Pharmacokinetics, pharmacodynamics and pharmacogenetics of immunosuppressants in liver transplant recipients

Ingjerd Sæves

Thesis for the degree of Philosophiae Doctor

Department of Medical Biochemistry and
Department of Pharmacology, Oslo University Hospital
Oslo, Norway

Department of Pharmaceutical Biosciences,
School of Pharmacy, University of Oslo
Oslo, Norway

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Ingjerd Sæves
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List of papers

Paper I

Sæves, Ingjerd; Vethe, Nils Tore; Bergan, Stein. Quantification of 6 Glucocorticoids in Human Plasma by Liquid Chromatography Tandem Mass Spectrometry: Method Development, Validation, and Assessment of Matrix Effect.
Ther Drug Monit 2011;33(4):402-10.

Paper II

Sæves, Ingjerd; Line, Pål-Dag; Bergan, Stein. The pharmacokinetics of prednisolone and prednisone in adult liver transplant recipients early after transplantation.
Ther Drug Monit 2012; 34(4):452-9

Paper III

Sæves, Ingjerd; Line, Pål-Dag; Bremer, Sara; Vethe, Nils Tore; Tveit, Ragnhild Gislefoss; Meltevik, Tore Jakob; Bergan, Stein. Tacrolimus and mycophenolate early after transplantation: pharmacokinetic, pharmacodynamic and pharmacogenetic characteristics.
Submitted for publication.

Errata

Paper II:

Table 1: Number of patients receiving methylprednisolone (i.v) should be *4*.

Parentheses in the units of dose/BW-adjusted AUC, C_0 and C_{max} are lacking. The units should be µg*h/L/(mg/kg) (AUC) and µg/L/(mg/kg) (C_0 and C_{max}).

Paper III:

First and second authors' names abbreviated wrongly in reference 30: *de Jonge H, de Loor H.*

Thesis:

Page 19: The enzymes 11β-HSD1 and 2 belong to the short-chain dehydrogenase/reductase *family*.

Page 39: Typing error in the unit for IMPDH activity. It should be pmol/10^6 *cells*/min.

Page 41: According to these guidelines the assay intra-day and inter-day precision should have coefficients of variation lower than 15% (20% at the lower limit of quantification, LLOQ), and the accuracy between 85% and 115% (*80-120%* at LLOQ).

Abbreviations

11β-HSD	11β–hydroxysteroid dehydrogenase
A_0	pre-dose activity
A_{min}	minimum activity
AcMPAG	MPA acyl-glucuronide
ACTH	adrenocorticotropic hormone
ALAT	alanine aminotransferase
AME	apparent mineralocorticoid excess
AP-1	activating protein 1
APC	antigen presenting cell
ASAT	aspartate aminotransferase
ATP	adenosine triphosphate
ATG	anti–thymocyte globulin
AUC	area under the time concentration curve
BPAR	biopsy proven acute rejection
BW	body weight
C_0	trough concentration
C_2	concentration after two hours
CBG	corticosteroid binding globulin
CD	cluster of differentiation
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
Cl	clearance
Cl/F	apparent clearance
C_{max}	maximum concentration
CMV	cytomegalovirus
CNI	calcineurin inhibitors
CRH	corticotropin releasing hormone
CsA	cyclosporine A
CV	coefficient of variation
CYP	cytochrome P450
DNA	deoxyribonucleic acid

EC–MPS	entero–coated mycophenolate sodium
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmatic reticulum
F	bioavailability
FKBP	FK506 binding protein
GC	glucocorticoid
GR	glucocorticoid receptor
GRE	glucocorticoid response element
H6PDH	hexose 6-phosphate dehydrogenase
HCC	hepatocellular carcinoma
HLA	human leukocyte antigen
HPA	hypothalamic-pituitary-adrenal
HPLC	high performance liquid chromatography
IFN-γ	interferon gamma
IgG	immunoglobulin G
IκB	inhibitor of κB
IKK	IκB kinase
IL	interleukin
IL-2	interleukin 2
IL2-R	interleukin 2 receptor
IRF3	interferon regulatory factor 3
I.V	intravenous
IMP	inosine 5'–monophosphate
IMPDH	inosine 5'–monophosphate dehydrogenase
JAK3	janus kinase 3
K_a	absorption rate constant
K_e	elimination rate constant
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
LLE	liquid liquid extraction
LLOQ	lower limit of quantification
LTx	liver transplantation
MAP	mitogen activated protein
MDR	multidrug resistance
MHC	major histocompatibility complex

MMF	mycophenolate mofetil
MPA	mycophenolic acid
MPAG	MPA glucuronide
MPAGl	MPA glucoside
MR	mineralocorticoid receptor
mRNA	messenger RNA
MRP2	multi-drug resistance protein 2
mTOR	mechanistic target of rapamycin
m/z	mass over charge
NAD	nicotinamide adenine dinucleotide
NADP(H)	nicotinamide adenine dinucleotide phosphate
nGRE	negative glucocorticoid responsive element
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor-κB
PBC	primary biliary cirrhosis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	pharmacodynamics
P-gp	p-glycoprotein
PI-3K	phosphoinositide 3 kinase
PK	pharmacokinetics
PKC	protein kinase C
P.O.	peroral
POMC	pre-opiomelanocortin
PSC	primary sclerosing cholangitis
PXR	pregnane X receptor
RNA	ribonucleic acid
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
SNV	single nucleotide variant
SPE	solid phase extraction
$T_{1/2}$	half-life
TCR	T-cell receptor
TDM	therapeutic drug monitoring

TGF-β	transforming growth factor beta
TGF-βR	transforming growth factor beta receptor
T_{max}	time to reach maximum concentration
T_{min}	time at minimum activity
TNF-α	tumour necrosis factor alpha
Tx	transplantation
UGT	uridine 5'-diphospho glucuronosyltransferase
V_D	volume of distribution
XMP	xanthosine monophosphate

1 Background

1.1 Liver transplantation

Dr. Thomas E. Starzl was the first surgeon to perform a successful deceased donor liver transplant procedure in 1963.[1] From being considered an experimental procedure in 1983 by the National Institute of Health, the number of liver transplantations increased steadily during the next decades.[2] Liver transplantation, the replacement of the native, diseased liver by a normal graft, is now accepted as a successful therapeutic option for patients with end-stage liver disease. Liver transplantation is indicated for acute liver failure, chronic liver failure, cirrhosis, cholestatic and non-cholestatic liver disorders, and metabolic disorders causing cirrhosis among others. It is also indicated for hepatocellular carcinoma and other selected hepatic malignancies.

Oslo University Hospital is the only solid organ transplantation centre in Norway. In 2011 86 liver transplantations (LTx) and three combined liver and kidney transplantations were performed at this centre. In Scandinavia, more than 300 annual liver transplantations are performed, and more than 5500 in Europe.[3] In the 1970s, the overall 1-year survival was approximately 30%.[4] Advances in surgical techniques, organ preservation, anaesthesia and immunosuppressive therapy have improved the long-term survival. The patient survival in the Nordic countries was 85% (1 year) and 66% (10 years) and the graft survival 83% (1 year) and 61% (10 years) in the years 2000 to 2009.[3]

1.2 Immunology in allograft transplantation

Allogeneic transplantation is transplanting an organ between genetic different individuals within the same species. The immune response protects the body against foreign attacks (i.e. bacteria, virus and cancer). In cases of allograft transplantation, the immune system recognizes the graft-antigens as foreign, and triggers a massive immune response with attempt to destroy the graft. Without adequate immunosuppressive therapy this response will result in either a hyper acute, acute or a chronic rejection.

Foreign antigens are recognized by the naïve T-lymphocyte through HLA (human leukocyte antigen) molecules present on an antigen presenting cell (APC). The HLA is the

major histocompatibility complex (MHC) in human, and its function is to present foreign peptide antigens (derived from infectious agents or an allograft) at the surface of the APC. The antigen presentation by the APC to the T-cell triggers activation and proliferation of the T-cell through three specific signals (Figure 1). The first interaction between the T-cell and APC is binding of the HLA-antigen complex to the T-cell receptor (TCR:CD3 complex) (signal 1). Co-stimulating molecules on the APC (CD80 and CD86) binds to CD28 on the T-cell and induce a stimulatory signal to the T-cell (signal 2). These signals activate three signal transduction pathways: the calcium-calcineurin pathway, the mitogen activated protein (MAP) kinase pathway and the nuclear factor-κB (NF-κB) pathway, which activates the transcription factors nuclear factor of activated T cells (NFAT), activating protein 1 (AP-1) and NF-κB, respectively. This in turn results in mRNA synthesis and expression of interleukin-2 (IL-2), CD154 and CD25. CD154 stimulates the APC, while IL-2 binds to the IL-2 receptor (CD25) on the T-cell (signal 3). This signal, in collaboration with cytokines, activates the mechanistic target of rapamycin (mTOR) pathway, leading to an activation of the cell cycle and proliferation of the T-cell.

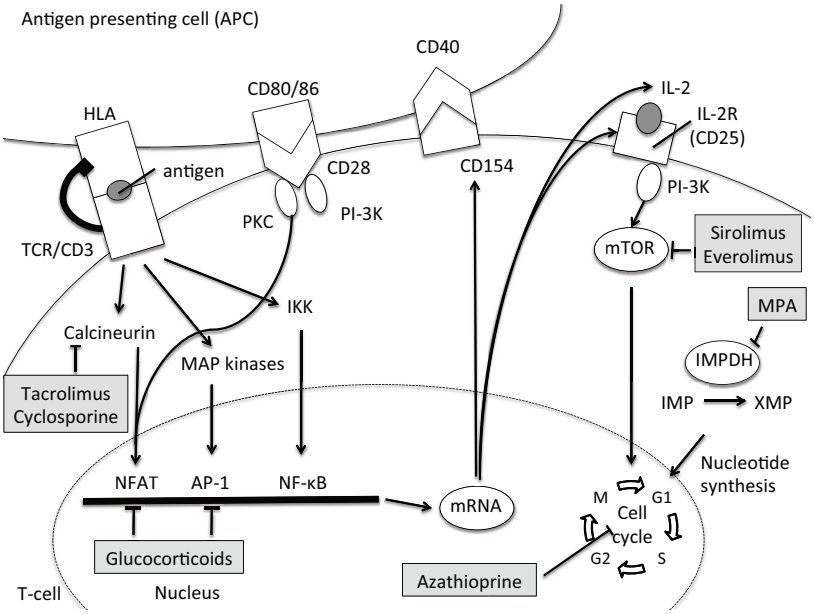

Figure 1. APC antigen presentation and T-cell activation (schematic and simplified). Site of action of immunosuppressive drugs are indicated. For abbreviations see page 5.

