FERDI SOMBOGAARD

pharmacodynamic monitoring of

IMPDH

A BASIS FOR OPTIMIZED AND INDIVIDUALIZED
MYCOPHENOLATE MOFETIL THERAPY

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Pharmacodynamic Monitoring of Inosine Monophosphate Dehydrogenase Activity

A Basis For Optimized and Individualized Mycophenolate Mofetil Therapy

Farmacodynamisch monitoren van inosine monofosfaat dehydrogenase activiteit

Een basis voor geoptimaliseerde en geïndividualiseerde mycofenolaat mofetil therapie

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General introduction

HISTORY OF MYCOPHENOLIC ACID

In 1896 Gosic isolated for the first time mycophenolic acid (MPA) from *Penicillium glaucum*.[1] It was subsequently isolated from *Penicillium stoloniferum* Thom (synonym *P. brevi-compactum* Dierckx) by Alsberg and Black in 1913 who gave the acid phenolic substance its name mycophenolic acid (MPA).[2] From 1931 to 1933 a series of studies in the biochemistry of micro-organisms was published by Clutterbuck, Raistrick and Oxford of the division of biochemistry from the University of London. These series described in detail the isolation and characterization of MPA.[3-6] The structure of MPA was finally established in 1952 (Figure 1).[7] In the first decades, MPA was eventually found to have antineoplastic, antibacterial, antifungal and antiviral properties and was investigated by a few groups.[8] In 1969, Planterose *et al* described an effect of MPA most likely to an immunosuppressive effect,[9] and Mitsui *et al* reported in the same year for the first time the immunosuppressive effect of MPA in mice, at the cellular and humoral levels of antibody formation.[10] In the following years, more and more research was done on the efficacy of MPA as an immunosuppressant.[11-14] Mycophenolate mofetil (MMF) was developed at Syntex Corporation to be a more bioavailable form of MPA.[15] The first human trials of MMF in kidney recipients were conducted by Sollinger *et al*,[16] leading to the registration of the pharmaceutical compound as immunosuppressant in 1995.

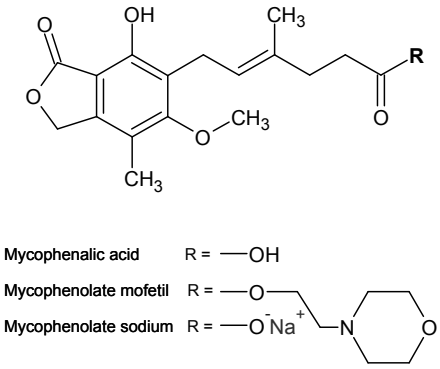

Figure 1. Molecule structures of mycophenolic acid and its drug derivatives.

MYCOPHENOLIC ACID AS IMMUNOSUPPRESSANT

Currently, two mycophenolate compounds are available as immunosuppressant: mycophenolate mofetil (MMF, CellCept®, Roche) and enteric-coated mycophenolate sodium (EC-MPS, Myfortic®, Novartis). In 1995 MMF was approved in the US for prophylaxis of organ rejection in kidney transplantation. Its approval was initially based on data from

three, randomized, double-blind, controlled, multicenter phase III trials performed in *de novo* kidney transplant recipients.[17-19] The enteric-coated, slow release MPA variant EC-MPS was approved in 2004. MPA has proven to be effective in the prevention of acute rejection in combination therapy consisting of MPA, a calcineurin inhibitor (CNI) such as ciclosporin or tacrolimus and corticosteroids. MPA has gained widespread acceptance as the antimetabolite immunosuppressant of choice in solid organ transplant regimens and has largely replaced azathioprine. An estimated 79% of kidney, 48% of liver and 75% of heart transplant recipients use MPA at hospital discharge.[20] The success of MPA in solid organ transplantation has also led to its application for prevention of graft-versus-host disease after haematopoietic stem cell transplantation,[21] as well as for treatment of autoimmune diseases such as systemic lupus erythematosus and psoriasis.[22] MMF is considered to be used with a fixed dose recommendation of 1000mg twice a day after kidney transplantation[23] and 1500mg twice a day after liver or heart transplantation.[24] An equimolar dose of EC-MPS can be obtained by multiplying the MMF dose with 0.720.[25] Distinctly adverse effects of MPA are gastrointestinal effects (diarrhea, vomiting, nausea, abdominal cramps), hematological effects (leukopenia, thrombocytopenia, anemia) and infections (viral: cytomegalovirus, herpes zoster, polyomavirus; bacterial: urinary tract, pneumonia).[24,25]

PHARMACOKINETICS OF MMF

After oral administration, MMF is extensively hydrolyzed presystemic to MPA by esterases in the stomach, small intestine, blood, and liver.[26,27] The absorption of MPA is fast and almost complete, with maximum plasma concentrations generally occurring within 1 hour after MMF administration.[27] It is possible that poor postoperative absorption or gastrointestinal tract metabolism may play a role in the initial disposition of MPA in some transplant recipients.[28] In whole blood, 99.99% of MPA is found in the plasma fraction with only 0.01% in cellular compartments.[29,30] MPA binds extensively to serum albumin in the order of 97-99% in patients with normal renal and liver function.[30-32] The unbound fraction of MPA is thought to be responsible for immunosuppressive activity.[30]

Uridine diphosphate glucuronosyltransferases (UGTs) metabolize MPA to the pharmacologically inactive metabolite 7-O-MPA-glucuronide (MPAG) in the liver, kidney, and intestinal mucosa.[33-35] MPAG is usually present in plasma at approximately 20- to 100-fold higher concentrations than MPA. The minor but active metabolite acyl-glucuronide (AcMPAG) is also formed and has a 10-fold lower affinity for inosine 5'-monophosphate dehydrogenase (IMPDH).[33,36-38] AcMPAG may contribute to hypersensitivity, drug toxicity and immune response in patients receiving MPA therapy.[39] High AcMPAG concentrations may also be associated with MPA-related gastrointestinal adverse effects that are

frequently observed, and which are an important reason for MMF dose reductions or for discontinuation of the drug.[40,41] MPA is excreted in the urine for 93%, of which approximately 87% as MPAG, and for 6% in the faeces.[27] Excretion of MPAG formed in the liver into the bile appears to involve by the enzyme multidrug resistant protein-2 (MRP-2).[42,43] In the intestine MPAG is deconjugated back to MPA and reabsorbed in the colon. This enterohepatic recirculation contributes 10% to 60% to the total MPA exposure.[27,31] The often-used immunosuppressant cyclosporine A inhibits the biliary excretion of MPAG by the MRP-2 transporter, which results in a reduction of the enterohepatic recirculation and a decreased MPA exposure.[44]

MPA is believed to undergo restrictive clearance, because only the unbound MPA fraction is presumed to be available for metabolism into MPAG.[38,45] Especially during renal insufficiency the unbound MPA fraction would increase, due to the uremic state and to displacement of MPA from its albumin binding sites by high concentrations of the MPAG that is cleared by the kidneys.[46] In patients with poor renal function, total MPA concentrations are low due to increased clearance, but at the same time the non-protein bound fraction is high (fractions up to 18.3% are reported[47]) and free MPA concentrations may be unaltered or even elevated.[48,49]

CLINICAL PHARMACOKINETIC STUDIES

The pharmacokinetics of MMF have been extensively investigated in renal transplant recipients. Several studies have investigated the MPA concentration-effect relationship in renal transplant recipients for the risk of acute rejection. Studies with patients receiving ciclosporin as CNI showed a higher risk for acute rejections with a low MPA exposure ($AUC_{0\text{-}12}$),[50-56] while in patients treated with tacrolimus as CNI a significant MPA concentration-effect relationship was not found.[57-60] The clinical studies also found a large between- and within-patient variability in the pharmacokinetics of MPA, up to 50-fold range in trough levels and up to 10-fold range in AUC.[51,56,58,60-63] The pharmacokinetics of MPA are further characterized by a gradual rise in MPA exposure of at least 30 to 50% from the first weeks relative to the stable period at 1 to 6 months after renal transplantation despite the use of fixed[32,58,62,64-66] or even reduced doses of MMF[51].

Even though the "one-size-fits-all" dose regimen may have contributed to the widespread use of MMF, a growing amount of data suggests that this may not be the optimal therapeutic option for MMF. It is suggested that individualization of MMF dose can further improve clinical outcome.[51,65,67] Based on the large between-patient variability in pharmacokinetics and on the repeatedly shown relationship between MPA exposure early after transplantation and acute rejection, therapeutic drug monitoring (TDM) strategies have been developed.[68,69] The added value of TDM has been tested in

two prospective randomized trials. The Apomygre-study showed that using a limited sampling strategy, adjusting the dose of MMF based on MPA exposure, the risk of treatment failure could be significantly reduced, without an increase in adverse events. The study showed the supremacy of a concentration controlled therapy above a fixed dose regimen in kidney transplant recipients.[70] The FDCC-trial was not able to show the same result. In the FDCC-trail the applied protocol of MMF dose adjustments based on target MPA exposure was not successful, partly because physicians seemed reluctant to implement substantial dose changes.[71]

PHARMACOLOGY OF MYCOPHENOLIC ACID

Inosine 5'-monophosphate dehydrogenase (IMPDH) is an enzyme that catalyzes the oxidation of inosine monophosphate (IMP) to the intermediate xanthine monophosphate (XMP), which is a metabolite for the synthesis of the purine nucleotide guanosine triphosphate (GTP). IMPDH is the key enzyme in the de novo synthesis of these nucleotides and induces the rate-limiting step in this synthesis (Figure 2).[14,65,72] The proliferation of lymphocytes depends on this pathway of synthesis of nucleotides, while other types of cells have a salvage pathway for the synthesis of nucleotides. MPA is a non-competitive inhibitor of IMPDH and it will react on the MPA/NAD binding pocket. This makes MPA a drug that specifically inhibits the proliferation of the lymphocytes.[73]

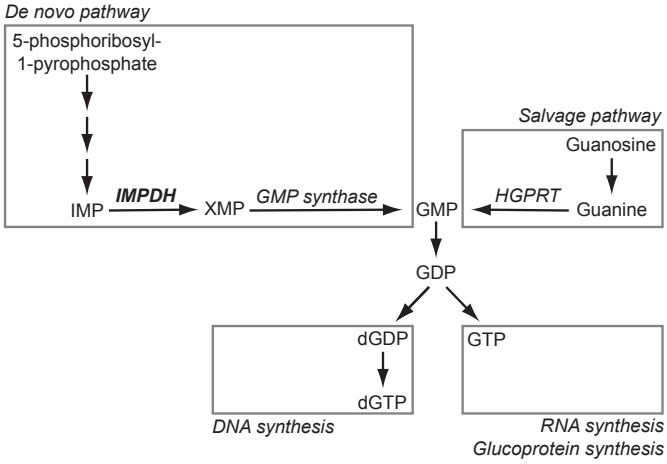

Figure 2. Schematic representation of the de novo and salvage pathways of guanine nucleotide biosynthesis. The enzymes involved in the biosynthesis are IMPDH, GMP synthase (both in the de novo pathway), hypoxanthine guanine phosphoribosyltransferase (HGPRT) and purine nucleoside phosphorylase (PNP, both in the salvage pathway).

In humans there are two isoforms of IMPDH, type I and II, which are encoded by two different genes, located on two different chromosomes, respectively chromosome seven and three. IMPDH type I is constitutively present in different cells, e.g. leukocytes, lymphocytes and erythrocytes. On the other hand, IMPDH type II is expressed in cells stimulated to proliferation, mainly in cells in the growth or differentiation phase.[74,75] The active site of IMPDH type II is Cys331.[73] In mature resting lymphocytes IMDPH type I is the dominant species. In activated lymphocytes IMPDH type II predominates over type I. This difference in expression suggests different regulatory mechanisms for IMPDH type I and type II.[72,76,77] Human IMPDH type I and type II cDNAs encode the same size proteins of 514 amino acids with 84% sequence identity.[78] Both isoforms show similar affinities for the substrates, with similar K_m values for nicotinamide adenine dinucleotide (NAD) and IMP and similar K_i values for the reduced form of NAD and XMP.[74,79] MPA was found to inhibit both IMPDH isoforms by the same mechanism.[77] However, IMPDH type II (K_i = 9.5nM) is 3.9 to 5.0-fold more sensitive to MPA than the type I isoform (K_i = 37nM).[72,74,76,77,80] The selective inhibition of IMPDH type II is thought to explain the relatively mild side effects of MMF in clinical trials.[74,75]

The overall activity of IMPDH increases in erythrocytes after exposure to MPA, but the IMPDH activity decreases in lymphocytes.[75,77] The regulation of IMPDH type I and type II mRNA and the levels of protein will not alter after MPA exposure in these cells. Hence, to measure the IMPDH activity in patients treated with MMF, measuring the IMPDH activity in peripheral blood mononuclear cells (PBMCs) is abided.[77,80,81] Lymphocytes are primarily responsible for the induction or rejection of the transplanted organ, so measuring the activity of IMPDH in these cells may best reflect the relevant biologic activity.

PHARMACOGENETICS OF IMPDH

IMPDH type I

Several studies have demonstrated pathogenicity correlated with polymorphisms of the IMPDH type I gene in patients with retinitis pigmentosa.[82-84] A group of seven single nucleotide polymorphisms (SNPs) was investigated on a pathogenic relationship at autosomal dominant retinitis pigmentosa (313C>T, 349C>T, 594T>G, 671C>G, 676G>A, 971G>A, 1115A>C). Though there was a correlation between some of the polymorphisms and clinical outcome, none of them affected enzyme activity.[84] An altering in folding and aggregation of the enzyme was noticeable,[82] but obviously there was not an alteration in activity. Other investigated SNPs had no effect on enzyme activity, protein stability, or protein aggregation.[83]

In a group of inflammatory bowel disease (IBD) patients resistant to azathioprine twelve IMPDH type I SNPs were characterized (-91-83ins9bp, 76T>C, 69A>G, 66A>G,

11401G>C, 14805C>T, 15328G>A, 15489G>A, 17493G>A, 17509T>C, 17529C>T, 17535A>C).[85] There was no measurement of IMPDH activity, only alteration in promoter activity was measured using luciferase reporter vector for only mutations in the promoter P3 sequence. Because mutations in P3 were not found in non-IBD patients, the authors concluded that the promoter activity decreasing mutations in P3 only occur in IBD patients. Although, this mutations collectively produced a relatively small decrease in IMPDH type I P3 promoter activity in vitro, it suggests the variant promoter has altered function which may be important in vivo.[85]

One study performed in renal transplant patients showed an association between two IMPDH type I SNPs (IVS7+125G>A, IVS8-106G>A) and biopsy proven acute rejection in the first year post-transplantation. One possible explanation is their linkage to other SNPs that may control IMPDH type I mRNA expression, enzyme activity, and ultimately lymphocyte proliferation.[86]

IMPDH type II

Different SNPs for IMPDH type II were investigated. The IMPDH type II 4999C>T SNP was also described in the group IBD patients resistant to azathioprine, but no effect on IMPDH activity was reported.[85] Digits *et al* have constructed and characterized three mutants in the human IMPDH type II gene with the goal of identifying structural features that determine species selectivity of MPA (322Arg>Lys, 441Gln>Glu, 322Arg>Lys/441Gln>Glu). The activity of these mutants was too low (less then 0.045% of wild type IMPDH type II activity) to thoroughly characterize them. The mutant 441Gln>Ala increased the MPA sensitivity with 20-fold, and also the activity decreased with 20-fold.[87] An MPA-resistant neuroblastoma cell line showed two mutant enzymes (333Thr>Ile and 351Ser>Tyr). This mutation is less sensitive to MPA, and also much less active than wild type.[75] The CEASAR study presented the IMPDH type II 3757T>C mutation, located in an intron of the mRNA. This mutation showed a significant (P = 0.012) correlation with biopsy proven acute rejection in kidney transplant recipients.[88] The results of the FDCC trial did not prove this correlation, although a trend was noticeable. The FDCC-trial investigated also five other mutations (192A>G, 915C>G, 1253A>G, 720C>T and 460T>C). None of these SNPs were presented in the included patients.[89] Another polymorphism, which was investigated by kidney transplant recipients was 787C>T. This SNP reduced the IMPDH activity in the variant carrier group to 10% of the wild type, but had an allele frequency of only 1.0%. In the same study eight other SNPs were investigated, but none of them showed any significance with clinical outcomes.[90] So far, only two IMPDH type II polymorphisms are correlated to decreased IMPDH activity and increased risk of biopsy proven acute rejection (BPAR) in kidney transplant recipients.

IMPDH ACTIVITY ASSAYS

History of the IMPDH activity assay

Already in 1957 Magasanik *et al* published the first IMPDH activity assay. Their method was based on the oxidation of IMP or the formation of reduced diphosphophyridine nucleotide (DPN), followed by observing the increase in optical density of XMP or DPNH by a spectophotometric ultraviolet (UV)-assay.[91] The major aim of the paper was not to describe an IMPDH activity method; the first aim was to isolate and to identify the enzyme in *Aerobacter aerogenes* which was responsible for the irreversible enzymatic conversion of IMP into XMP. A reaction mix that contained IMP, DPN serving as hydrogen acceptor, K^+ or NH_4^+ and glutathione or cysteine in Tris buffer was used. The enzyme reaction occurred at room temperature and was unattached to pH.[91] Nevertheless, the main disadvantage was that this method was only usable in bacterial extracts. Although the main goal was to converse IMP into XMP, nowadays this method has inspired many people to develop IMPDH activity assays.

Radiochemical assays

Saccoccia *et al* discussed the spectrophotometric IMPDH activity technique in 1969 and concluded that the assay based on the change in optical density was incapable of detecting the rapid degradation of IMP by IMP phosphatase activity, an enzyme frequently present in mammalian tissues.[92] Therefore, a new method based on a radiochemical assay to measure IMPDH activity was developed. The used reaction mixture contained Tris buffer (pH 7.5), magnesium chloride, potassium chloride (KCl), NAD as hydrogen acceptor and the radioactive agent [^{14}C]IMP. After adding the IMPDH enzymes the reaction was inhibited at room temperature. After thin layer chromatography (TLC) the radioactivity of the separated XMP and IMP spots were measured.[92] Although this radiochemical method greatly increased the sensitivity of detecting IMPDH activity, it was still limited to tissues such as liver, kidney and heart.

In 1983 Proffitt *et al* modified the method of Saccoccia *et al*. Their assay incorporated inhibitors of several interfering enzyme activities and allowed the accurate measurement of IMPDH activity in crude tissue supernatants. The used reaction mixture contained Tris buffer (pH 8.0), KCl, EDTA, α-β-methylene adenosne 5'-diphosphate, allopurinol, NAD and [^{14}C]IMP. The enzyme suspension was incubated with the mixture at 37°C . After TLC the radioactivity of the separated XMP and IMP spots were measured.[93] This was the first assay showing a linear increased XMP formation over time and extreme purification of the enzyme suspension was no longer needed.

The last interesting modification to this [^{14}C]-assay for determination of IMPDH activity was made by Ikegami *et al* in 1985. Due to using a high-voltage electrophoresis, the separation of IMP and XMP has improved and the assay has become faster (45 minutes),

more sensitive, and required less tissue sample.[94] Nowadays, this [^{14}C]-IMPDH activity assay is used incidentally in published papers.

In 1983 Cooney *et al* published the radioactive method to measure IMPDH activity using the isotope [^{3}H]IMP. Approximately 95% of the formed [^{3}H]XMP was countable, versus 30% to 80% of the [^{14}C]XMP when crude extracts were used. For this reason, this new method was exceptionally uncomplicated and well-suited for pharmacodynamic – pharmacokinetic studies. The major disadvantage of this technique was the commercial unavailability of the required isotope.[95] A few years later, [^{3}H]IMP became commercially available and in 1992 Balzarini *et al* developed a useful, convenient and sensitive method for measuring IMPDH activity in intact human lymphocytes.[96] Langman *et al* measured the IMPDH activity using a modification of this assay in the first pharmacodynamic studies of MPA induced immunosuppression.[72,97,98] After these studies, this radioactive [^{3}H]-assay became the first choice in radioactive IMPDH activity assays.

Spectrophotometric assays

While the accuracy of the radioactive assays is high, these techniques tend to be cumbersome. Therefore, Anderson *et al* used the IMPDH activity method of Magasanik *et al* to improve the spectrophotometric-assay in 1968. The reaction mixture was optimized for mammalian cells and the incubation temperature was increased to the physiologically temperature of 37°C. The main finding of this study was that K^{+} stimulates IMPDH activity by a direct interaction with the enzyme, so a potassium salt should be part of the reaction mixture.[99] Nevertheless, the non-radioactive assay for measuring IMPDH activity was developed slowly, because of the disadvantages of the spectrophotometric assay compared to the established [^{3}H]-assay. Also the publications of methods by Gilbert *et al* in 1979[100] and Proffitt *et al* in 1983[93] did not give a new impulse for development, despite the fact that Proffitt used a mixture of enzyme inhibitors, so the required purification of the enzymes was no longer needed.

The real break-through of the spectrophotometric assay was started by Montero *et al* in 1995. At that time, current methods were less sensitive and required a large volume of blood. [93,94,100,101] Montero *et al* isolated IMPDH enzymes from erythrocytes obtained from whole blood. Erythrocyte lysate was added to the reaction mixture and was incubated for 2 hours at 37°C. After the incubation was terminated and the protein precipitate had been removed, the supernatant was extracted five times and analyzed using an ion-pair reversed-phase high-performance liquid chromatography (HPLC) method. The formed XMP was monitored using UV absorption at 254 nm and normalized to the protein concentration of the sample.[102] Montero *et al* described for the first time a non-radioactive IMPDH activity assay for enzymes easily isolated from bacterial as mammalian blood cells. The assay provided a sensitive and accurate method of measuring even low IMPDH activity in normal human erythrocytes. The assay was dealing with the degradation of

IMP by IMP phosphatase activity and other enzyme activities. The spectrophotometric assay by Montero *et al* had shown its superiority compared to the assay of Magasanik *et al*.

Whole blood or isolated blood cells

As soon as this reproducible and sensitive non-radioactive method of determining IMPDH activity was described, other blood compartments containing IMPDH were measured for IMPDH activity based on the method Montero *et al* had described for erythrocytes.[102] In 1997 Griesmacher *et al* published a modification of the method described by Montero for measuring IMPDH activity in isolated human lymphocytes (exhibiting mainly type I activity) and lymphoblasts (exhibiting mainly type II activity). After isolating the different cell types, the cell suspension was incubated 2.5 hours for lymphocytes and 1.5 hours for lymphoblasts, in a phosphate buffered reaction mixture (pH 7.5) containing IMP, NAD and KCl.[103] Albrecht *et al* described a modified method for measuring IMPDH activity of whole blood cells (WBC) lysate and PBMC lysate. WBCs and PBMCs were pre-treated with a mixture of Tris buffer (pH 8.0), EDTA and allopurinol, just like Proffitt *et al* did in 1983[93] and were ultrasonically treated. The reaction mixture contained no potassium salts; only NAD and IMP were added to the pre-treated lysates. The incubation time was 0.5 or 1 hour at 37°C. The formed XMP was measured using a reversed phase HPLC method and was normalized to the used volume of whole blood.[104] The authors discussed the importance of whole blood inhibition profiles. IMPDH activity is predominantly located in erythrocytes while the contribution of IMPDH activity from PBMC remained less than 10% of the total IMPDH activity in patients who received MMF. The authors validated therefore the assay only for pharmacodynamic monitoring of MPA in WBC.[104]

In 2006 Vethe *et al* described a modified IMPDH assay to measure the activity in CD4+ cells. The major fraction of the circulating CD4+ cells is constituted of T-helper cells, which play important roles in graft rejection and inflammation. These cells were directly isolated from whole blood using paramagnetic beads coated with antibodies. The great advantage of this method is the minimal manipulation of cells during isolation. After the intracellular MPA concentration was restored by incubation in ultrafiltrated plasma obtained from the original whole blood sample, cells were resuspended in phosphate buffered saline. The cell suspension was incubated for 2 hours at 37°C in the same reaction mixture used by Albrecht. XMP was measured using a reversed phase HPLC method and was normalized to the number of CD4+ cells.[105]

In 2001 Glander *et al* published a modified method for PBMCs based on the method of Montero *et al*. PBMCs were isolated using Ficoll-Paque and the cells were lysated. The obtained lysate was incubated for 2.5 hours and the produced XMP was measured by a validated ion-pair reversed-phase HPLC method. XMP was normalized to the protein

concentration in the used lysate sample. The authors showed that the isolation of the PBMCs was a critical step in the assay. Reducing the washing procedure resulted in a decrease of IMPDH activity, because less MPA was washed out the cells. The measured IMPDH activity comes closer to the *in vivo* IMPDH activity, using a less intensive washing procedure. Unavoidable dilution of the samples may lead to an underestimation of the inhibition of IMPDH activity in MMF treated patients.[106] In 2002 Daxecker *et al* published a slightly different method. Despite the proven critical isolation step, this method had an extensively washing procedure. The concentrations of the compounds in the reaction mixture was different and the produced XMP was normalized to the amount of cells in the used samples.[107]

Comparison of the methods

Since the first description of a method to measure IMPDH activity by Magasanik *et al* in 1957, many methods and modifications to measure IMPDH activity have been published. Three main methods can be distinguished, namely the radioactive [^{3}H]IMP and [^{14}C]IMP methods and the non-radioactive spectrophotometric method. The development of these assays has led to precise and accurate methods. The laboratory equipment and facilities used for non-radioactive spectrophotometric methods are more standard and less complicated than for the radioactive methods. For that reason, the non-radioactive spectrophotometric methods are preferred for measuring IMPDH activity above radioactive methods. Table 1 shows the specifications of the different currently used non-radioactive methods.

For a good reflection of the *in vivo* activity measured *in vitro,* it is necessary to make the right choice in the compartment where IMPDH activity will be measured. The easiest way is to measure in whole blood (Albrecht *et al*[104]) or erythrocytes (Montero *et al*[102]), because no additional isolation of particular cells is necessary. The great disadvantage of whole blood is the increased IMPDH activity and GTP levels under prolonged MMF therapy in erythrocytes.[108,109] Even during a one dose interval the IMPDH activity increased after inhibition by MPA to a level above pre-dose IMPDH activity.[81] The effects of increased IMPDH activity under prolonged MMF therapy are characteristic for IMPDH activity measurements in whole blood and erythrocytes.

Because the target for MPA therapy is the lymphocyte compartment, measuring IMPDH activity in this compartment seems more appropriate.[77,80,81] This is enforced by data showing that MPA causes a significantly reduction in GTP in PBMC, with a paradoxical simultaneous elevation of GTP in erythrocytes.[108,110] A few authors discussed the relevance of measuring IMPDH activity in PBMCs or circulating lymphocytes compared with erythrocytes.[77,111] Nevertheless, it is widely accepted that PBMCs or subpopulations of circulating lymphocytes are the most relevant cell population for measuring IMPDH activity. Glander *et al* and Daecker *et al* have published IMPDH activity assays in PBMCs.[106,107]

Cells were isolated using the relative simple technique of Ficoll-Paque gradient. The measured activity corresponds better with the immunosuppressive effect of MMF. However, due to the isolation of PBMCs using a Ficoll-Plaque gradient and the necessary wash steps afterwards, the MPA concentration in the PBMCs was altered and thereby the IMPDH activity.[106] Griesmacher *et al* isolated lymphocytes and lymphoblasts from PBMCs, which resulted in even more washing steps.[103] Accurate and appropriate isolation of PBMCs appears to be a limiting feature of any of the IMPDH activity isolation methods presented.[112]

Another potentially useful approach would be to measure IMPDH activity in the CD4+ cell population – which is mainly T-helper cells – described by Vethe.[105] Monitoring IMPDH activity in this compartment is supposed to be approaching *in vivo*. Nevertheless, the used method is a time-consuming process.[112] However, T- and B-lymphocytes are the targets of inhibition and therefore it would be the most appropriate compartment to evaluate.

The perfect IMPDH activity assay

Several IMPDH activity assays are being used for preclinical and clinical studies, but a golden standard to measure IMPDH activity is still not pointing out.[112] Three major steps are noticed in all assays. The first step is the isolation of the cells and the IMPDH enzymes. The fragile balance has to be found in isolating the most proper cells in a less manipulating way. Furthermore, small sampling volume is preferred to use the assay in clinical practice. Because lymphocytes play important roles in the acute rejection episodes isolating lymphocytes seems to be the best choice. However, for high-quality isolation, the cells must be isolated without manipulation of the cells and without washing out the intercellular MPA concentration.

The second step in the IMPDH activity assay is the *ex vivo* incubation of the IMPDH enzymes and the measurement of the enzyme product XMP. A reaction mixture close to the *in vivo* environment would be the most natural environment for the enzymes. IMPDH enzymes are dependent on potassium,[72,99] so the incubation mixture should be a physiologically buffered mixture containing at least potassium, IMP as substrate and NAD as hydrogen acceptor. The incubation temperature should be 37°C. After the incubation, the enzymatic reaction has to be terminated without harming XMP. The enzymatic product XMP has to be determined with a validated HPLC method. An IMPDH activity assay will be more useable in clinical and research practice when the time of incubation and the time of determination of XMP are short.

The third step of the IMPDH activity assays is the way to express the activity. The measured product XMP has to be normalized to the number of enzymes that participated in the conversion of IMP into XMP. The used volume of whole blood, protein concentration or cell count of the measured sample can be used, provided that there is a correlation with the involved IMPDH enzymes in the incubation.

Table 1. Comparison of the different currently used non-radioactive IMPDH activity assays

Author	**Montero, 1995[102]**	**Griesmacher, 1997[103]**	**Albrecht, 2000[104]**
Compartment	erythrocytes	Lymphocytes, lymphoblasts	whole blood
Sample blood volume (mL)	10	N.A.	7.5
Reaction mixture			
IMP (mmol/L)	1	1	0.25
NAD (mmol/L)	0.5	0.5	0.25
KCl (mmol/L)	100	200	-
EDTA (mmol)	-	-	3.0
Allopurinol (mmol/L)	-	-	0.058
Buffer; concentration (mmol/L)	Phosphate; 40	Phosphate; 80	Tris; 0.1
pH	7.4	7.5	8.0
IMPDH assay			
Incubation time (h)	2	2.5 or 1.5	0.5 and 1
Incubation temperature (°C)	37	37	37
Incubation termination	40% trichloroacetic acid	perchloric acid	4 mol/L perchloric acid
Neutralization of sample	Extracted, water-saturated diethyl ether pH > 5.0	potassium carbonate	1h heated 100°C, 4M KOH pH 2-3
HPLC specifications			
Injection volume (µL)	50	100	200
Column	125x4.6mm 3µm ODS2 Hypersil	CNU-010	2x 150x4.6mm 5µm ID C18 Nucleosil
Type of HPLC	Ion exchange	Reversed phase	Reversed phase
Flow rate (mL/min)	1.0	1.2	1.0
Oven temperature (°C)	RT	RT	RT
UV-detection wavelength (nm)	254 and 280	N.A.	260
Eluens	70% 0.1 mol/L KH_2PO_4 (pH 5.5) / 8 mmol/L TBAS; 30% MeOH	0.015 mol/L KH_2PO_4; 0.5 mol/L KH_2PO_4 (pH 3.45)	4% MeOH in water H_3PO_4 pH 1.8
Runtime (min)	21	33	> 25
Activity unit	pmol / h / mg protein	pmol / min / 10^6 cells	nmol / min / ml WB(C)

N.A., not available; TBAS, tetrabutylammonium hydrogen sulphate; TEA, triethylamine; RT, room temperature; WB(C), whole blood (cells).

An ideal IMPDH activity assay should have optimized all these three steps. So far, the known assays are not optimized for all three steps. A more robust and reproducible IMPDH activity assay has to be found for multicenter and longitudinal studies to evaluate the additional value of any pharmacodynamic monitoring among a diversity of patients treated with MPA.

Glander, 2001[106]	Daxecker, 2002[107]	Vethe, 2006[105]
PBMC	PBMC	CD4+ cells
5	30	4
1	0.273	1.79
0.5	0.273	0.38
100	200	-
-	-	3.0
-	-	0.058
Phosphate; 40	Phosphate; 80	Tris; 0.1
7.4	7.4	8.0
2.5	2	2
37	37	37
4 mol/L perchloric acid	2.5 mol/L perchloric acid	4 mol/L perchloric acid
5M potassium carbonate	3M K_2HPO_4 pH > 7.2	1h heated 100°C, 4M KOH pH 2-3
15	20	100
250x3.1mm 5µm ODS AQ Prontosil 120	250x4.0mm ID RP-18 LiChroCART Superspher 100	100x4.6mm Chromolith + 150x4.6mm 5 µm C18 Nucleosil
Ion exchange	Ion exchange	Reversed phase
0.7	1.0	1.0
45	22	RT
254	254	260
4% MeOH in 96% 50 mmol/L KH_2PO_4 + 7 mmol/L TBAS (pH 5.50)	5.5 mmol/L $MgSO_4$ + 100 mmol/L H_3PO_4 + TEA (pH 6.40)	4% MeOH in water H_3PO_4 pH 1.8
35	54	20
nmol / h / mg protein	pmol / min / 10^6 cells	pmol / min / 10^6 cells

IMPDH mRNA GENE EXPRESSION

An alternative method to monitor the IMPDH activity could be measuring IMPDH mRNA, using the relative easier to use quantitative real-time polymerase chain reaction (RT-PCR) method. The regulation of expression of the two IMPDH genes differs between the two isoforms. IMPDH type I mRNA is expressed in a complex manner with three transcripts arising from three alternate promoters.[113,114] IMPDH type II is strongly up-regulated at the transcriptional level by a promoter region that responds directly to growth stimuli.[115-117] The gene expression of these two IMPDH isoforms has a negative

feedback regulation by intra- and extracellular guanine nucleotides.[118-120] An increased expression of IMPDH type I and II genes leads to an increased IMPDH activity.[77,111] It is known that an increased IMPDH activity has been correlated with an increased cellular proliferation and transformation.[121,122]

Recent studies have evaluated the expression of the IMPDH type I and II gene after kidney transplantation. The expression of the IMPDH type I gene was up-regulated 0.5-fold for CD4+ and 20-fold for PBMC under MMF therapy compared with values before transplantation without MMF therapy. IMPDH type II mRNA was increased after transplantation 15% for CD4+ and 121% for PBMC.[113,118,123,124] Induction of IMPDH activity after prolonged exposure to MPA may be partially explained by the up-regulation of IMPDH type I and II mRNA in PBMCs.[124] Although, a significant difference was seen in IMPDH type II gene expression in CD4+ cells between patients with and without a BPAR, this difference was probably related to the high-dose glucocorticoids, according the authors.[123]

It is not yet comprehensible that measuring IMPDH mRNA is a useable alternative to monitoring IMPDH activity. A correlation between mRNA and IMPDH activity is necessary to adopt mRNA measurements an alternative approach for IMPDH activity measurement.

CLINICAL PHARMACODYNAMIC STUDIES

Even though IMPDH activity assays are complex analyses, they are more and more used in research studies. Pharmacodynamic monitoring is a novel approach for individualization of the MPA therapy. It may better reflect biological response to the drug.[30,67]

Early studies already showed an inverse correlation between MPA concentration and IMPDH activity. In general, the maximum inhibition of IMPDH was achieved 1 to 2 hours after dosing.[97,125-127] The concentration-effect correlation was evaluated by Langman *et al* in 22 healthy persons and showed an IC_{50} (the concentration of MPA where 50% of the maximum IMPDH activity is measured) of 2.0 to 5.0mg/L. Complete inhibition of IMPDH was not observed but modeled at MPA concentrations of 20mg/L.[128] Besides using whole blood, the correlation was evaluated when lymphocyte membranes were disrupted and an *in vitro* setting of unbound MPA was created. The unbound MPA IC_{50} was 0.5 to 1.0mg/L.[128] In a number of studies, large inter-patient variability was observed in IMPDH activity, with a relative small intra-patient variability in respectively healthy persons, patients on dialysis and kidney transplant recipients. The inter-patient variability of more than 10-fold followed a Gaussian distribution, while the intra-patient variability measured over one month varied 14%.[106,127-130] The large inter-patient variation in healthy volunteers could not be attributed to age or gender. Genetic differences may

account for some of this variablility.[129] The causes of this large inter-patient variability in pre- and post-transplant IMPDH activity need to be investigated.[28]

A few studies have investigated the effect of time on the inhibition of IMPDH. In a population of five patients treated with MMF for more than two years, the inhibition of IMPDH activity was dramatically reduced when the length of the MPA therapy increased.[81] In a cohort of 29 patients the IMPDH activity was 5-fold higher than at baseline and occurred at a median of 40 days post-transplantation.[109] However, these studies were preformed in whole blood and therefore the results are difficult to interpret. Pharmacodynamic studies of IMPDH activity performed in PBMCs are few. In a study of 8 long-term MMF treated patients the IMPDH activity measured in PBMCs did not cause induction, despite a 2-fold higher MPA exposure after two years.[129] All these findings suggest IMPDH activity changes over time, which stresses that IMPDH activity measurement could help to optimize the individually MPA treatment.

One of the most interesting studies on the pharmacodymanics of MPA found a correlation between pre-transplant IMPDH activity and post-transplant dose adjustments, MPA related side effects and episodes of acute rejections. A cohort of 48 kidney transplant recipients treated with 1000 mg MMF twice a day was followed the first year after transplantation. Patients with high pre-transplant IMPDH activity had a 3.6-fold higher risk for acute rejection compared with patients with low pre-transplant IMPDH activity ($P = 0.009$). The risk for MMF dose reduction – due to MMF related clinical side-effects – was even 4.6-fold higher in patients with a high pre-transplant IMPDH activity ($P = 0.005$).[130] This study emphasizes that patient-specific measurements of IMPDH activity may optimize the MMF efficacy and toxicity.

It is not yet known whether IMPDH activity increases prior to rejection or if other promoters of lymphocyte activation, such as infection, also induce IMPDH activity. Only an increase in IMPDH activity appearing a few days before rejection has been described in a rabbit heart transplant model.[98] More research is needed to investigate whether differences in IMPDH activity can predict efficacy and dose requirements of MPA, and how differences in unbound MPA concentration may affect IMPDH activity. The observed high inter-patient variability in MPA exposure and pre- and post-transplant IMPDH activity strengthen the idea that therapeutic drug monitoring could optimize the use of MMF and improve the rejection outcome. Post-transplant IMPDH activity while on MPA therapy needs to be related to clinical outcome.[28,129] Further investigations with a large number of patients are needed to fully explore the pharmacodynamic – pharmacokinetic relationship between MPA and IMPDH activity. The measurement of pharmacodynamic drug effects by determination of IMPDH activity may provide a more direct insight of the functional activity of MMF therapy *in vivo* and may help to increase the efficacy and safety of MMF therapy.[131]

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Chapter 1.2

Scope and aims of this thesis

SCOPE AND AIMS OF THIS THESIS

Since the introduction of MMF in clinical practice, research has taken place to optimize and individualize MMF therapy in transplanted patients to improve the clinical outcome. The high observed inter-patient variability in MPA exposure and the small therapeutic window of MPA plead for good therapeutic drug monitoring regimens. Many studies monitored the pharmacokinetics of MPA concentrations for this purpose, sometimes with different outcomes. Pharmacodynamic monitoring of the response to MPA could be an attractive approach to individualization, because it integrates pharmacokinetic and pharmacodynamic variability.

Therefore, the overall aim of this thesis was to explore the usefulness of pharmacodynamic monitoring of IMPDH activity in MPA treated transplant recipients to optimize and individualize the MPA therapy. The obtained findings are supposed to make a foundation for clinical research strategies aiming at individualized MPA therapy in transplant recipients. More specifically, separate objectives were also formulated and were as follows:

1. To improve the robustness and reproducibility of the method for the determination of IMPDH activity in order to allow for the method to be used in multicenter longitudinal studies and in different patient groups (chapter 2).
2. To investigate the pharmacodynamic correlation between IMPDH activity and unbound and total MPA concentration (chapter 3).
3. To investigate the inter- and intra-patient variability in IMPDH activity in correlation to genetic polymorphisms, demographic characteristics and clinical laboratory parameters (chapters 3 and 4.1).
4. To determine the predictive performance of IMPDH activity for the incidence of acute rejection and adverse effects (chapters 3.1 and 4).
5. To study the correlation between IMPDH activity and IMPDH mRNA gene expression (chapter 4.2).
6. To explore IMPDH activity in haemotopoietic stem cell transplant recipients (chapter 5.1).

Chapter 2

IMPDH activity method

Chapter 2.1

Improved assay for the non-radioactive determination of IMPDH activity in peripheral blood mononuclear cells

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ABSTRACT

Mycophenolic acid (MPA) inhibits the enzyme inosine monophosphate dehydrogenase (IMPDH). Thus, the measurement of IMPDH activity could serve as a specific pharmacodynamic (PD) tool for monitoring MPA therapy. At present, however, monitoring of pharmacokinetic (PK) parameters is preferred over that of PD parameters because in general, PD assays are labor-intensive and poorly reproducible. Currently, cell count or protein concentration is widely accepted as methods to normalize enzyme activity. In the present study we have attempted to further improve a method for the determination of IMPDH activity to increase the robustness and reproducibility of the IMPDH activity assay itself, without making the assay more labor-intensive. Therefore, several aspects of the IMPDH method were investigated regarding their influence on the reproducibility and also modified to increase the feasibility and consistency of the assay. The isolation of peripheral blood mononuclear cells (PBMC) of whole blood samples was found to be the most variable step. Normalization on cell count is labor-intensive and at the same time has a poor reproducibility. Determination of the protein content in cell extracts is impaired by contamination with extra-cellular proteins and non-PBMC. Alternatively, the intracellular substance adenosine monophosphate (AMP) was investigated to normalize the newly generated xanthosine monophosphate. Among various subject groups, no significant differences in mean AMP concentration were found. To simplify the procedure, PBMCs were diluted to a fixed volume after isolation from sample of whole blood, and the IMPDH activity was normalized to the AMP concentration quantified in the same high-performance liquid chromatography run as xanthosine monophosphate was quantified. The within-run and total imprecision (coefficient of variation) ranged from 4.2 to 10.6% and from 6.6% to 11.9%, respectively. In conclusion, the modified method described here for the measurement of IMPDH activity can be used reliably in multicenter trials and in longitudinal studies to evaluate the additional value of any PD monitoring among a diversity of patients treated with MPA.

INTRODUCTION

The immunosuppressive drugs mycophenolate mofetil (MMF, CellCept) and enteric-coated mycophenolic sodium (Myfortic) have been registered for the prevention of acute rejection after solid organ transplantation. The active compound mycophenolic acid (MPA) is responsible for the reversible inhibition of inosine 5'-monophosphate dehydrogenase (IMPDH, EC 1.1.1.205). IMPDH is an nicotinamide adenine dinucleotide (NAD^+)-dependent dehydrogenase that converts inosine monophosphate (IMP) into xanthosine monophosphate (XMP).[1] MMF and enteric-coated mycophenolic sodium were each developed originally as fixed-dose drugs.[2] Reasons to consider individualization of dosage rather than using fixed-dose therapy include the large inter-individual variability in the pharmacokinetics (PK) and pharmacodynamics (PD) of this drug.[3-5] A recently published randomized trial showed that dose adjustment based on therapeutic drug monitoring using MPA levels reduced acute rejections in comparison with that using fixed-dose MMF therapy. In this study, a Bayesian estimation based on 3-point sampling was used to calculate the MPA exposure.[6]

On pure mechanistic grounds, measurement of PD parameters might correlate more closely with clinical outcome than would the measurement of PK parameters.[7] Recently we presented the first study showing a relationship between pretransplant IMPDH activity and posttransplant outcome.[8] At present, however, monitoring of PK parameters is preferred over PD parameters because, in general, PD assays are deemed to be labor-intensive and to have poor reproducibility. For measurement of the activity of IMPDH in tissue or blood samples, either the chromatographic or the radiometric quantification of enzyme-catalyzed product formation are used. Advantages and disadvantages of the different methods are summarized in a recently published review.[9] Because the target for MPA therapy is the lymphocyte, measuring IMPDH activity in isolated peripheral blood mononuclear cells (PBMCs) seems to be more appropriate.[10-12] A few years ago, Glander *et al*[13] introduced a method for the determination of IMPDH activity in PBMC extracts obtained after an accelerated cell isolation technique and using a nonradioactive procedure based on chromatographic determination of the XMP produced. This assay has been used in several clinical trials.[8,14-18]

To express an enzyme activity, the enzyme product measured has to be normalized. Currently, 2 potential methods are widely accepted, namely cell count and protein concentration. In the present study, the method of Glander *et al*[13] was modified to increase the robustness and reproducibility of the IMPDH activity assay, without rendering the assay more labor-intensive. We investigated how the different steps of the assay influence the variability and to what extent any contamination of the extract may influence the IMPDH activity derived. Modifications of the isolation of PBMCs, the preparation of lysate, and the normalization of the XMP improved the consistency and reproducibility

of the IMPDH activity assay. Moreover, it was evaluated whether the adenosine monophosphate (AMP) concentration in the PBMC extract could be used as normalization factor for IMPDH activity.

