Inosine Monophosphate Dehydrogenase:
The Molecular Target of Mycophenolate

Doctoral Thesis by
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# Table of Content

Table of Content 3  
Acknowledgements 4  
List of Papers 6  
Abbreviations 7  
Errata 10  
1. Introduction 11  
   1.1 Immunosuppressive Therapy in Transplantation 11  
   1.2 Inosine Monophosphate Dehydrogenase 17  
      1.2.1 Biosynthetic pathways 17  
      1.2.2 Genes and regulation 19  
      1.2.3 IMPDH isoenzymes 23  
      1.2.4 Catalytic cycle 24  
      1.2.5 Protein structure 25  
      1.2.6 IMPDH and pathophysiology 26  
      1.2.7 Inhibitors of IMPDH 27  
   1.3 Mycophenolic Acid 29  
      1.3.1 Mechanisms of action 29  
      1.3.2 Pharmacokinetics 33  
   1.4 Individualization of Mycophenolate Therapy 38  
      1.4.1 Monitoring; Why, when and how? 38  
      1.4.2 Pharmacodynamic biomarkers of mycophenolate effect 42  
      1.4.3 IMPDH and clinical outcome 45  
      1.4.4 Potential induction of IMPDH during MPA therapy 48  
2. Objectives of the Thesis 55  
   2.1 Paper I 55  
   2.2 Paper II 55  
   2.3 Paper III 55  
   2.4 Paper IV 56  
3. Methods 57  
   3.1 Paper I 57  
   3.2 Paper II 59  
   3.3 Paper III 59  
   3.4 Paper IV 60  
   3.5 Ethics 61  
   3.6 Data Analysis and Statistics 61  
4. Results and Discussion 63  
   4.1 Paper I 63  
   4.2 Paper II 66  
   4.3 Paper III 70  
   4.4 Paper IV 73  
   4.5 Limitations 76  
5. Conclusions of the Thesis 77  
6. Future Perspectives 79  
7. References 81
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Sara Bremer
List of Papers

I.

II.

III.

IV.
Abbreviations

Ab                Antibody
ABCC2             ATP binding cassette subfamily C member 2
AcMPAG            Mycophenolic acid acyl glucuronide
adRP              Autosomal dominant retinitis pigmentosa
ALAS1             Aminolevulinate delta-synthase 1
AMP               Adenosine 5'-monophosphate
AP2               Activating enhancer-binding protein 2
APC               Antigen-presenting cell
ATF-2             Cyclic AMP-dependent transcription factor
ATP               Adenosine 5'-triphosphate
AUC               Area under the variable versus time curve
AZA               Azathioprine
B2M               Beta-2-microglobulin
BH₄               Tetrahydrobiopterin
bp                Base pair
C₀                Predose concentration
C₂                Concentration 2 hours postdose
CBS               Cystathionine β-synthase
CD                Cluster of differentiation
CDK               Cyclin dependent kinase
cGMP              Cyclic guanosine 5'-monophosphate
CKI               CDK inhibitor
Cₘax              Maximum concentration
Cₘin              Minimum concentration
c-Myc             Myc proto-oncogene protein
CNI               Calcineurin inhibitor
Cp                Crossing point
CREB              cyclic AMP response element-binding protein
CsA               Cyclosporine A
CTLA-4            Cytotoxic T lymphocyte antigen 4
CV  Coefficient of variation
CYP  Cytochrome P450
dGN  Deoxyguanine nucleotide
E  PCR efficiency
EC-MPS  Enteric coated mycophenolate sodium
Egr-1  Early growth response protein 1
EHC  Enterohepatic circulation
ELP  Elongation complex protein
FKBP12  FK506 binding protein 12
GDP  Guanosine 5’-diphosphate
GI  Gastrointestinal
GMP  Guanosine 5’-monophosphate
GN  Guanine nucleotide
G6PD  Glucose-6-phosphate dehydrogenase
GTP  Guanosine 5’-triphosphate
HPRT  Hypoxanthine-guanine phosphoribosyltransferase
IL-2  Interleukin-2
IL-2R  Interleukin-2 receptor
IMP  Inosine 5’-monophosphate
IMPDH  Inosine 5’-monophosphate dehydrogenase
iNOS  Inducible form of nitric oxide synthase
IRF-1  Interferon regulatory factor 1
kb  Kilobase
LC  Liquid chromatography
mAb  Monoclonal antibody
MAPK  Mitogen activated protein kinase
MHC  Major histocompatibility complex
MMF  Mycophenolate mofetil
MPA  Mycophenolic acid
MPAG  Mycophenolic acid 7-O-glucuronide
MPAGIs  Mycophenolic acid 7-O-glucoside
MRP2  Multidrug resistance-related protein 2 (encoded by ABCC2)
mTOR  Mammalian target of rapamycin
NAD  Nicotinamide adenine dinucleotide (oxidized, NAD⁺; reduced, NADH)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>Nm23</td>
<td>Nucleoside diphosphate kinase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>OATP</td>
<td>Organic anion transporting polypeptide</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Pharmacodynamic</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetic</td>
</tr>
<tr>
<td>pRb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>PRPP</td>
<td>5-phosphoribosyl-1-pyrophosphate</td>
</tr>
<tr>
<td>RGI</td>
<td>Reference gene index</td>
</tr>
<tr>
<td>RIN</td>
<td>RNA integrity number</td>
</tr>
<tr>
<td>RPL 13A</td>
<td>Ribosomal protein L13a</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>Sp1</td>
<td>Transcription factor Sp1</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TDM</td>
<td>Therapeutic drug monitoring</td>
</tr>
<tr>
<td>t_{max}</td>
<td>Time to C_{max} within dose interval</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>UGT</td>
<td>UDP-glucuronosyltransferase or UDP-glucosyltransferase</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>XMP</td>
<td>Xanthosine 5’-monophosphate</td>
</tr>
</tbody>
</table>

Abbreviations are given in italicized upper case letters when representing gene symbols.
Errata

Page 60, second paragraph: “The IMPDH activity was determined in lysates of CD4+ cells and whole blood as described in Paper II” has been corrected to “The IMPDH activity was determined in lysates of CD4+ cells as described in Paper II”.

Paper IV:
Page 16, third paragraph: “for CD4+ and CD8+ cells (n=8)” has been corrected to “for CD4+ and CD8+ cells (n=6)”.
Page 30, Table 1: “DD, diseased donor “has been corrected to: “DD, deceased donor.”
1. Introduction

1.1 Immunosuppressive Therapy in Transplantation

Following solid organ transplantation, most patients require lifelong immunosuppressive therapy to prevent allograft rejection. The emergence of novel and more effective immunosuppressive agents has dramatically reduced the incidence of acute rejection. However, long-term outcomes are still challenged by the adverse effects of immunosuppressants, contributing to late graft failure, cardiovascular morbidity, opportunistic infections and malignancies. Therefore, optimization of immunosuppressive regimens is needed.

Allograft rejection is primarily mediated by activated T cells. Full activation and proliferation of naïve T cells in response to alloantigens require three separate but complementary signals (Figure 1). The interaction between the T cell receptor (TCR)-CD3 complex and a peptide-MHC II (major histocompatibility complex class II) expressed on antigen-presenting cells (APCs) provides the first signal (Signal 1). The second costimulatory signal is delivered through the binding of T cell surface receptors (CD28, CD154) to their respective ligands on the APC (CD80/86, CD40) (Signal 2). Together, these two signals promote T cell activation and induce expression and secretion of interleukin-2 (IL-2) and the expression of high-affinity IL-2 receptors. Binding of IL-2 to the IL-2 receptor initiates the third autocrine growth signal required for T cell proliferation (Signal 3). Most immunosuppressants target one or more of these signals (Figure 1). By using combination regimens of drugs that act on different stages of T cell activation, the dosing and toxicity of each agent can be minimized without compromising the total immunosuppressive effect.

With the introduction of cyclosporine (CsA) in the early 1980s, posttransplant outcomes improved significantly, and calcineurin inhibitors (CNIs) still provide the foundation for most immunosuppressive regimens. The use of tacrolimus has increased gradually, and is now the dominant CNI in clinical transplantation (Figure 2).\(^1\) CsA and tacrolimus bind to immunophilins (cyclophilin and FKBP12, respectively), forming complexes that inhibit the phosphatase calcineurin.\(^2,3\) This suppresses the production of NFAT regulated genes like IL-2, thereby inhibiting the
Introduction – Immunosuppressive Therapy in Transplantation

first signal of T cell activation. Tacrolimus displays a greater molar potency than CsA, but current dosing strategies result in similar immunosuppressive efficacy. Both agents display considerable nephrotoxicity and a risk of hemolytic-uremic syndrome. Other non-immune effects differ between the CNIs. CsA is associated with significantly more hirsuitism, hypertension and hyperlipidemia, while diabetes mellitus is reported to be more frequent with tacrolimus. Antimetabolites like azathioprine (AZA) and mycophenolic acid (MPA) inhibit cell proliferation through interference with DNA and RNA synthesis. Since its introduction in the mid 1990s, MPA has largely replaced AZA, and is now included in most immunosuppressive regimens after transplantation (Figure 2). MPA inhibits inosine 5’-monophosphate dehydrogenase (IMPDH) and affects mainly activated lymphocytes. This offers increased selectivity and decreased toxicity compared to AZA. The major non-immune effects of MPA are gastrointestinal (GI) and hematological, which generally respond to dose reductions. Sirolimus and everolimus bind to the immunophilin FKBP12, yielding complexes that inhibit the mammalian target of rapamycin (mTOR). This blocks cell proliferation induced by growth factors and cytokines (Signal 3). The principal adverse effects include hyperlipidemia, anemia, thrombocytopenia and impaired wound healing. Compared to CNI based regimens, mTOR inhibitors display lower efficacy against acute rejections, and are thus not regarded as first-line therapy in organ transplantation. Still, sirolimus and everolimus constitute valuable therapeutic options, e.g. for patients that cannot tolerate CNIs. Furthermore, as mTOR inhibitors are associated with antineoplastic effects, their use may be of benefit in patients with a high risk of posttransplant malignancies.

Corticosteroids are used for induction and maintenance therapy, as well as for reversal of established allograft rejection. They alter the gene expression of a wide range of genes and exert multiple anti-inflammatory and immunomodulatory effects. The major immunosuppressive effects include suppressed cytokine production, increased apoptosis of lymphocytes, altered macrophage migration and inhibition of dendritic cells. Owing to their multifarious effects on gene expression and cellular metabolism,
Activation and proliferation of naïve T cells require three independent signals. **Signal 1:** Interaction of the T cell receptor (TCR) and a peptide-MHC class II (major histocompatibility complex) expressed on antigen-presenting cells (APCs) transmits an antigen specific signal. **Signal 2:** Binding of T cell surface receptors (e.g. CD28) to their respective ligands on the APC (e.g. CD80/86) provides costimulation. **Signal 3:** Autocrine stimulation by interleukin-2 (IL-2) provides proliferative signals involving mammalian target of rapamycin (mTOR) and cyclin/cyclin-dependent kinases (CDKs). Examples of immunosuppressive agents that target one or more of these signals are shown in white boxes.

AZA, azathioprine; CsA, cyclosporine; IL-2R, interleukine-2 receptor; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MPA, mycophenolic acid; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; TCR, T cell receptor; TF, transcription factor
corticosteroid treatment is associated with a myriad of side effects that increase the 
risk of cardiovascular disease and metabolic disturbances.\textsuperscript{19}

Induction therapy with antibody (Ab) preparations has increased substantially during 
the last decades. Depleting agents like polyclonal antithymocyte globulin and 
muromonab-CD3 (mouse monoclonal Ab (mAb) against CD3) act by binding to 
lymphocyte cell surface receptors resulting in phagocytosis, cell lysis, apoptosis or 
downregulation of cell surface antigens. This leads to effective immunosuppression 
through long-lasting lymphocyte depletion. Adverse effects include cytokine-release 
syndrome, anaphylactic reactions, thrombocytopenia and an increased risk of 
infections and malignancies. More recently, non-depleting mAbs against the $\alpha$-chain 
(CD25) of IL-2 receptor (IL-2R) were introduced and are available as daclizumab 
(humanized) and basiliximab (chimeric). These agents target only activated T cells 
while resting T cells are spared. Compared to the depleting agents, IL-2R mAbs 
demonstrate minimal toxic effects but might be less effective.\textsuperscript{20,21}

At Rikshospitalet University Hospital in Oslo, the current standard 
immunosuppressive regimen after renal transplantation consists of a low-dosed CNI, 
mycophenolate, glucocorticoids and induction therapy (anti-IL-2R mAb).

The successful reduction in acute rejection episodes has shifted the focus towards 
minimizing drug toxicities to improve the long-term outcomes. Current 
immunosuppressive agents are usually characterized by narrow therapeutic indexes 
and broad pharmacokinetic and pharmacodynamic variability. Strategies for improved 
outcomes involve individualized therapy, considering both type of regimen and 
dosing to address the unique immune versus toxic responses of a particular patient. 
Therapeutic drug monitoring (TDM) is a valuable tool for individualization of therapy 
and is routinely used to guide dosing of CNIs and mTOR inhibitors. However, the 
strategy for potential monitoring of MPA is still debated (Section 1.4).\textsuperscript{22} Further 
efforts focus on protocols that taper or withdraw corticosteroids or CNIs. MPA seems 
to be devoid of nephrotoxic and metabolic side effects and displays potential 
beneficial effects on long-term outcomes, and is therefore frequently used to facilitate 
drug sparing regimens.\textsuperscript{23} However, the results of drug sparing regimens are so far 
conflicting, and careful selection of patients and monitoring is required. Moreover,
development of novel, potent agents with increased specificity against alloimmune responses could improve long-term outcomes. A T cell costimulation blocker, belatacept, is currently undergoing phase III clinical trials in renal transplantation. Belatacept is a second generation CTLA-4 IgG fusion protein, which binds to CD80 and CD86 on APCs and thereby inhibits the costimulatory signal through CD28 on T cells (Figure 1). Data from a phase II trial demonstrated similar efficacy and less adverse effects compared to CsA, implying that belatacept might be a promising alternative to CsA or corticosteroids.

![Figure 2. Immunosuppressive agents in renal transplantation.](image)

Clinical use of maintenance immunosuppression in renal transplant patients prior to discharge from 1995 to 2005 (Based on US data from the 2006 OPTN/SRTR Annual Report)

AZA, azathioprine; CsA, cyclosporine; MPA, mycophenolic acid

The use of MPA is steadily growing in transplantation (Figure 2), as well as for several autoimmune diseases. However, the utilization of this drug is hampered by GI and hematological toxicities and an increased risk of opportunistic infections, which requires frequent dose reductions or withdrawal. Consequently, there is an increasing focus on individualization of MPA therapy to improve the tolerability and thereby enhance the therapeutic potential of this agent.
The establishment of feasible strategies for individualization of MPA therapy requires substantial insight into MPA pharmacokinetics and pharmacodynamics. This includes investigations of the relation between MPA, the molecular target IMPDH, and finally the clinical outcome. Further knowledge of IMPDH and its regulation is also important within fields like antiviral and anticancer therapy, as well as in development of novel IMPDH inhibitors.
1.2 Inosine Monophosphate Dehydrogenase

1.2.1 Biosynthetic pathways

Adequate levels of purine nucleotides are essential for cell proliferation, cell signaling and as a biochemical energy source. Because the intracellular pools of nucleotides (except ATP) are relatively small, continuous generation of nucleotides is required. Purine nucleotide levels are maintained through a combination of de novo and salvage biosynthesis pathways (Figure 3). Both pathways utilize the activated sugar 5-phosphoribosyl-1-pyrophosphate (PRPP), which is generated from ribose 5’-phosphate and ATP. The de novo pathway converts PRPP through ten biosynthetic steps into inosine 5’-monophosphate (IMP). This represents a branch point in the purine nucleotide synthesis and IMP is further converted to either guanine or adenine nucleotides. Inosine 5’-monophosphate dehydrogenase (IMPDH, EC 1.1.1.205) catalyzes the conversion of IMP to xanthosine 5’-monophosphate (XMP), which is the rate-limiting step in de novo synthesis of guanine and deoxyguanine nucleotides (Figure 3). GMP synthetase converts XMP to guanosine 5’-monophosphate (GMP), which is further phosphorylated to guanosine di- (GDP) and triphosphates (GTP). Furthermore, ribonucleotide reductase converts GDP to deoxy GDP (dGDP), which is subsequently phosphorylated to dGTP.

Salvage pathways recycle free purine bases and nucleosides, originating from nucleic acid breakdown or cellular uptake, into their corresponding nucleotides (Figure 3). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) is a principal enzyme in the salvage of guanine and hypoxanthine into GMP and IMP, respectively. PRPP serves as a ribose phosphate donor. Dependence on de novo and salvage pathways is largely cell type and cell cycle specific. Salvage pathways seem to be the predominant source of purine nucleotides in most cell types. In contrast, lymphocytes are critically dependent on de novo purine synthesis for initiation of proliferative responses. The relative contributions of de novo and salvage pathways remain to be definitively elucidated in various cell populations and conditions.
Figure 3. Schematic overview of purine nucleotide biosynthesis.

The main steps of de novo (continuous arrows) and salvage pathways (dashed arrows) of adenine nucleotide (grey) and (deoxy) guanine nucleotide synthesis (black). Both pathways utilize 5-phosphoribosyl-1-pyrophosphate (PRPP), which is synthesized by PRPP synthetase. Inosine 5'-monophosphate dehydrogenase (IMPDH) and hypoxanthine guanine phosphoribosyltransferase (HPRT) are pivotal enzymes in de novo and salvage GMP synthesis, respectively.

A, adenine; AMP, adenosine 5'-monophosphate; AR, adenosine; dGDP, deoxyguanosine diphosphate; dGTP, deoxyguanosine triphosphate; G, guanine; GMP, guanosine 5'-monophosphate; GDP, guanosine 5'-diphosphate; GR, guanosine; GTP, guanosine 5'-triphosphate; Hx, hypoxanthine; HxR, inosine; IMP, inosine 5'-monophosphate; XMP, xanthosine 5'-monophosphate
The biosynthesis of purine nucleotides is tightly controlled through feedback regulation of rate-limiting enzymes. The control mechanisms are widely studied in prokaryotes and lower eukaryotes (e.g. yeast), but are less characterized in mammals. In human lymphocytes, PRPP synthetase is reported to be under allosteric control by adenine and guanine nucleotides, leading to suppressed and increased activity, respectively. The activity of ribonucleotide reductase is reported to be reduced by excess levels of dATP, while dGTP binding stimulated the enzyme. Furthermore, IMPDH is suggested to be subject to both enzymatic and transcriptional feedback control. *E.coli* enzyme models have demonstrated allosteric or competitive feedback regulation of IMPDH activity by GMP. However, the contributions of these mechanisms *in vivo* in human cells are poorly defined. On the other hand, studies in human cells have reported feedback regulation at the transcriptional level (Sections 1.2.2 and 1.4.4). Pimkin *et al.* speculated that regulation at the enzymatic level might be the first-line control while transcriptional regulation is a slower and secondary control mechanism.

