Molecular Pharmacodynamics of the Immunosuppressant Mycophenolic Acid:

A Basis for Monitoring and Individualized Treatment

Doctoral Thesis by

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List of Papers

(I) Vethe NT, Mandla R, Line PD, Midtvedt K, Hartmann A, Bergan S. Inosine monophosphate dehydrogenase activity in renal allograft recipients during mycophenolate treatment. *Scand J Clin Lab Invest*. 2006;66(1):31-44.

(II) Vethe NT, Bergan S. Determination of inosine monophosphate dehydrogenase activity in human CD4+ cells isolated from whole blood during mycophenolic acid therapy. *Ther Drug Monit*. 2006;28(5):608-613.

(III) Vethe NT, Bremer S, Bergan S. IMP dehydrogenase basal activity in MOLT-4 human leukaemia cells is altered by mycophenolic acid and 6-thioguanosine. *Scand J Clin Lab Invest*. 2008;68(4):277-285.

(IV) Vethe NT, Bremer S, Rootwelt H, Bergan S. Pharmacodynamics of mycophenolic acid in CD4+ cells: A single-dose study of IMPDH and purine nucleotide responses in healthy individuals. *Ther Drug Monit*. 2008;Epub ahead of print.

Abbreviations

AcMPAG	mycophenolic acid acyl glucuronide
A_{min}	minimum enzyme activity
A_t	enzyme activity at time t (hours)
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
AUC_{0-12h}	area under the variable vs. time curve between time zero and 12 hours
AZA	azathioprine
b.i.d.	*bis in die* (twice daily)
CD4+ cells	cells that express the surface protein CD4 (T lymphocyte subset)
C_{max}	maximum concentration
C_0	pre-dose concentration (or concentration at time zero)
C_t	concentration at time t (hours)
CsA	cyclosporine A
CV	coefficient of variation (standard deviation relative to the mean)
CYP	cytochrome P-450
DNA	deoxyribonucleic acid
EC_{50}	concentration required for 50 % inhibition of enzyme activity
EC-MPS	enteric-coated mycophenolate sodium
EHC	entero-hepatic circulation
EDTA	ethylene diamine tetraacetic acid
E_{min}	minimum gene expression
E_t	gene expression at time t (hours)
GI	gastrointestinal
GDP	guanosine 5'-diphosphate
GMP	guanosine 5'-monophosphate
GTP	guanosine 5'-triphosphate
$IC_{50\ (or\ 90)}$	concentration required for 50 % (90 %) inhibition of cell proliferation
IMP	inosine 5'-monophosphate
IMPDH	inosine monophosphate dehydrogenase (IMP dehydrogenase)
IMPDH1	IMP dehydrogenase type 1 gene

IMPDH2	IMP dehydrogenase type 2 gene
K_i	inhibition constant
LC	liquid chromatography
MMF	mycophenolate mofetil
MOLT-4	specific cell line of human acute T-lymphoblastic leukemia cells
MPA	mycophenolic acid
MPAG	mycophenolic acid 7-O-glucuronide
Mrp-2	multidrug resistance-associated protein 2
NAD	nicotinamide adenine dinucleotide (oxidized, NAD^+; reduced, NADH)
P value	the probability of observing a difference strictly by chance
PBS	phosphate-buffered saline
PBMC	peripheral blood mononuclear cells
SE	standard error of the mean
SNP	single nucleotide polymorphism
Tac	tacrolimus
TDM	therapeutic drug monitoring
6-TGN	6-thioguanine nucleotides
tGuO	6-thioguanosine
$t_{1/2}$	half-life
t_{max}	time to maximum of the observed variable
UGT	uridine diphosphate-glucuronosyltransferase
UV	ultraviolet
XMP	xanthosine 5'-monophosphate

Errata

Paper II

In the *Cell preparation* section it should read “The tube was incubated at **2 to 8 °C** for 30 min on a rotating-tilting device.”

The full name of NAD should be nicotin**amide** adenine dinucleotide.

Background

Mycophenolic Acid: Antibiotic and Immunosuppressant

Toward the end of the 19th century, the Italian scientist Bartolomeo Gosio searched for the cause of pellagra among toxic substances in infected maize. In 1896, he reported the isolation of a *Penicillium* strain from which a crystalline metabolic substance was obtained. The metabolite was shown to inhibit the growth of *Bacillus anthracis*.[1] Some years later, in 1913, the substance was re-isolated and named mycophenolic acid (MPA) by Alsberg and Black.[2] Clutterback and Raistrick isolated MPA from *Penicillium brevi-compactum* and reported tentative structural characteristics of the molecule in 1933.[3] Its structural formula (**Figure 1**) was, however, not confirmed until 1952.[4,5]

Investigations of the antibiotic activity of MPA were carried out in the 1940s. The fungal metabolite demonstrated growth inhibition of certain microorganisms.[6] Antiviral and antitumour activities of MPA were characterized during the 1960s. However, the in vivo antiviral activity appeared to be lower than expected from in vitro studies, possibly due to the counteracting immunosuppressive effect that was recognized at the same time.[7-9 quoted in 10] The Lilly Research Laboratories caught interest in the pharmacodynamics of MPA and studied its antiviral and antitumour activities,[11-13] extending into clinical trials of MPA therapy in cancer patients. Only a slight antitumour effect was demonstrated.[14] The Eli Lilly and Company's most promising program for taking MPA into clinical practice aimed at the antiproliferative effect of MPA in the treatment of psoriasis in the 1970s. Several clinical studies demonstrated that MPA (dose range 2400 to 7200 mg/day) reduced the severity of psoriasis, though adverse effects like nausea, abdominal cramps, diarrhea, viral infections and leukopenia were reported.[15-20] Ultimately, the adverse effect profile and immunosuppressive potential of MPA caused the discontinuation of the multicenter psoriasis trials in 1977-78.[19,21]

Still, the immunosuppressive activity of MPA became the pharmacological success of this *Penicillium* metabolite. In 1972 Ohsugi et al. reported that MPA mediated prolonged survival time of skin allografts in mice.[22 quoted in 23] In the 1980s, Allison at Syntex Research recognized the immunosuppressive potential of MPA in rejection prophylaxis following organ transplantation, and a drug development program was initiated.[24] The pharmacokinetics of MPA was improved by morpholinoethyl ester derivatization. Based on studies in monkeys, the morpholinoethyl ester prodrug (named mycophenolate mofetil:

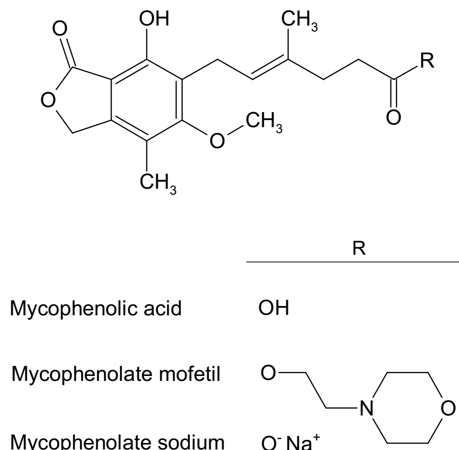

Figure 1. Molecule structures of mycophenolic acid and its drug derivatives.

MMF, RS-61443, molecule structure in **Figure 1**) demonstrated shorter time to reach maximum concentration (t_{max}), higher bioavailability and less between-subject variability compared to MPA.[25] Pre-clinical studies on MMF demonstrated prolonged survival of pancreatic islet allografts in mice,[26] heart allografts in rats and monkeys[27,28] and renal allografts in dogs.[29] Also, reversal of heart allograft rejection in rats[27] and renal allograft rejection in dogs[30] was demonstrated. A two-center dose-finding phase I/II clinical trial was performed in human renal allograft recipients in order to investigate the safety, pharmacokinetics and efficacy of MMF maintenance therapy combined with cyclosporine A (CsA) and corticosteroids. Acceptable tolerability with daily oral doses up to 3500 mg MMF and significant correlation between rejection episodes and dosage was reported.[31] Subsequently, a five-center clinical trial addressing reversal of acute refractory cellular rejection in renal transplant patients showed beneficial efficacy of MMF rescue therapy.[32] The results from these early clinical studies opened for three multicenter, prospective, randomized, double-blinded clinical trials that were initiated in 1992: the U.S. Renal Transplant Mycophenolate Mofetil Study, the European Mycophenolate Mofetil Cooperative Study and the Tricontinental Mycophenolate Mofetil Renal Transplantation Study. The large-scale phase III trials in de novo renal transplant patients demonstrated superior efficacy and acceptable safety of oral MMF maintenance therapy (1000 mg b.i.d.), as part of a triple immunosuppressive regimen with CsA and corticosteroids, compared to placebo and azathioprine (AZA).[33-35] A pooled data analysis of MMF 1000 mg b.i.d. in the

three trials showed 50 % reduced incidence of acute rejection and a one-year graft survival that was comparable to the combined placebo/AZA group.[36]

During the pre-marketing period MMF was included in Roche's product line when Syntex Research was acquired by F. Hoffmann-La Roche Ltd. As a curiosity, the MMF brand name – CellCept® – is an acronym of the words "select" and "accept".[37] On May 3, 1995, about 100 years after the first isolation of MPA, regulatory authorities approved the immunosuppressant MMF in rejection prophylaxis after renal allograft transplantation (U.S. Food and Drug Administration, www.fda.gov/cder, Drugs@FDA).

Mycophenolic Acid in Transplant Immunosuppression

Following its approval in renal transplant patients (1000 mg b.i.d.), MMF was also approved in maintenance rejection prophylaxis after transplantation of cardiac and liver allografts (1500 mg b.i.d) (European Medicines Agency, U.S. Food and Drug Administration). According to the label, MMF should be used concomitantly with CsA and corticosteroids. However, since the mid-1990s, post-transplant immunosuppressive regimens have increasingly turned into protocols favoring tacrolimus (Tac) as the cornerstone calcineurin inhibitor. Consequently, in current transplant immunosuppression MMF is frequently combined with Tac and corticosteroids. MMF has mostly replaced AZA in triple regimens including calcineurin inhibitors and corticosteroids, and in quadruple regimens including antibody-based induction, calcineurin inhibitors and corticosteroids (US data).[38] The superiority of MMF compared to AZA has later been questioned. A study in renal transplant recipients showed similar efficacy of MMF and AZA, combined with CsA microemulsion (Neoral®), in preventing acute rejection.[39,40] However, the encouraging efficacy and safety of MMF have probably facilitated the evolution of regimens that allow reduced steroid or calcineurin inhibitor dose in attempts to decrease drug-related complications.[41] Although MMF has not improved the one-year patient or graft survival, the ester prodrug has become a frequent constituent in immunosuppressive protocols following transplantation of kidney, pancreas, liver, heart and lung.[38,41]

Mycophenolate mofetil does not induce irreversible organ toxicity or lipid abnormalities. Nevertheless, in the three multicenter trials preceding its clinical launch, a total of 113 of the 910 patients discontinued MMF. Gastrointestinal (GI) and hematological symptoms dominate the adverse effect profile; primarily diarrhea, abdominal pain, nausea,

vomiting, leukopenia and anemia. The incidence of these adverse events is reported to be dose-dependent, and symptoms resolve with dose reduction or splitting, or discontinuation. Increased risk of lymphomas and lymphoproliferative disease, though of low incidence, and possibly tissue-invasive cytomegalovirus infection, are reported in relation to the use of MMF (adverse events reviewed by Mele and Halloran[41]). With respect to teratogenic effects, the drug label was recently revised and now includes that MMF treatment during pregnancy is associated with increased risk of pregnancy loss and congenital malformations.

Gastrointestinal intolerance has been the Achilles' heel of the MMF therapy, and enteric-coated mycophenolate sodium (EC-MPS, Myfortic®, molecule structure in **Figure 1**) was developed in an attempt to reduce possible MPA-induced upper GI irritation. This delayed-release tablet was approved in rejection prophylaxis following renal transplantation, combined with CsA and corticosteroids, by the Swiss Agency for Therapeutic Products in 2002. However, EC-MPS demonstrated similar efficacy and safety to near-equimolar doses of MMF in de novo and maintenance renal transplant recipients, and no significant improvement of GI adverse effects was achieved with the enteric-coated formulation.[42-44]

Mechanism of Action of Mycophenolic Acid

In 1969, Franklin and Cook reported that MPA-mediated inhibition of DNA synthesis in cultured cells was reversed by the addition of guanine, but not by hypoxanthine, xanthine or adenine. They demonstrated that MPA inhibited the conversion of [^{14}C]hypoxanthine into xanthine and guanine fractions, and ultimately revealed that preparations of inosine monophosphate dehydrogenase (IMP dehydrogenase, IMPDH, EC 1.1.1.205) were strongly inhibited by MPA, with K_i values between 3.0×10^{-8} and 4.5×10^{-8} mol/L.[45]

IMPDH catalyzes the oxidation of inosine 5'-monophosphate (IMP) to xanthosine 5'-monophosphate (XMP) in the de novo synthesis of guanine and deoxyguanine nucleotides (**Figure 2**). This branch-point enzyme is usually referred to as rate-limiting in the guanine nucleotide synthesis pathway, a suggestion originally based on studies in rat liver.[46] Nicotinamide adenine dinucleotide (NAD) is electron acceptor during the IMPDH-catalyzed reaction, and the enzyme is dependent on potassium ion bound near the active site for catalytic activity. A suggested reaction mechanism involves binding of IMP to IMPDH followed by a nucleophilic attack on the C2 position of IMP by an active-site cysteine thiol (Cys-331). The oxidation is accompanied with hydride transfer to NAD^+, and a covalent