MATERIALS AND METHODS

Experiments were performed at 2 research centers: the Department of Internal Medicine, Nephrology of the University Medical Center Charité, Berlin, and the Department of Hospital Pharmacy, Erasmus University Medical Center, Rotterdam. Where necessary, referral will be made here to research center B (Berlin, Germany) or center R (Rotterdam, The Netherlands).

Patients and samples

Blood was obtained from healthy volunteers (n = 12), dialysis patients (n = 11), *de novo* renal transplant patients (n = 25) and stable renal transplant patients (n = 31) treated with calcineurin inhibitors and MMF with or without corticosteroids. The local ethics committees of Charité – Universitätsmedizin, Berlin, Germany and Erasmus University Medical Center, Rotterdam, The Netherlands have approved the blood sampling for this purpose. All subjects gave their informed consent. Before dosing, blood samples were collected in lithium heparin tubes stored at room temperature (15°C–25°C) and processed within 12 hours after collection.

Reagents and chemicals

IMP, XMP, AMP, NAD^+ and bovine serum albumin were obtained from Sigma-Aldrich Chemicals (Deisenhofen, Germany; Zwijndrecht, The Netherlands). Phosphate-buffered saline (PBS, pH 7.4) was purchased from PAA Laboratories GmbH (Linz, Austria). All other chemicals were obtained from Merck (Darmstadt, Germany; Zwijndrecht, The Netherlands) and were of analytical grade; the ion-pair reagent tetra-n-butylammonium hydrogen sulfate (TBAS) and methanol were of high-performance liquid chromatography (HPLC) quality (LiChropur, LiChrosolv). Leucosep tubes with Ficoll-Paque solution were obtained from Greiner Bio-One (Frickenhausen, Germany; Alphen a/d Rijn, The Netherlands).

Isolation of human PBMCs

PBMCs were isolated from lithium heparin-anticoagulated whole blood using Leucosep tubes with Ficoll-Paque according to the manufacturer's protocol with slight modifications. A biologically inert barrier on top of the separation medium Ficoll-Paque allowed a direct dilution of the 2.5 mL blood with an equal volume of PBS within the tube. Tubes

were centrifuged at 1200*g* at room temperature for 20 minutes. PBMCs were harvested and diluted with 5 ml of PBS. After centrifugation of the suspension for 10 minutes at 1200*g* at 4°C, the cell pellet was resuspended in 250 µL of water (HPLC grade, 4°C) and frozen at -80°C. Insoluble fragments of disrupted cells were removed by centrifugation at 1000*g* for 2 minutes after thawing. This supernatant lysate was used for the enzyme assay and protein determination. Protein concentrations were determined with BCA (bicinchoninic acid) Protein Assay Reagent (Pierce Chemical Co., Rockford, IL) according to the instructions of the manufacturer. In some experiments, cells were counted in an aliquot using a Neubauer counting chamber or a CASY cell counter (Casy Technology, Reutlingen, Germany) and resuspended to yield a final cell concentration of 1 x 10^7 cell per milliliter with cold HPLC water.

IMPDH activity assay

The incubation buffer consisted of 1 mmol/L IMP, 0.5 mmol/L NAD^+, 40 mmol/L sodium dihydrogen phosphate, and 100 mmol/L potassium chloride with a pH of 7.4, similar to that used in the previous method.[13] The incubation was initiated by the addition of 50 µL of lysate to 130 µL of incubation buffer in a 1.5-mL microtube maintained at 37°C and moderately shaken at 800 rpm (Thermomixer, Eppendorf, Hamburg, Germany). After 150 min, the enzymatic reaction was terminated by adding 20 µL of 4 mol/L perchloric acid, mixing, and placing the samples on ice. Precipitated proteins were removed by centrifugation for 5 minutes. The supernatant (170 µL) was transferred to a second 1.5-mL microtube containing 10 µL of 5 mol/L potassium carbonate and then mixed. Subsequently, samples were stored for at least 30 minutes at -80°C or for 2 hours at -20°C. After thawing and centrifugation, the supernatant was transferred into HPLC injection vials.

HPLC analysis

At research center B, AMP and XMP were quantified using a Shimadzu LC-2010 system (Shimadzu, Kyoto, Japan) with a built-in UV-VIS detector set to a wavelength of 256 nm. For chromatographic separations, a ProntoSIL AQ C18 column (particle size 3 µm, 3 mm inner diameter x 150 mm (Bischoff chromatography, Leonberg, Germany) was employed, and for data acquisition and reprocessing, Class VP software (Shimadzu) was used. The isocratic eluent consisted of a 6:94 (v/v) mixture of methanol and buffer containing 50 mmol/L KH_2PO_4 and 7 mmol/L TBAS (pH 5.50).

In center R, the HPLC system included a continuous mobile-phase degasser, an HPLC pump, an auto injector and an UV-VIS spectrophotometric detector with the wavelength set to 254 nm (Surveyer, Thermo Finnigan, Breda, The Netherlands). Data was captured and processed by ChromQuest software (Thermo Electron, Waltham, MA). Separation was achieved by using a ChromSpher C18, 5 µm particle size, 150 x 4.6 mm column (Varian Inc, Lake Forest, CA). For elution, a 50 mmol/L potassium dihydrophosphate buffer

(pH 5.6) with 7 mmol/L TBAS in 100% methanol was employed as a gradient. Over 0–4 minutes, the eluant consisted of 90% ion-pair buffer and 10% methanol, after 4 minutes methanol was increased linearly from 10% to 50% over 1 minutes, was then kept constant at 50% for 6 minutes, returned to 10% over 1 minute and then finally equilibrated for 5 minutes prior to the next injection.

In both centers, a 5-µL sample was injected onto the column, which was maintained at 40°C. Using a flow rate of 1.0 mL/min the cycle time amounted to 25 minutes for the isocratic mode, and 17 minutes for the gradient mode.

Calibrators and quality control samples

Quantification was performed using external standard calibrators and XMP and AMP with four calibration levels of 1, 5, 10 and 25 µmol/L for both components. Two calibrators (0.75 and 50 µmol/L) were added for the validation of the method. Because of the natural occurrence of AMP and XMP in PBMCs, no spiked blank samples could be used as calibrators. Therefore, calibrators were prepared by dissolving XMP and AMP in incubation buffer (without IMP and NAD^+) which contained 1 mg/mL of bovine serum albumin. The calibrators were treated with perchloric acid, neutralized and analyzed in the same manner as the incubation samples were.

For the final HPLC method, calibration curves and linearity, imprecision according to the NCCLS EP5-T guidelines[19], analytical specificity, recovery, and the lower limits of detection and quantification were determined for AMP and XMP. Quality control samples (QCSs) for the IMPDH assay were prepared by pooling several cell extracts of healthy volunteers, dialysis patients, or patients under MMF treatment. Both centers made QCSs of their own pool of subjects. The average activity and its standard deviation of the QCSs were determined in duplicate on 5 different days. The coefficient of variation accepted had to be less than 10%. Aliquots of this pool were stored at -80°C until analysis.

Sample stability

Investigations with regard to the stability of IMPDH activity in stored samples were carried out with the following components: whole blood at room temperature over 24 hours, lysate samples stored at -20°C and -80°C over 6 months, and samples that had been incubated to completion at room temperature and then stored at -20°C and -80°C over 6 months.

Data analysis

The results shown here are expressed as mean ± SD. Correlations between variables were assessed using univariate linear regression analysis for continuous data or X^2 test for categorical data. Paired 2-tailed Student *t* tests or Mann-Whitney *U* tests were preformed for the comparison of two means and 1-way analysis of variance when dealing

with more than 2 means. Statistics were processed by SPSS 12.0.1 for Windows (SPSS Inc, Chicago, IL). Results were considered significant if $P < 0.05$.

RESULTS

PBMC isolation variability

Sample aliquots after different intermediate steps of PBMC isolation were used to assess the variability for the different steps of the assay. The coefficient of variation for XMP production in samples isolated from the same volumes of identical blood was 29% (n = 6). The variation of XMP production in PBMC aliquots after harvesting from the interface was 19% (n = 6). Coefficients of variation for multiple cell counts (n = 6) of one and the same PBMC suspension ranged between 6%–28% (median 8%) for the CASY cell counter and 6%–19% (median 16%), and 8%–17% (median 11%) for 2 experienced researchers utilizing manual counting with the hemacytometer. Multiple incubations of the same lysate after removing undisrupted and insoluble cell fragments revealed a variation of 4.8% (n = 8). XMP determination by HPLC in identically incubated samples showed a variation of 0.3% (n = 8).

MPA concentrations in lysates and the corresponding total MPA plasma levels were measured in 25 randomly chosen samples from renal transplant recipients. All patients had been receiving MMF treatment with 1000 mg twice a day. The MPA concentration in the lysates was correlated with the total MPA concentration in plasma (Fig. 1; $r^2 = 0.94$; $P < 0.0001$). It is thought that the unbound fraction is responsible for immunosuppressive activity.[20] Accordingly, an unbound fraction of MPA in plasma of 1.38% ± 0.045% (the slope of curve) will represent the total MPA concentration in the lysate, equating to the unbound MPA concentration in plasma.

Contamination of cell extracts

It is possible that after only 1 step of washing, residual supernatant may still introduce extracellular protein to the sample. A nearly plasma free cell extract was prepared from an extensively washed cell pellet and resuspended in different volumes of water (50, 250 and 1000 µL). To this suspension, variable volumes of supernatant (10–150 µL) containing a mixture of plasma and PBS were added. Although XMP production was the same in all measured samples, calculated enzyme activity based on normalization with protein concentration was dramatically reduced when protein concentration was increased, and the volume of water for preparation of cell extracts was decreased (Fig. 2).

An erythrocyte suspension was used as an artificial contamination of the PBMC extract via an increasing number of erythrocytes (0.05×10^7 to 30×10^7 erythrocytes per 1×10^7 PBMC). Figure 3 illustrates the relative trueness of the IMPDH activity when compared

to that of samples measured without any additional contamination using erythrocytes. Calculated IMPDH activity decreased more than 10% with increasing contamination by erythrocytes due to increasing AMP and protein content. But in contrast to normalization using protein concentrations, the influence of increasing AMP on trueness seems to be significant only when contamination is observable.

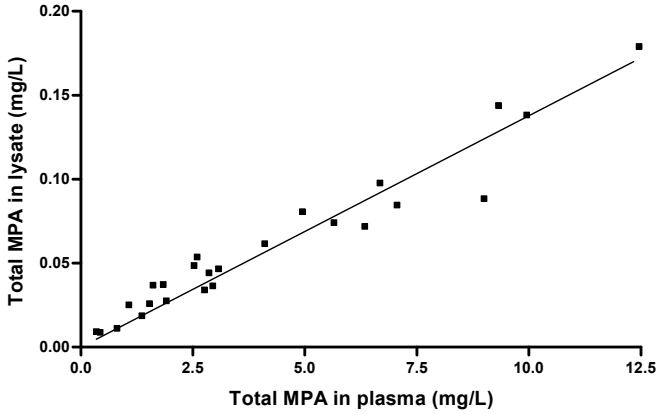

Figure 1. The correlation between the MPA level in lysate sample and its corresponding total MPA plasma levels was found to be significant after the isolation of PBMCs described in this paper ($r^2 = 0.94$; $P < 0.0001$). Accordingly, an unbound faction of MPA in plasma of 1.38% ± 0.045%–corresponding to the slope of the curve–will yield a total MPA concentration in lysate equal to the unbound MPA concentration in plasma.

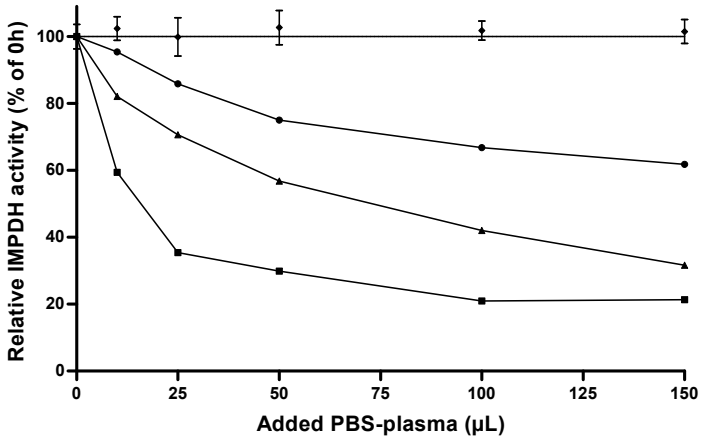

Figure 2. Influence of extracellular protein contamination on the calculated IMPDH activity. Different volumes of plasma-PBS dilution were added to cell pellets, and these were then resuspended in 50 µL (squares), 250 µL (triangles), or 1000 µL (circles) HPLC water for lysis. Although XMP production (diamonds) is the same in all samples, calculated enzyme activities based on normalization with protein concentration are influenced significantly. The relative trueness of the activity was at least 62% after adding 1000 µL (circles) or 21% after adding 50 µL (squares) of water to resuspend the cell extracts.

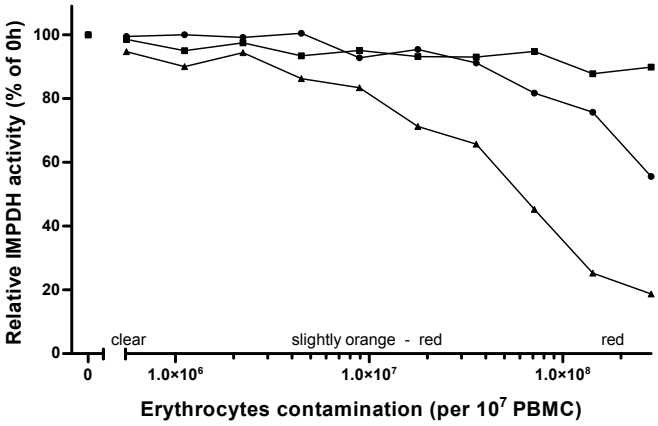

Figure 3. Influence of erythrocytes' contamination on the calculated IMPDH activity. Relative trueness of the calculated activity was compared in samples with increasing erythrocyte contamination based on normalization of XMP production after utilizing cell counts (squares), AMP concentrations (circles), and protein concentrations (triangles). Protein concentration is most sensitive to contamination with erythrocytes. In the usual working range (<3.0 x 10^7 erythrocytes per 107 PBMC) IMPDH activity, which was related to the protein concentration, was decreased to 71% (triangles). In contrast, IMPDH activity normalized to cell count (squares) and AMP concentration (circles) were only decreased to 93% and 95%, respectively.

Sample stability

Storage of lithium heparin blood samples at room temperature for 24 hours before to PBMC isolation did not lead to any significant changes in the IMPDH activity measured, when the activity was normalized to the AMP concentration. Samples of 5 patients (1 healthy volunteer, 2 renal transplant recipients and 2 dialysis patients) were used. Comparison of the results during the first 5 hours with those after 22 hours showed that only random fluctuations occurred. AMP and XMP were altered in the same manner reflecting only the variability in the yield of cells, whereas protein concentrations were additionally influenced to varying degrees by contamination and via performance of the protein assay. AMP concentrations followed XMP fluctuations more closely than protein concentrations. No activity was lost when cell extracts were kept at -20°C or -80°C for up to 6 months (data not shown).

Samples obtained from 1 healthy volunteer, 2 dialysis patients and 3 renal transplant recipients were used for to investigate the influence of incubation time on AMP stability and XMP production. Figure 4A shows a small increase of AMP in all investigated samples over time, but it still appears to be quite small when compared to the variability between the samples. The XMP production of the enzymes increased linearly over the time of incubation (Fig. 4B; $r^2 = 0.96 \pm 0.04$; $P < 0.001$) similar to the ratio of XMP/AMP (Fig. 4C; $r^2 = 0.83–0.99$; $P < 0.001$).

When the patients' samples were incubated to completion, neutralized, and then stored at room temperature, the AMP signal was found to remain stable only over the first 24 hours. Thereafter, AMP concentrations increased, whereas the XMP concentra-

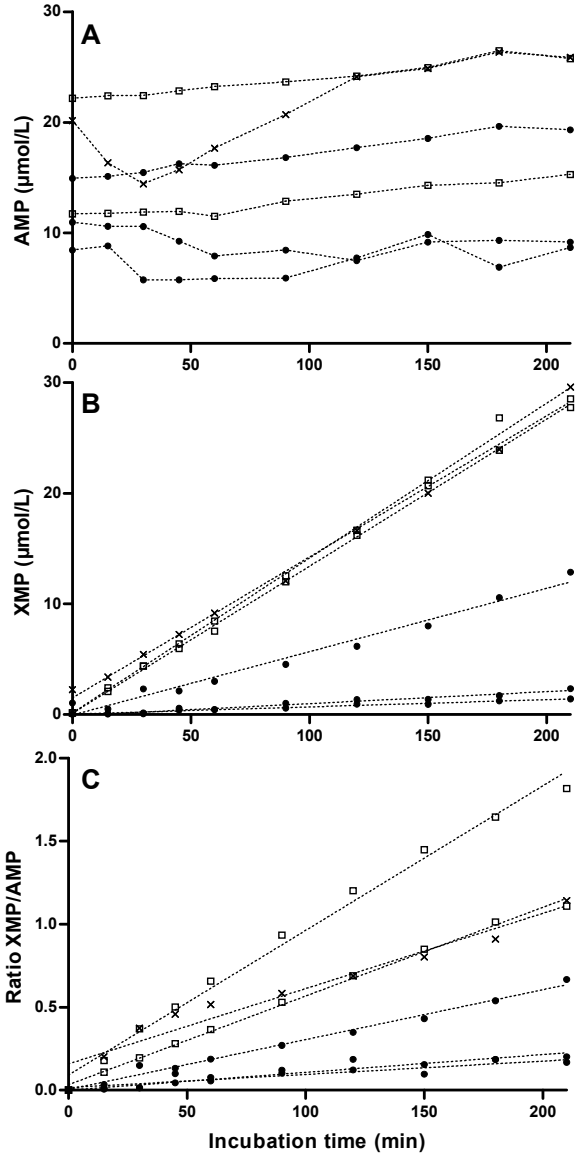

Figure 4. Time profiles of (A) AMP, (B) XMP, and (C) the ratio of XMP/AMP in different patients. Samples of 2 dialysis patients (open squares), 3 renal transplant recipients (solid circles), and pooled lysate samples from healthy volunteers (crosses) are displayed. AMP shows a small increase in all samples investigated over time, but remains very small when compared to the variability between the samples. The XMP production of the enzymes increased linearly with the time of incubation ($r^2 = 0.96 \pm 0.04$; $P < 0.001$) similar to the ratio of XMP/AMP ($r^2 = 0.83–0.99$; $P < 0.001$).

tions remained constant. There were no significant differences observed in the content of AMP or XMP in completely incubated and neutralized patient samples when they were stored at -20 °C or -80 °C for up to 6 months; when compared to initial values,

these 2 temperature conditions represented values of 106 and 102% respectively. Thus, samples should be analyzed within 24 hours or kept frozen until analysis.

Linearity with sample dilution

Lysate samples from 2 healthy volunteers were diluted stepwise in order to obtain 5 different enzyme concentrations. These samples were incubated in duplicate and XMP and AMP activities were then determined. The correlation was linear with a regression coefficient of 1.00 ($P < 0.001$) and 0.996 ($P < 0.001$). The enzyme activities of the original nondiluted lysate were 117.9 and 69.39 $\mu mol \cdot s^{-1} \cdot mol^{-1}$ AMP in the 2 healthy subjects. The activities of all of the measured samples (n = 10) from the same two subjects were 117.7 ± 8.89 and 72.35 ± 4.31 $\mu mol \cdot s^{-1} \cdot mol^{-1}$ AMP. The coefficients of variation were 7.6 and 6.0%, and the accuracies 99.8 % and 104.3% (Fig. 5).

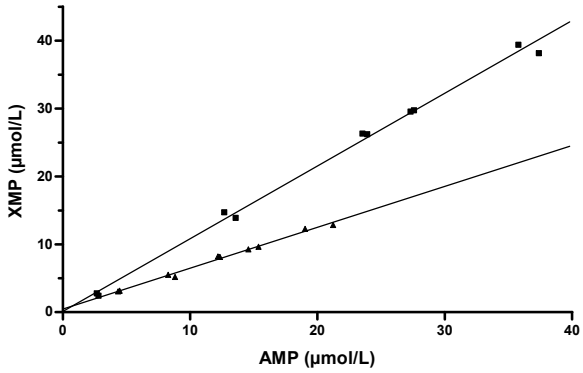

Figure 5. Linearity between XMP produced and AMP present in the sample after diluting 2 different patient samples. Both lines showed linearly [$r^2 = 1.00$; $P < 0.001$ (square) and $r^2 = 0.996$; $P < 0.001$ (triangle)] with a calculated activity out of the each 10 diluted samples of 117.7 ± 8.89 and 72.35 ± 4.31 $\mu mol \cdot s^{-1} \cdot mol^{-1}$ AMP respectively.

AMP in PBMC of patients and healthy subjects

Samples of 3 different subject groups were isolated, and then, AMP and protein concentrations were determined (Fig. 6). No significant differences were noticed in regard to the AMP concentration among the patient groups ($P = 0.63$), but a significant difference in protein concentration ($P < 0.001$) in the patient groups was observed.

HPLC Determination of AMP and XMP

The chromatograms of incubation buffer without the addition of cell extracts did not reveal any interfering peaks at the retention times seen for AMP or XMP (Fig. 7A). Cell extracts incubated in incubation buffer but without IMP and NAD^+ were apparently free of XMP (Fig. 7B). No interfering peaks in the vicinity of the retention time of AMP were found in 10 different batches of patient plasma. Isocratic elution (in research center B) resulted in reten-

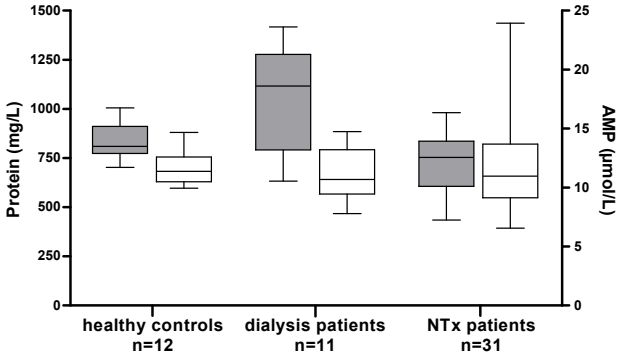

Figure 6. Box-Whisker plots of protein concentrations (filled) and AMP concentration (empty) within and between 3 different subject groups. No differences in AMP concentrations were found between the groups ($P = 0.63$), whereas there was a difference in protein concentration ($P < 0.001$). There was no difference in XMP production between the groups which were not undergoing MMF treatment (data not shown). Normalizing the activity of AMP concentrations makes it possible to compare the calculated IMPDH activity between different patient groups.

tion times for AMP and XMP of 5.9 and 7.6 minutes respectively (Fig. 7C). With gradient elution (in center R), retention times for AMP and XMP were found to be 3.5 and 5.6 minutes, respectively (Fig. 7D). The resolutions of the peaks were 1.5 ± 0.06 (range 1.44–1.63) and 3.6 ± 1.5 (range 1.40–6.05), respectively. These values were all assessed in 24 patient samples.

Calibration curves of XMP and AMP were constructed by relating peak area to the concentration of the calibrators. For XMP, the calibration curve was linear in the concentration range of 0.75–50.0 µmol/L. The correlation coefficient (r^2) ranged from 0.996 to 1.000. The imprecision and accuracy for XMP determination of 4 levels measured separately is summarized in Table 1. The accuracy of determination (as percentage of the theoretical value) ranged from 99.2% to 103.3%. The lower limit of quantification and determination was 0.031 µmol/L and 0.010 µmol/L, respectively. The recovery of XMP ranged from 76% ± 1.9% to 108% ± 3.9%.

For AMP, the calibration curve was linear in the concentration range of 0.75–50.0 µmol/L. The correlation coefficient (r^2) ranged from 0.986 to 1.000. The imprecision and accuracy for AMP determination measured at 4 different levels is summarized in Table 1. The accuracy of determination (as percentage of the theoretical value) ranged from 99.7% to 103.3%. The lower limit of quantification and detection was 0.067 µmol/L and 0.040 µmol/L, respectively. The recovery of AMP ranged from 102% ± 1.1% up to 118% ± 3.9%.

Imprecision of IMPDH activity measurement

There is not yet a gold standard for the measurement of IMPDH activity. To compare imprecision of 3 alternative procedures for the normalization of IMPDH activity, PBMCs were isolated, 10-fold, from whole blood samples taken from 2 normal healthy controls. In these 20 samples, cells were counted during isolation, protein concentrations were

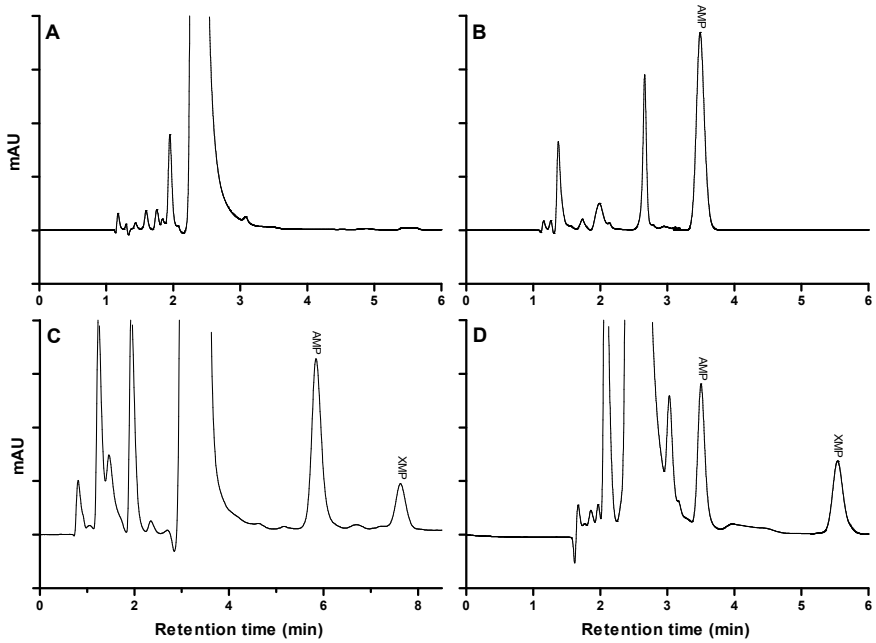

Figure 7. Chromatograms of different samples involving (A) incubation buffer used for the in vitro IMPDH enzyme reaction; (B) cell extract incubated in reaction buffer without IMP and NAD^+; and (C) patient sample incubated according to the new method used in research center B. The retention times of AMP and XMP are 5.9 and 7.6 minutes, respectively, and (D) patient sample incubated according to the new method used in center R. The retention times here of AMP and XMP are 3.5 and 5.6 minutes, respectively.

determined, and subsequently the lysates were incubated. Finally, the concentrations of AMP and XMP were determined. The IMPDH activity was normalized according to the following 3 procedures. Cell counts, protein concentration and AMP concentration. The coefficients of variation ranged between 11.2% and 39.8%, 11.8% and 12.4%, and 6.6% and 8.5%, respectively.

Multiple aliquots of cell extracts with 3 different activity levels were measured over 20 days (10 days for the high level) whereby 4 samples a day were used for each estimation of within-run, within-day, between-runs, between-days, and total standard deviation. Results are summarized in Table 2. Lysate samples of 3 patients treated with MMF were incubated and measured six-fold to calculate the IMPDH activity. The coefficient of variation differed between 0.6% and 3.4% (Table 3). The total within-run imprecision for the complete assay including PBMC cell isolation and incubation was 109.7%, 106.4%, and 104.0% (n = 6) for 3 different activity levels (114.1, 78.5 and 18.9 $\mu mol \cdot s^{-1} \cdot mol^{-1}$ AMP). The limited stability of whole blood unfortunately did not permit any estimation of between-runs imprecision of the complete assay including the PBMC isolation step.

Table 1. Imprecision among determinations of AMP and XMP using 4 different SCSs

	SCS (1.0 µmol/L)	SCS (5.0 µmol/L)	SCS (10.0 µmol/L)	SCS (25.0 µmol/L)
XMP				
Mean (µmol/L)	0.99	5.05	10.33	25.67
Accuracy (%)	99.2	100.9	103.3	102.7
SD within-run	0.16 (16.6)	0.49 (9.6)	0.78 (7.6)	0.71 (2.8)
SD between-runs	<0.01	<0.01	<0.01	0.59 (2.3)
SD total imprecision run	0.16 (16.6)	0.49 (9.6)	0.78 (7.6)	0.93 (3.6)
SD within-day	0.15 (15.3)	0.46 (9.0)	0.77 (7.4)	0.73 (2.9)
SD between-days	<0.01	<0.01	0.064 (0.6)	0.58 (2.3)
SD total imprecision day	0.15 (15.3)	0.46 (9.0)	0.77 (7.4)	0.93 (3.6)
AMP				
Mean (µmol/L)	1.02	4.99	10.23	25.82
Accuracy (%)	102.0	99.7	102.3	103.3
SD within-run	0.11 (11.2)	0.40 (8.0)	0.69 (6.8)	0.39 (1.5)
SD between-runs	0.015 (1.5)	<0.01	0.30 (2.9)	0.67 (2.6)
SD total imprecision run	0.12 (11.3)	0.40 (8.0)	0.75 (7.4)	0.78 (3.0)
SD within-day	0.11 (10.9)	0.39 (7.9)	0.70 (6.8)	0.41 (1.6)
SD between-days	0.034 (3.3)	<0.01	0.29 (2.9)	0.67 (2.6)
SD total imprecision day	0.12 (11.3)	0.39 (7.9)	0.76 (7.4)	0.79 (3.0)

Imprecision was evaluated according to NCCLS EP5-T.[19] For each concentration, 80 samples were determined: 2 samples per run, 2 runs per day for 20 days. Imprecision data are presented as follows: standard deviation [relative standard deviation (%)].
SCS, spiked control sample.

Table 2. Imprecision of IMPDH activity determined in I-QCS at 3 different IMPDH activity levels

	I-QCS (Low level)	I-QCS (Medium level)	I-QCS (High level)
Mean (µmol·s^{-1}·mol^{-1} AMP)	31.5	89.2	124.5
SD within-run	3.35 (10.6%)	3.79 (4.2%)	6.98 (5.6%)
SD between-runs	1.65 (5.3%)	4.44 (5.0%)	4.62 (3.7%)
SD total imprecision run	3.75 (11.9%)	5.84 (6.6%)	8.37 (6.7%)
SD within-day	3.42 (10.8%)	4.60 (5.2%)	7.80 (6.3%)
SD between-days	1.55 (4.9%)	3.62 (4.1%)	3.08 (2.5%)
SD total imprecision day	3.76 (11.9%)	5.86 (6.6%)	8.38 (6.7%)

Imprecision was evaluated according to NCCLS EP5-T.[19] For the low and medium levels, 80 samples were determined, and for the high level, 40 samples: 2 samples per run, 2 runs per day for 20 days (10 days for the high level). Imprecision data are presented as follows: standard deviation [relative standard deviation (%)].
I-QCS, incubation quality control samples.

Table 3. Imprecision of the IMPDH activity determination using 3 different patient samples

	Parameters analyzed	Patient 1 (n = 6)	Patient 2 (n = 6)	Patient 3 (n = 6)
MPA	mean (mg/mL)	1.95	0.94	1.29
AMP	mean (µmol/L)	17.14 ± 0.59	19.52 ± 0.38	17.98 ± 0.16
	CV (%)	3.4	1.9	0.9
XMP	mean (µmol/L)	8.77 ± 0.09	4.06 ± 0.04	30.97 ± 0.24
	CV (%)	1.0	1.0	0.8
IMPDH activity	mean (µmol·s^{-1}·mol^{-1} AMP)	56.9 ± 1.9	23.1 ± 0.4	191.4 ± 1.1
	CV (%)	3.4	1.6	0.6

CV, coefficient of variation (SD/mean * 100%).

DISCUSSION

Following publication of the HPLC-based measurements by the German group,[13] other laboratories also set up this assay. Continued experience with the assay has shown that a number of steps require further assay improvement. In a mutual project, 2 laboratories actively involved in monitoring kidney transplant patients with this assay have modified the method. In Table 4, all modifications described in this article–in comparison with those of the first publication–are summarized. The modifications have led to an IMPDH assay which is less labor-intensive, more robust in general, and capable of yielding a better reproducibility.

Table 4. Summary of all modifications of the assay in comparison with the previous publication

Parameters analyzed	Glander *et al* 2001[13]	Glander *et* Sombogaard 2009
Sample preparation		
Volume of whole blood (mL)	5	2.5
Aliquot resuspension	To a cell concentration of about 1 x 10^7 cells/mL	In 250 µL ice-cold HPLC water
HPLC method		
Column temperature	45°C	40°C
Eluent	Isocratic	Isocratic (research center B) Gradient (center R)
Flow rate (mL/min)	0.7	1.0
Injected sample volume (µL)	15	5
Run time (min)	35	25 (center B) 17 (center R)
Normalization		
Expression of concentration in calibrators	pmol/incubation sample	µmol/L
Normalization of XMP production	Protein concentration	AMP concentration
Expression of IMPDH activity	nmol·h^{-1}·mg^{-1} protein	µmol·s^{-1}·mol^{-1} AMP

Currently, there are 2 widely accepted ways to normalize enzyme activity, namely cell count and protein concentration. We here introduce a third method, that of measuring the AMP concentration. When comparing these 3 methods, the following advantages and disadvantages can now be envisaged. At first glance, the counting of cells after harvesting from the interface seems to be an easy and reproducible method, but it also accounts for the variability of yield and number of cells in the whole blood sample. Even experienced technicians are faced with high intraobserver and interobserver variability when using automatic and manual methods for counting PBMCs harvested from the interface. In a recent article concerned with PD monitoring of calcineurin inhibitor therapy, it was shown that leukocyte subset cell count variation could also affect the calcineurin activity measured. Variability in sample composition could also affect other PD monitoring strategies for immunosuppressive drugs.[21] An additional source of error in the proposed IMPDH activity method is found in the process of cell disruption by osmosis after adding water. During this process, the cytoplasm–including the IMPDH enzymes–will be released into the lysis medium while nondisrupted cells are removed after centrifugation. The rate of the cell disruption will be less when samples contain more cells. Measuring the cell count prior to cell disruption however does not indicate the actual number of cells that really do disrupt. The poor reproducibility of cell counting, combined with the uncertainty of any further steps for the final preparation of cell extracts, may thus explain why laborious cell counting has no advantage in regard to optimizing reproducibility of the composition of PBMC extracts.

In yet a different IMPDH method, the cell pellet is resuspended in the original patient plasma.[22] This method will result in an MPA concentration in the lysate, which is identical to the total MPA concentration in plasma. However we found that the MPA concentration in the lysate is much lower in comparison with the total MPA concentration in plasma. The MPA concentration in lysates correlates with the total MPA plasma concentration and is similar to the theoretical unbound MPA plasma concentration, which is thought to be responsible for its immunosuppressive activity.[20] Therefore, our method reflects much better the in vivo situation and results in a more accurate determination of the IMPDH inhibition by MPA.

Traditionally, enzyme activity has been related to protein content. For the calculation of enzyme activity per amount of protein, intracellular as well as extracellular proteins from the sample matrix have been included. Despite a careful isolation of the PBMCs, it is in our opinion still impossible to obtain a supernatant-free PBMC pellet just before the lysis step. The remaining supernatant on the pellet always contains a variable amount of dissolved (extracellular) proteins. These proteins will also be present in the final sample, and therefore, they too will be measured in the protein determination. Contamination with erythrocytes during the isolation of PBMCs will further increase the total protein concentration measured in the final samples, thereby decreasing the calculated IMPDH

activity. The storage time of whole blood before isolation has some influence on the yield of cells. Although the yield of mononuclear cells often decreases, the contamination with erythrocytes increases due to aggregation of erythrocytes and lymphocytes. The magnitude of this variability differs between samples and is a random occurrence.

The solution which we have chosen for the normalization of the enzymes' products makes use of an intracellular compound instead of cell count or protein concentration. By using an intracellular compound, a correction is made for the number of cells that are actually lysed and have released their contents including the enzymes into the solution. AMP is a nucleotide of the cell matrix and derives from the same cells from which IMPDH enzymes originate. When enzyme activity is normalized to AMP content, this should better reflect the enzyme activity of disrupted cells. AMP concentrations can easily be determined in the same run as newly synthesized XMP. The IMPDH activity is expressed as the number of moles of XMP produced per second per mole of detected AMP, in short $mol \cdot s^{-1} \cdot mol^{-1}$ AMP or katal/mol AMP. The activity can be calculated from the measured concentrations of AMP and XMP according to the following equation:

$$\text{IMPDH activity } (\mu mol \cdot s^{-1} \cdot mol^{-1} \text{ AMP}) = \frac{\text{produced XMP } (\mu mol \cdot L^{-1}) \cdot 10^6}{\text{incubation time (s)} \cdot \text{measured AMP } (\mu mol \cdot L^{-1})}$$

We consider that the variation in precision and accuracy of the AMP and XMP determination in the present work is on an excellent level, with coefficients of variation residing below 15%. The amount of AMP present in the final sample of an assay seems to be influenced neither by the supernatant above PBMCs during the isolation nor by contamination with erythrocytes in the usual working range. One disadvantage of using AMP concentrations is the amount of time it takes before a stable level of AMP is reached in the incubation step. An incubation time of at least 120 minutes is necessary in order to obtain a stable concentration of AMP.

The factor for normalization must also be comparable in different subject groups. In contrast to the usage of protein concentrations, it has been shown here that AMP concentrations did not differ between normal healthy volunteers, dialysis patients and renal transplant patient treated with MMF. The large variation for AMP in renal transplant recipients is possibly due to a substantial variability in harvested PBMCs from patients who received antithymocyte globulin induction therapy. Even patients after a transplantation who were treated early with an antithymocyte globulin induction therapy exhibited similar IMPDH activities.

CONCLUSIONS

We present here a method capable of reducing the variability and at the same time improving the practicability of the enzymatic assay for the measurement of IMPDH activity in PBMCs. The protocol requires only 2.5 mL of fresh blood and the usual standard laboratory equipment. Using AMP concentrations for normalization of the newly generated XMP results in a smaller variability when compared with that of other normalization factors used previously, such as protein concentrations and cell counts. The within-run and within-day reproducibilities of the IMPDH activity values as gained by our modified HPLC method are, in our opinion, for such a complex method really within acceptable limits (<11%).

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Chapter 3

Pharmacokinetic / pharmacodynamic correlation

Chapter 3.1

MMF therapy in renal transplant patients: relationship between pharmacokinetics, IMPDH activity and clinical outcome

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ABSTRACT

Mycophenolic acid (MPA), the active metabolite of mycophenolate mofetil (MMF), exerts its immunosuppressive effect by inhibiting inosine 5´-monophosphate dehydrogenase (IMPDH). A cohort of 101 de novo kidney transplant patients was followed prospectively for 12 months. MPA concentrations were related to IMPDH activity at day 6, 21, 49 and 140 post-transplantation using NONMEM. An inhibitory E_{max} model described the relationship between MPA concentration and IMPDH activity. At day 6 the area under the 12-hour time profile (AUC_{0-12h}) of MPA concentration of patients with a biopsy proven acute rejection (BPAR) (n=23) was comparable to non-BPAR patients (n=69) (p=0.73), whereas the AUC_{0-12h} of IMPDH activity was higher with median values of 241 and 201 h·µmol/s/mol AMP (p=0.086), respectively. An a posteriori analysis indicated that BPAR patients exhibited a significantly higher E_0 and IC_{50} at day 6 than non-BPAR patients; corresponding increases were 23% (p=0.025) and 75% (p=0.011). Our data suggest that the inhibition of IMPDH activity during the first week of MMF treatment is associated with the rate of rejection. Combined monitoring of MPA concentration and IMPDH activity may optimize MMF therapy.

INTRODUCTION

Mycophenolate mofetil (MMF) is an immunosuppressant used to prevent allograft rejections in solid organ transplant patients.[1] MMF is a prodrug of mycophenolic acid (MPA) – a noncompetitive reversible inhibitor of inosine 5´-monophosphate dehydrogenase (IMPDH). IMPDH is the rate-limiting enzyme in the *de novo* pathway of guanine nucleotide synthesis. Inhibition of IMPDH retards the proliferation of T- and B-lymphocytes, which are dependent on the guanine nucleotide synthesis pathway.[2] There are two isoforms of IMPDH that are encoded by two distinct genes: type I and type II.[3]

After oral administration MMF is rapidly hydrolyzed to MPA, which is metabolized in the liver by UDP-glucuronosyltranferases to three metabolites 7-O-mycophenolic acid glucuronide (MPAG), MPA-acyl-glucuronide and MPA-phenyl-glucuronide.[4] The glucuronides are excreted in urine and bile. Biliary excretion of metabolites in the gut lumen contributes to enterohepatic recirculation.[5]

The existence of a therapeutic window and the highly variable pharmacokinetics of MPA have led investigators to evaluate the potential benefit of therapeutic drug monitoring using MPA plasma concentrations.[6] In a prospective randomized study it was demonstrated that therapeutic monitoring using a limited sampling strategy to determine the area under the 12 hour MPA plasma concentration versus time curve (MPA $AUC_{0\text{-}12h}$) reduced the risk of treatment failure and acute rejection in the first year after renal transplantation.[7]

So far, individualization of MMF therapy has been focused primarily on the pharmacokinetics of MPA. However, since IMPDH is the molecular target of MPA, IMPDH activity may serve as a surrogate pharmacodynamic marker for immunosuppression and clinical outcome. By monitoring of MPA-induced IMPDH inhibition it is conceivable that a more accurate prediction of therapeutic response will be obtained than by monitoring of MPA plasma concentrations alone.[8]

In the present study a prospective cohort of 101 *de novo* renal transplant recipients on MMF therapy was followed for 12 months after transplantation. Time profiles of plasma MPA concentration and IMPDH activity in peripheral blood mononuclear cells (PBMC) were assessed at 4 occasions (days 6, 21, 49 and 140 post-transplant) in order to develop an integrated pharmacokinetic-pharmacodynamic model. Population pharmacokinetic and pharmacodynamic parameters was assessed as well as their relationship with clinical outcome.

RESULTS

Patients and clinical outcome

The patient characteristics are summarized in Table 1. At day 140 post-transplant 37 patients had discontinued the study due to primary non-function of graft or transplantectomy (n=4), problems with repetitive blood sampling (n=6), withdrawal of informed consent (n=13), missed clinical visits (n=9) and other reasons (n=5).

