T-cells that is not reflected in the PBMC population in general. If NFAT regulated cytokine expression is correlated with concentrations within these T-cell subpopulations, this could explain the reason why NFAT appears to be inhibited without seeing an increase in intracellular

concentrations in PBMC. It has also been shown that NFAT-RGE measured in whole blood is more sensitive towards calcineurin inhibition than when measured in PBMC [224], making it overall difficult to compare intracellular concentrations of tacrolimus in PBMC to NFAT-RGE measured in whole blood.

Pharmacodynamic monitoring of MPA was performed in Paper III. The inhibition of the target enzyme (IMPDH) and alteration in the pool of nucleotides (guanine and adenine) in PBMC were examined longitudinally in a cohort of renal transplant recipients. One of the most surprising observations was that IMPDH appeared less sensitive towards MPA over time, with IMPDH-capacity in non-stimulated PBMC reaching pre-transplantation levels after one year of treatment. Few reports on longitudinal IMPDH-measurements during MMF or MPA treatment have been published, but increasing IMPDH-capacity over time has been shown by both Chiarelli *et al* [225] and Molinaro *et al* [196]. Chiarelli demonstrated that this increase occurred in stable renal recipients (median three years after transplantation, minimum 6 months) and Molinaro saw a similar increase between 2 and 24 weeks after transplantation, whilst MPA plasma concentrations did not change. Others have demonstrated similar differences when early (<1 year post-transplant) and late (>2 years post-transplant) recipients were compared regarding IMPDH-inhibition by MPA [226]. A possible explanation for this increase could be a shift towards more IMPDH 1 and less IMPDH 2. Since MPA inhibits IMPDH 2 more than it inhibits IMPDH 1, an increase in IMPDH 1 proportion could explain why total IMPDH-capacity appears to increase. However, no clear shift towards IMPDH 1 has been shown in studies measuring IMPDH 1 and 2 expressions longitudinally after transplantation. Some studies show that both isoform have reduced expressions within the first week after transplantation before expression increases to above pre-transplantation expression over the next four months [227]. Others show an initial increase in expression of IMPDH 1 and 2 from pre-transplantation to day 2 after transplantation followed by a reduction and stabilization during the first two weeks [228]. No change in either IMPDH 1 or 2 from the second week to month 6 after transplantation has also been reported [196]. An alternative explanation could be increased expression of the transporter protein MRP2 in lymphocytes after transplantation. MPA is a substrate of MRP2 [229] and an increased expression of this transporter protein could be part of the reason why IMPDH-capacity appears to increase over time, since less MPA would distribute to lymphocytes. To date, no studies

describing alteration of MRP2 expression after transplantation has been published, so this mechanism remains unexplored.

The rate of rejection episodes within the first year has been greatly reduced over the last decades, but the rate of late rejections appears to be unchanged. Antibodies has been suggested as an important contributor in late (often termed chronic) rejection [230] and can lead to late stage graft failure, occurring years after transplantation [231]. The formation of *de novo* donor specific antibodies (dnDSA) occurs median 4.6 years after transplantation [232] and has been implicated in loss of function or graft loss in stable renal recipients [233]. While calcineurin inhibitors are mostly T-cells specific, MPA inhibits proliferation of both T and B cells. Antibodies are produced via the B-cell linage and a failure in MPA treatment could therefore lead to increased risk of dnDSA, resulting in rejection or loss of function. The increasing IMPDH-capacities seen after transplantation, illustrated in Paper III, could potentially be a contributor to the issue of late stage rejections.

Paper III also showed that mitogen stimulation increased the amount of both guanine and adenine nucleotides, as expected in activated lymphocytes. Nucleotide levels also decreased after initiation of MMF treatment and from before to 1.5 hours after MMF dose. This contrasted with non-stimulated PBMC where nucleotides remained largely unaltered. These findings underline the concept that nucleotide levels are maintained through the IMPDH independent salvage pathway in resting lymphocytes, but are synthesized *de novo* during activation. Under the current understanding of *de novo* purine synthesis, IMPDH is not a central step in the formation of adenine nucleotides. However, the synthesis of IMP, the common precursor for both adenine and guanine nucleotides, is believed to be regulated by guanine nucleotides [234] and the further conversion of IMP to AMP could be guanosine phosphate dependent [235]. A depletion of guanine nucleotides could therefore reduce the availability of IMP for synthesis of adenine nucleotides and explain the correlation seen between adenosine nucleotides and guanosine nucleotides in Paper III. Qiu *et al* demonstrated similar findings [198], but did also measure the alteration in cytidine and uridine phosphates following stimulation. They demonstrated that although uridine phosphate synthesis appeared unaffected by MPA, expansion of the cytidine nucleoside pool was restricted by MPA, possibly explained by CTP-synthetase being GTP dependent

[235]. This highlights that the purine and pyrimidine synthesis is co-regulated, and depletion of guanosine nucleotides may not be the sole effect of MPA treatment.

4.5 Clinical outcomes

In Paper I, a cohort of renal transplant recipients was followed for one year after transplantation and the occurrence of BPAR was recorded. The relationship between tacrolimus concentration in whole blood, in PBMC, and the degree tacrolimus was distributed to PBMC (measured as the ratio between the concentrations in the different matrices), did not differ between those with and without BPAR. The large variation in PBMC concentrations of tacrolimus and the relative low number of patients in Paper I make the data underpowered to determine whether there is a relationship between BPAR and intracellular concentration. In addition, the other immunosuppressants given to these patients are expected to be important confounders, making it difficult to assess the isolated effect of tacrolimus in PBMC (or whole blood) on the BPAR risk. Few studies have shown clear association between PBMC concentration of tacrolimus and the risk of BPAR, but Capron *et al.* showed that liver transplant recipients with BPAR had lower PBMC concentration of tacrolimus (14.2 – 33.8 pg/10^6 cells) compared to those without BPAR (24.6 – 90.9 pg/10^6) in the first week after transplantation while on tacrolimus monotherapy [178], illustrating that intracellular tacrolimus measurements may have merit in this patient population.

In Paper III, none of the pharmacodynamic measurements could be related to the risk of BPAR. The relative low number of patients, the variability in both IMPDH and purines and the confounding effect of other immunosuppressant are also expected to obscure any correlation between the biomarkers and BPAR. However, IMPDH-capacity in non-stimulated PBMC 6-9 days after transplantation was significantly associated with the need for dose-reduction within the first year after transplantation. Although non-significant, similar relationships were observed 0-4 days prior to transplantation and in *ex vivo* stimulated PBMC. The principle of IMPDH capacity in unstimulated cells as predictor of MMF dose reduction is supported by others [197]. If these findings are confirmed in larger studies with precise quantitative relationships between IMPDH capacity and risk of needing to reduce MMF doses, early IMPDH measurements could be used to identify patients who could benefit from an alternative drug regimen.

5 Future perspectives

In this thesis, three different assays have been used in a novel way to measure immunosuppressant drugs used after transplantation. In Paper I, the intracellular concentration of tacrolimus in PBMC was assessed using liquid chromatography and mass spectrometry. Traditional whole blood concentrations had limited correlation with the concentrations in PBMC, and with the latter being more representative of the target cell population (lymphocytes, particularly $CD4^+$ T-helper cells) this should intuitively be a better method. However, some aspects should be considered before applying this assay. Paper I demonstrated a large intra-individual variation in intracellular tacrolimus concentrations. TDM may be limited by intra-individual variation since it weakens the correlation between a measured concentration and future concentrations, making it difficult to assess what dose is required to attain a given concentration. In addition, the validity of PBMC as a representative matrix should be carefully considered since a large part of this population is not the target of tacrolimus. The sensitivity of the assay presented in Paper I does allow for tacrolimus to be measured in sub-populations of PBMC, *e.g.* $CD4^+$ T-cells and this could be the subject of further research. Paper I also illustrated a phenomenon of altered tacrolimus distribution seen in PBMC, where there was an increase in intracellular concentration from trough to peak whole blood concentration at 6-9 days after transplantation, but not at 5-7 weeks or 1 year. This time-dependent alteration of distribution into mononuclear cells should be considered when whole blood measurements at different times after transplantation are compared.