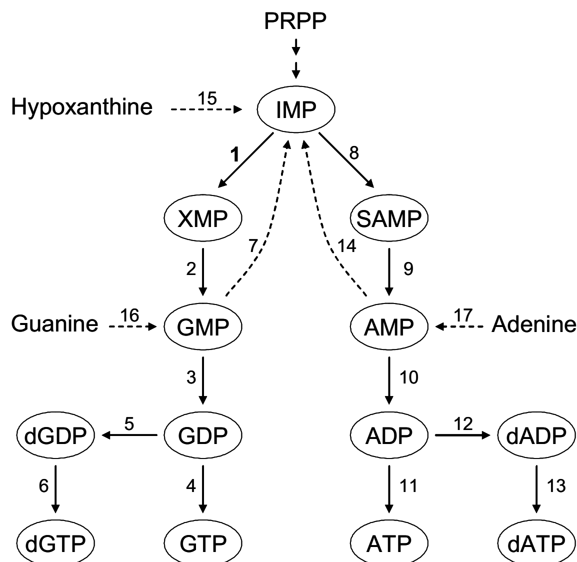

Figure 2. Purine nucleotide synthesis. Phosphoribosyl pyrophosphate (PRPP), inosine monophosphate (IMP), xanthosine monophosphate (XMP), guanosine mono-/di-/triphosphate (GMP, GDP, GTP), deoxyguanosine di-/triphosphate (dGDP, dGTP), succinyl-adenosine monophosphate (SAMP), adenosine mono-/di-/triphosphate (AMP, ADP, ATP), deoxyadenosine di-/triphosphate (dADP, dATP). (1) IMP dehydrogenase (IMPDH), (2) GMP synthase, (8) adenylosuccinate synthase, (9) adenylosuccinate lyase, (3, 4, 6, 10, 11, 13) kinases, (5, 12) ribonucleotide reductases, (7) GMP reductase, (14) AMP deaminase, (15, 16) hypoxanthine phosphoribosyltransferase, (17) adenine phosphoribosyltransferase.

thioimidate intermediate is formed. NADH is then released and a catalytic water molecule hydrolyzes the intermediate to produce XMP. The ring system of MPA is hypothesized to mimic the nicotinamide ring of the cofactor, while the phenolic hydroxyl antagonizes the catalytic water molecule. Thus, MPA blocks the hydrolysis of the covalent nucleotide-enzyme intermediate and consequently the dissociation of XMP (**Figure 3**).[47,48] With respect to pharmacodynamics at the conformational level of the protein, MPA was reported to induce a mobility shift of yeast IMPDH (IMD) upon gel electrophoresis.[49] A similar mobility shift was reported for human IMPDH exposed to MPA.[50,51] It was further demonstrated that MPA induced large intracellular IMPDH aggregates that to some extent

attained the form of ring structures, and this aggregate formation was reversed by the addition of guanosine 5'-triphosphate (GTP).[50]

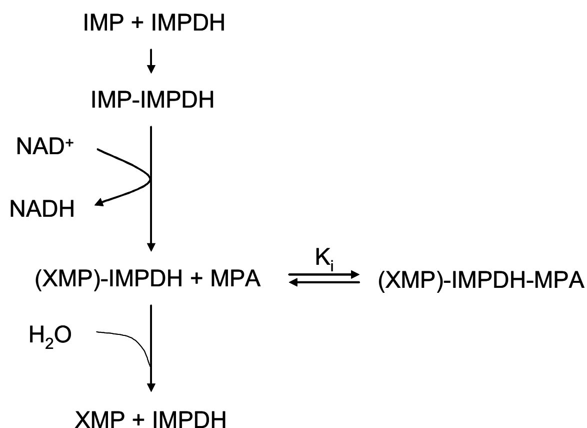

Figure 3. Mycophenolic acid (MPA) blocks the dissociation of xanthosine monophosphate (XMP) by uncompetitive, reversibel, tight-binding inhibition of IMP dehydrogenase (IMPDH). Inosine monophosphate (IMP), nicotinamide adenine dinucleotide (NAD), inhibition constant (K_i).

The human IMPDH protein is a tetramer composed of 56 kDa monomers.[47] The enzyme is expressed in two isoforms, IMPDH 1 and 2, which are encoded on distinct genes and show 84 % identity in the peptide sequences of 514 amino acids.[52] Substrate affinities and catalytic turnover rates are comparable for IMPDH 1 and 2,[47,53] and both isoforms are expressed in most tissues.[54] In peripheral blood mononuclear cells (PBMCs) the gene expression of *IMPDH1* is reported to be higher than the expression of *IMPDH2*.[54,55] Up-regulation of the *IMPDH2* gene expression is related to the elevated IMPDH activity seen in malignant transformation,[56] while both *IMPDH 1* and *2* mRNAs are induced upon lymphocyte activation.[55]

Studies showed that MPA inhibits both IMPDH 1 and 2 in an uncompetitive manner with respect to IMP and NAD (i.e. MPA binds non-competitively to the enzyme, after the binding of substrate).[47,53] The K_i values for MPA against IMPDH 1 and 2 were 3.3×10^{-8} and 7.0×10^{-9} M, respectively, thereby classifying MPA as a tight-binding inhibitor of both isoforms, and rendering IMPDH2 almost 5-fold more sensitive to MPA inhibition.[47] MPA binds non-covalently to IMPDH,[48] and several studies have demonstrated the reversibility of

the interaction in vivo.[57-60] It has been reported that MPA also inhibits guanosine monophosphate synthase (GMP synthase, EC 6.3.5.2), the enzyme catalyzing the conversion of XMP to GMP, with a K_i value in the order of 10^{-8} mol/L.[12] In the literature, however, IMPDH is generally recognized as the specific target of MPA.

The immunosuppressive effect of MPA is mainly caused by its antiproliferative activity against activated T and B lymphocytes undergoing clonal expansion. Proliferating lymphocytes are selectively targeted by MPA because they are highly dependent on the de novo purine synthesis. The MPA-mediated reductions of (deoxy)guanine nucleotides lead to cell cycle arrest in the synthesis (S) phase due to blocking of DNA synthesis.[61] Next to the depletion of these purine nucleotides, other mechanisms may also contribute to the immunosuppressive effect. The following processes are suppressed by MPA: Antibody production, glycosylation and expression of certain adhesion molecules that facilitate recruitment of mononuclear cells into sites of inflammation, maturation of dendritic cells and production of nitric oxide by inducible nitric oxide synthase. In addition, MPA presumably induces apoptosis of activated T lymphocyte subsets (extensive review of mechanisms of action by Allison and Eugui[24,62]).

Pharmacokinetics of Mycophenolic Acid

Following oral administration, MMF is efficiently absorbed and rapidly hydrolyzed to the active agent MPA. The mean oral bioavailability of MPA (derived from MMF) was reported to be 94 %, 81 % and 95 % relative to intravenous administration in healthy volunteers,[63] renal[64] and heart transplant recipients,[65] respectively. As the in vivo plasma half-life ($t_{1/2}$) appeared to be approximately 2 minutes for intravenous MMF, essentially complete presystemic deesterification of oral MMF was demonstrated in healthy volunteers.[63] Maximum plasma concentration (C_{max}) of MPA occurs 1 to 2 hours after oral MMF administration, and frequently a secondary peak attributed to entero-hepatic circulation (EHC) appears 4 to 12 hours post-dose.[66] It is suggested that the excretion of the phenolic glucuronide metabolite of MPA into bile is facilitated by the multidrug resistance-associated protein 2 (Mrp-2) in hepatocytes, and that reabsorption of MPA occurs after deglucuronidation in the intestine.[67,68] The contribution of EHC to the total MPA exposure ranges between 10 % and 61 %.[66]

According to the label, the absolute bioavailability of MPA is 72 % after oral administration of EC-MPS in renal transplant recipients. With respect to the area under the plasma concentration vs. time curve between time zero and 12 hours post-dose ($AUC_{0\text{-}12h}$), EC-MPS 720 mg b.i.d. demonstrates similar MPA exposure as MMF 1000 mg b.i.d. in maintenance renal transplant patients. However, it should be noted that the pharmacokinetics differ between the two formulations. The MPA C_{max} is slightly lower, the time to reach peak concentration (t_{max}) is longer and more variable, and the predose concentration (C_0) of MPA is higher and more variable with EC-MPS than with MMF.[69-71]

Ex vivo studies have shown that MPA is highly recovered in the plasma fraction of the blood compartment.[72,73] The drug is extensively bound to albumin as the unbound fraction usually ranges between 1 % and 3 % relative to the total plasma concentration.[73-75] While the MPA unbound fraction was fairly stable over a wide range of total MPA concentrations, a non-linear inverse relationship was demonstrated between the MPA unbound fraction and the concentration of albumin itself. Certain substances were reported to potentially increase the MPA unbound fraction by competitive displacement. Importantly, the MPA-mediated inhibition of IMPDH activity and cell proliferation was related to the unbound drug concentration in vitro.[73]

According to clinical pharmacokinetic observations and consistent with its tight binding to albumin, MPA is characterized as a restrictively cleared drug (i.e. the clearance is limited by the unbound concentration).[76] In healthy volunteers, the mean apparent terminal $t_{1/2}$ was reported to be approximately 17 hours.[63] However, estimation of the plasma $t_{1/2}$ of MPA may be complicated by the EHC. MPA is almost entirely eliminated by metabolism. The liver is the major metabolizing site, but the gut wall and the kidneys are presumably also contributing. Most of the MMF dose is excreted in urine as the phenolic MPA 7-O-glucuronide (MPAG).[63] This major metabolite appears in plasma at concentrations 20- to 100-fold higher than MPA[66] with an albumin-bound fraction ranging from 60 % to 80 %.[75,77] Minor metabolites are identified as the MPA 7-O-glucoside and the MPA acyl glucuronide (AcMPAG),[78] and the trace amount oxidation product 6-O-desmethyl MPA[79] (molecule structures of the metabolites in **Figure 4**). Average exposure of AcMPAG is approximately 10 % relative to MPA in renal transplant patients.[74,80] The uridine diphosphate-glucuronosyltransferase (UGT) enzyme family is primarily responsible for the clearance of MPA. MPAG is mainly formed by UGT1A9-catalyzed conjugation, and also by UGT1A7, 1A8 and 1A10, whereas AcMPAG is formed by UGT2B7.[81] Formation of the MPA 7-O-glucoside is presumably catalyzed by uridine diphosphate-

glucosyltransferase(s),[78] while cytochrome P-450 (CYP) 3A4/5 is involved in the production of 6-O-desmethyl MPA.[78,79] It has been suggested that Tac increases the bioavailability of MPA by inhibition of its glucuronidation.[82]

The main metabolite MPAG is pharmacodynamically inactive,[83] whereas AcMPAG is reported to inhibit IMPDH[84] and the proliferation of in vitro stimulated PBMCs,[85] though the antiproliferative effect was partially attributed to free MPA as deglucuronidation occurred during the incubation. Nevertheless, the carboxyl-linked glucuronide has showed reactivity towards proteins. Covalent AcMPAG adduct formation has been demonstrated for human albumin,[80] and for certain proteins in rat liver and colon.[86] It has been speculated whether such adducts might have a causal role in MPA-related adverse effects (e.g. GI intolerance).

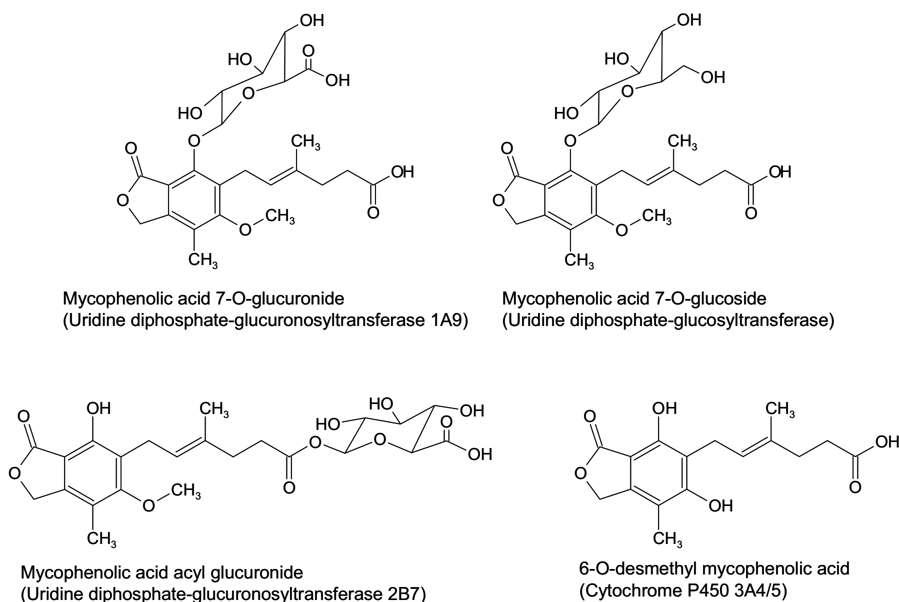

Figure 4. Identified metabolites of mycophenolic acid, and enzymes involved in their formation.

A characteristic feature of the MPA pharmacokinetics is the increase of the dose-normalized AUC_{0-12h} during the first months after transplantation in some patients.[87,88] Parallel in time, decreasing concentration of MPAG and unbound fraction of MPA have been demonstrated.[74] This is partially attributed to gradually improving renal function in the early post-transplant period. The observation of impaired renal function increasing the

apparent clearance of MPA may be explained in light of MPA being a restrictively cleared drug. Renal impairment leads to accumulation of MPAG, and possibly other substances, which displaces MPA from its albumin-binding sites. Then a higher MPA unbound fraction is available for elimination through metabolism.[76] In a population pharmacokinetic meta-analysis, the time-dependent decrease of the apparent clearance of MPA turned out to be inversely related to renal function (i.e. estimated creatinine clearance by the Cockcroft and Gault formula), as well as to albumin and hemoglobin concentrations, whereas the MPA exposure was inversely related to the CsA pre-dose concentration.[88,89] The albumin concentration in plasma will influence the fraction of MPA that is available for glucuronidation. Since CsA interrupts the EHC of MPAG/MPA by inhibition of Mrp-2,[67,68] reduction of the CsA dose over time will increase the recycling of MPA.

In addition to the intraindividual alterations over time that are related to physiological characteristics and interacting co-medication, the clinical pharmacokinetics of MPA also shows high variability between individuals. More than 10-fold variation has been reported for the dose-normalized MPA $AUC_{0\text{-}12h}$.[90] Also, the concentration-time profiles within the MMF oral dose intervals differ between patients. Variable occurrence of a lag time in absorption, and variable timing and extent of EHC make the pharmacokinetics complex. Administration of MMF or EC-MPS in the fed state is reported to cause delayed t_{max} and reduced C_{max} of MPA, though the $AUC_{0\text{-}12h}$ appears to be unaltered.[66] The highly variable pharmacokinetics of MPA that is observed between individuals is apparently related to intrinsic and extrinsic factors influencing absorption, EHC, albumin-binding and metabolic capacity.