Table 1. Demographic, baseline biochemical and molecular patient characteristics

	Visit 1 (Day 6)	**Visit 2 (Day 21)**	**Visit 3 (Day 49)**	**Visit 4 (Day 140)**
Days post transplant (days)	6 (3-16)	21 (15-42)	49 (40-76)	140 (133-168)
Number of patients (n)	101	82	72	64
Gender male/female (n)	73/28	62/20	53/19	47/17
Race Caucasian/Black/Asian/Hispanic (n)	83/13/3/2	69/8/3/2	59/9/3/1	51/9/3/1
Patients with pre-Tx diabetes (n)	14	9	9	8
IMPDH type II 3757T>C WT/het/hom (n)	89/10/2	71/9/2	63/7/2	54/8/2
Age (years)	54 (19-76)	54 (19-76)	55 (19-76)	54 (20-77)
Weight (kg)	80 (44-145)	79 (40-131)	80 (42-126)	82 (44-134)
IMPDH act pre-Tx (µmol/s/mol AMP)	44 (6-178)	43 (6-178)	43 (6-178)	45 (6-178)
Serum creatinine (µmol/L)	213 (61-1190)	149 (74-1013)	134 (78-525)	132 (81-816)
Creatinine clearance (mL/min)	28 (4-90)	43 (5-90)	49 (10-90)	47 (6-82)
Plasma albumin (g/L)	34 (21-43)	39 (10-46)	42 (24-50)	43 (26-48)
Serum AST (U/L)	34 (9-236)	17 (9-58)	17 (8-66)	23 (12-73
Serum ALT (U/L)	44 (2-534)	26 (7-162)	17 (5-114)	19 (1-83)
Haemoglobin (mmol/L)	6.2 (3.3-9.4)	6.1 (4.2-8.8)	6.8 (4.5-9.5)	7.6 (4.9-9.5)
Thrombocytes (10^9/L)	194 (32-407)	254 (40-568)	232 (131-455)	245 (7-338)
Leukocytes (10^9/L)	8.1 (0.3-29.0)	8.4 (3.1-18.3)	7.7 (3.3-24.0)	6.2 (0.4-12.3)
Patients with first Tx (n)	90	72	65	57
Donor living/deceased (n)	64/37	55/27	51/21	46/18
HLA mismatch 0 / 1-3 / 4-6 (n)	6/50/45	5/41/36	3/38/31	3/33/28
PRA 0 / 1-10 / 11-50 / 50-100 / n.a. (n)	66/12/5/4/14	55/10/4/3/10	49/10/3/3/7	46/8/3/3/4
Delayed graft function (n)	31	23	17	13
Dose MMF (mg)	1000 (500-1500)	500 (500-1500)	500 (250-1500)	500 (250-1000)
Daily dose tacrolimus (mg)	11 (4-20)	9 (2-20)	8 (2-20)	4 (1-20)
Tacrolimus trough level (µg/L)	11 (2-30)	11 (5-28)	10 (4-21)	9 (4-16)
ATG induction therapy (n)	29	21	15	14

Values are expressed as median (range) except for (n).

ALT, alanine transaminase; AMP adenosine monophosphate; AST, aspartate transaminase; ATG, anti-thymocyte globulin; het, heterozygote; HLA, human leukocyte antigen; hom, homozygote; IMPDH, inosine monophosphate dehydrogenase; MMF, mycophenolate mofetil; n.a., not available; PRA, panel-reactive antibodies; Tx, transplantation; wt, wild type; XMP, xanthine monophosphate.

Twenty three patients exhibited a biopsy proven acute rejection (BPAR) within 12 months post-transplant. Acute rejection was presumed for 4 patients and a primary non-function of the graft was observed in 5 patients. BPAR occurred at a median of 11 days (range 6 – 210) post-transplant. Incidence rates of abdominal cramps, diarrhoea, nausea and vomiting were less than 21%, 15%, 16% and 8%, respectively. Anemia decreased from 68% on day 6 to 16% on day 140, whereas thrombocytopenia decreased from 32% to 8%. The incidence of leukopenia was less than 13%.

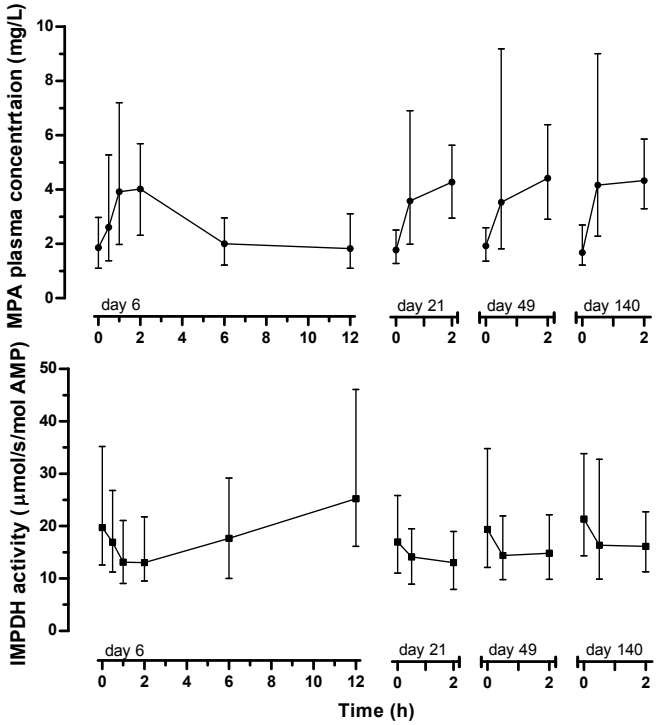

Figure 1. Time profiles of mycophenolic acid (MPA) plasma concentration (circles) and inosine monophosphate dehydrogenase (IMPDH) activity (squares) following an oral administration of mycophenolate mofetil (MMF). Data are expressed as median plus 25% and 75% percentiles. Data were obtained in 101 (day 6), 82 (day 21), 72 (day 49) and 64 (day 140) patients.

Pharmacokinetics

Figure 1 depicts the time profiles of MPA plasma concentrations at day 6, 21, 49 and 140. A two-compartment pharmacokinetic model with first-order absorption and elimination adequately described the data. Time dependency was detected for both the central volume of distribution (V_1/F) and apparent oral clearance (CL/F) (Table 2). CL/F was 62% and 25% higher on day 6 and 21 compared to day 49 and 140; corresponding values for V_1/F were 273% and 73%. Diagnostic plots (e.g predicted versus observed

concentrations, weighted residuals versus time) confirmed the adequacy of the population pharmacokinetic model (plots not shown). Furthermore, the population estimates of the model were well within the 95% confidence interval as produced by the bootstrap procedure (Table 2).

Table 2. Population pharmacokinetic parameter estimates for mycophenolic acid

Population parameters	**Estimated value (%CV)**	**1000 bootstrap replicates Median (95% interval)**
Typical parameter		
T_{lag} (h)	0.36 (7%)	0.35 (0.32-0.39)
K_a (h^{-1})	3[1]	3[1]
V_1 visits 3 and 4 (L)	65 (13%)	66 (47-90)
Θ_{V1}-visit 1	3.7 (16%)	3.6 (2.5-4.0)
Θ_{V1}-visit 2	1.7 (16%)	1.7 (1.1-2.3)
CL visits 3 and 4 (L/h)	15 (6%)	15 (13-18)
Θ_{CL}-visit 1	1.6 (7%)	1.5 (1.3-1.8)
Θ_{CL}-visit 2	1.3 (6%)	1.3 (1.1-1.6)
V_2 (L)	1200 (125%)	1300 (570-2350)
Q (L/h)	38 (11%)	37 (29-49)
Between-patient variability		
T_{lag} (%)	45 (86%)	52 (23-102)
K_a (%)	102 (108%)	98 (66-150)
V_1 (%)	59 (53%)	65 (34-89)
CL (%)	32 (23%)	33 (21-51)
Correlation		
CL – V_1	1[2]	1[2]
CL – K_a	0.14	0.18 (0.05-0.35)
CL – T_{lag}	0.25	0.25 (0.12-0.46)
V_1 - K_a	0.14	0.11 (0.03-0.21)
V_1- T_{lag}	0.25	0.26 (0.12-0.35)
K_a – T_{lag}	0.88	0.81 (0.46-0.95)
Within-patient variability		
K_a (%)	143 (23%)	150 (86-200)
V_1 (%)	56 (59%)	52 (35-88)
CL (%)	35 (16%)	35 (24-49)
Residual error		
Additive (mg/L)	0.44 (4%)	0.44 (0.40-0.50)

[1] K_a was fixed to 3 h^{-1}; [2] Correlation between CL and V_1 was high and fixed to 1.

CL/F day 49 and 140, apparent oral clearance on days 49 and 140; K_a, absorption rate constant; Q/F, intercompartmental clearance; T_{lag}, lag time; V_1/F day 49 and 140, central volume of distribution on days 49 and 140; V_2/F, peripheral volume of distribution; $\Theta_{CL/F}$-day 6, fractional increase of CL/F on day 6 compared to days 49 and 140; $\Theta_{V1/F}$-day 6, fractional increase of V_1/F on day 6 compared to days 49 and 140.

On the basis of the individually derived pharmacokinetic parameters MPA exposure was calculated. Median (range) MPA $AUC_{0\text{-}12h}$ was 29 (9 – 70), 28 (11 – 85), 27 (11-103) and 27 (10 – 63) h·mg/L on day 6, 21, 49 and 140, respectively (n=101, 82, 72 and 64). No significant differences were detected between the exposures on the different days.

Pharmacodynamics

The median pretransplant IMPDH activity in PBMC was 44 µmol/s/mol AMP (range 6-178 µmol/s/mol AMP). The predose IMPDH activity at the first sampling day was significantly lower than the pre-transplant activity with a median value of 20 µmol/s/mol AMP (range 7-119 µmol/s/mol AMP) ($p<0.01$). As depicted in Figure 1 IMPDH activity decreased from the predose value of 20 µmol/s/mol AMP to 13 µmol/s/mol AMP at 1 hour after dose ingestion. With decreasing MPA concentrations IMPDH activity restored to pre-administration values.

The relationship between IMPDH activity and MPA plasma concentration was adequately modeled with an inhibitory E_{max} model (Equation 3, Table 3, Figure 2). The baseline IMPDH activity (E_0) increased from 55 µmol/s/mol AMP at day 6 to 76 µmol/s/mol AMP at day 21 and to 92 µmol/s/mol AMP at days 49 and 140 ($p<0.01$). The MPA concentration at half maximal inhibition of IMPDH activity (IC_{50}) was 0.76 mg/L at day 6 and 0.17 mg/L at the subsequent days ($p<0.01$). The maximum reduction of IMPDH activity was 88%.

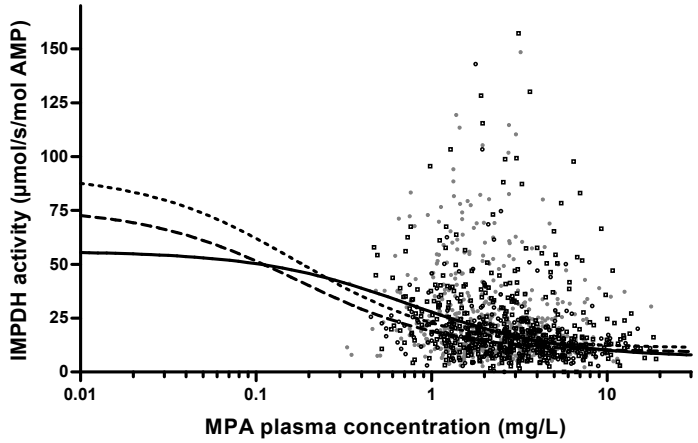

Figure 2. Inosine monophosphate dehydrogenase (IMPDH) activity versus mycophenolic acid (MPA) plasma concentration. The data points represent the observed IMPDH activity at bayesian predicted MPA concentrations for day 6 (closed circles), day 21 (open circles) and day 49 and 140 (open squares). The respective solid, dashed and dotted line represent the relationship on basis of the population parameters presented in Table 3 for day 6, day 21 and day 49/140. The baseline IMPDH activity (E_0) increases from 55 µmol/s/mol AMP at day 6 to 76 µmol/s/mol AMP at day 21 and to 92 µmol/s/mol AMP at days 49 and 140 ($p<0.01$), whereas the MPA concentration at half maximum inhibition (IC_{50}) decreases from 0.76 mg/L at day 6 to 0.17 mg/L at the subsequent days ($p<0.01$).

Table 3. Population pharmacodynamic parameter estimates

	Basic model Estimate (%CV)	Final model Estimate (%CV)	1000 bootstrap replicates median 95% interval
Typical parameter			
E_0 (µmol/s/mol AMP)	60 (13%)	-	-
E_0 visit 3 and 4 (µmol/s/mol AMP)	-	92 (21%)	89 (65-112)
$\Theta_{visit\ 1}$	-	0.61 (10%)	0.54 (0.42-0.85)
$\Theta_{visit\ 2}$	-	0.83 (9%)	0.84 (0.68-0.96)
Θ_{black}	-	0.88 (6%)	0.90 (0.77-0.98)
$\Theta_{homozygous}$	-	1.55 (13%)	1.43 (1.12-2.01)
$\Theta_{leukocytes}$	-	0.20 (35%)	0.18 (0.11-0.42)
IC_{50} (mg/L)	0.44 (26%)	-	-
IC_{50} visit 2, 3 and 4 (mg/L)	-	0.17 (32%)	0.18 (0.09-0.31)
$\Theta_{visit\ 1}$	-	4.4 (45%)	4.3 (1.4-7.9)
$\Theta_{albumin}$	-	1.14 (36%)	1.18 (0.36-1.98)
E_{max}	0.85 (3%)	0.88 (4%)	0.87 (0.80-0.99)
Between-patient variability			
E_0 (%)	8 (89%)	9 (91%)	10 (0 - 28)
IC_{50} (%)	102 (41%)	99 (52%)	95 (45 – 176)
Correlation			
E_0-IC_{50}	-1[1]	-1[1]	
Within-patient variability			
Baseline activity (%)	41 (45%)	39 (41%)	42 (22-85)
Residual error			
Additive error	0.48 (3%)	0.48 (3%)	0.48 (0.41-0.52)
MVOF	-72.5	-110.4	

[1] Correlation between E_0 and IC_{50} was high and fixed to -1.

AMP adenosine monophosphate; E_0 day 49 and 140, baseline inosine monophosphate dehydrogenase (IMPDH) activity at day 49 and 140; IC_{50} day 21, 49 and 140, MPA plasma concentration at half maximum inhibition at day 21, 49 and 140; Θ_{E0}-black, fractional decrease of E_0 in black patients compared to Caucasian, Asian and Hispanic patients; Θ_{E0}-day 6, fractional decrease of E_0 on day 6 compared to days 49 and 140; Θ_{E0}-homozygous, fractional increase of E_0 in patients homozygous for *IMPDH type II* 3757T>C compared to wild type and heterozygous patients; Θ_{E0}-leukocytes, exponent in relationship between E_0 and number of leukocytes ($E_0 = 92$ x (leukocytes/8.1 $10^9)^{0.20}$); Θ_{IC50}-albumin, exponent in relationship between IC_50 and albumin ($IC_{50} = 0.17$ x (albumin/34)$^{1.14}$); Θ_{IC50}-day 6, fractional increase of IC_{50} on day 6 compared to days 21, 49 and 140.

E_0 of black patients was 12% lower than for Caucasian, Asian and Hispanic patients ($p<0.05$; Figure 3). Patients homozygous for the *IMPDH type II* 3757T>C genotype exhibited an E_0 55% higher than heterozygous and wild type patients ($p<0.05$; Figure 3). No difference was detected between the latter two genotypes. A significant correlation was observed for E_0 and the number of leukocytes ($p<0.01$). Compared to the median leukocyte count of $8.1 \cdot 10^9$ cell, E_0 decreased with 34% when leukocytes dropped to $1 \cdot 10^9$ cells and E_0 increased with 20% with a leukocyte count increased to $20 \cdot 10^9$. IC_{50} correlated with albumin ($p<0.05$). An increase of albumin from 34 (median value) to 40 g/L

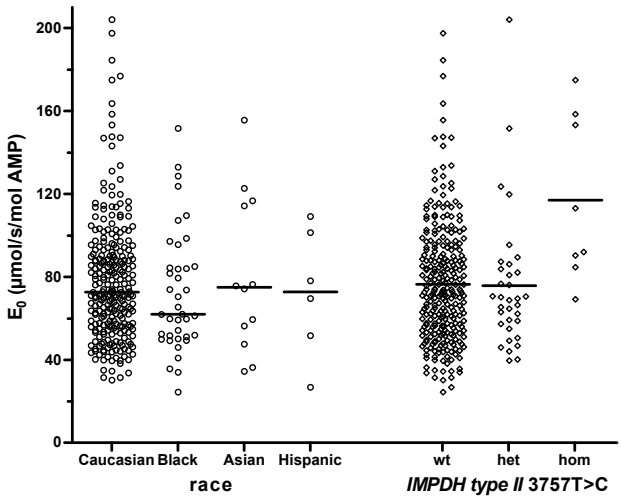

Figure 3. Bayesian predicted E_0 versus race and IMPDH type II 3757T>C genotype. E_0 of Black patients was 12% lower than for Caucasian, Asian and Hispanic patients ($p<0.05$). E_0 was 55% higher in homozygous (hom) patients compared to heterozygous (het) and wild type (wt) patients ($p<0.05$). Data (n=319) are presented for all patients and for all visits. The solid lines represent the median baseline inosine monophosphate dehydrogenase (IMPDH) activity (E_0).

produced a 20% higher IC_{50} value, whereas a reduction to 20 g/L produced a 45% lower IC_{50} value.

Between-patient variability in IC_{50} was high (99%) whereas between-patient variability in E_0 was low (8%). Within-patient variability in E_0 was however considerable (39%).

The diagnostic plots confirmed the adequacy of the population pharmacodynamic model (plots not shown). The population estimates of the final model were well within the 95% confidence interval as produced by the bootstrap procedure (Table 3).

The area under the 12 hour time curve of IMPDH activity (IMPDH activity $AUC_{0\text{-}12h}$) was calculated using the individual pharmacokinetic and pharmacodynamic parameters. Median (range) values were 210 (80 – 710), 187 (64 – 690), 195 (96-837) and 217 (76 – 908) h·µmol/s/mol AMP for day 6, 21, 49 and 140, respectively (n=101, 82, 72 and 64). IMPDH activity $AUC_{0\text{-}12h}$ on day 21 was significantly lower than on the other days ($p<0.01$).

Relationships with clinical outcome and adverse effects

An *a posteriori* analysis indicated that BPAR patients exhibited a significantly higher E_0 and IC_{50} at day 6 than non-BPAR patients; corresponding increases were 23% (p=0.025) and 75% (p=0.011). No differences were detected for the other days. At day 6 the IMPDH activity $AUC_{0\text{-}12h}$ of BPAR patients was higher than in non-BPAR patients with respective median (range) values of 241 (103-710, n=23) and 201 (80-618, n=69) h·µmol/s/mol AMP (Figure 4). This difference almost reached statistical significance (p=0.086). No relationships were found between clinical outcome and pharmacokinetic parameters

like CL/F and MPA $AUC_{0\text{-}12h}$. At day 6 MPA $AUC_{0\text{-}12h}$ of BPAR patients was comparable to the AUC of non-BPAR patients with respective median values of 29 (13- 59) h·mg/L and 29 (9- 70) h·mg/L (p=0.73) (Figure 4). Pre-transplant IMPDH activity was not associated with clinical outcome. Pre-transplant IMPDH activity in BPAR and non-BPAR patients was 38 (10-105) and 45 (6-178) µmol/s/mol AMP, respectively (p=0.54).

IMPDH activity $AUC_{0\text{-}12h}$ was significantly lower in case of leukopenia grade 1 to 4 compared to grade 0; corresponding median values were 163 and 212 h·µmol/s/mol AMP (n=18 and n=293, p<0.01). IMPDH activity $AUC_{0\text{-}12h}$ was significantly lower in case of anemia grade 2 to 4 compared to grade 0 and 1 with median values of 176 and 210 h·µmol/s/mol AMP, respectively (n=65 and n=250, p<0.05). No significant relationships were detected between the incidence rates of thrombocytopenia, abdominal cramps, diarrhoea, nausea and vomiting and IMPDH activity $AUC_{0\text{-}12h}$ or MPA $AUC_{0\text{-}12h}$.

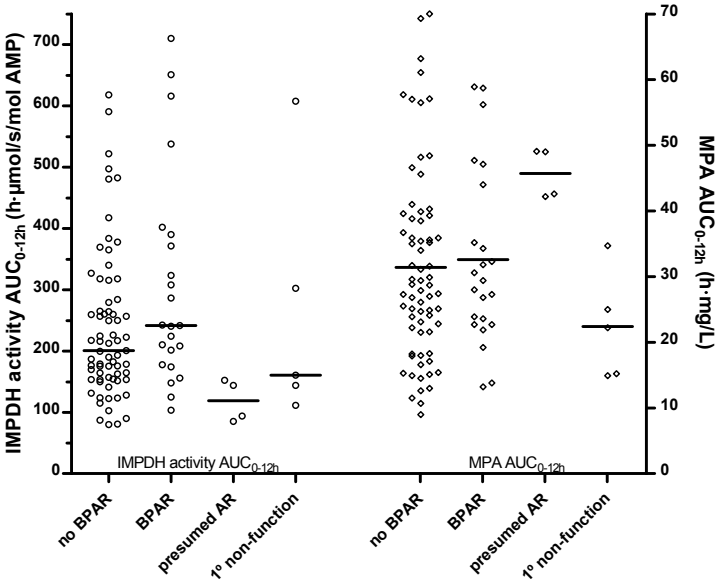

Figure 4. Area under the inosine monophosphate dehydrogenase (IMPDH) activity versus time profile curve during 12 hours (IMPDH activity $AUC_{0\text{-}12}$h) at day 6 and area under the mycophenolic acid (MPA) plasma concentration versus time profile during 12 hours (MPA $AUC_{0\text{-}12}$h) for patients with no acute rejection (no BPAR), a biopsy proven acute rejection (BPAR), a presumed AR and a primary non-functioning graft. Median IMPDH activity $AUC_{0\text{-}12}$h of no BPAR and BPAR patients was 201 and 241 h·µmol/s/mol AMP, respectively (p=0.086). Median MPA $AUC_{0\text{-}12}$h of no BPAR and BPAR patients was similar 29 h·mg/L (p=0.73). The solid line represents the median.

DISCUSSION

IMPDH activity may serve as a surrogate pharmacodynamic marker for immunosuppression and clinical outcome. The combination of pharmacokinetics and pharmacodynamics could be used to optimize the dosing regimen of MMF, as well as to monitor therapy and allow for dosage adjustments to maximize immunosuppression and to minimize side effects. In the present study the relationship between MPA plasma concentrations, inhibition of IMPDH activity and clinical outcome was quantified in 101 *de novo* renal transplant patients.

The pharmacokinetics of MPA were adequately described with a two-compartment model. The estimated apparent oral clearance in the first week (24 L/h, Table 2) corresponds well with the value of 29 L/h reported earlier by Staatz.[9] The time-dependency of the pharmacokinetics of MPA as observed in the present study has been studied earlier by Van Hest[10] who reported that following renal transplantation clearance was reduced from 34 L/h in the first week to 20 L/h in week 20 (41% reduction). In the referred study the decrease of clearance was explained by the improvement of both the renal function and albumin in time over time. The inverse correlation between MPA clearance and both creatinine clearance and albumin may be explained by their influence on protein binding of MPA. Impaired renal function may reduce protein binding of MPA by uremia as well as MPAG accumulation.[11] Consequently, impaired renal function and low plasma albumin may produce an increased free fraction of MPA, which in turn may lead to increased metabolism of MPA, since MPA is believed to have a low to intermediate extraction ratio.[12]

The relationship between the IMPDH activity in PBMC and MPA plasma concentration was successfully described with an inhibitory E_{max} model (Figures 2 and 3). Vethe[13] used a similar pharmacodynamic model to relate IMPDH activity in CD4+ cells to MPA plasma concentrations of healthy volunteers receiving single doses of MMF. In the referred study an E_{max} value of approximately 85% was observed, which corresponds well with the value estimated in the present study (88%, Table 3). In our renal transplant patients baseline IMPDH activity increased from 55 (day 6) to 92 µmol/s/mol AMP at day 49 and 140 (Table 3). The increase of baseline activity may reflect the induction of enzyme activity. Long-term MPA therapy has been associated with an induction of IMPDH activity which is suggested to be mediated through feedback regulation by guanine nucleotides.[14] Induction of enzyme activity has been described to occur in renal transplant patients taking MMF for a prolonged period of time.[15,16] Interestingly, a parallel increase was observed for both IMPDH type I and type II mRNA in the patients of the present study.[17]

The presence of considerable predose inhibition of IMPDH activity has also been reported by Vethe.[13] In healthy volunteers taking single doses of 500 and 1000 mg MMF IMPDH activity in CD4+ cells at 12 hours after ingestion was still reduced with 32% and

40%, respectively, compared to pre-administration levels.[13] In the referred study a value of 2.3 mg/L was reported for the IC_{50} which was higher than the value estimated in the present study (Table 3). In *in vitro* experiments the MPA concentration required for 50% inhibition of IMPDH activity was approximately 2.0-5.0 mg/L for both whole blood and isolated lymphocytes.[18] It is difficult to compare the reported IC_{50} values with our results since the inhibitory potency may be dependent on the studied cell type (whole blood cells, lymphocytes, monocytes), the applied IMPDH assay (single or multiple washing steps), single versus multiple MMF dosing (enzyme induction) and the applied immunosuppressive drug regimen.[16,18-20]

The covariate analysis demonstrated that patients homozygous for the *IMPDH type II* 3757T>C single-nucleotide polymorphism exhibited a baseline activity that was 55% higher than heterozygous and wild type patients. For the two homozygous patients IMPDH activity $AUC_{0\text{-}12h}$ was 522 and 710 h·µmol/s/mol AMP at visit 1 which was in the upper 10 percentile of the whole population [median 210 h·µmol/s/mol AMP (n=101)]. One of the two homozygous patients experienced an acute rejection. Increased rejection rates have been reported for patients with a variant allele.[21] The decreased baseline activity in black patients may be explained by a genetic polymorphism as well. However, so far no information is available in the literature with respect to ethnic differences in IMPDH activity.

It is difficult to explain the observed association between the leukocyte count and the baseline IMPDH activity, since both are influenced by multiple factors like the clinical status of the patient and the administered immunosuppressive drugs. The positive correlation between IC_{50} and albumin may be explained by a decreased MPA free fraction in case of high albumin. With less unbound MPA available for inhibiting the IMPDH enzyme, the whole IMPDH activity versus total MPA plasma concentration curve will be shifted to the right producing an increased IC_{50}. On the basis of the observed association between IC_{50} and albumin it may be speculated that the inhibition of IMPDH activity is primarily determined by unbound MPA concentration instead of total MPA concentrations. With an assumed free fraction of MPA of 3%, the observed IC_{50} of 0.17 mg/L corresponds to a unbound IC_{50} of 5.1 µg/L or 16 nM, which is in the same order of magnitude as the reported K_i for IMPDH type I and II with respective values of 37 nM and 9.5 nM.[3]

Since estimates of between- and within-patient variability of pharmacokinetic and pharmacodynamic parameters are available (Tables 2 and 3), one may speculate on the feasibility to adjust the individual MMF dose in order to obtain a specific targeted inhibition of IMPDH activity. Bayesian adaptive dosing may be used to reduce the influence of between-patient variability whereas within-patient variability cannot be reduced. The observed within-patient variability of 39% for E_0 in the present study indicates that a patient with a typical baseline IMPDH activity of 55 µmol/s/mol AMP may actually exhibit an E_0 value in the range from 12 to 98 µmol/s/mol AMP (95% CI = mean $\pm$ $t_{0.95}$*$\sigma/\sqrt{n}$ = 55

± 2·0.39·55) at a specific visit. The 8-fold within-patient variability in E_0 will presumably produce major variability in the observed IMPDH activity $AUC_{0\text{-}12h}$. As a result it is difficult at this point to predict whether it will be possible to target IMPDH activity $AUC_{0\text{-}12h}$ at a certain value.

By monitoring of MPA-induced IMPDH inhibition it is conceivable that a more accurate prediction of therapeutic response will be obtained than by monitoring of MPA plasma concentrations alone. In the present study at day 6 post-transplant no difference in pharmacokinetic exposure was detected between patients with or without BPAR; both median values were 29 h·mg/L (n=23 and n=69, respectively). It has been reported earlier that targeting $AUC_{0\text{-}12h}$ in the range of 30 – 60 h·mg/L reduces the risk of acute rejection.[6] In our study however, MPA exposure did not predict clinical outcome. A BPAR was observed in 12 of 51 patients (24%) with an $AUC_{0\text{-}12h}$ less than 30 h·mg/L, whereas in the 41 patients with an $AUC_{0\text{-}12h}$ greater than 30 h·mg/L 11 patients (27%) experienced a BPAR (p=0.54). Glander has reported that high pre-transplant activity and MMF dose reductions were associated with high rejection rates.[22] We can not confirm this in the present study as pre-transplant IMPDH activity was comparable in patients with and without BPAR. Interestingly, IMPDH activity $AUC_{0\text{-}12h}$ at day 6 was greater [although not statistical significant (p=0.086)] in patients that experienced a BPAR when compared to patients without a BPAR, with respective values of 241 (103-710, n=23) and 201 (80-618, n=69) h·µmol/s/mol AMP [median (range)]. Since IMPDH activity $AUC_{0\text{-}12h}$ ranges are overlapping (Figure 4) it is not possible to define a target value for IMPDH activity. Nevertheless, it can be concluded that IMPDH activity in PBMC may be a more sensitive parameter to predict transplantation outcome than MPA plasma concentration. Consequently, combined monitoring of MPA plasma concentration and IMPDH activity during MMF therapy may more accurately predict the therapeutic response than monitoring of MPA plasma concentrations alone. This needs however to be confirmed in a prospective study.

In summary, an integrated pharmacokinetic-pharmacodynamic model was developed which described the relationship between MMF dose, MPA plasma concentrations and IMPDH activity in PBMC in renal transplant patients. Our data suggest that the inhibition of IMPDH activity during the first week of MMF treatment is associated with the rate of rejection. As a result, combined monitoring of MPA plasma concentration and IMPDH activity may allow the optimization of MMF therapy.

PATIENTS AND METHODS

Patients

A prospective cohort of 101 *de novo* renal transplant recipients was followed for 12 months after transplantation. Before transplantation a blood sample was obtained for the determination of pre-transplant IMPDH activity and pharmacogenetic analysis. On days 6, 21, 49 and 140 days after transplantation blood samples were drawn for assessment of MPA concentrations and IMPDH activity. On day 6 samples were taken pre-dose, 0.5, 1, 2, 6, and 12 hours after oral intake of MMF. On the other days samples were taken at pre-dose, 0.5 and 2 hours after oral intake of MMF. The study was approved by the local ethics committee of Erasmus MC, Rotterdam, The Netherlands and complied with the Declaration of Helsinki. All patients gave written informed consent.

Immunosuppressive therapy

Immunosuppressive therapy was started directly after the transplant surgery. Patients started with MMF 1000 mg twice daily. MMF dose adjustments were dependent on the clinical status of the patient and at the physician's own discretion. Tacrolimus was dosed to obtain the following trough levels: 10-15 µg/L (first month), 8-12 µg/L (second month), 6-10 µg/L (months 3, 4 and 5) and 5 - 8µg/L (month 6 and further). The start dose of prednisone was 20 mg once daily after two days of 50 mg prednisone i.v. twice daily; the dose was tapered at the physician's own discretion. Patients that received a kidney from a deceased donor were considered for anti-thymocyte globulin (ATG) induction therapy.

Clinical outcome

The efficacy of the immunosuppressive therapy was evaluated by monitoring the occurrence of biopsy proven acute rejections (BPAR) within 12 months post-transplant using the Banff classification.[23] Kidney biopsies were taken on clinical grounds of suspicion of a rejection episode. No routine kidney biopsies were taken. Adverse events were graded according to the standard response and toxicity criteria.[24] Delayed graft function was defined as more than 3 days depending on dialysis starting in the first two weeks after transplantation.

Assays

IMPDH activity in peripheral blood mononuclear cells (PBMC) was assessed by using a non-radioactive HPLC method reported earlier.[25] The total and within-day imprecision of the IMPDH activity assay was less than 8.4% and 7.8%, respectively, in the IMPDH activity range of 32 to 125 µmol/s/mol AMP. MPA plasma concentrations were measured with a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method.[26] The total and within-day imprecision of the method was less than 5% and 3%, respectively, for plasma

MPA concentrations in the range from 0.1 to 50 mg/L. Genotyping of the *IMPDH type II* 3557T>C eight polymorphism was performed as described by Sombogaard.[26]

Population pharmacokinetic-pharmacodynamic modeling

All data were analyzed simultaneously using the nonlinear mixed effects modeling software program NONMEM (double precision, Version VI, level 2.0, GloboMax LLC, Hanover, USA). The concentration data were logarithmically transformed. Parameters were estimated using the first order (FO) estimation method, since the first order conditional estimate method (FOCE) did converge successfully. The pharmacokinetics of MPA were described with a two-compartmental model with first-order absorption (K_a) with a lag time (T_{lag}). The following parameters were estimated: apparent oral clearance (CL/F), intercompartmental clearance (Q/F) and central and peripheral volumes of distribution (V_1/F and V_2/F). Between- and within-patient variability was characterized using exponential models. For example, CL/F for the i^{th} individual on the j^{th} visit (CL/F_{ij}) was estimated using equation 1:

$$CL/F_{ij}\ (L/h) = \theta_{pop} * \exp(\eta_i + \kappa_j) \qquad \text{(Eq. 1)}$$

where θ_{pop} represents the population value for CL/F. η_i and κ_j represent the between- and within-patient random effect, respectively, with mean 0 and variance ω^2 and π^2. In case of two or more random effects covariance was estimated as well. Within-patient variability corresponded to the variability of a pharmacokinetic parameter within a patient between the sampling days.

The difference between the k^{th} observed concentration of the i^{th} individual ($C_{obs,ik}$) and the corresponding model predicted value ($C_{pred,ik}$) was modeled using an additive error model:

$$\ln C_{obs,ik} = \ln C_{pred,ik} + \varepsilon_{ik} \qquad \text{(Eq. 2)}$$

where ε is the residual random error with mean 0 and variance σ^2.

In population pharmacodynamic analysis effect IMPDH activity data were logarithmically transformed. The FOCE method was used in combination with NONMEM´s INTERACTION option to allow for interaction between random effects. In analysis individual pharmacokinetic parameters were fixed at posthoc values. The relationship between MPA concentration (C) and IMPDH activity in PBMC (E) was described with an inhibitory E_{max} model:

$$E = E_0 \bullet \left(1 - \frac{E_{max} \bullet C}{IC_{50} + C}\right) \qquad \text{(Eq. 3)}$$

where E_0 is the baseline IMPDH activity [i.e. with no MPA present (C=0)], E_{max} is the maximal fractional inhibitory effect and IC_{50} is the MPA concentration at half E_{max}. Between- and within-subject variability was modeled using exponential models (equation 1) and residual variability was modeled using an additive error model (equation 2).

Relationships between both demographic and pathophysiological factors and pharmacodynamic parameters were evaluated. The tested covariates included age, gender, ethnicity, *IMPDH type II* 3557T>C genotype, weight, pretransplant diabetic status, plasma albumin concentration, plasma protein concentration, liver enzymes aspartate transaminase (AST) and alanine transaminase (ALT), bilirubin, alkaline phosphatase, gamma-glutamyltransferase (GGT), urea, creatinine clearance (CrCl), calculated using the 4-variable MDRD formula, C-reactive protein (CRP), hemoglobin, hematocrite, thrombocytes, leukocytes, pretransplant IMPDH activity, delayed graft function, tacrolimus daily dose, tacrolimus concentration, corticosteroid dose and anti-thymocyte globulin (ATG) induction therapy (Table 1).

Continuous covariates, for instance leukocytes, were modeled in an exponential manner:

$$E_{0,ij} = \theta_{pop} * (\text{leukocytes}/8.1\ 10^9)^{\theta leuko} \qquad \text{(Eq. 4)}$$

in which θ_{pop} is the baseline IMPDH activity in an individual with $8.1\ 10^9$ leukocytes (median of the population) and θ_{leuko} is an exponent determining the shape of the relationship. Categorical variables, like for example *IMPDH type II* 3557T>C genotype, were modeled as shown in equation 6:

$$E_{0,ij} = \theta_{pop} * \theta_1^{\theta heterzygote} * \theta_2^{\theta homozygote} \qquad \text{(Eq. 5)}$$

where θ_{pop} is the population value for E_0 in wildtype patients ($\theta_{heterozygote} = 0$ and $\theta_{homozygote} = 0$), θ_1 is the fractional change of E_0 in heterozygous patients ($\theta_{heterozygote} = 1$ and $\theta_{homozygote} = 0$) and θ_2 is the fractional change of E_0 in homozygous patient ($\theta_{heterozygote} = 0$ and $\theta_{homozygote} = 1$).

The log-likelihood test was used to assess the statistical signifcancy of the relationship between the covariate and the pharmacodynamic parameters.[27] In an univariate analysis a decrease of the minimum value of objective function (MVOF) of >3.8 units indicated $p<0.05$ (1 degree of freedom). Furthermore, a reduction in between- or within-patient variability was used as a criterion for covariate selection, as well as the biological plausibility of a relationship. All covariates selected during the univariate analysis were included in an intermediate model. Subsequently, covariates were excluded separately in a backward elimination procedure. An increase of MVOF (>3.8 units) was necessary to remain the parameter in the population model.

The stability and the performance of the final model were checked with an internal validation of the final model, using the bootstrap resample technique.[28]

The area under the concentration versus time curve (MPA $AUC_{0\text{-}12h}$) and IMPDH activity versus time curve (IMPDH activity $AUC_{0\text{-}12h}$) were calculated over a period of 12 hours on basis of individual Bayesian parameter estimates.

Statistical analysis

Statistical tests were performed using SPSS software version 15.0 (SPSS, Chicago IL, USA) and a p-value less than 0.05 was considered significant. Normally distributed data were analyzed by a t-test or ANOVA, and non-normally distributed data by a Mann-Whitney U test or Kruskal-Wallis test.

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Chapter 3.2

Inhibition of IMPDH activity in MMF treated renal transplant patients is better correlated to unbound MPA concentrations

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Submitted

ABSTRACT

The active moiety of the immunosuppressivum mycophenolate mofetil – mycophenolic acid (MPA) – is extensively bound to plasma albumin. It is thought that the unbound concentration of mycophenolic acid is responsible for the inhibition of inosine monophosphate dehydrogenase (IMPDH). From 99 kidney transplant patients blood samples were drawn 6 days after transplantation. IMPDH activity was correlated with total and unbound MPA concentrations and albumin in causing the biological effect of MPA.

Significant poor correlations were found between total and unbound MPA concentration pre-dose and IMPDH activity pre-dose using an E_{max} model. The calculated E_0 and IC_{50} parameters showed a large confidence interval. The therapeutic window of total MPA AUC (30 to 60 h•mg/L) was not significant correlated to IMPDHact AUC, but unbound MPA AUC (2.1 to 4.2 h•mg/L; 7.0% unbound fraction) was. IMPDH activity was significantly lower in patients with low serum albumin compared to patients with higher levels. Nevertheless, no significantly differences were found between patients with low and high albumin for unbound MPA AUC, but a significantly difference was found in total MPA AUC.

We conclude that patients with decreased albumin levels have lower total MPA AUC and decreased IMPDH activity despite having similar unbound MPA concentrations.

INTRODUCTION

Mycophenolic acid (MPA) has become a frequently used drug to prevent acute rejection in de novo kidney transplant recipients. Although this success has been largely accomplished with standard dose therapy, there is now increasing evidence that other strategies than the standard fixed dosing recommendation may further improve the efficacy of MPA therapy.[1]

Based on the large between-patient variability in pharmacokinetics and on the repeatedly shown relationship between MPA plasma concentrations early after transplantation and incidence of acute rejection, therapeutic drug monitoring (TDM) strategies have been developed.[2] The APOMYGRE-study showed that by using a limited sampling strategy to adjust the dose of mycophenolate mofetil the risk of treatment failure could be significantly reduced, without an increase in adverse events.[3]

The pharmacokinetics of MPA are characterized by an extensive binding to plasma albumin with a non-protein bound fraction reported to be less than 3%.[4] It is the unbound fraction that is thought to be responsible for immunosuppressive activity.[5] One would expect that observational studies would show a better correlation between efficacy and non-protein bound MPA plasma concentrations compared to total MPA concentrations.[6]

MPA is believed to undergo restrictive clearance. Only the unbound MPA fraction is presumed to be available for elimination from the body through metabolism to the 7-O-MPA-glucuronide metabolite (MPAG) in liver, kidney, and intestinal mucosa.[7,8] Especially during renal insufficiency the unbound MPA fraction would increase, due to the uremic state and to displacement of MPA from its albumin binding sites by high concentrations of MPAG that is cleared by the kidneys.[9,10]

In patients with poor renal function, due to increased MPA clearance, total MPA concentrations are low, but at the same time the non-protein bound fraction is high (fractions up to 18.3% are reported[11]) and free MPA concentrations may be unaltered or even elevated.[12,13] If one would believe in the concept that free MPA is the biologically active moiety, then dose increases would not be indicated and the low total MPA levels should be ignored.[14]

In order to investigate the relevance of non-protein bound MPA plasma concentrations in causing the biological effect of MPA we decided to monitor MPA therapy by measurement of the activity of the target enzyme inosine monophosphate dehydrogenase (IMPDH).[15] We correlated total as well as unbound MPA concentrations to IMPDH activity and serum albumin levels in order to evaluate the relevance of the unbound MPA concentration in generating the biological effect of MMF.

METHODS AND PATIENTS

Patients

A prospective cohort of *de novo* kidney transplant patients (n=101) was followed for 12 months after transplantation. At 6 days after transplantation blood samples were drawn for pharmacokinetics (PK) and pharmadynamics (PD). A full area under the curve (AUC, 0-12 hr) was obtained with samples taken at pre-dose, 0.5, 1, 2, 6, and 12 hours after oral intake of MMF. Patients with a *de novo* adult kidney transplant with an uncomplicated post-operative recovery on the first day following transplantation and who were being treated with MMF were asked to participate in this study. Patients with seriously compromised peripheral venous vasculature (for example due to repetitive dialysis access surgery), complicating venous blood sampling, were not approached. The study was approved by the local ethics committee of Erasmus MC, Rotterdam, The Netherlands and complied with the Declaration of Helsinki. All patients gave written informed consent.

Immunosuppressive therapy

All patients received triple immunosuppressive therapy, consisting of tacrolimus (FK, Prograf®, Astellas Pharma, Leiderdorp, The Netherlands), mycophenolate mofetil (CellCept®, Roche, Welwyn Garden City, United Kingdom) and prednisone according to the local protocol. Medication was started directly after the transplant surgery. Patients were treated with 1000 mg MMF twice a day. Dose adjustments could take place depending on the clinical status of the patient. Tacrolimus was dosed to achieve target trough levels, while the target levels decreased over time after transplantation. Prednisone was given once daily, started with 20 mg and was discontinued after 4 months. Patients who had received a kidney from a deceased donor could be considered for anti-thymocyte globulin (ATG) induction therapy. In total, 29 of all patients (29%) received ATG induction therapy.

Measurement of MPA plasma concentrations and IMPDH activity

To sample of 50µL plasma, 200 µL zinksulfate 0.1 mol/L and 500 µL acetonitril with 3 mg/mL MPAC was added for determine the total plasma concentrations of MPA. For the determination of the unbound plasma concentration of MPA, plasma was first ultrafiltrated using 30 KDa centrigugal filter devices (Microcon Ultracel YM-30, Millipore, Amsterdam, The Netherlands). To 50 µL ultrafiltrate 50 µL acetonitril with 60 mg/mL MPAC was added. The UPLC-MS/MS system consisted of a Waters Acquity UPLC instrument coupled to a Quattro Premier XE tandem-quadrupole mass spectrometer (Waters Corp., Milford, MA). The analytical column was an Acquity UPLC BEH C18 2.1-mm by 50-mm column with a 1.7-µm particle size (Waters Ltd., Dublin, Ireland). The mobile phase was a gradient of solution A (2.0 mmol/L ammonium acetate and 0.1% formic acid in water) and solution

B (2.0 mmol/L ammonium acetate and 0.1% formic acid in methanol), with an initial composition of 25% solution B. The mobile phase composition changed linearly from initial 25% B to 80% B at 2.0 min and onward to 100% B at 3.0 min. The composition was switched back to 25% B at 4.0 min and maintained until 5.5 min. The flow rate was 0.5 ml/min, with a column temperature of 50°C. From each sample, 5 µL was injected onto the column. Analytes were detected via MS with an electrospray ionization interface in positive multiple reaction monitoring mode. Optimized multiple reaction monitoring settings were for MPA: parent-daughter ion mass 338.10>206.90 m/z; cone voltage, 15.0 V; collision energy, 22.0 eV; and for MPAC: parent-daughter ion mass 438.10>206.90 m/z; cone voltage, 10.0 V; collision energy, 30.0 eV. The acquisition settings were as follows: capillary voltage, 3.0 kV; source temperature, 130°C; desolvation temperature, 300°C; desolvation gas flow, 600 L/h; cone gas flow, 1 L/h; and dwell time, 50 ms. The coefficient of variation of the inter- and intraday precision of the used method was less than 5% and 3% respectively for the total plasma MPA (range 0.1 – 50 mg/L). For the unbound plasma concentration these values were less than 13% and 7% (range 0.001 – 1.0 mg/L).