Prior to Paper II, no assay for measuring belatacept was available. The assay for determining belatacept concentrations in serum in Paper II therefore makes belatacept TDM possible. Although both literature [142-144] and pharmacokinetic data in Paper II show stable and predictable belatacept pharmacokinetics (*i.e*. indicators of TDM not being warranted), TDM has shown beneficial effects with other biological drugs with stable pharmacokinetics [236]. Future alteration in belatacept treatment, like prolonged times between infusions or a sub-cutaneous formulation could warrant monitoring since some patients could be under-exposed in such settings. In addition, since the assay in Paper II utilized drug target to capture and measure belatacept, the assay could be used to identify patients where belatacept is hindered to bind to the drug target, *e.g.* due to neutralizing anti-belatacept antibodies.

Paper III showed that IMPDH-monitoring early after renal transplantation could potentially identify patients that later needed reduction of MMF dose, a known risk-factor for rejection. This relationship could be elaborated on further by proactively reduce the MMF dose in patients with low IMPDH-capacity, and see if this can reduce the incidence of adverse effects without increasing rates of rejections. The increase in IMPDH-capacity seen from early to late after transplantation could also be compared to the development of dnDSA.

6 Conclusion

To conclude, this thesis has demonstrated alternative methods of monitoring of the immunosuppressive treatment given to renal transplant recipients.

An assay for measuring tacrolimus inside a lymphocyte-enriched cell population (PBMC) was developed and validated. Longitudinal monitoring of renal transplant recipients showed weak correlation between tacrolimus in PBMC and in whole-blood. PBMC concentrations had larger inter- and intra-individual variation than whole-blood concentrations. PBMC-tacrolimus kinetics appeared to become altered during the first year after transplantation. The increase from trough to 1.5 hours after dose seen the first week after transplantation was not evident at five weeks and later, where PBMC concentrations were similar before and after dose. Limited correlation between PBMC concentration normalized to number of cells and to amount of cellular protein shows that these two methods are not comparable.

Pharmacokinetic measurements of belatacept are now possible through a newly developed assay, suitable for routine concentration measurements. Belatacept pharmacokinetics appears to have low variability, but adjusting dose according to total body weight might result in different exposure in light and heavy patients.

MPA appear to be a stronger inhibitor of stimulated PBMC compared to non-stimulated PBMC. The levels of guanine and adenine in resting PBMC do not appear to be altered by MPA, possibly due to salvage pathway recirculation. IMPDH-capacity appears to increase over time after renal transplantation. A low IMPDH-capacity early after transplantation could be associated with increased risk for needing reduced MMF doses due to adverse events.

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Paper III

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ORIGINAL ARTICLE

Pharmacodynamic assessment of mycophenolic acid in resting and activated target cell population during the first year after renal transplantation

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Aims: To explore the pharmacodynamics of mycophenolic acid (MPA) through inosine monophosphate dehydrogenase (IMPDH) capacity measurement and purine levels in peripheral blood mononuclear cells (PBMC) longitudinally during the first year after renal transplantation (TX).

Methods: PBMC were isolated from renal recipients 0–4 days prior to and 6–9 days, 5–7 weeks and 1 year after TX (before and 1.5 hours after dose). IMPDH capacity and purine (guanine and adenine) levels were measured in stimulated and nonstimulated PBMC.

Results: Twenty-nine patients completed the follow-up period, of whom 24 received MPA. In stimulated PBMC, the IMPDH capacity (pmol 10^{-6} cells min^{-1}) was median (interquartile range) 127 (95.8–147) before TX and thereafter 44.9 (19.2–93.2) predose and 12.1 (4.64–23.6) 1.5 hours postdose across study days after TX. The corresponding IMPDH capacity in nonstimulated PBMC was 5.71 (3.79–6.93), 3.35 (2.31–5.62) and 2.71 (1.38–4.08), respectively. Predose IMPDH capacity in nonstimulated PBMC increased with time, reaching pre-TX values at 1 year. In stimulated PBMC, both purines were reduced before (median 39% reduction across days after TX) and after (69% reduction) dose compared to before TX. No alteration in the purine levels was observed in nonstimulated PBMC. Patients needing dose reductions during the first year had lower pre-dose IMPDH capacity in nonstimulated PBMC (1.87 *vs* 3.00 pmol 10^{-6} cells min^{-1}, $P = .049$) at 6–9 days.

Conclusion: The inhibitory effect of MPA was stronger in stimulated PBMC. Nonstimulated PBMC became less sensitive to MPA during the first year after TX. Early IMPDH capacity appeared to be predictive of dose reductions.

The authors confirm that the Principal Investigator for this paper is Karsten Midtvedt MD PhD and that he had direct clinical responsibility for patients.

KEYWORDS
biomarkers, immunosuppression, therapeutic drug monitoring, transplantation

1 | INTRODUCTION

Proliferation of activated B and T cells is a vital step in an adaptive immune response, and the purine nucleotides of **guanine** and **adenine** are in this respect essential for the (deoxy)ribonucleic acid synthesis. Mammalian cells with a low proliferation rate rely mainly on the salvage pathway for these purines,[1] whilst rapidly dividing lymphocytes are more dependent on *de novo* purine synthesis.[2] **Mycophenolic acid** (MPA) is a reversible inhibitor of **inosine monophosphate dehydrogenase** (IMPDH), a rate-limiting enzyme in the *de novo* synthesis of guanine nucleotides (Figure 1). MPA therefore inhibits proliferating lymphocytes and is frequently used as an immunosuppressant to prevent rejection after solid organ transplantation[3,4] and to treat certain autoimmune disorders.[5-7]

After renal transplantation, either the sodium salt of MPA or the prodrug mycophenolate mofetil (MFF) is used together with other immunosuppressants, commonly a calcineurin inhibitor and prednisolone. By including MMF in the immunosuppressive regimen after renal transplantation, the incidence of biopsy-proven acute rejections (BPAR) within the first year was reduced from 56 to 30%.[8] Reduction in dose, therapy interruption, or discontinuation of MMF or MPA treatment is associated with increased risk of BPAR.[9] Nevertheless, it has been reported that up to half of patients starting on MMF need dose reductions within the first year after renal transplantation, most often due to haematological toxicities.[10]

An MPA area under the concentration *vs* time curve (AUC) below 30–40 mg h L^{-1},[11-15] or an MPA trough concentration below 1.6 mg/L[16-18] is associated with the risk of BPAR. Although some studies have shown a relationship between AUC[19] or trough concentrations and MPA-related adverse events,[18] including leucopenia and anaemia,[20] a clear relationship between MPA exposure and toxicity has not been determined.[21,22] As an alternative to MPA concentration-based monitoring, assays for pharmacodynamic monitoring of MPA have been developed[23-27] with the rationale being that there is an interindividual variation in the pharmacodynamic response to the same MPA exposure. While most assays quantify the effect of MPA on the target enzyme IMPDH, some assays monitor the levels of lymphocyte purine nucleotides.[24,28] Although the adenosine-5′-triphosphate (ATP) levels in *ex-vivo* activated CD4+ cells have been suggested to reflect the immune status with the ability to predict BPAR,[29] the application of purines as selective biomarkers for MPA has not been widely explored in a clinical setting.

In this study, we aimed to explore the potential applicability of molecular pharmacodynamic markers for monitoring MPA therapy. We performed a longitudinal study during the first year after renal transplantation. Samples were collected pre- and post-transplant at the time of MPA trough and peak concentrations, and both IMPDH capacity and levels of adenine and guanine nucleotides were assessed in nonstimulated and *ex vivo*-stimulated lymphocytes. In this explorative setting, associations between these biomarkers and 2 central clinical end points—BPAR and dose reduction—were examined.