Individualization of Mycophenolate Therapy

Rationale and Strategies for Dose Individualization

Individualized drug treatment implies consideration of factors that directly or indirectly influence the variability of responses between and within patients to provide a rationale for drug dose or drug choice. Direct or surrogate response measurement, blood concentration of drug, physiological characteristics, expression or activity of endogenous molecules specifically related to drug pharmacodynamics or pharmacokinetics, or genetic information with impact on these, may ultimately guide the pharmacotherapy into improved clinical outcome. However, certain criteria should be met to justify the implementation of drug individualization based on laboratory methodologies. Some important criteria are as follows:

Failure of drug treatment has serious consequences for the patient
A narrow therapeutic window exists for the drug
Considerable interindividual pharmacokinetic or pharmacodynamic variability
A relationship between the observed variable and clinical outcome
A reasonable balance between benefit and cost of individualization

Mycophenolate mofetil was approved with fixed dose recommendations. However, a consensus panel early proposed that therapeutic drug monitoring (TDM, i.e. dose individualization based on measurement of drug concentration) could optimize the use of MMF and improve the rejection outcome.[91] During more than a decade pharmacologists and clinicians have put a great effort in the development of MMF individualization strategies. Although MPA meets most of the criteria for dose individualization and some TDM guidelines are suggested for MMF/MPA,[92] this is still controversial in routine practice.[93] Possible reasons may be the relatively low rejection rates obtained with standard MMF dosing in combination with other efficacious immunosuppressants, the use of variable concomitant immunosuppressants, the absence of irreversible organ toxicity related to MPA, the vague relationship between pharmacokinetic variables of MPA and adverse effects, and the complex pharmacokinetics of MPA (e.g. time-dependent clearance, EHC, variable concentration-time profiles).[92,93] It is, however, generally accepted that low MPA exposure in the early post-transplant period is related to the incidence of acute rejection,[92-95]

and it is likely that emerging immunosuppressive regimens with reduced calcineurin inhibitor and steroid dose will increase the need for variability-controlled MPA therapy.

Although MPA AUC_{0-12h} demonstrated a significant relationship to acute rejection in a study of renal transplant patients, considerable overlap was reported for this variable between those patients experiencing and not experiencing rejection.[87] This observation indicates that variable pharmacodynamics exist between patients, and opens for the search of pharmacodynamic markers with stronger predictive power than MPA exposure. Individualization of MPA therapy based on IMPDH-related variables is yet at the experimental stage. Current research focuses on examination of the pharmacokinetic-pharmacodynamic relationship between MPA and IMPDH. No studies have prospectively used IMPDH as a biomarker to guide dose individualization. However, reports of association between IMPDH and clinical outcome are emerging. It is conceivable that measurement of MPA-mediated IMPDH inhibition could serve as a surrogate pharmacodynamic marker for the therapeutic response to MPA. The integration of pharmacokinetic and biological variability may potentially provide a more accurate prediction of efficacy and adverse effects than MPA exposure alone. Also, cellular guanine or deoxyguanine nucleotide concentrations may reflect the in vivo inhibition of IMPDH and thereby the efficacy of MPA. The choice of bioanalytical matrix, sampling time relative to transplantation and within the dose interval, and the choice of absolute levels vs. relative alterations will influence the potential success of demonstrating a strict relationship between the pharmacodynamic marker and clinical outcome. Differentiation between the two IMPDH isoforms could also be influential. In combination with pharmacokinetic measurements, the monitoring of IMPDH activity or inhibition in a relevant cell matrix may be valuable in future strategies of individualized MPA therapy.

The Pharmacokinetic Approach

Plasma concentrations of MPA may be quantified in the laboratory by liquid chromatography (LC)-based methods coupled to UV detection or mass spectrometry, or by the enzyme-multiplied immunoassay technique (EMIT). While the LC methods specifically determine MPA, the EMIT assay shows cross-reactivity between MPA and AcMPAG.[96] Recently, the validation of an IMPDH inhibition assay for the quantification of MPA plasma concentration was presented.[97]

The MMF phase I/II study in renal transplant recipients showed a significant relationship between acute rejection and MMF dose.[31] Thereafter, an open-label multicenter study demonstrated association between acute rejection and low MPA $AUC_{0\text{-}12h}$ following renal transplantation.[98] The pharmacokinetic-pharmacodynamic relationship was further investigated in a randomized, double-blinded, multicenter, concentration-controlled study of MMF treatment in 150 deceased donor renal transplant patients receiving CsA and corticosteroids. In this study, a logistic regression analysis demonstrated that the MPA $AUC_{0\text{-}12h}$ was significantly related to the incidence of acute rejection, and $AUC_{0\text{-}12h}$ 15 mg×h/L predicted 50 % of maximum efficacy. The pre-dose concentration of MPA had lower predictive value. Tolerability was apparently not related to systemic MPA exposure.[87,99] Based on the results in this study, a tentative therapeutic window of MPA $AUC_{0\text{-}12h}$ between 30 and 60 mg×h/L was suggested.[100] It should be noted that the suggested upper limit was based on maximum efficacy, and not adverse events. However, later retrospective studies indicated that adverse events could be related to MPA exposure (total MPA C_0, $C_{0.5}$, C_1, $AUC_{0\text{-}12h}$) in renal transplant patients receiving CsA[101] or Tac.[102] An MPA peak concentration above 8 mg/L was suggested to be a reasonable discriminator for adverse events in patients receiving MMF in combination with Tac, and also C_0 3 mg/L or $AUC_{0\text{-}12h}$ 38 mg×h/L were proposed as adverse event thresholds. A study in pediatric renal transplant patients receiving CsA indicated that high unbound MPA $AUC_{0\text{-}12h}$ (above 0.4 mg×h/L), but not total AUC, was associated with MPA-related leukopenia or infections. The authors reported total MPA $AUC_{0\text{-}12h}$ below 34 mg×h/L or total MPA C_0 below 1.2 mg/L as potential predictors of acute rejection.[103] Other investigators have also reported a relationship between unbound MPA exposure and hematological or infectious events.[104,105] Pharmacokinetic-pharmacodynamic investigations in 94 renal transplant patients receiving CsA (of whom 72 received basiliximab induction therapy) demonstrated that MPA $AUC_{0\text{-}12h}$ significantly predicted acute rejection. In this study, $AUC_{0\text{-}12h}$ 22 mg×h/L at day 3 post-transplant appeared as the best threshold level for efficacy, whereas the MPA exposure did not predict adverse effects.[106] Another study in 100 renal transplant patients receiving Tac (of whom 31 received daclizumab induction therapy) did not demonstrate any significant relationship between MPA exposure alone and acute rejection. However, high $AUC_{0\text{-}12h}$ or C_0 were significantly related to anemia and leukopenia.[107]

Although the exposure-efficacy/safety reports have been somewhat conflicting, the total MPA $AUC_{0\text{-}12h}$ has been regarded as the most plausible single variable in therapeutic

monitoring of MPA.[92-94,100] MPA C_0 may be a poorer discriminator between rejecters and non-rejecters.[108] Because full AUC_{0-12h} sampling is expensive in routine practice, estimation of AUC_{0-12h} by limited sampling strategies based on three-point algorithms have emerged as practical approaches. Yet, prospective studies are of a limited number.[92] A 12-month, multicenter, open-label, randomized study in 130 de novo renal transplant patients receiving basiliximab induction, CsA and corticosteroids (the APOMYGRE study) assessed concentration-controlled vs. fixed dose MMF treatment. MPA AUC_{0-12h} was estimated by a three-point limited sampling strategy using a Bayesian estimator.[109] The clinical outcome was significantly improved by targeting MPA AUC_{0-12h} 40 mg×h/L in the concentration-controlled group.[110] Concentration-controlled vs. fixed dose MMF treatment was also examined in a large-scale, multicenter, open-label, randomized study in 901 de novo renal transplant patients receiving CsA or Tac (the FDCC study). MPA AUC_{0-12h} was estimated by a three-point limited sampling strategy using linear regression algorithms. According to a preliminary report, the MPA exposure was comparable between the two groups, and consequently the concentration-controlled strategy did not improve the clinical outcome.[111] Based on data from this study, it was recently reported that high risk patients (i.e. delayed graft function, second or third transplantation, panel reactive antibodies > 15 %, or four or more HLA mismatches) were at greater risk of acute rejection if MPA AUC_{0-12h} at day 3 after transplantation was below 30 mg×h/L. Such a relationship was not observed for low risk patients.[112]

Although in their early stages, indirect pharmacogenetic-pharmacokinetic approaches might prove valuable for MMF dose individualization. Two single nucleotide polymorphisms (SNPs) of the *UGT1A9* gene promoter region, associated with increased expression and glucuronidation rate,[113] were related to significantly lower MPA exposure in transplanted patients receiving MMF 1000 mg b.i.d. in combination with Tac. In the same cohort of patients, a SNP of the *UGT1A9* coding region, linked to substrate-dependent decreased glucuronosyltransferase activity,[113,114] was associated with numerically higher MPA exposure.[115] Also SNPs in the *UGT1A9* gene promoter region were recently reported to be associated with higher MPA exposure than the wild type genotype in renal transplant patients receiving CsA.[116] Two SNPs in the *Mrp2* gene, coding the organic anion transporter involved in biliary excretion of MPAG, influenced the MPA pharmacokinetics in renal transplant patients receiving Tac. Non-carriers with mild liver dysfunction demonstrated decreased MPA exposure in the early post-transplant period. One of the SNPs was

associated with higher MPA C_0 levels in stable patients, and also with more episodes of diarrhea during the first year after transplantation.[117]

The Pharmacodynamic Explorations:

Current Status – Including Discussion on Findings of the Doctoral Thesis

The methodologies for determination of IMPDH activity and IMPDH inhibition in clinical samples have evolved from methods based on IMPDH-catalyzed conversion of radiolabelled substrate, into LC-based quantification of endogenous substrate-to-product conversion. Balzarini and De Clercq developed assay conditions that demonstrated reduced [^{3}H] release from [2,8-^{3}H]IMP in cells exposed to IMPDH inhibitors.[118] Montero et al. developed a non-radiolabel assay allowing measurement of the XMP production rate in erythrocyte lysate by ion-pair reversed-phase LC with UV detection after liquid-liquid extraction.[119] Later, LC-UV methods without the requirement of liquid-liquid extraction were established,[57,120,121] and those methods of Albrecht et al. and of Glander et al. were thoroughly validated for the determination of IMPDH activity in lysates of whole blood cells or PBMCs and in lysates of PBMCs, respectively. We modified the LC-UV method of Albrecht et al. and developed an assay for the determination of IMPDH activity in CD4+ cells. This assay was specifically validated for MPA-mediated IMPDH inhibition.[122] An assay for the measurement of IMPDH activity in PBMCs using LC coupled to mass spectrometry was recently reported.[123]

Langman et al. were apparently the first to demonstrate the immediate and reversible MPA-mediated inhibition of whole blood IMPDH activity in vivo following administration of MMF to rabbits[124] and transplanted patients.[59] An inverse relationship between MPA plasma concentration and whole blood IMPDH activity was indicated during the 12 hours dose interval in the patients receiving MMF. The immediate, reversible, inverse pharmacokinetic-pharmacodynamic relationship in vivo was later confirmed in whole blood cells,[57,60] in PBMCs[58] and in CD4+ cells.[122,125]

A pertinent question has been whether MPA induces the underlying IMPDH activity during long-term MPA therapy. Such an induction could potentially counteract the effect of MPA over time, and would complicate the approach of pharmacodynamic monitoring of IMPDH-related variables. Sanquer et al. reported that prolonged MMF treatment (>1 year) was associated with increased IMPDH activity in whole blood cells.[126] High whole blood

cell IMPDH activity in MMF patients was also observed by Albrecht et al.[57] Subsequently, Weigel et al. reported that the long-term MMF-associated induction of IMPDH activity was located to erythrocytes.[127] We described the longitudinal alterations of whole blood cell IMPDH activity in renal transplant patients receiving MMF. A few days post-transplant the pre-dose enzyme activity decreased, and thereafter the activity gradually increased during 6 to 8 weeks, reaching almost 5-fold of baseline activity. Despite the elevated IMPDH activity, the MPA exposure caused considerable IMPDH inhibition in the whole blood cell fraction.[60] Furthermore, it was demonstrated that increased *IMPDH 1* and *2* gene expressions in reticulocytes were associated with MMF treatment.[128] Currently, no relationship between whole blood cell IMPDH activity and the clinical immunosuppressive effect of MPA has been described. A dose interval study in dialysis patients receiving the first MMF dose vs. long-term (>1 year) transplanted patients on repeated MMF dosing indicated that long-term MMF treatment does not induce the IMPDH activity in PBMCs. The IMPDH pharmacodynamics was similar between the two groups. However, the MPA AUC_{0-11h} was approximately 2-fold higher in the long-term patients.[58] In heart transplant patients switched from AZA to MMF, the IMPDH activity in PBMCs was significantly reduced at 3, 6 and 12 months after MMF onset, and the pre-dose MPA concentration was reported to be stable during the observation period.[129] Our group recently reported that transplantation and initiation of immunosuppressive therapy caused an initial transient increase of the *IMPDH 1* and *2* gene expressions and the underlying IMPDH activity in CD4+ cells. The increase was associated with high dose corticosteroid administration. The *IMPDH 1* expression in CD4+ cells remained slightly above baseline two weeks post-transplant, though the elevation was not specifically related to MMF.[128] In an in vitro MOLT-4 human leukemia cell model, we demonstrated that MPA influenced the underlying IMPDH activity in a biphasic concentration-dependent manner. The underlying IMPDH activity increased, in association with induced gene expression, at MPA concentrations causing less than 50 % inhibition of the cell proliferation. On the other hand, the underlying activity was reduced at a proliferation-blocking concentration of MPA.[130] The impact of MPA on the underlying IMPDH activity in different cell populations is apparently complex. Concomitant immunosuppressants, immune status and time after transplantation may also be influential in the clinical setting. These aspects should be integrated in the considerations of sampling time and choice of cell matrix when pharmacodynamic monitoring is approached.