As GMP constitutes the smallest of purine and pyrimidine ribonucleotide pools, the guanine nucleotide levels are particularly sensitive to biosynthetic modulation. Production of dGTP and GTP is required for DNA and RNA synthesis, respectively. Guanine nucleotides are further essential for cellular signaling and trafficking. Moreover, the activity of IMPDH has been reported to be a critical determinant of p53-dependent growth control. This implies that IMPDH plays an important role in the regulation of cell proliferation, differentiation and death.

### 1.2.2 Genes and regulation

Genes encoding IMPDH are found in all eukaryotes and most prokaryotes, and are highly conserved across species. Human IMPDH activity is constituted by two isoenzymes, IMPDH type 1 and type 2, which are encoded by *IMPDH1* and *IMPDH2*, respectively. The genes are located on chromosomes 7q31.3-q32 and 3p21.2-p24.2 and demonstrate similar coding exon structures with highly conserved exon-intron boundaries and approximately 76% nucleotide sequence identity in coding regions. The *IMPDH2* gene is 5.8 kb in length with 14 exons (49 to 207 bp) and introns ranging from 73 to 1065 bp, while the *IMPDH1* gene is considerable.
larger (>18 kb) with introns from 88 to longer than 3200 bp. Furthermore, the IMPDH1 gene includes three additional exons at the 5’ end (A, B, C), a recently discovered exon (13b) located between exon 13 and 14 and an extra noncoding region of exon 14 at the 3’ end (Figure 4). The distinct intron sizes and sequence divergence of the genes, suggests that the isoforms have arisen from an early gene duplication event, while the conservation of two isoenzymes with similar catalytic activity implies separate and essential functions of both proteins. Multiple processed pseudogenes have been generated from IMPDH1, whereas none are identified to origin from IMPDH2. The IMPDH1P11 pseudogene (previously IMPDH1L) displays 90% sequence identity to IMPDH1 and 72% identity to IMPDH2.

**Figure 4. The genomic structure of IMPDH1 and IMPDH2.**

Organization of the IMPDH1 (A) and IMPDH2 (B) genes. Coding and noncoding exons/regions of exons are represented by grey and black boxes, respectively. Black/white striped boxes show IMPDH1 exons/regions that are protein coding in retinal cells. Potential promoters (P) are illustrated as white rectangles. Genetic variants that have been associated with outcomes after transplantation or IMPDH activity (Section 1.4.3) are indicated with arrows. The locations and lengths of the target sequences of the quantitative reverse transcription-PCR assay (Section 3.1 and Paper I) are indicated.
The IMPDH genes are regulated at the transcriptional level, but the detailed mechanisms are not known. The 5’ flanking region of IMPDH2 has been reported to contain several transcription binding motifs including two cAMP response elements (CRE), an Sp1 site, an overlapping Egr-1/Sp1 site, an Nm23 motif and a palindromic octamer sequence (POS). Because promoter sites have been shown to be occupied in unstimulated as well as stimulated cells, the regulation of IMPDH2 appears to involve protein-protein interactions or posttranslational modifications of the bound transcription factors. The transcription factors ATF-2 and Sp1, and a POS-binding protein are suggested to be involved in T cell regulation.

The level and pattern of IMPDH1 expression is generally more variable than that of IMPDH2. This may be attributed to alternative splicing and utilization of at least three IMPDH1 promoters (P1, P2 and P3; Figure 4), resulting in the generation of various IMPDH1 transcripts. Of these, a 4.0 kb transcript is reported to be expressed mainly in activated T cells and monocytes, while a 2.5 kb transcript predominates in most tissues. Another 2.7 kb transcript was primarily found in transformed cells. These transcripts differ only in the 5’ untranslated regions (Exons A, B, C), contain identical coding sequences from exons 1–14 and encode identical proteins of 514 amino acids, denoted as canonical IMPDH1. More recent findings revealed novel IMPDH1 transcripts and proteins in human and mouse retinal cells, probably deriving from a combination of alternative splicing, different transcription/translation initiation sites and/or inclusion of the 13b exon (17 bp). The predominant retinal variants are proteins of 546 (IMPDH1α) and 595 (IMPDH1γ) amino acids. Both variants include 5 alternative and 32 additional amino acids (exon 13b+14) at the C-terminus compared to the canonical IMPDH1. The IMPDH1γ variant additionally includes 49 amino acids (exon A) at the N-terminus resulting from an alternative initiation site. Potential binding sites for Sp1, AP2, Ets-1, PuF, SIF, ELP, Nm23, CREB and NF-κB have been identified at the IMPDH1 promoter sequences.

The factors regulating expression of the IMPDH genes are poorly characterized. Myc proto-oncogene protein (c-Myc) is a key regulator of cell cycle progression and has been demonstrated to induce expression of both IMPDH1 and IMPDH2. In contrast, the tumor antigen p53 is reported to downregulate expression of IMPDH2,
IMPDH activity and consequently guanine nucleotide levels, and this is considered a critical part of growth regulation in p53-dependent processes.\textsuperscript{37,52}

Studies in yeast (\textit{S. cerevisiae}) and human blood cells suggest feedback regulation of \textit{IMPDH} gene expressions by guanine nucleotides.\textsuperscript{31,34,35,53} A model in yeast recently proposed GTP-dependent start site selection and transcription attenuation as a mechanism for IMPDH regulation.\textsuperscript{32} In human, \textit{in vitro} studies in transformed cells demonstrated upregulation of \textit{IMPDH 1} and 2 expression during exposure to guanine nucleotide depleting agents,\textsuperscript{34,53} whereas addition of salvage precursors increased guanine nucleotide levels and reduced gene expression.\textsuperscript{34} Dayton \textit{et al.} reported similar feedback regulation of \textit{IMPDH2} in normal human T cells.\textsuperscript{35} However, the feedback regulation of IMPDH in human cells is debated, the underlying molecular mechanisms have not been determined and \textit{in vivo} data are sparse. In a study of healthy individuals exposed to escalating MMF doses, \textit{IMPDH1} expression tended to be inversely correlated to GTP concentrations (Paper III). The MPA mediated changes of \textit{IMPDH} expression are further discussed in Section 1.4.4 and in the Papers II-IV.

\textbf{Figure 5. Regulation of \textit{IMPDH1} and/or \textit{IMPDH2} expression.}

Schematic view of factors that may regulate the expression of \textit{IMPDH1} and/or \textit{IMPDH2} in human cells. Arrows indicate stimulation while T-bars represent inhibition.

dGN, deoxyguanine nucleotides; GN, guanine nucleotides; IMPDH, inosine 5'-monophosphate dehydrogenase
Introduction – Inosine Monophosphate Dehydrogenase

The p53-dependent mechanisms that regulate IMPDH gene expression for growth control are suggested to be distinct from the feedback regulation by guanine nucleotides. Such a bipartite regulation might be expected for genes which encode products that are required both for constitutive anabolic processes (e.g. RNA and DNA synthesis) and for conditional molecular regulation (e.g. growth control).\textsuperscript{54}

1.2.3 IMPDH isoenzymes

Both IMPDH 1 and 2 constitute proteins of 56 kDa with 84\% identity at the amino-acid level.\textsuperscript{41} Moreover, substrate affinities and catalytic activities are comparable between the isoenzymes.\textsuperscript{55} Despite substantial similarities, IMPDH 1 and 2 are differently expressed in various tissues and are subject to distinct mechanisms of regulation.\textsuperscript{56,57} Both isoenzymes are widely expressed, and most cells types display higher expression of IMPDH2 than of IMPDH1. The highest levels of IMPDH1 are observed in resting and activated peripheral blood lymphocytes.\textsuperscript{47,57,58} Furthermore, variants of IMPDH1 are highly expressed in retinal cells, displaying 10-fold higher expression than of IMPDH2 (Section 1.2.2).\textsuperscript{59} This suggests a critical role of IMPDH1 for photoreceptor function.

Malignant transformation is associated with an upregulation of IMPDH activity and the gene expression of IMPDH2 (Section 1.2.6).\textsuperscript{36,60} Both isoenzymes are involved in lymphocyte proliferation. Ex vivo stimulation of normal peripheral T cells induced the expression of IMPDH 1 and 2 up to 10 times the levels in resting cells. This was accompanied by a 15-fold increase in enzyme activity and 6-fold elevation of GTP within 72 hours after activation.\textsuperscript{35,58}

The relative biological roles of IMPDH1 and IMPDH2 have been investigated in gene knockout mouse models. Loss of both IMPDH2 alleles resulted in early embryonic lethality, while heterozygous IMPDH2 knockouts displayed no significant phenotype changes. However, in combination with homozygous HPRT knockout, heterozygous IMPDH2 lymphocytes demonstrated impaired proliferative responses to mitogens and decreased cytolytic function.\textsuperscript{56} In contrast, homozygous IMPDH1 knockout mice seemed to have normal development and fertility, and lymphocytes demonstrated normal proliferative responses, also with combined HPRT knockout.\textsuperscript{57} These findings suggest that IMPDH2 is essential for normal development and fertility and that the
collective actions of IMPDH1, IMPDH2 and HPRT provide the increase in guanine nucleotides that is required for lymphocyte activation.\textsuperscript{56,57}

1.2.4 Catalytic cycle

The two IMPDH isoenzymes display common catalytic mechanisms and indistinguishable activities.\textsuperscript{55}

\textbf{Figure 6. Catalytic reaction of IMPDH.}

Potential mechanism for the catalytic conversion of inosine 5’-monophosphate (IMP) to xanthosine 5’-monophosphate (XMP) by the enzyme IMP dehydrogenase (IMPDH). The catalytic loop containing the active site cysteine is shown in black. The mobile flap is shown in dark grey and folds into the nicotinamide adenine dinucleotide (NAD) site after NADH is released. Mycophenolic acid (MPA) competes with the flap for the NAD site. Binding of MPA causes an allosteric conformational change that traps the enzyme-XMP* intermediate.
IMPDH catalyzes two distinct chemical transformations, a dehydrogenase reaction and a hydrolysis reaction, which are accompanied by a shift between an open and a closed conformation (Figure 6). IMPDH binds IMP at its active site and the reaction is initialized by a nucleophilic attack of an active site cysteine (Cys 331) on the C2 position of IMP to form a covalent intermediate (E-IMP). Binding of nicotinamide-adenine dinucleotide (NAD\(^+\)) results in hydride transfer, release of NADH and an oxidized intermediate covalently bound to IMPDH (thioimidate, E-XMP\(^*\)). A mobile loop structure, called a flap, moves into the vacant NAD site and activates water for E-XMP\(^*\) hydrolysis, and finally free XMP is released.\(^{38,61,62}\) The enzyme requires the presence of potassium,\(^{63}\) which is probably involved in stabilization of the NAD site.\(^{38}\)

### 1.2.5 Protein structure

Several crystal structures of IMPDH 1 and 2 from various organisms have provided the basis for models of structure-function relations of the isoenzymes. The human IMPDH1 and IMPDH2 proteins exist as homotetramers of 56 kDa monomers.\(^{64}\) Each monomer consists of two structurally discrete domains, a catalytic core domain and a subdomain. The catalytic domain constitutes approximately 400 amino acids in an eight-stranded \(\alpha/\beta\)-barrel fold. A cleft in the barrel, close to the interface between two monomers, forms an active site. The IMP and NAD\(^+\) binding sites are located within this cleft and are partly covered by a mobile flap of 50 residues (Figure 7).\(^{38,62,65,66}\)

The subdomain, also referred to as a Bateman domain, constitutes about 120 residues within the sequence of the catalytic domain and appears to be appended to the protein core as an independent folding unit. The Bateman domain includes two tandem repeats of a cystathionine \(\beta\)-synthase (CBS) like motif.\(^{38,67}\)

Although the Bateman domain is highly evolutionary conserved, no specific functions have been established for the subdomain. Amino acid substitutions, as well as complete deletion of this structure does not impact catalytic activity \textit{in vitro}.\(^{59,68}\) The subdomain has been reported to bind non-sequence-specific single-stranded nucleic acids and to be associated with polyribosomes.\(^{68-70}\) These observations suggest a
Introduction – Inosine Monophosphate Dehydrogenase

direct role in gene transcription or translation. Furthermore, a recent study proposed a function in the regulation of adenine nucleotide levels and purine nucleotide turnover. This implies that the physiological importance of IMPDH may extend beyond its primary role in de novo guanine nucleotide biosynthesis.

**Figure 7. Schematic illustration of the IMPDH homotetramer structure.**
The catalytic core domains are presented in light grey. The active site contains a catalytic loop, shown in black, and a mobile flap illustrated in grey. Binding sites for inosine 5'-monophosphate (IMP) and nicotinamide adenine dinucleotide (NAD$^+$) are located at the monomer interfaces.

1.2.6 IMPDH and pathophysiology

The activity and expression of IMPDH2 has been reported to be greatly increased in solid neoplastic and leukemic cells. Furthermore, treatment response and resistance to cancer chemotherapy has been correlated to IMPDH activity and gene expression. This has made the enzyme an attractive target for development of anticancer agents, as well as for monitoring the clinical response to cancer therapy.
Missense mutations within the \textit{IMPDH1} gene cause the RP10 form of autosomal dominant retinitis pigmentosa (adRP), accounting for 2–3\% of adRP cases, and are also a cause of rare cases of Leber congenital amaurosis.\(^{59,77}\) The pathogenic mutations are located within or proximate to the CBS coding sequence and do not affect enzymatic activity. However, the mutations seem to alter the nucleic acid binding and polyribosome association properties of IMPDH1.\(^{43,68-70}\) These observations suggest that IMPDH1 has a role in RNA metabolism that is crucial for photoreceptor function. The pathophysiology of retinal degeneration may be mediated through the presence of unique retinal IMPDH1 isoforms (Section 1.2.2).\(^{43}\) However, the detailed mechanism of the retinal degradation remains to be elucidated. The association between \textit{IMPDH1} polymorphisms and adRP has further increased the interest in IMPDH biology.

\subsection*{1.2.7 Inhibitors of IMPDH}

The pivotal role of IMPDH in \textit{de novo} guanine nucleotide biosynthesis makes this enzyme essential in cell proliferation and differentiation, and thus a potential target for immunosuppressive, anticancer, antiviral, antiparasitic or antimicrobial effects.\(^{78}\) Several classes of IMPDH inhibitors are now either in use or under development. On a structural basis the inhibitors in current clinical use can be divided into nucleoside (ribavirin, mizoribine and tiazofurin) and non-nucleoside inhibitors (MPA). Ribavirin and mizoribine undergo intracellular phosphorylation to their respective 5’-monophosphates, which bind competitively at the IMP site of IMPDH. Ribavirin displays broad antiviral activity and is used for the treatment of respiratory syncytial virus (RSV) and in combination with interferon-\(\alpha\), for the treatment of chronic hepatitis C viral (HCV) infection.\(^{79}\) Mizoribine is widely used in Japan for prevention of rejection after renal transplantation, and for treatment of lupus nephritis, rheumatoid arthritis and nephritic syndrome.\(^{80}\) Tiazofurin is activated to the NAD analog, tiazofurin adenine dinucleotide (TAD), which binds competitively at the NAD site of IMPDH. It demonstrates widespread antineoplastic activity and was tested in clinical phase I/II trials, but considerable toxicity prevented further clinical testing. The agent has been used “off-label” in patients for treatment of blast crisis of chronic myelogenous leukemia (CML).\(^{81}\)
Mycophenolic acid (MPA, Figure 9) is a non-nucleoside agent, which mediates uncompetitive and reversible inhibition of human IMPDH type 1 and 2.\textsuperscript{55,64} MPA binds to the NAD site after NADH release and causes an allosteric modification of the enzyme that traps the E-XMP* intermediate, preventing release of XMP (Figure 6).\textsuperscript{65} MPA is nearly 5 times more potent as inhibitor of IMPDH2 than of IMPDH1, displaying inhibition constants ($K_i$) of $7.0 \times 10^{-9}$ and $3.3 \times 10^{-8}$ M, respectively.\textsuperscript{64}

None of the IMPDH inhibitors in current use are strictly isoenzyme selective. Traditionally, attention has been directed at development of selective IMPDH2 inhibitors for anticancer and immunosuppressive actions.\textsuperscript{64} More recently, both isoenzymes have been demonstrated to be involved in lymphocyte proliferation and thus potential targets for immunosuppressive effects.\textsuperscript{35,58} Although IMPDH2 is the predominant isoenzyme in malignant cells and drug development has been targeted at this isoenzyme, expression of \textit{IMPDH1} has recently been associated with tumor angiogenesis,\textsuperscript{82} suggesting that both isoenzymes could be potential targets also for anticancer therapy.
1.3 Mycophenolic Acid

Mycophenolic acid (MPA) was probably first discovered by Gosio in 1896 and is a fermentation product of several *Penicillium* species. Early investigations demonstrated antineoplastic, antifungal, antiviral, anti-inflammatory and immunosuppressive activity of this compound. Despite promising antitumor activity in experimental models, the susceptibility of MPA to metabolic inactivation rendered it ineffective in clinical anticancer therapy. It is now widely used as an immunosuppressant in transplantation and has been established in therapy of autoimmune disorders like lupus nephropathy.

1.3.1 Mechanisms of action

Inhibition of IMPDH by MPA leads to reduced levels of intracellular guanine and deoxyguanine nucleotides. This induces a range of cellular effects, depending on cell type, differentiation and cycle status. The direct relationship between biologic activity and guanine nucleotide depletion was established *in vitro* by the reversibility of MPA effects with repletion of the guanine nucleotide pools. Furthermore, MPA has also been reported to affect adenine nucleotide levels. Cellular effects of MPA are illustrated in Figure 8.