What is already known about the subject

- The prodrug mycophenolate mofetil (MMF) and its metabolite mycophenolic acid (MPA) are used after solid organ transplantation, impeding the lymphocyte proliferation through inhibition of inosine monophosphate dehydrogenase (IMPDH) and the *de novo* purine synthesis.
- IMPDH monitoring in renal transplant patients has shown potential utility, but longitudinal studies are lacking and there are few studies on the pharmacodynamic monitoring in activated lymphocytes.
- Adverse effects are a common reason for MMF dose reduction and have been associated with impaired outcome.

What this study adds

- MPA is a more potent inhibitor of IMPDH in activated peripheral blood mononuclear cells compared with nonactivated cells.
- Over time, circulating lymphocytes become less sensitive to MPA.
- The IMPDH capacity in peripheral blood mononuclear cells is low early after renal transplantation in patients needing later MMF dose reduction.

2 | METHODS

2.1 | Nomenclature of targets and ligands

Key drugs and targets are named according to the Concise Guide to PHARMACOLOGY 2017/2018 source.[30]

2.2 | Patients, treatment protocol and sampling

Patients admitted for renal transplantation, aged 18 years or older and receiving transplant from living donors, were included. The study was approved by the Regional Committee for Medical and Health Research Ethics (reference 2011/1282) and performed in accordance

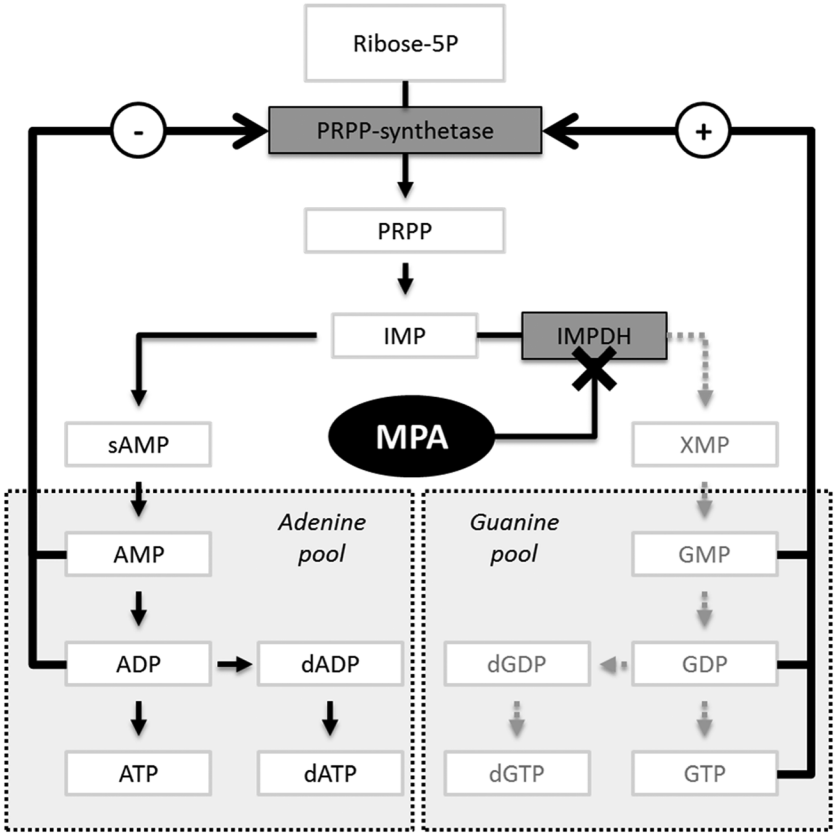

FIGURE 1 Ribose-5P is synthesized to phosphoribosyl pyrophosphate (PRPP) by PRPP-synthetase and further to inosine monophosphate (IMP). Mycophenolic acid (MPA) inhibits inosine monophosphate dehydrogenase (IMPDH) and thereby the conversion of IMP to xanthosine monophosphate (XMP), guanosine-5′-monophosphate (GMP), guanosine-5′-diphosphate (GDP), 2′-deoxyguanosine-5′-diphosphate (dGDP), guanosine-5′-triphosphate (GTP) and 2′-deoxyguanosine-5′-triphosphate (dGTP). AMP, (d)ADP, and (d)ATP are corresponding adenosine nucleotides

with the declaration of Helsinki. Written informed consents were provided from the participants. Data on tacrolimus (TAC) concentrations in peripheral blood mononuclear cells (PBMC)[31] and the nuclear factor of activated T-cell-regulated gene expression[32] are previously reported.

All patients received TAC and prednisolone. Patients with 1 or more human leucocyte antigen (HLA)-mismatches were given 750 mg MMF twice daily. Standard risk patients (i.e patients without donor specific antibodies, panel reactive antibodies or ABO mismatch) were given TAC initially administered at 0.04 mg kg^{-1} twice daily and adjusted to obtain a predose concentration within 3–7 μg L^{-1} in whole blood during the first 180 days post-transplant and thereafter 3–5 μg L^{-1}. Standard risk patients received 250 mg methylprednisolone (350 mg if body weight was >90 kg) intravenously on the day of transplantation followed by peroral prednisolone once daily with a gradual reduction to 5 mg daily at 6 months. Basiliximab was given at the day of transplantation and at day 4 (20 mg each day). TAC, MMF and prednisolone were administered simultaneously.

One patient with pretransplant donor-specific antibodies was classified as high risk with increased risk of graft rejection. This patient received enhanced immunosuppression according to protocol: TAC target level was initially 8–12 μg L^{-1} (day 0–30) and 6–10 μg L^{-1} thereafter. Methylprednisolone (350 mg) and prednisolone (80 mg) was given on the day of transplantation. Prednisolone was tapered to 10 mg by week 8. Rituximab (375 mg m^{-2}) was given 30 days prior to transplantation and human normal immunoglobulin (400 mg kg^{-1} day^{-1}) was given day 0–4 after transplantation. MMF treatment was the same for standard and high-risk patients.

Blood samples were collected in the morning on 4 occasions: 0–4 days before transplantation, and 6–9 days, 5–7 weeks and 1 year after transplantation. On each post-transplant occasion, blood samples were collected immediately prior to (t_0) and 1.5 hours ($t_{1.5}$) after the morning dose. At each sampling, blood was collected in 4-mL EDTA tubes (Vacuette, Greiner Bio-One, Monroe, NC, USA) for determination of MPA plasma concentration and in 4-mL heparinized tubes (Vacuette) for isolation of PBMCs and subsequent measurement of the IMPDH capacity and the levels of guanine and adenine nucleotides.

As per hospital protocol, surveillance graft biopsies were collected and classified according to Banff criteria,[33] at week 6, at 1 year or when a rejection was clinically suspected. The MMF dose was adjusted according to clinical judgment in standard practice.

2.3 | Determination of MPA plasma concentration

Plasma was isolated from EDTA whole blood by centrifugation at 1500 *g* for 10 minutes at 4°C and stored at −80°C until analysis. Determination of plasma MPA has been described previously.[34] Briefly, plasma samples were thawed and 200 μL of acidified methanol was added to 100 μL of sample for protein precipitation. After centrifugation at 4°C, the supernatant was diluted with equal volume of water. Separation and analysis were performed using reverse phase high performance liquid chromatography (Ultimate 3000, Thermo Fisher, Waltham, MA, USA). A 15-cm phenyl column with 3-μm particles (ACE Phenyl, Advanced Chromatography Technologies, Aberdeen, UK) was applied with mobile phase containing 53% methanol

and phosphate buffer (pH 2.5). Detection was performed with UV absorbance at 215 nm.