The high interindividual variability of pre-transplant IMPDH activity underlines the rationale for exploring the possibilities for individualized MPA exposure. In renal transplant patients, the pre-transplant IMPDH activity varied 8.5- to 17-fold,[60,128] 41-fold[128] and approximately 8-fold[131] in whole blood cells, CD4+ cells and PBMCs, respectively. In a cohort of renal transplant patients receiving MMF in combination with Tac or CsA, a retrospective analysis showed that low pre-transplant IMPDH activity predicted MMF dose reduction due to intolerability. On the other hand, acute rejection was significantly associated with combined high pre-transplant IMPDH activity and MMF dose reduction.[131] We did not disclose any relationship between pre-transplant IMPDH activity in CD4+ cells and acute rejection in renal transplant patients during the early post-transplant period.[128] However, the follow-up was only 2 weeks, and there were also some technical concerns about the protocol for determination of the underlying IMPDH activity (c.f. the Paper II discussion). Recently, it was reported that high IMPDH activity (AUC_{0-12h}) in PBMCs at day 6 after renal transplantation was significantly related to acute rejection in MMF-treated patients, and there was no difference with respect to the MPA exposure between rejecters and non-rejecters.[132] Another recent study demonstrated less MPA-mediated inhibition of IMPDH in lymphocytes during the first week following renal transplantation in those patients experiencing acute rejection.[133]

Quantification of IMPDH activity in PBMCs throughout the dose interval has been used to support the hypothesis of bioequivalence between near-equimolar doses of MMF and EC-MPS in renal transplant patients. Although the activity-time profiles differed according to the different pharmacokinetics of MMF and EC-MPS, the IMPDH activity AUC_{0-12h} was comparable for the two formulations.[69,70] In these studies, the MPA concentrations required for 50 % inhibition of IMPDH (EC_{50}) were reported to be approximately 3.5 to 4 mg/L[70] and 4.5 to 6 mg/L.[69] We performed an MMF single-dose crossover exposure-response study in healthy individuals to investigate the pharmacodynamics of MPA in CD4+ cells. The non-linear relationship between IMPDH inhibition and MPA concentration was characterized. Plasma concentrations of MPA above 6 mg/L did not decrease the IMPDH activity any further, and the EC_{50} was 2.3 mg/L by a pooled data analysis. The overall IMPDH activity (i.e. AUC_{0-12h}) approached maximum reduction at a total MPA exposure beyond AUC_{0-12h} 22 mg×h/L (corresponding to the median MPA exposure at 500 mg MMF in this study).[125] These findings support the previous suggestions that early MPA AUC_{0-12h} above 22 mg×h/L might be adequate for rejection prophylaxis,[106] and that MPA peak concentrations above 8 mg/L might cause

intolerability related to overexposure.[102] The exposure-response relationship that was revealed in the study of healthy individuals also explains the observation that significantly different MPA exposure after administration of EC-MPS and MMF (61 vs. 41 mg×h/L, respectively) in a cohort of patients with progressive IgA nephritis did not cause any substantial difference with respect to the overall IMPDH suppression.[134]

A limited number of studies have addressed the potential alterations of cellular guanine nucleotides in relation to MPA therapy. Increased GTP concentration in erythrocytes was demonstrated, in association with elevated IMPDH activity, during prolonged MMF administration.[127,135] Conflicting results are reported for the GTP levels in PBMCs during long-term MMF treatment. While a study in renal transplant patients demonstrated MMF-related reduction of GTP in PBMCs,[136] another study in heart transplant patients indicated no significant alteration of GTP in PBMCs after MMF treatment for 3, 6 and 12 months, suggesting that the observed parallel induction of hypoxanthine guanine phosphoribosyltransferase (HPRT) counteracted the effect of IMPDH suppression.[129] A somewhat surprising effect has been observed for the GTP levels in lymphocytes throughout the MMF dose interval. Compared to transplanted patients on AZA, the general lymphocyte GMP concentrations were reduced and the GDP concentrations tended to be reduced, whereas the GTP concentrations increased during the MMF dose interval.[59] We investigated the effect of MPA, exclusively, on the concentrations of GDP and GTP in CD4+ cells. No immediate reduction of neither of the molecules was observed following escalating single MMF doses in healthy volunteers. On the contrary, a trend toward increased GTP was observed also in this specific lymphocyte subset.[125] No relationship between clinical outcome and cellular guanine nucleotide concentrations, or relative alterations of these, has yet been described in transplanted patients receiving MPA therapy. Although reduced guanine nucleotides obviously are related to the antiproliferative effect of MPA in vitro and ex vivo,[130,137] the pharmacodynamics may be more complex in vivo. It could be that the compartment of circulating lymphocytes does not reflect the real requirement and capacity of purine synthesis in immunoactivated, proliferating lymphocytes. Also, it could be speculated that deoxyguanine nucleotides might be better pharmacodynamic markers than guanine nucleotides, since the former have demonstrated a closer relationship to the antiproliferative effect of MPA in vitro.[61]

Pharmacogenetic-pharmacodynamic studies have demonstrated that certain pharmacogenetic characteristics of IMPDH are associated with clinical outcome, although no specific relationship to the immunosuppressive efficacy of MPA has been reported. A

trend toward elevated *IMPDH2* gene expression in PBMCs was observed in association with gastrointestinal and hematological complications in liver transplant patients receiving MMF.[138] In renal transplant patients receiving MMF, the *IMPDH1* gene expression in PBMCs was 2- to 3-fold increased during the first 3 months post-transplant, and a 20-fold increase during acute rejection was observed for this gene in 2 patients.[139] A longitudinal study of the gene expressions of *IMPDH 1* and *2* in renal transplant patients during the early post-transplant period showed that patients who experienced acute rejection had significantly higher pre-transplant *IMPDH2* expression in CD4+ cells than the non-rejecters.[128] Also, variants of the *IMPDH* genes may influence the IMPDH phenotype, and possibly the efficacy or adverse effects of MPA. A non-frequent SNP in the *IMPDH2* exon 7, leading to substitution from leucine to phenylalanine at residue 263, generated a variant that demonstrated 10-fold decreased catalytic activity compared to the wild-type IMPDH2 protein.[51] Supported by observations of leukopenia in patients with the variant genotype, it could be speculated that this variant will predispose for MMF-related adverse effects.[51,140] On the contrary, another SNP in the *IMPDH2* gene was recently related to elevated IMPDH activity in PBMCs during MPA exposure.[141] Two intronic SNPs in the *IMPDH1* gene have been associated with the incidence of acute rejection in renal transplant patients receiving MMF treatment.[140] The clinical impact of *IMPDH* expressions and gene variants needs further characterization in future studies to develop potential algorithms for individualized MPA exposure.

In summary, MMF is frequently included with standard dosing in immunosuppressive regimens following solid organ transplantation. The exposure-dependent efficacy of MPA combined with its highly variable pharmacokinetics between patients triggered the interest in therapeutic monitoring of the drug. A therapeutic window for MPA $AUC_{0\text{-}12h}$ in the range 30 to 60 mg×h/L has been suggested. However, the MPA $AUC_{0\text{-}12h}$ between rejecters and non-rejecters show considerable overlap, the pharmacokinetics of MPA show a rather vague relationship to adverse events, and the activity of the drug target IMPDH demonstrates high interindividual variability. These findings have encouraged the exploration of pharmacodynamic monitoring of MPA. The integration of pharmacokinetics and pharmacodynamics may provide a more accurate prediction of efficacy and adverse effects than MPA exposure alone. IMPDH phenotype, gene expression and genotype have separately been associated with clinical outcome in transplanted patients receiving MMF treatment. Monitoring of the IMPDH activity (i.e. phenotype) or inhibition would represent

the most direct approach of targeting the response to MPA. The induction of IMPDH activity in erythrocytes during prolonged MPA therapy makes whole blood cells less attractive as pharmacodynamic compartment, whereas specific lymphocyte subsets (as CD4+ cells) or PBMCs seem to constitute appropriate cell matrices for the assessment of MPA pharmacodynamics. Reliable assays for the determination of IMPDH activity and MPA-mediated IMPDH inhibition have been developed, and the pharmacokinetic-pharmacodynamic relationship at the molecular level has been thoroughly described throughout the MMF dose interval. Immediate and extensive suppression of IMPDH in CD4+ cells was demonstrated after the administration of single MMF doses. Potential alterations of the underlying IMPDH activity in relation to drug administration or immune state should be considered in the design of clinical pharmacodynamic studies. The relationship between IMPDH activity or inhibition and clinical outcome needs further characterization before prospective validation of individualized MPA therapy based on pharmacodynamic monitoring may be initialized.

Objectives of the Thesis

Individualization of the MPA therapy in transplanted patients may ultimately improve the clinical outcome. Pharmacodynamic monitoring of the response to MPA is an attractive approach to individualization since it allows integration of pharmacokinetic and pharmacodynamic variability. The main objectives of this thesis were to develop reliable methods for the assessment of MPA pharmacodynamics in clinical samples, and to provide in-depth knowledge of the pharmacokinetic-pharmacodynamic characteristics of MPA at the molecular level. The obtained methods and information were supposed to make a foundation for clinical research strategies aiming at individualized MPA therapy in transplanted patients.

Specific objectives of the projects were as follows:

Paper I

The aims of this study in renal transplant patients were to describe the acute and long-term effects of MPA on the IMPDH activity. Specifically, the pharmacokinetic-pharmacodynamic relationship for MPA (1) within the MMF dose interval in stable patients and (2) longitudinally during the first 2 months post-transplant were investigated. The study was designed to allow detailed description of time-dependent alterations of the IMPDH activity following transplantation and initiation of immunosuppressive therapy including MMF.

Paper II

The method for the determination of IMPDH activity, and specifically for the quantification of MPA-mediated IMPDH inhibition, in CD4+ cells was developed in order to provide a reliable assay that was relevant for the assessment of MPA immunopharmacodynamics in clinical samples.

As CD4+ cells play important roles in the acute rejection immune response,[142] and because MPA inhibits the clonal expansion of these cells, we found it attractive to examine the pharmacodynamics of MPA in this specific cell subset mostly constituted of T lymphocytes. The cell isolation procedure based on anti-CD4 antibody-coated paramagnetic beads involves gentle treatment of the cells. Previous studies indicated that efflux of MPA

was a concern during cell isolation.[57,121,124] Rather than attempting to maintain the intracellular MPA concentration during processing of the cells, we decided to restore the intracellular concentration of MPA after cell isolation, thereby re-establishing the impact of the in vivo intracellular MPA concentration.

Paper III

The pharmacodynamics of MPA and 6-thioguanine nucleotides (6-TGN, metabolites of azathioprine or mercaptopurine) involve interference with the DNA replication in proliferating cells. While MPA inhibits the de novo synthesis of guanine and deoxyguanine nucleotides, the thiopurines cause cytotoxic effects by incorporation into the DNA. Depletion of the guanine nucleotides could potentially influence the gene expression and protein level of IMPDH. However, conflicting results have been reported on this issue.[55,143,144]

The aim of the study was to characterize the impact of MPA and 6-thioguanosine (tGuO) on the basal (i.e. underlying) IMPDH activity in proliferating cells. The study specifically focused on the potential concentration-dependencies of these relationships. The relative gene expressions of the *IMPDH* isoforms were investigated as potentially explanatory factors, and the cellular guanine and adenine nucleotides were determined to describe effects on the purine pools.

Paper IV

The integrated pharmacokinetic-pharmacodynamic approach of measuring MPA plasma concentration and IMPDH activity or purine nucleotides in relevant cell subsets might provide surrogate markers for the therapeutic response to MPA.

The aim of the study was to characterize the molecular exposure-response relationship for MPA in order to explore the possibilities and guide the development strategies for individualized therapy based on pharmacodynamic monitoring.

Methods

Paper I

Fifteen stable renal transplant patients attending the outpatient clinic were recruited for the MMF dose interval study (group 1). The patients received a CsA-based immunosuppressive regimen. Four of the patients served as controls as they did not receive MMF (which was according to the standard protocol for renal transplantation between haploidentical siblings). Two 12-h dose intervals at one-week interval were planned for each patient. Venous blood samples were collected pre-dose and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, and 12.0 hours post-dose.

Thirtythree patients undergoing renal transplantation were consecutively recruited for the longitudinal study (group 2). Four of the patients received renal grafts from their siblings and served as controls as they did not receive MMF, according to the standard protocol. Venous blood samples were collected pre-transplant and thereafter pre-dose two to three times a week during the first two weeks post-transplant, and one to two times a week until eight weeks post-transplant.

Venous blood samples were collected from 22 healthy individuals in order to obtain an estimate of the normal range of IMPDH activity.

The unbound and total MPA plasma concentrations were determined by a validated reversed-phase LC-UV method.[75] The unbound MPA assay was based on dialysis using the Gilson ASTED (automated sequential trace enrichment of dialysates) equipment (Villiers-le-Bel, France).

At this time, a reliable assay for IMPDH activity in lymphocytes isolated from clinical samples, maintaining the actual intracellular MPA concentration, had not been published. In order to minimize that problem, we chose to initiate the investigations of MPA pharmacodynamics with whole blood cells as matrix. The assay published by Albrecht et al.[57] was slightly modified and used for the determination of IMPDH activity in whole blood cells. Samples were incubated with saturating amounts of IMP and NAD, and the XMP production rate (µmol/L cell lysate/min) was quantified by measuring xanthine derived from XMP with LC-UV. The assay modification involved replacement of a Nucleosil C18 column by a Chromolith Performance column. This modification allowed a shorter elution time for xanthine (8 vs. 22 minutes), and the total run time was reduced by 50 % (20 vs. 40

minutes). Additionally, the xanthine peak height was increased by approximately 40 % (data not shown).

Linear regression of peak heights vs. concentrations was used for the calibration of MPA and xanthine in the LC assays. The MPA and IMPDH activity AUC_{0-12h} were calculated by the trapezoid method. The results are presented as medians with range, or with lower and upper quartiles. Differences in medians between groups were assessed by the Mann-Whitney U-test, and differences between time-points within the same group were tested by the Wilcoxon signed ranks test. Statistical significance was considered at the $P < 0.05$ level. Assessment of correlation was performed by bivariate analyses using Pearson's correlation. The software SPSS 11.0 was used for the statistical calculations.