The procedure for determination of the IMPDH activity in PBMC has been described in detail elsewhere.[16] Enzyme activity was expressed as produced xanthosine monophosphate (XMP) (µmol) per time unit (s) per present adenosine monophosphate (AMP) (mol) for normalizing the activity to the destructed cells. AMP and XMP concentrations were validated in the range from 0.75 to 50.0 µmol/L. The precision of the IMPDH activity assay was 6.6 to 11.9% and the coefficient of variation ranged from 0.6 to 3.4%.

Data analyses

A linear trapezoidal model was used to calculate the area under the concentration – time curve of MPA (MPA AUC) and the effect – time curve of IMPDH activity ($IMPDH_{act}$ AUC). Data was only used from patients that had complete AUC curves, containing at least 6 samples.

The relation between IMPDH activity and MPA plasma concentrations was evaluated using an inhibitory E_{max}-model according to the following equation:

$$E = E_0 \bullet \left(1 - \frac{E}{IC_{50} + C}\right)$$

where E_0 is the baseline IMPDH activity with no MPA present, C is the concentration of MPA and IC_{50} is the concentration of MPA where 50% of the maximal inhibition of IMPDH activity is measured. E_{max} presents the maximal decrease in IMPDH activity and was set equal to E_0.

Statistical analysis

All statistical tests were performed using SPSS software version 16.0 (SPSS, Chicago, IL) and a P-value < 0.05 was considered significant. Correlations coefficients between normally distributed data were analyzed with by Pearson's correlation, non-normally distributed data by Spearman's rang. All normally distributed data were analyzed by an independed-samples *t* test and non-normally distributed data by a Mann-Whitney U test. A one-way ANOVA test was used to analyze data with more than two variables. Values are reported as mean ± standard deviation, unless otherwise stated.

RESULTS

Baseline characteristics

From April 2006 to September 2007, a total of 101 patients were recruited for the study. On day 6.1 ± 1.7 post transplantation blood samples were drawn. From 99/101 patients blood samples were collected for the first two hours and from 82/101 patients a full twelve hours AUC consisting of six blood samples was collected. Patient baseline characteristics are summarized in Table 1 for the 99 patients of whom full data in the first two hours were available. The laboratory results and the daily dose of tacrolimus and MMF on the day of sampling are shown in Table 2.

Table 1. Patient characteristics of included patients

Characteristics	**n=99**
Age (years)	51.2 ± 14.4
Weight (kg)	80.9 ± 18.2
Gender Male	72 (73%)
Ethnicity	
Caucasian	82 (83%)
Black	13 (13%)
Asian	2 (2%)
Hispanic	2 (2%)
Donor Living / Deceased	62 / 37
Delayed graft function	30 (30%)
ATG induction therapy	29 (29%)
Patients with previous Tx	11 (11%)
Diabetes Melitus present pre-Tx	14 (14%)
HLA – mismatch 0 / 1-3 / 4-6	6 / 50 / 43
PRA 0 / 1-10 / 11-50 / 50-100 / N.A. (%)	64 / 12 / 4 / 5 / 14
BPAR / presumed AR / primary non-function	23 / 4 / 5

ATG, anti-thymocyte globulin; (BP)AR, (biopsy proven) acute rejection; HLA, human leukocyte antigen; N.A., not available; PRA, panel-reactive antibodies; Tx, transplantation.

Table 2. Laboratory results and immunosuppressive treatment on day of sampling

Clinical laboratory	**n=99**
Albumin (g/L)	34.0 ± 4.0
Urea (mmol/L)	19.5 ± 10.8
Creatinine (µmol/L) median (range)	338 ± 281 213 (61 – 1190)
Creatinine clearance[1] (mL/min) median (range)	33.2 ± 25.2 28.0 (4.1 – 122.0)
Hemoglobine (mmol/L)	6.3 ± 1.0
Trombocytes (·10^9/L)	195.4 ± 74.1
Leukocytes (·10^9/L)	9.0 ± 4.6
Tacrolimus daily dose (mg)	10.8 ± 3.7
Tacrolimus trough level (µg/L)	12.5 ± 6.7
MMF daily dose (mg)	1970 ± 224

[1] Estimated using 4-variable MDRD equation.

IMPDH activity and MPA levels

Figure 1 shows the IMPDH activity time profiles of total MPA (A) and unbound MPA (B) on day 6. IMPDH activity decreased after MMF intake and reached its nadir after 2 hours. After this a full recovery of the activity to pre-dose levels was established. Both MPA curves reach a maximal concentration 1 hour post-dose.

MPA plasma concentrations and pharmacodynamic parameters of the patients are summarized in Table 3.

Table 3. Pharmacokinetic en pharmacodynamic parameters of MPA on day 6 after transplantation

Parameter	t=0h (n=99)	t=2h (n=99)
Total MPA (mg/L)	1.86 (0.233-10.6)	4.02 (0.769-16.8)
Unbound MPA (mg/L)	0.039 (0.002-0.846)	0.098 (0.003-1.32)
IMPDH activity (µmuol/s/mol AMP)	19.7 (7.2-119.3)	13.0 (2.2 – 57.5)
	0-12h (n=82)	
Total MPA AUC (h·mg/L)	34.0 (8.93-127)	
Unbound MPA AUC (h·mg/L)	1.88 (0.038-9.09)	
Free fraction MPA (%)	7.0 (0.25-24)	
$IMPDH_{act}$ AUC (h·µmol/s/mol AMP)	226 (58-868)	

Values are presented as median (range).

A significant correlation was found between total and unbound pre-dose MPA concentrations and IMPDH activity pre-dose (Figures 2A and 2B respectively). Though the correlations are significant, the correlation coefficients are poor ($r = 0.33$; $P < 0.001$ and $r = 0.20$; $P < 0.001$ for total and unbound MPA respectively). The calculated E_0 was 47.2 µmol/s/mol AMP (95% CI: 29.2 – 65.3 µmol/s/mol AMP) using the total MPA concentra-

tion and 32.3 µmol/s/mol AMP (95% CI: 25.7 – 38.8 µmol/s/mol AMP) using the unbound MPA concentration. The IC_{50} was calculated at 2.6 mg/L (95% CI: 0 – 5.4 mg/L) and 0.7 mg/L (95% CI: 0 – 1.7 mg/L) for total and unbound MPA concentrations respectively.

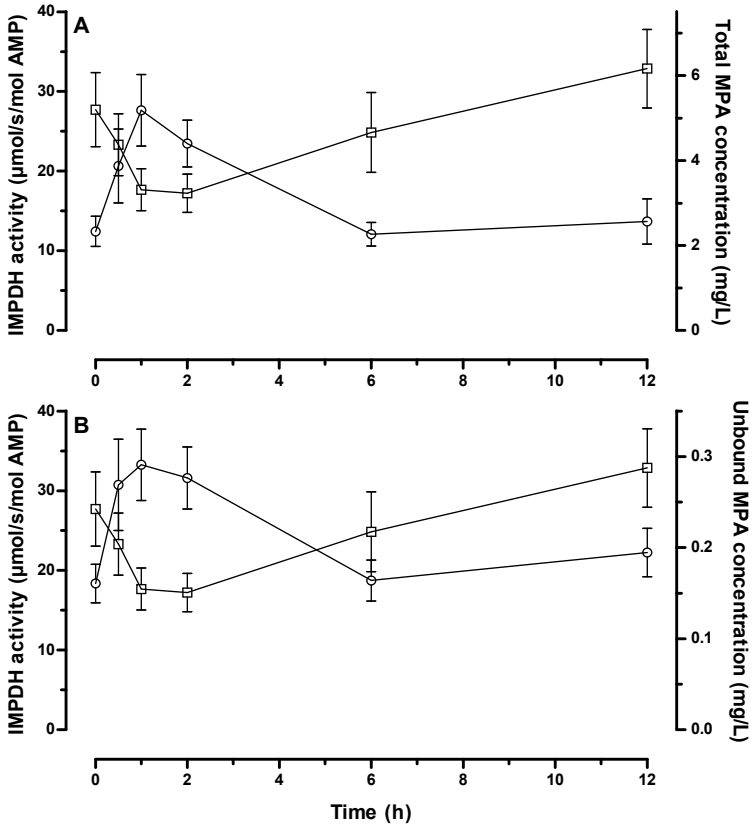

Figure 1. IMPDH activity (square) on day 6 as a function of time after oral MMF intake (A and B). The total MPA plasma concentration (circle) is shown in plot A and the unbound MPA plasma concentration (triangle) is shown in plot B. Values are means; the error bars reflect the standard deviations of the means.

Figures 2C and 2D show the correlation between the AUC_{0-12} of total and unbound MPA and IMPDH activity pre-dose. Both correlations are significant, but MPA AUC is not better correlated compared to MPA trough levels ($r = 0.20$; $P < 0.001$ and $r = 0.22$, $P < 0.001$ respectively). The calculated E_0 was 41.6 µmol/s/mol AMP (95% CI: 18.0 – 65.2 µmol/s/mol AMP) for the total MPA concentration and 36.7 µmol/s/mol AMP (95% CI: 27.0 – 46.5 µmol/s/mol AMP) for the unbound MPA concentration. The IC_{50} was calculated at 75 h·mg/L (95% CI: 0 – 215 mg/L) and 7 h·mg/L (95% CI: 0 – 15 mg/L) for total and unbound MPA concentrations respectively. No significant correlations were found between total or unbound MPA AUC and IMPDH activity AUC (data not shown).

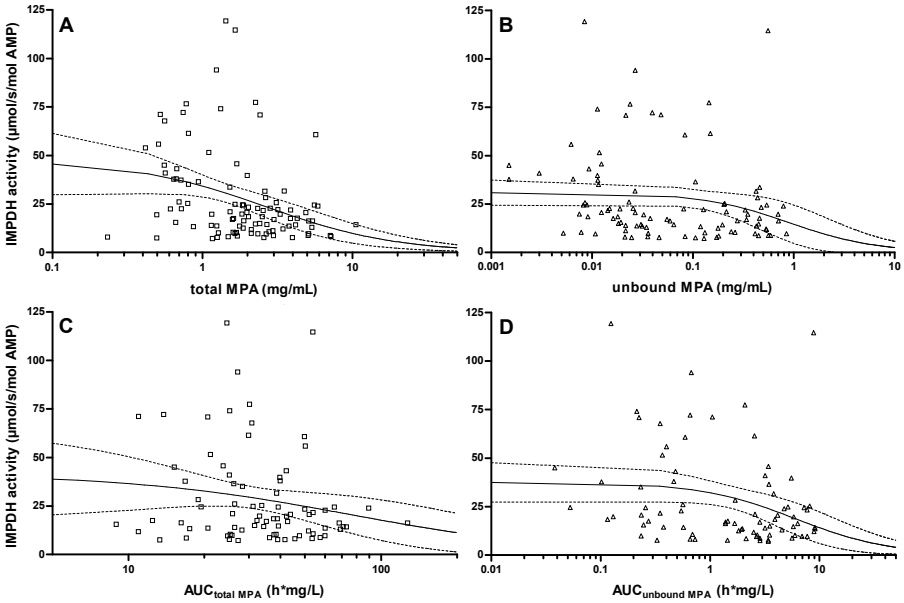

Figure 2. A and B show the IMPDH activity pre-dose correlated to the corresponding total and unbound MPA concentration respectively. While both correlations are significant, the correlation coefficients were poor ($r = 0.33$; $P < 0.001$ and $r = 0.20$; $P < 0.001$ for total and unbound MPA concentrations respectively). The IMPDH activity pre-dose versus the AUC of total and unbound MPA over 12 hours are shown in plot C and D ($r = 0.20$; $P < 0.001$ and $r = 0.23$; $P < 0.001$ for total and unbound MPA AUC respectively). The correlation coefficient for total MPA AUC was lower compared to the MPA trough level, and a slightly higher correlation coefficient was found for the unbound MPA.

The presumed therapeutic window for the exposure to total MPA (total MPA AUC) is set at 30 to 60 h·mg/L.[17,18]. Figure 3 shows the exposure to IMPDH activity ($IMPDH_{act}$ AUC) for patients with a total MPA AUC below 30, between 30 and 60 and above 60 h·mg/L. In this cohort, on day 6 post-transplant 31 patients (40%) had a total MPA AUC below the lower threshold of the therapeutic window and 9 patients (12%) had a total MPA AUC above 60 h·mg/L. There was no significant difference in the $IMPDH_{act}$ AUC between the three groups ($P = 0.76$). For unbound MPA we have also divided the patients into three groups and correlated the unbound MPA AUC to $IMPDH_{act}$ AUC. The lower limit for unbound MPA AUC was set at 2.1 h·mg/L and the upper limit at 4.2 h·mg/L. These limits of unbound MPA AUC were calculated by multiplying the limits of the total MPA AUC with the median unbound fraction in this cohort (7.0%). For unbound MPA AUC, 41 patients (53%) were below the lower threshold and 17 patients (22%) above the upper threshold. In contrast to the data shown for total MPA AUC there was a significant difference in the $IMPDH_{act}$ AUC for the three unbound MPA AUC groups ($P = 0.038$). The difference in $IMPDH_{act}$ AUC between patient below and patients above the therapeutic window defined for the unbound MPA AUC was also significantly ($P = 0.025$).

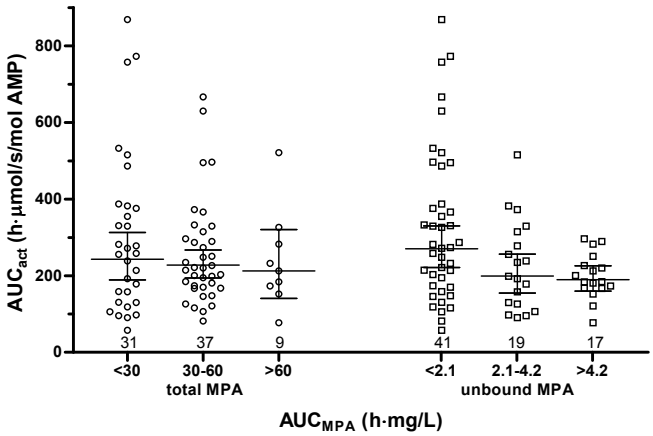

Figure 3. The distribution of the exposure to IMPDH activity ($IMPDH_{act}$ AUC) for patients below, within or above the therapeutic window of total (circles) or unbound (squares) MPA AUC0-12. There was no significant difference between the total MPA AUC groups for $IMPDH_{act}$ AUC (P = 0.76), but a significant difference in $IMPDH_{act}$ AUC was found for the unbound MPA AUC (P = 0.038). The difference in $IMPDH_{act}$ AUC between patients below and above the therapeutic window for unbound MPA was also significant (P = 0.025). The figures below the plots represent the number of patients. The lines are geometric means; the error bars reflect the 95% confidence interval of the geometric mean.

Influence of serum albumin on unbound MPA concentration and on IMPDH activity

Because MPA is highly bound to serum albumin, patients with low serum albumin levels may have higher unbound MPA concentrations and more inhibition of IMPDH activity[5] Figure 4 shows that patients with albumin levels below 32 g/L have lower total MPA concentrations (P = 0.011), but unbound MPA concentrations are not different (P = 0.34). Figure 5 shows the pre-dose IMPDH activity for patients with a serum albumin concentration below 32 g/L or a serum albumin equal and above 32 g/L. IMPDH activity is significantly lower in patients with low serum albumin compared to patients with high serum albumin (20.4 ± 16.3 vs. 30.6 ± 25.0 µmol/s/mol AMP; P = 0.030).

DISCUSSION

For more than a decade MMF is used to prevent acute rejection after solid organ transplantation. Despite extensive clinical experience with this drug there is uncertainty about the importance of the non-protein bound MPA concentration. Theoretically, and in line with other drugs, the unbound fraction of MPA would be responsible for the immunosuppressive activity.

The inhibitory effect of unbound MPA on IMPDH activity was investigated *in vitro*,[19] but there have been no reports on the relevance of the unbound MPA concentration on IMPDH inhibition *in vivo* in renal transplant patients using MMF. We here present the first prospective pharmacodynamic study in 99 renal transplant recipients correlating the *in vivo* inhibition of IMPDH to both total and unbound MPA concentrations.

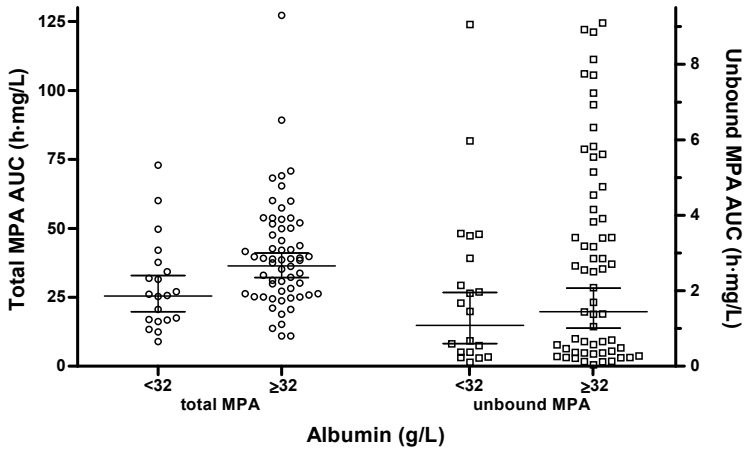

Figure 4. Patients with a serum albumin below 32 g/L had significantly lower total MPA AUC (circles) compared to patients with a serum albumin level equal and above 32 g/L (P = 0.011). A difference between patients with albumin levels below or equal and above 32 g/L was not found for unbound MPA AUC (squares; P = 0.34). The lines are geometric means; the error bars reflect the 95% confidence interval of the geometric mean.

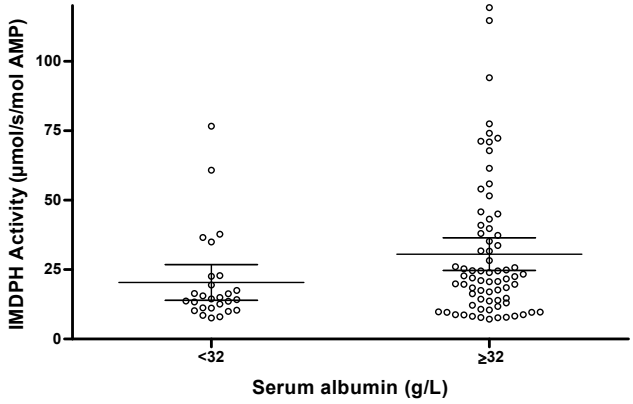

Figure 5. Patients with a low serum albumin (<32 g/L) have a significantly lower IMPDH activity compared to patients with a serum albumin level of 32 g/L or higher (P = 0.030).

We confirm the inverse relationship between IMPDH activity and MPA plasma levels, with maximal inhibition of the IMPDH activity almost coinciding with the MPA peak levels.[20] The changes in IMPDH activity follow the pharmacodynamics of total and unbound MPA concentrations closely, and there is a striking recovery of IMPDH activity to pre-dose levels over the 12 hour dosing interval.

To investigate the pharmacodynamic – pharmacokinetic relationship of IMPDH, we used an inhibitory E_{max}-model. Previously Glander *et al* have shown a 10-fold inter-patient variability in IMPDH activity and a relatively small intra-individual variability

of 14% over a one month interval.[19,21] In this study the large inter-patient variability is confirmed; patients with equal MPA AUC showed different IMPDH activities, as displayed in figure 2. For example, a total MPA concentration of 1.48 mg/L resulted in one patient in an IMPDH activity of 8.15 µmol/s/mol AMP, but another patient who had a total MPA concentration of 1.43 mg/L showed an IMPDH activity of 119.3 µmol/s/mol AMP, almost 15-fold higher IMPDH activity with comparable MPA concentrations. Although there were significant correlations between the IMPDH activity and the total and unbound MPA concentration and exposure, the correlations were weak (ranged from 0.20 to 0.33). Langman *et al* showed in *in vitro* tests an IC_{50} for total MPA of 2.0-5.0 mg/L and 0.5-1.0 mg/L for unbound MPA.[22] The IC_{50} calculated in this study were 2.64 mg/L for total MPA concentration and 0.73 mg/L for the unbound MPA concentration, but with large confidence intervals. Nevertheless, the calculated IC_{50} values corresponded to earlier finding. Values of E_0 could not be compared to previously reported values, as there are no known publications reporting E_0 values using the same IMPDH activity assay. Using an E_{max} model, the calculated 95% confidence interval values of E_0 were largely ranged between 18.0 and 65.3 µmol/s/mol AMP.

Considering the wide range in the 95% confidence intervals of the estimated pharmacodynamic values, it is reasonable these analyses were unable to describe the pooled data of the whole cohort in a correct way. Given the large inter-patient variability, a separate estimation for the individual pharmadynamic parameters could improve the estimation of these population parameters. A possible reason could be the large inter-patient variability in the concentration – effect relation. Introducing more patient characteristics in the pharmacokinetic-pharmacodynamic model could improve the explanation of the variability in IMPDH activity under MMF therapy. Some factors which partly explaining the inter-patient variability in pharmacokinetics of MMF could also be used to explain the inter-patient variability in IMPDH activity. It is likely different races difference in IMPDH gene polymorphisms and expression of the IMPDH enzyme. It also could be that co-morbidity influences the IMPDH activity in the same way. Time after transplantation could have any influence on the sensitivity or recovery of the inhibition of MPA on IMPDH, and perhaps co-medication has an inhibitory or inductive influence on the activity of IMPDH. It is not entirely unreasonable to expect that these factors affect IMPDH enzymes and IMPDH activity. Presumable, also other factors affect the individual pharmacodynamic of IMPDH. This could be pre-transplant IMPDH activity; hence the higher IMPDH has to be inhibited after transplantation. Glander *et al* have also shown a correlation between pre-transplant IMPDH activity and clinical outcome after transplantation.[21] Genetic polymorphisms in the IMPDH genes could directly or indirectly influence the stability or functionality of the IMPDH enzymes. Alterations in leukocytes differentiation could give a different distribution of the cells and thereby the number and type of IMPDH enzymes. The mentioned factors are only suggestions; it is

also possible that other factors and patient characteristics have any influence on the IMPDH activity.

Based on a number of observational studies the presumed therapeutic window for total MPA AUC was set at 30 to 60 h·mg/L.[17,18] The upper and lower limit of the therapeutic window for unbound MPA AUC are unknown. To compare the effects of total and unbound MPA exposure on IMPDH activity, we defined an arbitrary window for unbound MPA AUC by multiplying the window of 30 to 60 h·mg/L of total MPA with the median of the unbound fraction of MPA (7%). As shown in Figure 3, a significant difference in IMPDH activity for the three groups was found for unbound MPA, but not for total MPA. We also found that patients with a low serum albumin level had a significantly lower IMPDH activity compared to patients with a normal serum albumin. In this study we used the cut-off value for albumin levels of 32 g/L that was previously reported to be clinically relevant.[12] Surprisingly however, despite the lower IMPDH activity in patients with a low serum albumin we did not find high unbound MPA concentrations in these patients. It remains unclear how the stronger inhibition of IMPDH activity in patients with low albumin levels is explained. Possibly patients with low albumin levels are in a poor general clinical condition and perhaps this poor condition induces a state of reduced immunoreactivity that is reflected in reduced IMPDH activity. It would be interesting to study the IMPDH activity in transplant patients not treated with MPA and see if IMPDH activity is a reflection of immune status. The lower total MPA AUC values in patients with a low albumin are not surprising. Our group has previously described that low plasma albumin levels are associated with decreased total MPA AUC.[23] A population pharmacokinetic study found that decreased albumin levels have a minimal to no effect on the unbound MPA AUC, while a significantly increased unbound fraction was seen.[24] The concept is that in patients with low albumin levels the unbound MPA will immediately be cleared by metabolism in the lever or may be transferred into intra-cellular compartments. Due to the increased clearance the bound MPA concentrations decrease and the unbound MPA concentrations do not alter which results in decrease total MPA.

We conclude that compared to patients with a normal albumin level, patients with decreased albumin levels have lower total MPA AUC and decreased IMPDH activity despite having similar unbound MPA concentrations. For clinical practice we would recommend that patients with low albumin levels in whom low total MPA AUC values are found, no MMF dose increments are performed, as in those patients IMPDH activity is already at a low level. Measurement of unbound MPA concentrations is not likely to further contribute to patient management, as we show that in patients with low albumin levels the unbound concentrations are not significantly increased.

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Chapter 4

Pharmacogenetics and mRNA expression

Chapter 4.1

Inter-patient variability in IMPDH activity in MMF treated renal transplant patients is correlated with *IMPDH type II* 3757T>C polymorphism

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ABSTRACT

Objectives

The active metabolite of mycophenolate mofetil (MMF), mycophenolic acid, inhibits the activity of the target enzyme inosine monophosphate dehydrogenese (IMPDH). The aim of this study was to correlate eight different single nucleotide polymorphisms of the IMPDH type II gene to the activity of the IMPDH enzyme to explain between-patient differences in IMPDH activity.

Methods and results

In a prospective study we measured IMPDH activity, mycophenolic acid plasma concentrations and eight polymorphisms of IMPDH type II in de novo kidney transplant recipients, 6 days post-transplantation while on MMF treatment. Polymorphisms in the IMPDH type II gene were only observed for the *IMPDH type II* 3757T>C (rs11706052) single nucleotide polymorphism. Ten of 101 patients (10%) were heterozygous and two of 101 patients (2%) homozygous for *IMPDH type II* 3757T>C. The allele frequency was 6.9%. The IMPDH activity over 12 hours (AUC_{act}) was 49% higher for patients with an *IMPDH type II* 3757C variant [n=12 vs. n=68; 336 (95% confidence interval: 216 – 512) vs. 227 (95% confidence interval: 198 – 260) hμmol/s/mol adenosine monophosphate; P=0.04]. The IMPDH activity measured before transplantation ($Act_{pre\text{-}Tx}$) was not significantly different between *IMPDH type II* 3757TT wild type and variant carrier patients (p=0.99).

Conclusion

We report that the *IMPDH type II* 3757T>C polymorphism is associated with an increased IMPDH activity in MMF-treated renal transplant patients. This polymorphism explains 8.0% of the interpatient variability in IMPDH activity.

INTRODUCTION

Mycophenolate mofetil (MMF), mostly combined with calcineurin inhibitors and corticosteroids, is the most frequently used immunosuppressive drug in newly transplanted kidney recipients.[1] The official dose recommendation for patients after kidney transplantation is 1000 mg MMF twice daily (b.i.d.). Although MMF has been on the market for more than 10 years and is mostly used as a fixed dose drug, there is ongoing debate whether or not fixed-dose therapy is the optimal strategy to treat patients.[2] One possibility to individualize therapy would be to adjust the MMF dose based on plasma concentrations of mycophenolic acid (MPA), the active metabolite of MMF. The interindividual variability in exposure to MPA, and the correlations between drug concentrations and outcome are strong arguments in favor of MPA monitoring.[3] A recently published randomized trial showed that concentration-controlled renal transplant patients indeed had lower rates of acute rejection compared to patients on fixed-dose MMF therapy.[4]

Another possibility would be to monitor the pharmacodynamic effect of MPA, by measuring the activity of the target enzyme inosine monophosphate dehydrogenase (IMPDH).[5] The advantage of using pharmacodynamic measures is that, in contrast to drug concentrations, they do take into account inter-individual differences in susceptibility for the drug.[6,7] IMPDH is the key enzyme in the de-novo synthesis of nucleotides and induces the rate-limiting step in this synthesis.[8] The proliferation of lymphocytes depends on the synthesis of nucleotides by IMPDH, while other types of cells have a salvage pathway for the synthesis of nucleotides. This makes MPA a drug that specifically inhibits the proliferation of the lymphocytes. MPA is a noncompetitive inhibitor of IMPDH and it will react on the MPA/nicotinamide adenine dinucleotide (NAD)-binding pocket.[9]

Two isoforms of IMPDH exist, derived from different genes.[10] In mature resting lymphocytes, IMDPH type I is the dominant species. In activated lymphocytes IMPDH type II predominates over type I. This difference in expression suggests different regulatory mechanisms for IMPDH type I and type II.[8,11,12] The selective inhibition of IMPDH type II is thought to explain the relatively mild side effects of MMF in clinical trials.[13,14]

Human IMPDH type I and type II cDNAs encode the same size proteins of 514 amino acids with 84% sequence identity. Both isoforms show similar affinities for the substrates, with K_m values for NAD and inosine 5′-monophosphate (IMP) and K_i values for the reduced form of NAD (NADH) and xanthosine monophosphate (XMP) that are also similar.[14,15] MPA was found to inhibit both IMPDH isoforms by the same mechanism.[12] However, IMPDH type II (K_i = 9.5 nmol/l) was 3.9-fold more sensitive to MPA than the type I isoform (K_i = 37 nmol/l).[14]

Assays of IMPDH activity have produced conflicting results.[5] Assays using whole blood typically showed increasing IMPDH activity and increased guanosine triphos-

phate (GTP) levels under prolonged MMF therapy.[16,17] The target cells for MMF therapy are in the white blood cell compartment, and not in erythrocytes. Therefore, measuring IMPDH activity in isolated peripheral blood mononuclear cells (PBMCs) seems more appropriate.[12,18,19] This is enforced by data showing that MMF causes a significant reduction in GTP in PBMCs, with a paradoxical simultaneous elevation of GTP in erythrocytes.[16,20] Another potentially useful approach would be to measure IMPDH activity in the CD4+ cell population.[21,22]

Glander *et al*[23] have used a validated high-performance liquid chromatography (HPLC) method that measures the rate of XMP production by PBMCs under controlled in-vitro conditions. With this assay, the combined IMPDH activity of type I and II is measured. After oral administration of MMF, IMPDH activity is decreased by 65–100 %, persisting for 4–8 h, and returned to the level of activity before the intake of MMF after 12 h.[24] They also showed that equimolar doses of MMF and enteric-coated mycophenolate sodium resulted in similar inhibition of IMPDH activity.[25] In a preliminary study using this pharmacodynamic assay, correlations between preoperative IMPDH results and posttransplantation outcome were shown.[26]

All studies have found considerable interpatient variability in IMPDH activity, with relatively small intraindividual variability. This has generated speculations that single-nucleotide polymorphisms (SNPs) in the gene encoding for IMPDH could explain part of the variability in IMPDH activity. Potentially, such pharmacogenetic variability could be linked to the occurrence of adverse drug events or to reduced efficacy, and transplant patients would then benefit from individualization of immunosuppression, guided by pharmacogenetic data.[27] Several studies have shown pathogenicity correlated with polymorphisms of the IMPDH type I gene in patients with retinitis pigmentosa.[28-31] One study conducted in renal transplant patients proved an association between two IMPDH type I SNPs and acute rejection.[32] The *IMPDH type II* 787C>T polymorphism reduces the IMPDH activity in variant carrier group to 10% of the wild type, but had an allele frequency of only 1.0%.[33] In this study, we correlate, for the first time, eight currently known IMPDH type II SNPs directly to IMPDH activity pre transplantation and posttransplantation with the aim to explain the between-patient differences in IMPDH activity.

METHODS AND PATIENTS

Patients

For the purpose of this study in a prospective cohort of kidney transplant patients, we performed an MPA pharmacokinetic assessment 6 days after transplantation. From April 2006 to September 2007, a total of 101 patients were recruited for this study. All patients were transplanted in Erasmus University Medical Center, Rotterdam, The Netherlands.

All samples obtained were measured for IMPDH activity at the laboratory of the Department of Hospital Pharmacy and genotyped at the Pharmacogenetics laboratory of the Department of Clinical Chemistry, both in Erasmus MC. A full area under the curve (AUC, 0-12 h) was obtained, with samples taken at predose, 0.5, 1, 2, 6, and 12 h after oral intake of MMF. The pharmacodynamic effect of MPA was assessed on the same day and at the same sample times by measuring the IMPDH activity. Patients with a *de novo* adult kidney transplant with an uncomplicated postoperative recovery on the first day after transplantation and who were being treated with MMF were informed to participate in this study. Patients with seriously compromised peripheral venous vasculature (e.g. because of repetitive dialysis access surgery), complicating venous blood sampling, were not approached. The study was approved by the Local Ethics Committee of Erasmus MC, Rotterdam, The Netherlands, and complied with the Declaration of Helsinki. All patients gave written informed consent.

Immunosuppressive therapy

All patients received triple immunosuppressive therapy, consisting of tacrolimus (FK, Prograf, Astellas, Leiderdorp, The Netherlands), mycophenolate mofetil (MMF, CellCept, Roche, Woerden, The Netherlands) and prednisone. Medication was started directly after the transplant surgery. All patients were treated with 1000 mg MMF b.i.d., except two patients in the wild type group who received 500 mg MMF b.i.d. at the time of sampling. Doses and trough levels of tacrolimus are listed in Table 1. All patients received a fixed dose prednisone of 20 mg once daily. In 23 patients, anti-thymocyte globulin (ATG) induction therapy was given (Table 1).

Measurement of mycophenolic acid plasma concentrations

For the purpose of this study, the active compound MPA was measured with a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method.[34] The LC-MS/MS system consisted of a Waters Acquity Ultra Performance LC coupled to a Quattro Premier XE tandem quadrupole mass spectrometer. Data were acquired using Masslynx V4.1 and processed using Quanlynx V4.1 software (Waters Inc., Etten-Leur, The Netherlands). The analytical column was an Acquity UPLC BEH C18 2.1 x 50 mm with 1.7 µm particle size (Waters Inc.). Of each sample, 5 µL was injected onto the column. Detection was performed via MS with an ESI interface in positive MRM mode. Optimized MRM-settings for MPA and its metabolites and carboxy butoxy ether of MPA are according the setting Brandhorst *et al*,[35] reported earlier, and were optimized for our equipment. The coefficient of variation of the interday and intraday precision of the used method was less than 5 and 3% respectively. A linear trapezoidal model was used to calculate manually the area under the time-concentration curve of MPA (AUC_{MPA}), for those curves that contains 6 points.

Table 1. Patient baseline characteristics and demographics

	IMPDH type II 3757T>C		
Characteristics	**Wild type (n = 68)**	**Variant carrier (n = 12)**	**P value**
Age (years)	49.8 ± 14.4	53.5 ± 12.8	0.41[2]
Weight (kg)	80.2 ± 16.2	81.5 ± 11.4	0.79[2]
Height (cm)	174 ± 10	174 ± 6	0.90[2]
Male sex, n (%)	50 (74%)	11 (92%)	0.17[3]
Ethnicity, n (%)			
Caucasian	57 (84%)	11 (92%)	
Black	9 (13%)	1 (8%)	0.73[3]
Oriental	2 (3%)	0	
Donor characteristics, *n* (%)			
Living donor	44 (64%)	8 (67%)	0.90[3]
Deceased donor	25 (36%)	4 (33%)	
Patients previously transplanted	7 (10%)	2 (17%)	0.52[3]
Delayed graft function	19 (28%)	4 (33%)	0.70[3]
ATG induction therapy	20 (29%)	3 (25%)	0.76[3]
Mycophenolate mofetil daily dose (mg)	1971 ± 170	2000 ± 0	0.55[2]
Tacrolimus daily dose (mg)	11.2 ± 3.4	10.4 ± 4.1	0.48[2]
Range (mg)	4-20	4-18	
Trough level (µg/l)	13.2 ± 6.9	12.5 ± 8.2	0.78[2]
Albumin (g/l)	34 ± 3.6	33 ± 4.0	0.39[2]
Creatinine clearance[1] (ml/min)	31 ± 21	39 ± 30	0.41[2]
Leukocytes (10^9/l)	9.0 ± 4.6	9.0 ± 5.1	0.99[2]

Data are expressed as mean ± SD.

[1] Estimated using MDRD equation; [2] t-test; [3] Pearson's χ^2 test.

ATG, antithymocyte globulin; IMPDH, inosine monophosphate dehydrogenase.

Measurement of IMPDH activity

A validated nonradioactive HPLC method by Glander *et al*[36] was used to measure the IMPDH activity. The rate of XMP production by IMPDH from PBMCs was measured and was normalized to the measured intracellular adenosine monophosphate (AMP).

In summary, PBMCs were isolated from lithium heparin-anticoagulated whole blood using Leucosep tubes with Ficoll-Paque (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) according to the manufacturer's protocol with slight modifications. After one washing step, the aliquot was resuspended in 250µl ice-cold water and stored at –20°C to lysate. To 50µl mononuclear cells lysate 130µl reaction buffer with IMP and β-NAD^+ was added to start the incubation of enzyme reaction. The reaction tubes were placed in a Thermomixer (Eppendorf Ltd., Cambridge, UK) and were incubated at 37°C and 800 rpm for 2.5 h. The reaction was stopped by adding 20µl 4 mol/l ice-cold perchloric acid. After centrifugation, 10µl of 5 mol/l potassium carbonate was added to 170µl superna-

tant to neutralize the solution. The supernatant was transferred into HPLC injection vials for the determination of AMP and of produced XMP. Enzyme activity was expressed as produced XMP (µmol) per time unit (s) per amount of AMP (mol). The precision of the IMPDH activity assay was 6.6-11.9% and the coefficient of variation ranged from 0.6 to 3.4%. A linear trapezoidal model was used to calculate manually the area under the time effect curve for the activity (AUC_{act}), for those curves that contained 6 points.

IMPDH type II genotyping

For all the patients, 4 ml EDTA blood was drawn within 3 weeks after transplantation for analysis of polymorphisms in the IMPDH gene. Genotyping of eight different polymorphisms (Table 2) was performed using a TaqMan allelic discrimination assay (assay ID C1842928-10; Applied Biosystems, Foster City, California, USA) on an ABI Prism 7000 sequence detection system (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). PCR were performed in a reaction volume of 12.5 µl containing assay-specific primers, allele-specific TaqMan MGB probes (either FAM or VIC labeled), TaqMan Universal PCR Master Mix No AmpErase UNG (2X), and 10 ng genomic DNA. The thermal profile consisted of an initial incubation at 50°C for 2 min, denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 92°C for 15 s, and annealing and extension at 60°C for 1 min. Genotypes were scored by measuring allelic-specific fluorescence using the SDS 1.2.3 software for allelic discrimination (Applied Biosystems). The assay was validated by sequencing a wild type, a heterozygote and a homozygote variant sample. Wild type, heterozygote and homozygote variant quality control samples validated by direct sequencing were added in each run.

Table 2. Genetic variants of IMPDH type II in renal transplant patients detected by bidirectional DNA sequencing

Variant	Position	Flanking sequence	Amino Acid change	Minor allele frequency (%)	Reference
-95T>G	Promoter / 5'UTR	CGCAGCGCAG[T/G]GACGAAATCG			
192A>G	Exon 3	TTAAGACCCC[A/G]CTGGTTTCCT	Phe64Phe	N.A.	rs11557547
460T>C	Exon 5	GGGGAGCCGC[T/C]TGGTGGGCAT	Leu154Leu	N.A.	rs11557542
720C>T	Exon 7	CCAAAGATGC[C/T]AAGAAACAGC	Ala240Ala	N.A.	rs11557544
787C>T	Exon 7	GCTGGACTTG[C/T]TCGCCCAGGC	Leu263Phe	1.0	33
915C>G	Exon 9	CCATAGTGGT[C/G]ACTGCTGCCC	Val305Val	N.A.	rs11557546
1253A>G	Exon 11	GGTTCTCTCG[A/G]TGCCATGGAC	Asp418Gly	N.A.	rs11557540
3757T>C	Intron 7	GGTGGACACA[T/C]CGTCGAGTGG		10.4[1] 6.8	rs11706052 37

[1] HapMap-CUE: European Caucasian population.

IMPDH, inosine monophosphate dehydrogenase; N.A., not available; UTR, untranslated region.

Statistical analysis

All statistical tests were performed using SPSS software Version 16.0.1 (SPSS, Chicago, Illinois, USA) and a *P* value of less than 0.05 was considered significant. IMPDH activity data and AUC_{MPA} were log-transformed and were reported back-transformed. Data was tested for normal distribution by one-sample Kolmogorov-Smirnov test. Data were analyzed by an independent-samples *t*-test (t-test; normal distributed) or by a Mann-Whitney *U* test (MW-test; effect data). Related data was tested with a Wilcoxon signed-ranks test. Categorical data and genotype and allele distribution as well as the presence of Hardy–Weinberg equilibrium were analyzed by a Pearson χ^2 test. Values are reported as mean ± standard deviation or as mean (95% confidence interval), unless otherwise stated.

RESULTS

Baseline characteristics

From April 2006 to September 2007, 177 adult patients have received a single blood group ABO-compatible kidney transplantation in our center; 101 patients were recruited for the study. Seventy-six patients were not included in the study because of (i) patient refusal (38), (ii) seriously compromised peripheral venous vasculature complicating frequent blood sampling (23), and (iii) other reasons (15). From 80 of 101 patients (79%), a full 12 hours curve consisting of six blood samples on day 6.0±1.3 was available for measurement of both MPA plasma concentrations and IMPDH activity. From the other 21 patients, incomplete 12 h curve samples had been collected. For calculating the area under the effect (IMPDH activity) – time curve (AUC_{act}) and the area under the MPA plasma concentration – time curve (AUC_{MPA}) from 0 to 12 h, these patients were withdrawn from the analyses. Polymorphisms in the IMPDH type II gene were only observed for the *IMPDH type II* 3757T>C polymorphism (Table 2). In 12 patients, at least one *IMPDH type II* 3757C allele was found: 10 of 101 patients (10%) were heterozygous and two of 101 patients (2%) homozygous for this allele. The allele frequency of the *IMPDH type II* 3757T>C for this patient group was 6.9%. The *IMPDH type II* 3757T>C polymorphism was approaching the limit of Hardy Weinberg disequilibrium (*P*=0.07). Patients with at least one *IMPDH type II* 3757C allele were pooled together (*n*=12) and compared with patients with the wild type SNP (*n*=68). Patient baseline characteristics for the 80 patients of whom full data (full MPA-AUC and genotype) were available are summarized in Table 1. None of the characteristics showed significant differences between the two groups.

IMPDH activity pre-transplantation

The IMPDH activity measured from samples taken just before the transplantation (Act_{pre-Tx}) showed no difference between the two groups [40.7 (95% CI: 33.0–50.1) vs. 42.8 (95% CI: 26.6–68.9) µmol/s/mol AMP; *P*=0.99, MW-test; Table 3).

Table 3. Comparisons of Act and C parameters between *IMPDH type II 3757* T>C wild type and variant carrier groups

Parameter	*IMPDH type II* 3757T>C		*P* value[3]
	Wild type (*n* = 68)	Variant carrier (*n* = 12)	
$Act_{bas(pre-Tx)}$ (µmol/s/mol AMP)[1]	40.7 (33.0–50.1)	42.8 (26.6 – 68.9)	0.99
AUC_{act} (h·µmol/s/mol AMP)[1]	227 (198–260)	336 (216 – 521)	0.042
IMPDH activity (µmol/s/mol AMP)[1]			
Act_0	19.8 (16.4–24.0)	26.5 (15.6–45.0)	0.31
$Act_{0.5}$	18.9 (16.0–22.4)	17.4 (10.3–29.6)	0.62
Act_1	14.3 (12.3–16.6)	15.4 (10.3–23.0)	0.67
Act_2	13.0 (11.2–15.1)	16.7 (9.1–30.5)	0.56
Act_6	16.5 (13.8–19.8)	22.0 (12.0–40.3)	0.51
Act_{12}	24.5 (20.8–29.0)	40.8 (23.8–69.8)	0.021
AUC_{MPA} (h·mg/L)[1]	31.0 (27.2–35.3)	38.9 (30.2–50.2)	0.18
MPA levels (mg/L)[2]			
C_0	2.39 ± 1.92	2.54 ± 1.30	0.22
$C_{0.5}$	3.84 ± 4.79	4.42 ± 3.49	0.32
C_1	4.94 ± 4.08	4.55 ± 3.10	0.92
C_2	4.21 ± 2.38	5.08 ± 4.27	0.80
C_6	2.14 ± 1.29	3.07 ± 1.57	0.038
C_{12}	2.23 ± 1.67	2.23 ± 1.19	0.62

[1] Data are expressed as mean (95% confidence interval); [2] Data are expressed as mean ± SD; [3] Mann–Whitney U test.