2.4 | PBMC isolation and stimulation

PBMCs were isolated from 4 mL heparinized whole blood with slight modifications to the protocol of the LeucoSep manufacturer (Greiner Bio-One, Kremsmünster, Austria). The plasma was first separated by centrifugation (1000 *g*, 10 min, 4°C). Remaining blood cells were mixed with 6 mL cold phosphate-buffered saline (PBS) without calcium or magnesium (BioWhittaker; Lonza, Basel, Switzerland) and thereafter transferred to prefilled LeucoSep-tubes and centrifuged (1000 *g*, 10 min, 4°C). The resulting PBMC layer was transferred to a 14 mL round-bottom polystyrene tube and washed by resuspension in 5 mL cold PBS and centrifuged (300 *g*, 10 min, 4°C). The supernatant was discarded and the remaining PBMC pellet was resuspended in 1 mL Roswell Park Memorial Institute medium (RPMI; Sigma Aldrich, St. Louis, MO, USA). An aliquot of 10 μL was used for cell counting on a Coulter counter (Counter Z-series, Beckman Coulter, Brea, CA, USA) with particle size range 5–15 μm. The cell suspension was diluted with heparinized plasma from the original sample and RPMI, resulting in a final concentration of 20% ($^{v}/_{v}$) plasma and 1.6×10^6 cells mL^{-1}. Diluted cell suspensions (200 μL aliquots) were transferred to 2 5 mL round-bottom tubes. One aliquot was to added 200 μL of RPMI containing mitogens for activation of lymphocytes (100 ng mL^{-1} phorbol-12-myristate-13-acetate, 2.5 μg L^{-1} ionomycin, 0.030% $^{v}/_{v}$ DMSO, 200 U L^{-1} penicillin–streptomycin), and the other aliquot was added to RPMI without mitogens (0.030% $^{v}/_{v}$ DMSO, 200 U L^{-1} penicillin–streptomycin). The cell suspensions were thereafter incubated for 72 hours in a humid environment (37°C, 5% CO_2) with cap allowing for gas exchange. After incubation, the cells were centrifuged (1000 *g*, 4°C, 5 min) and the supernatant was removed. The cells were resuspended in 500 μL cold PBS and transferred to 1.5 mL microcentrifuge tubes (LoBind; Eppendorf, Hamburg, Germany). The PBS supernatant was removed after centrifugation (2350 g, 2 min, 4°C) and the resulting cell pellet was lysed in 125 μL de-ionized water (Milli-Q; Merck, Darmstadt, Germany) during vortex. The lysates were stored at −80°C until assaying IMPDH capacity and purines.

2.5 | Determination of IMPDH capacity and purines in PBMC

We used a previously reported assay for the simultaneous determination of guanine, adenine and IMPDH capacity.[24] The samples were thawed at room temperature and homogenized by vortexing (30 s) and ultrasonication (120 s). Following centrifugation (1150 *g*, 2 min), 50 μL supernatant was added to each of 2 1.5-mL polypropylene microcentrifuge tubes (aliquots A and B). To both A and B, 50 μL of aqueous buffer solution (250 μmol L^{-1} trishydrochloride, 7.5 mmol L^{-1} EDTA, 250 μmol L^{-1} KCl, 5.0 mmol L^{-1} DTT) was added. To A, 25 μL of an aqueous solution containing 9.0 mmol L^{-1} IMP and 2.0 mmol L^{-1} NAD+ was added, and 25 μL of deionized water was added to B. Both aliquots were briefly vortexed and aliquot A was incubated in a heated water bath (37°C) for 120 minutes to allow IMPDH-mediated production of xanthosine monophosphate (XMP). Aliqout B was kept on the laboratory bench during incubation. The enzyme reaction was terminated by adding 20 μL of 4.0 mol L^{-1} perchloric acid and a brief vortex (added to aliquot A and B). A 20-μL volume aqueous solution containing internal standards (25 μmol L^{-1} 1,3-^{15} N_2-xanthine, 25 μmol L^{-1} 8-^{13}C-7,9-^{15} N_2-guanine, 25 μmol L^{-1} $^{13}C_5$-adenine) was added to both aliquots. After vortex and centrifugation (9400 g, 4 min, 4°C) the supernatants were transferred to flat-bottom glass inserts in liquid chromatography vials. The vials were placed on a heating block (60 minutes 100°C) to hydrolyse XMP, guanosine nucleotides (guanosine-5′-monophophate, guanosine-5′-diphosphate, guanosine-5′-triphosphate, 2′-deoxy-guanosine-5′-diphosphate and 2′-deoxy-guanosine-5′-triphosphate) and adenosine nucleotides (adenosine-5′-monophophate, adenosine-5′-diphosphate, adenosine-5′-triphosphate, 2′-deoxy-adenosine-5′-diphosphate and 2′-deoxy-adenosine-5′-triphosphate) to xanthine, guanine and adenine respectively. After cooling, 15 μL aqueous solution containing 4.0 mol L^{-1} potassium acetate was added to each vial. The vials were centrifuged (2000 g, 5 min, 4°C) and placed in the autosampler maintained at 4°C. Details regarding the liquid chromatography–tandem mass spectrometry conditions are described in the method publication.[24] The IMPDH capacity was calculated using the following formula:

$$IMPDH\ capacity = \frac{\frac{(XAN_A - XAN_B)}{N}}{t} \left(units : pmol\ 10^{-6}\ cells\ min^{-1} \right)$$

XAN_A and XAN_B was the amount of xanthine (pmol) in aliquot A and B, respectively, while N was the number of lysed cells (in millions) in the reaction and t was the duration of the enzyme reaction (min).

Since the assay measures the rate of XMP production in a setting where both substrate and co-factor are added to saturate the enzyme reaction, the term IMPDH capacity was applied to underline that this was the maximum *ex vivo* production rate (not the *in vivo* IMPDH activity).

Guanine and adenine were measured in aliquot B and expressed as nmol 10^{-6} cells, reflecting the corresponding purine nucleotide pools.

2.6 | IMPDH, purines and clinical outcome

Results from biopsy evaluations as well as any need for MMF dose reduction were collected from patient records. To assess the predictability of IMPDH-capacity, guanine and adenine on the risk for BPAR these measurements were compared before transplantation between patients with and without BPAR within the first year after transplantation. To assess predictability whilst under MMF treatment we divided patients who had been BPAR free until 5–7 weeks into those who later did and did not have BPAR, and compared the biomarkers between these groups.

To explore the predictability of biomarkers measured early after transplantation on the need for later reduction of MMF dose, the

biomarkers and MPA plasma concentrations 0–4 days before and 6–9 days after transplantation were compared between patients who later needed MMF dose reduction and patients who remained on the initial dose during the whole study.

2.7 | Statistics and data sharing

The study was designed as an exploratory study to describe the molecular pharmacodynamics of MPA and to identify the potential associations between the investigated biomarkers and clinical outcome.

Continuous variables were compared using a *t*-test when data was normally distributed or when ln-transformation resulted in normal distribution. Continuous variables not being normally distributed were compared using Wilcoxon signed rank test for paired data or Mann–Whitney U test for unpaired data. Pearson correlation was used to correlate IMPDH capacities in stimulated and nonstimulated PBMC and to correlate adenine and guanine. Spearman correlation was used to correlate IMPDH capacity and MPA plasma concentrations.

Multigroup comparison between continuous variables collected at different times was performed using Skillings–Mack test to allow for non-normal distribution and missing data.

Statistical significance was considered with 2 tails at $P < 0.05$.

All statistical tests were performed in R v.1.1.447.

Research data are not shared.

3 | RESULTS

3.1 | Patients and clinical outcome

Out of 33 patients initially included in the study, 4 were excluded before the first sampling (2 transplantations were postponed, 1 switched to cyclosporine, 1 was lost to study follow-up). Among the remaining 29 patients, 5 patients received a zero HLA-mismatched graft and, according to protocol, were not started on MMF at the time of transplantation. From patients receiving MMF at the time of sampling, IMPDH capacity and purine levels were measured in 94% (n = 158) of the maximum possible number (n = 168) of samples. In addition, a total of 29 samples from patients not receiving MMF (n = 5) were analysed for IMPDH and purines. Complete datasets from 13 patients using MMF throughout the study, and not receiving MMF before the pretransplant sample, were available. A total of 8 BPARs (2 with clinical manifestations) were recorded in 7 patients. One patient was diagnosed with BPAR at day 4 and 1 year, 1 patient at day 5, 1 patient at 60 days, 1 patient at day 34 and 3 patients at 1 year. One of the patients with BPAR at 5–7 weeks received a zero HLA-mismatch graft and did not receive MMF, but was started on MMF after the BPAR episode.