1.3 Immunosuppressive therapy in liver transplantation

Immunosuppression is the prevention or interference with the development of the normal immunologic response. After liver– and other solid organ transplantation the recipient needs life-long immunosuppressive therapy to avoid an immunological mediated rejection of the transplanted graft. The balance between adequate immunosuppression (preventing rejection episodes) and avoiding adverse effects is delicate. Under-immunosuppression increases the risk of graft rejection episodes, while over-immunosuppression increases the risk of opportunistic infections, malignancies and drug-specific adverse effects. The immunosuppressive regimens combine drugs with different modes of action. In general, the immunosuppressive drugs used after solid organ transplantation can be classified as follows:

- Anti-proliferative agents (azathioprine and mycophenolic acid)
- Glucocorticoids (prednisolone and methylprednisolone)
- Calcineurin inhibitors (cyclosporine and tacrolimus)
- mTOR inhibitors (sirolimus and everolimus)
- Monoclonal antibodies (basiliximab, daclizumab, alemtuzumab and belatacept)
- Polyclonal antibodies (anti-thymocyte globulin)

In Norway the current immunosuppressive protocol after liver transplantation is a triple regimen consisting of corticosteroids, mycophenolic acid and low dose calcineurin inhibitor (tacrolimus), while a quadruple regimen is used in renal transplantation (IL-2 receptor antagonist is added). The rationale behind a multiple regimen is that synergistic effects of the drugs are achieved, and the doses of the individual drugs might be reduced, resulting in a lower risk of dose-dependent drug specific adverse effects. The risk of rejection is highest in the immediate phase after transplantation, therefore a more intensive therapy is required during the first days post-transplant with further tapering of the drugs according to protocol.[5] The immunosuppressive therapy is a life-long treatment, and the lowest effective dose of each drug should be used in order to maintain an active immune response against infections and to keep side effects at a minimum.

The glucocorticoid methylprednisolone is administered intravenously pre–, peri– and post-operative, and the first day after surgery. Glucocorticoid treatment is switched to per oral prednisolone from day two, and then tapered (see Table 1). In the case of an acute

rejection episode methylprednisolone (i.v.) is administered. Mycophenolic acid (mycophenolate mofetil, MMF) is administered per orally in doses of 1 g twice daily. Tacrolimus doses are adjusted according to the whole blood concentration of the drug according to the TDM protocol shown in Table 1. In cases of steroid resistant rejection episodes anti thymocyte globulin (ATG) is administered intravenously.

Day post-transplant	**Glucocorticoids** Methylprednisolone (Solu-Medrol™) Prednisolone (generic)	**Tacrolimus** (Prograf™)	**Mycophenolic acid** Mycophenolate mofetil (CellCept™)
Transplantation	Methylprednisolone i.v.: 40 mg at start 500 mg before reperfusion 40 mg post-operative		
1	80 mg (methylprednisolone i.v.)	Starting dose: 0.1 mg/kg/day further dose adjustment according to concentration: 5-15 ng/mL	1 g x 2
2	80 mg (prednisolone p.o.)		
3	70 mg		
4	60 mg		
5	50 mg		
6	40 mg		
7	30 mg		
8-30	20 mg		
31-60	15 mg	5-10 ng/mL	
61-90	10 mg	5-10 ng/mL	
91-180	7.5 mg	5-10 ng/mL	
181-360	5 mg	5-8 ng/mL	
>360	0 mg (tapering)	3-8 ng/mL	0.5 g x 2

Table 1. Standard triple immunosuppressive protocol after adult (>16 years) liver transplantation at Oslo University hospital (per June 2012). Doses given as daily doses. Solu-Medrol™ (Pfizer), Prograf™ (Astellas), CellCept™ (Roche)

1.4 Glucocorticoids

Glucocorticoids are a group of steroid hormones (corticosteroids) synthesized in the adrenal cortex. The biosynthesis of glucocorticoids in the adrenal cortex is regulated by the adrenocorticotropic hormone (ACTH) from the pituitary, which in turn is regulated by the corticotropin releasing hormone (CRH) from the hypothalamus. The synthesis and secretion of CRH and ACTH is regulated through negative feedback from the glucocorticoids on the hypothalamic-pituitary-adrenal axis (HPA-axis), controlling the circulating levels of corticosteroids. The endogenous ligand of the glucocorticoid receptor (GR) is cortisol. The metabolic and regulatory effects of glucocorticoids are mediated mainly via genomic mechanisms. They influence the balance of carbohydrates (reduced uptake and utilization, increased gluconeogenesis), proteins (increased catabolism, reduced anabolism) and redistribution of fat. They have anti-inflammatory and immunosuppressive

effects. Glucocorticoids exert their effect on several inflammatory and immunological mediators and cells, the vascular system and the HPA-axis. They suppress pro-inflammatory cytokines (e.g. IL-1β, IFN-α), induce anti-inflammatory cytokines (e.g. TGF-β, IL-10) and anti-inflammatory cytokine receptors (e.g. TGF-βR, IL-10R). The glucocorticoid also reduce the expression of interleukins (IL-1, IL-2, IL-6, IL-12), interferon γ (IFN-γ), tumour necrosis factor α (TNF-α), which results in suppression of activated T-cells. The production of eicosanoids and immunoglobulin G is also inhibited. Furthermore, glucocorticoids reduce the migration of immune cells to the site of inflammation by repression of adhesion molecules. Dendritic cells are switched to IL-10 production instead of IL-12 by administration of glucocorticoids, which limits the differentiation of Th0 to Th1 cells. Glucocorticoid might also have apoptotic effects, which is suggested as the mechanism of intravenous methylprednisolone pulse therapy. In allograft transplantation the glucocorticoids inhibit the differentiation and antigen presentation of macrophages and dendritic cells, and thereby inhibit the initiation of an immune response.[6]

Glucocorticoids are administered in a wide range of conditions, ranging from those that require anti-inflammatory or immunosuppressive treatment (asthma, allergy, rheumatoid arthritis, systemic lupus erythematosus, and rejection prophylaxis after organ transplantation) and malignancies (leukaemia) to substitution therapy (Addisons disease). Prednisolone (Figure 2) is a synthetic glucocorticoid and plays an important role in rejection prophylaxis after solid organ transplantation. Prednisolone is well absorbed after administration and the oral bioavailability is reported to be 60-100%. Time to reach maximum concentration (T_{max}) for prednisolone is approximately 1.5 hours.[6] Prednisolone is bound in plasma to corticosteroid binding globulin (CBG) with high affinity and low capacity, and to albumin with low affinity and high capacity. A non-linear reduction in prednisolone protein binding from 95% to 60-70% when the serum concentration increases from 200 to 800 ng/mL is reported.[7] Prednisolone is mainly eliminated by hepatic metabolism and renal excretion. It is degraded in the liver and conjugated mainly with glucuronic acid and to a lesser degree with sulphates. Cortisol is metabolized to 5α-tetrahydrocortisol and 5β-tetrahydrocortisol by 5α-reductase and 5β-reductase, respectively. The latter also converts cortisone into tetrahydrocortisone. These metabolites are excreted into the urine. [8]

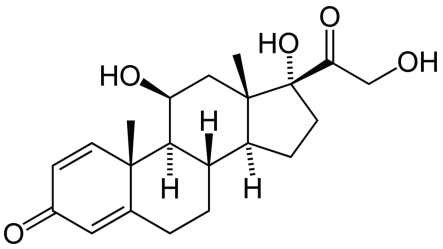

Figure 2. Molecular structure of prednisolone.

The two enzymes 11β-hydroxysteroid dehydrogenase (11β-HSD) 1 and 2 play an important role in the pre-receptor regulation of glucocorticoid and mineralocorticoid receptor activation. They catalyse the interconversion between the hormonally active cortisol (hydroxysteroid) and inactive cortisone (ketosteroid), see Figure 3.[9] These two enzymes possess different catalytic activities, 11β-HSD1 is mainly a NADP(H) dependent reductase (dehydrogenase *in vitro*) with its catalytic site in the ER-lumen (endoplasmatic reticulum). The co-factor NADP(H) is generated in the same cell compartment by the hexose 6-phosphate dehydrogenase (H6PDH) and is crucial for the reductase activity of 11β-HSD1.[10] The 11β-HSD2 is a dehydrogenase using NAD as a co-factor with its catalytic site facing the cytosol.[11,12] The biological activity of glucocorticoids relates to the presence of a hydroxyl group at position C11 (e.g. cortisol) of the steroid structure. Oxidation of this group to an 11-keto group inactivates the steroid (e.g. cortisone). Synthetic glucocorticoids like prednisolone and prednisone are also substrates for 11β-HSD.[13] The 11β-HSD1 enzyme is expressed in liver, lungs, gonads, pituitary, adrenal cortex, central nervous system and adipose tissue and supplies the glucocorticoid receptor with cortisol.[14,15] The function of 11β-HSD2 is to protect the mineralocorticoid receptor (MR) against high circulating concentrations of cortisol, and this enzyme is expressed in kidneys, colon, salivary glands and placenta.[16-20]

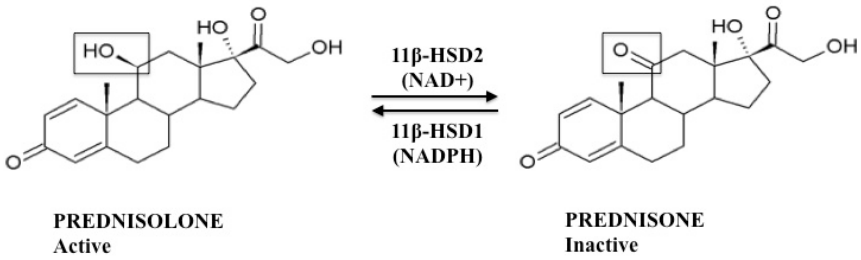

Figure 3. Interconversion between prednisolone (active) and prednisone (inactive) via 11β-hydroxysteroid dehydrogenase 1 and 2 (11β-HSD1 and 2).