Act, IMPDH activity; Act_{pre-Tx}, IMPDH activity measured before transplantation; AMP, adenosine monophosphate; AUC_{MPA}, area under the time–concentration curve of mycophenolic acid; C, mycophenolic acid concentration; IMPDH, inosine monophosphate dehydrogenase.

IMPDH activity and MPA levels post-transplantation

Figures 1 and 2 show the IMPDH activity and the MPA levels on day 6 posttransplantation for the *IMPDH type II* 3757TT wild type and the *IMPDH type II* 3757C carriers, respectively. The IMPDH activity 12 h after MMF intake was significantly higher in the variant carrier group compared to the *IMPDH type II* 3757TT wild type group [40.8 (95% CI: 23.8–69.8) vs. 24.5 (95% CI: 20.8–29.0) µmol/s/mol AMP; *P* = 0.02, MW-test; Table 3]. The area under the time–effect curve for the IMPDH activity over 12 h was significantly higher in the 12 patients with an *IMPDH type II* 3757C variant compared to the 68 patients with the *IMPDH type II* 3757TT wild type [336 (95% CI: 216–521) vs. 227 (95% CI: 198–260) h·µmol/s/mol AMP; *P* = 0.04, MW-test; Table 3]. The MPA exposure (AUC_{MPA}) was not significantly different between these groups (*P* = 0.18, MW-test; Table 3). Patients with a variant SNP

were randomly distributed among the overall population (Fig. 3). The mean AUC_{MPA} values are in close proximity in a highly variable range of MPA exposure. Excluding the two patients who received 500 mg MMF b.i.d. did not alter the results. Therefore, those two patients are included in all presented results. A bivariate correlation coefficient *r* of 0.29 (P = 0.01) was found between AUC_{act} and the analyzed SNP. The square of the correlation coefficient is 0.080 (8.0%) and displays the variation in AUC_{act} explained by the analyzed SNP. Restricting the patient group to only Caucasian patients resulted in a similar coefficient of correlation. In 68 Caucasian patients, 57 patients with the *IMPDH type II* 3757TT wild type and 11 patients with at least one C-allele were found. The AUC_{act} in the *IMPDH type II* 3757C variant carrier group was also significantly higher compared to the wild type patients [347 (95% CI: 215–561) vs. 229 (95% CI: 197–266) hμmol/s/mol AMP; *P* = 0.04, MW-test].

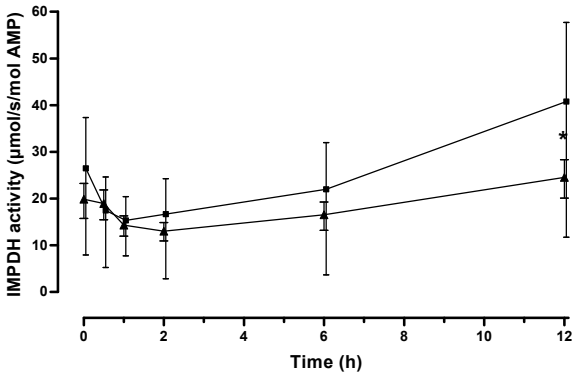

Figure 1. Inosine monophosphate dehydrogenase (IMPDH) activity versus time curve. Triangles represent the *IMPDH type II* 3757T>C wild type group (n=68), and the squares the variant carrier group (n=12). The marked activity after 12 h is significantly higher in the variant carrier group compared with the wild type group (*P = 0.02, Mann–Whitney U test).

One of the two patients who were homozygous for the variant allele had the highest IMPDH activity of the entire patient cohort (AUC_{act} of 1218 hμmol/s/mol AMP; Fig. 4). The two patients with the *IMPDH type II* 3757CC homozygote variant genotype had a slightly higher IMPDH activity in comparison with the 10 *IMPDH type II* 3757TC heterozygote variant patients [495 and 1218 vs. 284 (95% CI: 186–435) hμmol/s/mol AMP].

The IMPDH activity expressed as AUC_{act} is lower when the AUC_{MPA} is higher. The decreasing AUC_{act} over the three AUC_{MPA} subgroups (< 30, 30-60 and > 60 hmg/l respectively[38,39]) was, however, not significantly different: 264 (95% CI: 207–336) versus 227 (95% CI: 193–268) versus 204 (95% CI: 121–345) μmol/s/mol AMP (*P* = 0.35, MW-test).

In 16 of the 80 of the patients (20%), a biopsy proven acute rejection (BPAR) was reported within 1 year after transplantation. Of these patients, 15 had a wild type IMPDH genotype and one was homozygous for the variant allele. This implies that 22% of the *IMPDH type*

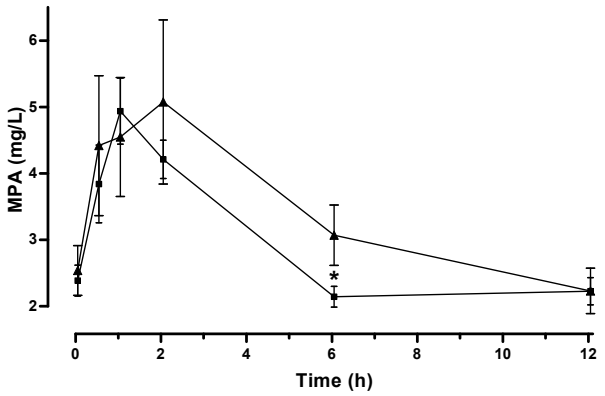

Figure 2. Mycophenolic acid (MPA) plasma concentration versus time curve. Triangles represent the *IMPDH type II* 3757T>C wild type group (n=68), and the squares the variant carrier group (n=12). The marked MPA concentration after 6 h is significantly higher in the variant carrier group compared with the wild type group (*P = 0.04, Mann–Whitney U test).

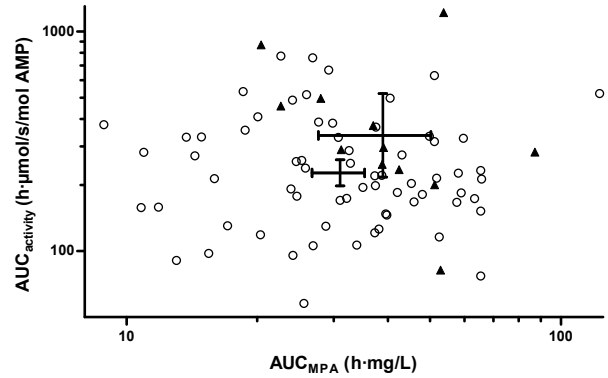

Figure 3. Patients with a variant single nucleotide polymorphism (SNP; solid triangles) were distributed equally throughout the patients with a wild type SNP (open circles). The error bars present the 95% confidence interval of the area under the time-effect curve for the activity (AUC_{act}) and area under the time-concentration curve of mycophenolic acid (AUC_{MPA}) for both patient groups.

II 3757TT wild type patients and 8% of the variant genotype patients had a BPAR, which was not significantly different ($P = 0.27$, MW-test). The interesting homozygous variant allele patient with the highest measured AUC_{act} (1218 hµmol/s/mol AMP) was the patient suffering from a BPAR (Banff classification I-b) 4.5 months after transplantation. Figure 5 shows the IMPDH activity and MPA plasma levels over 12 h after MMF oral dosing, of all 80 patients on day 6 posttransplantation. An inverse relationship between IMPDH activity and MPA plasma levels is observed, with maximal inhibition of IMPDH activity coinciding with the MPA peak levels.[40] The inhibition of the IMPDH activity is significantly lower up to 6 h after intake of MMF compared to the predose activity. The MPA plasma levels increase after MMF intake and are also significantly higher compared to predose until 6 h after MMF intake (Table 4).

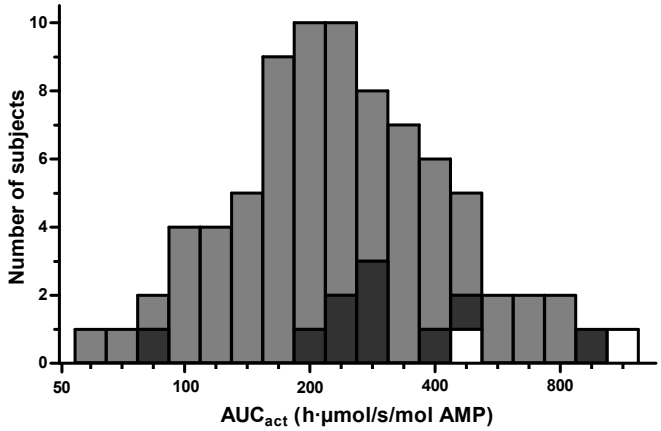

Figure 4. Histogram of the area under the time-effect curve for the activity (AUCact) of the inosine monophosphate dehydrogenase (IMPDH) activity of the *IMPDH type II* 3757T>C polymorphism. Gray filled bars presenting the *IMPDH type II* 3757TT wild type patients, dark filled bars *IMPDH type II* 3757TC variant carrier patients, and the blank filled bars *IMPDH type II* 3757CC variant carrier patients.

Table 4. Mean values of IMPDH activity and MPA concentrations at sample time points.

Time (h)	Activity (µmol/s/mol AMP)	*P* value[1] (compare to t = 0h)	MPA concentration (mg/l)	*P* value[1] (compare to t = 0h)
0	20.72 (17.39 – 24.70)		2.41 ± 1.84	
0.5	18.72 (15.99 – 21.92)	0.053	3.92 ± 4.61	< 0.001
1	14.46 (12.58 – 16.61)	< 0.001	4.88 ± 3.93	< 0.001
2	13.53 (11.63 – 15.74)	< 0.001	4.34 ± 2.73	< 0.001
6	17.25 (14.48 – 20.56)	0.013	2.28 ± 1.37	0.88
12	26.44 (22.46 – 31.12)	0.009	2.23 ± 1.60	0.22

P values are calculated for each sample point compared to the sample point on 0 h. Data are expressed as mean (95% confidence interval) or mean ± SD.

[1] Wilcoxon signed-ranks test.

AMP, adenosine monophosphate; IMPDH, inosine monophosphate dehydrogenase; MPA, mycophenolic acid.

Anti-thymocyte globulin induction therapy

Induction therapy with ATG was given to 20 of 68 patients (28%) in the *IMPDH type II* 3757TT wild type and to three of 12 patients (25%) in the *IMPDH type II* 3757TT variant genotype group. The AUC_{act} of all patients who received ATG induction therapy was not significantly different compared with patients who did not receive ATG [210 (95% CI: 155–283) vs. 254 (95% CI: 220–294) hµmol/s/mol AMP; $P = 0.15$, MW-test]. Lymphocyte counts were significantly different in patients who did and did not receive ATG (0.17±0.19 vs. 1.15±0.87 10^9/l; $P < 0.0001$, MW-test). No significant correlation between AUC_{act} and lymphocytes counts was found ($r = 0.34$; $P = 0.32$).

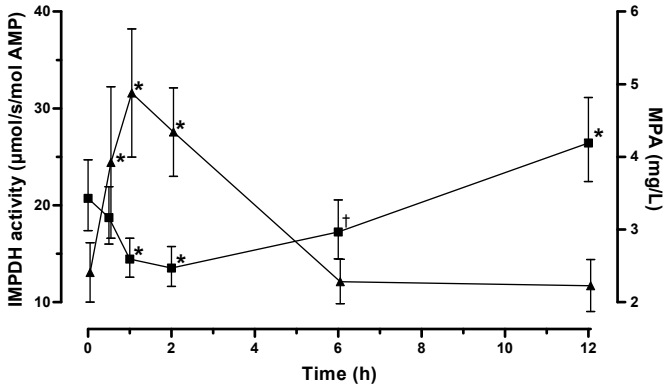

Figure 5. Inosine monophosphate dehydrogenase (IMPDH activity; squares) and mycophenolic acid (MPA plasma levels; triangles) of the whole study population on day 6 posttransplantation. Sample points that are significantly different compared with the sample point at 0 h *P < 0.01, Wilcoxon signed-ranks test or a †P=0.01, Wilcoxon signed-ranks test.

DISCUSSION

We here report that the *IMPDH type II* 3757T>C polymorphism is associated with an increased IMPDH activity in MMF-treated renal transplant patients. Patients with at least one variant *IMPDH type II* 3757C allele had a mean IMPDH activity that was 48% higher compared with *IMPDH type II* 3757TT wild type patients [336 (95% CI: 216–521) vs. 227 (95% CI: 198–260) h·µmol/s/mol AMP, respectively]. This polymorphism explains 8.0% of the variation in IMPDH activity between patients. Remarkably, the patient with the highest IMPDH activity in this cohort of 80 patients was one of the two patients homozygous for the variant C-allele. The allele frequency of this polymorphism was 6.9%. The *IMPDH type II* 3757T>C polymorphism in the *IMPDH type II* gene was not associated with IMPDH activity pretransplantation. This may be explained by the constitutive expression of mainly IMPDH type I in nonactivated lymphocytes, and induction of expression of IMPDH type II following allo-recognition. IMPDH activity in pretransplantation samples mainly reflects type I activity, and as a result the type II polymorphism does not lead to substantial change in overall IMPDH activity pretransplantation. Despite the significant difference in lymphocyte counts between patients who did and did not receive ATG induction therapy, a difference in IMPDH activity was not found. This can be explained by the method used to measure IMPDH activity that expresses XMP production per the amount of AMP present, and thus corrects for the number of cells from which IMPDH was isolated.

As shown in Fig. 4, and despite the statistically significant difference, there is extensive overlap between the ranges of IMPDH activity results from patients with the *IMPDH type II* 3757TT versus the *IMPDH type II* 3757CT/CC genotype. All IMPDH activity measurements were made while the patient was on MMF therapy. Though the pharma-

cokinetics of MPA is not stabilized until several months after transplantation, all activity measurements have taken place at the same time (6±1.3 days post transplantation). MPA exposure, that is, both predose plasma concentrations as well as the AUC_{MPA}, was not different between the two *IMPDH type II* 3757T>C genotype groups. Although the mean AUC_{MPA} was 20% higher in patients with a variant allele, the mean AUC_{MPA} in patients with a variant allele was not significantly different from wild type patients. Individual data points of patients with a variant SNP were randomly distributed among the overall population. In view of the inhibitory effect of MPA on IMPDH, the IMPDH activity would have been higher in the absence of MPA, but as all patients were on MMF therapy we cannot show untreated posttransplant IMPDH activities from our patients. In line with the aim of this study, the similarity of AUC_{MPA} between the two groups strengthens our conclusion that the *IMPDH type II* 3757T>C polymorphism is primarily responsible for less inhibition of IMPDH activity with comparable MPA exposure.

We did find an inverse relationship between MPA concentrations and IMPDH activity. First, IMPDH activity was significantly higher in samples drawn pre-MMF dosing (Act_0) compared with samples drawn at a half ($Act_{0.5}$), 1 (Act_1), 2 (Act_2) and 6 (Act_6) h postdose (20.72 µmol/s/mol AMP vs. 18.72, 14.46, 13.53 and 17.25 µmol/s/mol AMP, respectively). MPA levels were significantly higher compared to pre-MMF dosing at half ($C_{0.5}$), 1 (C_1) and 2 (C_2) h postdose. We found no significant differences in IMPDH activity between patients with low versus high AUC_{MPA} on day 6 after transplantation. It is remarkable that, despite the influence of MPA on IMDPH activity, we still found a correlation between the *IMPDH type II* 3757T>C polymorphism and IMPDH activity. Along these correlations, the ultimate effect on the IMPDH enzyme of this intronic polymorphism of the IMPDH type II gene is still not clarified. In general, intronic polymorphisms would be associated with the susceptibility and stability of the mRNA. The consequences are presumably a shorter peptide or a decreased amount of encoded protein.[41,42]

In this study, there was no increased incidence of BPAR in the patients with at least one C-allele. Given the fact that these patients have a higher IMPDH activity, one would expect them to be more allo-reactive and have more rejections. In the Caesar study, a statistically significant association between rejection incidence and the *IMPDH type II* 3757T>C polymorphism was found in a cohort of 237 genotyped patients.[37] With a patient population of 80 patients and only 12 patients with at least one *IMPDH type II* 3757C allelle, our study was underpowered to show a significant difference in rejection incidence in the different genotype groups. Given the low numbers of BPARs for each genotype, this finding cannot directly be interpolated to a clinically relevant conclusion. Grinyo *et al*[37] suggested that further studies are needed to determine a possible correlation between *IMPDH2 type II* 3757T>C polymorphism and IMPDH enzyme activity. In our study, we have support this correlation. The previously reported *IMPDH type II* 787C>T polymorphism was not present in our patient group. The authors reported an

allele frequency of 1.0 %.[33] This low allele frequency could explain the absence of this polymorphism and its association with a lower IMPDH activity in our patient population of 80 patients. All performed tests were univariate tests. A multivariate test would be superior, but with the small sample size of 12 patients with at least *IMPDH type II* 3757C allelle the results were underpowered.

The main finding of this study is that between-patient variability in IMPDH activity measured in blood samples drawn from renal transplant patients treated with MMF is explained for 8.0% by the *IMPDH type II* 3757T>C polymorphism. This effect of genotype on posttransplantation phenotype cannot be identified before transplantation by measuring IMPDH activity, because of the differences in expression of IMPDH type I and type II.

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Chapter 4.2

IMPDH mRNA expression is correlated to clinical outcomes in MMF treated kidney transplant patients whereas IMPDH activity is not

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ABSTRACT

Measurement of the pharmacodynamic biomarker inosine monophosphate dehydrogenase (IMPDH) activity in renal transplant recipients has been proposed to reflect the biological effect better than using pharmacokinetic parameters to monitor mycophenolate mofetil therapy. The IMPDH assays are however labor intensive and this complicates implementation into patient care. Quantification of IMPDH messenger RNA (mRNA) could form an attractive alternative. This study was designed to correlate IMPDH mRNA levels with IMPDH activity and clinical outcome in renal transplant recipients.

From a cohort of 101 renal transplant patients, blood samples were drawn pre transplantation and at 4 times after transplantation. IMPDH activity, IMPDH type I and type II mRNA levels, and mycophenolic acid concentrations were measured and correlated to clinical outcomes.

No correlation was found between IMPDH type I and type II mRNA levels and IMPDH activity in pre- and posttransplant samples. A significant increase in IMPDH mRNA levels was found between day 6 and day 140 after transplantation. IMPDH type I and type II mRNA levels before transplant showed a trend towards statistical significantly higher levels in patients with an acute rejection ($P = 0.052$ and $P = 0.058$). After transplant, the IMPDH type I and type II mRNA levels were significantly lower in patients with an acute rejection ($P = 0.026$ and $P = 0.007$).

We conclude that IMPDH mRNA levels do not correlate with IMPDH activity but are nevertheless correlated with acute rejections. Furthermore, although the regulation of the expression of the 2 isoforms is presumed to be different, in this study, the changes in the expression of type I mRNA closely paralleled those of type II.

INTRODUCTION

Inosine monophosphate dehydrogenase (IMPDH) is the rate-limiting enzyme in the de novo synthesis of guanine nucleotides. It catalyzes the conversion of inosine monophosphate to xanthosine monophosphate (XMP) at the purine metabolic branch point. There are 2 isoforms of IMPDH, type I and type II, and each consists of 514 amino acids with 84% sequence identity.[1] Type I and type II are encoded by 2 distinct genes, located at 2 different chromosomes.[2,3] IMPDH type I is constitutively present in the majority of cells, including resting lymphocytes.[4,5] Proliferation of cells is related to upregulation of the type II isoform, whereas lymphocyte activation under normal physiological conditions is associated with increased expression of both isoforms. The regulation of expression of the 2 IMPDH genes differs according to the 2 isoforms. IMPDH type I messenger RNA (mRNA) is expressed in a complex manner with 3 transcripts arising from 3 alternate promoters.[6,7] The IMPDH type II gene is strongly upregulated at the transcriptional level by a promoter region that responds directly to growth stimuli.[8-10] The gene expression of the 2 IMPDH isoforms has a negative feedback regulation by intra- and extracellular guanine nucleotides.[11-13] The increased expression of IMPDH genes leads to increased IMPDH activity,[14,15] and an increased IMPDH activity has been correlated with an increased cellular proliferation and transformation.[16,17]

Inhibition of IMPDH leads to depletion of guanine nucleotide pools and retards the proliferation of T en B lymphocytes, which are dependent on this de novo synthesis pathway of the nucleotides.[18] Mycophenolic acid (MPA), the active metabolite of mycophenolate mofetil (MMF), is a clinically used inhibitor of IMPDH as part of immunosuppressive therapy after solid organ transplantation and hematopoietic stem cell transplantation and varied autoimmune diseases. MPA is a 3.9-fold more potent inhibitor of IMPDH type II compared to IMPDH type I.[19] The activity of IMPDH shows a large inter-patient variability.[20] The sensitivity for MPA to inhibit IMPDH also differs between individuals, even when MPA levels are equal. Pharmacokinetic (PK) monitoring of MPA concentrations does not take this interpatient variability into account, whereas the pharmacodynamic (PD) approach (ie, measurement of IMPDH activity) does. For this reason, a number of different IMPDH activity assays have been developed, each having its own advantages and disadvantages.[21,22] The major disadvantage of all IMPDH assays is the time-consuming and labor-intensive procedure. An alternative method to monitoring the IMPDH activity could be the measurement of IMPDH mRNA using a relatively easy-to-use quantitative real-time polymerase chain reaction (RT-PCR) method.[23] Preferably, for the use of IMPDH mRNA as a biomarker for the IMPDH activity and MPA treatment, IMPDH mRNA must be correlated with the IMPDH activity.

Recent studies have evaluated the expression of the IMPDH type I and type II gene after kidney transplantation. Transplantation and the initiation of immunosuppressive

therapy were associated in peripheral blood mononuclear cells (PBMCs) with a significantly increased IMPDH type I mRNA expression of 50%–88% and with a significantly decreased IMPDH type II mRNA expression of 42%–56% within 2 weeks after transplantation. In CD4+ T cells, however, IMPDH type II increased significantly by 15%.[24] It has been demonstrated in in vitro experiments that IMPDH type II mRNA is correlated with the IMPDH activity in transplanted tissue.[25] Patients suffering from acute rejection during follow-up demonstrated significantly higher IMPDH type II mRNA expression in $CD4^+$ T cells pre transplant than nonrejectors.[24] The authors concluded that IMPDH type II mRNA expression could be an indicator of immune activation.[24] Another study showed a 2- to 3-fold elevation of IMPDH type I mRNA during the first 3 months following transplantation.[25] IMPDH type I mRNA expression reached its maximal level during acute rejection episodes, whereas IMPDH type II mRNA was stable in PBMCs. A 20-fold increase of IMPDH type I mRNA was observed in the 2 patients who experienced acute rejection. *In vitro* experiments confirmed that IMPDH type I is inducible but more in monocytes than in lymphocytes. This finding suggests that the measurement of IMPDH mRNAs may provide reliable information to predict acute rejection.[25]

The aim of this prospective longitudinal study was to correlate IMPDH type I and type II mRNA levels to MPA concentrations, IMPDH activity, and clinical outcomes after kidney transplantation. Potentially, IMPDH mRNA levels could be an interesting biomarker to monitor and optimize MMF treatment after kidney transplantation.

PATIENTS AND METHODS

Patients

A prospective cohort of *de novo* kidney transplant patients (n = 101) was longitudinally followed for 12 months after transplantation. Before transplantation and on day 6, 21, 49 and 140 after transplantation, blood samples were drawn for PK (MPA concentrations) and PD (IMPDH activity) monitoring to follow IMPDH activity over time. A full area under the curve (AUC, 0-12 hours) was obtained on day 6, when samples were taken pre dose, 0.5, 1, 2, 6, and 12 hours after oral intake of MMF. An abbreviated AUC was taken on the second (day 21), third (day 49) and fourth (day 140) visit, when samples were taken pre-dose, 0.5 and 2 hours after oral intake of MMF. At all visits, a sample was drawn pre dose for determination of IMPDH mRNA levels. Patients were included into the study from April 2006 to September 2007. Due to logistic problems with the mRNA samples, no post-transplant mRNA data were available from the period of April 2006 to August 2006. Adult patients receiving a kidney transplant with an uncomplicated postoperative recovery in the first days after transplantation and who were being treated with MMF were informed to participate in this study. Patients with seriously compromised periph-

eral venous vasculature, complicating venous blood sampling, were not approached. The study was approved by the local Ethics Committee of Erasmus University Medical Center, Rotterdam, The Netherlands, and complied with the Declaration of Helsinki. All patients gave written informed consent.

Immunosuppressive therapy

All patients received triple-drug immunosuppressive therapy, consisting of tacrolimus (FK, Prograf; Astellas Pharma, Leiderdorp, The Netherlands), mycophenolate mofetil (MMF, CellCept; Roche, Welwyn Garden City, United Kingdom) and prednisone according to protocol. Therapy was started directly after transplant surgery. Patients were treated with MMF twice a day, starting with 2000 mg daily. Dose adjustments were performed depending on the clinical status of the patient and at physician's own discretion. MPA plasma concentrations were available but were not used for MMF dose adjustment. Tacrolimus was dosed on trough levels, although the trough target levels decreased over time after transplantation (first month after transplantation, 10-15 µg/L; second month, 8-12 µg/L; 6-10 µg/L after month 3; and 5-8 µg/L after month 6). Methylprednisolone was given intravenous for the first 3 postoperative days, in a dose of 100 mg daily. Thereafter, prednisone was given orally once daily, starting with 20 mg and was tapered at physician's own discretion and to be discontinued at month 3. In total, 22 recipients of a kidney from a deceased donor and 1 recipient of a kidney from a living unrelated donor received antithymocyte globulin (ATG) induction therapy [23 patients of all included patients (30%)].

Quantitative mRNA expression analysis

Total RNA was isolated using the High Pure RNA Isolation kit (Roche Applied Science, Almere, The Netherlands), according to the manufacturer's instructions. RNA concentrations were measured by using the Nanadrop ND-8000 Spectrophotometer (Isogen Life Science, De Meern, The Netherlands). Samples containing at least 500 ng RNA per sample were used. The purity of the used samples was examined using UV spectral ratios. For RNA purity, a cutoff value of the 260/280nm ratio of 1.8 was used, and for the organic purity of the samples, a cutoff value of the 260/230nm ratio of 1.0 was used. Impure samples with a ratio below the cutoff value were rejected. RNA was denatured for 5 minutes at 80°C and then chilled on ice. First-strand complementary DNA (cDNA) reaction was performed from 500 ng of the isolated RNA at 42°C for 90 minutes in a volume of 50 µL. The reaction mixture contained 10 µL of 5x first-strand buffer, 500 U of Moloney murine leukemia virus reverse transcriptase (M-MLV RT), 0.5 nmol/L of dithiothreitol (Life Technologies, Gaitherburg, MD), 25 pmol of each dNTP (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands), 0.25 µg of random primers, and 20 U of RNAse inhibitor (Promega Benelux, Leiden, The Netherlands). After the cDNA

reaction, the M-MLV reverse transcriptase was inactivated by a 5-minute incubation at 95°C.

A quantitative RT-PCR was used to quantify the amount of IMPDH type I and type II expression in the samples. Assay-on-demand products for the detection and quantification of IMPDH type I (hs00992210_m1) and IMPDH type II (hs01021353_ma) mRNA were designed by Applied Biosystems (Foster City, CA).

A 5 µL sample of cDNA was added to 20 µL PCR mixture containing 12.5 µL Universal PCR Master Mix (Applied Biosystems), 0.625 µL of each specific primer and probe assay-on-demand mix, and 6.875 µL water. The RT-PCR reaction was performed after a first step of 2 minutes 50°C and 10 minutes 90°C followed by cycles of 15 seconds at 95°C and 1 minute at 60°C using the ABI Prism 7700 sequence detector (Applied Biosystems). Each run contained several negative controls (no template), and 2 positive reference samples. The amount of each target molecule was quantified by measuring the threshold cycles (C_t) on a TaqMan Real-Time PCR system (Applied Biosystems) and was transformed to the number of cDNA copies [$2^{(40-Ct)}$] as described by Bustin *et al*[26] The absolute values of the number of IMPDH mRNA copies were log transformed.

IMPDH activity assay

The validated nonradioactive high-performance liquid chromatography method by Glander *et al*[22] was used to measure the IMPDH activity. In summary, PBMCs were isolated from lithium heparin-anticoagulated whole blood using Leucosep tubes with Ficoll-Paque (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). After one washing step, the aliquot was resuspended in 250 µL ice-cold water and stored at –20°C to lysate. To 50 µL PBMC lysate 130 µL reaction buffer with inosine monophosphate and β-NAD^+ was added to start the incubation of enzyme reaction at 37°C and moderately shaken at 800 rpm (Thermomixer; Eppendorf, Hamburg, Germany) for 2.5 hours. The reaction was stopped by adding ice-cold perchloric acid. After centrifugation, potassium carbonate was added to the supernatant to neutralize the solution. The supernatant was transferred into high-performance liquid chromatography injection vials for determination of produced XMP and present adenosine monophosphate (AMP). Enzyme activity was expressed as produced XMP (micromoles) per time unit (s) per present AMP (moles) for normalizing the activity to the destructed cells. The precision of the IMPDH activity assay was 6.6% to 11.9%, and the coefficient of variation ranged from 0.6 to 3.4%.

Measurement of MPA concentrations

For the purpose of this study, the active compound MPA was measured using a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method[27] optimized for our equipment. To a sample of 50 µL plasma, 200 µL zinc sulfate (0.1 mol/L) and 500 µL acetonitrile with 3 mg/mL of the internal standard carboxy butoxy ether of MPA was

added for determination of the total plasma concentrations of MPA. For the determination of the unbound free plasma concentration of MPA, plasma was first ultrafiltrated using 30 KDa centrifugal filter devices (Microcon Ultracel YM-30, Millipore, Amsterdam, The Netherlands). The coefficient of variation of the inter- and intraday precision of the used method was less than 5% and 3%, respectively, for the total plasma MPA concentrations (range: 0.2–50 mg/L) and were less than 13% and 7%, respectively, for the unbound plasma MPA concentration (range: 0.001–1.0 mg/L).

Clinical outcome

Patients were suspected of an acute rejection episode based on clinical grounds and all acute rejections were confirmed by a core biopsy. All biopsies were read by a pathologist and graded according to the Banff classification.[28] Adverse events were graded according to the standard response and toxicity criteria.[29] Gastrointestinal adverse events (nausea, vomiting, diarrhea, and abdominal cramps) were pooled and evaluated together. Hematological adverse events (leukopenia, thrombocytopenia, and anemia) were also pooled and evaluated together. Leukopenia was defined as a leukocyte count less than 3.0×10^9 cells per liter, thrombocytopenia as a thrombocyte count less than 75.0×10^9 cells per liter, and anemia as a hemoglobin concentration below 5.8 millimoles per liter for men and women.

Statistical analysis

All statistical tests were performed using SPSS software version 16.0 (SPSS, Chicago IL) and a *P* value less than 0.05 was considered significant. Bivariated correlations coefficients between data were analyzed using Pearson. Unrelated data was tested by a Mann-Whitney *U* test, related data by a Wilcoxon signed ranks test. A one-way analysis of variance test was used to analyze data with more than 2 variables. Values are reported as mean ± SD for normally distributed data or as geometric mean (95% confidence interval) for non-normally distributed data, unless otherwise stated.

RESULTS

Patient characteristics

In our cohort of patients, 101 individuals were included presenting a total of 319 visits. Due to logistic problems, no mRNA samples were drawn on 83 visits, mostly at visit 1 (day 6). Seventy-eight samples were excluded from the analyses because of impurities in the samples. In total, 158 patient visits were included in the analyses, representing 77 patients. Table 1 shows the patient characteristics of the 77 included patients whose mRNA data was used for the analysis. Laboratory results of each visit of the included

patients and the analyzed number of samples at each visit are summarized in Table 2. Biopsy proven acute rejection (BPAR) was diagnosed in 13 patients. In 10 of these patients, BPAR occurred within the first month after transplantation.

Table 1. Characteristics of included patients

Characteristics	**n=77**
Age (years)	51.8 ± 14.9
Weight (kg)	80.6 ± 17.1
Height (cm)	174 ± 10
Gender Male	57 (74%)
Ethnicity	
Caucasian	64 (83%)
Black	10 (13%)
Hispanic	2 (3%)
Asian	1 (1%)
Diabetes Mellitus present pre-TX	12 (16%)
Donor Living/Deceased	48/29 (62/38%)
Mismatches 0/1-3/4-6	4/39/34 (5/51/44%)
Patients with previous Tx	6 (8%)
PRA 0/1-25/26-100	59/12/6 (77/16/8%)
Delayed graft function	22 (29%)
ATG induction therapy	23 (30%)

ATG, anti-thymocyte globulin; PRA, panel-reactive antibodies; Tx, transplantation.

IMPDH activity and MPA concentrations

The daily MMF dose decreased from 2000 ± 224mg on day 6 to 1115 ± 401 mg on day 140, whereas the total plasma MPA trough level did not alter (Table 2). Total IMPDH activity pre dose was correlated to IMPDH gene expression of both type I and type II, measured pre transplantation and at all 4 posttransplant visits separately using linear regression. The correlation coefficients were weak (ranged from -0.28 to 0.058) and none of the correlations were significant (*P* values are ranged between 0.11 and 0.77). IMPDH activity pre dose was also correlated to the sum of both types of IMPDH gene expression, but no significant linear correlations were found (correlation coefficients ranged from 0.055 to 0.24; *P* values ranged from 0.13 to 0.75). Significant correlations were also not found at any visit between total and unbound predose MPA levels and the IMPDH type I and type II gene expression (correlation coefficients ranged from -0.30 to 0.10; *P* values ranged from 0.073 to 0.95).

Table 2. Immunosuppressive therapy and laboratory results of the analyzed patients on each of the four visits

	Day 6 (n=41)	Day 21 (n=37)	Day 49 (n=41)	Day 140 (n=39)	*P*[1]
Immunosuppressiva					
Daily dose MMF (range, mg)	2000 ± 224 (1000-3000)	1459 ± 505 (1000-2000)	1220 ± 419 (500-2000)	1115 ± 401 (500-2000)	<0.001
Daily dose tacrolimus (range, mg)	11.4 ± 3.7 (4-18)	9.3 ± 4.7 (2-20)	8.8 ± 4.1 (3-20)	6.6 ± 4.4 (2-20)	<0.001
Daily dose prednisolon (range, mg)	20.0 ± 0.0 (20-20)	16.6 ± 2.4 (15-20)	11.4 ± 3.7 (5-20)	3.4 ± 3.8 (0-20)	<0.001
Tacrolimus trough level (µg/L)	12.6 ± 6.0	10.8 ± 3.3	11.1 ± 4.2	9.0 ± 3.0	0.008
Total plasma MPA trough level (mg/L)[2]	2.08 ± 1.53	1.77 ± 1.38	1.99 ± 1.47	1.93 ± 1.55	0.83
Unbound plasma MPA trough level (mg/L)[2]	0.15 ± 0.19	0.10 ± 0.20	0.16 ± 0.16	0.095 ± 0.093	0.18
Clinical laboratory					
Serum albumin (g/L)	34 ± 4	38 ± 6	42 ± 4	43 ± 4	<0.001
AST (U/L)	50 ± 50	19 ± 8	19 ± 11	25 ± 11	<0.001
ALT (U/L)	80 ± 110	36 ± 30	21 ± 18	22 ± 15	<0.001
Serum creatinine (µmol/L)	255 ± 210	148 ± 63	160 ± 95	140 ± 40	<0.001
Estimated glomerulair filtration rate[3] (mL/min)	39.1 ± 24.2	50.9 ± 18.2	49.8 ± 20.2	52.9 ± 17.2	0.012
Haemoglobin (mmol/L)	6.3 ± 1.1	6.3 ± 0.9	6.8 ± 0.8	7.6 ± 0.9	<0.001
Haematocrit (%)	0.31 ± 0.053	0.32 ± 0.045	0.34 ± 0.038	0.38 ± 0.043	0.40
Thrombocytes (10^9/L)	199 ± 67	237 ± 94	251 ± 79	238 ± 53	0.013
Leukocytes (10^9/L)	9.9 ± 5.1	8.6 ± 2.9	8.8 ± 3.6	6.3 ± 2.1	0.002

[1] One-way analysis of variance; [2] Data presented ad median (range); [3] Estimated using MDRD equation.
ALT, alanine (amino)transminase (ALAT); AST, aspartate (amino)transaminase (ASAT).

Clinical outcomes

Pretransplant IMPDH type I and type II mRNA levels were higher in patients with a BPAR (n = 13) compared with patients without a BPAR episode (n = 47) after transplantation, and this difference showed a trend towards statistical significance (Fig. 1; $P = 0.052$ for IMPDH type I and $P = 0.058$ for IMPDH type II). On day 6 after transplantation, IMPDH mRNA type I and type II were significantly lower in patients with a BPAR (n = 13) compared to patients without a BPAR (n = 28) [Fig. 2; IMPDH type I: 4.7 (95%CI: 1.9–11.3) versus 11.5 (95%CI: 6.8–19.4) x 10^3 copies; $P = 0.026$ and IMPDH type II: 2.3 (95%CI: 0.9–5.9) versus 7.9 (95%CI: 4.5–13.8) x 10^3 copies; $P = 0.007$]. This difference was also significant when only patients with a BPAR within the first 30 days post-transplantation (n = 10) were analyzed [IMPDH type I: 6.1 (95%CI: 3.0–12.5) x 10^3 copies; $P = 0.044$ and IMPDH type II: 3.1 (95%CI: 1.4–6.9) x 10^3 copies; $P = 0.018$]. No significant differences were found in total and unbound MPA trough levels between patients with and without a BPAR [total MPA 1.98 (95%CI: 1.36–2.86) versus 1.84 (95%CI: 1.30–2.61) mg/L; $P = 0.57$ and unbound MPA 0.08 (95%CI: 0.04–0.17) versus 0.03 (95%CI: 0.01–0.06) mg/L; $P = 0.28$]. The IMPDH activity pre dose on day 6 was also not different between patients with and without a BPAR [23.4 (95%CI: 15.3–35.7) versus 20.3 (95%CI: 15.8–26.1) $\mu mol \cdot s^{-1} \cdot mol^{-1}$ AMP; $P = 0.26$]. The ROC analysis of the IMPDH type I mRNA level showed a significant AUC of 0.72 ($P = 0.027$). A cutoff value for IMPDH type I mRNA of 10.4 x 10^3 copies had a specificity of 67.9% (95% CI: 47.7–84.1%) and a sensitivity of 76.9% (95% CI: 46.2–95.0%) with a likelihood ratio of 2.39. The ROC analysis of the IMPDH type II mRNA level showed a significant AUC of 0.76 ($P = 0.008$). A cutoff value for IMPDH type II mRNA of 2.49 x 10^3 copies had a specificity of 89.3% (95% CI: 71.8–97.7%) and a sensitivity of 61.5% (95% CI: 31.6–86.1%) with a likelihood ratio of 5.74.

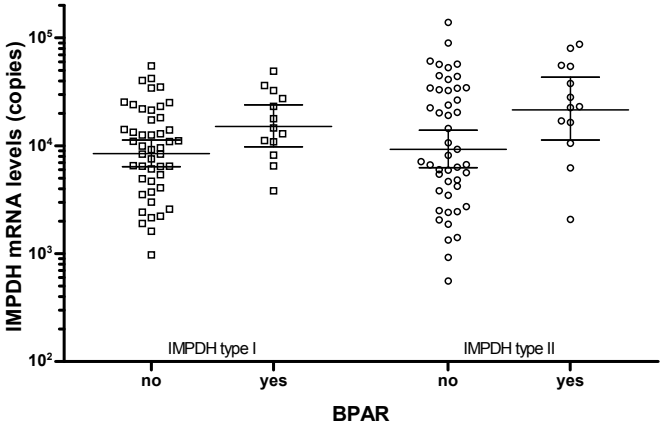

Figure 1. The expression of IMPDH mRNA type I (squares) and type II (circles) before transplantation in patients with a BPAR (n = 13) and without a BPAR (n = 47) episode after transplantation. The bars present the log-transformed mean with 95% confident interval.

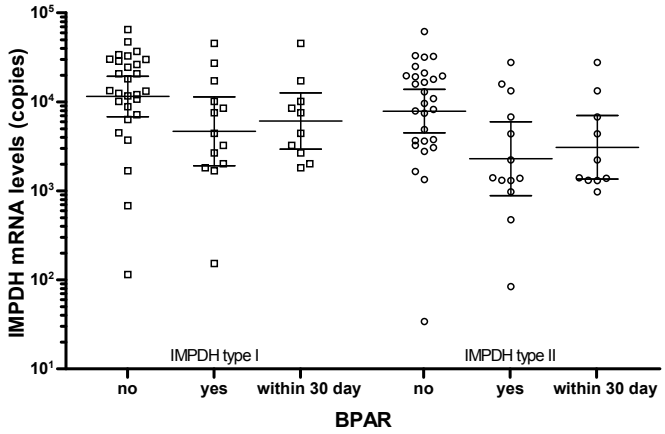

Figure 2. The expression of IMPDH mRNA type I (squares) and type II (circles) on day 6 after transplantation in patients with a BPAR (n = 13; within 30 days after transplantation n = 10) and without a BPAR (n = 28) episode after transplantation. The bars present the log-transformed mean with 95% confident interval.

At visit 4, patients who had lower IMPDH type I and type II mRNA levels had significantly more hematological adverse events (n = 8) compared to patients without these adverse events (n = 31) [IMPDH type I: 7.7 (95% CI: 3.5–16.7) versus 23.7 (95% CI: 13.3–42.3) x 10^3 copies; *P* = 0.012 and IMPDH type II: 6.9 (95% CI: 2.16–18.6) versus 22.3 (95% CI: 11.9–41.9) x 10^3 copies; *P* = 0.027]. No significant correlations were found for type I or type II for adverse events at all other visits (*P* values ranged from 0.17 to 0.94).

IMPDH gene expression over time

IMPDH gene expression changed over time after transplantation as shown in Figure 3. IMPDH type I and type II mRNA levels decreased directly after transplantation on day 6 compared with the mRNA levels before transplantation. The IMPDH type II mRNA expression decreased significantly from 12.1 (95% CI: 8.6–17.0) x 10^3 copies pre transplant to 5.3 (95% CI: 3.2–8.8) x 10^3 post transplant on day 6 (*P* = 0.005). After transplantation, the mRNA expression of IMPDH type I and type II increased from day 6 to day 140 (Table 3; *P* = 0.061 and *P* = 0.004, respectively). Predose IMPDH activity was higher before transplantation compared to all post transplant visits but did not change over time at the 4 visits after transplantation (Table 3; *P* = 0.49). The IMPDH mRNA levels reached a maximum level at day 140 and were not significantly different compared to the mRNA levels pre transplant. ATG induction therapy directly after transplantation did not influence the IMPDH mRNA type I and type II levels on day 6 [type I: 9.6 (95% CI: 2.6–35.9) versus 8.3 (95% CI: 5.2–13.4); *P* = 0.33 and type II: 3.9 (95% CI: 0.9–17.6) versus 5.9 (95% CI: 3.5–9.8); *P* = 0.82 for patients who received ATG versus those that not received ATG, respectively].