*Cell cycle arrest*

The immunosuppressive actions of MPA are primarily related to the inhibition of T and B cell proliferation. Proliferating lymphocytes and monocytes largely depend on *de novo* guanine nucleotide synthesis because the salvage pathways cannot alone provide the required guanine nucleotides in these cells. *In vitro* experiments proposed that the suppressed proliferation was related to the depletion of dGTP pools. Moreover, MPA displays antiproliferative properties in non-immune cells, including renal tubular cells, mesangial cells, vascular smooth muscle cells, endothelial cells and fibroblasts. The mechanisms underlying the antiproliferative effects have been investigated in many cell models and are reported to involve altered expression of cell cycle regulatory proteins, inhibited phosphorylation of retinoblastoma protein (pRb) and finally cell cycle arrest in G1 phase. Furthermore, inhibition of RNA primed DNA synthesis has been demonstrated in leukemic T cell lines.
Progression of cell cycle is controlled by the activity of a series of cyclin-dependent kinases (CDKs), which in turn are regulated by the binding of cyclins and the expression of CDK inhibitors (CKIs). The cyclin-CDK complexes phosphorylate pRb, resulting in the liberation of the transcription factor E2F and subsequently activation of genes required for cell proliferation to proceed (Figure 8). The upstream mechanisms that sense guanine nucleotide depletion and trigger cell cycle arrest are still not clear and seem to differ between cell types. Studies in human cell lines (osteosarcoma and normal fibroblasts) demonstrated that MPA resulted in p53-dependent G\(_0\)/G\(_1\) cell cycle arrest, mediated through induction of p53 and p21\(^{\text{Cip1/Waf1}}\) (CKI) and subsequent dephosphorylation of pRb.\(^{94,96}\) The MPA induced activation of p53 might be caused by suppressed pre-rRNA synthesis, nucleolar stress and enhanced interaction of MDM2 with ribosomal proteins L5 and L11.\(^{96,97}\) In contrast to this p53-dependent mechanism, stimulated T cells demonstrated an MPA induced G\(_1\) block that was only associated with a modest increase of p53, whereas p21\(^{\text{Cip1/Waf1}}\) (CKI) was not altered.\(^{93,98}\) The cell cycle arrest in these cells involved inhibited induction of cyclin D3, and to a lesser extent of cyclin D2, CDK6 and CDK2 levels, in conjunction with reduced degradation of p27\(^{\text{Kip1}}\) (CKI), thereby resulting in reduced pRb phosphorylation.\(^{93,98}\)

**Cell differentiation**

In some cell types, the MPA mediated reduction of GTP levels also influences differentiation and maturation. Transformed myeloid and erythroid cell lines and leukemic cells from patients were induced to differentiate by MPA,\(^{99,100}\) and this emphasizes the potential of IMPDH inhibitors in anticancer therapy. On the other hand, MPA has been reported to suppress the maturation and functions of normal dendritic and CD8+ cells, which could contribute to the immunomodulative effects of this agent.\(^{101,102}\)

**Cell death**

In addition to the cytostatic effects, MPA also mediates cytotoxic effects depending on cell type and activation state. It is reported to induce apoptosis in lymphocyte- and macrophage-monocyte lineage cell lines.\(^{86}\) The cytotoxic effects probably occur through both caspase-dependent\(^{103}\) and caspase-independent\(^{104}\) processes, but the
Figure 8. Summary of established and potential cellular effects of mycophenolic acid (MPA).
The effects depend on cell type and proliferation status. Relevant cell types are indicated in the white boxes. Arrows represent stimulation while T-bars represent inhibition.

\( \times \) indicates processes that might be suppressed by MPA.

BH4, tetrahydrobiopterin; CDK, cyclin-dependent kinase; dGN, deoxyguanine nucleotides; GN, guanine nucleotides; IMPDH, inosine 5'-monophosphate dehydrogenase; iNOS, inducible nitric oxide synthase; MPA, mycophenolic acid; pRb, retinoblastoma protein
Introduction – Mycophenolic acid

detailed mechanisms are still poorly defined. A recent report proposes that the major
cytotoxic action of MPA in lymphocytes is mediated through activation of a caspase-
independent necrotic signal.\textsuperscript{105}

\textit{Glycoprotein synthesis}

MPA also acts at the level of leukocyte recruitment, antigen presentation and
leukocyte effector functions. Depletion of GTP inhibits the transfer of fucose and
mannose during synthesis of glycoproteins,\textsuperscript{78} which include several adhesion and
costimulatory molecules. This decreases the recruitment of leukocytes into sites of
inflammation and suppresses both the initiation and the effector phase of immune
responses.\textsuperscript{106}

\textit{Nitric oxide production}

Inducible nitric oxide synthetase (iNOS) catalyzes the conversion of L-arginine to
NO. The enzyme is involved in several inflammatory pathways and iNOS activation
is correlated with renal allograft rejection.\textsuperscript{107} MPA is reported to inhibit iNOS,\textsuperscript{108}
probably through cell type specific mechanisms. In endothelial cells, reduced GTP
levels suppressed synthesis of the tetrahydrobiopterin (BH\textsubscript{4}), which is an essential
cofactor for enzyme activity.\textsuperscript{108} In fibroblasts, however, the regulation is probably due
to impaired activation of the iNOS transcription factor IRF-1.\textsuperscript{109}

\textit{Cellular signal transduction}

Other immune modulating effects of MPA include interference with cellular signaling
pathways that are involved in immunological or pathological processes. Decreased
availability of GTP has been reported to interfere with the function of guanine
nucleotide binding proteins (G proteins).\textsuperscript{78,110,111} This might impair signal pathways
important for T cell activation and function,\textsuperscript{111,112} including activation of T cell
surface antigens.\textsuperscript{113} Furthermore, alterations of G protein activity has been proposed
as an underlying molecular and sensing mechanism for the antiproliferative and
cytotoxic effects of MPA.\textsuperscript{114,115} Moreover, cyclic GMP (cGMP) is generated from
GTP and inhibition of IMPDH has been associated with reduced cGMP
concentrations.\textsuperscript{116} This might also impact cellular signaling pathways.
**Aggregate formation**

In addition to the direct inhibition of enzyme activity by MPA, it was reported to induce the intracellular formation of IMPDH aggregates, which were observed as linear or ring structures.\(^{117}\) The macrostructures were localized in the perinuclear area of the cytoplasm and did not seem to be associated with organelles. Addition of GTP reversed the aggregate formation\(^{117}\) and the structures appear to be regulated in response to changing intracellular GTP levels.\(^{48}\) While both IMPDH 1 and 2 formed macrostructures with GTP depletion, the IMPDH2 isoenzyme seemed to be more susceptible to aggregation.\(^{48}\) Indeed, the functional impact of this aggregation remains to be elucidated.

**Clinical effects**

The several mechanisms of actions of MPA provide a wide range of immunosuppressive, antiproliferative and anti-inflammatory effects. As well as preventing allograft rejection, MPA favours long-term posttransplantation outcomes by suppressing proliferation of non-immune cells and reducing inflammation. Reported beneficial effects include reduced graft fibrosis, amelioration of CNI mediated nephrotoxicity, retarded progression of atherosclerosis and reduced risk of malignancies.\(^{118-121}\) Furthermore, the immune modulator function of this drug emphasizes the potential of MPA in the treatment of immunologically driven inflammatory disorders.

\subsection*{1.3.2 Pharmacokinetics}

MPA is available as the morpholinoethyl ester mycophenolate mofetil (MMF; CellCept\textsuperscript{®}) or as enteric-coated mycophenolate sodium (EC-MPS; Myfortic\textsuperscript{®}). The drugs are usually administered in fixed doses of 1000 mg MMF or 720 mg EC-MPS (~equimolar quantity of MPA) twice daily when used with CsA in adult renal transplant patients, while doses of 1500 mg MMF twice daily is recommended when administered to cardiac or liver allograft recipients.\(^{122,123}\) MMF has improved oral bioavailability, compared to MPA, and is rapidly hydrolyzed by esterases to MPA and absorbed in the upper GI, reaching maximum concentration approximately 1–2 hours postdose.\(^{124}\) The EC-MPS formulation delays the release of MPA until the drug reaches the small intestine.\(^{125}\) The absorption is high from both formulations, resulting
Introduction – Mycophenolic acid

in bioavailabilities of approximately 81–95%\textsuperscript{126,127} and 72% for MMF and EC-MPS, respectively.\textsuperscript{122} Administration of EC-MPS with a high-fat meal was associated with a delay in absorption and a reduction of the maximum MPA concentration.\textsuperscript{122} Thus, it is recommended to give EC-MPS on an empty stomach.

MPA displays tight and extensive binding to serum albumin, approximately 97–99% in patients with normal renal and liver function.\textsuperscript{128-131} The binding is reduced by renal impairment due to a direct effect of uremia and the accumulation of the 7-O-glucuronide metabolite of MPA (MPAG), which can displace MPA from albumin binding sites.\textsuperscript{128,132,133} Furthermore, hyperbilirubinemia and hypoalbuminemia might decrease the plasma protein binding of MPA.\textsuperscript{130,134,135} Only the unbound fraction of MPA is pharmacologically active.\textsuperscript{136} Measurement of the free MPA concentration is thus suggested in patients with renal or liver impairment or low serum albumin levels (≤31 g/L) to achieve a correct interpretation of unbound MPA exposure.\textsuperscript{134}

MPA is metabolized by uridine diphosphate-glucuronosyltransferases (UGTs) primarily in the liver, but also in the intestine and kidneys.\textsuperscript{137,138} The conversion to the predominant and pharmacologically inactive MPAG metabolite is catalyzed mainly by UGT 1A9, but other UGTs (1A1, 1A6, 1A7, 1A8 and 1A10) are also involved.\textsuperscript{139,140} A minor acyl glucuronide (AcMPAG) metabolite is generated primarily by UGT 2B7 and demonstrates pharmacological activity comparable to MPA.\textsuperscript{138,141} The AcMPAG metabolite has also been suggested to contribute to the MPA related gastrointestinal toxicity.\textsuperscript{141,142} Other minor metabolites include phenolic and acyl glucosides, probably generated by uridine diphosphate-glucosyltransferases, and a 6-O-desmethyl metabolite, which is generated by cytochrome P-450 (CYP) 3A isoenzymes.\textsuperscript{138-140} The metabolic pathway of MPA is depicted in Figure 9.
Figure 9. Pharmacokinetic (PK) processes of mycophenolic acid (MPA).

Chemical structures of MPA, the administered compounds mycophenolate mofetil (MMF) and mycophenolate sodium (MPS), and three metabolites are depicted. The major enzymes involved in generation of the 7-O-glucuronide (MPAG, major, inactive), acyl glucuronide (AcMPAG, minor, active) and 7-O-glucoside (MPAGls, minor, active) metabolites of MPA are given. Factors with potential impact on PK processes are illustrated.

CsA, cyclosporine; GI, gastrointestinal; MRP2, multidrug resistance-related protein 2; UGT, uridine diphosphate-glucuronosyltransferase (MPAG, AcMPAG) or uridine diphosphate-glucosyltransferase (MPAGls).
Introduction – Mycophenolic acid

The MPAG metabolite undergoes extensive biliary secretion via the ATP binding cassette (ABC) transporter, multidrug resistance-associated protein 2 (MRP2) in hepatocytes. MPAG can be hydrolyzed back to MPA by bacterial glucuronidases in the intestine and then reabsorbed into the systemic circulation (Figure 9). This enterohepatic circulation (EHC) of MPA can be observed as secondary concentration peaks at 4–12 hours postdose and account for approximately 40% (range 10–60%) of the total MPA area under concentration-time curve (AUC). Other transporters, e.g. breast cancer resistance protein (BCRP/ABCG2) and organic anion-transporting polypeptides (OATP, SLCO) have also been associated with MPAG transport.

The MPA metabolites are cleared primarily by renal excretion while a small proportion is eliminated by the fecal route after secretion into bile. The terminal elimination half-life is estimated to be in the range of 9 to 17 hours.

The variability of MPA pharmacokinetics is large within and between individuals. More than 10-fold differences have been reported for the dose-normalized MPA AUC from 0 to 12 hours. In addition to the mentioned impact of renal and hepatic function and albumin levels, factors like comorbidities, comedication, genetics and times since transplant might contribute to the PK variability of MPA.

Drug interactions

Concomitant intake of drugs like antacids, ferrous sulphate, cholestyramine and sevelamer, has been reported to impair the absorption of MPA. Corticosteroids and rifampicin could induce the synthesis of UGT enzymes and possibly drug transporters and thereby increase the clearance of MPA. Exposure to MPA when given in combination with CsA is approximately 30–40% lower than when given alone or in combination with tacrolimus or sirolimus. CsA is reported to mediate dose-dependent inhibition of the MRP2 transporter. This suppresses the excretion of MPAG into bile and consequently the EHC of MPAG, thus reducing plasma concentrations of MPA. Antibiotic therapy has also been shown to reduce EHC in healthy individuals and transplant patients, probably mediated through disruption of the normal gut flora and a reduction in bacterial glucuronidase activity.
Time dependent pharmacokinetics

The MPA pharmacokinetics is reported to change during the first months after transplantation. Renal transplant patients display 30–50% lower MPA exposure (AUC$_{0-12h}$) the first few weeks, compared to 1–6 months posttransplant. The change in MPA concentrations is multifactorial and could be related to improvement in renal function and hypoalbuminemia, and tapered doses of corticosteroids and CNIs.

Genetics

There are considerable differences between individuals in both protein levels (17-fold) and enzymatic activity of UGT enzymes. Single nucleotide polymorphisms (SNPs) of the UGT1A9 gene have been associated with protein expression and enzymatic activity, suggesting that genetic polymorphisms might contribute to the variable MPA metabolism. A recent study in renal allograft recipients reported that a significantly higher proportion of MPA concentration measurements was in the low MPA target range in patients carrying the UGT1A9 upstream SNPs −275T>A (rs6714486) and/or −2152C>T (rs17868320). Genetic variability has also been reported for ABCC2, encoding MRP2, but the clinical relevance is not known.
1.4 Individualization of Mycophenolate Therapy

1.4.1 Monitoring; Why, when and how?

The use of MPA in transplantation is steadily increasing. Beneficial effects on long-term outcomes have promoted the use in novel immunosuppressive regimens and to facilitate CNI or corticosteroid sparing. Over the last decade, MPA has also emerged as a therapy for autoimmune diseases. However, the therapeutic potential is limited by the narrow therapeutic window and dose limiting toxicity of MPA. The most frequent adverse events are gastrointestinal (GI) effects including nausea, vomiting, abdominal pain and diarrhea.\(^\text{10-13}\) A formulation of enteric-coated mycophenolate sodium (EC-MPS) was launched to improve the upper GI tolerability of MPA. However, no significant improvements with respect to the frequency or severity of adverse effects were observed.\(^\text{173,174}\) Hematological toxicities, mainly leukopenia and anemia, and an increased risk of opportunistic infections are among other serious adverse events.\(^\text{10-12}\) The toxicity is mainly reversed with dose reductions or temporary discontinuation of therapy. However, MPA dose tapering is associated with an increased risk of acute graft rejection in renal allograft recipients.\(^\text{175,176}\) Studies of renal, cardiac and liver allograft recipients reported that approximately half of the patients receiving MMF in combination with CsA were underexposed to MPA early posttransplant according to recommended target ranges,\(^\text{177}\) thus being of higher risk of rejection.\(^\text{175,178}\) Furthermore, reaching an adequate MPA exposure might be of particular importance when MPA is used in CNI or corticosteroid sparing regimens.

The concentration related efficacy, large PK and PD variability and narrow therapeutic window of MPA provide a rationale for therapeutic drug monitoring (TDM). Individualized MPA therapy could enhance the efficacy and minimize the toxicity of the drug and thereby improve outcomes in transplantation and other fields of immune modulation.

There is a particular value for monitoring MPA therapy in the early posttransplant period, during major changes in immunosuppression (e.g. tapering of CNI or corticosteroids) and at the time of major clinical events (rejection, infection, adverse events or malignancy). Individualization of MPA therapy could be guided through
pharmacokinetic (PK), pharmacodynamic (PD) and/or pharmacogenetic approaches (Figure 10).

**Pharmacokinetic monitoring**

TDM has traditionally been pharmacokinetically oriented, using drug concentrations as surrogate markers for drug activity. To justify TDM, there should be a clear relation between the measured parameter and clinical events (toxicity, acute rejections). Common PK parameters include measurements at single time points, area under concentration-time curve (AUC) and various limited sampling strategies for estimation of AUC. Methods for measurement of MPA concentrations include immunoassays, liquid chromatography (LC) methods with UV or mass spectrometric (MS) detection and a novel enzymatic assay based on IMPDH inhibition.\(^{179,180}\) The immunoassays are less laborious and time consuming compared to LC methods. However, the antibody used in the immunoassays cross reacts with the AcMPAG metabolite, thus overestimating MPA concentrations.\(^{181}\) The LC methods are more specific and allow accurate determination of both free and total concentrations of MPA and metabolites.

The potential of PK guided dosing was first demonstrated in a concentration controlled trial in renal transplant patients\(^{163,182}\) and later confirmed in studies in pediatric patients.\(^{164,183}\) The full 12-hour MPA AUC was reported to be the best predictor for acute rejection episodes\(^{182-184}\) and a therapeutic range of MPA AUC\(_{0–12h}\) between 30 and 60 mg×h/L has been suggested in patients on CsA based immunosuppression.\(^{182}\) Optimal therapeutic ranges may depend on the type of transplant, time since transplantation, immunosuppressive regimen and the presence of risk factors for acute rejection and should therefore be investigated for these conditions. The full AUC\(_{0–12h}\) parameter is impractical for routine monitoring and several limited sampling strategies are evaluated for the best estimation of MPA AUC\(_{0–12h}\).

Two recent randomized clinical trials, the Fixed Dose Versus Concentration Controlled (FDCC) and the Apomygre, evaluated the value of TDM of MMF in *de novo* renal allograft recipients during a 12 months posttransplantation period. The
Apomygpre trial included 137 patients receiving MMF in combination with CsA, corticosteroids and basiliximab. The MMF doses in the concentration controlled group were guided using a Bayesian estimator based on a three point limited sampling strategy. The concentration controlled MMF group demonstrated a reduced risk of treatment failure and acute rejections without any increase in adverse effects, supporting the use of TDM.\textsuperscript{185} In contrast, the FDCC trial (n=901) did not show any benefit of TDM. The patients received CsA or tacrolimus based immunosuppression and the MPA AUC\textsubscript{0−12h} was estimated by a three point limited sampling scheme in combination with a linear regression algorithm. The MPA exposure was similar between the groups, which might be a result of nonadherence to the recommended dose increments in the concentration controlled group.\textsuperscript{178} This implies that no difference could be expected between the groups. Both studies found an association between MPA exposure and acute rejection.\textsuperscript{178,185}

While the efficiency of MPA is associated with drug exposure, the correlations between PK parameters and GI toxicity or infections are poor and inconsistent, and a clear cutoff between effective drug exposure and the appearance of adverse effects has not been established.\textsuperscript{163,171,178,182} An explanation could be that the reported adverse effects have multiple causes. Furthermore, GI toxicity is reported to be stronger related to MMF dose than to MPA plasma concentrations,\textsuperscript{182} suggesting an impact of local GI drug exposure. This theory is supported by a substudy of the FDCC trial, reporting more frequent GI toxicity in tacrolimus treated patients, which probably was related to a greater intestinal exposure to MPA metabolites in these patients.\textsuperscript{186} Measurements of free MPA concentration or concentrations of metabolites like AcMPAG have been proposed to better predict adverse effects, but the data are conflicting.\textsuperscript{183,186,187} Hematological toxicities are stronger correlated to MPA exposure, and a recent study in renal allograft recipients suggested that patients suffering from leukopenia or anemia might benefit from MMF dose adjustments based on PK measurements.\textsuperscript{171}

The poor correlation between MPA concentration and adverse effects, the limited value of PK monitoring the first days of treatment and the high interindividual variability of the drug target, IMPDH, indicates a potential for PD measurements as an alternative or supplemental approach for monitoring MPA therapy.
Figure 10. Monitoring of mycophenolate therapy.

Potential approaches to improve the clinical outcomes during mycophenolate therapy.

AUC, area under the concentration versus time curve; SNP, single nucleotide polymorphism.
**Pharmacodynamic measurements**

Monitoring of PD biomarkers integrates both PK and PD variability and provides more direct determination of the biological effects of drugs. Potential PD parameters of MPA efficacy include IMPDH activity, guanine nucleotides and lymphocyte proliferation. Different approaches and sample matrixes for PD monitoring are addressed in Section 1.4.2.