The enzymes 11β-HSD1 and 2 belong to the short-chain dehydrogenase/reductase. They share 21% homology, and are encoded by two different genes, *HSD11B1* and *HSD11B2*.[21—23] Increased and decreased 11β-HSD1 activity has been associated with the pathophysiology of common diseases. Cushing's syndrome (i.e. glucocorticoid excess) can cause symptoms of metabolic syndrome (central obesity, glucose intolerance and hypertension). Animal studies performed in transgenic rodents, with over-expression of 11β-HSD1 in liver and adipose tissue, show increased local glucocorticoid concentrations and features of metabolic syndrome.[24,25] Conversely, inhibition of 11β-HSD1 increases insulin sensitivity in humans.[26] The 11β-HSD1 is regulated by both hormonal and nutritional factors, but there is evidence that genetic factors can contribute to inter-individual variation in 11β-HSD1 activity. A polymorphism in the intronic enhancer (rs12086634) is associated with lower 11β-HSD1 transcriptional activity *in vitro*.[27] Polymorphisms in the *HSD11B1* gene (rs846910 and rs12086634) have been associated with type 2 diabetes and hypertension.[28-30] Two other *HSD11B1* variants (rs846910 and rs12086634) are associated with increased levels of 11β-HSD1 mRNA and activity in adipose tissue.[31] Malavasi *et al* described that the allelic variant of rs13306421 gave higher 11β-HSD1 expression and activity *in vitro*.[31,32]

The 11β-HSD2 enzyme plays an important role in regulating mineralocorticoid action, by inactivating cortisol, which has mineralocorticoid action, to cortisone. Thus, 11β-HSD2 protects the mineralocorticoid receptor (MR) against high circulating concentrations of cortisol. Inhibition or absence of this enzyme results in high local concentrations of cortisol in mineralocorticoid tissues, which again leads to hypertension and hypokalaemia. A previous study found that 16% of patients with essential

hypertension had an elevated cortisol/cortisone ratio, suggesting that a defect in 11β-HSD2 could be involved.[33] The single polymorphism G534A in the *HSD11B2* was reported by Brand *et al.*[34] Further studies of this variant could not report any correlation between the G534A polymorphism and hypertension.[35-37] Thus the importance of variants in the *HSD11B2* in essential hypertension is controversial. The rare syndrome of apparent mineralocorticoid excess (AME) is caused by inactivating mutations in the *HSD11B2* gene, and more than 30 mutations have been described.[38-40]

The glucocorticoid receptor (GR) is a cytosolic receptor, belonging to the nuclear hormone receptor super family and is encoded by the *NR3C1* gene. The endogenous ligand for GR in human is cortisol, but it is also the target for synthetic glucocorticoids used pharmacologically. Its primary mechanism of action is regulation of gene transcription. The binding of glucocorticoids to the glucocorticoid receptor induces a series of cellular events that results in activation or repression of a network of glucocorticoid responsive genes and produces a cellular response.[41] After entering the cell, the glucocorticoid (GC) binds to the ligand binding domain of GR and forms a GC-GR complex, with a conformational change in the GR revealing a DNA binding domain. The GC-GR complexes form homodimers and translocates into the nucleus. The complex binds to a glucocorticoid responsive element (GRE) in the promoter area of anti-inflammatory genes (e.g. lipcortin, inhibitor of κB, IκB), and induces the expression of these, a process called transactivation.[6] By transrepression the glucocorticoids suppress the expression of pro-inflammatory genes (e.g. interleukin 1, interleukin 2 and pre-opiomelanocortin) via activating protein 1 (AP-1), nuclear factor-κB (NF-κB) and interferon regulatory factor 3 (IRF3).[6,42] The repression of negatively regulated target genes is mediated by negative glucocorticoid responsive elements (nGREs).[43] In addition to these genomic mechanisms of action, the glucocorticoids exert a non-genomic action which is independent of the GR interaction. The glucocorticoid may directly interact with the cell membrane, and change the properties of the membrane and membrane associated proteins.[44]

Alternative splicing of the *NR3C1* gene generates two glucocorticoid isoforms (the functional GRα and GRβ with no hormone binding ability), where GRα is the predominant one, and is expressed in the cytoplasm of most cells. [45,46] A polymorphism in codon 363 in the glucocorticoid receptor gene has been associated with increased cortisol suppression and insulin response to exogenous glucocorticoids. [47] Other sequence variants in the *NR3C1* gene are associated with glucocorticoid resistant syndromes.[48,49]

1.5 Tacrolimus

In addition to cyclosporine A, tacrolimus (Figure 4) is a widely used calcineurin inhibitor (CNI) after solid organ transplantation, and now the preferred CNI in standard immunosuppressive protocols. Tacrolimus is a macrolide lactone type calcineurin inhibitor first isolated from soil containing the bacteria *Streptomyces tsukubaensis*. Tacrolimus forms a complex by binding to the immunophilin FKBP (FK506 binding protein), which inhibits the calcium dependent phosphatase calcineurin.[50,51] The tacrolimus-FKBP complex inhibits T-lymphocyte signal transduction and proliferation through inhibition of the calcineurin mediated de-phosphorylation of the transcription factor NFAT. This supresses the transcription of interleukin 2 (IL-2) and inhibits the signal 1 and T-cell activation.[52]

In organ transplantation, two distinct peroral formulations are available: once daily (Advagraf™, Astellas) and twice daily (Prograf™, Astellas; plus generic) tacrolimus. The twice daily formulation is approved for rejection prophylaxis after kidney, liver and heart transplantation while the once daily formulation is approved after kidney and liver transplantation. A topical formulation (Protopic™, Astellas) is approved for the treatment of atopic dermatitis.

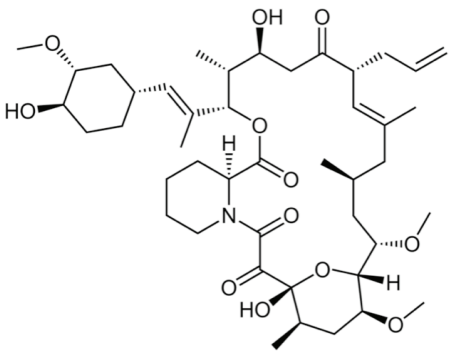

Figure 4. Molecular structure of tacrolimus.

The rate of absorption and bioavailability of orally administered tacrolimus are highly variable, and the bioavailability is poor (mean 25%, range 4-93%).[53] Maximum blood concentration is normally reached between 0.5 and 1 hour after dose.[54] Tacrolimus is substrate of both CYP3A4, CYP3A5 and P-glycoprotein, where CYP3A4 and CYP3A5 is

responsible for extensive first-pass metabolism in the liver and upper small intestine, while efflux pump P-glycoprotein will transport the drug back into the intestinal lumen.[55-57] The main route of elimination for tacrolimus and its metabolites is the biliary route, where up to 95% of the administered dose was excreted into the faeces as metabolites (only trace amounts of unchanged drug were detected in urine and faeces).[58]

Cytochrome P450 (CYP) is a heme containing family of metabolic enzymes, which mainly catalyses oxidation of organic substances (endogenous and exogenous). CYP enzymes are the major enzymes involved in drug metabolism and bioactivation, and are important in phase I metabolism. The CYP3A subfamily consists of several isoforms: CYP3A4, CYP3A5, CYP3A7 and CYP3A43, which have overlapping substrate specificities, where CYP3A4 and CYP3A5 are most abundant in adults.[59,60] CYP3A is involved in the metabolism of more than 50% of the drugs on the market and accounting 30% of hepatic CYP and more than 70% of small intestinal CYP. In adults CYP3A4 shows highly variable expression with 10 to 100-fold differences between individuals in liver and up to 30-fold in the intestines.[60] The intestinal CYP3A content is reported to be present at 10-50% of the content in liver.[60,61]

In general the CYP3A5 isoform is expressed in lower levels than CYP3A4, but it shows genetic variability. In expressers the CYP3A5 might constitute 6-99% of the total CYP content in the liver.[62] The wild type allele is assigned *CYP3A5*1*, while the *CYP3A5*3* allele is the most abundant and functionally important variant.[63,64] Only individuals carrying the *CYP3A5*1* allele produce high levels of full length CYP3A5 mRNA and thereby express functional CYP3A5 protein.[65] The *CYP3A5*3* allele, with allele frequencies of 85-95% among Caucasians, causes a splicing defect and thereby lack of functional CYP3A5 protein.[65,66] Individuals carrying the CYP3A5*1 allele have 3-fold higher CYP3A protein levels than CYP3A5*3 homozygotes.[65] The CYP3A5 expression (*1/*1 and *1/*3) has clinical impact, because it leads to more extensive metabolism of CYP3A substrates and higher dose requirements. CYP3A4 activity and *CYP3A5* genotype is reported to explain 56-59% of the variability in tacrolimus dose requirements and clearance, while hematocrit explains 4-14% after renal transplantation.[67]

Tacrolimus is extensively metabolised by the CYP3A4/5 in liver and intestines, forming the main metabolite 13-*O*-demethyl-tacrolimus.[68,69] Renal transplant recipients carrying CYP3A5*3/*3 required a lower dosage of tacrolimus than CYP3A*1 carriers.[70] Prednisolone is also a substrate for the CYP3A4/5 enzymes.[71]

P-glycoprotein (P-gp) is a member of the ATP-binding cassette super family and encoded by the multi-drug resistance gene (*MDR1* or *ABCB1).* P-gp is an adenosine triphosphate (ATP) dependent efflux pump and plays an important role in absorption, distribution and response of a drug. This transporter has a wide range of substrates, including glucocorticoids and tacrolimus and is often co-located with CYP3A4. P-gp is expressed in a variety of tissues including the adrenal glands, blood-brain-barrier, kidneys, liver, lungs, stomach, jejunum and colon.[60] The mRNA levels increase longitudinally along the intestine. The inter-individual variability in P-gp expression was more than eight-fold in intestinal biopsies from renal transplant recipients.[72] Diarrhoea is also a frequent adverse effect of the combination of tacrolimus and mycophenolic acid. An effect on P-gp has been reported in cases of diarrhoea, where the P-gp content may be reduced in the intestines. In cases of severe and prolonged diarrhoea, reduced P-gp activity in the intestines may be the most important explanation for the frequent and significant increase in tacrolimus exposure.[73]

1.6 Mycophenolic acid

Mycophenolate mofetil (MMF, CellCept™, Roche and generic) is the 2-morpholinoethyl ester of mycophenolic acid (MPA). MPA (Figure 5) is also available as the enteric coated sodium salt (EC-MPS, Myfortic™, Novartis). Mycophenolic acid is the active moiety of both MMF and EC-MPS. As rejection prophylaxis, it is approved after kidney, liver and heart transplantation.

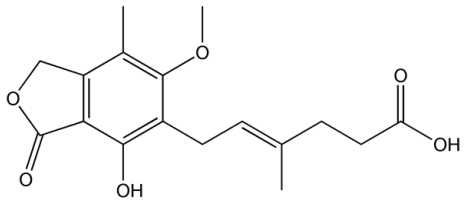

Figure 5. Molecular structure of mycophenolic acid.

After oral administration, mycophenolate mofetil (MMF) is rapidly hydrolyzed by esterases to MPA, and absorbed in the upper gastrointestinal tractus. The oral bioavailability was reported to be 94% in healthy volunteers and 81% in renal transplant

recipients.[74,75] MPA is highly bound to plasma albumin, approximately 97-99%.[76-78] Maximum plasma concentration after MMF administration usually occurs between 1 and 2 hours, while EC-MPS has lag-time of 0.25 to 1.25 hours.[79,80]

MPA is conjugated to glucuronic acid by UDP-glucuronosyltransferases (UGTs) in the liver, intestine and kidneys, and more than 90% of the administered dose is excreted in the urine as the inactive metabolite 7-O-MPA-glucuronide (MPAG).[80-82] MPAG is secreted into the bile by the multidrug resistance-associated protein 2 (MRP-2) in the hepatocytes.[83] In the intestines the MPAG is hydrolyzed back to MPA and reabsorbed. This enterohepatic circulation contributes 37% (range 10-61%) of the total MPA exposure.[74] MPAG is mainly formed by UGT1A9 in liver, kidney and GI, but other UGTs are also involved.[81,82] Uridine 5'-diphospho-glucuronosyltransferase (UGT) is a family of phase II conjugating, metabolizing enzymes, which are responsible for glucuronidation of endogenous and exogenous compounds, normally making them more water-soluble and more easily eliminated. There are two main families of UGT-enzymes, UGT1 and UGT2, where UGT1A, UGT2A and UGT2B are subfamilies. The pharmacologically active acyl-glucuronide (AcMPAG) is formed by UGT2B7, and is suggested as a contributor to the gastrointestinal toxicity related to MPA.[82,84,85] Additionally, another minor metabolite has been identified, the phenolic 7-O-glucoside (MPAGl), which is pharmacologically inactive.[86] See figure 6 for a summary of the metabolic pathway of MPA.