Paper II

Paramagnetic monodisperse polystyrene beads coated with anti-CD4 monoclonal antibodies (Dynabeads®) were utilized for the isolation of CD4+ cells from 4 mL EDTA-blood samples. Plasma from the sample was subjected to 0.1 μm filtration in order to remove platelets and platelet-derived vesicles. The captured cells were washed in phosphate-buffered saline (PBS), and thereafter they were incubated in the microfiltrated plasma from the original sample. The plasma was removed after centrifugation, and the cells were resuspended in PBS. Determination of the cell concentration was performed by counting the intact cell nuclei on a Coulter Counter® after lysis of the cell membranes. The remaining suspension was frozen.

The IMPDH assay was modified from the method published by Albrecht et al.[57] After thawing and homogenizing, the IMPDH-catalyzed conversion of IMP to XMP was carried out in microtubes at 37 °C with saturating amounts of IMP and NAD. Following termination of the enzyme reaction, XMP was hydrolyzed to xanthine. The samples were tested for the presence of pre-existing xanthine, xanthosine or xanthine nucleotides in one parallel aliquot by omitting NAD and incubation. Xanthine was quantified by reversed-phase LC-UV. The introduction of a Chromolith Performance column in series with the Nucleosil C18 column allowed adequate separation with 50 % shorter run-time. Linear regression of peak heights was used to calculate the amount XMP produced during incubation. The IMPDH activity was expressed as the XMP production rate (pmol XMP/10^6 CD4+ cells/min).

The total MPA plasma concentration was determined as described by Svensson et al.[145] The albumin plasma concentration was determined on the Modular analytics instrument (Roche Diagnostics).

Paper III

Human acute T-lymphoblastic leukemia cells (MOLT-4) were used as the in vitro model. RPMI-1640 / fetal bovine serum (9/1) containing penicillin, streptomycin and mycostatin constituted the growth medium. The cells were resuspended in fresh medium (250 000 cells/mL in 1 mL wells) 24 hours before addition of MPA or tGuO, and the exposure lasted for 72 hours. Cell counting was performed on a Coulter Counter®.

The cell viability was determined by detection of the dead cell fractions using ethidium homodimer-1. The IMPDH activity was determined according to the assay for CD4+ cells described in paper II. A wash-out protocol was utilized for removal of intracellular MPA, allowing the IMPDH basal activity to be measured. The IMPDH activity was adjusted for cell viability and expressed as pmol XMP/10^6 cells/min. Relative gene expressions of *IMPDH 1* and *2* were quantified by a previously described real-time reverse transcription PCR assay.[146] Target gene expressions were calculated relative to the geometric mean expression of aminolevulinate delta-synthase 1, β2-microglobulin and ribosomal protein L13. A linear gradient anion-exchange LC-UV method described by others[136,147] was applied for the quantification of cellular GDP, GTP and ATP. A minor modification involving the introduction of two reversed-phase Chromolith Performance columns in front of the anion-exchange Hypersil NH2 column improved the separation of the nucleoside triphosphates.

The IC_{50} values and slope factors (H) were estimated by non-linear regression of a 4-parameter equation using the GraFit software. Furthermore, the IC_{90} values were calculated by the equation $IC_{90} = 9^{1/H} \times IC_{50}$. The control value was defined as the mean of control replicates in an experiment. The t-test and calculation of 95 % confidence intervals were performed by the statistical software SPSS 13.0. Statistical significance was considered with two tails at $P < 0.05$.

Paper IV

Five healthy individuals (Caucasians, male/female 2/3) participated in this single-dose crossover exposure-response study of MPA molecular pharmacodynamics. Median age was

50 years (range 24 to 62), and median body mass index was 23.9 kg/m^2 (range 20.6 to 26.2). Single MMF doses (mycophenolate mofetil oral suspension, CellCept™) of 100, 250, 500 and 1000 mg were administered to each individual between 8:00 and 8:30 a.m. Wash-out periods of at least five days were established between the doses.

Venous blood samples were collected pre-dose and 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0 and 24.0 hours post-dose. MPA plasma concentration and IMPDH activity were determined at all sampling times, whereas (0.25, 0.5, 1.5, 3.0, 5.0, 10.0) hours and (0.25, 5.0, 10.0) hours did not include measurement of gene expression and purine nucleotides, respectively. Also, purine nucleotides were not determined following the 100 mg dose. Samples for baseline assessments of all variables (except MPA) were collected at 8:00 a.m., 10:00 a.m., 12:00 a.m., 2:00 p.m., 4:00 p.m., 6:00 p.m. and 8:00 p.m. on a single occasion without MMF administration.

The total plasma concentration of MPA was determined according to a previously published LC-UV method.[145] The IMPDH activity in CD4+ cells, expressed as the XMP production rate (pmol/10^6 cells/min), was measured by the assay described in Paper II. A validated real-time reverse transcription PCR assay was used to quantify the relative gene expressions of *IMPDH 1* and *2* in CD4+ cells.[146] Concentrations of GDP, GTP, AMP, ADP and ATP in CD4+ cells were determined by combined reversed-phase anion-exchange LC-UV, slightly modified from a previously described method.[136,147] The chromatographic separation of the nucleoside triphosphates was improved by coupling two Chromolith Performance columns in front of a Hypersil NH2 column. Plasma albumin, total bilirubin, creatinine, urea and alanine aminotransferase (ALAT) were determined on the Modular Analytics instrument (Roche Diagnostics).

The area under the variable vs. time curve (AUC) was calculated by the trapezoid method. Minimum and maximum values, and corresponding time points, were extracted as actual observed values. The software WinNonlin Version 5.2 was used to estimate the EC_{50} values by non-linear regression with the inhibitory effect E_{max} model (effect zero at infinite concentration). Differences were assessed by the Wilcoxon signed ranks test which was run on the software SPSS 15.0. Statistical significance was considered with two tails at $P < 0.05$.

Ethics

The clinical studies were approved by the Regional Committee for Medical Research Ethics.

Results and Discussion

Paper I

In patient group 1, twenty (nineteen for the IMPDH activity) and seven 12-h profiles were obtained from the patients receiving and not receiving MMF, respectively. The total MPA C_0 was 1.2 (range 0.36 to 6.0) mg/L, C_{max} was 13.3 (range 2.7 to 37.3) mg/L and t_{max} was 1.0 (range 0.5 to 3.0) hours, and AUC_{0-12h} was 40 (range 13 to 72) mg×h/L. The median of the unbound MPA fraction medians in the dose intervals was 1.6 (range 1.1 to 2.7) %. The median coefficient of variation (CV) of the unbound fraction within the dose interval was 14 (range 10 to 23) %. A good correlation was demonstrated between MPA AUC_{0-12h} and concentrations at 1.5 and 5.0 hours, alternatively at pre-dose and 1.5 hours. In the patients receiving MMF, the minimum IMPDH activity (A_{min}) was 8 (range 2 to 26) % compared to the pre-dose level and coincided with the MPA peak concentration. Approximately six hours after dose administration the IMPDH activity returned to pre-dose values. The IMPDH activity was stable throughout the 12-h interval in the patients not receiving MMF. The pre-dose IMPDH activity was significantly higher in the MMF patients than in the non-MMF patients and in the healthy individuals. Also, the IMPDH activity AUC_{0-12h} was significantly higher in the MMF patients compared to the non-MMF patients.

In patient group 2, all MMF patients except one were observed at least until week 6, thereafter the number of patients was 16 and 12 for week 7 and week 8, respectively. The MPA C_0 was fairly stable throughout the study period. The IMPDH activity was initially reduced following transplantation and MMF onset. A reduction of 76 (range 27 to 96) % relative to pre-transplant values was observed at median 4 days post-transplant. Thereafter the IMPDH activity gradually increased. The maximum IMPDH activity was 4.9-fold (range 2.0- to 17-fold) higher than at baseline and occurred at median 40 days post-transplant. A similar increase was not observed in the non-MMF patients.

A limitation of this study would be the use of whole blood cells as bioanalytical matrix. Lymphocytes, or even activated lymphocytes, would probably constitute a more representative matrix with respect to the pharmacodynamics of MPA. Nevertheless, uncontrolled manipulation of the intracellular MPA concentration during cell processing would limit the interpretation of the MPA-mediated IMPDH inhibition in isolated cell subsets. In the present study, the real-time in vivo impact of MPA was maintained and the

measured IMPDH activity should thereby reflect the clinical capacity for guanine nucleotide synthesis. It should be noted that the MPA concentration to some extent masks the longitudinal increase of IMPDH activity in the pre-dose samples.

With precautions regarding the cell matrix, it could be speculated that the relative alterations of whole blood cell IMPDH activity within the MMF dose interval might serve as a surrogate pharmacodynamic marker for MPA. The results suggest that the clinical effect may be related to the sharp MPA peaks, as the IMPDH activity was close to pre-dose values during the second half of the MMF dose interval. The transient IMPDH inhibition may be sufficient to arrest proliferating lymphocytes in the synthesis (S) phase. Furthermore, the results suggest that monitoring strategies should focus on the first half of the MMF dose interval.

The mechanisms leading to the observed longitudinal alterations of IMPDH activity in the MMF patients may be complex. Although the measured enzyme activity in whole blood was quantitatively dominated by erythrocytes, changes with respect to the fractions of cell subsets could potentially influence the total activity. The initial reduction of IMPDH activity could be a direct MPA effect. The gradually increasing activity could be explained by induced IMPDH expression, decreased IMPDH degradation or cell subset changes. In a recent study, we demonstrated that MPA therapy was associated with increased *IMPDH* gene expression in reticulocytes.[128] The clinical relevance of the considerably increased IMPDH activity in whole blood cells during prolonged MPA therapy is unknown. Despite this longitudinal increase, the IMPDH activity is still reduced to very low levels during the MPA peaks.

Paper II

The kinetics of the IMPDH-catalyzed reaction in the CD4+ cell matrix was saturated with substrate (IMP) and co-factor (NAD) concentrations of 1.79 μmol/L and 0.38 μmol/L, respectively. The XMP concentration increased linearly vs. incubation time during 240 minutes, and a standard incubation period of 120 minutes was implemented. The XMP production rate was considered to be linear in the interval 0.13 to 8.7 pmol/min.

The relevance of restoring the intracellular MPA concentration was investigated in samples from a healthy individual who received a single 1000 mg MMF dose. CD4+ cells were isolated from pre-dose (t_0) and 1.5 hours post-dose ($t_{1.5}$) samples. Incubation of t_0- and $t_{1.5}$-cells in t_0-plasma demonstrated the MPA wash-out effect. On the other hand, incubation of t_0- and $t_{1.5}$-cells in $t_{1.5}$-plasma demonstrated the principle of re-establishing the

intracellular MPA concentration. The recoveries of albumin and MPA in the microfiltrated plasma were 97 % and 96 %, respectively.

A wash-out procedure for complete removal of intracellular MPA was described. The two last washing steps were replaced by PBS volumes of 5 mL after transferal to a new tube. In the paper, it is stated that no IMPDH inhibition was observed if the cells were resuspended in PBS rather than plasma. However, empirical data indicate that the cells should be incubated in microfiltrated blank (MPA-free) plasma to obtain comparable levels of IMPDH activity between the wash-out and standard procedure.

The interseries CVs of the IMPDH assay in CD4+ cells at MPA plasma concentrations of 0, 2.2 and 8.6 mg/L were 25 %, 16 % and 13 % (n = 7 at each concentration), respectively.

The major fraction of circulating CD4+ cells is constituted by T helper lymphocytes. This class of cells plays an important role in allograft rejection and inflammation. Hence, the IMPDH activity or inhibition in this specific cell subset may predict the clinical immunosuppressive effect of MPA. A limitation of the cell matrix is that the requirement and capacity of (deoxy)guanine nucleotide synthesis, and furthermore the sensitivity to MPA, might differ between naïve and activated lymphocytes.

The measured IMPDH activity is the maximal reaction rate of the IMP-XMP turnover. In absolute terms, it does not directly express the real in vivo IMPDH activity. It will, however, correlate to the amount of active enzyme and thereby to the capacity of XMP and (deoxy)guanine nucleotide synthesis.

We demonstrated that MPA efflux and influx are dynamic processes that should be properly handled in IMPDH inhibition assays. The concept of restoring the intracellular MPA concentration by incubating the isolated cells in microfiltrated plasma separated from the original blood sample eliminated the previously described drug wash-out problem. Importantly, the plasma was removed and the cells were resuspended in PBS before cell lysis. Then the cellular concentrations of MPA and IMPDH were proportionally diluted, thereby preserving the impact of the intracellular MPA on the IMPDH activity. Because complete removal of MPA from the cell surface is impossible without washing, there is a risk for over-estimating the enzyme inhibition.

The presented imprecision of the assay reflects the actual methodological variation between samples as the CVs comprise the entire assay including cell counting, the IMPDH-catalyzed XMP production, hydrolysis of XMP and quantification of xanthine by LC-UV.

Paper III

With respect to growth inhibition of the MOLT-4 cells, the estimated IC_{50} values were 0.24 (SE 0.01) µmol/L and 0.19 (SE 0.02) µmol/L, and the calculated IC_{90} values were 0.54 µmol/L and 0.64 µmol/L for MPA and tGuO, respectively. Cells exposed to MPA showed numerically lower viability than cells exposed to equimolar tGuO concentrations.

MPA demonstrated a biphasic concentration-dependent effect on the IMPDH basal activity; the activity increased at MPA $\leq IC_{50}$, whereas the activity was significantly reduced at MPA 0.50 µmol/L. The IMPDH activity was stable in the low range of tGuO concentrations. However, the activity was significantly reduced at tGuO 0.50 µmol/L.

The *IMPDH2* gene demonstrated 23-fold higher expression than *IMPDH1* in the MOLT-4 cells (controls). MPA induced the gene expression of both isoforms at MPA 0.15 µmol/L and 0.50 µmol/L. During exposure to tGuO a modest increase in the expression of *IMPDH2* was observed, whereas the expression of *IMPDH1* was not consistent between experiments.

At low tGuO concentrations the cellular concentrations of GDP and GTP tended to increase, and ATP increased significantly. Steep reductions of these nucleotides were observed when tGuO was increased to 0.50 µmol/L. The nucleotides gradually decreased during increasing MPA exposure. A trend toward modestly increased ATP levels was observed at the low MPA concentrations.