**Pharmacogenetics**

Pharmacogenetic strategies for individualization of drug therapy include genotyping SNPs of e.g. drug targets, metabolizing enzymes or transporters. This approach offers an opportunity for initial selection of drug regimen and adjustment of dosing to ensure adequate immunosuppression during the critical first days posttransplant. Moreover, genotype information is valuable in the interpretation of PK or PD data. The genotype is a stable characteristic and only needs to be determined once for any given gene. In transplanted patients, the genotype of the graft might also be of interest. The individual drug response is determined by multiple genes and gene-to-gene interactions, thus haplotyping or pharmacogenomic approaches could offer better prediction of drug response. Microarray technology allows gene expression profiling at the genome level. However, these strategies require advanced methodology and data interpretation. Potential candidates for pharmacogenetic testing of MPA could be the two IMPDH isoenzymes (IMPDH1, IMPDH2), the MRP2 transporter (ABCC2) and UGT enzymes. However, the value of genotyping has not yet been assessed for MPA, and further studies are required to characterize the impact of genetics on MPA PK or PD (Section 1.4.3).

**1.4.2 Pharmacodynamic biomarkers of mycophenolate effect**

The efficacy of MPA therapy can be evaluated by drug specific and non-specific biomarkers. Non-specific PD parameters reflect the general activity of the immune system. Common biomarkers for evaluation of the overall immunosuppression include various lymphocyte proliferation assays, determination of lymphocyte surface antigens (e.g. CD11a, CD25, CD71, CD95, CD134 and CD154) and cytokine production. The methodology includes flow cytometric, reverse transcription (RT)-PCR and ELISA techniques. Barten et al. demonstrated that inhibition of lymphocyte
proliferation and antigen expression by MPA correlated with MMF dose, MPA level and with the histologic grade of graft rejection in a cardiac transplant rat model. More recently, a commercially available whole blood assay was introduced for assessment of overall immune function (Cylex ImmuKnow\textsuperscript{TM}). The assay is based on measurement of ATP levels in CD4+ cells after stimulation with phytohemagglutinin. However, the assay is not specific for immune status since ATP levels reflect the overall energy metabolism of cells. The potential of the assay in transplantation is debated and needs further evaluation. Moreover, an interferon-\(\gamma\) (INF-\(\gamma\)) enzyme-linked immunosorbent spot (ELISPOT) assay has been reported to be a highly sensitive tool for assessment of primed effector/memory T cells. In renal allograft recipients, the cellular alloreactivity as measured by the INF-\(\gamma\) ELISPOT assay, correlated with acute rejection and renal functional impairment posttransplant. Indeed, these findings need to be confirmed in prospective studies and larger cohorts of patients.

Measurement of the catalytic activity of IMPDH is investigated for more specific characterization of MPA effects. The techniques for determination of IMPDH activity comprise radioassays determining the IMPDH catalyzed conversion of radiolabelled substrate and several LC assays measuring the XMP production rate using UV or mass spectrometry (MS) for product detection. The initial assays determined IMPDH activity mainly in whole blood. However, as lymphocytes are the principal targets for immunosuppressive effects, IMPDH measurements in these cells are considered to be the most appropriate to ensure adequate immunosuppression (Sections 1.4.3 and 1.4.4). Several LC assays are validated for IMPDH analysis in isolated peripheral blood mononuclear cells (PBMCs). However, the isolation and pretreatment of cells might influence the measured IMPDH activity. Another IMPDH assay, based on the method published by Albrecht \textit{et al}., was developed for analysis in isolated CD4+ cells. Intracellular MPA concentrations were restored after cell isolation to avoid potential wash-out of MPA during the cell preparation steps. None of these activity measurements can distinguish between the IMPDH isoenzymes.

Gene expression assays have been used to characterize the relative importance of the isoenzymes, and furthermore to explore the mechanisms of IMPDH regulation.
Traditionally methods for determination of gene expression include Northern blotting and qualitative/competitive RT-PCR. The more recently introduced real-time RT-PCR technology allows more accurate quantification of mRNA over a broad dynamic range. However, the reliability of the results highly depends on assay design, optimization and the quantification strategy. Vannozzi et al. presented a real-time RT-PCR assay for quantification of IMPDH2 mRNA in human lymphocytes normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The calculations did not include correction for PCR efficiency and only limited validation of the method was presented. Another quantitative RT-PCR assay determined the expression of both IMPDH isoforms in various cells in sheep. Amplification products were monitored by SYBR Green I and the expression of target genes was normalized to a single reference gene, beta-actin. We developed and validated a real-time RT-PCR assay for specific quantification of IMPDH1 and IMPDH2 in human blood cells (Paper I). Expression of the target genes were normalized to a reference gene index (RGI) as described in Section 3.1.

Since the major effects of MPA are mediated through alterations of guanine nucleotide levels, measurement of guanine nucleotides could represent a biomarker closer related to MPA efficiency than IMPDH levels. In multiple myeloma patients treated with MMF, a positive correlation was observed between depletion of intracellular dGTP and clinical response. However, the relation between guanine nucleotide levels and clinical outcome in transplantation remains to be characterized. Furthermore, the relevance of the different nucleotide pools for immunosuppressive action is not definitively determined. Following administration of single MMF doses, no immediate reduction of guanine nucleotides (GDP and GTP) was observed in CD4+ cells from healthy individuals. However, this might not reflect the response in populations of activated cells during repeated dosing.

Based on the current knowledge, no conclusions can be drawn as to which are the most appropriate PD biomarkers and how best to monitor them.
1.4.3 IMPDH and clinical outcome

The high variability of IMPDH activity between individuals prior to transplantation emphasizes the rationale for individualized MPA exposure. Although IMPDH measurements are not implemented in clinical routine yet, several retrospective investigations in MMF treated patients support the clinical potential of IMPDH monitoring.

In renal transplant patients, high pretransplant IMPDH activity in PBMCs was associated with acute rejection episodes.\(^{209}\) This is in agreement with our finding that high pretransplant \textit{IMPDH2} expression in CD4+ cells predicted acute rejection episodes (Paper II). Furthermore, high IMPDH activity (AUC\(_{0-12h}\)) in PBMCs at day 6 posttransplant was significantly associated with acute rejection in renal allograft recipients, while no correlation was observed for MPA exposure.\(^{210}\) Another recent study reported less IMPDH inhibition in lymphocytes from patients with acute rejection during the first week posttransplant compared to patients without rejection.\(^{211}\)

The variable activity of this enzyme could also explain some of the adverse effects. Glander \textit{et al.} reported that low pretransplant IMPDH activity in PBMCs predicted MMF dose reduction because of intolerable adverse events.\(^{209}\) In contrast, a study in liver transplant patients on MMF therapy demonstrated a trend towards more gastrointestinal and hematological complications with elevated \textit{IMPDH2} gene expression in PBMCs.\(^{204}\)

These findings suggest that PD monitoring by IMPDH measurements might address some of the limitation of PK monitoring. Indeed, further studies are needed to characterize the relation between IMPDH parameters and the clinical outcome before prospective intervention studies based on IMPDH monitoring may be initiated. Assays should be standardized to allow comparisons between studies.

\textit{IMPDH and immune activity}

The relation between IMPDH levels in lymphocytes or mononuclear cells and outcome after transplantation might be explained by the immune status of the patient.
The mechanisms of immune activation and allograft rejection involve the expression and regulation of multiple genes. Activation of T cells is associated with a considerable increase in IMPDH enzyme activity, as well as upregulation of *IMPDH I* and 2 gene expression (Figure 11).\(^\text{35}\) In renal transplant patients, up to 20-fold upregulation of predose *IMPDH1* expression was observed in PBMCs during acute rejection episodes.\(^\text{212}\) Devyatko *et al.* demonstrated a close correlation between IMPDH predose activity in PBMCs and the proportion of activated (CD38+) CD8+ cells in heart transplant patients, suggesting that IMPDH plays a role in the regulation of CD8+ T cell activation.\(^\text{213}\) Diabetic renal transplant patients generally have lower innate immune status than non-diabetic transplant patients.\(^\text{214,215}\) This could explain the lower IMPDH activity that was observed in PBMCs from diabetic compared to non-diabetic renal transplant patients.\(^\text{215}\) The activity or gene expression of IMPDH might thus provide a surrogate marker for cellular immunity (Figure 11).

**Figure 11. Schematic illustration of T cell activation and IMPDH upregulation.**

The first exposure to foreign antigens may induce a primary immune response. Naïve T cells are activated by professional antigen-presenting cells (APCs) in lymphoid tissue. The subsequent proliferation and differentiation into effector T cells is driven by interleukin-2 (IL-2) and requires an upregulation of IMPDH activity. In addition to the generation of relatively short-lived effector cells, some cells become long-lived memory T cells, which may be sustained by cytokines.
The immune status pretransplant could be influenced by *e.g.* dialysis, infections, previous pregnancies, blood transfusions and previous transplantations. Exposure to dialysis is associated with non-specific cellular activation, particularly of T cells, that might contribute to increased alloreactivity and hence the risk of rejection.\textsuperscript{194,216} This is in agreement with our observations that the risk of rejection seemed to be higher among patients with dialysis pretransplant compared to patients without (50\% versus 21\%, ns, Paper II).

*Genetic polymorphisms*

Recently, polymorphisms within the *IMPDH1* and *IMPDH2* genes have been suggested to impact baseline IMPDH activity and outcomes after transplantation (location of variants in Figure 4, Section 1.2.2).\textsuperscript{217-219} Wang *et al.* reported two intronic *IMPDH1* single nucleotide polymorphisms (SNPs; rs2278293 and rs2278294) that were significantly associated with acute rejection episodes in renal transplant patients receiving MPA therapy.\textsuperscript{217} Grinyo *et al.* demonstrated that the presence of at least one *IMPDH2 3757T>C* (rs11706052) allele tripled the odds for developing rejection within 12 months posttransplant.\textsuperscript{219} The association between the genetic variants and posttransplant outcome may be correlated with enzyme activity. A recent study in renal allograft recipients showed that the presence of an *IMPDH2 3757T>C* allele correlated with significantly higher IMPDH activity in PBMCs during MPA exposure.\textsuperscript{220} A nonsynonymous c.787C>T variant (L263F) of *IMPDH2* resulted in a reduction of enzyme activity to 10\% of wild type activity. The allele frequency in a cohort of transplant patients was 1\%, and the variant genotype was suggested to predispose for MPA related leukopenia.\textsuperscript{217,218}

The clinical impact of the gene variants needs further characterization and evaluation before potential implementation in *e.g.* dosing algorithms for individualized MPA exposure.
1.4.4 Potential induction of IMPDH during MPA therapy

The pharmacodynamics of MPA has been reported to change with time in transplant patients. The findings are indeed conflicting and seem to depend on methodology, cell type, cell cycle status and degree of MPA exposure.

**Whole blood and erythrocytes**

Several studies report an induction of IMPDH activity in whole blood and erythrocytes during prolonged MMF therapy. In heart transplant patients, long-term MPA therapy (>1 year) was associated with an elevation of predose IMPDH activity (5-fold), GTP concentrations (2-fold) and to a lesser extent HPRT activity in erythrocytes. Similar findings were reported by Goldsmith *et al.*, demonstrating 3–4 fold higher GTP levels in predose erythrocyte samples from renal transplant patients receiving MMF (>3 months) compared to patients with AZA. An increase in predose IMPDH activity was also reported in whole blood samples from renal transplant patients. Following an immediate reduction early posttransplant, the enzyme activity increased gradually and was 5 times higher than pretransplant levels approximately 40 days posttransplant. Despite the elevated predose activity, IMPDH activity was considerably suppressed within the dose intervals. In another study in renal transplant patients, we observed an elevation of predose IMPDH 1 and 2 gene expression in reticulocytes (Figure 15, Section 4.2), which could be related to the MMF treatment (Paper II). The increase started the second week posttransplant and was accompanied by a similar rise in whole blood activity. This implies that the gradual upregulation of predose activity in whole blood might involve an induction at the transcriptional level in the early differential stages of erythrocytes. The IMPDH predose activity in erythrocytes is also reported to be induced by ribavirin, another IMPDH inhibitor. In immunodeficient children, considerable increments of IMPDH predose activity (up to 30-fold) were observed 3–4 weeks after introduction of ribavirin antiviral therapy.

A dose interval study in renal transplant patients on short-term (<1 year) versus long-term (>2 years) MMF treatment demonstrated that the inhibitory effect of MPA on whole blood IMPDH activity was reduced in the long-term treated patients. With prolonged therapy, individual patients demonstrated only weak inhibition and
subsequent increases of activity reaching 5 times the predose levels.\textsuperscript{224} Similar findings were also reported in a later study, showing 2- to 4-fold induction of whole blood activity postdose in patients on prolonged (18–24 months) MMF therapy.\textsuperscript{212} Despite the induction within dose intervals, no alteration was observed in predose activity.\textsuperscript{212} The rapid and transient induction of IMPDH activity suggests a considerable influence of IMPDH in nucleated cells. The authors reported that IMPDH in mononuclear cells accounted for 50\% of the measured whole blood activity.\textsuperscript{212} Langman \textit{et al.} did not report any induction of enzyme activity. Predose IMPDH activity in whole blood was comparable between MMF and AZA treated patients (>2 years posttransplant). Following dose, an immediate and reversible inhibition of IMPDH activity was observed only in the MMF patients. With respect to guanine nucleotides, intracellular levels of GMP and GDP were lower, while the GTP concentrations were higher in the MMF group compared to AZA patients.\textsuperscript{225} Indeed, both MMF and AZA might influence purine nucleotide levels and thereby complicate the interpretation of these findings.

\textit{Mononuclear cells}

In contrast to the elevation that was observed for predose IMPDH activity and GTP levels in erythrocytes and whole blood, the corresponding parameters seem to be relatively stable or declining with prolonged MPA therapy in various populations of mononuclear cells. In heart transplant patients switched from AZA to MMF, the IMPDH predose activity in PBMCs was significantly reduced after 3, 6 and 12 months on MMF therapy while MPA C\textsubscript{0} remained stable.\textsuperscript{226} No significant alterations were observed for concentrations of GTP in the corresponding samples. This could be explained by a concomitant increase in HPRT activity, which maintains the intracellular purine nucleotide pools.\textsuperscript{226} Similar results were observed by Sankatsing \textit{et al.} in renal transplant patients on MMF therapy. Predose levels of dGTP and GTP were stable during the 8 weeks posttransplant follow-up. Despite this, \textit{ex vivo} stimulated T cell proliferation was inhibited. Comparable findings were reported in HIV-1 infected patients receiving MMF.\textsuperscript{227} In contrast, Jagodzinski \textit{et al.} reported a significant decrease in GTP and total guanine nucleotide pools in PBMCs from renal transplant recipients with MMF therapy compared to dialysis patients and healthy controls. Guanine nucleotide concentrations were similar between patients with MMF treatment for less and more than 6 months.\textsuperscript{208}
Considering dose interval studies of renal transplant patients, Sanquer et al. reported transient inductions of \textit{IMPDH1} expression in PBMCs after MMF intake. The induction was highest three months after transplantation (mean 236%, SD ± 229%, compared to predose), but was considerable throughout the two years observation period. The predose samples demonstrated higher \textit{IMPDH1} expression the first three months, while expression was stable from 6 to 24 months posttransplant. Expression of \textit{IMPDH2} was stable throughout the study period, but increased postdose at month 24 (mean 50%, SD ± 45%).\textsuperscript{212} This is in agreement with our findings in a study of patients on belatacept or CsA based immunosuppression, including MMF (Paper IV). With increasing time since transplantation, we observed transient postdose inductions of \textit{IMPDH1} gene expression and IMPDH activity in CD4+ cells (Figure 17, Section 4.4). The rapid and transient induction observed in nucleated cells contrasts the gradual elevation in erythrocytes, which may originate from an induction in earlier differentiation stages that persists during erythrocyte maturation.

Other dose interval studies, however, did not observe altered PD response in mononuclear cells with time. Glander \textit{et al.} reported no significant difference in IMPDH inhibition in PBMCs from dialysis patients receiving their first MMF dose versus cardiac transplant patients on prolonged MPA therapy (>1 year),\textsuperscript{228} however such comparisons across populations must be interpreted cautiously. Another study in renal transplant patients also demonstrated an inverse relation between MPA concentration and IMPDH activity, with a mean inhibition of 87%, more than a year posttransplant.\textsuperscript{229} Furthermore, a study comparing EC-MPS and MMF formulations in renal transplant patients demonstrated considerable inhibition of IMPDH activity within the dose interval after more than 3 months of MPA therapy.\textsuperscript{230}

\textit{Feedback regulation}

Expression of the \textit{IMPDH} genes is suggested to be regulated by guanine nucleotides through negative feedback mechanisms in human cells (Figure 12 and Section 1.2.2).\textsuperscript{35,40} The apparent induction of IMPDH activity could thus be related to increased gene expression. However, feedback regulation at the enzymatic level cannot be excluded (Section 1.2.1).
Introduction – Individualization of Mycophenolate Therapy

The regulation of IMPDH during MPA exposure has been studied in various human \textit{in vitro} cell models. Glesne \textit{et al.} examined the effects of guanosine and MPA on \textit{IMPDH} mRNA in promyelocytic and melanoma cell lines. Guanosine supplementation increased the intracellular guanine nucleotide levels and subsequently reduced \textit{IMPDH} expression whereas exposure to MPA reduced guanine nucleotide levels and increased the IMPDH mRNA levels.\textsuperscript{34} IMPDH protein levels were influenced in a similar manner. The authors suggested \textit{IMPDH} to be regulated at the posttranscriptional level, and that the regulation occurred through alterations of guanine nucleotides rather than deoxyguanine nucleotides.\textsuperscript{34} An inverse regulation in response to guanine nucleotides was also observed in another study in cultured human acute T lymphoblastic leukemia (MOLT-4) cells. Following MPA exposure (0.15 and 0.5 µmol/L, 72 hours), a reduction of GTP (35% of control) levels was observed and accompanied by increased expression of \textit{IMPDH1} (40%) and \textit{IMPDH2} (2-fold).\textsuperscript{53} Dayton \textit{et al.} investigated normal human T cells during \textit{ex vivo} exposure to mizoribine, an IMPDH inhibitor, and guanosine (12 and 24 hours). Inhibition of IMPDH activity resulted in an upregulation of \textit{IMPDH2} expression (3-fold), probably mediated through reduced guanine nucleotides.\textsuperscript{35} On the other hand, a study in \textit{ex vivo} stimulated PBMCs and three different transformed cell lines did not demonstrate any significant changes in \textit{IMPDH 1} or \textit{2} mRNA or protein levels after exposure to MPA (<1 µmol/L, 72 hours).\textsuperscript{58} The relationship between guanine nucleotides and expression of \textit{IMPDH} isoforms is poorly characterized \textit{in vivo} in humans. In a study of healthy individuals exposed to MPA, we observed that \textit{IMPDH1} expression tended to be inversely related to GTP concentrations. Indeed, the purine nucleotide responses were variable and the correlation was not statistically significant (Paper III).