Table 3. IMPDH activity and mRNA expression of IMPDH type I and type II summarized for each visit

	Pre-Tx (n=60)	Day 6 (n=41)	Day 21 (n=37)	Day 49 (n=41)	Day 140 (n=39)	*P* value,[1] All occasions	*P* value,[1] Visit 1–4
IMPDH type I mRNA (x 10^3 copies)	10.0 (7.9 – 12.8)	8.6 (5.5 – 13.6)	14.8 (9.2 – 23.9)	17.3 (11.8 – 25.5)	18.8 (11.4 – 30.9)	0.019	0.061
IMPDH type II mRNA (x 10^3 copies)	12.1 (8.6 – 17.0)	5.3 (3.2 – 8.8)	11.6 (7.0 – 19.3)	14.5 (9.5 – 22.1)	17.6 (10.2 – 30.3)	0.005	0.004
IMPDH Activity pre-dose (μmol s^{-1} mol^{-1} AMP)	45.0±29.3	28.5±20.2	22.4±11.2	28.3±24.0	27.2±19.7	N.A.	0.49

Values are presented as geometric mean (95% confidence interval).

[1] One-way analysis of variance.

N.A., not applicable.

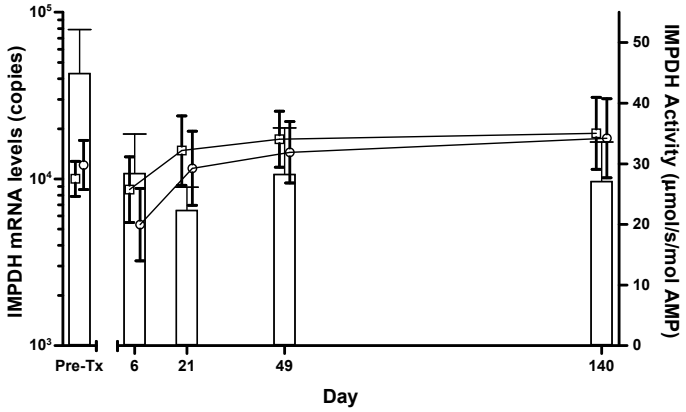

Figure 3. Changes in total IMPDH activity pre dose (bars), IMPDH mRNA type I (squares) and type II (circles) expression in patients over time after transplantation. The bars present the mean with 95% confident interval (log transformed for mRNA data).

DISCUSSION AND CONCLUSION

In this prospective longitudinal study, we have investigated the correlation between mRNA levels of the IMPDH type I and type II enzymes and total IMPDH activity and clinical outcomes in renal transplant recipients treated with MMF. The incorporation of PK and PD variability by monitoring of the MPA-mediated IMPDH activity might introduce a more accurate prediction of the therapeutic response than monitoring of the MPA plasma concentration alone. Monitoring IMPDH mRNA expression levels might be a more attractive method of monitoring the biologic effect of MMF treatment,[23] as RT-PCR is less labor intensive and less time consuming compared to the IMPDH activity assays.

This study confirms the upregulated expression of IMPDH type I mRNA over time after transplantation.[24,25] For IMPDH type II, mRNA conflicting data have been reported. Some studies showed stable IMPDH type II mRNA levels after transplantation,[25] whereas others found a decrease over time.[24] Our study showed a significant increase in IMPDH type II mRNA levels over time after transplantation, and the changes in type II paralleled those in type I mRNA. Our findings are in line with previously reported results that induction of IMPDH activity after prolonged exposure to MPA may be partially explained by the upregulation of both IMPDH type I and type II mRNA in PBMC.[25] The decreased expression of IMPDH mRNA levels of both types on day 6 compared with pretransplant expression may be due to the administration of high dose corticosteroids, as also suggested by others.[24] In a study in healthy volunteers, MPA induced a decrease in IMPDH type I expression but stable type II expression.[30] The rise in mRNA expression after day 6 in our study is presumably due to the MPA-induced inhibition of IMPDH activity. IMPDH mRNA expression is regulated by a negative feedback mechanism by guanine

nucleotides. The inhibition of IMPDH activity by MPA leads to a decrease in the intracellular depots of guanine nucleotides.[11] According to the studies performed by Jain *et al*,[15] IMPDH inhibitors do not seem to alter the regulation of IMPDH type I or type II mRNA or protein levels directly but may have an indirect role in the regulation. Considering the role of MPA on this feedback mechanism on IMPDH expression, it is not surprising that we could not find a correlation between mRNA levels and IMPDH activity in the blood samples drawn after transplantation. However, we expected to find a better correlation before transplantation, as at that time point no MPA was present, and IMPDH activity should be better correlated to gene expression. Also pretransplantation mRNA levels for IMPDH type I and type II were not related to IMPDH activity.

It was thought that IMPDH type I enzymes were constitutively present in the cells and that IMPDH type II would appear after immune activation.[4,5] However, in this study, IMPDH type I was also upregulated in PBMCs after immune activation by organ transplantation. Hence, both isoforms of IMPDH are responsible for the proliferation of the lymphocytes after transplantation and, therefore, both isoforms should be inhibited to decrease the proliferation of the lymphocytes and to prevent the graft for acute rejections. Also Dayton *et al*[14] concluded that both type I and type II IMPDH should be considered important targets for immunosuppressive therapy.

Previous studies used area under the mRNA–time curve (AUC) as variable for the IMPDH gene expression,[25,31] whereas other studies expressed the IMPDH gene expression as relative numbers compared to the expression before transplantation.[24,32] We chose here to use IMPDH mRNA predose levels because we were mostly interested in the changes in IMPDH mRNA gene expression over time. IMPDH mRNA levels fluctuate less over a dose interval than over a period of several months after transplantation.

In this same patient cohort, we did find a correlation between exposure to IMPDH activity over 12 hours and the incidence of BPAR,[33] with higher IMPDH activity being a risk factor for more BPAR. On day 6, IMPDH type I and type II mRNA levels were lower in patients with a BPAR compared with patients without a BPAR, and this difference was statistically significant ($P = 0.026$ and $P = 0.007$, respectively). It would seem plausible that patients with less mRNA have higher IMPDH activity and therefore higher immunoreactivity and higher rejection rates. However, the already mentioned MPA-induced inhibition of IMPDH activity and the resulting upregulation of mRNA expression obscures the correlation between mRNA levels and IMPDH activity. For the pre transplant time point, in the absence of MPA, the IMPDH mRNA levels in rejectors were higher compared to nonrejectors and this difference showed a trend towards statistical significance ($P = 0.052$ and $P = 0.058$ for IMPDH type I and type II, respectively). As shown in Figures 2 and 3, there is a considerable overlap in mRNA levels in rejectors and nonrejectors and it would be difficult to propose a cutoff level for use in clinical practice. Although the ROC analyses recommended cutoff values for IMPDH type I and type II mRNA levels,

the sensitivity and specificity for the cutoffs was poor (ranged from 62% to 89%). More studies are needed to evaluate the added value of monitoring IMPDH mRNA levels in patient management. We would also suggest performing such studies in patients not treated with MPA as especially in these patients IMPDH mRNA levels may reflect immune activation.

Only at visit 4, lower IMPDH type I and type II mRNA expression were significantly correlated to hematological adverse events (leukopenia, thrombocytopenia and/or anemia). No other significant correlations with IMPDH mRNA levels were found for hematological and gastrointestinal adverse events. MPA-related adverse events have also been found to have a poor correlation with MPA plasma concentrations.[34] Unfortunately, mRNA monitoring is not going to solve the unmet need for a biomarker that may assist in preventing MPA-induced toxicity.

The present study has also some limitations. From a cohort of 101 patients, only 77 patients were analyzed of whom mRNA samples were obtained. Due to logistic problems and impurity of samples, only a half of all samples was included and analyzed. This resulted in a maximum of up to 41 samples per post transplant visit and 60 samples pre transplant. This unintentional selection of the samples has introduced a selection bias. Because an overlap in the results of IMPDH mRNA expression between rejectors and nonrejectors was noticed, a patient cohort with less selection bias is needed to confirm our results.

In conclusion, in this prospective longitudinal study, IMPDH type I and type II mRNA levels were found to increase from day 6 to day 140 after renal transplantation. IMPDH activity was not correlated to mRNA levels, possibly due to the fact that the lower guanine nucleotide levels caused by MPA-related enzyme inhibition induce the expression of the IMPDH gene. However, also before transplantation in an MPA-free situation, mRNA levels were not correlated to IMPDH activity. Nevertheless, we did find a trend towards significantly higher IMPDH mRNA levels pre transplantation in rejectors compared with nonrejectors, and post transplant significantly lower IMPDH mRNA levels in rejectors. Although the regulation of the expression of the 2 isoforms is presumed to be different, in this study, the changes in the expression of type I mRNA closely paralleled those of type II.

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Chapter 5

Hematopoietic stem cell transplant recipients

Chapter 5.1

Pharmacodynamic monitoring of MMF therapy by measuring IMPDH activity after allogeneic hemaetopoietic stem cell transplantation: a pilot study

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ABSTRACT

Mycophenolate mofetil (MMF) is used more frequently in hematopoietic SCT (HSCT) recipients to prevent acute GVHD. The active metabolite mycophenolic acid (MPA) inhibits the activity of the target enzyme inosine monophosphate dehydrogenase (IMPDH). In this pilot study we aim to explore for the first time the pharmacodynamics of MPA by measuring IMPDH activity in a prospective, unselected cohort of allogeneic HSCT recipients.

Patients suffering from serious neutropenia, thrombocytopenia and anemia had a significantly lower IMPDH activity. No significant differences were found in IMPDH activity compared to patients developed GVHD.

However IMPDH activity was not significantly different, MPA concentrations in renal transplant were significantly higher than in HSCT recipients. IMPDH enzymes could be more sensitive for MPA inhibition in HSCT patients. Over all visits it is shown that the inter-patient variability for the concentration-effect correlation is large.

We showed significant correlations between IMPDH activity and the toxicity of MMF therapy in HSCT patients. We hypothesis IMPDH enzymes are more sensitive for the inhibition of MPA in HSCT patients compared with renal transplant patients. The clinical relevance of the results of this pilot study has to be confirmed in a more selected and larger cohort of HSCT patients.

INTRODUCTION

Mycophenolate mofetil (MMF) is a prodrug for the pharmacologically active compound mycophenolic acid (MPA). MMF has been used for more than a decade as a immunosuppressive agent in the treatment after kidney transplantation to prevent acute rejection.[1] MPA is rapidly absorbed from the gut, maximum MPA concentrations generally occur within 1 hour after oral MMF administration.[2] MPA is primarily metabolized in the liver into the inactive metabolite phenolic MPA glucuronide (MPAG) and into the lesser extent but active metabolite acyl MPA glucuronide (AcMPAG). The glucuronide metabolites are excreted into the bile, and in the intestine MPAG is deconjugated back to MPA. In the colon, MPA is reabsorbed to complete the enterohepatic recirculation (EHC), which causes a second MPA peak concentration approximately 6 to 12 hours after administration.[2,3] Finally, the metabolites are mainly excreted by the kidneys.[2]

MPA is a non-competitive, reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH), which has a crucial role in the proliferation of lymphocytes. IMPDH is the key enzyme in the de novo synthesis of the purine nucleotide guanosine triphosphate (GTP) and induces the rate-limiting step in the synthesis.[4,5] Previous studies in renal transplantation recipients have shown that determination of IMPDH activity may be a relevant pharmacodynamic parameter.[6-10] An inverse relation between MPA concentration and IMPDH activity was seen in renal transplant recipients.[11,12] There is a great inter-individual variability in the activity of IMPDH pre and post transplantation.[6,7] A high IMPDH activity pre-transplant was associated with MMF dose reduction and acute rejection after renal transplantation.[7] Post-transplant, an elevated exposure to IMPDH activity was also correlated to acute rejection.[9]

MMF has been shown to be a potent immunosuppressive drug with beneficial effects on acute rejection rates and on long-term outcomes in comparison with azathioprine in kidney transplantation.[1,13,14] Due to this success, MMF is also used in allogeneic hematopoietic stemcell transplantation (HSCT) recipients to prevent acute and chronic graft-versus-host disease (GVHD) as well as to promote engraftment.[15-17] For this indication, it is supported that MMF has a more favorable toxicity profile then methotrexate, which induces mucositis.[15,17,18] Pharmacokinetic research showed that standard MMF dosing in HSCT patients based on data from renal transplantation recipients resulted in low MPA trough levels compared to levels from renal transplantation patients.[15,16,19-22] Van Hest *et al* found that the total MPA exposure in HSCT recipients is low because of high MPA clearance and short apparent MPA half-life in comparison with reference values from renal transplantation.[23] MMF dose and dose interval applied in HSCT recipients are largely based on pharmacokinetic data from renal transplant studies. The starting daily MMF dose generally is 30 mg/kg orally divided over two or – as used more

frequently – over three doses, which in most HSCT patients results in the standard MMF dose recommended in renal transplantation (1000 mg twice daily).[19,20,22-25]

MPA is extensively bound to albumin (97–99%) in healthy patients;[26] however, binding is altered in patients with significant renal dysfunction, liver disease, or other cause of hypoalbuminemia. Increased percentage unbound MPA has been associated with decreased plasma albumin levels in pediatric renal transplant recipients.[27-28] Similarly, *in vitro* studies have demonstrated increased percentage unbound MPA when plasma albumin decreased.[29] The pharmacological activity of MPA, which is the inhibition of IMPDH, is a function of unbound MPA. However, for this crucial role for unbound MPA, only *in vitro* data are currently available.[28,29] The measurement of free, pharmacologically active MPA would therefore appear to be the correct approach for therapeutic drug monitoring.

In this pilot study we aim to explore for the first time the pharmacodynamics by measuring IMPDH activity in an unselected cohort of allogeneic HSCT recipients. We studied the correlations of IMPDH activity to MPA concentrations, clinical outcome, and adverse events.

PATIENTS AND METHODS

Patients

For the purpose of this study in a prospective cohort of allogeneic hematopoietic SCT (HSCT) patients we preformed a MPA pharmacokinetic and pharmacodynamic assessment at one and two or at two and four weeks after transplantation. Patients were recruited in the Erasmus University Medical Center – Daniel den Hoed Cancer Center. Patients were included regardless of underlying disease, conditioning regimen, donor type, or patient condition. The study complied with the Declaration of Helsinki and all patients gave informed consent.

Conditioning regimens and co-medication

The day of the HSCT is designated day 0. Patients up to 40 years of age with a related HLA-identical siblings (Sib) donor received a standard myeloablative conditioning regimen consisting of cyclophosphamide 60 mg/kg i.v., on days –5 and –4 and TBI, 6 Gy on day –2 and –1. A non-myeloablative, reduced intensity conditioning (RIC) regimen was applied in patients older than 40 years. This regimen consisted of fludarabine 30 mg/m^2 i.v. on days –5 to –3 (total 3 doses) and TBI, 2 Gy on day –1. An alternative RIC regimen consisted of fludarabine 25 mg/m^2 i.v. and cyclophosphamide 500 mg/m^2 i.v. on days –7 to –3 (total 5 doses). Patients who received HSCT from a matched unrelated donor (MUD) underwent the same conditioning regimens as patients with a Sib donor. In ad-

dition, they were given rabbit anti-thymocyte (rATG) 2 mg/kg i.v. on days –7 to –4 (total 4 doses).

Patients with cord blood donor transplantation received a RIC regimen. This regimen consisted of fludarabine 40 mg/m^2 i.v. on days –5 to –2 (total 4 doses), cyclophosphamide 60 mg/kg i.v. on day –6 and TBI, 2 Gy on day –1.

For prevention of serum sickness, prednisone was administered in patients receiving rATG 0.5 mg/kg twice daily i.v. on days –7 to –4 (total 8 doses) and once daily on days –3 to +7.

Immunosuppressive therapy for prophylaxis of GVHD consisted of CsA and MMF. CsA was started on day –3. Patients who were undergoing the myeloablative conditioning, CsA i.v. (Sandimmune, Novartis Pharma BV, Arnhem, The Netherlands) was administered in doses of 1.5 mg/kg twice daily. When these patients could tolerate oral medication, they were converted to oral CsA (Neoral, Novartis, Arnhem, The Netherlands) in an identical dose. In patients receiving non-myeloablative conditioning, oral CsA was given twice daily in doses of 6.25 mg/kg. CsA trough levels, determined in whole blood by the EMIT assay, were targeted at 250-350 mg/L, but higher levels were accepted during the first week in patients treated with non-myeloablative conditioning. Oral MMF (CellCept, Roche, Woerden, The Netherlands) was started 5 to 10 hours after transplantation with a dose of 10 mg/kg three times daily oral (daily dose 30mg/kg), rounded up or down to the nearest 250 mg MMF increment. Patients with severe mucositis received MMF i.v. The higher frequency used in HSCT patients compared to the standard twice daily frequency in kidney transplant patients was used because several studies proved that the MPA concentrations and area under the curve (AUC) in HSCT patients were significant lower with the standard dosing frequency compared to in kidney transplant patients.[15,16,19-22] Higher dosing frequencies resulted in higher MPA concentrations.[22]

Infection prophylaxis consisted of ciprofloxacin, 500 mg orally twice daily, and fluconazole, 200 mg orally once daily, started on the first day of the conditioning regimen, until the granulocyte count was above 0.5x10^9/L. All patients received valcyclovir, 500 mg three times daily, from the first day of the conditioning regimen until 1 year post-transplantation. For the prevention of a *Pneumocystis carinii* infection, all patients received co-trimoxazole, 480 mg once daily, when the administration of ciprofloxacin and fluconazole was stopped, until 1 year after transplantation.

Sampling procedure

Samples for analysis MPA level were collected in EDTA-containing 4 mL tubes. Samples for pharmacodynamic analysis of IMPDH activity were collected in lithium heparin (Li-hep)-containing 4 mL tubes. Samples were drawn pre-dose and 2 hours after dosing, at days +7 and +14 or days +14 and +28. After collection, EDTA blood samples were centrifuged at 3000*g*, and plasma was stored frozen at –80 °C until analysis. PBMCs were

isolated form the Li-hep blood within 24 hours after collection. The PBMCs were lysated with ice-cold water and the cell lysate was stored at –80°C until analysis.[11]

Measurement of IMPDH activity and MPA concentrations

A validated non-radioactive HPLC method by Glander *et* Sombogaard[11] was used to measure the IMPDH activity. In summary, PBMC were isolated from lithium heparin-anticoagulated whole blood using Leucosep tubes with Ficoll-Paque (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). After one washing step, the aliquot was resuspended in 250µL ice-cold water and stored at –20°C to lysate. To 50µL PBMC lysate 130µL reaction buffer with inosine monophosphate (IMP) and β-nicotinamide adenine dinucleotide (β-NAD^+) was added to start the incubation of enzyme reaction at 37°C and moderately shaken at 800 rpm (Thermomixer, Epperndorf, Hamburg, Germany) for 2.5 hours. The reaction was stopped by adding ice-cold perchloric acid. After centrifugation potassium carbonate was added to the supernatant to neutralize the solution. The supernatant was transferred into HPLC injection vials for determination of produced xanthine monophosphate (XMP) and present AMP. The rate of XMP production by IMPDH from PBMCs was measured and was normalized to the measured intracellular AMP. Enzyme activity was expressed as produced XMP (µmol) per time unit (s) per present AMP (mol) for normalizing the activity to the destructed cells. The imprecision of the IMPDH activity assay was 6.6 to 11.9% and the coefficient of variation of the IMPDH activity ranged from 0.6 to 3.4%.

For the purpose of this study MPA plasma concentrations were measured with a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method optimized for our equipment.[30] To a sample of 50µL plasma 200µL zinc sulfate 0.1mol/L and 500µL acetonitrile with 3mg/mL of the internal standard carboxy butoxy ether of MPA (MPAC) was added for determination of the total plasma concentrations of MPA. The coefficient of variation of the inter- and intraday imprecision of the used method was less than 5% and 3% respectively for the total plasma MPA concentrations (range: 0.2 to 50 mg/L).

Clinical outcome

Patients were suspected of GVHD episode based on clinical grounds or biopsy proven were graded according to the standard response and toxicity criteria.[31] Hematological adverse events (neutropenia, leukopenia, thrombocytopenia and anemia) were also graded according this standard. Neutropenia grade 3 and 4 was defined as absolute neutrofil count less than 1.0 and less than $0.5x10^9$/L respectively. Leukopenia was defined as a leukocyte count less than 2.0 and less than $1.0x10^9$/L for grade 3 and 4 respectively. Thrombocytopenia was defined as a thrombocyte count less than 50.0 and less than $25.0x10^9$/L and anemia as a hemoglobin concentration below 5.0 and below 4.0mmol/L for men and women for grade 3 and 4 respectively.

Statistical analysis

All statistical tests were performed using SPSS software version 16.0 (SPSS, Chicago IL, USA) and a P-value less than 0.05 was considered significant. Bivariated correlations coefficients between data were analyzed with Pearson's. Unrelated data was tested by a Mann-Whitney U test for two variables and by a Kruskal-Wallis test for more than two variables. Related data was tested by a Wilcoxon Signed Ranks test. Post-hoc analyzes on the data was preformed by a Dunnett's test. Values are reported as median (range), unless otherwise stated.

RESULTS

Patient characteristics

From May 2007 until May 2008, a total of 24 patients were included into the study, from which in total on 45 occasions samples were taken. On 8/45 occasions (6 different patients) MMF was administered i.v. on the day of sampling and these data were excluded, because of different pharmacokinetics of the i.v. formulation. Two patients were only receiving MMF i.v. and not oral at the days of sampling, so these patients were also excluded from the study. Finally, data of 37 occasions from 22 patients were analyzed. Baseline characteristics of these patients and the kind of transplantation are summarized in Table 1. Due to the non-interfering inclusion of the patient, a disproportional distribution of patients over the visits was accomplished: 7 patients were sampled 7 and 14 days post-transplant, 8 patients at day 14 and 28, 5 patients only at day 14 and 2 patients only at day 28 post-transplant.

Laboratory data and adverse events

Patients who have received a HSCT could have seriously adverse events, some induced by medication such as MMF. Figure 1 shows the corresponding IMPDH activity for four common hematological adverse events after HSCT and the use of MMF. Laboratory results and severe or life-threatening hematological toxicity (grade 3 or 4) of each visit are summarized in Table 2. For each adverse event grade 3 and 4 (severe and life-threatening) were pooled together and compared to the pooled data of adverse effect grade 0 to 2. Patients suffering from serious neutropenia, thrombocytopenia and anemia had a significantly lower IMPDH activity compared to the group of patients with none or a mild grade of these adverse events (P-values <0.01, 0.02 and 0.03 respectively). There was no significantly difference in IMPDH activity observed for leukopenia (P = 0.43). Since there was a significant difference between the visit for leukopenia and neutropenia (table 2), these adverse events were also analyzed separately for each visit. At visit day 7 and day 14 IMPDH activity was also significantly lower in patients suffering from serious neutro-

penia (grade 3 and 4) compared to neutropenia grade 0 to 2 (P = 0.05 for both visits). At these visit days, no differences were found for IMPDH activity for leukopenia.

Table 1. Patient baseline and transplantation characteristics

Characteristics	**n=22**
Gender Male van (n)	15 (68%)
Age (yr)	53 (18 - 65)
Body weight (kg)	80 (58 - 105)
Diagnosis van (n)	
Acute myeloid leukemia / Myelodysplastic syndrome	10
Acute lymphoblastic leukemia	3
Non-Hodgkin's lymphoma	2
Waldenström's macroglobulinemia	2
Myelofibrosis	2
Chronic myeloid leukemia	1
Multiple myeloma	1
Aplastic anemia	1
Conditioning regimen van (n)	
Myeloablative	5
Non-myeloablative	17
Donor Sib/MUD/Cord van (n)	5/14/3

MUD, matched unrelated donor; Sib, sibling.

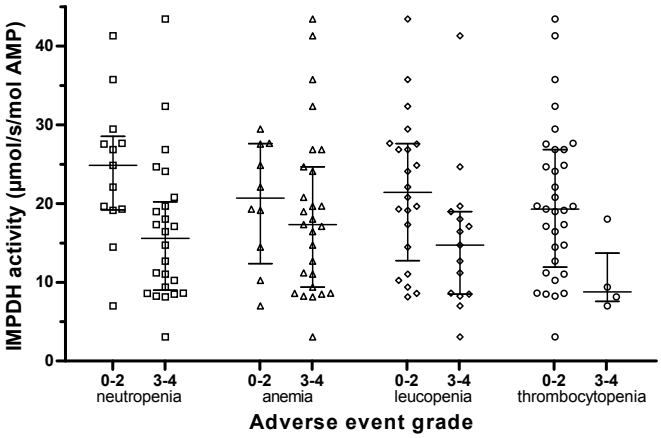

Figure 1. IMPDH activity in patients suffering from none to mild adverse events (grade 0-2) compared with patients suffering from severe to live-threatening (grade 3-4). Significant differences were seen for neutropenia, thrombocytopenia and anemia. The IMPDH activity was not significantly correlated to leukopenia. The bars are presenting the interquartile interval and the horizontal lines the median of the IMPDH activity of the groups.

Table 2. Laboratory results of the patients on each of the three visits

Visit (day)	7 (n=7)	14 (n=20)	28 (n=10)	P-value[2]
Serum albumin (g/L)	38 (30 - 40)	35 (25 - 42)	38 (30 - 41)	0.49
Bilirubin (µmol/L)	23 (17 - 56)	15 (7 - 132)	12 (6 - 51)	0.09
Alkaline phosphatase (U/L)	57 (37 - 151)	84 (50 - 553)	87 (25 - 111)	0.28
Y-glutamyl transferase (U/L)	54 (24 - 67)	42 (19 - 269)	43 (21 - 100)	0.96
Serum creatinine (µmol/L)	84 (53 - 94)	86 (38 - 141)	113 (56 - 271)	0.36
Glomerulair filtration rate (ml/min/1.73m^2)[1]	74 (60 -147)	68 (35 - 237)	59 (16 - 151)	0.37
Cyclosporin trough level (µg/L)	398 (300 - 551)	366 (234 - 722)[3]	277 (232 - 380)[3]	0.01
Haemoglobin (mmol/L)	6.0 (5.5 - 7.6)	5.4 (4.6 - 7.9)	6.0 (4.4 - 7.2)	0.21
Haematocrit (l/L)	0.27 (0.25 - 0.34)	0.24 (0.19 - 0.33)	0.28 (0.19 - 0.33)	0.09
Thrombocytes (10^9/L)	64 (6 - 297)	48 (12 - 503)	112 (30 - 334)	0.48
Leukocytes (10^9/L)	0.49 (0.06 - 6.0)[3]	0.65 (0.15 - 3.3)[4]	3.2 (1.2 - 10.2)[3,4]	<0.01
Neutrocytes (10^9/L)	0.38 (0.05 - 5.8)	0.37 (0.05 - 1.5)[4]	2.2 (0.6 - 9)[4]	<0.01
Neutropenia (grade 3 or 4)	5 (71%)[3]	17 (85%)[4]	2 (20%)[3,4]	<0.01
Leukopenia (grade 3 or 4)	6 (86%)[3]	18 (90%)[4]	3 (30%)[3,4]	<0.01
Thrombocytopenia (grade 3 or 4)	3 (43%)	10 (50%)	2 (20%)	0.30
Anemia (grade 3 or 4)	0 (0%)	3 (15%)	1 (10%)	0.55

[1] Estimated GFR using MDRD; [2] A Kruskal-Wallis test was used to compare the means over all three visits. Significant differences were tested post-hoc using a Dunnett's test; [3] $P < 0.01$ post-hoc analysis, compared to the value with the same symbol; [4] $P < 0.05$ post-hoc analysis, compared to the value with the same symbol.

In the first six months after transplantation grade I (mild moderate) acute GVHD was developed in 4 patients (18%), 10 patients (45%) had grade II (severe) and 1 patient (5%) developed grade III (life-threatening) GVHD. In the first month after transplantation 7 patients (32%) developed a GVHD and in the second month 4 patient (18%). The median time to GVHD was 39 days after transplantation and ranged from 12 to 177 days. IMPDH activity on day 14 in patients developed a GVHD grade II or higher within 40 days after sampling (n=7) was compared with patients without a GVHD (n=13) (Figure 2). There were no significant differences found in IMPDH activity measured pre-dose and 2 hours after administration of MMF comparing patients without GVHD to patient with GVHD.

IMPDH activity and MPA concentrations

IMPDH activity on t=2h was inhibited compared to t=0h as a result of the increased MPA concentrations on t=2h compared to t=0h (Table 3). Figure 3 showed the IMPDH activity for all three visits. On every visit the IMPDH activity at 2h post MMF dose is inhibited compared to 0h (pre-dose). The median IMPDH activities at 2h are 10.6, 10.5 and 13.0 µmol/s/mol AMP respectively for the three visits. No significant differences are observed pre- and post-dose over the visits for IMPDH activity levels and MPA concentrations and the daily MMF dose did not significantly changed over the visits. In Figure 3 it is notice-

able that patients with a relative high IMPDH activity pre-dose have a stronger inhibition (per cent) of IMPDH activity at t=2h. The slope of the curves of these patients is in Figure 3 steeper than patients with already a lower IMPDH activity, what results in a gentler to almost horizontal slope of the curve.

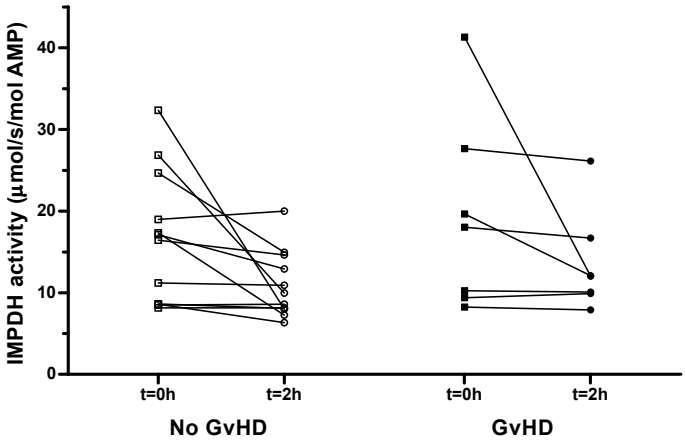

Figure 2. IMPDH activity in patients who developed GVHD grade II or higher (n=7) within 40 days after sampling on day 14 compared to patients who did not develop GVHD (n=13). IMPDH activity measured pre-dose are for each individual patient connected to the corresponding IMPDH activity measured 2 hours post-dose.

Table 3. IMPDH activity and MPA and MPAG pharmacokinetic of each visit

Visit (day)	**7 (n=7)**	**14 (n=20)**	**28 (n=10)**	**P-value[1]**
Daily dose MMF (mg)	2500 (1750 - 3000)	2500 (1500 - 3500)	2625 (2000 - 3500)	0.77
Dose MMF at t=0h (mg)	750 (750 – 1000)	750 (500 – 1500)	875 (500 – 1500)	0.75
IMPDH Activity t=0h (µmol/s/mol AMP)	19.7 (11.0 - 27.6)	17.2 (8.2 - 43.4)	20.0 (3.1 - 35.7)	0.75
IMPDH Activity t=2h (µmol/s/mol AMP)	10.6 (2.8 - 20.3)	10.5 (6.3 - 43.2)	13.0 (3.2 - 25.8)	0.98
P-value[2] t=0h vs. t=2h	0.001	<0.001	<0.001	N.A.
MPA concentration t=0h (mg/L)	0.73 (0.20 - 1.70)	0.54 (0.10 - 1.18)	0.76 (0.22 - 2.32)	0.40
MPA concentration t=2h (mg/L)	2.50 (0.71 - 5.27)	2.66 (1.08 - 11.45)	2.22 (0.92 - 20.23)	0.90
P-value[2] t=0h vs. t=2h	0.011	<0.001	0.009	N.A.

[1] Kruskal-Wallis test; [2] Wilcoxon Signed Ranks test.
N.A., not applicable.

In Figure 4 the concentration-effect curves of each visit are showed. Over all visits it is shown that the inter-patient variability for the concentration-effect correlation is large. Some patients showed small differences in MPA concentration between pre- and

post-dose, other large differences. This phenomenon was also seen in IMPDH activity. It was not obvious that patients with a small difference in MPA concentration also had a small difference in IMPDH activity.

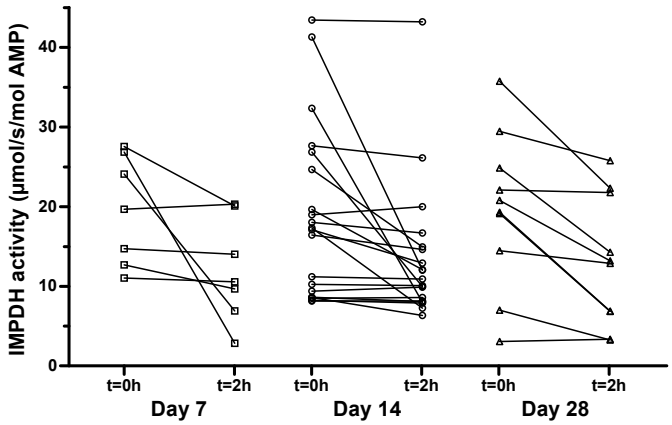

Figure 3. The inhibition of IMPDH activity per patient per visit after administration of MMF. Individual curves showed in general a lower IMPDH activity on 2h post-dose compared to 0h (pre-dose).

DISCUSSION

The last years MMF is used more frequently after HSCT for the prevention and treatment of acute GVHD with successful results.[15-17] The applied dose and dose interval of MMF were based on those used in renal transplant recipients. So, information from new pharmacokinetic studies of MMF in HSCT patients has been gathered to find a specific dose regimen of MMF for this patient group.[16,22,32] The interest in pharmacodynamic monitoring of MMF in renal transplant recipients by measuring IMPDH activity has been increased over the last years. Several correlations have been found in clinical outcomes and IMPDH activity.[7,9] Therefore, a pharmacodynamic approach to monitoring MMF therapy in HSCT patients may also applicable. This pilot study in 22 HSCT patients had the aim to investigate the pharmacodynamics of MMF by measuring IMPDH activity in HSCT patients under oral MMF therapy.

The present study showed a direct inverse relationship of MPA concentrations and IMPDH activity, which already have been seen in renal transplant recipients.[12] In a prospective study of 101 *de novo* renal transplant recipients the IMPDH activity at day 6 after transplantation was in the same range; pre-dose IMPDH activity was 20.7 µmol/s/mol AMP and 2 hours post-dose 13.5 µmol/s/mol AMP.[33] The observed IMPDH activities in HSCT patients in this study are similar to renal transplant patients, pre-dose IMPDH activity was 19.7 and 2h post-dose was 10.6 µmol/s/mol AMP at day 7 (p=0.22 and p=0.10

respectively). However, a comparison of total MPA concentrations in renal transplant and HSCT recipients showed a vast difference: pre-dose 2.41 mg/L and 2h post-dose 4.34 mg/L for renal transplant recipients and pre-dose 0.73 mg/L and 2h post-dose 2.50 mg/L for HSCT recipients ($p<0.001$ and $p<0.01$ respectively). It is remarkable that lower MPA levels in HSCT recipients resulted in the same IMPDH activity levels compared to

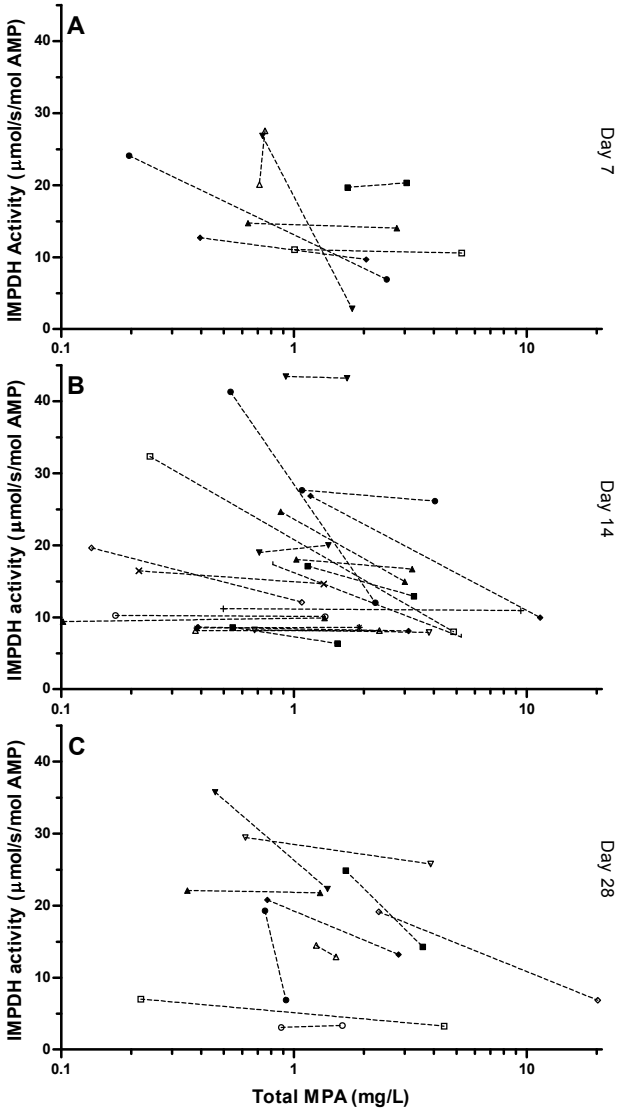

Figure 4. Concentration-effect curves for IMPDH activity vs. MPA concentrations at day 7 (A), day 14 (B) and day 28 (C) after transplantation. In each curve the individual patients are showed as dotted lines, connecting the pre-dose point with the 2h post-transplant point.

renal transplant recipients. A possible reason could be that the IMPDH enzymes in HSCT patients are more sensitive for MPA inhibition. To establish this hypothesis, more sophisticated analyzing methods are suggest for determining baseline IMPDH activity (E_0) and the sensitivity of IMPDH enzymes for MPA (IC_{50}) in MMF treated patients after renal and stem cell transplantation. The pharmacodynamic effect of both MMF regimens is the same; the inhibited IMPDH activity was equal in both patient groups using 1000mg twice daily in renal transplant recipients and 10mg/kg thrice daily in HSCT recipients.

In renal transplant recipients it is already confirmed that there is a large inter-patient variability in IMPDH activity.[6,7] This small cohort of HSCT patients showed also a large inter-patient variability in IMPDH activity and the concentration-effect correlation.

Because of the small sample size of this pilot study, adverse events of all three visits were pooled together for these analyzes. A significant correlation was found between IMPDH activity and hematological adverse events. Patients who suffered from severe (grade 3) or live-threatening (grade 4) neutropenia, thrombopenia or anemia had a significantly lower pre-dose IMPDH activity. IMPDH activity was measured in PBMCs (lymphocytes and monocytes) and corrected for lysated cells in the samples by using AMP concentration.[11] However, no significant relation was found for leukopenia with IMPDH activity. Based on this pilot study, IMPDH activity could be a useful tool to determine MMF toxicity in HSCT patients.

Of the 22 included patients, 11 patients developed GVHD grade II or III within six months after transplantation. For the comparison of IMPDH activity between patients with and without GVHD, data of day 14 after transplantation was used. Patients who developed GVHD 40 days after sampling were scored as non-GVHD patients. No significant correlations were found for IMDPH activity, neither with pre-dose IMPDH activity nor 2 hours post-dose IMPDH activity. It is still uncertain whether IMPDH activity – pre-dose and minimal activity – could be a tool to predict GVHD in HSCT recipients. A more selected and larger cohort of patients has to give more clarity if IMPDH monitoring in HSCT patients could be a useful tool to predict and establish the efficacy and toxicity of MMF therapy.

This pilot study is the first study in HSCT patients where MMF treatment was monitored using IMPDH activity. We showed significant correlations between IMPDH activity and the toxicity of MMF therapy in HSCT patients. A pharmacodynamic approach to monitoring the treatment of MMF in HSCT patients could be useful. IMPDH activity was similar to renal transplant recipients, but MPA levels were lower in HSCT patients. Therefore, we hypothesized that IMPDH enzymes are more sensitive for the inhibition of MPA in HSCT patients compared with renal transplant patients. The clinical relevance of the results of this pilot study has to be confirmed in a more selected and larger cohort of HSCT patients.

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Chapter 6

General discussion

INTRODUCTION

Immunosuppressive drugs are used after organ transplantation to prevent acute rejection of the graft by the immune system of the recipient. The balance between the efficacy and the toxicity of the used dosing regimens of immunosuppressive drugs is fragile and gives ample reasons to search for the optimal combination of drugs and dosing levels. Under-immunosuppression may lead to acute rejection and over-immunosuppression will lead to serious adverse events and toxicity, and often presents as renal failure, malignancies or infections. To find this balance, therapeutic drug monitoring (TDM) strategies measuring drug levels or dosing based on body weight are routinely used to find the optimum dose for the applied immunosuppressive drugs.[1] However, for one of the most frequently used immunosuppressive drugs – mycophenolate mofetil (MMF) – a fixed dose of 1000 mg twice daily is recommended by the manufacturer. If dose adjustments are made, they are typically based on the clinical situation of the patient when adverse events occur or when an acute rejection is suspected. Over the last decade, more and more research has been done to develop TDM strategies for MMF to balance efficacy and toxicity.[1,2] Pharmacokinetic (PK) research has shown that (i) an area under the concentration-time curve (AUC) of the active moiety mycophenolic acid (MPA) below 30 h·mg/L is associated with an increased risk for acute rejection,[3,4] (ii) standard dose therapy will result in a 10-fold variability in MPA exposure between patients[3,5,6] and (iii) despite a stable MMF dose an increase in MPA exposure is found over time after transplantation.[5-7] The high observed inter-patient variability in MPA exposure and the relatively narrow therapeutic window of MPA plead for good TDM strategies. Initially such strategies were strictly focused on the PK of MMF.

PHARMACODYNAMIC MONITORING OF MMF

A pharmacodynamic (PD) approach for monitoring MMF therapy may be useful besides PK monitoring. MPA exerts its immunosuppressive effect by inhibition of the enzyme inosine 5'-monophosphate dehydrogenase (IMPDH), which leads to a decreased proliferation of T- and B-lymphocytes.[8] Over the years various IMPDH activity assays have been developed to measure the IMPDH activity. A large inter-patient variability in IMPDH activity has been observed in several studies. In pre-transplant blood samples as well as in post-transplant samples the inter-patient variability in IMPDH activity is more than 10-fold measured in dialysis patients, renal transplant recipients and healthy volunteers. The intra-patient variability varied from 7.1 to 26.0% measured over one month. A 2-fold increase of IMPDH activity was observed over two years in long-term MMF treated patients.[9-11] It is thought that the non-protein bound MPA concentration

is the pharmacologically active fraction that inhibits IMPDH.[12] MPA is highly bound to plasma proteins for 97% to 99% in patients with normal renal and liver function,[12] but unbound fractions up to 18.3% in a patient who suffered from biliary obstruction have also been reported.[13] Therefore, measuring IMPDH activity could better reflect the immunosuppressive action than measurement of the total or unbound MPA plasma concentrations. Monitoring IMPDH activity could be an attractive approach to monitor MMF therapy, because it integrates the variability of the PK and the PD of MMF.

The measurement of PD effects of MMF by determination of IMPDH activity may provide a more direct insight in the immunosuppressive activity of MMF therapy *in vivo*. Dosing MMF based on IMPDH activity may help to increase the efficacy and decrease the toxicity of MMF therapy. We aimed to investigate whether differences in IMPDH activity could predict effectiveness and toxicity of MMF, and how total and unbound MPA concentration affects IMPDH activity. We here discuss how the combined main findings of the presented studies can contribute to novel methods that aim to optimize and individualize MMF therapy.

MEASURING IMPDH ACTIVITY

As a first requirement for the establishment of a prospective study in PD monitoring of IMPDH activity in transplanted patients we needed to develop a precise, accurate and practical method to measure the IMPDH activity. All published IMPDH activity assays are based on an *ex vivo* transformation of the added substrate IMP to the enzyme product xanthine monophosphate (XMP). Firstly, the IMPDH enzyme has to be isolated and purified from the patient's blood. Secondly, the isolated IMPDH enzyme facilitated the biotransformation of inosine monophosphate (IMP) into XMP and the product XMP has to be measured. Finally, the measured amount of product needs to be normalized for the amount of IMPDH enzyme and the reaction time. In order to develop a reliable method to measure the IMPDH activity, these three major steps of the IMPDH activity assay were optimized.