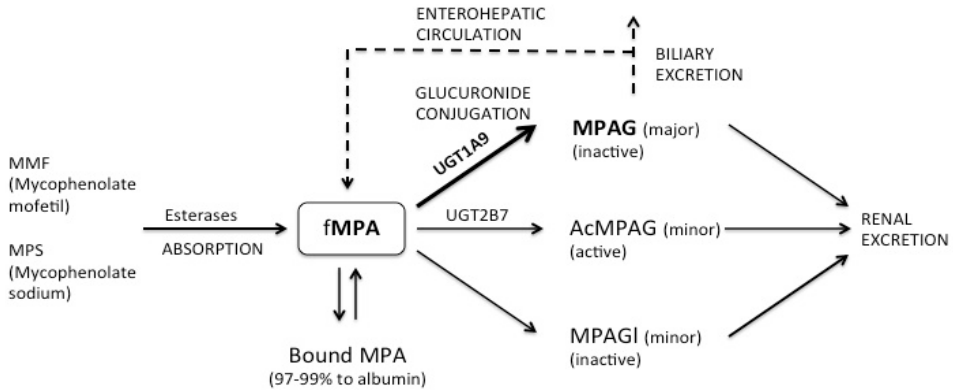

Figure 6. Pharmacokinetics of mycophenolic acid.
Free mycophenolic acid (fMPA), uridine diphosphate-glucuronosyltransferase (UGT), 7-O-glucuronide (MPAG), acyl glucuronide (AcMPAG), 7-O-glucoside (MPAGl)

UGT1A9 shows a large degree of sequence variability, which alters the expression and enzyme activity.[87] An increased glucuronidation capacity is observed in individuals carrying the c.-2152C>T (rs17868320) and c.-275T>A (rs6714486) variants, which gives lower MPA exposure and an increased risk of graft rejection.[87-93] The sequence variants *UGT2B7*2* (rs7439366), *UGT1A9* c.-440>T (rs2741045) and c.-331T>C (res2741046) are associated with reduced glucuronidation activity and increased concentrations of MPA, while UGT2B7*2 and UGT1A8*2 are associated with increased and reduced MPA related side effects, respectively.[94-98]

MPA is a selective and reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH). IMPDH catalyses the oxidation of inosine-5'-monophosphate (IMP) to zanthosine-5'-monophosphate (XMP), which is the rate-limiting step in the *de novo* synthesis of guanine and deoxyguanine (figure 7). While other cells more efficiently recirculate purines from a salvage pathway, T- and B-lymphocytes are relative dependent on the *de novo* synthesis for proliferation. This gives mycophenolic acid a potent cytostatic effect on lymphocytes.[99]

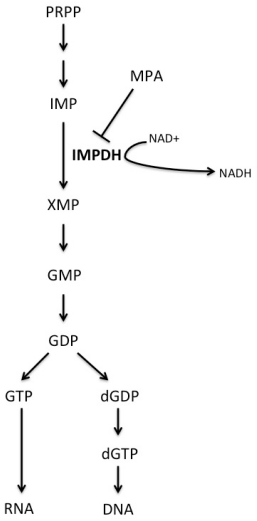

Figure 7. The *de novo* synthesis of guanine and deoxyguanine nucleotides. Phosphoribosyl pyrophosphate (PRPP), inosine monophosphate (IMP), mycophenolic acid (MPA), IMP dehydrogenase (IMPDH), nicotinamide adenine dinucleotide (NADH), reduced form of NADH (NAD+) dixanthosine monophosphate (XMP), guanosine mono-/di-/triphosphate (GMP, GDP, GTP), deoxyguanosine di-/triphosphate (dGDP, dGTP), ribonucleic acid (RNA) and deoxyribonucleic acid (DNA).

Two distinct isoforms of IMPDH have been reported (IMPDH 1 and 2), where MPA is a fivefold more potent inhibitor of the type 2 isoform (IMPDH2), which predominates in activated lymphocytes, but both IMPDH 1 and 2 mRNA are induced after lymphocyte activation.[100-102] IMPDH 1 and 2 are encoded by the *IMPDH1* and *IMPDH2* genes, respectively.[102] Intra- and inter-individual variability in the IMPDH activity (basal, without inhibitor) and the degree of enzyme inhibition under MPA therapy has been described.[103,104] Furthermore, activation of lymphocytes increases the IMPDH activity and changes the immune status, resulting in variable IMPDH activity and MPA response.[103-105] Sequence variants in the *IMPDH 1* and *IMPDH 2* genes can add further variability between individuals, resulting in an altered pharmacodynamic response. Two *IMPDH1* single nucleotide polymorphisms (SNPs: rs227893 and rs2278294) were reported by Wang *et al*. that were significantly associated with the incidence of biopsy proven acute rejection (BPAR) in the first year after renal transplant recipients receiving MPA therapy.[105] The presence of a *IMPDH2* 3757 T>C variant allele (rs11706052) is associated with an increased IMPDH activity in MMF-treated renal transplant patients, and triples the odds for BPAR within 12 months after renal transplantation.[106,107] Large inter-individual variation of IMPDH enzyme activity pre-transplant has been observed.[108] The *IMPDH2* 3757 T>C variant has been reported to explain 8% of the inter-patient variability in IMPDH activity.[106]

1.7 Principles of therapeutic drug monitoring

The main purpose of therapeutic drug monitoring (TDM) is to individually adjust the dose of a drug to improve the outcome of the therapy. Criteria for drugs considered for therapeutic drug monitoring are as follows:

- Narrow therapeutic window (small changes in dose and exposure can result in toxicity or loss of efficacy)
- Failure of drug treatment has serious consequences for the patient
- Relationship between dose and blood concentration is poorly predictable
- The clinical effect is difficult to quantify
- Considerable pharmacokinetic and pharmacodynamic variability between individuals
- The observed variable is associated with pharmacological effect and clinical outcome

- Therapeutic range of the measured variable must be established
- Assays for monitoring must be available
- Cost and benefit must be reasonable

There are several approaches for monitoring of drug therapy. It can be based on individualization before the therapy is started (pharmacogenetics, demographic and clinical information) or after (the pharmacokinetic or pharmacodynamic approach). TDM is a valuable tool for establishing the optimal drug concentration short-term after therapy initiation and in cases of drug interactions.

A simple definition of pharmacokinetics is what the body does to the drug. This includes the time course of absorption, distribution, metabolism and elimination of the administered drug. Several factors are known to affect the pharmacokinetics of a drug: absorption, tissue and body fluid mass and volume, genetic factors, elimination (e.g. renal function) drug metabolism and drug interactions. Pharmacokinetic monitoring is the most widespread form of TDM and is based on measurements of blood concentrations of the drug. These measurements are based on either single point concentrations (C_0 or C_2) or drug exposure (area under the time-concentration curve, AUC). The trough concentration (C_0) is the drug concentration at the end of a dosing interval just before the next dose, while the C_2 is the drug concentration two hours after administered dose. A full AUC is considered as the best marker for drug exposure. Compared to a single point measurement a full AUC requires several sampling time points to cover the dosing interval (normally 12 hours), which is both time- and labour-consuming. The use of single point measurements assumes that there is a correlation between e.g. C_0 and the AUC, and that C_0 can predict the exposure. Abbreviated AUC (e.g. $AUC_{0\text{-}2}$) and limiting sampling strategies (normally 3 time points and a mathematical algorithm) have been proposed as an alternative to a full AUC, but they are still time-consuming. C_0 is therefore the most common variable to measure in terms of pharmacokinetic monitoring. For cyclosporine A (CsA) the C_2 concentration correlates better with drug exposure, and is an established way for monitoring monitor CsA. A newer strategy in therapeutic drug monitoring is the use of population pharmacokinetics combined with Bayesian estimators.

Pharmacodynamics is defined as what the drug does to the body, and refers to the relationship between drug concentration at the site of action and the efficacy. Pharmacodynamic monitoring uses biological surrogate or end-point markers for effect (e.g. target enzyme activity), and reflect the biological response of the drug more closely to

the site of action than the pharmacokinetic approach. Factors that may affect the pharmacodynamics of a drug are drug receptor status, genetic factors, pharmacodynamic drug interactions and tolerance.

1.8 Therapeutic drug monitoring of immunosuppressive drugs

The goal of the monitoring of immunosuppressive therapy after solid organ transplantation is a treatment individualized to each patient. This therapy is a delicate balance in order to avoid over- or under-immunosuppression. Overexposure of immunosuppressive drugs increases the risk of drug related adverse effects, opportunistic infections or malignancies, while underexposure might cause acute or chronic rejection and graft loss. Both cases might result in impaired quality of life and high costs. The optimal dosing of immunosuppressive drugs can be achieved by therapeutic drug monitoring. The risk of rejection episodes after transplantation is highest short-term after transplantation.[5] Reaching the recommended target concentrations of the immunosuppressive drugs as shortly as possible after transplantation is crucial for optimal, clinical outcome.

The maintenance immunosuppressive therapy after liver transplantation consists of prednisolone, tacrolimus and mycophenolic acid. These drugs are associated with a broad range of adverse effects. Glucocorticoids (e.g. prednisolone) have a large number of side effects: risk of infection, diabetes mellitus, hypertension, dyslipidaemia, weight gain, osteoporosis, Cushingoid symptoms, glaucoma, suppression of the adrenal cortex, growth retardation in children, skin atrophy and neurological side effects like insomnia, irritability, psychosis and mood changes.[6] The most abundant side effects of tacrolimus are nephrotoxicity, diabetes mellitus, tremor, headache, alopecia, diarrhoea, nausea and vomiting.[62] Drug-specific adverse effects related to mycophenolic acid include leukopenia, diarrhoea, nausea, vomiting and an increased risk of CMV-infection (cytomegalovirus).[77]

Today, the rejection rate in liver transplant recipients is relatively low (see section 1.1). One of the main purposes of therapeutic drug monitoring nowadays is to optimize the therapy to improve quality of life, reduce the drug related toxicity and to reach the lowest dose possible while maintaining the optimal protection against graft rejection.

In the standard immunosuppressive regimen after liver transplantation, only tacrolimus is subject for routinely therapeutic drug monitoring. Tacrolimus pharmacokinetics is highly

variable between individuals. The established TDM of tacrolimus is based on single point pharmacokinetic measurements, by measuring the pre-dose concentration (i.e. concentration at the end of the dosing interval, right before the next dose = C_0) in whole blood. The recommended target trough concentration, according to the protocol at our transplantation centre, is 5-15 ng/mL (1-30 days post-transplant), 5-10 ng/mL (31-180 days post-transplant), 5-8 ng/mL (181-360 days post-transplant) and 3-8 ng/mL a year post-transplant. In patients with elevated creatinine, the clinicians aim at the lower end of the recommended concentration range (5-15 ng/mL) in the early post-operative period, in order to manage the renal function of the patient, due to tacrolimus nephrotoxicity.