Interpretation of the relation between MPA exposure, IMPDH activity and finally guanine nucleotide pools might be complicated by various degrees of compensating mechanisms like induction of the IMPDH isoenzymes and HPRT. Furthermore, the most relevant nucleotide pools with respect to the IMPDH regulation remain to be elucidated.
Figure 12. Negative feedback regulation of the IMPDH genes.
Inhibition of inosine 5’-monophosphate dehydrogenase (IMPDH) by mycophenolic acid (MPA) reduces de novo synthesis of guanine- and deoxyguanine nucleotides (GN and dGN, respectively). This could attenuate a suppressive mechanism of GN/dGN on gene expression and consequently induce the expression of IMPDH1 and/or IMPDH2. The relative contributions of de novo (via IMPDH) and salvage pathways differ between cell type and cell cycle status. Arrows represent stimulation while T-bars represent inhibition. ❎ indicates steps that are suppressed by MPA.

Clinical implications
The IMPDH activity in whole blood is probably predominated by erythrocytes, which constitute the largest blood cell population. Alterations in blood cell numbers and populations might also impact whole blood measurements. Furthermore, the clinical relevance of the observed enzyme induction of erythrocyte predose activity is not known. Thus, IMPDH measurements in lymphocytes might better predict MPA efficacy. Activated lymphocytes play a pivotal role in allograft rejection and are among the target cells for MPA effects. Indeed, the activity in the general population of circulating lymphocytes or mononuclear cells might not reflect the IMPDH status in a relatively low fraction of activated, proliferating lymphocytes.

The requirement of guanine nucleotides is considerably higher in proliferating versus resting, non-proliferating lymphocytes and this is reflected in different basal levels of IMPDH activity. It could be speculated that inhibition of IMPDH in cells with
Introduction – Individualization of Mycophenolate Therapy

relatively low basal IMPDH activity to a higher degree initiates compensating mechanisms, like induction of IMPDH and HPRT enzymes, to maintain the guanine nucleotide levels that are required for constitutive processes. The relative importance of IMPDH versus HPRT enzymes depend on cell type. Such induction has been reported in vivo and in vitro and may completely mask the inhibition of IMPDH activity and even induce IMPDH and guanine nucleotide levels. Budde et al. reported that two dialysis patients with low basal IMPDH demonstrated a small elevation of IMPDH protein 6 hours after the first MMF dose. In a study of healthy individuals, we also observed an “overshoot” of IMPDH activity and gene expressions appearing 8–24 hours after administration of MMF 1000 mg (Section 4.3 and Paper III). In contrast, IMPDH inhibition in cells with higher enzyme levels might not suppress guanine nucleotide levels sufficiently to induce IMPDH and/or HPRT. Alternatively, even if compensatory mechanisms are induced, this might not be adequate to supply the required guanine nucleotides in proliferating cells. Hence, MPA may increase IMPDH in resting cells and still inhibit the proliferation of activated cells. This could provide an explanation for why induction of IMPDH does not translate into reduced clinical efficacy of MPA. This present hypothesis is further supported by studies in renal transplant and HIV-1 infected patients on MMF therapy. Although dGTP and GTP levels in PBMCs were stable, ex vivo induction of T cell proliferation was inhibited.

The apparent loss of inhibitory action of MPA on IMPDH in PBMCs and CD4+ cells within dose intervals might thus reflect an effect in a population of mainly non-proliferating cells and may not be relevant for MPA efficiency. Indeed, further studies are needed to explain these findings. The hypothesis could be addressed by relating the IMPDH results to lymphocyte activation status (e.g. by flow cytometric assessment) and by investigations of specific cell populations.

The activity of IMPDH depends on cell type, cell cycle and differentiation status. Hence, the results of the reported studies might be impacted by the cell subsets investigated. Furthermore, the contrasting findings between studies could be influenced by differences in methodology (e.g. Northern blotting versus real-time PCR), control groups (AZA treated, dialysis patients and healthy individuals), comedication and time since transplantation.
The feedback regulation of the *IMPDH* genes seems to differ from the regulation in response to inducers of cell proliferation and growth. While both isoforms are considerably upregulated with lymphocyte activation, the MPA induced regulation in PBMCs and CD4+ cells was most pronounced for *IMPDH1* (Paper IV). This might be related to different functions of the isoenzymes.
2. Objectives of the Thesis

In the process of optimizing MPA therapy in transplanted patients, better knowledge of the pharmacodynamic-pharmacokinetic relations of MPA is warranted. The main objective of this thesis was to characterize the two IMPDH isoenzymes during exposure to MPA.

Specific objectives of the projects were as follows:

2.1 Paper I
The aim of the first project was to establish and validate a method for reliable quantification of \textit{IMPDH1} and \textit{IMPDH2} gene expression in human blood cells to explore the regulation of the IMPDH isoenzymes.

2.2 Paper II
The primary objective of this study was to investigate whether a reported induction of whole blood IMPDH activity in renal allograft recipients\textsuperscript{223} was due to altered gene expression, and to determine the relative contributions of IMPDH1 and IMPDH2 in relevant blood cell populations. A second objective was to identify possible associations between the \textit{IMPDH} expression in transplanted patients and factors like acute rejection episodes, dialysis pretransplant or the current immunosuppressive treatment, including MPA.

2.3 Paper III
The primary goal of this study was to investigate the regulation of \textit{IMPDH1} and \textit{IMPDH2} in blood cells from healthy individuals during \textit{in vivo} exposure to MPA. A secondary objective was to investigate the gene expressions in association with the corresponding IMPDH activities and concentrations of MPA and purine nucleotides.
2.4 Paper IV

The aim of this study was to investigate MPA pharmacokinetics and pharmacodynamics in recipients of extended criteria donor (ECD) renal allografts, receiving MMF in belatacept or CsA based immunosuppressive regimens.
3. Methods

3.1 Paper I

Development and validation of the real-time RT-PCR assay was based on whole blood and isolated cells from healthy volunteers and patients. Furthermore, the assay was adapted for measurement in cultured lymphoblasts. PAXgene Blood RNA Tubes® provided immediate stabilization of the expression profile in whole blood. Paramagnetic beads with CD4 antibodies were used to isolate CD4+ cells from EDTA blood. Red blood cells (reticulocytes and erythrocytes) were separated from whole blood by filtration through a column consisting of α-cellulose and microcrystalline cellulose.²³³

Figure 13. Overview of the steps of the gene expression assay.

Total RNA was extracted from lysed blood cells on the automated MagNA Pure® instrument. Concentration, purity and integrity of total RNA was evaluated by spectrophotometry on the NanoDrop® ND-1000 and electrophoresis on the Agilent Bioanalyzer® 2100. Reverse transcription (RT) was performed in a separate step, preceding real-time PCR on the LightCycler® 1.0 instrument (Figure 13). Reaction parameters were optimized for each step and the pre-PCR steps included a constant
Methods

cost of carrier RNA (MS2 RNA). The optimized RT was performed with Transcriptor reverse transcriptase, using random hexamer primers. Target and reference genes were amplified in separate reactions and amplification products were monitored by SYBR Green I during the optimizing stages, whereas hybridization probes were used for quantification. Gene specific primers and hybridization probes were designed using OLIGO 6.60 software and the LightCycler Probe Design software combined with manual evaluation. Primer and probe target sequences were checked for possible homologies with other sequences by Basic Local Alignment Search Tool (BLAST) searches. The approximate locations of the target sequences are illustrated in Figure 4 (Section 1.2.2).

Quantification

Five housekeeping genes, delta-aminolevulinate synthase1 (ALAS1), beta-2-microglobulin (B2M), glucose-6-phosphate dehydrogenase (G6PD), hypoxanthine phosphoribosyltransferase (HPRT1) and hydroxymethylbilane synthase (HMBS; previous name porphobilinogen deaminase), were evaluated as potential references for relative quantification. The former three genes were selected for normalization based on pilot data. However, further evaluations demonstrated regulation of G6PD in CD4+ cells. This gene was therefore omitted as a reference in these cells and subsequently replaced by ribosomal protein L13a (RPL13A).

The quantification principle was as follows: Relative concentrations of each target (IMPDH1 and IMPDH2) and reference (ALAS1, B2M and RPL13A or G6PD) gene were calculated by the obtained crossing point (Cp) and the gene specific PCR efficiency (E) as in Equation 1. The expression of each target gene was calculated relative to the geometric mean expression of the selected reference genes (Equation 2). This ratio was further normalized to the corresponding ratio calculated for the calibrator (Equation 3). The calibrator normalization corrects for different detection sensitivities of the target and reference amplicons and provides a constant calibrator point between PCR runs. Furthermore, a gene specific standard (10^4 copies) was included to serve as a positive control and allow estimation of absolute template concentrations using preformed calibration curves. Relevant software is presented in Section 3.6.
Methods

Relative concentration: \[ [\text{gene}] = E^{-Cp} \]  \hspace{2cm} (1)

Relative expression: \[ \frac{[\text{IMPDH}1] \text{ or } [\text{IMPDH}2]}{\sqrt[3]{([\text{ALAS}1] \times [\text{B2M}] \times [\text{RPL} 13\text{A} \text{ or } \text{G6PD}])}} \]  \hspace{2cm} (2)

Relative expression: \[ \frac{(\text{TG}/\text{RGI})_{\text{sample}}}{(\text{TG}/\text{RGI})_{\text{calibrator}}} \]  \hspace{2cm} (3)

The assay was validated according to specificity, sensitivity, limits of detection and quantification, dynamic range and imprecision (within and between assay).

3.2 Paper II

This was a prospective descriptive study of 30 adults receiving renal allograft from a living donor. The immunosuppression included MMF for 25 of the patients. Blood samples were collected on three occasions preceding transplantation, and predose on eight occasions during the first two weeks posttransplant.

The validated assay from Paper I was used to determine the gene expression of \textit{IMPDH} 1 and 2 in whole blood, isolated CD4+ cells and reticulocytes. The IMPDH activity was determined in CD4+ cells and whole blood cells by LC-UV as described elsewhere.\textsuperscript{197,201} Intracellular MPA was removed from the CD4+ cells to determine the basal IMPDH activity. Predose plasma concentrations of MPA were determined by LC-UV,\textsuperscript{234} while whole blood concentrations of CsA (2 hours postdose, \(C_2\)) and tacrolimus (predose, \(C_0\)) were monitored using immunoassays.

3.3 Paper III

Five healthy human volunteers were included in a crossover study involving different MMF doses. Blood was sampled predose (−24 and 0 hours) and at 1, 2, 4, 6, 8, 12 and
Methods

24 hours postdose for each of the single doses of 100, 250, 500 and 1000 mg MMF. A 12-hour baseline control interval without MPA was investigated for each individual with sampling every two hours.

The gene expressions of \textit{IMPDH 1} and 2 were quantified in CD4+ cells and whole blood as described for Papers I and II except that ribosomal protein L13a (\textit{RPL13A}) replaced \textit{G6PD} as a reference. The IMPDH activity was determined in lysates of CD4+ cells as described in Paper II except that intracellular MPA concentrations were restored in CD4+ cells by incubating the isolated cells in filtrated plasma originating from the same sample.\textsuperscript{201} Purine nucleotides (GDP, GTP, AMP, ADP and ATP) were quantified in CD4+ cells by reversed-phase anion-exchange LC-UV as described elsewhere.\textsuperscript{207,208}

3.4 Paper IV

The MPA PK and PD study was a non-sponsored substudy in patients included in the BENEFIT-EXT multicentre trial. Seven adult renal transplant patients were enrolled at Rikshospitalet University Hospital. The patients received grafts from extended criteria donors, defined as donor age above 60 years, donor age above 50 years and other donor co-morbidities, cold ischemia time above 24 hours or donation after cardiac death. Patients were randomized into three arms with CsA in one arm and belatacept (less intensive or more intensive, respectively) in the two others. Additional immunosuppression consisted of MMF 1000 mg twice daily, corticosteroids and induction therapy with basiliximab. Samples were collected at one occasion before transplantation and for 9-hour profiles at approximately 1, 2 and 13 weeks posttransplant.

The IMPDH activity in CD4+ cells (after reconstitution of MPA levels) and concentrations of MPA and CsA (\textit{C}_0) were determined as described in Papers II and III. The gene expression assay presented in Paper I was adapted to the LightCycler® 480 instrument allowing higher sample throughput. The geometric mean expression of \textit{ALAS1}, \textit{B2M} and \textit{RP L13A} was used for normalization of \textit{IMPDH1} and \textit{IMPDH2} expressions. Absolute quantification of T cell subsets, defined by the markers CD3, CD4, CD8, CD45RA and CD45RO, was performed by flow cytometric analyses on
Methods

the FACS Calibur® instrument using TruCount® tubes and a direct staining procedure.

3.5 Ethics

The studies in Papers II-IV were approved by the Regional Committee for Medical Research Ethics and all participants signed an informed consent form.

3.6 Data Analysis and Statistics

Results of the RT-PCR assays were analyzed using the LightCycler software v.3.5 and LightCycler 480 software v.1.5. The target gene expressions were normalized to an index comprising three reference genes using the Relative Expression Software Tool v.2 (REST-384) and LightCycler 480 software v.1.5. Calculations were also performed with the LightCycler Relative Quantification Software v.1. Flow cytometric data were analyzed using the CellQuest software. Data were presented as median (range) unless otherwise specified.

Statistical analyses were performed using SPSS versions 13, 15 and 16. Since our data involved a limited number of observations and were not always normally distributed, the non-parametric Wilcoxon signed rank test and Mann-Whitney U test were applied for statistical analysis of paired and independent data, respectively. Statistical significance was considered at P≤0.05.
4. Results and Discussion

4.1 Paper I

The present assay was the first RT-PCR assay for specific mRNA quantification of both human \textit{IMPDH 1} and 2, normalized to a reference gene index (RGI). Previous reported gene expression methods for \textit{IMPDH} were Northern blotting assays and two published real-time RT-PCR assays that normalized target gene expression to a single reference gene (Section 1.4.2). \textsuperscript{204,205}

The assay was developed primarily for gene expression measurements in transplanted patients. Variations in cell numbers and populations, because of immunosuppressive therapy or immune activation (\textit{e.g.} during rejection episodes) could result in variable RNA yield and gene expression. Quantitative real-time RT-PCR is a sensitive method that allows measurement of low amounts of mRNA. However, substantial assay validation is required to ensure reliable gene expression results.

\textit{Sample materials}

The present method was adapted for investigations in whole blood, CD4+ cells and reticulocytes, as well as cultured cells. Lymphocytes, and CD4+ cells in particular, play a pivotal role in graft rejection, and are among the main target cells for MPA effects (Section 1.4.2). Erythrocytes constitute the largest population of blood cells and probably dominate the whole blood IMPDH enzyme activity. Expression in reticulocytes, which contain some preformed mRNA, could provide a sensitive indicator for changes in the IMPDH expression and activity of erythrocytes since reticulocytes represent the most newly formed population.

\textit{Reference genes}

Normalization of the target gene expression to an expression ratio of several reference genes compensates for variations in the sample amount, RNA recovery, RNA integrity, efficiency of cDNA synthesis, presence of inhibitors and differences in the overall transcriptional activity of the tissues or cells analyzed. The selection of suitable reference genes is an important prerequisite to obtain reliable gene expression results. Reference genes should display highly uniform expression across the
investigated cells, developmental phases, environments and experimental treatments. However, no single gene can be defined as an omnipotent reference for a wide variety of biologic systems, and several potential genes should be validated to find an optimal set of reference genes for each experimental system. To strengthen the accuracy of the IMPDH assay, we normalized the gene expression of the target genes to a geometric mean of three reference genes; a reference gene index (RGI). The reference genes were selected among ubiquitously expressed genes from different functional classes to reduce the influence of possible regulation. Measurement of multiple reference genes with each experiment allowed ongoing validation of the reference genes, thus controlling for variables that might not have been accounted for in the initial validation. Although initial investigations suggested stable expression of G6PD in our samples from transplant patients, further validation revealed regulation of this gene in CD4+ cells. Expression data of G6PD were therefore omitted from the calculation of gene expression in these cells, and RPL 13A was included as reference in the subsequent studies. The regulation of G6PD was particularly pronounced the first days posttransplant and could be attributed to high-dose corticosteroids, the transplantation itself or to potential immune responses. Figure 14 illustrates the consequences of relating target gene expression to a coregulated G6PD gene.

![Figure 14: Impact of reference gene selection.](image)

**Figure 14. Impact of reference gene selection.**

Gene expression of IMPDH1 in CD4+ cells from a renal transplant recipient when separately normalized to the expression of three potential reference genes; aminolevulinate delta-synthase 1 (ALAS1), beta-2-microglobulin (B2M) and glucose-6-phosphate dehydrogenase (G6PD).
Results and Discussion

Validation

All samples were stabilized within an hour after sampling. RNA integrity numbers (RIN) showed isolation of intact RNA and the 260/280 nm absorption ratios (>1.9) indicated pure samples. The RT step is reported to be highly variable. Linear RT reactions (5×10^2–5×10^6 RNA copies) were obtained by the addition of carrier RNA (MS2 RNA) and using stable conditions for RNA isolation and the following RT step.

The high level of sequence identity between the two IMPDH isoforms, and also to several IMPDH1 derived pseudogenes, emphasized the need for careful selection of PCR primers. Specific quantification of target sequences was further ensured by using gene specific hybridization probes for real-time monitoring of amplification products. The specificity of the assay was confirmed by gel electrophoresis, melting curve analysis, sequencing of amplification products and inclusion of negative controls.

Calibration curves were linear over a dynamic range of at least six orders of magnitude (starting concentrations of 10^6 to 10 templates). Repeated generation of the calibration curves resulted in coefficients of variation (CVs) <1.7% and <0.8% for slope and intercept. The linearity of the calibration curves indicated constant amplification efficiencies over the concentration range studied, confirming minimal influence of PCR inhibitors. PCR efficiencies for IMPDH 1 and 2 were 2.03 and 1.99, while the efficiencies for ALAS1, B2M and G6PD were 1.99, 1.99 and 1.96, respectively. The gene specific PCR efficiencies were included in the calculations of relative expressions.

A majority of gene expression assays report imprecision levels calculated on the basis of Cp values. The log-linear inverse relationship between concentrations and Cp values implies that CVs based on relative concentrations will be nominally higher than CVs based on Cp values. We quantified the imprecision in terms of relative concentrations and the final normalized ratios, which are directly applicable and relevant for the interpretation of the results. Within-run and total imprecision were <25%, which may be an acceptable limit in this type of assay. Repeated analysis of the calibrator sample, already included in each run, demonstrated the stability of the cDNA material over a two year period. The total between-day RT-PCR CVs were 10.4% and 13.4% (normalized to calibrator) for IMPDH 1 and 2 respectively. Assay
Results and Discussion

Sensitivity was confirmed by the slopes of the linear regression curves used to measure the dynamic range. The detection limit (LOD) was 10 templates per reaction, while the lower limit of quantification (LLOQ) was set to $10^3$ templates per reaction since lower concentrations resulted in a significant increase in imprecision (CV) from $<12\%$ to $>26\%$.