The first step is to choose the right cell fraction to measure the effect of MPA and isolate these cells from whole blood. High-quality isolation is characterized by minimizing manipulation of the cells and preventing washing out intracellular MPA. We chose to isolate peripheral blood mononuclear cells (PBMCs), which contain most of the immune cells on which MPA has its immunosuppressive effect. We modified the isolation using Ficoll-Paque by reducing the number of washing steps mentioned in the manufacturer's protocol of the cells so that there was a limited manipulation of cells. For our assay only 2.5 mL heparinized whole blood was required per sample, which forms an advantage compared to previously developed assays that required higher volumes of blood (4 to 30

mL). The method we developed for isolation of the IMPDH enzyme from PBMCs is easier than the method isolating CD4+ T-cells (Vethe *et al*[14]) which needs more sophisticated laboratory equipment and trained employees. Using PBMCs approaches the target cells of MPA better than using whole blood (Albrecht *et al*[15]) or erythrocytes (Montero *et al*[16]). We found a strong correlation between the MPA concentration in the lysate samples and MPA plasma concentration. This demonstrates that the used enzyme lysates have a MPA concentration that is directly correlated to the MPA concentration in plasma. This correlation is necessary to correlate the IMPDH activity measured in an *ex vivo* setting to the *in vivo* MPA plasma concentration. Nevertheless, the correlation does not mean automatically that the intracellular MPA concentration is equal to the MPA concentration in the lysate samples.

The second step in the IMPDH activity assay is the *ex vivo* incubation of the IMPDH enzyme lysate and the measurement of the enzyme product XMP. The composition of the used reaction mixture and the reaction conditions were close to the *in vivo* environment regarding temperature, pH, salts and physiological buffer. The mixture contained surplus substrate, co-substrate and potassium to contribute the enzymatic reaction of IMPDH. Different assay conditions were tested to investigate the influence of the incubation time and the dilution of the samples on the reaction. The time of incubation of the enzyme lysate has no influence on the formation rate of the product XMP, but disturbed significantly the new used internal standard adenosine monophosphate (AMP). We therefore chose an incubation time of 2.5 hours. An important disadvantage of this method is this long incubation time. After terminating the incubation, an ion-pair high-performance liquid chromatography (HPLC) method was used for the determination of XMP and AMP. The sensitivity and lower limit of quantification was sufficient for the tested patients groups (healthy volunteers, dialysis patients and renal transplant recipients). By using gradient elution the runtime per sample was decreased to 17 min and is now the shortest runtime of all published IMPDH activity assays so far.[17]

The biggest contribution to the increase in the robustness and reproducibility was made in the last step of the assay with the introduction of the new normalization factor AMP for the IMPDH activity. We showed that the so far known methods to normalize the enzyme activity, namely cell count and protein concentration, introduce measurement errors in the estimated IMPDH activity. The intracellular substance AMP is released together with the IMPDH enzymes in the process of cell disruption by osmosis during the isolation of the enzymes from PBMCs. By using AMP as a so-called internal standard for the method to normalize the enzyme product XMP, IMPDH activity is only corrected for the number of cells that had released their content including the IMPDH enzyme. This is not the case when cells are counted or protein concentration is determined, since the not yet lysed cells are included as well in these procedures. The AMP concentration shows fewer fluctuations over time and is more equal over different patient groups

compared with protein concentration. Therefore, IMPDH activity normalized to AMP concentration is suitable to compare the IMPDH activity over different patients and different patient groups.

By making the above described changes, we have developed a method with less variability and at the same time improved practicability for the measurement of IMPDH activity in PBMCs. The within-day and between-days reproducibility of the IMPDH activity values as obtained by our modified HPLC method were less than 10.8%. Only the IMPDH activity assay of Albrecht *et al*[15] using whole blood and the assay of Glander *et al*[18] using PBMCs showed less variability between days (6.8% and 9.5%, respectively). The between-days reproducibility of all other known assays is higher and ranged from 12.8% to 25%.[14,16,19,20] Compared with these assays our assay is one of the most precise assays and only our assay allows comparison of values between different patient groups.

Despite the many advantages of this assay, the procedure is still time-consuming and labor-intensive in routine clinical practice. Therefore we have investigated the possibility to use the easier real-time polymerase chain reaction (RT-PCR) method to determine IMPDH mRNA levels as alternative biomarker for IMPDH activity. The correlations pre-transplantation and at all visits were examined between pre-dose IMPDH activity and IMPDH gene expression of both type I and type II. The correlation coefficients were weak and not significant. The rise in mRNA expression after day 6 in our study is presumably due to the MPA induced inhibition of IMPDH activity. IMPDH mRNA expression is regulated by a negative feedback mechanism by guanine nucleotides. The inhibition of IMPDH activity by MPA leads to a decrease in the intra-cellular depots of guanine nucleotides.[21-23] According to the studies performed by Jain *et al* IMPDH inhibitors do not appear to alter the regulation of IMPDH type I or II mRNA or protein levels directly,[24] but may have an indirect role in the regulation. Considering this role of MPA on the feedback mechanism of IMPDH mRNA expression, it was not unexpected we could not find a direct correlation. But also pre-transplantation – in the absence of MPA – we did not find a good correlation between IMPDH activity and IMPDH mRNA levels. Based on these findings we can conclude that more factors are involved in the regulation of IMPDH mRNA expression besides IMPDH activity and the inhibition of IMPDH enzymes by MPA alone. We conclude that IMPDH mRNA levels are not correlated to IMPDH activity, and therefore measuring IMPDH mRNA levels is not an alternative for IMPDH activity assays.

Using the improved IMPDH activity assay we have performed a longitudinal prospective study in 101 *de novo* renal transplant recipients, the IMPDH Activity Study. The data of this study was analyzed with the aim to create a basis for MMF dose optimization and individualization based on IMPDH activity measurements.

MPA CONCENTRATION – IMPDH ACTIVITY CORRELATION

We have investigated the correlation between IMPDH activity and PK parameters to determine the PK/PD relationship for MMF. Previous studies have shown that plasma MPA concentrations are well correlated to IMPDH activity,[25,26] and this was supported by the observation that the IMPDH activity–time curve is the inverse of the MPA concentration–time curve. Nevertheless, the correlation between MPA concentrations and IMPDH activity was never characterized in an integrated PK/PD model to describe the PD parameters of the IMPDH inhibition by MPA. Furthermore, it is generally thought that the non-protein bound fraction is responsible for the pharmacological effect.[12] For this reason the unbound MPA concentration should have a better correlation with IMPDH activity compared to total MPA concentration. However, a comparison between unbound and total MPA concentrations in correlation with IMPDH activity had never been done.

A nonlinear mixed effects modeling (NONMEM) was used to describe the concentration–effect relationship between total MPA concentration and IMPDH activity. The PK of MPA were described using a 2-compartment model. An inhibitory E_{max} model adequately described the relationship between total MPA concentration and IMPDH activity. The maximal inhibition of IMPDH activity (E_{max}) was 89%. Baseline activity (E_0) increased from 55 (day 6) to 92 µmol/s/mol AMP (day 49 and 140) and the MPA concentrations which inhibit E_{max} to 50% activity (IC_{50}) were decreased from 0.76 to 0.17 mg/L. As a reaction to the inhibition of IMPDH by MPA, the baseline activity of IMPDH increased over time after transplantation. But at the same time, IMPDH become more sensitive for the inhibition by MPA respected the decrease of IC_{50}. In clinical practice, MPA concentrations are generally far above the IC_{50} so therefore the maximal inhibition of IMPDH activity is still achieved. It is already reported that the exposure to MPA increases over time after transplantation due to a decreased MPA clearance.[2,3,7] Combining those PK results (decrease of MPA clearance) together with our PD results (decrease of IC_{50}), it is more reasonable to reduce the MPA doses over time after transplantation. Before the factor time after transplantation can be used to adjust MMF dose, more information is needed about the optimal window for IMPDH activity at different time intervals after transplantation. Thus far, our results confirm that IMPDH activity changes over time after transplantation in patients using MPA.

Besides using total MPA concentration to investigate the PK/PD relation with IMPDH activity, using the unbound MPA concentration is probably better to describe this relation, because the unbound fraction is presumed to be the pharmacologically active fraction. Therefore, unbound and total MPA concentrations were correlated with IMPDH activity by naïve pooling. A PK/PD analysis showed a significant correlation between IMPDH activity with total MPA and IMPDH activity with unbound MPA concentrations

measured at time points just before the next MMF dose administration. The 12-hours exposure to total and unbound MPA (AUC MPA) was also significantly correlated to IMPDH activity measured pre-dose. However, these correlation coefficients were poor and the estimated PD parameters E_0 and IC_{50} showed large confidence intervals. Based on these results, we can not determine whether or not IMPDH activity measured on single time points is better correlated with bound or unbound MPA. A significant correlation was found for the 12-hours exposure to IMPDH activity (AUC IMPDH) with unbound MPA AUC using the therapeutic window 2.1 to 4.2 h·mg/L. The upper and lower limit of this window were obtained by multiplying the limits of the therapeutic window of total MPA AUC of 30 to 60 h·mg/L with the median unbound fraction of 7.0% found in our cohort of patients. Patients with high unbound MPA AUC had a significantly lower IMPDH activity. At the same time, there was no significant correlation for total MPA AUC correlated to IMPDH activity. Based on these findings, it seems that the AUC of IMPDH activity is better correlated to unbound MPA AUC than to total MPA AUC. These findings strengthen the hypothesis that unbound MPA is responsible for the inhibition of IMPDH. Nevertheless, before accepting this hypothesis, more extended population PD analyzes using total and unbound MPA, MPAG and AcMPAG concentrations should be made for a better and more precise comparison between unbound and total MPA concentrations in relation to IMPDH activity inhibition.

In the same study, we also found that patients with a serum albumin level below 32 g/L had a significantly lower IMPDH activity compared to patients with a normal serum albumin. It is known from PK studies that patients with lower serum albumin have lower total MPA concentrations, and higher unbound MPA concentrations compared with patients with normal serum albumin.[12,27,28] In our analysis we also found lower total MPA concentrations and lower IMPDH activity in patients with a low serum albumin, but we did not find higher unbound MPA concentrations. Actually, the unbound MPA concentration was not different between patients with low or normal serum albumin level, despite lower IMPDH activity in patients with low serum albumin. The PK in the present study is confirming previously reported studies, but the PD is not confirming the pharmacology of MPA. It remains unclear how the stronger inhibition of IMPDH activity in patients with low albumin levels is explained. Possibly patients with low albumin levels are in a poor general clinical condition and perhaps this poor condition induces a state of reduced immunoreactivity that is reflected in reduced IMPDH activity. It would be interesting to study the IMPDH activity in transplant patients not treated with MMF and see if IMPDH activity is a reflection of the immune status.

This present study is the first study which compared unbound and total MPA concentrations with IMPDH activity. Unfortunately, we found conflicting PD results, thus it is still unclear how unbound and total MPA concentrations are related to the inhibition of IMPDH and which concentration should be measured in clinical practice for

appropriate MMF therapy monitoring. To enlarge the knowledge of unbound and total MPA concentrations in correlation with IMPDH activity more investigation in this subject is necessary. Data collected in this study could be used in extended multivariate PK/PD population analyses using also unbound MPA and MPAG concentrations and patient characteristics. These analyses could give more understanding which concentration of MPA – total or unbound – is responsible for the *in vivo* inhibition of IMPDH.

PATIENT VARIABILITY IN IMPDH ACTIVITY

The PK/PD analyses of our data showed large inter-patient variability in IMPDH activity. Previous studies have also shown more than 10-fold inter-patient variability in IMPDH activity,[9,11,18] but it is still unknown if any patient characteristics or clinical laboratory data can explain the variability. To optimize individual dose regimens it is necessary to determine which factors explain the inter-patient variability. Therefore, we have investigated if patient variability in IMPDH activity could be explained by genetic, demographic and pathophysiologic factors.

Single nucleotide polymorphisms (SNPs) are substitutions in the genetic DNA code of an enzyme and could change the amino acid sequence or folding of enzymes. In that way SNPs could change the activity of an enzyme. In humans there are two isoforms of IMPDH, type I and type II. IMPDH type I is constitutively present in cells, while type II is expressed in cells stimulated to proliferation.[29,30] We studied eight different SNPs in the IMPDH type II gene in blood samples drawn from 80 renal transplant patients treated with MMF. Only the polymorphism *IMPDH type II* 3757T>C SNP was present with an allele frequency of 6.9%. The seven other polymorphisms in this gene were not observed in our patients. Despite the fact that *IMPDH type II* 3757T>C SNP is located on an intron of the IMPDH gene, we showed a significant difference in the IMPDH activity over 12 hours (AUC IMPDH). Patients with a variant allele had a higher IMPDH activity compared to patients with a wild type allele, and one patient who was homozygous for this SNP had the highest IMPDH activity. This SNP explains 8.0% of the inter-patient variability in total IMPDH activity. Patients homozygous for *IMPDH type II* 3757T>C had 55% higher E_0 compared to wild type patients. The *IMPDH type II* 3757T>C polymorphism in the IMPDH type II gene was not associated with IMPDH activity pre-transplantation. This may be explained by the constitutive expression of mainly IMPDH type I in non-activated lymphocytes, and induction of expression of IMPDH type II following allo-recognition. IMPDH activity in pre-transplantation samples mainly reflects type I activity. It is remarkable that a polymorphism in the IMPDH type II gene showed significant differences in the IMPDH activity measured with an assay that can not distinguish between isoforms type I and type II. This fact, the similar MPA AUCs and the same proportion of use of

anti-thymocyte globulin (ATG) induction therapy in patients of the different genotypes strengthens the importance of polymorphisms in the IMPDH gene for clinical outcome after transplantation. The *IMPDH type II* 3757T>C polymorphism seems to be the most interesting polymorphism, because a recent study has already shown that this SNP is correlated to acute rejection.[31] Based on genotyping the IMPDH type II gene, the clinical response to MMF treatment could be enlarged by increasing the MMF doses in patients having a variant type of this polymorphism. A higher MMF dose will decrease the IMPDH activity and will give more protection from acute rejections, where this polymorphism is associated to.

The influence of demographic characteristics on inter- and intra-patient variability was evaluated using an inhibitory E_{max} model. The analysis showed a high inter-patient variability in IC_{50} of 99%. The inter-patient variability in E_0 was low (9%), however the intra-patient variability was considerable with a value of 39%. Introducing demographic patient parameters into the model showed a significant 12% lower E_0 value in black patients compared to other ethnicity. It is reported that black patients require higher MMF doses than Caucasians to have the same effect.[32] Our results showed a difference in the PD of MMF in black patients, but this difference is not explaining the higher required doses of MMF; a lower E_0 value implicates a lower MMF doses is needed. To explain both differences in PK and PD in black patients, other factors need to be investigated.

The covariate analysis showed a significant difference in the PD parameters for the clinical laboratory parameters leukocyte count and serum albumin. A decrease in leukocytes was associated with a decrease in E_0. As MMF decreases the proliferation of lymphocytes by inhibition of IMPDH enzymes in the same cells, a decreased number of leukocytes might be the result of the decreased IMPDH activity measured at the same time. Increased albumin levels resulted in increased IC_{50} values for total MPA concentration. Previous studies have shown that lower albumin levels are associated with higher unbound MPA concentration.[12,27,28] Nevertheless, no higher unbound MPA concentrations were measured in patients with low albumin levels. Although we found evidence for a relationship between albumin levels and IMPDH activity, the role for unbound MPA concentration remains uncertain, as discussed before.

No other patient or physiologic characteristics were significantly correlated to the PD parameters (E_0 and IC_{50}) characterizing the relationship between inhibition of IMPDH activity and MPA plasma concentration. Besides the discussed polymorphism *IMPDH type II* 3757T>C, no other characteristics could contribute to optimize and individualize MMF therapy after renal transplantation as shown in this study. The present analyses provide only more information to understand and explain the patient variability in IMPDH activity.

ACUTE REJECTIONS AND ADVERSE EVENTS

The clinical use of monitoring IMPDH activity would be strengthened if IMPDH activity is also a predictor of biopsy proven acute rejections (BPAR) and adverse events. Glander *et al*[10] showed that pre-transplant IMPDH activity is a predictor for post-transplant dose adjustments of MMF, mostly due to adverse effects of MMF. Therefore, we have investigated the correlation between IMPDH activity and the clinical outcomes BPAR and adverse events.

In our prospective observational study patients with a BPAR had a 23% significantly higher E_0 and a 75% significantly higher IC_{50} at the first visit. The IMPDH AUC was slightly but not significantly higher in rejectors compared to non-rejectors with median values of 241 and 201 h·µmol/s/mol AMP. Patients with higher E_0 and a higher IC_{50} are less sensitive for the inhibitory effect of MPA and have therefore higher IMPDH activity and hence an increased risk of BPAR. Patients who suffer from leukopenia and serious anemia had significantly lower IMPDH AUC. Lower IMPDH activity will cause a reduced proliferation of lymphocytes, because of a decrease in GTP synthesis. This can be the reason for leukopenia. Erythrocytes do not depend on IMPDH activity for *de novo* GTP synthesis, because they have a salvage pathway. However, given the low hemoglobin levels in patients with low IMPDH activity, we have also shown that the inhibition of IMPDH has a suppressive effect on erythrocytes.

In this study we showed PD monitoring of MMF therapy is related to several clinical outcomes. The IC_{50} and E_0 are increased at visit 1 in patients with a BPAR. These patients need more inhibition of IMPDH by a higher dose of MMF to prevent rejection of the transplanted kidney. Nevertheless, we could not propose a clear threshold value of IMPDH activity with sufficient sensitivity and specificity, as there was considerable overlap in IMPDH activity between patients with a BPAR and patients without a BPAR, despite the significant differences in IC_{50} and E_0.

No correlation was found between BPAR and the *IMPDH type II* 3757T>C SNP, despite the fact that Grinyo *et al*[31] recently reported that variant carriers for this SNP were 3.4 times more likely to experience BPAR. They used a cohort of 237 renal transplant recipients, so we concluded that our cohort of 80 patients was underpowered to show a significant difference.

Though IMPDH mRNA levels were not correlated to IMPDH activity, IMPDH mRNA levels were significantly correlated to BPAR. Increased IMPDH mRNA type I and type II levels were observed pre-transplantation, while both types IMPDH mRNA levels were significantly decreased in rejectors compared to non-rejectors post-transplantation. We suggest that patients with less IMPDH mRNA have higher IMPDH activity and therefore higher immunoreactivity and higher rejection rates. Nevertheless, there is a considerable overlap in mRNA levels in rejectors and non-rejectors. The sensitivity and specificity

for the calculated cut-off level for IMPDH mRNA were poor (62-89%). Only at visit 4 lower IMPDH type I and type II mRNA expression was significantly correlated to pooled hematological events. Since no other significant correlations were found for adverse events at other visits, unfortunately, mRNA monitoring is not going to solve the unmet need for a biomarker that may assist in preventing MPA induced toxicity.

Despite the fact that many studies have investigated the relationship between adverse events and pharmacodynamic parameters, there is still no parameter that has sufficient sensitivity and specificity to be of use for clinical practice.[4,5,33] We have correlated the PD parameters IMPDH activity and IMPDH mRNA with adverse events, but unfortunately also did not find a useful clinical tool to guide MMF dose adjustments in the management of drug-related toxicity. Despite the significant correlations between some parameters and adverse events, the clinical relevance is too low given the low sensitivity and specificity.

EXPRESSION OF mRNA OVER TIME

The expression of mRNA correlated to adverse events and BPAR showed no differences between type I and type II mRNA expression. It is thought that IMPDH type I is constitutively present in several cells, and IMPDH type II is expressed in cells stimulated to proliferation, mainly in cells in the growth or differentiation phase. Therefore, it is assumed that only expression of IMPDH type II is increased after immune activation by organ transplantation.[26,34] Studies have evaluated the expression of the IMPDH type I and type II gene after kidney transplantation. The expression of both isoforms was up-regulated within several weeks after transplantation using MMF treatment compared with values before transplantation and without MMF therapy. The expression of IMPDH mRNA over a longer time after transplantation has never been studied yet.

Changes in the expression of IMPDH type I mRNA in our study closely paralleled those of type II. We did not find any evidence that only IMPDH type II was increased after transplantation, as both levels altered. In this study decreased mRNA levels of both IMPDH type I and type II were shown directly after transplantation and both types increased over time after transplantation. Induction of IMPDH activity after prolonged exposure to MPA may be partially explained by the up-regulation of both IMPDH type I and type II mRNA in PBMCs. IMPDH mRNA expression is regulated by a negative feedback mechanism by guanine nucleotides. The inhibition of IMPDH activity by MPA leads to a decrease in the depots of guanine nucleotide. Based on these finding, we conclude that both IMPDH isoforms are induced after immune activation.

IMPDH ACTIVITY IN HSCT RECIPIENTS

MMF is a frequently used immunosuppressive drug after solid organ transplantation but is also more frequently used in hematopoietic stem cell transplant (HSCT) patients to prevent acute graft-versus-host disease (GvHD).[35] In HSCT patients we have found in previous studies that the clearance of MPA is higher compared to kidney transplant patients. In a 1000 mg twice daily dosing regimen the total MPA concentrations in HSCT patients are below what is considered efficacious. However, in many of the HSCT patients a low albumin level may result in high unbound MPA exposure. The high unbound MPA would then result in a substantial inhibition of IMPDH activity, which obviates the need for increased dosing.[36] To optimize the dose regimen in this patient group, a PD approach could be useful, and could solve the dilemma of adjusting the MMF dose or not.

In the HSCT patients of our pilot study, a large inter-patient variability in IMPDH activity was observed. IMPDH activity of HSCT patients compared to renal transplant patients did not show significant differences and was comparable at pre-dose time point and 2 hours after MMF dosing. However, a significant difference was found in MPA concentrations. HSCT patients showed 70% lower pre-dose concentrations and 60% lower 2 hours concentrations compared to renal transplant patients. Based on these findings we conclude that IMPDH in HSCT patients is more sensitive to the inhibitory effect of MMF compared to renal transplant patients. Whether this difference is the result of a lower E_{max} and/or a higher IC_{50} of IMPDH and a different unbound concentration of MPA needs to be investigated with more sophisticated analytical methods than used in this study. No significant differences were found in IMPDH activity in patients who developed GvHD compared to patients without GvHD. Nevertheless, significantly lower IMPDH activity was found in patients suffering from serious adverse events like neutropenia, thrombopenia and anemia.

Based on the significant correlations we have found between IMPDH activity and adverse events, this pilot study showed that PD monitoring of MMF therapy could be useful in HSCT patients to find a more accurate dose regimen for this patient group. Low total MPA exposure is associated with a higher incidence of GvHD,[37] and therefore an average MPA steady state concentration of 2.5 to 5.0 mg/l is suggested as the therapeutic range for MPA in these patients.[38] But still, there are potential inaccuracies and difficulties in interpreting total MPA concentrations into clinical dose adjustments.[38,39] It is also not possible to adopt doses regimens of renal transplant patients, because the physiologic differences peri-transplant between renal transplant and HSCT patients that alter drug disposition. These differences include renal function, chemotherapy effects, cyclosporine A co-treatment, prophylactic antibiotic use and higher severity of illness which result in large variability in PK between renal transplant and HSCT patients. By measuring the PD parameter IMPDH activity these differences will partly be eliminated

for it is representing the direct effect of MPA. Studying the PK/PD relationship of MMF will provide more specific information in HSCT patients which could help to optimize the treatment with MMF.

In the present pilot-study, significant correlations between IMPDH activity and the toxicity of MMF were observed. A reduction of the MMF dose could result in less toxicity in patients with low IMPDH activity, while there is still accurate prophylaxis to GvHD. Because it is already shown that low MPA exposure is associated with an increase of the incidence of GvHD, it is not rational to reduce in general the MMF dose for all HSCT patients. In our study large inter-patient and inter-disease variability has also been observed. Potential reasons for the variability could be found in the wide diversity of the characteristics of the patients, which include the diagnoses for transplantation, stadium of disease, conditioning regimens (chemotherapy and body irradiation), CMV status, and donor characteristics. The findings of the PD of IMPDH activity after MMF therapy of the present study have to be confirmed in more selected and larger cohorts of HSCT patients. Further studies are needed to show if there is any clinical relevance of monitoring IMPDH activity in these patient group to increase efficacy and decrease toxicity.

FURTHER INVESTIGATIONS

Besides the new relevant information that our studies have given on the PD monitoring of IMPDH activity after MPA treatment, several questions emerged which require further research. This could be carried out to get more insight in the relationship between the PK and PD of MPA. The clinical application of the extended PK/PD model should investigate whether it could help to optimize and individualize MMF therapy after transplantation and other diseases that are treated with MMF like autoimmune diseases.

The present thesis has enlarged the knowledge of the PK/PD relation between MPA concentration and IMPDH activity. Nevertheless, it is still not clear if unbound MPA concentrations are really better correlated to IMPDH activity compared with total MPA. Also, we have not investigated the role of the total and unbound concentration of the active metabolite acyl 7-O-MPA-glucuronide (AcMPAG) in the inhibition of IMPDH. In our studies, we have not sampled the patients during the enterohepatic circulation (EHC) between 6 and 12 hours after administration of MMF. When MMF is co-administrated with tacrolimus, the exposure to MPA is determined for 10 to 60% by the EHC[40,41] Therefore, sampling between those time points is necessary to have a correct IMPDH AUC. For a better understanding of the complicated inhibition of IMPDH more research on the PK/PD relationship could be done using total and unbound concentrations of MPA and AcMPAG in relation to IMPDH activity. This could be useful to find better targets to optimize and individualize MMF therapy. Another interesting field of further research

is the performance of IMPDH activity prior to rejection. It is still not known whether IMPDH activity alters only by inhibition of MPA or if other promoters of lymphocyte activation – e.g. infection, co-morbidity, and primary disease – also induce IMPDH activity. The present studies did not sample the patients prior to acute rejections, so it would be of great interest to investigate the behavior of IMPDH activity prior rejection. For a better understanding of transcription and translation of IMPDH mRNA in transplant recipients, we suggest studying IMPDH mRNA in renal transplant recipients who are not on MMF treatment. Combining the results of both patient groups, the effect of MPA on the complex pathway of transcription and translation of IMPDH mRNA could be clarified.

Besides IMPDH type II also IMPDH type I is increased after transplantation. Therefore, it is likely that overall IMPDH activity after transplantation is determined by IMPDH type I and type II. For that reason, pharmacogenetic polymorphisms in IMPDH type I gene could also contribute to the inter-patient variability of IMPDH activity. Research on IMPDH type I SNPs might be useful, as well haplotyping of all SNPs in the IMPDH type I and type II gene. More knowledge of the influence of these SNPs may help to comprehend the inter-patient variability of IMPDH activity.

The IMPDH activity assay used in this thesis was modified, but can still be improved further. Using the assay in clinical research did show that the lower limit of quantification could be further optimized to measure XMP and AMP also in lower concentrations. Especially in patients suffering from leukopenia – where harvesting PBMCs is difficult – a lower limit of quantification will improve the accuracy and precision of the measured IMPDH activity. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analytical techniques are the right choice of method for this purpose. The IMPDH activity assay has to be improved to monitoring IMPDH activity in daily clinical practice. For that purpose, a less labor-intensive and more rapid IMPDH activity assay is needed.

Besides research to enlarge the knowledge of the PK/PD relationship, further research could also be done with the purpose to optimize and individualize the MMF therapy based on this knowledge. Monitoring IMPDH activity does not have an excellent specificity and sensitivity for the clinical practice to define adverse events and acute rejections. Nevertheless, combining PD monitoring with PK monitoring could have the potency to improve MMF therapy by dose adjustments. Our data and conclusions, as well as new insights could be used for this clinical application.

FINAL CONCLUSIONS

In the studies presented in this thesis we have extensively investigated the PD of MMF therapy by monitoring IMPDH activity with the aim to find a basis for the optimization and individualization of MMF therapy after transplantation. Focused on the activity of

the enzyme IMPDH we have studied the correlations with several outcome parameters and we have expanded the knowledge of the PK/PD relationship between MPA concentration and IMPDH activity. Unfortunately, based on our data IMPDH activity monitoring can not yet be recommended to optimize and individualize the MMF therapy. In the previous paragraphs we have discussed that the studied IMPDH enzyme is inhibited by many more factors than we have investigated and shows a large unexplained variability within and between patients. Nevertheless, this thesis has given a basis of a better understanding of the IMPDH activity and IMPDH inhibition by MPA in patients treated with MMF. Our findings can serve as a starting point for future studies in the fascinating relationship between PK and PD. To understand all the details of the complicated way of inhibition of IMPDH, more information and studies are necessary. A better understanding of the PK/PD relation could be a first step to optimize and individualize MMF therapy based on the results of IMPDH activity studies.

Summarized, the main conclusions of this thesis are:

- Inter-patient variability in IMPDH activity is partly explained by the genetic polymorphism *IMPDH type II* 3757T>C, ethnicity, leukocyte count and serum albumin,
- Leukocyte count and serum albumin also partly explained the intra-patient variability in IMPDH activity,
- Baseline IMPDH activity (E_{max}) increases and the IC_{50} decreases over time after transplantation,
- Patients with increased IC_{50} and increased E_0 in the first week after transplantation have a higher risk of BPAR and patients with lower IMPDH activity have more MMF related adverse events, and
- Patients with decreased IMPDH mRNA type I and type II have a higher risk of BPAR, but IMPDH mRNA levels are not correlated to adverse events.
- Both IMPDH type I and type II isoforms are induced after immune activation,
- It seems that IMPDH activity is better correlated to the exposure of unbound MPA than to total MPA.
- We have improved the IMPDH activity assay to a more precise and practical assay, that can used to monitor MPA therapy in different patient groups, but
- Measuring IMPDH mRNA is not an alternative for IMPDH activity assays.
- IMPDH seems to be more sensitive to the inhibitory effect of MPA in HSCT patients compared with renal transplant patients, and
- In HSCT patients, IMPDH activity is not correlated to GvHD, but patients with low IMPDH activity suffer from serious adverse events.

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Chapter 7

Summaries

Chapter 7.1

Scientific summary

The enzyme inosine 5'-monophosphate hydrogenase (IMPDH) is the key enzyme in the *de novo* synthesis of nucleotides and induces the rate-limiting step in this synthesis. The proliferation of lymphocytes depends on this pathway of synthesis of nucleotides, which makes it an attractive pharmacological target of immunosuppressive therapy. Mycophenolic acid (MPA) – the active moiety of mycophenolate mofetil (MMF) – decreases the proliferation of T- and B-lymphocytes. For that reason MMF is used as immunosuppressive drug after solid organ transplantation to prevent rejection of the graft. MMF is also increasingly used after haematopoietic stem cell transplantation to prevent acute graft-versus-host disease. The recommended dose regimen for MMF is 1000mg twice daily, but more and more it is thought that a patient tailored regimen is better for the balance between efficacy and toxicity. Pharmacokinetic (PK) studies have shown that MMF exhibits large between- and within-patient variability in PK, whereas MPA plasma concentrations were found to correlate with efficacy. However, randomized trials comparing fixed dose versus concentration controlled regimens found contradicting results. An attractive approach to optimize and individualize the MMF therapy in transplant recipients could be pharmacodynamic (PD) monitoring by measuring IMPDH activity. IMPDH activity may better reflect the individual biological response to the drug, because it combines PK and PD variability. Several studies have demonstrated that IMPDH activity has a large inter-patient variability, a small intra-patient variability and that activity alters over time after transplantation. Only one study has correlated IMPDH activity with clinical endpoints and has demonstrated a correlation between pre-transplant IMPDH activity and post-transplant dose adjustments, MPA related adverse events and acute rejections. At the start of this project we aimed to investigate the added value of IMPDH monitoring in transplant patient management.

After a general introduction to PK monitoring of MPA and PD monitoring of IMPDH activity in **chapter 1.1**, the scope and aims of this thesis were formulated. The overall aim of this thesis was to explore the usefulness of monitoring IMPDH activity in MPA treated transplant patients to optimize and individualize the MPA therapy. More specifically, separate objectives were also addressed and formulated in **chapter 1.2**.

An important tool in the studies presented in this thesis is the newly modified IMPDH activity assay described in **chapter 2.1**. In the first step of the assay peripheral blood mononuclear cells (PBMCs) are isolated from 2.5 mL whole blood using a Ficoll-Paque gradient without manipulating the cells extensively and without washing out the intracellular MPA concentration which is responsible for the inhibition of IMPDH. Secondly, cell lysates containing the IMPDH enzyme are incubated *ex vivo* in a reaction mixture close to the *in vivo* environment containing potassium, IMP as substrate and nicotinamide adenine dinucleotide (NAD) as hydrogen acceptor at 37°C. One disadvantage of

the presented assay was the relatively long incubation time of 2.5 hours. After the reaction was terminated, xanthine monophosphate (XMP) and adenosine monophosphate (AMP) levels in the samples were measured using a validated high-performance liquid chromatography (HPLC) method. In other IMPDH activity assays, the production rate of XMP is expressed per cell or per amount protein. A new factor to normalize the formed IMPDH enzyme product XMP was introduced in the third step. Using the intra-cellular substance AMP to normalize XMP production produced better results for the assessed IMPDH activity than using cell count and protein concentration. In several experiments it has been shown that by using AMP the derived IMPDH activity is less sensitive to contamination of plasma, residual supernatant of the washing step, extra-cellular protein and erythrocytes. The reproducibility of IMPDH activity normalized to AMP is better compared to normalize to cell count, even in increased enzyme concentration in the samples and in different patient groups.

The modified assay is capable of reducing the variability and at the same time improving the practicability of the enzymatic assay for assessment of IMPDH activity in PBMCs. The within-run and within-day reproducibilities of the IMPDH activity values as produced by this modified HPLC method were less than 11% and are for such a complex method really within the generally accepted limit of 15%.

The modified IMPDH activity assay was thereafter used in the IMPDH Activity study, a prospective, longitudinal PK-PD study in 101 *de novo* kidney transplant patients. Blood samples were drawn pre-transplantation and on 4 visits after transplantation for PK and PD monitoring, determination of IMPDH mRNA levels and for pharmacogenetic purposes. Clinical outcome, clinical laboratory data and occurred adverse events were collected during all visits. Collected data was analyzed with different aims in chapters 3 and 4.

In **chapter 3.1** all patient data were analyzed simultaneously by using the nonlinear mixed effects modelling. Pharmacokinetic parameters for MPA were estimated using Bayesian analysis and the parameters were fixed in the second step where the pharmacodynamic parameters were estimated using an E_{max}-model. A large inter-patient variability (102%) was found for the IC_{50}, the concentration at half maximum inhibition (E_{max}) of IMPDH activity. Furthermore a considerable intra-patient variability of baseline activity (E_0) was detected (41%). A significant increase of E_0 from 55 to 92 h·µmol/s/mol AMP was observed over time, while IC_{50} decreased from 0.76 to 0.17 mg/L. E_0 of black patients was 12% lower that for Caucasian, Asian and Hispanic patients. Patients with a homozygous variant type of *IMPDH type II* 3757T>C exhibited an E_0 which was 55% higher than for homozygous wildtype and heterozygous patients. IC_{50} was significantly lower in patients with decreased leukocytes or decreased albumin. The inter- and intra-

patient variability in E_0 and IC_{50} was not explained by other patient characteristics or clinical laboratory outcomes. Variability in the pharmacodynamic parameters partly predicted clinical outcome and adverse events. Patients with a biopsy proven acute rejection (BPAR) had significantly a 23% higher E_0 and 75% higher IC_{50} 6 days after transplantation compared with patients without a BPAR. Patients with lower IMPDH AUC had significantly more leukopenia and/or anemia compared to patients with higher IMPDH AUC. These findings emphasize the importance of adequate inhibition of IMPDH to prevent acute rejections. Although high IMPDH activity was associated with the occurrence of BPAR and low IMPDH activity was correlated with the presence of adverse reactions, a target value for the inhibition of IMPDH activity has still to be defined. In our study we observed that BPAR was better associated with IMPDH activity than with MPA plasma concentrations. As a result, we conclude that monitoring of IMPDH activity could contribute to optimize MMF therapy.

To investigate the relevance of unbound MPA concentrations in causing the biological effect of MPA, we correlated total and unbound MPA concentrations to IMPDH activity and albumin levels in **chapter 3.2**. Using an E_{max} model, significant, but poor, correlations were found between IMPDH activity and MPA total and unbound concentrations and exposure on day 6. The pharmacodynamic parameters E_0 and IC_{50} showed large confidence intervals, due to the large inter-patient variability of IMPDH activity. Dividing MPA concentrations into three groups using the therapeutic window of total MPA AUC (30 to 60 h·mg/L) and of unbound MPA AUC (2.1 to 4.2 h·mg/L; limits were multiplied with 7.0% median unbound fraction) showed a significant difference in IMPDH activity for only unbound MPA AUC. A higher unbound MPA AUC gave a significantly higher inhibition of IMPDH activity, whereas no associated was detected for total MPA AUC. IMPDH activity was significantly lower in patients with low serum albumin compared to patients with high albumin levels despite the fact that both patient groups exhibited comparable unbound MPA AUC values. Patients with a low albumin level exhibited a significantly lower total MPA AUC. We conclude that unbound MPA AUC described inhibition of IMPDH activity better than total MPA concentrations. However, we have no explanation for the fact that IMPDH activity is decreased more in patients with low albumin levels despite the comparable unbound MPA concentrations.

The aim of **chapter 4.1** was to correlate eight different single-nucleotide polymorphisms (SNPs) of the IMPDH type II gene to IMPDH activity to explain inter-patient variability. In the studied cohort only the SNP *IMPDH type II* 3757T>C was observed with an allele frequency of 6.9%. Two percent of the patients were homozygous for variant type of the SNP. A comparison between wild type and variant carrier patient showed that IMPDH activity pre-transplantation was not significantly different between the patients. On day

6 post-transplantation the exposure to IMPDH activity was significantly 49% higher in variant carrier patients. We concluded that *IMPDH type II* 3757T>C polymorphism is associated with an increased IMPDH activity in MMF treated renal transplant patients and explains 8.0% of the inter-patient variability in IMPDH activity.

IMPDH activity assays are time-consuming and labor-intensive. In **chapter 4.2** we have therefore investigated the quantification of IMPDH mRNA as alternative method for monitoring MMF therapy. The study was designed to correlate IMPDH mRNA levels with IMPDH activity and clinical outcome in renal transplant recipients. No correlations were found between IMPDH type I and type II mRNA levels and IMPDH activity both pre- and post-transplantation. Determination of IMPDH mRNA levels is therefore no alternative for measuring IMPDH activity. Patients with an increased pre-transplantation IMPDH mRNA level exhibited an increased, but not significant, risk of BPAR. On day 6 post-transplantation, decreased IMPDH type I and type II mRNA levels were significantly correlated to the occurrence of BPAR. Although the regulation of the expression of the two isoforms is presumed to be different, IMPDH type I as well as type II mRNA levels increased in parallel over time after transplantation.

MMF is used more frequently in hematopoietic stem cell transplantation (HSCT) recipients to prevent acute graft-versus-host disease. In **chapter 5.1** we performed a pilot-study in HSCT patients to explore for the first time the pharmacodynamics after MMF treatment by measuring IMPDH activity. Patients with a higher IMPDH activity pre-dose had a stronger inhibition of IMPDH activity at 2 hours. The inter-patient variability for the concentration-effect relationship was large, similar as in renal transplant recipients. Inhibition of IMPDH activity in HSCT patient was similar as for renal transplant patients despite the fact that MPA levels were lower in the latter group. We hypothesize that IMPDH enzymes of HSCT patients are more sensitive for the inhibition of MPA than IMPDH enzymes of renal transplant recipients. No significant correlations were found between IMPDH activity and the occurrence of graft-versus-host disease (GvHD), but IMPDH activity was significantly lower in patients who suffered from serious neutropenia, thrombocytopenia and anemia compared to patients with none or mild grade adverse events. The clinical relevance of this pilot study has to be confirmed in a larger cohort of HSCT patients.

Chapter 6 discusses to what extent the combined main finding of the presented studies are in agreement with the specific aims formulated in chapter 1.2. In addition, some comments are given with regard to the applied study design.

We have improved the IMPDH activity assay, but the assay is still too time-consuming and labor-intensive for daily clinical use. Unfortunately, measuring IMPDH mRNA

levels is not an alternative for IMPDH activity assays. An integrated pharmacokinetic-pharmacodyanmic model was developed to describe the correlation between total MPA concentration and IMPDH activity. We have shown that IMPDH activity is better correlated to unbound MPA concentration than to total MPA concentrations. More extended population PD analyzes should be made for more precise comparisons between unbound and total MPA concentrations in relation to IMPDH activity inhibition. The genetic polymorphism *IMPDH type II* 3757T>C, patients of black ethnicity, leukocyte count and serum albumin explained some of the intra- and/or inter-individual variability in IMPDH activity. Patients with increased IC_{50} and increased E_0 had a higher risk on BPAR, and patients with lower IMPDH activity showed more MMF related adverse events. We found decreased levels of both IMPDH type I and type II directly after transplantation and both types increased over time after transplantation. Therefore, both isoforms should be inhibited to decrease the proliferation of the lymphocytes and to prevent the graft for acute rejection. Several new research questions have risen during the overview and discussion of the results presents in this thesis. Therefore, suggestions for further investigations are also given.

Based on our data, IMPDH activity monitoring can not yet be recommended to optimize and individualize the MMF therapy. In this thesis we have discussed that the studied IMPDH enzyme is inhibited by many more factors than we have investigated and shows a large unexplained variability within and between patients. To understand all the details of the complicated way of inhibition of IMPDH, more information and studies are necessary. This thesis has given a basis of a better understanding of the IMPDH activity and IMPDH inhibition by MPA in patients treated with MMF. Our findings can serve as a starting point for future studies in the fascinating relationship between PK and PD.

Chapter 7.2

Samenvatting voor niet-ingewijden

Wanneer bij een patiënt de nieren niet meer goed werken, is het soms mogelijk om een nier van iemand anders te krijgen door middel van een transplantatie. Het lichaam van de patiënt ziet echter deze nieuwe nier als een lichaamsvreemd orgaan en zal het proberen af te stoten. Daarom worden transplantatiepatiënten altijd behandeld met medicijnen die afstotingen proberen te voorkómen. De juiste dosering van deze medicijnen wordt bijna altijd per patiënt vastgesteld, om zo de optimale balans te krijgen tussen het gewenste effect en de bijwerkingen. De dosering van het veel gebruikte medicijn mycofenolaatmofetil (MMF, merknaam CellCept®) wordt echter niet per patiënt vastgesteld. De aanbevolen dosering voor MMF is tweemaal daags 1000 mg voor elke willekeurige patiënt. Maar steeds meer denkt men dat een dosering berekend voor elke patiënt afzonderlijk beter is om een juiste balans tussen werkzaamheid en bijwerking te krijgen. Het onderzoek beschreven in dit proefschrift heeft zich gericht op het optimaliseren en individualiseren van de dosering van MMF.

Het onderzoek heeft zich specifiek gericht op het enzym inosine 5'-monofosfaat dehydrogenase (IMPDH). Enzymen zijn eiwitten die zich in het lichaam bevinden en die zorgen dat stoffen omgezet worden in specifieke, andere stoffen. Het enzym IMPDH is een belangrijk enzym dat zorgt voor de aanmaak van nucleotiden: stoffen die nodig zijn bij het maken van cellen. De snelheid waarmee IMPDH deze stoffen maakt is de snelheidsbeperkende stap voor het ontstaan van deze nucleotiden. De ontwikkeling van de witte bloedcellen (lymfocyten) die het afweersysteem in het lichaam vormen, zijn afhankelijk van deze stoffen die door het enzym IMPDH gemaakt worden. Hoe actiever IMPDH is, hoe meer nucleotiden gemaakt kunnen worden en des te beter witte bloedcellen ontwikkeld kunnen worden en hoe sterker het afweersysteem is.

Na bijvoorbeeld een transplantatie is het nodig om de afweer van het lichaam juist te verminderen. Op die manier kan worden voorkomen dat het lichaam het getransplanteerde orgaan afstoot. Om dit te bereiken is het verminderen van de snelheid waarmee IMPDH stoffen maakt een aantrekkelijk doel voor geneesmiddelen. De behandeling om het afweersysteem te verminderen wordt immunosuppressieve therapie genoemd.