The results demonstrate that the IMPDH basal activity is influenced by MPA and tGuO, and suggest that reduced IMPDH basal activity is related to the proliferation-blocking effects of these agents. The reduced underlying IMPDH activity may enhance the antiproliferative activity of MPA and tGuO at high concentrations. If analogous reductions of the IMPDH basal activity occur during AZA and 6-mercaptopurine treatment, their antiproliferative and toxic effects may be limited by the IMPDH-dependent production of the thioguanine nucleotide antimetabolites. Since the IMPDH activity will not be a prerequisite for the conversion of 6-thioguanine to its corresponding nucleotides, this may contribute to the understanding of the excess toxicity that is seen with 6-thioguanine compared to 6-mercaptopurine in the treatment of childhood lymphoblastic leukemia.[148]

The elevated IMPDH basal activity at MPA $\leq IC_{50}$ was associated with increased *IMPDH2* gene expression. The *IMPDH2* gene expression was still increased at the proliferation-blocking concentration of MPA, whereas the IMPDH basal activity was reduced. According to a recent hypothesis, the MPA-mediated reduction of the GTP levels

could induce aggregate formation of IMPDH, which again may lead to trapping of less active conformations of the enzyme.[50] With respect to the tGuO experiments, the lack of correlation between the IMPDH gene expression and activity remains unexplained.

Conflicting results have been reported for the influence of MPA on the ATP levels in cell models.[137,144,149,150] Also, reports on the in vivo levels of cellular ATP during AZA and MMF treatment are diverse.[127,129,135,136] Our results indicate that ATP may be increased at low MPA and tGuO concentrations, thereafter gradually decreased during increasing MPA exposure and steeply decreased above a tGuO threshold concentration. This concentration-dependent biphasic effect might explain the diverse results previously reported.

Sensitivity to the MPA and tGuO exposure may be related to cell type and cell cycle status. Therefore, generalization of the effects observed in the MOLT-4 cell model has restrictions.

Paper IV

In the healthy individuals, the MPA C_{max} and AUC_{0-12h} increased linearly with dose. Maximum inhibition of the IMPDH activity in CD4+ cells occurred 0.25 to 3.0 hours after MMF administration and coincided with the MPA C_{max}. The IMPDH A_{min} medians were 94, 51, 15, 14 and 13 % relative to A_0 at baseline, 100, 250, 500 and 1000 mg MMF, respectively. Corresponding MPA C_{max} median values were 2.7, 6.4, 14.6 and 26.7 mg/L at 100, 250, 500 and 1000 mg, respectively. The EC_{50} was estimated to be 2.3 mg/L by a pooled data analysis, and the IMPDH activity was not further inhibited when the MPA concentrations exceeded approximately 6 mg/L. The overall IMPDH activity (AUC_{0-12h}) approached maximum reduction at MPA AUC_{0-12h} 22 mg×h/L. Maximum reduction relative to baseline IMPDH activity AUC_{0-12h} was in the order of 50 %. Although very low MPA concentrations were observed toward the end of the 12-h interval, the IMPDH A_{12} was still suppressed compared to A_0 and baseline A_{12}. An ad hoc experiment demonstrated that the direct IMPDH inhibition was 34 % at MPA C_{12} 1.1 mg/L (mean, n = 2).

The *IMPDH2* / *IMPDH1* gene expression (E_0) ratio in the CD4+ cells was median 3.6 (range 1.8 to 5.2). Minimum *IMPDH1* expression (*IMPDH1* E_{min}, normalized to E_0) within the 12-h interval was significantly reduced at 250 mg and 500 mg MMF. Also, the *IMPDH1* AUC_{0-12h} (% of E_0×h) showed a tendency toward reduction at 250 mg and 500 mg MMF. The *IMPDH2* E_{min} was significantly reduced at 500 mg MMF.

The guanine nucleotide concentrations in the CD4+ cells were not reduced following administration of MMF. On the contrary, GTP and ATP tended to increase. GDP, ADP and AMP were fairly stable.

This study in healthy individuals allowed the examination of MPA-mediated effects, exclusively, across a range of MMF doses. MPA mediated strong suppression of IMPDH in CD4+ cells, mainly by direct inhibition, but also in association with reduced *IMPDH* gene expression. It was demonstrated that total MPA AUC_{0-12h} exceeding 22 mg×h/L did not decrease the overall IMPDH activity any further. Accordingly, the 1000 mg MMF dose was in the upper range of the exposure-response curve. The results indicate that overexposure to MPA may occur with concentrations above approximately 6 mg/L. It may be hypothesized that the incidence of adverse effects could be reduced by identifying patients who are overexposed to MPA, and then reduce the MPA exposure according to IMPDH inhibition on an individual basis in these. Avoidance of high MPA C_{max} might improve the adverse effect profile. Dose reduction alone, or combined with more frequent administration, or controlled-release formulations could potentially overcome this.

The study does not support any immediate GDP or GTP reduction in circulating CD4+ cells following MMF administration. This finding suggests that these nucleotides lack potential as immediate response biomarkers to MPA. Future studies should rather address possible long-term alterations of guanine or deoxyguanine nucleotides and the potential relationship to clinical efficacy of MPA.

One limitation of this study is the single-dose design that did not allow investigation of steady state MPA concentrations. However, the MPA exposures at the high single doses were comparable to clinically relevant exposure. We could not assess the possible contribution of the active acyl glucuronide metabolite (AcMPAG) to the IMPDH inhibition, as this metabolite was not quantified.

Conclusions of the Thesis

Mycophenolic acid mediated a dual effect on the IMPDH activity in whole blood cells. Following transplantation and initiation of MMF treatment, the pre-dose IMPDH activity gradually increased during 6 weeks. At day 40 post-transplant the activity was nearly 5-fold higher than at pre-transplant. Still, a strong transient IMPDH inhibition coincided with the MPA C_{max} approximately 1 hour after MMF dose administration. The relevance of measuring IMPDH activity in whole blood cells is debated. It could be speculated that relative inhibition of whole blood cell IMPDH activity within the dose interval might predict the clinical response to MPA. The dose interval study in renal transplant patients indicates that monitoring strategies should focus on the first half of the MMF dose interval, as the IMPDH activity was close to pre-dose values during the second half of the interval.

In the MOLT-4 cell model, it was demonstrated that MPA influences the underlying IMPDH activity in a concentration-dependent manner. The proliferating cells possessed mechanisms for expression-associated induction of the IMPDH basal activity at moderate MPA concentrations, consistent with the observed induction of pre-dose activity in whole blood cells. However, the basal activity was reduced at MPA concentrations causing extensive inhibition of cell proliferation. These results suggest that reduced IMPDH basal activity may be related to the proliferation-blocking effects of MPA.

We developed and validated an assay for the determination of IMPDH activity and specifically the MPA-mediated IMPDH inhibition in CD4+ cells isolated from small-volume blood samples. This assay is suitable for the assessment of the direct molecular effect of MPA in clinical samples.

The exposure-response study in healthy individuals demonstrated strong reduction of the IMPDH activity in circulating CD4+ cells during clinically relevant MPA exposure. Reduced *IMPDH* gene expression was associated with the exposure to MPA. The pharmacokinetic-pharmacodynamic analysis revealed that MPA plasma concentrations above approximately 6 mg/L did not inhibit the IMPDH activity any further. The IMPDH activity $AUC_{0\text{-}12h}$ approached maximum reduction at MPA $AUC_{0\text{-}12h}$ 22 mg×h/L. As no guanine nucleotide reductions were observed in the circulating CD4+ cells after MMF administration, these nucleotides may not be used as immediate response biomarkers to MPA. The results suggest that the current standard dosing of MMF corresponds to MPA exposure in the upper range of the exposure-response curve, although a high variability was

observed. Still as a hypothesis, this could explain the frequent adverse effects that occur in transplanted patients receiving MPA therapy. Integrated monitoring of IMPDH activity and MPA concentration may help identifying patients who are over- or underexposed to MPA, and furthermore it may provide individualized dosing to improve the clinical outcome. Future studies are needed to characterize the potential relationship between IMPDH activity and clinical outcome during MPA therapy. Limited sampling strategies should be defined to allow the methodology in routine clinical practice.

Future Perspectives

A study of the pharmacokinetic-pharmacodynamic relationship for MPA in liver transplant patients has been initiated at Rikshospitalet University Hospital. The assay for determination of IMPDH activity in CD4+ cells will be utilized to examine the molecular response to MPA in this patient population. The first part of the study will be descriptive in order to assess the relationship between the biomarker and the clinical pharmacodynamics, and to define a limited sampling strategy. The second part will aim at prospective assessment of individualized MPA exposure based on monitoring of IMPDH activity or inhibition. One hypothesis is that adequate efficacy and less adverse effects may be obtained by reduced MPA exposure in a considerable proportion of the patients.

Similar pharmacokinetic-pharmacodynamic studies may be performed in other populations of transplanted patients. As the acute rejection and graft loss rates are relatively low with present immunosuppressive protocols, a general obstacle to prospective investigations is the great number of patients that will be needed to assess superiority of any intervention with reasonable statistical power. It could be appropriate to identify patients who are expected to benefit utmost by individualized immunosuppressive treatment (e.g. high risk patients), and then perform studies on the relationship between IMPDH activity and clinical outcome in such selected sub-populations. Prospective assessment of IMPDH monitoring should ultimately be accomplished. The importance of adverse effects due to excessive immunosuppression should be stressed as clinical endpoints in addition to acute rejection, graft loss and patient survival. Studies should ideally be designed to allow long-term follow-up. It remains to be elucidated whether relationships between IMPDH-related variables and clinical outcome are of sufficient predictive strength, and eventually whether it will be feasible to implement IMPDH monitoring in the routine laboratory service.

References

1. Gosio B. Ricerche batterologiche e chimiche sulle alterazioni del mais. Contributo all'etiologia della pellagra. *Rivista d'Igiene Sanitá Pubbl.* 1896;7:825-849.

2. Alsberg CL, Black OF. Contributions to the study of maize deterioration. Biochemical and toxicological investigations of Penicillium puberulum and Penicillium stoloniferum. *US Department of Agriculture Bureau of Plant Industry Bulletin.* 1913;7-48.

3. Clutterbuck PW, Raistrick H. Studies in the biochemistry of micro-organisms: The molecular constitution of the metabolic products of Penicillium brevi-compactum Dierckx and related species. II. Mycophenolic acid. *Biochem J.* 1933;27:654-667.

4. Birkinshaw JH, Bracken A, Morgan EN, et al. Studies in the biochemistry of micro-organisms. 78. The molecular constitution of mycophenolic acid, a metabolic product of Penicillium Brevi-compactum Dierckx. Part 2. Possible structural formulae for mycophenolic acid. *Biochem J.* 1948;43:216-223.

5. Birkinshaw JH, Raistrick H, Ross DJ. Studies in the biochemistry of micro-organisms. 86. The molecular constitution of mycophenolic acid, a metabolic product of Penicillium Brevi-compactum Dierckx. Part III. Further observations on the structural formula for mycophenolic acid. *Biochem J.* 1952;50:630-634.

6. Florey HW, Gilliver K, Jennings MA, et al. Mycophenolic acid: an antibiotic from Penicillium brevi-compactum dierckx. *Lancet.* 1946;46-49.

7. Herrmann EC, Jr., Gabliks J, Engle C, et al. Agar diffusion method for detection and bioassay of antiviral antibiotics. *Proc Soc Exp Biol Med.* 1960;103:625-628.

8. Mitsui A, Suzuki S. Immunosuppressive effect of mycophenolic acid. *J Antibiot (Tokyo).* 1969;22:358-363.

9. Williams RH, Lively DH, Delong DC, et al. Mycophenolic acid: antiviral and antitumor properties. *J Antibiot (Tokyo).* 1968;21:463-464.

10. Planterose DN. Antiviral and cytotoxic effects of mycophenolic acid. *J Gen Virol.* 1969;4:629-630.

11. Cline JC, Nelson JD, Gerzon K, et al. In vitro antiviral activity of mycophenolic acid and its reversal by guanine-type compounds. *Appl Microbiol.* 1969;18:14-20.

12. Sweeney MJ, Hoffman DH, Esterman MA. Metabolism and biochemistry of mycophenolic acid. *Cancer Res.* 1972;32:1803-1809.

13. Sweeney MJ, Gerzon K, Harris PN, et al. Experimental antitumor activity and preclinical toxicology of mycophenolic acid. *Cancer Res.* 1972;32:1795-1802.

14. Knudtzon S, Nissen NI. Clinical trial with mycophenolic acid (NSC-129185), a new antitumor agent. *Cancer Chemother Rep.* 1972;56:221-227.

15. Gomez EC, Menendez L, Frost P. Efficacy of mycophenolic acid for the treatment of psoriasis. *J Am Acad Dermatol.* 1979;1:531-537.

16. Jones EL, Epinette WW, Hackney VC, et al. Treatment of psoriasis with oral mycophenolic acid. *J Invest Dermatol.* 1975;65:537-542.

17. Lynch WS, Roenigk HH, Jr. Mycophenolic acid for psoriasis. *Arch Dermatol.* 1977;113:1203-1208.

18. Marinari R, Fleischmajer R, Schragger AH, et al. Mycophenolic acid in the treatment of psoriasis: long-term administration. *Arch Dermatol.* 1977;113:930-932.

19. McDonald CJ. The uses of systemic chemotherapeutic agents in psoriasis. *Pharmacol Ther.* 1981;14:1-24.

20. Spatz S, Rudnicka A, McDonald CJ. Mycophenolic acid in psoriasis. *Br J Dermatol.* 1978;98:429-435.

21. Epinette WW, Parker CM, Jones EL, et al. Mycophenolic acid for psoriasis. A review of pharmacology, long-term efficacy, and safety. *J Am Acad Dermatol.* 1987;17:962-971.

22. Ohsugi Y, Hirose S, Fujii G. Immunosuppressive effect of mycophenolic acid. Prolongation of survival time of skin allografts. *Transplant J.* 1972;7:257-259.

23. Ohsugi Y, Suzuki S, Takagaki Y. Antitumor and immunosuppressive effects of mycophenolic acid derivatives. *Cancer Res.* 1976;36:2923-2927.