The presented assay allowed specific and precise quantification of \textit{IMPDH 1} and 2 gene expression in blood cells from healthy individuals and transplant patients receiving immunosuppressive therapy. This offers an insight into the regulation of the two isoenzymes that might be important for immunosuppressive therapy as well as for disease states, like malignancies. The assay was later adapted for analysis on the LightCycler® 480 instrument, thereby allowing higher sample throughput.

\textbf{4.2 Paper II}

This is the first study of \textit{IMPDH 1} and 2 expression in relation to IMPDH activity in different blood cell populations from renal allograft recipients pre- and posttransplant. Twentyfive patients received immunosuppression with MMF while 5 patients without MMF treatment were included as controls for MPA effects. Acute rejection episodes were reported for 11 (37\%) patients during the follow-up and were treated with methylprednisolone (iv).

Both \textit{IMPDH} isoforms were expressed in all samples. The interindividual variability of IMPDH levels was large, demonstrating up to 8-, 3- and 17-fold differences in pretransplant gene expression for whole blood, CD4+ cells and reticulocytes, respectively. With respect to pretransplant enzyme activity, up to 17-fold and 41-fold differences were observed in whole blood and CD4+ cells, respectively. The gene expressions were relatively stable within individuals before the initiation of immunosuppressive therapy.

\textit{Regulation of IMPDH the first days posttransplant}

The transplantation and initiation of immunosuppressive therapy was associated with median 50\%, 57\% and 88\% upregulation of \textit{IMPDH1} expression in whole blood, CD4+ cells and reticulocytes, respectively (n=30, P<0.001). The increase was
Results and Discussion

followed by a gradual reduction the first week posttransplant nearly reaching baseline expression for whole blood, while the expression remained above baseline for CD4+ cells (26%, n=30, P<0.001) and reticulocytes (42%, n=30, P<0.001). Whole blood and reticulocytes demonstrated a transient 42% and 56% downregulation (n=30, P<0.001) of IMPDH2 expression, while CD4+ cells displayed a transient elevation of 15% (n=30, P=0.009). The predose activity in CD4+ cells (after removal of MPA) demonstrated a transient 90% increase (P<0.001) the first day after transplantation, which coincided with the upregulation of IMPDH 1 and 2 expression. In contrast, the corresponding predose activity of IMPDH in whole blood decreased the first couple of days posttransplant. This correlated with the regulation of IMPDH2 in reticulocytes (Figure 15).

The initial regulation of the IMPDH genes was observed in both patients with and without MMF and coincided with the administration of high-dose corticosteroids perioperatively. Further support of a relation to corticosteroids was provided by the appearance of similar gene expression changes in relation to corticosteroid based anti-rejection therapy. Corticosteroids demonstrate concentration-dependent effects and influence multiple signal transduction and gene expression pathways. This includes suppressing inflammatory genes, activating several anti-inflammatory genes and increasing the degradation of messenger RNA encoding inflammatory proteins.\(^19\) Still, gene expression effects provoked by the transplantation cannot be excluded. MPA did not appear to have a profound effect on IMPDH expression the first days after transplantation, although this cannot be ruled out since all patients were on a high glucocorticoid dosage during this period.

Potential induction of IMPDH expression

During the second week posttransplant, the patients with and without MMF displayed separate trends of IMPDH expression. Reticulocytes from the patients on MMF therapy demonstrated a gradual increase of both isoforms starting around one week posttransplant (Figure 15). Individual MMF patients showed IMPDH 1 and 2 increases reaching 8.7 and 2.4 times above baseline expression at the last observation day. When comparing the treatment groups, MMF treated patients demonstrated 2.9-fold higher (P<0.05) IMPDH2 expression than the patients without MMF at the last observation day. Although not statistically significant, also IMPDH1 expression
tended to be higher among MMF patients. Similar, but less pronounced upregulation was observed in whole blood. The increase in IMPDH 1 and 2 expressions was accompanied by a gradual increase in whole blood activity. These trends might result from enzyme induction under prolonged MMF treatment as discussed in Section 1.4.4. However, effects of basiliximab in the non-MMF patients cannot be excluded and the magnitude of a possible induction is uncertain due to the simultaneous influence of considerable corticosteroid dosage.

Figure 15. Potential induction of IMPDH 1 and 2 expression in reticulocytes.
Median expression of IMPDH 1 and 2 (normalized to day 0) in reticulocytes from patients with (n=25) and without (n=5) mycophenolate mofetil (MMF) treatment. * significant (P<0.05) difference from pretransplant. ¤ significant (P<0.05) difference between groups.
Results and Discussion

The whole blood samples reflect the total IMPDH expression and activity exhibited by the various blood cell populations. Erythrocytes, which constitute the largest blood cell population, probably dominate the IMPDH activity measured in whole blood. The clinical relevance of an induction in reticulocytes is not known and whole blood measurements might not reflect the activity in the cells of interest.

Acute rejection

A comparison of patients with (n=11) and without (n=19) acute rejection episodes during the follow-up, revealed higher (P=0.017) pretransplant IMPDH2 expression in CD4+ cells from the patients with rejection. Despite the significant influence of immunosuppressive therapy on gene expression posttransplant, the IMPDH2 expression among the patients with rejection seemed to be higher throughout the observation period (Figure 16). This correlation between IMPDH2 expression and the occurrence of rejection is in agreement with the correlations between high IMPDH activity pretransplant and at day 6 posttransplant and the risk of rejection as discussed in Section 1.4.4.209,210

![Figure 16. Higher IMPDH2 expression in CD4+ cells from patients with rejection.](image)

**Figure 16. Higher IMPDH2 expression in CD4+ cells from patients with rejection.**

Median IMPDH2 expression in CD4+ cells in patients with (n=11) and without (n=19) acute rejection. Vertical bars represent the range of observed expressions. * Significant (P<0.05) difference between groups.
The mechanisms of immune activation and graft rejection include expression and coregulation of multiple genes. High IMPDH2 expression may be a surrogate marker for increased activation of lymphocytes, which again could be caused by a higher level of T cell presensitization.\textsuperscript{194} The trend (ns, P=0.142) towards higher rejection rates in the population receiving dialysis pretransplant with 50% rejection episodes compared to 21% among patients without dialysis could also be explained by presensitization. Previous investigations have demonstrated higher risk of rejection in renal transplant patients receiving dialysis prior to transplantation compared to patients without dialysis.\textsuperscript{236,237} The initial elevation of IMPDH activity in CD4+ cells coincided with increased expression of IMPDH 1 and 2 in the same cells. This might be a response to immune activation and inflammation related to the transplantation, as well as the suggested impact of corticosteroids. The relation between IMPDH and immune activation is also addressed in Section 1.4.3.

The correlation between IMPDH2 expression and acute rejection episodes supports the potential of MPA individualization based on IMPDH measurements in lymphocytes. Indeed, additional studies are required to characterize the association between IMPDH, immune activation and occurrence of acute rejections. Furthermore, the influence of cellular presensitization suggests that monitoring of effector/memory T cells as discussed in Section 1.4.2, may be a promising approach to support rational therapeutic decisions.

4.3 Paper III

A majority of studies concerning IMPDH regulation have been performed \textit{ex vivo} in isolated cells or in immortalized cell lines. However, such studies may not reflect the \textit{in vivo} conditions and gene regulation mechanisms. Furthermore, samples from MPA treated patients often involve comedication, \textit{e.g.} corticosteroids, CNI and T cell depleting therapy, as well as lymphocyte populations of variable activation status. This could influence the IMPDH expressions and complicate the interpretation of specific MPA effects. We investigated the effects of MPA on IMPDH 1 and 2 expression in CD4+ cells and whole blood from healthy individuals receiving MMF doses of 100–1000 mg.
Results and Discussion

Regulation of gene expression in response to MPA exposure

The predose expression of IMPDH2 was generally higher than IMPDH1 in both CD4+ cells (3.6-fold) and whole blood (1.9-fold). The predose gene expressions displayed 2–3 fold variability between individuals, being highest for IMPDH1 expression in CD4+ cells. Furthermore, 12-hour intervals without MPA exposure demonstrated relatively stable expression of both IMPDH isoforms. Following intake of MMF, expression of IMPDH1 decreased rapidly in CD4+ cells, as well as in whole blood. In CD4+ cells, MMF doses of 250 and 500 mg caused median 29% (P=0.043) and 39% (P=0.043) suppression of IMPDH1 expression, respectively. The expression of IMPDH2 was less influenced by MPA. Still, a significant downregulation of IMPDH2 (10%, P=0.043) was observed in CD4+ cells following intake of 500 mg MMF. MMF doses of 1000 mg were associated with smaller, non-significant reductions of IMPDH1 and 2 expression. The initial reduction was mostly followed by increasing expression, which exceeded baseline expression within some of the intervals. This upregulation was most pronounced in whole blood where increments up to 2.4-fold and 75% above baseline were observed for IMPDH 1 and 2 expression, respectively, 24 hours after MMF doses of 1000 mg. The interindividual PK variability of MMF was substantial and the effects of MPA exposure were thus studied in terms of MPA plasma concentrations (C_{min}, C_{max} and AUC_{0−12h}). The gene expression responses appeared to be strongest correlated with MPA AUC_{0−12h}. We demonstrated a biphasic IMPDH1 expression response to MPA exposure not previously described. At MPA AUC_{0−12h} below 20 mg×h/L, increasing MPA exposure was associated with larger reductions of IMPDH1 expression in whole blood (r=−0.82, P<0.001) and CD4+ cells (r=−0.50, P<0.057). At MPA AUC_{0−12h} exposure exceeding 20 mg×h/L, the influence on IMPDH1 gene expression shifted and higher MPA exposure was correlated with smaller reductions of IMPDH1 in both CD4+ cells and whole blood (r=0.42, ns, and r=0.77, P<0.05). Inverse correlations between MPA concentration and time to minimum gene expression, further support an MPA exposure-dependent regulation of the IMPDH genes. The timescale for the changes suggests that the expression in whole blood is largely influenced by nucleated cells.
Gene expression in relation to enzyme activity and purine nucleotides

The enzyme activity of IMPDH showed a strong and immediate inhibition by MPA. In addition to this expected direct inhibition of MPA on IMPDH activity, reduced expression of \textit{IMPDH 1} and \textit{2} might contribute to the total reduction of enzyme activity. The maximum inhibition of IMPDH activity occurred at MPA AUC$_{0-12h}$ of 22 mg×h/L, approximately coinciding with the maximum reduction of \textit{IMPDH1} expression. No further reductions in enzyme activity were observed with MPA exposure exceeding this level, while the reductions of \textit{IMPDH1} expression were smaller with higher MPA concentrations. It could be hypothesized that these smaller reductions of gene expression might contribute to the plateau effect of IMPDH inhibition.

In contrast to an expected decrease, we observed elevated guanine nucleotide levels in CD4+ cells after MMF intake. The participants were exposed to single doses of MMF, and this limited duration of MPA exposure might not yield sufficient IMPDH inhibition for effective intracellular depletion of guanine nucleotides. Furthermore, activation of salvage enzymes in response to reduced \textit{de novo} guanine synthesis, or by increased substrate availability, could explain the elevations of purine nucleotides that were observed postdose in this study. Similar responses of ATP, not being a product of IMPDH, and GTP, support the hypothesis that alternative enzymatic pathways are involved. However, further studies, including multiple dosing, are necessary to characterize the mechanisms leading to purine nucleotide fluctuations.

The biphasic expression response to MPA could be explained by negative feedback regulation of \textit{IMPDH1} expression by guanine nucleotides. At MPA AUC$_{0-12h}$ below 20 mg×h/L, increasing MPA exposure was associated with elevated guanine nucleotides as well as larger reductions of \textit{IMPDH1} expression. At MPA exposure exceeding this threshold, the influence on \textit{IMPDH1} expression shifted and higher MPA exposure was correlated with smaller reductions of \textit{IMPDH1}, as well as a trend towards larger increases of expression 8–24 hours postdose. This was accompanied with decreased purine nucleotide pools in three of the individuals, reaching 47% of baseline GTP AUC$_{0-12h}$. Indeed, the guanine nucleotide response at high MPA
Results and Discussion

exposure was variable\textsuperscript{207} and not closely associated with either MPA exposure or gene expression response. This may be attributed to variable degrees of compensating mechanisms like induction of the HPRT and IMPDH enzymes. An alternative explanation might be that the biphasic response is mediated through two distinct mechanisms.

Expression of the \textit{IMPDH} genes during MPA exposure could also be regulated through other mechanisms than guanine nucleotide levels. The presence of MPA or the inhibition of IMPDH activity might directly influence the gene expression. Recently, the \textit{in vivo} generated MPA metabolite, AcMPAG, has been reported to regulate gene expression of IL-2 and nucleobindin 1, independent of guanine nucleotide levels.\textsuperscript{238}

Further studies are needed to confirm the concentration-dependent effects of MPA on \textit{IMPDH 1} and \textit{2} expressions and to identify the mechanism of this regulation. The single-dose design did not allow investigation of steady state MPA concentrations. Indeed, the MPA concentrations obtained at the high single doses were comparable to clinically relevant exposure. Future studies should rather address possible long-term alterations of guanine or deoxyguanine nucleotides and the potential relationship to clinical efficacy of MPA. Furthermore, since the majority of lymphocytes from healthy individuals are resting cells, studies in activated lymphocytes would probably be relevant for estimation of immunosuppressive effects.

4.4 Paper IV

This is the first study of MPA PK and PD among renal allograft recipients receiving belatacept compared to patients with CsA. All patients received allografts from deceased donors and pretransplant dose interval investigations were thus not feasible.

\textit{Pharmacokinetics}

Early posttransplant, belatacept patients showed higher MPA concentrations, as well as more pronounced secondary concentration peaks, than CsA patients. Despite MMF dose reductions for two belatacept patients, the MPA exposure increased significantly (\textit{P}=0.031, \textit{n}=6) from week 2 to week 13 when considering the whole population. The
Results and Discussion

elevation was especially pronounced among the CsA patients and might be related to the tapering of CsA and corticosteroid doses and improvement of renal function. These findings are in agreement with the previously reported increasing MPA levels after transplantation and the interaction between CsA and MPA (Section 1.3.2).

Pharmacodynamics

There was no tendency towards induction of predose IMPDH activity or expression in CD4+ cells with time since transplantation. However, the IMPDH response within dose intervals changed with time. Early posttransplant, there was an inverse correlation between MPA exposure and IMPDH activity. At week 13, IMPDH activity increased postdose within both treatment groups, reaching up to 7 times predose activities before returning towards predose levels. Considering the IMPDH \( \text{AUC}_{0-9h} \) activity, up to 3.6-fold higher activity was observed at week 13 compared to week 1. The MPA PK and PD profiles of a representative belatacept treated patient are given in Figure 17.

With respect to the \textit{IMPDH 1} and 2 gene expression in CD4+ cells, rapid and transient changes were observed for \textit{IMPDH1} while the expression of \textit{IMPDH2} was relatively stable. Increasing MPA exposure was associated with larger inductions of \textit{IMPDH1} expression (\( \text{AUC}_{0-9h} \): \( r=0.81, \ P=0.027, \ n=7 \)). The maximum \textit{IMPDH1} expression was 52 (13–177)% higher 13 weeks posttransplant compared to week 1 (\( P=0.031, \ n=6 \)). This might contribute to the associated elevation of IMPDH activity at week 13. The pretransplant expression of \textit{IMPDH2} was 2.1 (1.6–2.7) times higher than \textit{IMPDH1} in CD4+ cells. A relative increase of \textit{IMPDH1} versus \textit{IMPDH2} expression supports marked contributions of IMPDH1 to the measured activity within dosing intervals.

The present changes of IMPDH activity and \textit{IMPDH1} expression in CD4+ cells are consistent with previous observations in PBMCs from transplant patients as discussed in Section 1.4.4.\textsuperscript{212} The expression of \textit{IMPDH1} may be regulated by feedback mechanisms through changes in guanine nucleotide pools as suggested in Paper III. Concomitant measurement of guanine nucleotides and gene expression in a larger cohort is necessary to confirm this hypothesis.
Results and Discussion

Figure 17. PK-PD profiles of mycophenolic acid (MPA).
Representative MPA pharmacokinetic (PK) and pharmacodynamic (PD) profiles at 2 and 13 weeks posttransplant in a patient receiving mycophenolate mofetil in combination with belatacept. A: IMPDH activity (% of predose) in CD4+ cells and MPA plasma concentration versus time (hours) postdose. B: Gene expression of IMPDH1 (% of predose) in CD4+ cells versus time postdose.

We investigated a population of relatively old patients (median age; 71 years), receiving quadruple immunosuppressive regimens including induction therapy (basiliximab). The patients demonstrated relatively low predose IMPDH activity. According to the hypothesis presented in Section 1.4.4, the induction of IMPDH activity and IMPDH1 expression within dose intervals might be an MPA response that is primarily observed in resting lymphocytes with low basal IMPDH activity. However, the clinical relevance of the altered PD response to MPA remains to be elucidated. Even if the present regulation does not influence MPA efficacy, the findings should be considered when interpreting IMPDH measurements. Our data
suggest that assessment of basal IMPDH levels (pretransplant or predose after transplantation) might be a more feasible strategy for MPA monitoring than determination of relative changes after dose.

4.5 Limitations

The major limitation of the clinical studies (Papers II-IV) is the relatively low number of investigated individuals. This implies that the results should be interpreted with caution and that future prospective studies with larger cohorts are required to confirm the findings. Furthermore, the small groups in the study presented in Paper IV did not provide sufficient power to detect potential differences between the treatment groups. Moreover, heterogeneous patient populations with respect to immunosuppression and presensitization complicated the interpretation of the observations. The two weeks follow-up in the study presented in Paper II was based on a pilot study, but appeared to be relatively short for assessment of a potential IMPDH induction in erythrocytes. Moreover, potential effects of comedications like basiliximab, exogenous erythropoietin or the antiviral prophylaxis cannot be excluded. End stage renal disease, dialysis and the transplant surgery are other conditions possibly influencing gene expression. Since all patients received high-dose corticosteroids and underwent transplantation, we were not able to control for effects related to these parameters (Paper II and IV). Potential effects of the surgery could have been assessed by concomitant investigations in the donor population.
5. Conclusions of the Thesis

We developed and validated an assay for measurement of *IMPDH1* and *IMPDH2* gene expression in blood cells. The assay was further utilized for analysis in clinical samples from transplanted patients and healthy individuals.

A descriptive study in 30 renal allograft recipients suggested substantial impact of corticosteroids, and potentially also the surgery, on the expression of *IMPDH1* and *IMPDH2*. The regulation differed between isoforms and cell types. Furthermore, an induction of both isoforms in reticulocytes could be related to MPA. We also observed a significant association between pretransplant expression of *IMPDH2* in CD4+ cells and the risk of rejection.

The exposure-response study in five healthy individuals demonstrated that administration of clinically relevant MMF doses was associated with significant expression changes of *IMPDH1*, which traditionally is regarded as the constitutive isoform. Only smaller alterations were observed for *IMPDH2* expression. The regulation of *IMPDH1* seemed to depend on MPA exposure, and could be mediated through alterations in guanine nucleotide pools.