Of the 24 patients given MMF at transplantation, 6 (25%) required a dose reduction during the first year after transplantation. Three patients required a dose reduction at 8, 80 and 111 days after transplantation, but received full dose at 1 year. The remaining 3 patients had reduced dose at 1 year.

3.2 | MPA concentrations

Plasma concentrations of MPA are summarized in Table 1. Predose concentrations varied between study days ($P < .001$) and were lower at 6–9 days compared to the other study days ($P < .010$), whilst no difference between 5–7 weeks and 1 year could be shown ($P = .948$). No difference between MPA $t_{1.5}$ concentrations was observed with respect to time after transplantation ($P = .405$).

3.3 | IMPDH capacity

The IMPDH capacity in ex vivo stimulated and nonstimulated PBMC is shown in Table 1 and Table 2. In pretransplant samples taken before commencement of any immunosuppressant therapies (n = 23), mitogen stimulation increased the IMPDH capacity on average 22-fold (range 6–45-fold). In samples collected from patients receiving MMF after transplantation, mitogen stimulation increased predose IMPDH capacity on average 15-fold (range 1.1–60-fold) and postdose IMPDH capacity increased on average 7.1-fold (range 0.7–59-fold), compared to no stimulation. Before transplantation, the interindividual variation (CV %) in IMPDH capacity was 40% in stimulated PBMC and 62% in nonstimulated PBMC. In the stimulated PBMC post-transplantation, the interindividual variation was highest 6–9 days after transplantation being (before/after dose) 111/124%, and thereafter 82/112% at 5–7 weeks and 59% /72% at 1 year. The interindividual variations in IMPDH capacity in nonstimulated PBMC showed a similar pattern: 100/102% at 6–9 days, 71/68% at 5–7 weeks and 62% /74% at 1 year.

Predose IMPDH capacity varied between study days both in stimulated (p = 0.041) and nonstimulated PBMC ($P = .032$). At 1 year, the predose IMPDH capacity was higher compared to 6–9 days after transplantation, both in stimulated and nonstimulated PBMC ($P < .05$). Figure 2 shows the IMPDH capacity in stimulated and nonstimulated PBMC at the different sampling times after transplantation as a percentage of pretransplantation values. In patients receiving MMF after transplantation, the IMPDH capacity in nonstimulated PBMC measured before dose at 1 year after transplantation was comparable to pretransplant values measured in patient not receiving MMF at the pretransplant time point (Figure 2, Panel B).

After dose, the IMPDH capacity in nonstimulated PBMC was median 75, 73 and 57% of the predose levels at 6–9 days, 5–7 weeks and 1 year, respectively ($P < .033$). In the stimulated PBMC, the IMPDH capacity after dose was median 26, 34 and 21% of the predose levels at 6–9 days, 5–7 weeks and 1 year, respectively ($P < .001$).

Correlation between MPA plasma concentration and IMPDH capacity in either stimulated or nonstimulated PBMC was only observed at 1 year in stimulated cells. There was no correlation at any time point in nonstimulated cells (Figure S1).

TABLE 1 MPA plasma concentrations and biomarkers measured in peripheral blood mononuclear cells from 24 patients before and after renal transplantation (median, quartiles)

		0–4 days before treatment	6–9 days t0	6–9 days t1.5	5–7 weeks t0	5–7 weeks t1.5	1 year t0	1 year t1.5	*P*-value across study days (Skillings–Mack)	
Number of patients		19	22	22	24	24	23	24	t0	t1.5
Plasma MPA (mg/L)		-	1.47 (1.14–2.39)	5.27 (3.84–7.59)	2.75 (1.75–3.26)	7.02 (4.56–9.95)	1.98 (1.77–4.09)	6.47 (4.77–7.98)	<.001	.405
IMPDH capacity (pmol 10^{-6} cells min^{-1})	Stimulated	127 (95.8–147)	33.1 (13.4–50.7)	5.11*** (2.30–25.1)	35.3 (16.1–83.4)	10.7*** (5.72–19.5)	84.8 (43.6–99.3)	18.4*** (7.34–31.2)	.041	.526
	Nonstimulated	5.71 (3.79–6.93)	2.55 (1.78–4.83)	1.98* (1.27–3.66)	3.17 (2.41–4.37)	2.97* (1.73–3.90)	4.67 (3.06–8.32)	2.77*** (1.89–4.42)	.032	.343
Guanine (nmol 10^{-6} cells)	Stimulated	4.06 (2.68–5.69)	1.14 (0.61–2.14)	0.45*** (0.33–0.88)	1.06 (0.59–2.41)	0.56*** (0.34–1.25)	1.83 (1.26–2.51)	0.99*** (0.47–1.33)	.198	.171
	Nonstimulated	0.89 (0.74–1.16)	0.65 (0.49–0.88)	0.58ns (0.48–0.96)	0.91 (0.44–1.18)	0.69ns (0.46–1.29)	0.96 (0.55–1.10)	0.61ns (0.52–0.99)	.49	.57
Adenine (nmol 10^{-6} cells)	Stimulated	6.41 (4.63–7.19)	2.63 (1.47–3.63)	1.44*** (0.75–2.48)	2.59 (1.67–4.44)	1.37*** (1.12–2.97)	4.69 (3.71–5.53)	2.41*** (1.79–2.92)	.024	.009
	Nonstimulated	2.00 (1.67–2.51)	1.33 (1.21–1.97)	1.46ns (1.09–1.89)	1.62 (1.33–1.97)	1.90ns (1.50–2.70)	1.89 (1.28–2.66)	1.75ns (1.19–2.55)	.063	.381

IMPDH capacity and levels of purines measured in peripheral blood mononuclear cells from renal transplant recipients before and after transplantation. Stimulated; mitogens stimulated PBMC. IMPDH, inosine monophosphate dehydrogenase; MPA; mycophenolic acid; t0, immediately before next dose; t1.5, 1.5 hours after dose. Differences between t0 and t1.5 tested using *t*-test on ln-transformed values (IMPDH) or Wilcoxon signed rank test (guanine and adenine). Significantly lower than t0; *$P < .05$, ***$P < .01$, ns; not significant. Variations between days tested using Skillings–Mack test.

TABLE 2 Inosine monophosphate dehydrogenase (IMPDH) capacity and purines in peripheral blood mononuclear cells (PBMC) from renal transplant patients with zero human leucocyte antigen-mismatch graft and not receiving mycophenolate mofetil (median, range)

		0–4 days before transplantation	6–9 days t0	6–9 days t1.5	5–7 weeks t0	5–7 weeks t1.5	1 year t0	1 year t1.5
		(*n* = 5)	(*n* = 5)	(*n* = 5)	(*n* = 5*)	(*n* = 5)	(*n* = 4)	(*n* = 4)
IMPDH capacity (pmol 10^{-6} cells min^{-1})	Stimulated	**172** (41.0–208)	**134** (73.4–253)	**149** (116–180)	**186** (103–239)	**166** (93.5–216)	**194** (140–220)	**214** (118–269)
	Nonstimulated	**8.36** (2.88–20.6)	**5.15** (3.45–16.5)	**5.57** (2.32–7.47)	**11.1** (4.32–15.4)	**7.11** (1.74–12.6)	**10.7** (3.58–11.8)	**10.5** (2.79–27.3)
Guanine (nmol 10^{-6} cells)	Stimulated	**5.23** (4.06–6.84)	**5.27** (3.36–7.54)	**4.73** (3.87–5.74)	**5.27** (4.45–12.4)	**5.55** (3.95–11.1)	**6.65** (4.84–6.95)	**6.66** (2.75–8.65)
	Nonstimulated	**1.69** (0.52–1.90)	**0.79** (0.38–2.91)	**0.77** (0.34–1.14)	**1.32** (0.39–2.02)	**1.25** (0.32–3.76)	**1.08** (0.48–2.20)	**1.35** (0.41–2.96)
Adenine (nmol 10^{-6} cells)	Stimulated	**5.74** (4.14–7.89)	**7.7** (5.35–11.0)	**7.73** (4.73–9.41)	**8.13** (5.04–9.22)	**6.45** (4.61–9.77)	**6.45** (5.20–8.96)	**5.38** (5.12–9.06)
	Nonstimulated	**2.62** (0.92–4.88)	**1.75** (1.33–6.09)	**2.11** (1.22–2.53)	**2.81** (1.16–3.76)	**2.54** (1.14–5.04)	**2.67** (1.54–3.39)	**2.67** (1.51–4.20)

IMPDH capacity and levels of purines measured in PBMC from renal transplant recipients not receiving mycophenolate mofetil. Stimulated; mitogens stimulated PBMC. t0; immediately before next dose, t1.5; 1.5 hours after dose. *n = 4 for nonstimulated IMPDH.