24. Allison AC, Eugui EM. Mycophenolate mofetil and its mechanisms of action. *Immunopharmacology.* 2000;47:85-118.

25. Lee WA, Gu L, Miksztal AR, et al. Bioavailability improvement of mycophenolic acid through amino ester derivatization. *Pharm Res.* 1990;7:161-166.

26. Hao L, Lafferty KJ, Allison AC, et al. RS-61443 allows islet allografting and specific tolerance induction in adult mice. *Transplant Proc.* 1990;22:876-879.

27. Morris RE, Hoyt EG, Murphy MP, et al. Mycophenolic acid morpholinoethylester (RS-61443) is a new immunosuppressant that prevents and halts heart allograft rejection by selective inhibition of T- and B-cell purine synthesis. *Transplant Proc.* 1990;22:1659-1662.

28. Morris RE, Wang J, Blum JR, et al. Immunosuppressive effects of the morpholinoethyl ester of mycophenolic acid (RS-61443) in rat and nonhuman primate recipients of heart allografts. *Transplant Proc.* 1991;23:19-25.

29. Platz KP, Sollinger HW, Hullett DA, et al. RS-61443--a new, potent immunosuppressive agent. *Transplantation.* 1991;51:27-31.

30. Platz KP, Bechstein WO, Eckhoff DE, et al. RS-61443 reverses acute allograft rejection in dogs. *Surgery.* 1991;110:736-740.

31. Sollinger HW, Deierhoi MH, Belzer FO, et al. RS-61443--a phase I clinical trial and pilot rescue study. *Transplantation.* 1992;53:428-432.

32. Sollinger HW, Belzer FO, Deierhoi MH, et al. RS-61443 (mycophenolate mofetil). A multicenter study for refractory kidney transplant rejection. *Ann Surg.* 1992;216:513-518.

33. European Mycophenolate Mofetil Cooperative Study Group. Placebo-controlled study of mycophenolate mofetil combined with cyclosporin and corticosteroids for prevention of acute rejection. *Lancet.* 1995;345:1321-1325.

34. Sollinger HW, U.S. Renal Transplant Mycophenolate Mofetil Study Group. Mycophenolate mofetil for the prevention of acute rejection in primary cadaveric renal allograft recipients. *Transplantation.* 1995;60:225-232.

35. The Tricontinental Mycophenolate Mofetil Renal Transplantation Study Group. A blinded, randomized clinical trial of mycophenolate mofetil for the prevention of acute rejection in cadaveric renal transplantation. *Transplantation.* 1996;61:1029-1037.

36. Halloran P, Mathew T, Tomlanovich S, et al. Mycophenolate mofetil in renal allograft recipients: a pooled efficacy analysis of three randomized, double-blind, clinical studies in prevention of rejection. The International Mycophenolate Mofetil Renal Transplant Study Groups. *Transplantation.* 1997;63:39-47.

37. Sollinger HW. A few memories from the beginning.. *Transplantation.* 2005;80:S178-S180.

38. Meier-Kriesche HU, Li S, Gruessner RW, et al. Immunosuppression: evolution in practice and trends, 1994-2004. *Am J Transplant.* 2006;6:1111-1131.

39. Remuzzi G, Lesti M, Gotti E, et al. Mycophenolate mofetil versus azathioprine for prevention of acute rejection in renal transplantation (MYSS): a randomised trial. *Lancet.* 2004;364:503-512.

40. Remuzzi G, Cravedi P, Costantini M, et al. Mycophenolate mofetil versus azathioprine for prevention of chronic allograft dysfunction in renal transplantation: The MYSS follow-up randomized, controlled clinical trial. *J Am Soc Nephrol.* 2007;18:1973-1985.

41. Mele TS, Halloran PF. The use of mycophenolate mofetil in transplant recipients. *Immunopharmacology.* 2000;47:215-245.

42. Budde K, Curtis J, Knoll G, et al. Enteric-coated mycophenolate sodium can be safely administered in maintenance renal transplant patients: results of a 1-year study. *Am J Transplant.* 2004;4:237-243.

43. Gabardi S, Tran JL, Clarkson MR. Enteric-coated mycophenolate sodium. *Ann Pharmacother.* 2003;37:1685-1693.

44. Salvadori M, Holzer H, de MA, et al. Enteric-coated mycophenolate sodium is therapeutically equivalent to mycophenolate mofetil in de novo renal transplant patients. *Am J Transplant.* 2004;4:231-236.

45. Franklin TJ, Cook JM. The inhibition of nucleic acid synthesis by mycophenolic acid. *Biochem J.* 1969;113:515-524.

46. Jackson RC, Weber G, Morris HP. IMP dehydrogenase, an enzyme linked with proliferation and malignancy. *Nature.* 1975;256:331-333.

47. Carr SF, Papp E, Wu JC, et al. Characterization of human type I and type II IMP dehydrogenases. *J Biol Chem.* 1993;268:27286-27290.

48. Sintchak MD, Fleming MA, Futer O, et al. Structure and mechanism of inosine monophosphate dehydrogenase in complex with the immunosuppressant mycophenolic acid. *Cell.* 1996;85:921-930.

49. McPhillips CC, Hyle JW, Reines D. Detection of the mycophenolate-inhibited form of IMP dehydrogenase in vivo. *Proc Natl Acad Sci U S A.* 2004;101:12171-12176.

50. Ji Y, Gu J, Makhov AM, et al. Regulation of the interaction of inosine monophosphate dehydrogenase with mycophenolic acid by GTP. *J Biol Chem.* 2006;281:206-212.

51. Wang J, Zeevi A, Webber S, et al. A novel variant L263F in human inosine 5'-monophosphate dehydrogenase 2 is associated with diminished enzyme activity. *Pharmacogenet Genomics.* 2007;17:283-290.

52. Natsumeda Y, Ohno S, Kawasaki H, et al. Two distinct cDNAs for human IMP dehydrogenase. *J Biol Chem.* 1990;265:5292-5295.

53. Hager PW, Collart FR, Huberman E, et al. Recombinant human inosine monophosphate dehydrogenase type I and type II proteins. Purification and characterization of inhibitor binding. *Biochem Pharmacol.* 1995;49:1323-1329.

54. Senda M, Natsumeda Y. Tissue-differential expression of two distinct genes for human IMP dehydrogenase (E.C.1.1.1.205). *Life Sci.* 1994;54:1917-1926.

55. Jain J, Almquist SJ, Ford PJ, et al. Regulation of inosine monophosphate dehydrogenase type I and type II isoforms in human lymphocytes. *Biochem Pharmacol.* 2004;67:767-776.

56. Zimmermann AG, Gu JJ, Laliberte J, et al. Inosine-5'-monophosphate dehydrogenase: regulation of expression and role in cellular proliferation and T lymphocyte activation. *Prog Nucleic Acid Res Mol Biol.* 1998;61:181-209.

57. Albrecht W, Storck M, Pfetsch E, et al. Development and application of a high-performance liquid chromatography-based assay for determination of the activity of inosine 5'-monophosphate dehydrogenase in whole blood and isolated mononuclear cells. *Ther Drug Monit.* 2000;22:283-294.

58. Glander P, Hambach P, Braun KP, et al. Effect of mycophenolate mofetil on IMP dehydrogenase after the first dose and after long-term treatment in renal transplant recipients. *Int J Clin Pharmacol Ther.* 2003;41:470-476.

59. Langman LJ, LeGatt DF, Halloran PF, et al. Pharmacodynamic assessment of mycophenolic acid-induced immunosuppression in renal transplant recipients. *Transplantation.* 1996;62:666-672.

60. Vethe NT, Mandla R, Line PD, et al. Inosine monophosphate dehydrogenase activity in renal allograft recipients during mycophenolate treatment. *Scand J Clin Lab Invest.* 2006;66:31-44.

61. Eugui EM, Almquist SJ, Muller CD, et al. Lymphocyte-selective cytostatic and immunosuppressive effects of mycophenolic acid in vitro: role of deoxyguanosine nucleotide depletion. *Scand J Immunol.* 1991;33:161-173.

62. Allison AC, Eugui EM. Mechanisms of action of mycophenolate mofetil in preventing acute and chronic allograft rejection. *Transplantation.* 2005;80:S181-S190.

63. Bullingham R, Monroe S, Nicholls A, et al. Pharmacokinetics and bioavailability of mycophenolate mofetil in healthy subjects after single-dose oral and intravenous administration. *J Clin Pharmacol.* 1996;36:315-324.

64. Pescovitz MD, Conti D, Dunn J, et al. Intravenous mycophenolate mofetil: safety, tolerability, and pharmacokinetics. *Clin Transplant.* 2000;14:179-188.

65. Armstrong VW, Tenderich G, Shipkova M, et al. Pharmacokinetics and bioavailability of mycophenolic acid after intravenous administration and oral administration of mycophenolate mofetil to heart transplant recipients. *Ther Drug Monit.* 2005;27:315-321.

66. Staatz CE, Tett SE. Clinical pharmacokinetics and pharmacodynamics of mycophenolate in solid organ transplant recipients. *Clin Pharmacokinet.* 2007;46:13-58.

67. Hesselink DA, van Hest RM, Mathot RA, et al. Cyclosporine interacts with mycophenolic acid by inhibiting the multidrug resistance-associated protein 2. *Am J Transplant.* 2005;5:987-994.

68. van Gelder T, Klupp J, Barten MJ, et al. Comparison of the effects of tacrolimus and cyclosporine on the pharmacokinetics of mycophenolic acid. *Ther Drug Monit.* 2001;23:119-128.

69. Budde K, Glander P, Kramer BK, et al. Conversion from mycophenolate mofetil to enteric-coated mycophenolate sodium in maintenance renal transplant recipients receiving tacrolimus: clinical, pharmacokinetic, and pharmacodynamic outcomes. *Transplantation.* 2007;83:417-424.

70. Budde K, Bauer S, Hambach P, et al. Pharmacokinetic and pharmacodynamic comparison of enteric-coated mycophenolate sodium and mycophenolate mofetil in maintenance renal transplant patients. *Am J Transplant.* 2007;7:888-898.

71. Cattaneo D, Cortinovis M, Baldelli S, et al. Pharmacokinetics of mycophenolate sodium and comparison with the mofetil formulation in stable kidney transplant recipients. *Clin J Am Soc Nephrol.* 2007;2:1147-1155.

72. Langman LJ, LeGatt DF, Yatscoff RW. Blood distribution of mycophenolic acid. *Ther Drug Monit.* 1994;16:602-607.

73. Nowak I, Shaw LM. Mycophenolic acid binding to human serum albumin: characterization and relation to pharmacodynamics. *Clin Chem.* 1995;41:1011-1017.

74. Kuypers DR, Vanrenterghem Y, Squifflet JP, et al. Twelve-month evaluation of the clinical pharmacokinetics of total and free mycophenolic acid and its glucuronide metabolites in renal allograft recipients on low dose tacrolimus in combination with mycophenolate mofetil. *Ther Drug Monit.* 2003;25:609-622.

75. Mandla R, Line PD, Midtvedt K, et al. Automated determination of free mycophenolic acid and its glucuronide in plasma from renal allograft recipients. *Ther Drug Monit.* 2003;25:407-414.

76. Shaw LM, Korecka M, Aradhye S, et al. Mycophenolic acid area under the curve values in African American and Caucasian renal transplant patients are comparable. *J Clin Pharmacol.* 2000;40:624-633.

77. Bullingham RE, Nicholls AJ, Kamm BR. Clinical pharmacokinetics of mycophenolate mofetil. *Clin Pharmacokinet.* 1998;34:429-455.

78. Shipkova M, Armstrong VW, Wieland E, et al. Identification of glucoside and carboxyl-linked glucuronide conjugates of mycophenolic acid in plasma of transplant recipients treated with mycophenolate mofetil. *Br J Pharmacol.* 1999;126:1075-1082.

79. Picard N, Cresteil T, Premaud A, et al. Characterization of a phase 1 metabolite of mycophenolic acid produced by CYP3A4/5. *Ther Drug Monit.* 2004;26:600-608.

80. Shipkova M, Armstrong VW, Weber L, et al. Pharmacokinetics and protein adduct formation of the pharmacologically active acyl glucuronide metabolite of mycophenolic acid in pediatric renal transplant recipients. *Ther Drug Monit.* 2002;24:390-399.

81. Picard N, Ratanasavanh D, Premaud A, et al. Identification of the UDP-glucuronosyltransferase isoforms involved in mycophenolic acid phase II metabolism. *Drug Metab Dispos.* 2005;33:139-146.

82. Zucker K, Tsaroucha A, Olson L, et al. Evidence that tacrolimus augments the bioavailability of mycophenolate mofetil through the inhibition of mycophenolic acid glucuronidation. *Ther Drug Monit.* 1999;21:35-43.

83. Nowak I, Shaw LM. Effect of mycophenolic acid glucuronide on inosine monophosphate dehydrogenase activity. *Ther Drug Monit.* 1997;19:358-360.

84. Schutz E, Shipkova M, Armstrong VW, et al. Identification of a pharmacologically active metabolite of mycophenolic acid in plasma of transplant recipients treated with mycophenolate mofetil. *Clin Chem.* 1999;45:419-422.

85. Shipkova M, Wieland E, Schutz E, et al. The acyl glucuronide metabolite of mycophenolic acid inhibits the proliferation of human mononuclear leukocytes. *Transplant Proc.* 2001;33:1080-1081.

86. Shipkova M, Beck H, Voland A, et al. Identification of protein targets for mycophenolic acid acyl glucuronide in rat liver and colon tissue. *Proteomics.* 2004;4:2728-2738.

87. Hale MD, Nicholls AJ, Bullingham RE, et al. The pharmacokinetic-pharmacodynamic relationship for mycophenolate mofetil in renal transplantation. *Clin Pharmacol Ther.* 1998;64:672-683.

88. van Hest RM, Mathot RA, Pescovitz MD, et al. Explaining variability in mycophenolic acid exposure to optimize mycophenolate mofetil dosing: a population pharmacokinetic meta-analysis of mycophenolic acid in renal transplant recipients. *J Am Soc Nephrol.* 2006;17:871-880.

89. van Hest RM, van Gelder T, Bouw R, et al. Time-dependent clearance of mycophenolic acid in renal transplant recipients. *Br J Clin Pharmacol.* 2007;63:741-752.