Investigations of MPA PK-PD in belatacept versus CsA patients revealed significant changes of MPA PD with time since transplantation. Although no trend of induction was observed with respect to IMPDH predose activity in CD4+ cells, significant increases of enzyme activity were observed within the dose intervals at week 13 compared to week 1. This was observed both in cyclosporine and belatacept treated patients, and despite higher MPA exposure. This could be explained by a concomitant increase of *IMPDH1* expression, possibly mediated by reduced guanine nucleotide levels. Furthermore, the MPA exposure seemed to be lower early posttransplant among the CsA patients, which could be expected on the basis of the reported CsA induced reductions in MPA exposure. No pronounced effects were observed of belatacept per se on MPA PK or PD.
Conclusions of the Thesis

The validated real-time RT-PCR assay, and the use of a reference gene index for normalization, ensured reliable quantification of *IMPDH 1* and 2 expression. Altogether, our data support the potential of PD monitoring based on IMPDH levels in lymphocytes. However, the significant impact of corticosteroids and MPA should be considered when interpreting IMPDH measurements. We observed complex regulation of *IMPDH 1* and 2 gene expression and distinct mechanisms are probably involved during immune activation and in response to MPA exposure. Indeed, the detailed mechanisms of the gene expression changes remain to be elucidated.
Future Perspectives

6. Future Perspectives

Optimization of MPA therapy might increase the therapeutic potential of the drug in transplantation as well as within other fields of immune modulation. The best way to individualize MPA therapy has still not been defined, and monitoring of PD parameters like IMPDH activity is investigated as a complimentary tool to PK monitoring. Measurement of gene expression, as an alternative to enzyme activity, allows sensitive assessment of the two isoforms separately. Indeed, establishment of a feasible PD monitoring strategy requires further characterization of the relation between the potential IMPDH parameters and clinical outcome. Standardization of assays is required to allow comparisons between studies.

The selection of optimal PD markers and sampling strategy should be based on clinical studies in relevant populations. An ongoing descriptive study at Rikshospitalet University Hospital investigates the MPA PK-PD relationship in liver transplant patients to define eligible IMPDH parameters and a feasible sampling strategy for PD monitoring in these patients. Finally, the usefulness of IMPDH based PD monitoring strategies needs to be evaluated in randomized, controlled, prospective trials.

We and others have revealed significant alterations of the IMPDH response with prolonged MPA treatment. In PBMCs and CD4+ cells, a potential feedback regulation of the enzyme was observed, which attenuated the inhibitory action of MPA. However, the findings are conflicting and clinical evidence does not suggest reduced efficiency of MPA with time. It could be speculated that the variable IMPDH response between studies might be related to the proportion of proliferating versus non-proliferating lymphocytes in the analyzed samples. This hypothesis should be addressed in further studies. Moreover, the variable IMPDH responses within dose intervals suggest that the basal or predose activity of IMPDH might be a better parameter for PD monitoring than the relative IMPDH inhibition. Indeed, this needs further elucidation.
Other potential tools for individualization of MPA therapy might be assessment of non-specific biomarkers of immune status and pharmacogenetic measurements.

The IMPDH isoenzymes play a critical role in cell proliferation and differentiation and are considered important drug targets for anticancer, antiviral and immunosuppressive treatment. Characterization of the regulation and involvement of the specific isoenzymes in immunological and malignant proliferation might contribute to the processes of drug development and establishment of strategies for monitoring effect and disease activity. The gene expression assay we have developed is a useful tool for such applications.
7. References


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References


Mycophenolate Pharmacokinetics and Pharmacodynamics in Belatacept Treated Renal Allograft Recipients

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Running title: Mycophenolate PK/PD in belatacept treated patients

Abbreviations: AUC, area under the variable versus time curve; CNI, calcineurin inhibitor; CsA, cyclosporine; HPLC, high-performance liquid chromatography; IMPDH, inosine monophosphate dehydrogenase; MMF, mycophenolate mofetil; MPA, mycophenolic acid; PD, pharmacodynamic; PK, pharmacokinetic; XMP, xanthosine monophosphate

Key words: belatacept, inosine monophosphate dehydrogenase (IMPDH), mycophenolic acid (MPA), pharmacodynamic (PD), pharmacokinetic (PK)

Financial support: none.
Abstract

The objective of this study was to investigate the pharmacokinetics (PK) and pharmacodynamics (PD) of mycophenolate (MPA) in combination with belatacept (2nd generation CTLA4-Ig) or cyclosporine (CsA). Seven renal allograft recipients were randomized to either belatacept (n=4) or cyclosporine (n=3) based immunosuppression. Samples for MPA PK and PD evaluations were collected predose and at 1, 2 and 13 weeks posttransplant. Plasma concentrations of MPA were determined by HPLC-UV. Activity of inosine monophosphate dehydrogenase (IMPDH) and the expressions of two IMPDH isoforms were measured in CD4+ cells by HPLC-UV and real-time reverse-transcription PCR, respectively. Subsets of T cells were characterized by flow cytometry. The MPA exposure tended to be higher among belatacept patients than in CsA patients at week 1 (P=0.057). Further, MPA concentrations (AUC0−9h and C0) increased with time in both groups and were higher at week 13 than at week 2 (P=0.031, n=6). In contrast to the postdose reductions of IMPDH activity observed early posttransplant, IMPDH activity within both treatment groups was elevated throughout the dosing interval at week 13. Transient postdose increments were also observed for IMPDH1 expression, starting at week 1. Higher MPA exposure was associated with larger elevations of IMPDH1 (r=0.81, P=0.023, n=7 for MPA and IMPDH1 AUC0−9h at week 1). The maximum IMPDH1 expression was 52 (13–177)% higher at week 13 compared to week 1 (P=0.031, n=6). One patient showed lower MPA exposure with time and did neither display elevations of IMPDH activity nor IMPDH1 expression. No difference was observed in T cell subsets between treatment groups. The significant influence of MPA on IMPDH1 expression, possibly mediated through reduced guanine nucleotide levels, could explain the elevations of IMPDH activity within dosing intervals at week 13. The present regulation of IMPDH in CD4+ cells should be considered when interpreting measurements of IMPDH inhibition.
**Introduction**

The increasing understanding of T cell activation mechanisms reveals new promising targets for immunomodulation. Belatacept is a second generation cytotoxic T-lymphocyte antigen-4 (CTLA4)-Ig fusion protein that via high affinity binding to CD80 and CD86 provides T cell anergy and apoptosis.¹

The current immunosuppressive regimens in transplantation are mainly based on calcineurin inhibitors (CNI), which have lead to dramatic improvements in short-term outcome after transplantation. However, long-term use is associated with nephrotoxicity and cardiovascular morbidity that may lead to increased risk of late allograft loss and death. Belatacept is investigated as an alternative to CNI following transplantation. A phase 2 trial in renal allograft recipients (n=218) reports similar efficacy, higher glomerular filtration rates and less frequent chronic allograft nephropathy with belatacept compared to cyclosporine (CsA).²

Mycophenolic acid (MPA) is widely used in combination with CNI, corticosteroids, and frequently also induction therapy to prevent rejections posttransplant. Currently, two MPA formulations are available, the prodrug ester mycophenolate mofetil (MMF) and the enteric-coated mycophenolate sodium.

Inosine monophosphate dehydrogenase (IMPDH) catalyzes the rate-limiting step of de novo guanine nucleotide synthesis. The enzyme activity is constituted by two isoenzymes, encoded by *IMPDH1* and *IMPDH2*, which have similar kinetic properties and share 84 % identity at the amino acid level.³ However, the regulation and expression of the isoenzymes differ, and gene knockout models indicate distinct functions of IMPDH 1 and 2.⁴⁵ Lymphocyte activation is associated with elevation of both isoenzymes, while neoplastic cells display
marked up-regulation of \textit{IMPDH2}.\textsuperscript{6,7} MPA exerts its immunosuppressive action by inhibiting IMPDH, and thereby the proliferation of activated lymphocytes.\textsuperscript{8}

Despite substantial pharmacokinetic (PK) and pharmacodynamic (PD) variability of MPA, most protocols prescribe fixed doses ranging from 0.75 to 1.5 g MMF twice a day. Several strategies have been suggested to individualize MPA therapy and improve the clinical outcome after transplantation. The area under the MPA concentration versus time curve (AUC) from 0 to 12 hours correlates with clinical outcome after transplantation\textsuperscript{9} but is impractical for routine monitoring, and various limited sampling schemes are evaluated.\textsuperscript{10,11}

Measurement of IMPDH activity may provide a more direct estimation of drug efficacy, and is investigated as a PD approach for individualization of MPA therapy.\textsuperscript{12,13} Long-term MPA treatment has been associated with induced IMPDH activity and expression.\textsuperscript{14-18} However, the results are conflicting and depend on the investigated cell populations and methodology. Furthermore, concomitant medications (e.g. high doses of corticosteroids) and the transplantation surgery itself may influence the activity and expression of IMPDH.\textsuperscript{17} The clinical implications of these findings remain to be elucidated.

To date, only limited data are available concerning PK and PD of MPA in combination with belatacept. The present study is a supplemental study appended to the BENEFIT-EXT phase 3 trial in transplant patients receiving grafts from extended criteria donors (BMS protocol IM103027).\textsuperscript{19} This is an observational, pilot study in renal transplant patients receiving MMF in combination with either belatacept or CsA. The objective was to investigate PD and PK characteristics of MPA in the two treatment groups. Measurements of MPA concentrations
were used for PK evaluations, while PD investigations involved determination of IMPDH activity, analyses of *IMPDH 1* and 2 expression and characterization of T cell subpopulations.
Materials and methods

Study objects

From October 2006 to February 2007, seven adult patients receiving grafts from extended criteria donors were included in the BENEFIT-EXT study at Rikshospitalet University Hospital. Extended criteria donors were defined as donor age above 60 years, donor age above 50 years and other donor co-morbidities, cold ischemia time above 24 hours or donation after cardiac death. The inclusion and exclusion criteria are described in detail in the BENEFIT-EXT study protocol. Patients were randomized into three arms with CsA in one arm and belatacept (less intensive or more intensive, respectively) in the two others. Belatacept was administered as a 30 min intravenous (iv) infusion at intervals as described. Additional immunosuppression consisted of MMF (CellCept®, Roche, Basel, Switzerland) 1 g twice daily, corticosteroids and induction therapy with basiliximab (Simulect®, Novartis, Basel, Switzerland) 20 mg on day 0 (transplantation day) and day 4. Corticosteroids were given as iv methylprednisolone on day 0 and 1, followed by per oral prednisolone starting at 80 mg/day, tapered by 10 mg/day to 20 mg/day, maintained at 15 mg/day the second month and at 10 mg/day the third month. CsA was dosed according to protocol to reach target whole blood through concentrations ($C_0$) of 150–300 µg/L the first month posttransplant, and then lowered to 100–250 µg/L.

The protocol of both the BENEFIT-EXT trial and the present sub-study were approved by the regional committee for medical research ethics. The BENEFIT-EXT protocol was also approved by the Norwegian Medicines Agency. Written informed consent was obtained from all participants.
**Samples**

Samples were collected at one occasion before transplantation and for 9 hour-profiles at approximately 1, 2 and 13 weeks posttransplant (referred to as week 1, 2 and 13). Samples for 9 hour-profiles were drawn after an overnight fast before administration of the morning dose of immunosuppression, and at 0.5, 1, 1.5, 2, 3, 4, 5, 6 and 9 hours post-dose. *IMPDH* expressions were not determined at 0.5 and 1.5 h. Cell subsets were only characterized predose and 2 h post-dose. At each time point 10 ml whole blood was collected in EDTA tubes. Samples were immediately processed for CD4+ cell isolation, separation of plasma and staining of cells for flow cytometric characterization.

Enzyme activity and gene expression measurements were performed in CD4+ cells, which are relevant considering their role in allograft rejection as well as being a target for the action of MPA. The cells were isolated from whole blood within an hour after sampling by using paramagnetic beads with antibodies against CD4 (Dynabeads® CD4, Invitrogen, Carlsbad, CA) as described in detail elsewhere.\(^{20,21}\)

To evaluate the variability of IMPDH activity and gene expression without influence of medication or exposure to alloantigens, CD4+ cells from healthy individuals (n=5) were investigated. Samples were drawn every 2 hours over 6 hour intervals starting at 8 AM as described in detail elsewhere.\(^{13,22}\)

**Concentrations of immunosuppressive drugs**

Total plasma concentrations of MPA were measured by high-performance liquid chromatography assay with UV-detection (HPLC-UV).\(^{23}\) Routine measurement of whole
blood CsA C₀ was performed by the CEDIA® immunoassay (Microgenics corp., Fremont, CA) on a Modular analytics instrument (Roche Diagnostics, Mannheim, Germany).

**Enzyme activity**

For the quantification of IMPDH activity in CD4+ cells, intracellular MPA concentrations were restored by incubating the isolated cells in filtrated plasma originating from the same sample. The IMPDH activity was determined in cell lysates using an HPLC-UV assay for determination of xanthine derived from xanthosine monophosphate (XMP).²⁰ Activities were expressed as the XMP production rate (pmol XMP per 1.0 × 10⁶ CD4+ cells per min). For each dosing interval, predose (A₀), maximum (A_max) and minimum (A_min) enzyme activities were determined. Enzyme activity AUCs were calculated from 0–9 hours and 0–6 hours.

**Gene expression**

The gene expressions of IMPDH 1 and 2 in CD4+ cells were quantified by a validated reverse transcription-PCR method on a LightCycler® 480 instrument (Roche Applied Science) as previously described.²¹ Briefly, total RNA was extracted and reverse transcribed using random primers. Sequences of IMPDH1 and IMPDH2, and the reference genes aminolevulinate delta-synthase1, β2-microglobulin and ribosomal protein L13A, were amplified in separate reactions including hybridization probes for specific real-time product detection. Crossing points were defined by the second derivative maximum method and target gene expressions were calculated relative to the geometric mean expression of the reference genes. As for IMPDH activity, predose (E₀), maximum (E_max) and minimum (E_min) gene expression and AUCs were calculated for each profile.
Quantification of T-cell subsets

The numbers of total T cells (CD3+), as well as subpopulations of helper (CD4+) and cytotoxic (CD8+) T cells were determined by flow cytometry. These subsets were further characterized based on the expression of CD45RA and CD45RO isoforms indicating naïve and antigen experienced (activated/memory) lymphocytes, respectively.

Absolute quantification of T-cell subsets was performed using TruCount tubes according to the manufacturer’s instructions. Briefly, 50 µL EDTA blood was added to tubes containing a given number of beads and cells were stained with titrated amounts of anti-CD3-PerCP, anti-CD45 RO-PE, anti-CD45 RA-APC and anti-CD4-FITC or anti-CD8-FITC monoclonal antibodies (mAb). Isotype-matched control anti-mouse mAb and non-labeled cells were included for each sample. Erythrocytes were lysed by adding 450 µL FACS Lysing Solution. The tubes and all reagents were supplied by BD (Becton Dickinson Biosciences, Oxford, UK). Flow cytometric analyses were performed within 24 h after labeling on a FACSCalibur (BD) flow cytometer using the CellQuest Software (BD) for data acquisition. The bead population and CD3+ cell versus side scatter population were manually gated.

Data analysis and statistics

Results of the RT-PCR assays were analyzed using the LightCycler 480 Software v.1.5 (Roche Applied Science). All gene expression measurements were performed in triplicate. Absolute cell counts were calculated by the CellQuest Software based on the gated bead population.
Post-dose data of gene expression and enzyme activity were normalized to individual predose levels. AUCs were calculated by the linear trapezoid method. All results are presented as median (range) unless otherwise specified.

Statistical tests were performed using SPSS statistical software version 16.0 (SPSS Inc., Chicago, IL). The Mann-Whitney test was used for comparisons of unpaired data, while the Wilcoxon signed rank test was used for paired data. Pearson's $r$ was used for correlation analyses. Statistical significance was considered at $P<0.05$ (two-tailed).
Results

Patient population

The planned enrolment for the BENEFIT-EXT trial at Rikshospitalet University Hospital was 12 patients. However, only 7 patients receiving allografts from extended criteria donors were recruited at our center within the inclusion period. Out of these, 3 patients were randomized to receive CsA, while 4 patients received belatacept regimens.

Demographic and clinical data were collected from medical records and baseline characteristics are summarized in Table 1. There were no significant demographic differences between the treatment groups. One of the belatacept patients withdrew from the study after the 6 h post-dose sampling at week 2. Data from this profile were omitted from the AUC calculations.

All patients received prophylactic antiviral therapy consisting of valganciclovir or valaciclovir. No cytomegalovirus breakthrough disease was identified during follow-up. Biopsies were performed in cases of suspected rejection (Banff ‘97 grading system). No cases of biopsy verified acute rejection, graft loss or death were observed within the 13 weeks follow-up. Plasma concentrations of albumin, total bilirubin, and ALAT were stable throughout the study period.

MPA pharmacokinetics

Two patients, both in the belatacept arm, had their MMF dosing reduced to 1.5 g/day between weeks 2 and 13, both due to drops in leukocyte count. The other patients remained on MMF
doses of 1 g twice a day throughout the follow-up. Pharmacokinetic data of MPA are summarized in Table 2 and concentration profiles are depicted in Figure 1. The interindividual variability in MPA concentration was substantial and highest early posttransplant. Within the whole group, up to 4- and 7-fold differences were observed for MPA $C_0$ (week 2) and $AUC_{0-9h}$ (week 1), respectively. The first week posttransplant, MPA $C_0$ seemed to be higher among belatacept patients ($P=0.057$, $n=4$ and $n=3$) and 3 of 4 belatacept patients demonstrated higher MPA $AUC_{0-9h}$ than the CsA patients.

The maximum plasma concentrations ($C_{\text{max}}$) of MPA appeared 1 (0.5–2) hour postdose. Following $C_{\text{max}}$, secondary MPA concentration peaks were observed 5 (2–9) hours postdose and were more pronounced for belatacept patients than for CsA patients. Limited MPA concentration profiles were calculated from 4 to 9 hours to estimate potential impact of enterohepatic circulation. The MPA $AUC_{4-9h}$ was numerically higher among belatacept patients than for CsA patients at week 1, being 15.2 (10.4–27.1) mg x h/L and 7.8 (6.2–13.3) mg x h/L, respectively ($P=0.114$, $n=4$ and $n=3$).

Doses of CsA were tapered according to CsA $C_0$ measurements and were median 550 (450–825) mg, 550 (400–575) mg and 300 (300–350) mg at week 1, 2 and 13, respectively. The corresponding CsA $C_0$ were median 190 (160–380) µg/L, 265 (180–295) µg/L and 175 (140–180) µg/L. The reduction of CsA exposure was accompanied by increasing MPA concentrations. The association between MPA $C_0$ and CsA $C_0$, as well as CsA dose, displayed correlation coefficients ($r$) of $-0.74$ ($P=0.023$, $n=9$; pooled CsA data) and $-0.79$ ($P=0.012$, $n=9$), respectively.
Considering the entire study population, the lowest MPA exposure was observed at week 2 and then increased with time. At week 13, MPA $C_0$ was 60 (26–200)% higher ($P=0.031$, n=6), while MPA AUC$_{0-9h}$ was 43 (11–67)% higher ($P=0.031$, n=6) compared to week 2. The elevation seemed to be most pronounced in CsA patients, although no significant difference was detected between groups (Table 2).