At each sampling time point, there was a positive correlation between the IMPDH capacity in stimulated and nonstimulated PBMC (P < .036). The IMPDH capacity in nonstimulated PBMC explained between 22 and 51% of the capacity in stimulated PBMC.

The IMPDH capacity in patients with no HLA-mismatch and not using MMF (n = 5) is shown in Table 2. After tacrolimus and prednisolone administration, the IMDPH capacity was on average (10–90 percentile) 102% (76–134%) of the predose capacity in stimulated PBMC (n = 14 comparisons) and 99% (50–141%) in nonstimulated PBMC (n = 13 comparisons).

3.4 | Guanine and adenine

The results on guanine and adenine pools in PBMC are summarized in Table 1 and Table 2. Before transplantation and initiation of MMF, the mitogenic stimulation increased the guanine level mean 4.6-fold (range 2.3–7.9, n = 19, P < .001) and the adenine level increased mean 3.3-fold (range 1.4–6.6, n = 19, P < .001), compared to no stimulation. Following transplantation, the overall predose levels of guanine and adenine increased in a similar manner upon stimulation; mean 2.3-fold (range 0.4–16-fold) for guanine and 2.1-fold (range 0.4–12 fold) for adenine (n = 69). After dose, the only significant increase after stimulation was seen in adenine at 1 year (mean 1.5-fold increase, P= .008).

Compared to pretransplant levels, guanine and adenine was lower in stimulated PBMC at all sampling times after transplantation (P < .007). In nonstimulated PBMC, no reduction from baseline was observed following transplantation (guanine; P = .96, adenine; P = .36). In nonstimulated PBMC, no change in guanine or adenine was observed after dose (P > .055) relative to predose. In stimulated PBMC, the levels of both purines were reduced from predose to postdose at all sampling days (P < .001). Correlation between adenine and guanine is shown in Figure 3. In both stimulated and nonstimulated PBMC, guanine and adenine were positively correlated (R^2 0.49–0.82, P < .001) at all time-points.

3.5 | Biomarkers and clinical outcome

IMPDH capacity measured prior to transplantation did not differ between patients with and without BPAR, either in stimulated (p = 0.71) or nonstimulated (P = .49) PBMC (Table S1). Different aspects of IMPDH-capacity measured 5–7 weeks after transplantation (absolute capacity, capacity as a percentage of pretransplant and capacity postdose as a percentage of predose) was compared between patients who had been BPAR free until that time point, but who later did or did not have a BPAR episode. No differences were observed between the groups (Table S1). Similarly, neither adenine nor guanine was associated with BPAR (Table S2).

Pretransplantation measurements were available for 5 of the 6 patients who later needed reduced MFF dose. At 6–9 days, these were available for all 6. All dose reduction occurred after these

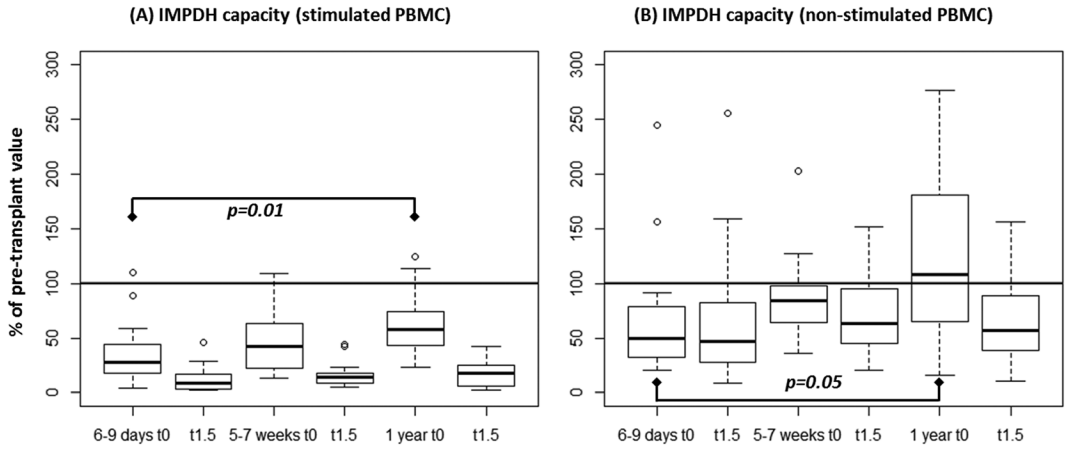

FIGURE 2 (A) Mitogen-stimulated peripheral blood mononuclear cells (PBMC); (B) nonstimulated PBMC. t0; before morning dose of mycophenolate mofetil, t1.5; 1.5 hours after administration of mycophenolate mofetil (n = 13). IMPDH, inosine monophosphate dehydrogenase