De stof mycofenolzuur (MPA) is de actieve vorm van het medicijn MMF en vermindert de ontwikkeling van twee soorten witte bloedcellen, de T- en B-lymfocyten. Om die reden wordt MMF gebruikt als immunosuppressief geneesmiddel na orgaantransplantatie om afstoting van het getransplanteerde orgaan tegen te gaan. Ook wordt MMF steeds meer gebruikt na transplantatie van bloedstamcellen (ook wel beenmergtransplantatie genoemd) om acute graft-versus-host (transplantaat tegen patiënt) reacties te voorkomen. Zoals elk geneesmiddel heeft ook MMF bijwerkingen. De meest voorkomende bijwerkingen zijn misselijkheid, braken, diarree, bloedarmoede, verminderde hoeveelheid witte bloedcellen, diverse infecties en zelfs bepaalde soorten kwaadaardige gezwellen.

Wat het líchaam doet met het medicijn wordt farmacokinitiek genoemd. Farmacokinetische studies hebben aangetoond dat tussen verschillende patiënten (inter-patiënt) en in één dezelfde patiënt (intra-patiënt) grote verschillen zijn in wat het lichaam doet met de stof MPA. Dit heeft bijvoorbeeld als gevolg dat er grote verschillen zijn in de concentratie van MPA in het bloedplasma bij een gelijke dosering van het geneesmiddel. En dat terwijl de MPA concentratie juist samenhangt met de werkzaamheid van het geneesmiddel. Toch zijn de uitkomsten van wetenschappelijke onderzoeken, waar een vaste dosering van het geneesmiddel is vergeleken met een dosering aangepast op de individuele patiënt, tegenstrijdig met elkaar. Een andere aanpak om de behandeling met MMF bij transplantatie patiënten te optimaliseren en te individualiseren is door te kijken wat het míddel met het lichaam doet. Dit wordt farmacodynamiek genoemd. Het meten van de activiteit van het enzym IMPDH is een manier om de behandeling met MMF farmacodynamisch te volgen.

Veronderstelt wordt dat het meten van IMPDH activiteit beter de individuele reactie van het lichaam op het geneesmiddel MMF weergeeft. Het combineert de veranderlijkheid van farmacokinetiek en farmacodynamiek in en tussen patiënten (intra- en inter-patiënt variabiliteit). Diverse farmacodynamische studies hebben aangetoond dat IMPDH activiteit een grote inter-patiënt variabiliteit en een kleine intra-patiënt variabiliteit heeft. Er is ook gezien dat de IMPDH activiteit verandert in de loop van de tijd na transplantatie. Er is slechts één onderzoek dat gekeken heeft naar de samenhang van IMPDH activiteit met klinische eindpunten, zoals afstoting van het getransplanteerde orgaan. Dit onderzoek heeft aangetoond dat patiënten die al voor de transplantatie een hoge IMPDH activiteit hebben vaker een verlaging van de dosis MMF na transplantatie krijgen. Ook hadden patiënten met een hoge IMPDH activiteit gemeten voor transplantatie vaker last van bijwerkingen en hadden meer acute afstotingen van het getransplanteerde orgaan. Bij de start van dit promotieonderzoek hebben we als doel gesteld om te kijken of het meten van IMPDH activiteit bij transplantatiepatiënten een toegevoegde waarde heeft op de behandeling met het geneesmiddel MMF.

Na een algemene inleiding over het farmacokinetisch monitoren van de stof MPA en het farmacodynamisch monitoren van IMPDH activiteit in **hoofdstuk 1.1**, is de strekking en zijn de doelstellingen van dit proefschrift verwoord in **hoofdstuk 1.2**. Het algemene doel van dit proefschrift is het onderzoeken van de bruikbaarheid van IMPDH activiteit monitoring om de behandeling met MPA te optimaliseren en te individualiseren in patiënten na een transplantatie. Dit algemene doel is verder uitgewerkt in meer specifieke en afzonderlijke doelstellingen, die ook in hoofdstuk 1.2 zijn vermeld.

Een belangrijk instrument dat in de studies in dit proefschrift is gebruikt, is de gewijzigde analysemethode die de IMPDH activiteit meet. Deze methode is beschreven in

hoofdstuk 2.1. De eerste stap van deze analysemethode bestaat uit het halen van bepaalde witte bloedcellen – perifeer bloed mononucleaire cellen (PBMCs) – uit 2,5 ml bloed. Hiervoor is gebruik gemaakt van een Ficoll-Paque gradiënt, een soort vloeibare suikerlaag waarbij de PBMCs gescheiden worden van de rode bloedcellen, bloedplaatjes en het bloedplasma. Door deze techniek zo aan te passen, hebben we ervoor gezorgd dat de cellen zo min mogelijk bewerkt en gewassen werden. Op deze manier wordt de MPA concentratie in de cellen die verantwoordelijk is voor het remmen van de IMPDH activiteit minimaal veranderd. De cellen zijn vervolgens kapot gemaakt, zodat het IMPDH enzym vrijgekomen is.

De tweede stap is het plaatsen van het IMPDH enzym in een reactiemengsel van 37°C dat sterk lijkt op de natuurlijke, lichamelijke omgeving. In het reactiemengsel zet het IMPDH enzym de stof inosine monofosfaat (IMP) om in de stof xanthine monofosfaat (XMP). Hoe meer van dit XMP wordt gemaakt, hoe actiever het IMPDH is. Het IMPDH kreeg 2,5 uur de tijd om XMP te maken. Deze lange periode was nodig om een goede herhaalbaarheid van de metingen te krijgen, maar is tevens een nadeel van de beschreven analysemethode. Na deze periode is de concentratie van de gevormde stof XMP en de concentratie van de stof adenosine monofosfaat (AMP) – een stof uit de gebruikte cellen – gemeten met een gevalideerde High Performance Liquid Chromatography (HPLC) methode.

De derde stap is om de gemeten concentratie van het gevormde product XMP weer te geven per cel die gebruikt is in de meting. Hierdoor kan de gemeten IMPDH activiteit vergelijken worden met IMPDH activiteit gemeten in andere monsters. In andere beschreven IMPDH activiteit analysemethodes is deze productiesnelheid uitgedrukt per getelde cel in het totale monster of per hoeveelheid eiwit in het totale monster. Een nieuwe factor om de door het IMPDH enzym gevormde stof XMP weer te geven is de stof AMP. Deze stof komt uit dezelfde cellen waaruit de gebruikte IMPDH enzymen komen. Deze nieuwe manier van het berekenen van IMPDH activiteit geeft daarom beter aan wat de totale activiteit is van het IMPDH enzym per cel in vergelijking met het totale aantal cellen in het monster of de totale hoeveelheid eiwit in het monster, waar tot nu toe mee werd gerekend. In diverse experimenten is vervolgens aangetoond dat door het gebruik van AMP de berekende IMPDH activiteit minder gevoelig is voor verontreiniging, eiwitten en rode bloedcellen die ook in het monster kunnen zitten. Zelfs wanneer het monster hoge concentraties enzym bevat is de reproduceerbaarheid van de gemeten IMPDH activiteit juist en precies. Ook is het nu mogelijk om IMPDH activiteit van verschillende patiëntengroepen met elkaar te vergelijken.

De gewijzigde analysemethode om IMPDH activiteit te meten in PBMC's geeft minder variabiliteit tussen de metingen en is tegelijkertijd praktisch beter uitvoerbaarheid. De reproduceerbaarheid van de IMPDH activiteit gemeten op dezelfde dag en op verschillende dagen was kleiner dan 11%, en is zeker voor een zo'n complexe analyse-

methode goed te noemen omdat ze binnen de algemeen aanvaarde grens van 15% vallen.

Vervolgens is de gewijzigde analysemethode om IMPDH activiteit te meten gebruikt in de IMPDH Activity study (IMP-Act study); een prospectieve, longitudinale farmacokinetiek-farmacodynamiek studie in 101 nieuwe niertransplantatie patiënten. Bloedmonsters werden vóór transplantatie en op 4 momenten na transplantatie afgenomen voor het monitoren van de farmacokinetiek en farmacodynamiek van MPA, het meten van IMPDH mRNA concentraties en voor farmacogenetische doeleinden. Klinische resultaten, klinisch laboratorium gegevens en het optreden van bijwerkingen werden verzameld tijdens alle bezoeken. De verzamelde data zijn vervolgens met verschillende doelstellingen geanalyseerd en beschreven in de hoofdstukken 3 en 4.

In **hoofdstuk 3.1** zijn alle gegevens van de patiënten gelijktijdig geanalyseerd met behulp van het computerprogramma NONMEM (nonlinear mixed effects modelling). Eerst zijn de farmacokinetische variabelen aan de hand van de gemeten MPA concentraties geschat met behulp van Bayesiaanse analyse. Hierna zijn met gebruik van deze farmacokinetische variabelen de farmacodynamische variabelen geschat aan de hand van de gemeten IMPDH activiteit. Hiervoor is gebruik gemaakt van een zogenoemd maximaal effect model, het E_{max}-model. Dit model beschrijft hoe het basale effect (E_0) van een enzym –hier de maximale IMPDH activiteit van een patiënt– wordt geremd naarmate de concentratie van het remmende geneesmiddel –hier de MPA concentratie– toeneemt. De concentratie waarbij 50% van de maximale remming (E_{max}) van het enzym wordt bereikt is de IC_{50}-waarde. Onze studiegroep liet een grote inter-patiënt variabiliteit van 102% zien voor IC_{50}. Verder werd een aanzienlijke variabiliteit van 41% per patiënt gezien in E_0 in de periode na transplantatie. E_0 steeg van 55 tot 92 µmol/s/mol AMP in de tijd na transplantatie, terwijl de IC_{50} tegelijkertijd daalde van 0,76 tot 0,17 mg/L. De E_0 van patiënten van het negroïde ras was 12% lager in vergelijking met blanken, Aziatische en Ibero-Amerikaanse patiënten.

Tevens is er gekeken naar de genetische code van het gen dat codeert voor het IMPDH enzym. Op plaats 3757 in het gen werd bij sommige patiënten in plaats van de gebruikelijke 'T' een 'C' gezien. Dit type van verschillende verschijningsvormen van een gen wordt een enkelvoudige nucleotide polymorfisme (SNP) genoemd. Door een SNP kan de werking van het enzym worden versneld, vertraagd of zelfs geheel geïnactiveerd worden. De hier onderzochte SNP wordt aangegeven als *IMPDH type II* 3757T>C. Patiënten waarvan beide genen deze variatie hadden, vertoonden een 55% hogere E_0 in vergelijking met patiënten waarvan één of geen gen deze variatie had. De IC_{50}-waarde was significant lager bij patiënten met een laag aantal witte bloedcellen of een lage albumine concentratie in het bloed. De variabiliteit tussen patiënten en in individuele

patiënten in E_0 en IC_{50} werd niet verklaard door andere kenmerken van patiënten of klinisch laboratorium resultaten.

De variabiliteit in de farmacodynamische variabelen E_0 en IC_{50} voorspelde deels de klinische resultaten en bijwerkingen. Patiënten met een zogenoemde biopsie bewezen acute afstoting (BPAR) van het getransplanteerde orgaan hadden een 23% significant hogere E_0 en een 75% significant hogere IC_{50} op dag 6 na de transplantatie in vergelijking met patiënten zonder een BPAR. Patiënten met een lagere blootstelling aan IMPDH activiteit over 12 uur hadden meer bijwerkingen van MMF in vergelijking met patiënten met een hogere blootstelling aan IMPDH activiteit. Deze patiënten leden vaker aan een te kort aan witte bloedcellen en/of bloedarmoede.

Deze bevindingen onderstrepen het belang van voldoende remming van het IMPDH enzym om acute afstotingen te voorkomen. Maar ook dat het IMPDH enzym niet te veel geremd moet worden om zo bijwerkingen te verminderen. Hoewel de hoge IMPDH activiteit samenhangt met het optreden van afstotingen en lage IMPDH activiteit met het optreden van bijwerkingen, moet een bruikbare streefwaarde voor de remming van de IMPDH activiteit nog worden vastgesteld. In ons onderzoek hebben we waargenomen dat afstoting beter samenhing met IMPDH activiteit dan met MPA concentraties in het bloed. Daarom concluderen we dat het monitoren van IMPDH activiteit kan bijdragen om de behandeling met MMF te optimaliseren.

In het bloed kunnen stoffen –en dus ook het geneesmiddel MPA– gebonden en niet-gebonden zijn aan eiwitten die in het bloed zitten. Men gaat er van uit dat alleen het gedeelte dat ongebonden is ook daadwerkelijk werkzaam is. Om de relevantie van de ongebonden MPA concentratie te onderzoeken, hebben we in **hoofdstuk 3.2** de totale (gebonden plus ongebonden) en de ongebonden MPA concentraties gecorreleerd aan IMPDH activiteit en albumine concentraties. Met behulp van het E_{max}-model werd op dag 6 een significante, maar zwakke samenhang gevonden van IMPDH activiteit met totale en ongebonden MPA concentraties en met de blootstelling aan IMPDH activiteit over 12 uur. De farmacodynamische variabelen E_0 en IC_{50} waren moeilijk te schatten vanwege de grote variabiliteit van IMPDH activiteit tussen de patiënten. Vervolgens zijn de patiënten verdeeld over drie groepen met als grens de grenzen die aan de blootstelling van totaal MPA over 12 uur zijn gesteld (30 tot 60 h·mg/L) en aan de blootstelling van ongebonden MPA zijn gesteld (2,1 tot 4,2 h·mg/l; de grenzen van totaal MPA zijn vermenigvuldigd met 7,0%, de gevonden mediaan ongebonden fractie). Nu bleek dat er een significant verschil in IMPDH activiteit waarneembaar was voor de blootstelling aan ongebonden MPA. Een hogere blootstelling aan ongebonden MPA gaf een significant hogere remming van de IMPDH activiteit, terwijl dit niet gezien werd bij de blootstelling aan totaal MPA.

De IMPDH activiteit was significant lager bij patiënten met een lage albumine concentratie in vergelijking met patiënten met een hoge albumine concentratie. De ongebonden MPA concentratie vertoonde geen verschil tussen beide groepen, terwijl verwacht werd dat patiënten met een lage albumine concentratie juist een hogere ongebonden concentratie zouden hebben. Deze veronderstelde hogere ongebonden concentratie zou het IMPDH meer kunnen remmen, dat de significant lagere IMPDH activiteit zou verklaren bij deze patiënten. Echter, er werd bij patiënten met een lage albumine concentratie juist een lagere totale MPA concentratie gevonden. Dit is zeer opmerkelijk omdat hier de niet-logische verklaring uit volgt dat een lagere MPA concentratie juist een verlaging van de IMPDH activiteit zou veroorzaken. We concluderen daarom ook dat ongebonden MPA beter de remming van IMPDH activiteit beschrijft dan totale MPA. We hebben echter geen verklaring voor het feit dat IMPDH activiteit sterker daalt bij patiënten met een lage albumine concentratie, terwijl de ongebonden MPA concentraties niet verschilden.

Het doel van **hoofdstuk 4.1** is acht verschillende SNP's –de verschillende verschijningsvormen van een gen– van het IMPDH type II gen te correleren aan IMPDH activiteit om de variabiliteit tussen patiënten verklaren. Door het voorkomen van een SNP kan IMPDH actiever of juist minder actief zijn. In de bestudeerde patiëntengroep werden alleen variaties van de SNP *IMPDH type II* 3757T>C waargenomen en deze variatie kwam met een frequentie van 6,9% voor. Van de patiënten had 2% de variatie van het gen op beide genen. Een vergelijking tussen patiënten zonder variatie en patiënten met een variatie van het gen liet geen significant verschil in IMPDH activiteit vóór transplantatie zien. Op dag 6 na de transplantatie was de blootstelling aan IMPDH activiteit over 12 uur significant 49% hoger bij patiënten met een variatie van het gen. Wij concluderen dat het *IMPDH type II* 3757T>C polymorfisme is geassocieerd met een verhoogde IMPDH activiteit in niertransplantatie patiënten die worden behandeld met MMF. Deze SNP verklaart voor 8,0% de variabiliteit in IMPDH activiteit tussen de patiënten.

IMPDH activiteit analysemethoden zijn tijdrovend en arbeidsintensief. In **hoofdstuk 4.2** hebben we daarom onderzoek gedaan of het bepalen van IMPDH mRNA als alternatieve methode kan dienen bij het monitoren van MMF therapie. IMPDH mRNA (messenger –'boodschapper'– RNA) wordt afgelezen van het DNA in de cel en zorgt vervolgens dat het enzym IMPDH gemaakt kan worden. mRNA is dus de boodschapper tussen het erfelijke DNA in de kern en het gedeelte in de cel dat de enzymen maakt. De studie was ontworpen om IMPDH mRNA concentraties in de cel te correleren met IMPDH activiteit en klinische uitkomsten bij niertransplantatie patiënten. Er werden geen correlaties gevonden tussen IMPDH type I en type II mRNA concentraties en IMPDH activiteit gemeten

vóór en na transplantatie. De bepaling van IMPDH mRNA concentraties is dan ook geen alternatief voor het monitoren van IMPDH activiteit.

Patiënten met een verhoogde IMPDH mRNA concentratie vóór transplantatie hadden een verhoogd risico op een acute afstoting, maar dit was niet significant. Op dag 6 na transplantatie waren verlaagde IMPDH type I en type II mRNA concentraties significant gecorreleerd met het optreden van acute afstotingen.

Het aflezen van de twee verschillende types IMPDH genen verloopt via andere routes en daarom werd aangenomen dat ook een verschil in het verloopt van de beide concentraties zou worden gezien. Maar in ons onderzoek stegen de concentraties van zowel IMPDH type I als type II mRNA gelijk ten opzicht van elkaar na transplantatie.

MMF wordt behalve na orgaantransplantaties steeds vaker gebruikt na transplantatie van bloedstamcellen om acute graft-versus-host reacties te voorkomen. In **hoofdstuk 5.1** hebben we een verkennend onderzoek uitgevoerd bij patiënten na een bloedstamcel transplantatie. De behandeling van MMF is in deze studie voor het eerst farmacodynamisch gemonitord door het meten van de IMPDH activiteit. Patiënten met een hogere IMPDH activiteit voor inname van MMF hadden een sterkere remming van de IMPDH activiteit 2 uur na inname. De variabiliteit tussen patiënten in de concentratie-effect relatie was groot, zoals reeds gezien is bij niertransplantatie patiënten. Ook de remming van de IMPDH activiteit in patiënten na bloedstamcel transplantatie was vergelijkbaar met de remming in patiënten na een niertransplantatie. Maar ondanks de gelijke IMPDH activiteit waren de MPA concentraties hoger in patiënten na een niertransplantatie. We veronderstellen daarom dat het IMPDH enzym bij patiënten na bloedstamcel transplantatie gevoeliger is voor het remmende effect van MPA dan bij niertransplantatie patiënten; er is minder MPA nodig om dezelfde remming van IMPDH te krijgen in vergelijking met niertransplantatiepatiënten.

Er werden geen significante correlaties gevonden tussen IMPDH activiteit en het ontstaan van graft-versus-host reacties. De IMPDH activiteit was wel significant lager in patiënten die leden aan ernstige bijwerkingen van MMF in vergelijking met patiënten met geen of milde bijwerkingen. Bijwerkingen die werden waargenomen waren een te kort aan bloedplaatjes, neutrofielen (een bepaalde soort witte bloedcellen) en/of bloedarmoede. Gezien de kleine groep onderzochte patiënten en de grote verscheidenheid van patiënten in deze pilot-studie, dient de klinische relevantie van onze conclusies bevestigd te worden in een studie met meer patiënten.

Hoofdstuk 6 bespreekt de belangrijkste resultaten en conclusies van de gepresenteerde onderzoeken in dit proefschrift. In dit hoofdstuk is ook gekeken in hoeverre deze een antwoord geven op de specifieke doelstellingen die zijn geformuleerd in hoofdstuk 1.2. Tijdens het samenvatten en bespreken zijn naar aanleiding van de verrichte

onderzoeken ook verschillende nieuwe onderzoeksvragen gesteld. Suggesties voor vervolgonderzoek naar het IMPDH enzym zijn in dit hoofdstuk weergegeven.

Op basis van onze resultaten kan het monitoren van IMPDH activiteit nog niet worden aanbevolen om de behandeling van MMF te optimaliseren en te individualiseren. Het IMPDH enzym wordt geremd door veel meer factoren dan wij hebben onderzocht. Toch heeft dit proefschrift een basis gegeven om IMPDH activiteit en de remming van IMPDH door MPA beter te begrijpen in patiënten die worden behandeld met MMF. Onze bevindingen kunnen dienen als uitgangspunt voor toekomstige studies naar de fascinerende relatie tussen farmacokinetiek en farmacodynamiek van het geneesmiddel MMF.

Chapter 8

Appendices

Chapter 8.1

Dankwoord

Drie en een half jaar IMPDH activiteit meten, analyseren en bediscussiëren betekent heel veel activiteit. In deze jaren hebben vele mensen naast mij ook veel activiteit geleverd om dit onderzoek tot een goed einde te brengen. De overeenkomsten met deze activiteit en IMPDH activiteit zijn groot: er is een grote inter-individuele variabiliteit en de activiteit is de sleutel tot het eindproduct. Het is mij daarom ook een groot genoegen dat ik hier een aantal mensen mag bedanken voor de activiteit die ze hebben geleverd voor het onderzoek en mijn proefschrift.

Allereerst wil ik mijn promotor Prof. Dr. A.G. Vulto en mijn co-promotoren Dr. T. van Gelder en Dr. R.A.A. Mathôt bedanken. Beste Arnold, bedankt voor het faciliteren van mijn promotieonderzoek in de apotheek van het Erasmus MC. Je schat aan informatie en parate kennis (je weet altijd feilloos het juiste artikel uit de immens grote stapels te halen) heeft me de complexiteit van het wetenschappelijk onderzoek meer doen inzien. Jouw inzicht gaf soms een hele andere kijk op de zaken. Bedankt voor de tijd die je hebt genomen in het begeleiden, zelfs tot mijn stellingen toe.

Beste Teun, volgens mij zijn wij elkaar steeds meer gaan waarden en respecteren in ons zijn. De snelheid waarmee je jouw scherpe, maar terechte commentaar op onderzoeksvragen en manuscripten leverde, heb ik zeer gewaardeerd. Je wist mij op de juiste momenten te motiveren en te activeren door soms slechts hele kleine subtiele opmerkingen of mailtjes. En tot op heden blijft het voor mij nog steeds een raadsel waar jij de energie vandaan haalt om na een zware congresdag nog tot diep in de nacht te stappen. Daar waar ik halverwege al afhaakte. Beste Ron, in twee grote projecten binnen mijn onderzoek heb ik van jouw expertise gebruik mogen maken. Dit heeft geleidt tot twee mooie papers die de basis en de kroon van mijn proefschrift zijn geworden. Een andere passie die we delen is het motorrijden. Maar helaas is het nooit gekomen tot een gezamenlijke motorrit.

Prof. Dr. J. Lindemans, Prof. Dr. W. Weimar en Dr. K. Budde dank ik voor hun bereidwilligheid om zitting te nemen in mijn kleine commissie en voor de inhoudelijke beoordeling van dit proefschrift.

Prof. Dr. H.J. Guchelaar, Prof. Dr. J.N.M. IJzermans en Prof. Dr. J.J. Cornelissen wil ik bedanken voor hun deelname aan de grote commissie.

Ontzettend veel dank ben ik verschuldigd aan alle patiënten die hebben deelgenomen aan deze studie. Ik waardeer het zeer dat ik deelgenoot mocht zijn van de spannende tijden rondom hun transplantatie en de tijd erna, om zo ook de praktische en emotionele kant van een transplantatie van dichtbij mee te kunnen maken. Het was een bevoorrechte positie waarin ik alle ups (eindelijk weer haring eten) en downs (toch weer mogelijk een afstoting) mee mocht maken.

Voor de afname van de vele bloedmonsters heb ik ontelbaar veel hulp gekregen van researchverpleegkundige Margaret Nierop. Ik weet zeker dat zonder jouw hulp en enthousiasme een stuk minder patiënten meegedaan zouden hebben. Je bent een meester in het bloed afnemen, waar alle patiënten je om roemen en waar ik veel van geleerd heb. Voor mij was het altijd weer een feest om even langs te komen op je kamer op 9 Zuid om naast alle afdelingsperikelen ook even te horen hoe het in Engeland was.

Ook de researchverpleegkundigen Bianca van der Velde, Evelien van Beugen en Marjolein Gerrits van de onderzoekskamer Bd 248 hebben ontzettend veel betekend in dit onderzoek. Jullie toegewijde aandacht voor de patiënten maakten de lange poli-bezoeken voor hen een stuk aangenamer. Jullie waren zeer flexibel wanneer afspraken verschoven werden of wanneer patiënten spontaan bij jullie aan de balie stonden. Mede daardoor zijn er slechts weinig patiënten uitgevallen in de loop van de studie. Het was fantastisch om met jullie samen te werken, en heerlijk om de beslommeringen rondom het onderzoek bij jullie kwijt te kunnen.

Tijdens het includeren van de patiënten heb ik veel hulp gehad van de zaalartsen Robert Nette, Marcia Kho, Mario Korte, Han Yo Ie en Madelon van Agteren en nurse practitioner Mirjam Tielen. Bedankt voor jullie helpende hand in het vragen van de patiënten en de klinische lessen die ik van jullie kreeg rondom transplantaties. Voor het poli-klinische gedeelte van het onderzoek ben ik veel dank schuldig aan de dames van de poli interne geneeskunde – niertransplantatie, en in het bijzonder aan Marja Swijnenburg. Bedankt voor het plannen van de afspraken op de juiste momenten, het klaarleggen van de statussen en het meedenken wanneer er weer eens logistieke problemen waren.

Veel tijd heb ik doorgebracht in het laboratorium van de apotheek. Mijn dank gaat daarom uit naar de analisten Angela, Bart, Chelesta, Christien, Frans, Joram, Manuel, Mark, Regina, Ruben, Ruud, Sandra en Wilfred. Door jullie gastvrijheid en gezelligheid is mijn tijd op het lab een geweldige tijd geweest. Het heeft mij goed gedaan tijdens het opzetten van de methoden en het meten van de monsters. Speciale dank gaat uit naar Bart, Ruben, Frans en Mark voor hun hulp bij het opzetten van de methoden. Jullie kennis over de apparaten en analysetechnieken hebben mij enorm geholpen.

Carla Baan, Annemiek Peeters en Monique Quaedackers, bedankt dat jullie alle IMPDH mRNA concentraties in mijn monsters hebben gemeten.

Ron van Schaik en Marloes van der Werf, bedankt voor de farmacogenetische analyse van alle polymorfismen in mijn patiëntenpopulatie.

De studenten Maurits de Rotte en Marianne Vos wil ik bedanken voor hun bijdrage in het onderzoek bij het stemcel transplantatie patiënten tijdens hun onderzoeksstage.

Petra Glander, Klemens Budde und Pia Hambach, ich bedanke mich sehr für die Gastlichkeit. Während meinen Aufenthalt bei Ihnen im Labor hat es mir sehr gut gefallen. Sie haben mir eine riesige Erklärung über der IMPDH Aktivität assay gegeben. Außerdem habe ich zusammen mit dir beiden auch sehr genossen. Die Stattführung durch Berlin und das Schlittschuhlaufen stellte sicher dass ich der Trip als sehr besonderes erfuhr.

Ik bedank mijn mede-onderzoekers Reinier, Maurice en Brenda voor onze samenwerking. Hoewel het voor velen leek of wij altijd zeeën van tijd hadden om koffie (of thee voor Maurice) te drinken, hebben we ook vele discussies met elkaar gehad over onze onderzoeken. Maar ook waren we er voor elkaar om de onvermijdelijke onderzoeksdippen (of software problemen) te bespreken, zodat we weer met frisse moed verder konden gaan. Brenda, jij speciaal bedankt voor de hulp en steun tijdens mijn onderzoek als 'sparring-partner', en alle gezellige tijden buiten het onderzoeken als goede vriend. Vandaag sta je me bij als paranimf, wat een mooie weerspiegeling is van onze samenwerking. En hierna weer heerlijk skiën en feesten met elkaar, want 'jij bent zo'.

Ook mijn andere kamergenoten, Anouk, Bregje, Laureen, Liselotte en Maren bedanken voor de gezellige tijd met elkaar op de kamer. Mensen maken voor mij het werk, en jullie hebben dat voor mij zeer aangenaam gemaakt. Hopelijk zijn jullie ook nog aan jullie werk toegekomen op onze niet-altijd-rustige kamer.

Samen met alle andere jonge apothekers Anna, Asmar, Carolien, Claartje, Delia, Jan-Dietert, Lyonne, Matthijs, Mila, Monique, Ryan, Satu, Savita, Sonja, Tessa, Valentina, Vincent en Yves heb ik een prachtige werksfeer gehad. Onze jaarlijks weekendje weg en de spontane vrijdagmiddagborrels bij Coenen hebben zeker bijgedragen aan de gezelligheid onderling. Verder wil ik alle andere collega's in de apotheek (ziekenhuisapotheker, apothekersassistenten en administratief, secretarieel, kwaliteits, logistiek, farmaceutisch en ICT medewerkers) bedanken voor mijn tijd in het Erasmus MC.

In het verlengde hiervan wil ik ook alle nieuwe collega's van mij in het OLVG bedanken, en speciaal Eric Franssen. Bedankt voor je interesse in mijn onderzoek, de ruimte die je gaf en de motivatie die je bij mij hoog hield om ook het laatste gedeelte van mijn promotie af te maken. Ik ben bevoorrecht dat jij mijn opleider bent voor mijn opleiding tot ziekenhuisapotheker.

Een promotieonderzoek is vaak een lange en eenzame gebeurtenis. Eén ding is zeker, het kost ongelofelijk veel tijd, tijd die ik helaas daardoor al te vaak niet aan mijn vrienden, familie en schoonfamilie kon besteden. Vrienden, jullie zeker ook bedankt voor jullie morele steun en toeverlaat in deze tijden. Ik hoop dat er wat dat betreft weer rustigere tijden aanbreken om elkaar weer meer te zien en te spreken. Erik, in de tijd dat we collega's waren heb ik ooit tegen je gezegd dat je mijn paranimf moest zijn als ik zou promoveren, toen nog niet gedacht dat die dag ook echt zou aanbreken. Ik ben blij

dat je die taak op je hebt genomen. En het is een mooi resultaat dat ik je tijdens onze avonden in de kroeg zo enthousiast voor het onderzoek heb gemaakt, dat jij nu ook bezig bent met jouw promotieonderzoek. Daniël, bedankt voor het ontwerpen van de voorkant van dit proefschrift. Judith, vielen Dank für Ihre Eingabe.

Lieve mam, bedankt voor je onvoorwaardelijke support en je moederlijke bezorgdheid tijdens mijn proefschrift, ondanks dat het in Rotterdam was. Zonder je positief bemoedigende woorden en je kaartjes op het juiste moment zou het een stuk zwaarder geweest zijn. Lieve Cynthia en Merik, bedankt voor jullie interesse in wat ik nu precies allemaal deed en waar ik nu weer naar toe moest voor congres.

En wat is een man zonder sterke, lieve en zorgzame vrouw naast zich? Marijke, wat ben ik ongelofelijk gezegend met jou naast me! Dit werk hebben we samen gedaan, want jij hebt hier ook veel tijd in zitten. Je hebt mij vaak moeten missen -meer dan ik wilde- wanneer ik avonden en weekenden lang bezig was het mijn onderzoek. Bedankt voor je liefde en begrip hiervoor. Samen met jou ben ik compleet, en daar hoef ik echt geen doctor voor te zijn om dat zeker te weten. Ik houd van je!

Chapter 8.2

List of publications

FULL PAPERS

Glander P, **Sombogaard F**, Budde K, van Gelder T, Hambach P, Liefeldt L, Lorkowski C, Mai M, Neumayer HH, Vulto AG, Mathot RA. Improved assay for the nonradioactive determination of inosine 5'-monophosphate dehydrogenase activity in peripheral blood mononuclear cells.

Ther Drug Monit. 2009;31:351-359

Sombogaard F, van Schaik RH, Mathot RA, Budde K, van der Werf M, Vulto AG, Weimar W, Glander P, Essioux L, van Gelder T. Interpatient variability in IMPDH activity in MMF-treated renal transplant patients is correlated with *IMPDH type II* 3757T>C polymorphism.

Pharmacogenet Genomics. 2009;19:626-634

Sombogaard F, Peeters AM, Baan CC, Mathot RA, Quaedackers ME, Vulto AG, Weimar W, van Gelder T. Inosine monophosphate dehydrogenase messenger RNA expression is correlated to clinical outcomes in mycophenolate mofetil-treated kidney transplant patients, whereas inosine monophosphate dehydrogenase activity is not.

Ther Drug Monit. 2009;31:549-56.

de Winter BC, van Gelder T, **Sombogaard F**, Shaw LM, van Hest RM, Mathot RA. Pharmacokinetic role of protein binding of mycophenolic acid and its glucuronide metabolite in renal transplant recipients.

J Pharmacokinet Pharmacodyn. 2009;36:541-564

Sombogaard F, Mathot R, Kho M, Vulto A, Weimar W, van Gelder T. Inhibition of IMPDH activity in MMF treated renal transplant patients is better correlated to unbound MPA concentrations.

Submitted

Sombogaard F, de Rotte M, Cornelissen J, Mathot R, van Gelder T, Doorduijn J. Pharmacodynamic monitoring of MMF therapy by measuring IMPDH activity after allogeneic hemaetopoietic stem cell transplantation: a pilot study.

Submitted

Mathot RAA, **Sombogaard F**, van Schaik RHN, Vulto AG, Weimar W, van Gelder T. Mycophenolate mofetil therapy in renal transplant patients: relationship between pharmacokinetics, inosine monophosphate dehydrogenase activity and clinical outcome.

Submitted

ABSTRACTS

Sombogaard F, Budde K, van Schaik RHN, Glander P, van der Werf M, van Gelder T.
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Br J Clin Pharmacol. 2009;68:134

Sombogaard F, Mathot RAA, Glander P, Weimar W, van Gelder T.
Br J Clin Pharmacol. 2009;68:135

Sombogaard F, Mathot RAA, Weimar W, van Gelder T.
Am J Transplant. 2010;10:(suppl2)30-31

Chapter 8.3

Abbreviations

AcMPAG	Acyl MPAG
ALAT	Alanine aminotransminase (ALT)
AMP	Adenosine monophosphate
ASAT	Aspartate aminotransaminase (AST)
ATG	Anti-thymocyte globulin
AUC	Area under the curve
b.i.d.	Lat: bis in die; twice daily
BCA	Bicinchoninic acid
BPAR	Biopsy proven acute rejection
cDNA	Complementary DNA
CNI	Calcineurin inhibitor
C_t	Cycles threshold
CL	Clearance
CrCl	Creatinine clearance
CRP	C-reactive protein
CsA	Ciclosporine A
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
DPN	Diphosphophyridine nucleotide
E_0	Baseline activity
EC-MPS	Enteric-coated mycophenolate sodium
EHC	Enterohepatic circulation
E_{max}	Maximum inhibitory effect
F	Bioavailability
FK	Tacrolimus
FO	First order
FOCE	First order conditional estimate
GFR	Glomerulair filtration rate
GGT	γ-glutamyltransferase
GTP	Guanosine triphosphate
GvHD	Graft-versus-host disease
HLA	Human leukocyte antigen
HPLC	High-performance liquid chromatography
HSCT	Hematopoietic stem cell transplant
IBD	Inflammatory bowel disease
IC_{50}	Concentration which inhibits E_{max} to 50%
IMP	Inosine monophosphate
IMPDH	Inosine 5′-monophosphate dehydrogenase
K_a	Absorption rate constant

KCl	Potassium chloride
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
Li-Hep	Lithium heparin
MMF	Mycophenolate mofetil
M-MLV RT	Moloney murine leukemia virus reverse transcriptase
MPA	Mycophenolic acid
MPAG	7-O-MPA-glucuronide
mRNA	Messenger ribonucleic acid
MRP-2	Multidrug resistant protein-2
MUD	Matched unrelated donor
MVOF	Minimum value of objective function
N.A.	Not available
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced NAD
NONMEM	Nonlinear mixed effects modeling
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PD	Pharmacodynamics
PK	Pharmacokinetics
PRA	Panel-reactive antibodies
Q	Intercompartmental clearance
QCS	Quality control sample
r^2	Correlation coefficient
RIC	Reduced intensity conditioning
RT-PCR	Real-time polymerase chain reaction
SCS	Spiked control sample
Sib	Siblings
SNP	Single nucleotide polymorphisms
T_{lag}	Lag time
TBAS	Tetra-N-butylammonium hydrogen sulfate
TDM	Therapeutic drug monitoring
Tx	Transplantation
UGT	Uridine diphosphate glucuronosyltransferase
UTR	Untranslated region
UV	Ultraviolet
V	Volume of distribution
WBC	Whole blood cell
XMP	Xanthine monophosphate

Chapter 8.4

Curriculum Vitea

Ferdi Sombogaard was born on 10 January 1979 in Bangert, The Netherlands. He completed secondary school (Athenaeum) at the Marcus College in Grootebroek in 1997. Subsequently, he started his Pharmacy study at the University of Groningen. During his study, he performed a research project titled 'Pharmacokinetics and toxicity of oxaliplatin and irinotecan in the treatment of patients with advanced colorectal carcinoma' at the Isala Clinics in Zwolle under supervision of Prof.Dr. J.R.B.J. Brouwers and Dr. F.G.A. Jansman. In 2003 he obtained his Master of Science degree in Pharmacy, main subject pharmacokinetics and drug delivery, followed by his Pharmacist's degree in 2004. Thereafter, he worked as a pharmacist in hospital in Het Groene Hart Hospital in Gouda.

Ferdi started in 2005 his PhD research at the department of Hospital Pharmacy, unit Clinical Pharmacology of the Erasmus University Medical Center in Rotterdam. Under supervision of Prof.Dr. A.G. Vulto, Dr. T. van Gelder and Dr. R.A.A. Mathôt he worked on the research project 'Pharmacodynamics of inosine monophosphate dehydrogenase' of which the results are described in this thesis. This project was in close affiliation with the departments of Internal Medicine – Nephrology and Clinical Chemistry. He received in 2006 the ESOT-Novartis study grant of the European Society of Transplantation for a co-research project with the department Internal Medicine – Nephrology of the Charité University Medical Center Berlin, Germany. In 2008 he received the Young Investigator Award of the American Society of Transplantation for parts of his research.

Ferdi is currently working in the Onze Lieve Vrouwe Gasthuis in Amsterdam as hospital pharmacist trainee. In June of this year, Ferdi will marry Marijke.

Chapter 8.5

PhD portfolio

Name:	Ferdi Sombogaard
PhD Period:	December 2005 – May 2009
Promotor:	Prof. Dr. A.G. Vulto, Dept. of Hospital Pharmacy
Copromotors:	Dr. Ron Mathôt, Dept. of Hospital Pharmacy
	Dr. Teun van Gelder, Dept. of Hospital Pharmacy, Dept. of Internal Medicine

1. PhD TRAINING

1.1 General academic skills

- Biomedical English Writing and Communication — Aug-Nov 2008
- Methodology of patient research and preparation of allowance requests — Mar 2006

1.1 Research skills

- Biomedical Research Techniques VII — Oct 2008
- Masterclass Clinical Research — Oct 2006

1.1 In-depth courses

- Hesperis course of the ESOT (Malmö, Sweden) — May 2007
  (Lyon, France) — Nov 2006
- 6th European Transplant Fellow Workshop ESOT (Wroclaw, Poland) — Dec 2005

1.1 Presentations

- IMPDH type I and II gene expression and IMPDH activity correlated to clinical outcomes in MMF treated kidney transplant patients. — oral
  NVKF&B, Mar 2009
- IMPDH type I and II gene expression and IMPDH activity correlated to clinical outcomes in MMF treated kidney transplant patients. — oral
  Annual Meeting NTV, Mar 2009
- IMPDH activity on day 6 after kidney transplantation is significantly re lated to the risk of acute rejection in MMF treated patients. — oral
  NVKF&B, Oct 2008
- IMPDH activity is correlated with the 3757T>C polymorphism in the IMPDH type II gene in MMF treated kidney transplant patients. — poster
  FIGON medicines Days, Oct 2008
- IMPDH activity on day 6 after kidney transplantation is significantly related to the risk of acute rejection in MMF treated patients. — oral
  ImmunoTDM - IATDMCT, Sept 2008

- IMPDH activity on day 6 after kidney transplantation is significantly related to the risk of acute rejection in MMF treated patients. *8th ATC Jun 2008* — oral
- IMPDH activity is correlated with the 3757T>C polymorphism in the IMPDH type II gene in MMF treated kidney transplant patients. *8th ATC, May 2008* — poster
- IMPDH activity on day 6 after kidney transplantation is significantly related to the risk of acute rejection in MMF treated patients. *Annual Meeting NTV, Mar 2008* — oral
- IMPDH activity is correlated with the 3757T>C polymorphism in the IMPDH type II gene in MMF treated kidney transplant patients. *Annual Meeting NTV, Mar 2008* — oral
- IMPDH genotype-phenotype correlation in lymphocytes drawn from healthy volunteers. *2nd NZVA Day, Oct 2007* — poster
- The Influence of Albumin on the Activity of IMPDH in Haematopoietic Stem Cell Transplant Recipients Treated with Mycophenolate Mofetil. *2nd NVZA Day, Oct 2007* — poster
- IMPDH genotype-phenotype correlation in lymphocytes drawn from healthy volunteers. *8th Congress of EACPT, Aug 2007* — poster
- Pharmacokinetics of Mycophenolate Mofetil in Haematopoietic Stem Cell Transplant Recipients. *6th European Transplant Fellow Workshop ESOT, Dec 2005* — oral

1.1 (inter)national Conferences

- Biannual meeting NVKF&B — Mar 2009
- Annual meeting NTV — Mar 2009
- 3rd NVZA Day — Nov 2008
- Biannual meeting NVKF&B — Oct 2008
- FIGON Medicines Days — Oct 2008
- ImmunoTDM IATDMCT (Warsaw, Poland) — Sept 2008
- 8th ATC (Toronto, Canada) — Jun 2008
- Annual meeting NTV — Mar 2008
- 2nd NVZA Day — Oct 2007
- 8th Congress of EACPT (Amsterdam, The Netherlands) — Aug 2007
- Biannual meeting NVKF&B — Mar 2007
- FIGON Medicines Days — Sept 2006

1.1 Seminars and workshops

- Pharmacogenetics in clinical practice — Sep 2007
- Pharmacokinetic future of MPA — Jan 2007
- World Transplantation Congress Review — Sep 2006

1.1 Prizes and awards

- FIGON poster prize — Oct 2008
- Poster of distinction ATC — Jun 2008
- Young Investigator Award ASTS/AST — May 2008
- ESOT Novartis Study Grant — Apr 2006

2. TEACHING ACTIVITIES

1.1 Lecturing

- Graduate level Medical students, 'Practical Pharmacotherapy' — 2006-2009
- Monitoring mycophenolate mofetil therapy by IMPDH activity measuring; interim results of a PK/PD study, dept. of nephrology — 2008

1.1 Supervising MSc-theses

- M. Vos – MSc student Medicine - Erasmus University Rotterdam — 2008
- M.C.F.J. de Rotte – MSc student Pharmacy - University of Groningen — 2007

AST	American Society of Transplantation
ASTS	American Society of Transplant Surgery
ATC	American Transplantation Congress
EACPT	European Association for Clinical Pharmacology and Therapeutics
IATDMCT	International Association of Therapeutic Drug Monitoring and Clinical Toxicology
NTV	Nederlandse Transplantatie Vereniging (Dutch Transplantation Society)
VKF&B	Nederlandse Vereniging van Klinsiche Farmacologie & Biofarmacie (Dutch Society of Clinical Pharmacology & Biopharmacy)
NVZA	Nederlandse Vereniging voor Ziekenhuis Apothekers (Dutch Association for Hospital Pharmacists)