Several of the marketed immunosuppressive drugs have a narrow therapeutic window, which increases the risk of complications in cases of clinical relevant drug interactions. When a drug interaction is likely to occur, monitoring might be of value to evaluate whether individual dose adjustments are necessary. As described in section 1.5 tacrolimus is mainly metabolized by the CYP3A enzyme, hence inhibition or induction of CYP3A4-mediated metabolism is a clinically important drug interaction for tacrolimus. Some relevant interactions of clinical importance are caused by grapefruit juice and co-administration of antifungal drugs. Grapefruit juice contains an array of furanocoumarins responsible for the inhibition of CYP3A,[109] which increases the oral bioavailability of tacrolimus. This drug interaction is sometimes used intentional to improve the uptake of orally administered tacrolimus. Due to the immunosuppressive state of the transplanted patients, antifungal drugs are frequently administered. The antifungal drugs fluconazole, itraconazole and ketoconazole increase the exposure of tacrolimus through inhibition of CYP3A with variable potency.[110] Co-administration of ketokonazole almost doubles the bioavailability of tacrolimus.[111] In cases of this drug interaction, close monitoring is essential for dose adjustment after initiation and discontinuation of these drugs. The human pregnane X receptor (PXR), encoded by *NR1I2*, regulates the expression of the *CYP3A* and *MDR1* genes.[112,113] Glucocorticoids induce CYP3A expression through PXR in hepatocytes and enterocytes.[112] Induction of CYP3A expression increases the metabolism of CYP3A substrates, which results in increased dose requirements of these drugs (e.g. tacrolimus). The clinically relevant drug interactions between tacrolimus and other frequently administered drugs after transplantation and the potential complications highlight the importance of controlling the tacrolimus levels and dosing.

Several studies have demonstrated that patients carrying the *CYP3A5*1* allele require higher doses of tacrolimus than the *CYP3A5*3* carriers to reach the same blood

concentrations.[114-116] Renal transplant recipients with at least one *CYP3A5*1* allele achieved only half the dose-normalized tacrolimus blood concentrations compared to *CYP3A5*3/*3* homozygotes, with a significant delay in reaching target blood concentrations in the *CYP3A5*1* carriers.[115] CYP3A5 genotyping in renal transplant recipients is predictive of the tacrolimus dose, and may help determine the initial daily dose of tacrolimus needed by the individual patient for adequate immunosuppression.[117] These findings might indicate that an individualized immunosuppressive therapy based on pharmacogenetics is promising after solid organ transplantation.

Considerable inter-individual variability in the pharmacokinetic parameters of MMF has been reported.[118] Considering the correlation between MPA plasma concentration and risk of acute rejection and the variability in MPA pharmacokinetics, individualizing the dose regimen of MMF may improve clinical outcome. Higher MPA plasma concentrations are correlated with a reduced risk of acute rejection in renal transplant recipients, hence controlling this variability is of clinical importance.[119] In the same study, the pharmacokinetic-pharmacodynamic relationship was investigated showing a significant relationship between MPA $AUC_{0\text{-}12h}$ and the risk of rejection and that pre-dose concentrations of MPA (C_0) has less predictive value of acute rejection than $AUC_{0\text{-}12h}$. A therapeutic range of MPA $AUC_{0\text{-}12h}$ between 30 and 60 mg*h/L has been suggested.[120] The value of therapeutic drug monitoring of MPA has been widely discussed, but single point C_0 measurements are performed by several centres, despite poorer correlation with clinical outcome than AUC. As therapeutic drug monitoring by full AUC sampling is both time and labour consuming in daily routine, another approach has arisen. Two large multicentre trials (APOMYGRE and FDCC) investigated a potential benefit in clinical outcome in renal transplant recipients, by individualizing MMF dosing by using three-point limited sampling strategies.[121,122] The APOMYGRE-trial demonstrated, by using a Bayesian estimator, that there was a significantly lower incidence of biopsy proven acute rejections in the concentration-controlled group than the fixed-dose group. The FDCC-trial found no difference in the incidence of treatment failure between the concentration-controlled group and the fixed-dose group. The lack of difference in MPA exposure between the concentration-controlled group and the fixed-dose group may partly be explained by failure to apply MMF dose changes based on target MPA exposure. Although conflicting results in these studies, the results in the APOMYGRE-trial showed that clinical outcome after renal transplantation might be improved by individualizing MMF dosing.

As described in section 1.8, sequence variants in the *UGT1A9* gene might describe some of the pharmacokinetic variability of MPA. Although further documentation is needed, determination of *UGT1A9* genotype might prove valuable as a supplement in further individualization of MPA treatment. Further discussion of this topic is presented in section 4.3.

As MPA inhibits the IMPDH in lymphocytes, measuring IMPDH activity in these cells might serve as a surrogate marker for MPA-induced immunosuppression. The pharmacokinetic approach to therapeutic drug monitoring of MPA uses the plasma concentration as the marker for the clinical effect. Pharmacodynamic monitoring measures the pharmacological effect more closely at the drug target, and will predict the efficacy and toxicity of MPA more directly. Pharmacodynamic monitoring of MPA is further discussed in section 4.8.

Glucocorticoid therapy is a cornerstone in the immunosuppressive regimens after organ transplantation. As mentioned in section 1.8 these drugs have a broad range of serious side effects. Despite the serious side effect profile of glucocorticoids and the long-term therapy, no concentration monitoring or individualized dosing is performed after adult solid organ transplantation. Several studies aiming to avoid or withdraw steroids in the immunosuppressive regiment have been published with positive results, but the results are conflicting.[123-126] Knight *et al.* reported that steroid avoidance or withdrawal decreases the risks of various side effects, but increases the risk of acute rejection.[127,128] In the APOMYGRE-study mentioned above, they demonstrated a significant reduction in treatment failure in the concentration-controlled group (of MPA) combined with steroid withdrawal.[121]

Although the single point pharmacokinetic monitoring as performed today is a valuable tool in therapeutic drug monitoring, it is only a surrogate marker for the drug exposure and predicted efficacy of the drug. The primary end-point of immunosuppressive therapy is the degree of immunosuppression and avoidance of graft rejection. Pharmacodynamic monitoring is measuring the biological response to a drug, which in addition to pharmacokinetic monitoring offers an improved method for optimization of drug dosing. As a supplement to established TDM, monitoring of immune status can give an indication whether the patient has a low, moderate or strong immune response, and identify patients at risk of acute rejection, infection or cancer. Rejection episodes, infections and cancer development are important sources of morbidity and mortality in immunosuppressed patients. An FDA approved commercial analysis kit has been marketed

(ImmuKnow®, Cylex™ Inc., Columbia, MD), which is an immune cell function assay and quantifies intracellular ATP (adenosine triphophate) in stimulated CD4 positive lymphocytes. Although the predictive value of this kit has been debated, a meta-analysis performed by Rodrigo *et al.* concludes that the ImmuKnow test is a valuable tool to predict the risk of further infections in adult liver transplant patients, but the identification of the risk for rejection is inconclusive.[129]

2 Objectives of the thesis

2.1 Overall objective

The liver plays a crucial role in the pharmacokinetics of immunosuppressive drugs. The overall objective of this thesis was to investigate the pharmacokinetics, pharmacodynamics and pharmacogenetics of the immunosuppressive drugs used after liver transplantation; glucocorticoids, mycophenolic acid and tacrolimus. Furthermore, the aim was to describe the intra– and inter–individual variability of these drugs in liver transplant recipients and to study which underlying factors contribute to the large variability in the clinical effect of these drugs.

2.2 Objective paper I

This paper aimed to develop a reliable LC-MS/MS assay for quantifying six relevant glucocorticoids (prednisolone, prednisone, cortisol, cortisone, methylprednisolone and dexamethasone) used after solid organ transplantation. An in-depth validation study should be performed according to the U.S. Food and Drug Administration guidelines.[130] Furthermore, the matrix effects should be assessed and the clinical application demonstrated.

2.3 Objective paper II

The objective of this second paper was to investigate the pharmacokinetics of prednisolone and prednisone in the first weeks following liver transplantation. The impact of the metabolizing enzymes 11β-hydroxysteroid dehydrogenase 1 and 2 on the pharmacokinetics of prednisolone and prednisone should be studied. Furthermore, the study aimed to investigate the ratio between prednisolone and prednisone as a potential marker in therapeutic drug monitoring. Additionally, the pharmacokinetics of methylprednisolone and endogenous cortisol and cortisone should be described.

2.4 Objective paper III

The aim of this study was to examine the pharmacokinetics, –dynamics and –genetics of mycophenolic acid early after liver transplantation, with respect to IMPDH activity and *UGT1A9*, *IMPDH1* and *IMPDH2* sequence variants. Furthermore, the study aimed to describe the pharmacokinetics and pharmacogenetics of the calcineurin inhibitor tacrolimus in the same patient population. By genotyping both donors and recipients for sequence variants in the *CYP3A5* gene, the association between *CYP3A5* genotype and the tacrolimus pharmacokinetics should be investigated.

3 Methods

3.1 Study design and patient recruitment

The study population reported in paper II and III were recruited at Oslo University Hospital in the period between February 2008 and July 2009. Sixteen liver transplant recipients were included. The inclusion criteria were liver transplant recipients at ages above 18 years, immunosuppressive therapy according to the standard protocol after liver transplantation, consisting of a triple regimen with prednisolone, mycophenolic acid and tacrolimus and no former use of these immunosuppressants. The study was performed in accordance with the Declaration of Helsinki and approved by the Regional Committee for Medical Research Ethics. Informed written consent from the study participants was obtained.

The study period included the first three weeks following liver transplantation. Full 12-hour pharmacokinetic profiles were obtained on up to four follow-up days for each patient. The first follow-up day was between day one and five post-transplant, the second between day six and ten, the third between day 11 and 17, while the fourth dosing interval was after day 17. The four follow-up days are reported as period I, II, III and IV, respectively. All four follow-up days were completed in 8 and 9 of the 16 recipients for tacrolimus and mycophenolic acid, respectively. Two of the recipients had complications with the central venous catheter, which resulted in only two and three intervals for these patients. Because of medical conditions at the inclusion time one patient missed the first period. Four of the patients were recovering fast and were discharged from the hospital prior to period IV. One of the patients started anti-thymocyte globulin (ATG) treatment during one of the follow-up days, and these samples were unsuitable for the IMPDH-assay, due to eradication of T-lymphocytes. For the tacrolimus study, one of the patients was excluded from the pharmacokinetic analysis due to administration of once-daily tacrolimus (Advagraf), which was not in accordance with the study protocol. Lastly, one of the intervals was excluded due to assay failure (IMPDH) and another one because of inappropriate timing of tacrolimus and MMF dose.