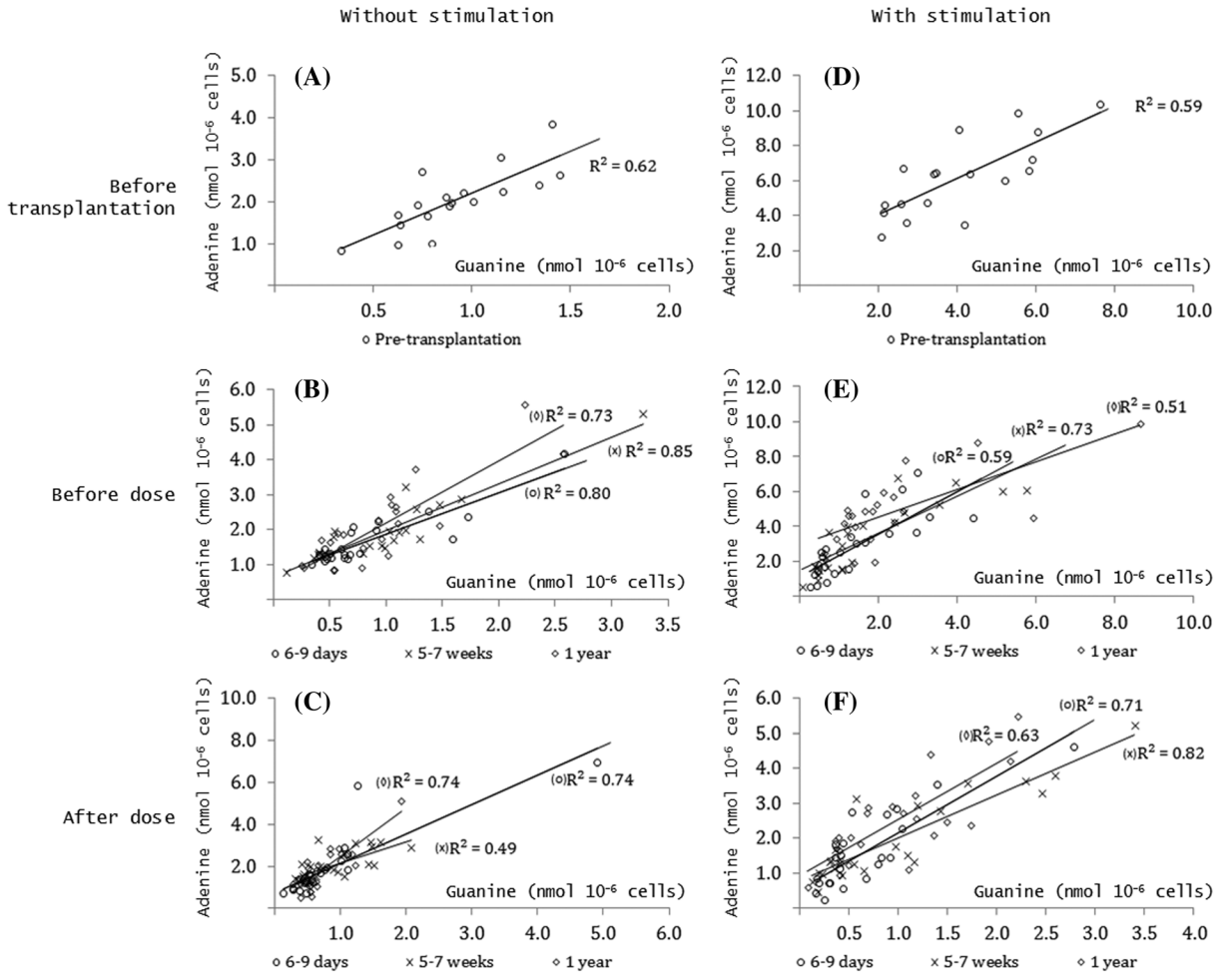

FIGURE 3 Guanine and adenine measured in peripheral blood mononuclear cells (PBMC) with mitogen-stimulation (D, E, F) and without stimulation (A, B, C) from renal transplant patient measured 0–4 days before transplantation (A, D) and before (B, E) and after dose (C, F) at 6–9 days (o), 5–7 weeks (x) and at 1 year (◊) after transplantation. All correlation; $P < .001$ (Pearson)

sampling times. Although patients who later needed dose reduction trended towards lower IMPDH-capacity in stimulated cells both 0–4 days before (median 69 *vs* 131 pmol 10^{-6} cells min^{-1}, $P = .13$) and 6–9 days after transplantation (19.0 *vs* 38.1 pmol 10^{-6} cells min^{-1}, $P = .13$) as well as higher MPA C_0 levels 6–9 days after transplantation (2.46 *vs* 1.37 mg L^{-1}, $P = .224$), these were not significantly different. In the nonstimulated PBMC, a similar difference in the IMPDH capacity was observed before transplantation (3.56 *vs* 5.88 pmol 10^{-6} cells

min^{-1}, P = .19), but reached statistical significance in predose samples 6–9 days after transplantation (1.87 *vs* 3.00 pmol 10^{-6} cells min^{-1}, P = .049). Receiver operating characteristic curve analysis for these time points is shown in Figure 4. At these time points, plasma MPA concentrations, guanine or adenine levels did not have a predictive value for prospective dose reduction (Figure S1).

To explore if there were overall differences during the first year after transplantation in IMPDH-capacity, guanine, adenine or MPA levels between patients with and without the need of MMF dose reduction a singular value was calculated for each variable. A predose and postdose average was calculated by averaging these values across 6–9 days, 5–7 weeks and 1 year for each patient. Patients needing dose reduction had a higher average MPA serum concentration and also lower average IMPDH-capacity and adenine in both stimulated and nonstimulated PBMC, whilst guanine was lower in the stimulated PBMC only (Table 3).

4 | DISCUSSION

In this exploratory study, we have characterized potential biomarkers for MPA, the active metabolite of the immunosuppressive drug MMF. We have measured its direct pharmacodynamic effect on the target enzyme and the downstream alterations of purine levels. A novel aspect of our clinical study is that biomarkers were measured in both mitogen-stimulated and nonstimulated PBMC. For all biomarkers, MPA showed a stronger inhibitory effect in stimulated PBMC compared to nonstimulated PBMC: The IMPDH capacity was only reduced 20–44% in nonstimulated PBMC when patients were given MMF, whilst the reduction in stimulated PBMC was 66–79%. An explanation for this difference in inhibition could be that MPA inhibits IMPDH2 more potently than IMPDH1[35] and the former is more abundantly expressed in activated PBMC.[36,37] Also, MPA may indirectly inhibit the expression of IMPDH during activation. One limitation of the study was the timing of cell counting. For both stimulated and nonstimulated PBMC this was done prior to incubation. Some cell proliferation may have occurred during the incubation period, with less proliferation taking place in the postdose sample where MPA is present at higher concentration. The IMPDH-capacity and purines are normalized to the preincubation cell number, therefore the apparent stronger inhibitory effect MPA had on stimulated PBMC at 1.5 hours postdose could be related to subdued proliferation.

During PBMC isolation, resuspension and 72 hour incubation, MPA could redistribute to the extracellular space and underestimate the inhibitory effect of MPA. However, the reduction in IMPDH-capacity from pre- to postdose of 25–43% in nonstimulated cells is comparable to other studies without prolonged

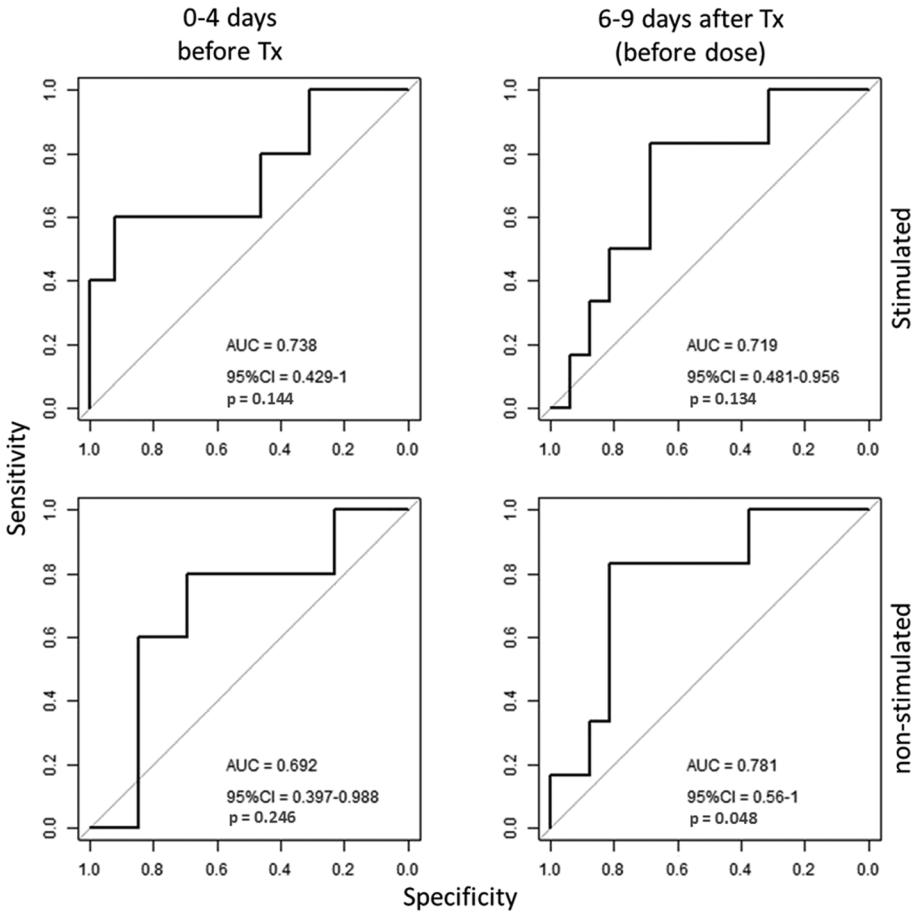

FIGURE 4 Receiver operating characteristic curve analysis of inosine monophosphate dehydrogenase capacity measured in mitogen-stimulated and nonstimulated peripheral blood mononuclear cells (PBMC) collected 0–4 days prior to and 6–9 days (before dosage) after transplantation to predict the need for dose reduction within the first year after transplantation (Tx)