At week 1, MPA exposure was inversely correlated to bodyweight, with correlation coefficients of $-0.90$ ($P=0.005$, n=7) and $-0.80$ ($P=0.031$, n=7) for MPA $C_0$ and AUC$_{0-9h}$, respectively. However, no significant relations were detected at later observations. Adjusted for bodyweight normalized doses, patients with belatacept displayed numerically higher MPA $C_0$ than CsA patients, being 0.22 (0.18–0.23) mg/L per mg/kg and 0.13 (0.07–0.17) mg/L per mg/kg at week 1 ($P=0.057$, n=4 and n=3). The MPA exposure did not seem to be associated with plasma albumin, ALAT or bilirubin.

**Enzyme activity**

Summarized data of IMPDH activity are presented in Figure 1 and Table 2. Pretransplant activity was variable and tended to be higher among CsA patients compared to belatacept patients. Following transplantation, predose activities ($A_0$) seemed to be influenced by the present MPA $C_0$, and no consistent trends were observed for $A_0$ versus time since transplantation (Table 2).

The postdose activities of IMPDH were strongly influenced by MPA exposure. At week 1, the activity profiles for 6 of the patients were inversely related to MPA concentrations with maximum 57 (42–75)% enzyme inhibition around MPA $C_{max}$ (Figure 1). The AUC$_{0-9h}$ activity displayed inverse correlations to MPA $C_0$ ($r=-0.91$, $P=0.012$, n=6) and MPA $C_{max}$.
(r=−0.86, P=0.028, n=6), implying greater inhibition of IMPDH with higher MPA exposure. However, this relation changed with time posttransplant. At week 13, IMPDH activity increased postdose within both treatment groups, reaching up to 7-times A₀ before returning towards predose activities (Figure 1). Considering AUC₀−₉h activity, 4 of 6 patients demonstrated substantial increases reaching 3.6 times the activity of week 1 (Figure 3). Compared to week 2, the AUC₀−₉h activity was 81 (25–322)% higher at week 13 (P=0.063, n=5). Higher MPA Cₘₐₓ was associated with increasing IMPDH activity, expressed as AUC₀−₉h (r=0.80, P=0.058, n=6) and Aₘₐₓ (r=0.88, P=0.051, n=6). Compared to healthy controls (n=5), the CsA treated patients (n=3) showed higher IMPDH AUC₀−₆h activity at week 13 (P=0.036). Within the belatacept group, 2 of 3 patients displayed higher activity than the controls.

**Gene expression**

The pretransplant expression of IMPDH2 was 2.1 (1.6–2.7) times higher than IMPDH1 in CD4+ cells. Predose expressions (E₀) of IMPDH 1 and 2 were highest and most variable the first week posttransplant, being 104 (20–150) % and 18.8 (7.2–75) % above the levels at week 13, respectively (P=0.031, n=6 for both). Predose expressions were comparable at week 2 and 13 (Table 3).

The 9 h profiles showed rapid changes of IMPDH1 expression postdose, while IMPDH2 expression was relatively stable (Figure 2). At week 1, IMPDH1 expression was transiently upregulated for belatacept patients, while CsA patients displayed downregulation. With longer time on immunosuppressive therapy, including higher MPA exposure, increasing transient inductions of IMPDH1 expression were observed postdose for both treatment groups (Table 3). At week 13, the maximum expression (Eₘₐₓ, % of E₀) of IMPDH1 was 52
(13–177)% higher than at week 1 (n=6, P=0.031). A similar trend was observed for IMPDH1 AUC\(_{0-9h}\) expression (n=6, P=0.094). Compared to healthy controls (n=5), the patients (n=6) demonstrated higher IMPDH1 \(E_{\text{max}}\) at week 13 (P=0.004), being 101 (100–116)% and 167 (118–193)%, respectively. Considering IMPDH1 AUC\(_{0-6h}\) expression, CsA patients (n=3) displayed higher levels at week 13 than controls (P=0.036). Among belatacept patients (n=3), IMPDH1 AUC\(_{0-6h}\) expression was elevated at week 1 (P=0.032) and tended to be increased at week 13 (P=0.071), compared to healthy controls. One of the patients with MMF dose reduction experienced lower MPA exposure with time, and did neither display elevations of IMPDH activity nor IMPDH1 expression (Figure 3). The first week posttransplant, IMPDH1 AUC\(_{0-9h}\) expression correlated with MPA \(C_0\) (r=0.76, P=0.047, n=7) and MPA AUC\(_{0-9h}\) (r=0.81, P=0.027, n=7). An association was also observed between minimum IMPDH1 expression (\(E_{\min}\)) and MPA AUC\(_{0-9h}\) (r=0.82, P=0.023, n=7). This implies that higher MPA exposure is associated with larger increases of IMPDH1 expression post-dose.

The IMPDH1 isoform demonstrated stronger correlations to IMPDH activity than IMPDH2. At week 1, there was an inverse correlation of −0.88 (P=0.02, n=6) between IMPDH1 \(E_{\text{max}}\) and IMPDH \(A_{\text{max}}\) indicating that lower IMPDH activity was accompanied by larger elevations of IMPDH1 expression. This relation changed with time, and 13 weeks posttransplant IMPDH1 AUC\(_{0-9h}\) expression displayed positive correlations with IMPDH AUC\(_{0-9h}\) activity (r=0.94, P=0.005, n=6) and \(A_{\text{max}}\) (r=0.90, P=0.038, n=5). Although IMPDH2 was the dominant isoform predose, the ratio of IMPDH2 to IMPDH1 expression declined after dosing toward ratios of about 1 for some patients.

No significant associations were observed between activity or gene expressions of IMPDH and age, time since transplantation, dialysis, infections or HLA-DR mismatches.
**T-cell subsets**

Characterization of T-cell subsets was only performed in 6 of the 7 patients, for technical reasons.

Before transplantation, patients demonstrated a wide range of T cell counts, with up to 2.2- and 2.8-fold variation for both CD4+ and CD8+ cells. Following transplantation, the number of both subpopulations tended to decrease among belatacept patients while the T cell profiles for CsA patients were more variable. At week 2, two of three CsA patients displayed up to 2-fold increases of CD4+ and CD8+ T cells, while reductions of 16.5 (7.7–49.5)% and 31.7 (32.0–49.6)% were observed for belatacept patients.

The proportions of naïve (CD45RA) and memory (CD45RO) T cells were comparable in both treatment groups, displaying CD45RA to CD45RO ratios of 0.61 (0.37–1.0) and 1.7 (1.1–3.0) for CD4+ and CD8+ cells (n=6), respectively, before transplantation. The percentage of CD4+ cells with memory phenotype tended to decline posttransplant within both groups. At week 13, the proportion of memory CD4+ cells was 12.3 (3.5–22)% (P=0.063, n=6) lower than pretransplant.

The largest alteration in T-cell subsets from pre- to post-dose, was observed for CD4+ cells at week 13 with reductions of 45.8 (24.6–52.8)% (n=6, P=0.063). However, the proportions of naïve and memory cells were comparable before and after dose.
Discussion

This is the first study of MPA PK and PD among renal allograft recipients receiving belatacept compared to patients with CsA. Data from healthy individuals were included to account for possible diurnal or random variability of IMPDH.

Although standard MMF doses were applied, there was a considerable variability of MPA exposure among individuals. Early posttransplant, belatacept patients showed higher MPA concentrations, as well as more pronounced secondary concentration peaks, than CsA patients. Other comedication and parameters of renal and hepatic function were similar between the groups, and the inverse correlation between CsA and MPA concentrations suggest an effect of CsA on MPA exposure. Despite MMF dose reductions for two belatacept patients, the MPA exposure increased significantly from week 2 to week 13 when considering the whole population. The elevation was especially pronounced among the CsA patients and might be related to the tapering of CsA and corticosteroid doses and improvement of renal function.

The PK of MPA is reported to be influenced by renal function, albumin levels and concomitant medications. Genetic polymorphisms of transporters, e.g. multidrug resistance-associated protein 2 (MRP2), and UDP-glucuronosyltransferases may also contribute to variable MPA exposure. Several studies have reported lower MPA concentrations when used in combination with CsA than used with tacrolimus, sirolimus or alone. This is probably due to CsA mediated inhibition of MRP2, which is involved in enterohepatic circulation of MPA. Furthermore, MPA exposure is reported to increase with time posttransplant. The mechanisms are multifactorial and may include changes in comedication, protein binding, renal function, liver disease and red blood cell counts.
In contrast to the inverse relation between MPA concentrations and IMPDH activity in CD4+ cells early posttransplant, prolonged MPA administration was associated with transient elevations of activity within dose intervals. This shifting IMPDH response is supported by the opposite correlations at week 1 and 13 between MPA exposure and IMPDH activity, and may provide an explanation for why higher concentrations of MPA do not result in markedly higher inhibition.\(^{13}\)

The regulation of the two IMPDH isoenzymes was further investigated by gene expression analysis. Following dosing, the expression of \textit{IMPDH1} displayed rapid and transient changes. Increasing MPA exposure was associated with larger inductions of \textit{IMPDH1}. This might contribute to the associated elevation of IMPDH activity at week 13. The relative increase of \textit{IMPDH1} versus \textit{IMPDH2} expression supports marked contributions of IMPDH1 to the measured activity within dosing intervals.

The present changes of IMPDH activity and \textit{IMPDH1} expression in CD4+ cells are consistent with previous observations in mononuclear cells from transplant patients.\(^{18}\) In addition, a study in healthy volunteers receiving different doses of MMF reported that regulation of \textit{IMPDH1} expression was associated with MPA exposure.\(^{22}\) The \textit{IMPDH1} gene may be regulated through changes in guanine nucleotides, or potentially by direct effects of MPA. Previous reports suggest negative feedback regulation of IMPDH by guanine nucleotides in cultured human cells and in yeast.\(^{825,7}\) In CD4+ cells from healthy individuals, low MPA exposure seemed to be associated with elevations of guanine nucleotides and subsequent reductions of \textit{IMPDH1} expression.\(^{13,22}\) In contrast, higher and repeated MPA exposure may lead to depletion of intracellular guanine
nucleotides and subsequent upregulation of IMPDH1 expression as was observed in the present study. Concomitant measurement of guanine nucleotides and gene expression in a larger cohort is necessary to confirm this hypothesis. Furthermore, potential effects of comedications like corticosteroids, basiliximab or the antiviral prophylaxis cannot be excluded.

Prolonged MPA administration has been associated with increased predose IMPDH activity in whole blood and erythrocytes but not lymphocytes.\textsuperscript{14-17} The rapid and transient induction of IMPDH in CD4+ cells contrasts the gradual elevation in erythrocytes, which may originate from an induction in earlier differentiation stages that persists during erythrocyte maturation.

Traditionally, IMPDH1 has been regarded constitutive, while IMPDH2 was considered to be the inducible isoenzyme and primary target for immunosuppression.\textsuperscript{35} More recent findings reveal that both isoenzymes are essential for lymphocyte proliferation and potentially important for immunosuppressive effects.\textsuperscript{6} Furthermore, associations between genetic variants of IMPDH1 and a form of autosomal dominant retinitis pigmentosa have increased the interest in this isoform.\textsuperscript{36} The current study emphasizes different genetic control of the isoenzymes in CD4+ cells. Although the detailed mechanisms are unknown, IMPDH1 is reported to be subject to complex regulation involving three promoters and various transcripts.\textsuperscript{37} Because IMPDH2 is approximately 5 times more sensitive to MPA than IMPDH1,\textsuperscript{38} a relative increase of IMPDH1 could have implications for the MPA effect.

Previous studies have described reduced CD4+ cell counts after initiation of immunosuppression.\textsuperscript{39} This was also observed for the belatacept patients in the present study. In contrast, the increased CD4+ cell counts for two CsA patients at week 2 may be attributed
to immune activation. Furthermore, the tendency towards reduced proportions of CD4+ memory cells within both treatment groups at week 13 may be explained by the current immunosuppression. It has generally been accepted that memory T cells do not require CD28-CD80/CD86 costimulation for recall responses. Recent studies have suggested that T cell costimulation is required for optimal IL-2 production and proliferation of both naïve and memory CD4+ T cells.\textsuperscript{40} Despite having different mechanisms of action, both belatacept and CsA interfere with the IL-2 pathway, supporting the similar effects on T cell subsets. However, several exogenous (e.g. other immunosuppressants) and endogenous factors (e.g. circadian rhythm, stress) may also influence lymphocyte subsets and should be accounted for in further studies.

The isolation of variable numbers of CD4+ cells in each sample was compensated by relating IMPDH activity to cell counts and gene expressions to a reference gene index. However, various subsets of peripheral CD4+ T-cell may display different levels of IMPDH activity and gene expressions. Alterations in these subsets could thereby influence the measured activity and gene expression. Although CD4+ cell counts changed, the proportions of naïve and memory cells remained stable after dose, indicating that IMPDH changes are not an effect of altered CD4+ cell populations.

The potential of a PD approach for MPA individualization has been supported by correlations between IMPDH levels and posttransplant outcomes. Sanquer et al. reported an up-regulation of predose \textit{IMPDH1} expression in mononuclear cells at acute rejection episodes.\textsuperscript{18} Moreover, high pretransplant IMPDH activity in mononuclear cells and \textit{IMPDH2} expression in CD4+ cells have been associated with acute rejection episodes.\textsuperscript{12,17} Recently, polymorphisms within the \textit{IMPDH1} and \textit{IMPDH2} genes have been suggested to impact baseline IMPDH activity
and outcomes after transplantation.\textsuperscript{41,42} Indeed, further investigations of IMPDH activity and regulation of the two isoenzymes are essential to elucidate the level of IMPDH inhibition that yields adequate immunosuppression. The present study suggests that MPA has a significant influence on \textit{IMPDH1} expression within the dose interval. This is an important aspect to consider when interpreting measurements of IMPDH inhibition.

The major limitation of this study is the low number of enrolled patients. This implies that the results should be interpreted with caution and that future prospective studies with larger cohorts are required to confirm the findings. The clinical outcome, including renal function, is investigated in detail in the ongoing BENEFIT-EXT trial.\textsuperscript{19}
Conclusion

In the present study, the IMPDH activity in CD4+ cells throughout dose intervals was significantly increased by week 13 compared to early posttransplant. This was observed both in cyclosporine and belatacept treated patients, and irrespective of higher MPA exposure. A marked increase of IMPDH1 expression within dose intervals, possibly mediated by reduced guanine nucleotide levels, may explain this paradox. The differences in MPA exposure between CsA and belatacept treated patients were as anticipated with reference to the documented CsA induced reductions in MPA exposure. No pronounced effects were observed of belatacept per se on MPA PK or PD.

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References


Figure legends

Figure 1

Median inosine monophosphate dehydrogenase (IMPDH) activity (% of predose) and mycophenolic acid (MPA) concentrations among renal allograft recipients. The vertical lines represent the range of total observations. Profiles of patients in the belatacept group (n=3) at weeks 1, 2 and 13 (A, B and C) and the cyclosporine group (n=3) at weeks 1, 2 and 13 (D, E and F). (Observe scale on right y-axis of C.)

Figure 2

Median gene expressions of *IMPDH1* and *IMPDH2* (% of predose) among renal allograft recipients. The vertical lines correspond to the range of total observations. Profiles of patients in the belatacept group (n=3) at weeks 1, 2 and 13 (A, B and C) and the cyclosporine group (n=3) at weeks 1, 2 and 13 (D, E and F).

Figure 3

Individual 0–9 h area under the curve (AUC) for 6 renal transplant patients at week 13 compared to week 1. Solid lines denotes belatacept patients (n = 3) while broken lines represent CsA patients (n = 3). Data are provided for A: mycophenolic acid (MPA) AUC<sub>0−9h</sub>, B: inosine monophosphate dehydrogenase (IMPDH) activity AUC<sub>0−9h</sub> and C: *IMPDH1* expression AUC<sub>0−9h</sub>. 
### Table 1. Patient characteristics

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<th>Belatacept (n=4)</th>
<th>CsA (n=3)</th>
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<td>Week 2</td>
<td>14.5 (13 - 15)</td>
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<tr>
<td>Week 13</td>
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CMV, cytomegalovirus; D, donor; DD, deceased donor; LD, living donor; R, recipient
<table>
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<tr>
<th>Table 2. MPA exposure and IMPDH activity</th>
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<tr>
<td><strong>MPA plasma concentration</strong></td>
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<td><strong>Week</strong></td>
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<tr>
<td>C₀ (mg/L)</td>
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<tr>
<td>AUC₀-₉h (mgxh/L)</td>
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<tr>
<td>C_max (mg/L)</td>
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<tr>
<td>IMPDH activity in CD4+ cells</td>
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<tr>
<td><strong>A₀</strong> (pmol/10⁶ cells/min)</td>
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<tr>
<td>AUC₀-₉h (% of A₀)xh</td>
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<tr>
<td>A_min (% of A₀)</td>
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<td></td>
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<tr>
<td>A_max (% of A₀)</td>
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</tbody>
</table>

Data are given as median (range). The belatacept group includes 3 patients at week 13 and for the maximum, minimum and AUC calculations at week 2. A₀, predose activity; A_max.
maximum activity; $A_{\text{min}}$, minimum activity; AUC, area under the variable versus time curve;

$C_0$, predose concentration, $C_{\text{max}}$, maximum concentration; $C_{\text{min}}$, minimum concentration,

IMPDH, inosine monophosphate dehydrogenase; MPA, mycophenolic acid.
<table>
<thead>
<tr>
<th>Table 3. <em>IMPDH1</em> expression</th>
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<tbody>
<tr>
<td><strong>IMPDH1</strong></td>
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<td>Eₘin (%) of E₀</td>
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<td>Eₘax (%) of E₀</td>
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</table>

Data are given as median (range). The belatacept group includes 3 patients at week 13 and for the maximum, minimum and AUC calculations at week 2. E₀, predose expression; Eₘax, maximum expression; Eₘin, minimum expression; AUC, area under the variable versus time curve.
Figure 1

Belatacept

A week 1

B week 2

C week 13

Cyclosporine

D week 1

E week 2

F week 13

IMPDH relative activity (%)

MPA concentration (mg/L)

Hours post-dose

IMPDH activity

MPA

IMPDH relative activity (%)

MPA concentration (mg/L)
Figure 2

Belatacept

A week 1

- IMPDH1 expression
- IMPDH2 expression

B week 2

- IMPDH1 expression
- IMPDH2 expression

C week 13

- IMPDH1 expression
- IMPDH2 expression

Cyclosporine

D week 1

- IMPDH1 expression
- IMPDH2 expression

E week 2

- IMPDH1 expression
- IMPDH2 expression

F week 13

- IMPDH1 expression
- IMPDH2 expression

Relative gene expression (%)

Hours post-dose