3.2 Sampling and pre-analytical preparation

Samples were collected peripherally pre-transplant (pre-Tx) and from a central venous catheter at four follow-up days in four distinct periods during the three weeks after surgery. Biological samples consisted of whole blood for genotype analyses, isolated CD4+ lymphocytes for gene expression analyses and IMPDH activity measurement, whole blood for tacrolimus concentration assessment and plasma for quantification of mycophenolic acid and glucocorticoid concentrations. The venous blood samples were drawn into tubes containing EDTA (ethylene diamine tetraacetic acid). Each follow-up day was a twelve hour dosing interval with thirteen samples (pre-dose, 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3, 4, 5, 6, 8, 10 and 12 hours post-dose). CD4+ lymphocytes for IMPDH activity, plasma and whole blood for drug concentration measurements were collected at all time points. Pre-transplant, samples were drawn for genotyping and basal IMPDH activity. In addition, whole blood from the respective liver donors was collected for genotype determination.

At the sampling days, a large effort had to be made isolating and providing the right biological material for the separate analyses. Whole blood collected pre-Tx for genotyping was frozen at -70 °C directly after sampling. After freezing an aliquot of whole blood for tacrolimus analysis at -20 °C, the remaining sample was centrifuged at 1500 g in 12 minutes to separate the plasma in aliquots for glucocorticoid and MPA analyses. The plasma samples were frozen at -20 °C until analysis.

For the IMPDH activity assay, CD4+ lymphocytes were isolated from whole blood using paramagnetic monodisperse beads coated with anti-CD4 monoclonal antibodies (Dynabeads® M-450 CD4, Life Technologies). EDTA (ethylenediaminetetraacetic acid) whole blood was incubated with beads, plasma removed and the captured cells were washed with phosphate buffered saline (PBS) sequentially. The plasma from the samples was subjected to 0.1 μm filtration. To restore the intracellular MPA concentration, the isolated lymphocytes were re-incubated in the micro-filtrated original plasma or drug-free plasma, depending on whether the inhibited or the basal IMPDH activity was to be measured. After lysis of the cell membranes the cell nuclei from the isolated lymphocytes were counted using a Coulter Counter® (Beckman Coulter, Inc.), which was set at a diameter range of 3 to 10 μm. The remaining suspension was frozen at -20 °C until analysis.

3.3 Pharmacokinetic analyses

The pharmacokinetic variables and parameters for glucocorticoids (paper II), tacrolimus (paper III), mycophenolic acid (paper III) were derived from plasma (MPA and glucocorticoids) and whole blood (tacrolimus) as follows. Maximum concentration (C_{max}), pre-dose concentration (C_0) and time to reach C_{max} (T_{max}) were read directly from the concentration versus time curves. The elimination rate constant (k_e) was estimated by log–linear regression of the terminal part of the concentration–time profile. Elimination half–life is calculated as $\ln 2/k_e$. By using the linear trapezoidal rule the area under the concentration–time curve ($AUC_{0\text{-}12h}$) was calculated. $AUC_{12\text{-}\infty}$ was extrapolated by C_{12} divided by k_e. Total $AUC_{0\text{-}\infty}$ is the sum of $AUC_{0\text{-}12h}$ and $AUC_{12\text{–}\infty}$, minus the contribution from previous dose of tacrolimus or mycophenolic acid (C_0/k_e). The apparent total clearance from plasma after an oral dose (Cl/F) was determined from the dose divided by the $AUC_{0\text{-}\infty}$. Apparent volume of distribution (V_D/F) was calculated as $(Cl/F)/k_e$. The data are based on single-compartmental pharmacokinetics. The pharmacokinetic data of tacrolimus, glucocorticoids and MPA were normalized to dose per bodyweight (dose/BW).

3.4 Statistical analyses

The statistical analysis, calculation and figure preparation were carried out using SPSS 18.0 (SPSS Inc., Chicago, IL) and Microsoft Excel (Microsoft Corp., WA). All continuous variables were reported as median and range, unless otherwise stated. To compare changes in pharmacokinetic parameters and variables between the four periods Related-Samples Wilcoxon Signed Rank Test was used. Statistical bivariate correlation was investigated by Spearman's rank correlation coefficient. In paper III the Kruskal Wallis Test was used in order to test whether there were differences in MPA pharmacokinetics between the three groups of *UGT1A9* genotypes. P-values less than 0.05 were considered statistical significant.

3.5 Paper I

To investigate glucocorticoid pharmacokinetics in clinical samples, an LC-MS/MS (tandem mass spectrometry coupled to high performance liquid chromatography) assay for quantifying prednisolone, prednisone, cortisol, cortisone, methylprednisolone and

dexamethasone was developed. The LC-MS/MS method was validated according to the bioanalytical guidelines published by U.S. Food and Drug Administration, validating stability, precision, accuracy, sensitivity, selectivity and linearity. [130] Additionally, matrix effects were validated both qualitatively and quantitatively. Quantification of plasma concentrations in plasma were performed by reversed phase chromatography, coupled to positive electrospray ionization with multiple reaction monitoring in the mass spectrometer. The chromatographic column in use was a Luna C18, 3 μm, 150 mm x 4.60 mm (Phenomenex, Torrance, CA), with a gradient elution with methanol and 2 mmol/L ammonium acetate with 0.1% formic acid (v/v). Sample preparation and pre-treatment consisted of protein precipitation with acetonitrile with isotope labelled internal standards, followed by liquid/liquid extraction with dichloromethane, evaporation under nitrogen (40 °C, 15 min) and re-constitution in methanol. The assay was developed using a HPLC (Alliance HT 2795, Waters, Manchester, UK) coupled to a tandem mass spectrometer of the triple quadropole type (Micromass Quattro Micro, Waters, Manchester, UK) using positive electrospray ionization (ESI+) with multiple reaction monitoring (MRM). Data were processed using the MassLynx^{TM} and QuanLynx^{TM} software supplied by Waters. Linear least-squares regression of peak area was used for calibration of each analyte, with 1/(analyte concentration)^2 weighting of the calibration curve.

3.6 Paper II

Determination of plasma concentrations of prednisolone, prednisone, cortisol, cortisone and methylprednisolone in the liver transplant population were determined by the validated LC-MS/MS method presented in paper I. The pharmacokinetic analyses were performed according to section 4.3. In addition, the ratio between active and inactive glucocorticoids (i.e. prednisolone and prednisone) was calculated as the $AUC_{0-\infty}$, C_0 or C_{max} of prednisolone divided by that of prednisone.

3.7 Paper III

Quantification of mycophenolic acid concentrations in plasma was performed by a LC-UV (HP series 1100 and HP Chemstation, Agilent Technologies, CA) assay published earlier, and used in the daily routine in our laboratory.[131] This is a reversed phase LC-method, using a Zorbax SB-C18 column, 3.5 μm, 74 x 4.6 mm with a Zorbax Eclipse XDB-SB-C8

guard cartridge, 5 μm, 12.5 x 2.1 mm (Agilent Technologies, CA). Isocratic elution with a mobile phase containing 30% acetonitrile and 40 mM phosphoric acid (pH 2.1) was performed. Protein precipitation with acetonitrile is used as sample purification. For detection the UV-absorption is measured.

Determination of inosine monophosphate dehydrogenase (IMPDH) activity was performed in isolated CD4+ cells and quantified by HPLC with UV-detection (HP Series 1100, Agilent Technologies, Palo Alto, CA), expressed as the XMP (xantosine monophosphate) production rate (pmol/10^6/cells/min).[131] The lymphocytes were isolated from whole blood by utilization the use of of paramagnetic beads coated with anti-CD4 antibodies. To restore the intracellular concentration of MPA after the sequential washing steps, the cells were incubated in micro filtrated plasma from the original sample. Additionally, at four time points the MPA was washed out and restored in drug free plasma, to measure the basal IMPDH activity. Inosine 5'-monophosphate (IMP) is the substrate for IMPDH. Together with nicotinamide adenine dinucleotide (NAD), which is the co-factor for IMPDH, IMP was added to the lysate of the CD4+ cells, and IMPDH activity was quantified as the xanthosine 5'monophosphate (XMP) production rate (pmol/10^6cells/min). The concentration of XMP was determined by HPLC after hydrolytic cleavage to xanthine, using a Chromolith Performance column, 100 x 4.6 mm (Merck, KgaA, Darmstadt, Germany) coupled in series with a Nucleosil C18 column, 5 μm, 150 x 4.6 mm (Supelco Inc., Bellefonte, PA). The mobile phase contained methanol 4% in o-phosphoric acid with pH 1.8, where the analytes were eluted using isocratic flow.

Concentration assessment of the calcineurin inhibitor tacrolimus was carried out by an LC-MS/MS assay developed, validated and published from our laboratory.[132] Instead of using the reported standards and quality controls in this method, a commercial kit was used (Mass Trak Immunosuppressants Kit, Waters, Manchester, UK). Whole blood samples were cleaned up by protein precipitation with 0.1 mol/L zinc sulphate and acetonitrile containing internal standard. The prepared samples were analysed with reversed phase chromatography on a Luna C18(2) cartridge 3μm, 20 x 2.0 mm with a guard cartridge C8 4.0 x 2.0 mm (Phenomenex, Torrance, CA), using mobile phases containing 2.0 mmol/L ammonium acetate and 0.1% formic acid (v/v) in methanol and water, respectively. The mass spectrometer was set in the positive electrospray mode with multiple reaction monitoring.

Samples for clinical biochemistry variables were collected and analysed as a part of the standard post-transplant follow-up on a daily basis in the study period. The biochemical parameters alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), total bilirubin and albumin were analysed in heparin plasma on the Modular Analytics analyser (F. Hoffmann- La Roche Ltd, Basel, Switzerland).

Genotyping of *CYP3A5*, *UGT1A9*, *IMPDH1* and *IMPDH2* was performed by PCR and melt curve analysis with hybridization probes on the LightCycler® 480 instrument (Roche Applied Science, Penzberg, Germany) after DNA extraction from EDTA anti-coagulated blood using the MagNA Pure instrument (Roche Applied Science, Penzberg, Germany). The following sequence variants were determined:

CYP3A5 (rs 776746; g.12083A>G, A=*CYP3A5*1* and G=*CYP3A5*3*)

UGT1A9 (rs17868320, c.-2152C>T)

UGT1A9 (rs2741046, c-440C>T)

UGT1A9 (rs2741045, c-331T>C)

UGT1A9 (rs6714486, c.-275T>A)

IMPDH1 (rs2278293, c.579+119G>A)

IMPDH1 (rs2278294, c.580-106G>A)

IMPDH2 (rs11706052, c.819+10T>C)

In order to investigate the pharmacodynamics of mycophenolic acid, the IMPDH activity was examined as follows. The pre-dose, minimum and maximum enzyme activities were read directly from the IMPDH activity versus time curve, and called A_0, A_{min} and A_{max}, respectively. The time points for A_{min} and A_{max} were assigned T_{min} and T_{max}. The maximum IMPDH inhibition was calculated by the formula $(1-A_{min}/A_0) \times 100\%$.