90. Shaw LM, Kaplan B, Denofrio D, et al. Pharmacokinetics and concentration-control investigations of mycophenolic acid in adults after transplantation. *Ther Drug Monit.* 2000;22:14-19.

91. Shaw LM, Sollinger HW, Halloran P, et al. Mycophenolate mofetil: a report of the consensus panel. *Ther Drug Monit.* 1995;17:690-699.

92. Shaw LM, Figurski M, Milone MC, et al. Therapeutic drug monitoring of mycophenolic acid. *Clin J Am Soc Nephrol.* 2007;2:1062-1072.

93. Arns W, Cibrik DM, Walker RG, et al. Therapeutic drug monitoring of mycophenolic acid in solid organ transplant patients treated with mycophenolate mofetil: review of the literature. *Transplantation.* 2006;82:1004-1012.

94. Jeong H, Kaplan B. Therapeutic monitoring of mycophenolate mofetil. *Clin J Am Soc Nephrol.* 2007;2:184-191.

95. van Gelder T, Shaw LM. The rationale for and limitations of therapeutic drug monitoring for mycophenolate mofetil in transplantation. *Transplantation.* 2005;80:S244-S253.

96. Elbarbry FA, Shoker AS. Therapeutic drug measurement of mycophenolic acid derivatives in transplant patients. *Clin Biochem.* 2007;40:752-764.

97. Marquet P, Premaud A, Sauvage F-L, et al. Comparison of a new enzymatic assay with LC-MS/MS for the determination of plasma mycophenolate in patients with different graft types and associated immunosuppressants. *World Transplant Congress, Boston, Massachusetts.* 2006;Abstract no. 2886.

98. Takahashi K, Ochiai T, Uchida K, et al. Pilot study of mycophenolate mofetil (RS-61443) in the prevention of acute rejection following renal transplantation in Japanese patients. RS-61443 Investigation Committee--Japan. *Transplant Proc.* 1995;27:1421-1424.

99. van Gelder T, Hilbrands LB, Vanrenterghem Y, et al. A randomized double-blind, multicenter plasma concentration controlled study of the safety and efficacy of oral

mycophenolate mofetil for the prevention of acute rejection after kidney transplantation. *Transplantation.* 1999;68:261-266.

100. van Gelder T, Meur YL, Shaw LM, et al. Therapeutic drug monitoring of mycophenolate mofetil in transplantation. *Ther Drug Monit.* 2006;28:145-154.

101. Mourad M, Malaise J, Chaib ED, et al. Correlation of mycophenolic acid pharmacokinetic parameters with side effects in kidney transplant patients treated with mycophenolate mofetil. *Clin Chem.* 2001;47:88-94.

102. Mourad M, Malaise J, Chaib ED, et al. Pharmacokinetic basis for the efficient and safe use of low-dose mycophenolate mofetil in combination with tacrolimus in kidney transplantation. *Clin Chem.* 2001;47:1241-1248.

103. Weber LT, Shipkova M, Armstrong VW, et al. The pharmacokinetic-pharmacodynamic relationship for total and free mycophenolic acid in pediatric renal transplant recipients: a report of the German study group on mycophenolate mofetil therapy. *J Am Soc Nephrol.* 2002;13:759-768.

104. Atcheson BA, Taylor PJ, Mudge DW, et al. Mycophenolic acid pharmacokinetics and related outcomes early after renal transplant. *Br J Clin Pharmacol.* 2005;59:271-280.

105. Cattaneo D, Gaspari F, Ferrari S, et al. Pharmacokinetics help optimizing mycophenolate mofetil dosing in kidney transplant patients. *Clin Transplant.* 2001;15:402-409.

106. Kiberd BA, Lawen J, Fraser AD, et al. Early adequate mycophenolic acid exposure is associated with less rejection in kidney transplantation. *Am J Transplant.* 2004;4:1079-1083.

107. Kuypers DR, Claes K, Evenepoel P, et al. Clinical efficacy and toxicity profile of tacrolimus and mycophenolic acid in relation to combined long-term pharmacokinetics in de novo renal allograft recipients. *Clin Pharmacol Ther.* 2004;75:434-447.

108. Mandla R, Midtvedt K, Line PD, et al. Mycophenolic acid clinical pharmacokinetics influenced by a cyclosporine C2 based immunosuppressive regimen in renal allograft recipients. *Transpl Int.* 2006;19:44-53.

109. Premaud A, Le Meur Y, Debord J, et al. Maximum a posteriori bayesian estimation of mycophenolic acid pharmacokinetics in renal transplant recipients at different postgrafting periods. *Ther Drug Monit.* 2005;27:354-361.

110. Le Meur Y, Buchler M, Thierry A, et al. Individualized mycophenolate mofetil dosing based on drug exposure significantly improves patient outcomes after renal transplantation. *Am J Transplant.* 2007;7:2496-2503.

111. van Gelder T, Silva HT, de Fijter H, et al. A prospective, randomised study comparing fixed dose vs concentration controlled MMF regimens for de novo patients following renal transplantation (the FDCC trial). *World Transplant Congress, Boston, Massachusetts.* 2006;Abstract no. 819.

112. van Gelder T, Gafner N. Mycophenolic acid plasma concentrations and acute rejection rate in high risk versus low risk renal transplant patients receiving mycophenolate mofetil. *Supplement to Transplantation.* 2008;86:39.

113. Girard H, Court MH, Bernard O, et al. Identification of common polymorphisms in the promoter of the UGT1A9 gene: evidence that UGT1A9 protein and activity levels are strongly genetically controlled in the liver. *Pharmacogenetics.* 2004;14:501-515.

114. Villeneuve L, Girard H, Fortier LC, et al. Novel functional polymorphisms in the UGT1A7 and UGT1A9 glucuronidating enzymes in Caucasian and African-American subjects and their impact on the metabolism of 7-ethyl-10-hydroxycamptothecin and flavopiridol anticancer drugs. *J Pharmacol Exp Ther.* 2003;307:117-128.

115. Kuypers DR, Naesens M, Vermeire S, et al. The impact of uridine diphosphate-glucuronosyltransferase 1A9 (UGT1A9) gene promoter region single-nucleotide polymorphisms T-275A and C-2152T on early mycophenolic acid dose-interval exposure in de novo renal allograft recipients. *Clin Pharmacol Ther.* 2005;78:351-361.

116. Baldelli S, Merlini S, Perico N, et al. C-440T/T-331C polymorphisms in the UGT1A9 gene affect the pharmacokinetics of mycophenolic acid in kidney transplantation. *Pharmacogenomics.* 2007;8:1127-1141.

117. Naesens M, Kuypers DR, Verbeke K, et al. Multidrug resistance protein 2 genetic polymorphisms influence mycophenolic acid exposure in renal allograft recipients. *Transplantation.* 2006;82:1074-1084.

118. Balzarini J, De Clercq E. Assay method for monitoring the inhibitory effects of antimetabolites on the activity of inosinate dehydrogenase in intact human CEM lymphocytes. *Biochem J.* 1992;287 (Pt 3):785-790.

119. Montero C, Duley JA, Fairbanks LD, et al. Demonstration of induction of erythrocyte inosine monophosphate dehydrogenase activity in ribavirin-treated patients using a high performance liquid chromatography linked method. *Clin Chim Acta.* 1995;238:169-178.

120. Daxecker H, Raab M, Muller MM. Influence of mycophenolic acid on inosine 5'-monophosphate dehydrogenase activity in human peripheral blood mononuclear cells. *Clin Chim Acta.* 2002;318:71-77.

121. Glander P, Braun KP, Hambach P, et al. Non-radioactive determination of inosine 5'-monophosphate dehydro-genase (IMPDH) in peripheral mononuclear cells. *Clin Biochem.* 2001;34:543-549.

122. Vethe NT, Bergan S. Determination of inosine monophosphate dehydrogenase activity in human CD4+ cells isolated from whole blood during mycophenolic acid therapy. *Ther Drug Monit.* 2006;28:608-613.

123. Kalsi K, Marinaki AM, Yacoub MH, et al. HPLC/tandem ion trap mass detector methods for determination of inosine monophosphate dehydrogenase (IMPDH) and thiopurine methyltransferase (TPMT). *Nucleosides Nucleotides Nucleic Acids.* 2006;25:1241-1244.

124. Langman LJ, LeGatt DF, Yatscoff RW. Pharmacodynamic assessment of mycophenolic acid-induced immunosuppression by measuring IMP dehydrogenase activity. *Clin Chem.* 1995;41:295-299.

125. Vethe NT, Bremer S, Rootwelt H, et al. Pharmacodynamics of mycophenolic acid in CD4+ cells: A single-dose study of IMPDH and purine nucleotide responses in healthy individuals. *Ther Drug Monit.* 2008;Epub ahead of print.

126. Sanquer S, Breil M, Baron C, et al. Induction of inosine monophosphate dehydrogenase activity after long-term treatment with mycophenolate mofetil. *Clin Pharmacol Ther.* 1999;65:640-648.

127. Weigel G, Griesmacher A, Zuckermann AO, et al. Effect of mycophenolate mofetil therapy on inosine monophosphate dehydrogenase induction in red blood cells of heart transplant recipients. *Clin Pharmacol Ther.* 2001;69:137-144.

128. Bremer S, Mandla R, Vethe NT, et al. Expression of *IMPDH1* and *IMPDH2* after transplantation and initiation of immunosuppression. *Transplantation.* 2008;85:55-61.

129. Devyatko E, Zuckermann A, Bohdjalian A, et al. Activation of the purine salvage pathway in mononuclear cells of cardiac recipients treated with mycophenolate mofetil. *Transplantation.* 2006;82:113-118.

130. Vethe NT, Bremer S, Bergan S. IMP dehydrogenase basal activity in MOLT-4 human leukaemia cells is altered by mycophenolic acid and 6-thioguanosine. *Scand J Clin Lab Invest.* 2008;68:277-285.

131. Glander P, Hambach P, Braun KP, et al. Pre-transplant inosine monophosphate dehydrogenase activity is associated with clinical outcome after renal transplantation. *Am J Transplant.* 2004;4:2045-2051.

132. van Gelder T, Sombogaard F, Glander P, et al. IMPDH activity on day 6 after kidney transplantation is significantly related to the risk of acute rejection in MMF treated patients. *Supplement to Transplantation.* 2008;86:250.

133. Raggi M, Stangl M, Siebert S, et al. IMPDH-inhibition as a key marker in renal transplantation. *Supplement to Transplantation.* 2008;86:334.

134. Czock D, Rasche FM, Carius A, et al. Pharmacokinetics and pharmacodynamics of mycophenolic acid after enteric-coated mycophenolate versus mycophenolate

mofetil in patients with progressive IgA nephritis. *J Clin Pharmacol.* 2007;47:850-859.

135. Goldsmith D, Carrey EA, Edbury S, et al. Mycophenolate mofetil, an inhibitor of inosine monophosphate dehydrogenase, causes a paradoxical elevation of GTP in erythrocytes of renal transplant patients. *Clin Sci (Lond).* 2004;107:63-68.

136. Jagodzinski P, Lizakowski S, Smolenski RT, et al. Mycophenolate mofetil treatment following renal transplantation decreases GTP concentrations in mononuclear leucocytes. *Clin Sci (Lond).* 2004;107:69-74.

137. Daxecker H, Raab M, Cichna M, et al. Determination of the effects of mycophenolic acid on the nucleotide pool of human peripheral blood mononuclear cells in vitro by high-performance liquid chromatography. *Clin Chim Acta.* 2001;310:81-87.

138. Vannozzi F, Filipponi F, Di Paolo A, et al. An exploratory study on pharmacogenetics of inosine-monophosphate dehydrogenase II in peripheral mononuclear cells from liver-transplant recipients. *Transplant Proc.* 2004;36:2787-2790.

139. Sanquer S, Maison P, Tomkiewicz C, et al. Expression of inosine monophosphate dehydrogenase type I and type II after mycophenolate mofetil treatment: A 2-year follow-up in kidney transplantation. *Clin Pharmacol Ther.* 2008;83:328-335.

140. Wang J, Yang JW, Zeevi A, et al. IMPDH1 gene polymorphisms and association with acute rejection in renal transplant patients. *Clin Pharmacol Ther.* 2008;83:711-717.

141. van Gelder T, Sombogaard F, van Schaik R, et al. IMPDH activity is correlated with the 3757T>C polymorphism in the IMPDH type 2 gene in MMF treated kidney transplant patients. *Supplement to Transplantation.* 2008;86:37.

142. Afzali B, Lechler RI, Hernandez-Fuentes MP. Allorecognition and the alloresponse: clinical implications. *Tissue Antigens.* 2007;69:545-556.

143. Collart FR, Huberman E. Expression of IMP dehydrogenase in differentiating HL-60 cells. *Blood.* 1990;75:570-576.

144. Glesne DA, Collart FR, Huberman E. Regulation of IMP dehydrogenase gene expression by its end products, guanine nucleotides. *Mol Cell Biol.* 1991;11:5417-5425.

145. Svensson JO, Brattstrom C, Sawe J. A simple HPLC method for simultaneous determination of mycophenolic acid and mycophenolic acid glucuronide in plasma. *Ther Drug Monit.* 1999;21:322-324.

146. Bremer S, Rootwelt H, Bergan S. Real-time PCR determination of *IMPDH1* and *IMPDH2* expression in blood cells. *Clin Chem.* 2007;53:1023-1029.

147. Ruckemann K, Fairbanks LD, Carrey EA, et al. Leflunomide inhibits pyrimidine de novo synthesis in mitogen-stimulated T-lymphocytes from healthy humans. *J Biol Chem.* 1998;273:21682-21691.

148. Vora A, Mitchell CD, Lennard L, et al. Toxicity and efficacy of 6-thioguanine versus 6-mercaptopurine in childhood lymphoblastic leukaemia: a randomised trial. *Lancet.* 2006;368:1339-1348.

149. Stet EH, De Abreu RA, Bokkerink JP, et al. Inhibition of IMP dehydrogenase by mycophenolic acid in Molt F4 human malignant lymphoblasts. *Ann Clin Biochem.* 1994;31 (Pt 2):174-180.

150. Qiu Y, Fairbanks LD, Ruckemann K, et al. Mycophenolic acid-induced GTP depletion also affects ATP and pyrimidine synthesis in mitogen-stimulated primary human T-lymphocytes. *Transplantation.* 2000;69:890-897.