TABLE 3 Across 1 year, mycophenolic acid (MPA) plasma concentration and biomarkers in peripheral blood mononuclear cells (PBMC) in patients requiring reduction of the mycophenolate mofetil dose (median, quartiles)

		Patients with dose reduction (n = 6)	Patients without dose reduction (n = 18)	P*
Trough plasma MPA (mg L^{-1})		3.22 (2.11–4.38)	1.79 (1.50–2.79)	.036
Stimulated PBMC	IMPDH capacity (pmol 10^{-6} cells min^{-1})	26.0 (11.2–43.5)	65.3 (4.2–96.0)	.042
	Guanine (nmol 10^{-6} cells)	0.91 (0.62–1.37)	2.18 (1.12–2.65)	.042
	Adenine (nmol 10^{-6} cells)	2.27 (1.34–3.27)	4.50 (2.40–5.15)	.042
Nonstimulated PBMC	IMPDH capacity (pmol 10^{-6} cells min^{-1})	2.96 (2.34–3.12)	4.50 (3.30–6.16)	.025
	Guanine (nmol 10^{-6} cells)	0.78 (0.54–0.85)	0.85 (0.70–1.20)	.48
	Adenine (nmol 10^{-6} cells)	1.45 (1.33–1.49)	2.08 (1.52–2.34)	.042

Median (quartiles) of average predose measurements at 6–9 days, 5–7 weeks and 1 year. Omitting samples taken at time points when the mycophenolate mofetil dose was reduced. *Differences in cross-year averages between patients with and without dose reduction tested using Mann–Whitney U test
IMPDH, inosine monophosphate dehydrogenase

incubation.[38-40] This indicates that incubation of cells with 20% of a patient's own plasma may adequately counter the efflux of MPA, although it is important to note that measuring IMPDH-capacity *ex vivo* will not represent the IMPDH-activity in vivo directly, but has to be considered as a model.

Although MPA lowered IMPDH capacity in nonstimulated PBMC, no alteration of adenine or guanine in these cells could be shown, indicating that purine levels are maintained in the resting lymphocyte by the salvage pathway and less by the *de novo* synthesis.[41] We have previously shown unaltered levels of guanine nucleotides in resting lymphocytes following a single MMF dose in healthy individuals[42] and this has also been observed in heart transplant recipients.[41]

Although IMPDH is not directly involved in the synthesis of adenosine phosphates (see Figure 1), there was a markedly decrease in adenine levels in stimulated PBMC after initiation of MMF-treatment and a further decrease from predose to postdose. Allison *et al*. described a feed-back mechanism were GMP, GDP and GTP stimulated further synthesis of IMP from ribose-5-phosphate via 5-phosphoribosyl pyrophosphate synthetase. When this induction of 5-phosphoribosyl pyrophosphate synthetase is removed due to depletion of guanosine phosphates, the level of IMP may also be reduced causing depletion of adenosine phosphates.[43] In addition, the conversion of IMP to AMP has previously been shown to be GMP dependent[44] and depletion of GMP by IMDPH inhibition is therefore expected to result in simultaneous decrease in adenosine phosphate levels. Qui *et al*. observed a similar parallel decrease in a primary human T-cell model *in vitro*[45] and we have previously shown this to be the case in MOLT-4 leukaemia cells.[46] The current study supports that this also occurs in PBMC *in vivo* (see Figure 3). However, inhibition of proliferation due to higher MPA content in the postdose sample could also explain the differences in purine levels in stimulated cells.

Our study indicated that patients needing reduction of the MMF dose within the first year have lower IMPDH capacity and levels of purines (Figure 4 and Table 3), in concordance with previous studies.[47] These biomarkers appeared with potentially relevant predictive values, measured before and 1 week after transplantation. They should be further investigated as possible biomarkers to identify those patients who could benefit of less MMF to avoid adverse effects (e.g. diarrhoea or leucopenia). Although patients who needed a reduction of MMF dose had generally higher plasma MPA concentrations throughout the year (Table 3), plasma concentrations were not predictive of the need for dose reduction when measured early (6–9 days) after transplantation (Figure S2). However, IMPDH capacity measured at 6–9 days was related to dose reduction.

There was no clear association between MPA concentration in plasma and IMPDH-capacity (Figure S1). A possible explanation could be that MPA concentrations in plasma poorly reflects the concentration inside PBMC, and that MPA concentration measured in PBMC might correlate with IMPDH-capacity. Md Dom *et al*. measured IMPDH-capacity, as well as MPA concentration in plasma and PBMC.[48] There was no clear correlation between MPA concentrations in PBMC, MPA concentration in plasma, or IMPDH-capacity, indicating that the varying IMPDH-capacity during MMF/MPA therapy is due to varying enzyme capacity or sensitivity, rather than distribution kinetics.

Unfortunately, we could not demonstrate any association between BPAR and IMPDH or purine levels. MPA is given as part of a multiple drug regimen after renal transplantation and under-immunosuppression with MPA could be compensated for with sufficient exposure to tacrolimus or prednisolone. Our study was designed as an exploratory study and was not powered to document differences in rejection rates. Given the limited number of patients and wide variation in IMPDH capacity and purines there is a risk for Type

II statistical error. Other studies have shown an association between low IMPDH inhibition and increased risk of BPAR,[47,49] demonstrating the value of IMPDH measurements.

Currently, there is no consensus on how to measure the effect of MPA on IMPDH. Most assays measure the rate of XMP production where both substrate (IMP) and co-substrate (NAD+) are in saturated concentrations, but there are several different ways of normalizing the results to amount of sample material. We have shown that adenine and guanine pools are highly regulated (Figure 3) indicating that adenine levels are also influenced by IMPDH activity. Several studies normalize the XMP production to AMP[50-56] since this is technically attractive. However, careful interpretation is suggested in light of co-regulation between the guanine and adenine pools in lymphocytes. Normalization to protein amount[38-40,47,49,57-59] or number of cells[60] are other options.

In the present study, there was an increase in IMPDH capacity in both *ex vivo*-stimulated and nonstimulated PBMC from 1 week to 1 year after transplantation (Figure 2 and Table 1). Chiarelli *et al*.[39] reported a similar increase in nonstimulated PBMC when stable renal transplant recipients were monitored for 15 months. Tang *et al*. observed no increase in predose IMPDH-capacity from day 6 to week 20 after transplantation,[50] suggesting that this increase mainly occurs after week 20.

Since there are 2 IMPDH enzymes (IMPDH1 and 2), and MPA inhibits IMPDH2 more potently than IMPDH1, an explanation for decreased MPA sensitivity over time could be due to a larger proportion of total IMPDH being IMPDH1 than IMPDH2. However, we[61] and others[62] have shown that expression of IMPDH1 decreases in the post-transplantation time frame, possibly related to decreasing doses of methylprednisolone or prednisolone. The predose IMPDH capacity in nonstimulated PBMC at 1 year was comparable to the pretransplant level (Figure 2 B), suggesting that patients could have insufficient immunosuppressive treatment. However, the IMPDH capacity in stimulated PBMC was still markedly decreased at 1 year. Since immunological rejection is mediated through the activated lymphocyte, this may explain why MMF still has a therapeutic value after prolonged treatment.

In conclusion, by measuring the molecular pharmacodynamic response to MPA in renal transplant patients over a prolonged period, we have shown that MPA inhibits activated lymphocytes to a larger degree than resting lymphocytes. In resting lymphocytes, the nucleotide pool appeared to be unaffected by MMF treatment, as it is probably being maintained through the salvage pathway. Low IMPDH capacity before and early after initiation of MMF treatment appeared to predict the need for dose reduction, suggesting that this biomarker should be further investigated in relation to patients at risk for MPA overexposure.

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COMPETING INTERESTS

The authors have no conflicts of interest to declare.

CONTRIBUTORS

St.B., Sa.B. and N.T.V. participated in research design. R.A.K. performed data analysis and wrote the manuscript. N.T.V., K.H., A.M.A. and C.B.N. participated in performance of research. K.M. and M.H.S. recruited patients. All authors were involved in the discussion of results, critical revision of the manuscript and approval of the final version.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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