4 Results and discussion

4.1 Paper I

This paper presents a quantitative assay for determination of prednisolone, prednisone, cortisol, cortisone, methylprednisolone and dexamethasone in plasma samples. The validation results are in accordance with the bioanalytical guidelines published by U.S. Food and Drug Administration. [130] According to these guidelines the assay intra-day and inter-day precision should have coefficients of variation lower than 15% (20% at the lower limit of quantification, LLOQ), and the accuracy between 85% and 115% (80-20% at LLOQ). The inter-day accuracy range was 92.1% to 104.1%. The inter-day imprecision for the six analytes was between 4.0% and 15.6%, with poorest performance for dexamethasone (15.6%). An isotope labelled internal standard specific for dexamethasone may be required to obtain a more precise quantification of this compound. The lower limits of quantification, LLOQs (i.e. the lowest concentration with CV<20% and accuracy within 80-120%), were sufficient for application in pharmacokinetic studies, ranging from 1.5 to 4.0 µg/L, covering the low concentrations in a dosing interval.

A frequent problem in mass spectrometry is matrix effects. Matrix effects are changes in the ionization efficiency in the presence of co-eluting components in the electrospray interface, either from the biological matrix (e.g. phospholipids), sample components (e.g. salts, surfactants), xenobiotics or additives in the mobile phase.[133,134] This results in either ion suppression or ion enhancement. It has been demonstrated that the main cause of ion suppression is a change in the droplet solution properties in the presence of non-volatile solutes in the electrospray ionization of the analytes.[135] The chemical properties of the compounds are important for causing ion suppression. Molecules with high molecular weight are likely to suppress smaller molecules, while polar compounds are more prone to ion suppression than non-polar.[136] Matrix effects have an impact on the performance of the mass spectrometer, and might affect the accuracy, precision and the lower limit of quantification of the assay. This emphasizes the need for investigation of matrix effects when developing mass spectrometry assays. According to the FDA bioanalytical validation guidelines, the validation of matrix effects are mandatory, but no procedure has been specified.[130] There are two established methods for evaluation of the

significance of the matrix effects: post-extraction addition and post-column infusion, which are quantitative and qualitative approaches, respectively.[136-138] In mass spectrometry matrix effects are unavoidable, but an adequate sample preparation and chromatography and the use of isotope labelled internal standard may minimize their impact. Paper I presents validation of matrix effect using both approaches. The results from the matrix effect validation in this paper show that there is substantial ion suppression, but this is corrected by the internal standards and does not affect the performance of the assay.

Ideally, each analyte should have its respective isotope labelled internal standard for optimal quantification. Addition of compounds to the assay affected the data sampling and resolution, resulting in fewer data points for each chromatographic peak. As a compromise the compounds with similar retention time and molecular structure had a common internal standard (prednisolone-cortisol, prednisone-cortisone). Dividing the data sampling into time segments further improved the resolution. Another problem with introducing more internal standards is the interferences between compounds in a narrow *m/z* (mass over charge) range with closely resembling molecular weight, chemical properties and fragmentation patterns.

This assay presents some limitations. The sample preparation (liquid liquid extraction) is quite laborious and time consuming, and includes some use of organic extraction solvents. The sample volume required for analysis is relatively large (500 µL). Smaller volumes were tested, but to achieve adequate signal intensity in the mass spectrometer this volume was necessary. In addition, the extracted sample had to be evaporated under nitrogen for further up-concentration and improvement in signal intensity. The analysis run time for each sample is 12 minutes, which in daily routine analysis is relative long. This was required to achieve chromatographic separation of analytes with similar *m/z* and fragmentation to avoid analytical interferences and cross-talk. With more sensitive instrumentation, this method might have been optimized, with shorter run-time, smaller sample volumes and potential for automation. Retrospectively, another extraction procedure (e.g. solid phase extraction) might have been chosen to avoid organic solvents.

So far, quantification of these glucocorticoids is not performed on a routinely basis, but for research purposes only. In pharmacokinetic studies, the assay determines all relevant concentrations in a dosing interval after administrations of these glucocorticoids. For this application, the assay performance was satisfactory.

4.2 Paper II

The glucocorticoid pharmacokinetics of 16 liver transplant recipients was studied with up to four 12-hour dosing intervals (period I-IV) within the first three weeks after transplantation. The intra- and inter-individual variability in prednisolone and prednisone pharmacokinetics was large in this liver transplant population. There were significant increases in dose per body weight (dose/BW) adjusted $AUC_{0-\infty}$ from period I to period II, III and IV for both prednisolone (median 5222, 6957, 8665 and 7660 µg*h/L/(mg/kg), respectively) and prednisone (495, 824, 897 and 782 µg*h/L/(mg/kg), respectively). The median dose/BW adjusted C_0 for prednisolone were 336, 258, 544 and 252 µg/L/(mg/kg) in period I, II, III and IV, respectively. The elimination half-lives of prednisolone were stable in the four periods, ranging from 3.2 to 3.6 hours. Median volume of distribution (V_D) of prednisolone was 78, 50, 49 and 47 L in period I to IV, respectively. The increments in exposure were reflected in a significant reduction in apparent clearance (Cl/F) of prednisolone between the same periods (median 14.8, 11.5, 9.6 and 8.8 L/h, respectively). The median elimination rate was constant during the follow-up periods, and hence the elimination half-life. As $Cl = V_D * k_e$ one can assume that the body weight adjusted apparent volume of distribution (V_D/F) declined in a similar manner as the apparent clearance (Cl/F). These findings might indicate that the variable $AUC_{0-\infty}$ may be caused by changes in bioavailability or the distribution volume.

In a previous study, large inter-individual variability in prednisolone pharmacokinetics was also found in young patients with systemic lupus erythematosus (SLE).[139] Full 9-hour PK profiles were analysed in 8 SLE patients. Mean prednisolone dose/BW adjusted AUC_{0-9} were 4361 ng*h/L/(mg/kg) (range 1136-9580). They also demonstrated a correlation between prednisolone pharmacokinetics and clinical effect. Prednisolone Cl/F and V_D were significantly lower in cushingoid patients than non-cushingoid. Another pharmacokinetic study of prednisolone was performed in lung transplant recipients.[140] Prednisolone exposure in 52 lung transplant recipients was measured by six hours AUC. This study population show wide inter-individual variation, with a significant increase in AUC/mg in patient with cystic fibrosis (511±82 nmol*h/L/mg) compared to non-cystic fibrosis patients (349±27 nmol*h/L/mg). Furthermore, they reported that female patients had a significantly higher AUC_{0-6} than male patients. Taken all this together, the pharmacokinetics of prednisolone is highly variable between individuals in several patient populations, including the liver transplant

population reported in this paper. In addition, there are differences in prednisolone pharmacokinetics between genders. This may indicate a need for a more individualized dosing of glucocorticoids.

A non-linear reduction of prednisolone protein binding from 95% to 60-70% when the serum concentration increases from 200 to 800 μg/L has been reported.[7] Prednisolone is bound in plasma to corticosteroid binding globulin (CBG) with a high affinity and low capacity, and to albumin with low affinity and high capacity.[6] This results in a saturation of the CBG and a higher free fraction of the drug when total concentration increases. An elevated free concentration will result in an increased elimination. The glucocorticoid doses administered the following days post-transplant resulted in maximum concentrations in this non-linear range of CBG binding in this study, which complicates the interpretation of the pharmacokinetic data. Measurements of the free concentration of the glucocorticoids could add further information, in order to investigate whether the increases in dose-BW-adjusted AUC also is a result of an increased clearance due to a potential increase in free fraction. Although the free fraction was not measured, one may assume that reduced protein binding contributed to the higher V_D/F and Cl/F and thereby to the lower dose-BW-adjusted $AUC_{0-\infty}$ and C_{max} in the first period after transplantation. According to this interpretation, one might speculate if a moderate initial dose of prednisolone, which gives concentrations within the linear range of CBG binding, could be equally effective with a reduced incidence of adverse effects due to lower unbound concentration.

As the plasma proteins albumin and CBG are both synthesized in the hepatocytes, the protein synthesis is dependent of the liver function after transplantation. Table 2 summarizes biochemical parameters in the study population, where the median albumin concentrations were below the lower reference limit in the four follow-up periods, with a gradually increase with time. The increasing albumin concentrations with time after transplantation might reflect the ability of the liver to synthesize protein, and may also affect the CBG synthesis. Increasing concentrations of CBG will affect the capacity to bind glucocorticoids and thereby the free fraction and clearance. In this study the CBG levels were not monitored, but it could add further information. One might speculate if the CBG concentrations could be a part of the explanation why the dose-adjusted exposure of prednisolone and prednisone increased during the post-operative phase.

	Follow-up period			
	I	II	III	IV
Albumin (g/L)	29 (20-36)	30 (23-37)	32 (28-40)	36 (25-40)
Total bilirubin (μmol/L)	17 (4-137)	15 (7-55)	12 (7-67)	11 (7-34)
Aspartate aminotransferase (IU/L)	75 (29-487)	43 (19-74)	18 (8-62)	17 (10-37)
Alanine aminotransferase (IU/L)	363 (72-1136)	178 (50-428)	81 (22-270)	35 (20-198)

Table 2. Biochemical parameters (serum) in period I-IV in the study population (n=16). Data given as median (range). Reference intervals in serum: albumin (36-48 g/L), total bilrubin (5-25 μmol/L) aspartate aminotransferase (women: 10-35 IU/L, men: 10-45 IU/L) and alanine aminotransferase (women: 10-45 IU/L, men: 10-70 IU/L).[141]

The median ratio of the AUC of prednisolone versus prednisone was stable through the four periods (range 9.2 to 10.1). Compared to the median ratio, one of the sixteen patients had a consistently higher ratio in period I, II and III (missed period IV), with ratios of 15.2, 24.2 and 52.7. The latter was in the presence of methylprednisolone administration. In this case an intravenous bolus dose (500 mg) of methylprednisolone influenced the relationship between prednisolone and prednisone by suppressing the plasma concentration of prednisone, resulting in an increased $AUC_{0-\infty}$ ratio (52.7). This phenomenon was seen at all follow-up days where methylprednisolone was administered. This patient also had a consistently higher ratio than median, even in the absence of methylprednisolone. The marked reduction in prednisone concentration coincided with high methylprednisolone concentrations. One explanation may be a saturation of 11β-HSD2, perhaps combined with an unsaturated 11β-HSD1, factors that would indicate nonlinear kinetics and increasing risk of adverse effects. The mechanism behind this finding and the impact of it should be investigated. In cases of extremely and consistently high prednisolone/prednisone ratio, one can speculate if genetic variants in *HSD11B1* or *HSD11B2*, the genes encoding 11β-HSD1 and 11β-HSD2, could be involved. This needs further investigation.

Given the broad range of serious adverse effects and toxicity, long-term glucocorticoid treatment is problematic. Individual patients differ in the response to the

same dose of prednisone.[142] In order to individualize glucocorticoid therapy a potential predictor of the risk of side effects could be of value. One of our hypotheses is that the ratio between the active prednisolone and inactive prednisone might be a potential marker in therapeutic drug monitoring to predict an increased risk of drug-related side effects of prednisolone. This study was too small and not powered and designed to establish significant association between ratio and side effects. The purpose was to describe the pharmacokinetics in this liver transplant population short-term after transplantation and to investigate relationship between the active and inactive forms of the glucocorticoids. As described in section 1.4 the expression of 11β-HSD 1 and 2 is tissue specific. A limitation of this ratio is that it is based on plasma concentration measurements, which does not necessarily reflect the prednisolone and prednisone concentrations in the various tissues. Whether a high or a low plasma ratio might be associated with a risk of adverse effects, rejection episodes or the glucocorticoid effects should be investigated. Furthermore, variability in the pharmacodynamics of glucocorticoids should be investigated with respect to the glucocorticoids receptor. Single nucleotide polymorphisms in the *NR3C1*, encoding the glucocorticoid receptor, might be a factor contributing to pharmacodynamic variability.[143] As prednisolone is substrate for both CYP3A4, CYP3A5 and P-glycoprotein, genetic variants altering the expression and function of these may impact the prednisolone pharmacokinetics. Furthermore, synthetic glucocorticoids induce gene expression of CYP3A and P-gp by activation of the human pregnane X receptor (PXR). Miura *et al.* investigated the influence of polymorphisms in *CYP3A5, ABCB1* (P-glycoprotein) and *NR1I2* (PXR) genes on the prednisolone pharmacokinetics in 95 renal transplant recipients.[144] They found that patients carrying the *NR1I2* 7635GG or 7635AG allele had significantly lower $AUC_{0\text{-}24}$ and C_{max} values than patients having the 7635AA allele. Furthermore, no significant differences in prednisolone pharmacokinetics between the *CYP3A5*3* and *CYP3A5*1* genotypes were revealed. There were no significant differences in prednisolone exposure between the different *ABCB1* genotypes. However, the combination of the *ABCB1* 3455CC and *CYP3A5**3/*3 genotypes revealed significant differences in mean C_{max} of prednisolone, but not for the $AUC_{0\text{-}24}$. Further explorations in the genetics affecting the pharmacokinetic variability of glucocorticoids should be performed in order to individualize glucocorticoid therapy.

To our best knowledge, this is the first study investigating the relationship between prednisolone and prednisone pharmacokinetics. The results in this study indicate a potential for individualization of glucocorticoid dosing after liver transplantation.

However, there are examples of extreme intra-individual day-to-day variability in the early post-transplant phase, which highlight the challenges for implementation into clinical practice. In future studies, the relationship between appropriately timed concentration measurements and the effects of glucocorticoid treatment must be addressed.

4.3 Paper III

Tacrolimus

The $AUC_{0\text{-}12h}$ for tacrolimus increased significantly from follow-up period I (day 1-5 post-transplant) to period II (day 6-10), III (day 11-17) and IV (> 17 days). Median trough concentrations (C_0) were 5.2, 6.5, 8.5 and 8.5 µg/L in the four periods. From period I to period III and IV the tacrolimus doses were doubled. The dose/BW-adjusted $AUC_{0\text{-}12h}$ increased significantly from period I to period II, III and IV. Within two weeks after transplantation, four patients experienced an episode of acute rejection. The pooled (period I-IV) median tacrolimus C_0 were 6.5 µg/L and 7.9 µg/L in the non-rejection group and rejection group, respectively. The median tacrolimus $AUC_{0\text{–}12h}$ (pooled period I-IV) was comparable between the non-rejection group (106 µg*h/L) and the rejection group (107 µg*h/L).

In this study population one of the patients was heterozygous expresser of *CYP3A5**1/*3, while 14 patients were non-expressers (*CYP3A5*3/*3*). The CYP3A5 expresser was transplanted with liver graft from a donor heterozygous for *CYP3A5*1/*3*, while the other donors carried the *CYP3A5*3/*3* genotype. The recipient carrying the CYP3A5*1/*3 variant had lower dose/BW-adjusted $AUC_{0\text{-}12h}$ and C_0, but not outside the range of the non-expresser group.

According to recently published work by De Jonge *et al.* the *CYP3A5* genotype contributes to approximately 30% of the variability in tacrolimus dose requirement and clearance in renal transplant recipients.[67] Furthermore, they describe that hematocrit explains additional 4-14%. In our study, the hematocrit was not investigated in terms of pharmacokinetic variability. Tacrolimus is most importantly metabolized by CYP3A in the hepatocytes. The metabolic capacity of the transplanted liver is affected by the graft function, which may be variable after transplantation. Lock *et al.* showed that the initial graft function after liver transplantation influences the pharmacokinetics of tacrolimus, and is a predictor of tacrolimus trough levels the first week after transplantation.[145] An impaired hepatic CYP3A metabolism due to a delayed graft function post-transplant may

affect the systemic exposure of tacrolimus. When the metabolic capacity of the liver is impaired, the intestinal CYP3A4 might be of higher importance. In addition to the impact of graft function, the *CYP3A5* genotype in liver and intestines affect the tacrolimus exposure and can cause variability in the tacrolimus pharmacokinetics. Recipients with a *CYP3A5*1* carrying liver graft have a reduced concentration/dose ratio, and require a higher dose to reach the target concentration.[146] A liver transplant recipient might receive a graft from a donor with a different *CYP3A5* genotype, which changes the recipient's metabolism post-transplant. Different *CYP3A5* variants in the liver and the intestines make genotyping of the recipient inconclusive because the major contributor to tacrolimus metabolism is the hepatic CYP3A4/5. Ji *et al.* recently investigated the combined effect of the *CYP3A5* genotypes in liver and intestines on the tacrolimus pharmacokinetics after liver transplantation.[147] They found that in the early phase after transplantation, the *CYP3A5* genotype in the native intestines was more important than the genotype in the transplanted liver. With time post-transplant the recipient-donor effect on the dose requirement changed. The benefit of pre-transplant *CYP3A5* genotyping was demonstrated in renal transplant recipients, where the initial tacrolimus dosing was adjusted according to *CYP3A5* genotype.[148] The increased dose requirement to achieve the target trough concentration of tacrolimus carrying the *CYP3A5*1* allele and the fact that this genotype explain a major part of the variability in tacrolimus pharmacokinetics, makes genotyping an attractive approach for further individualization of tacrolimus dosing. However, this is more complex in the liver transplant population since the genotype of the graft (the liver) is of particular importance.

The efflux pump P-glycoprotein functions as an absorption barrier to orally administered drugs.[149] Goto *et al.* investigated the relationship between the intestinal *MDR1* mRNA expression and the *CYP3A5* genotype in the grafted liver in 38 liver transplant recipients. They found that recipients with a high *MDR1* intestinal expression combined with the *CYP3A5*1* genotype had an increased tacrolimus dose requirement the first week after transplantation. [146] Thus, intestinal *MDR1* mRNA expression and *CYP3A5* genotypes explain some of the varibility in tacrolimus pharmacokinetics. As described in section 1.8, the expression of the *CYP3A* and *MDR1* genes is regulated by the human pregnane X receptor, which is activated by glucocorticoids. Due to high doses of prednisolone early after transplantation, induction of *MDR1* and *CYP3A* expression may contribute to the relatively low tacrolimus exposure observed in the first follow-up periods

described in this paper. When the prednisolone doses are tapered, this induction is reduced, and tacrolimus exposure will increase.

Another source of variability in the tacrolimus pharmacokinetics is the administration of grapefruit juice. A prospective study performed in 120 liver transplant recipients by Liu *et al.* demonstrated that co-administration of grapefruit juice increased the bioavailability of tacrolimus.[150] In our study 0, 4, 5 and 3 patients were administered grapefruit juice (200 mL twice daily) in follow-up period I, II, III and IV, respectively. As described in section 1.8, grapefruit juice inhibits CYP3A5, and is used to increase the oral bioavailability of tacrolimus, hence the concentration/dose ratio. This will affect both the $AUC_{0\text{-}12h}$ and dose/body weight adjusted $AUC_{0\text{-}12h}$ observed in this study.

Mycophenolic acid

The medians of MPA $AUC_{0\text{-}12h}$ and C_0 were stabile in the four follow-up periods, ranging from 21.9 mg*h/L to 27.8 mg*h/L and 1.2 mg/L to 1.6 mg/L, respectively. Medians of dose/BW-adjusted $AUC_{0\text{-}12h}$ and C_0 were in the range 1.83-2.25 mg*h/L/(mg/kg) and 0.094-0.116 mg/L/(mg/kg). The recommended target range of MPA $AUC_{0\text{–}12h}$ is suggested to be 30-60 mg*h/L in renal transplant recipients.[120] According to this target range, although this study presents data from liver transplant recipients, 67%, 69%, 53% and 60% of the patients fell below the lower limit of the suggested target in period I, II, III and IV, respectively. The $AUC_{0\text{-}12h}$ was in the range 8.6-57.4 mg*h/L, with median in each period ranging from 21.9 to 27.8 mg*h/L.

The pre-dose IMPDH activity (A_0) showed high inter-individual variability, ranging from 2.2 to 41.2 pmol/10^6cells/min between individuals, with medians of 12.7, 10.1, 11.4 and 6.4 pmol/10^6cells/min in period I, II, III and IV, respectively. Median minimum IMPDH activity (A_{min}) in the study population was between 2.3 and 2.9 pmol/10^6cells/min (range 0.0-12.1) in the four follow-up periods. The median IMPDH inhibition spanned 63% and 77% (range 10-100%) in the four periods. The median pre-transplant IMPDH activity was 14.9 pmol/10^6cells/min (range 9.4-40.6). Three of the patients showed a maximum IMPDH activity 4-fold higher within the dosing interval than the pre-dose concentration. One of these patients was heterozygous for the *IMPDH1* SNPs rs227893 and rs2278294 and the *IMPDH2* variant rs11706052, while one was homozygous for both *IMPDH1* variants. The third patient was heterozygous for rs227893 and rs11706052. These findings might indicate a potential for increasing the immunosuppressive effect of MPA by higher dosing, under monitoring of IMPDH activity,

in selected patients. Alternatively, in patients with low degree of IMPDH inhibition, other immunosuppressive drugs might be considered to achieve adequate immunosuppression.

For MPA the median AUC_{0-12h} was comparable between the non-rejection group (median 25.6 mg*h/L, period I-IV pooled) and the rejection group (median 24.2 mg*h/L). There was a trend towards a lower degree of IMPDH inhibition in the rejection group (median IMPDH inhibition 64.0%, period I-IV pooled) compared to the non-rejection group (median IMPDH inhibition 73.0%).

Although the median MPA exposure and pre-dose concentrations were stable during the four periods, considerable variability was observed between the patients. This inter-individual variability in MPA pharmacokinetics was consistent with findings in other studies.[151-155] In the study performed by Jain *et al*. in eight liver transplant recipients, the median MPA AUC_{0-12h} of 32.3 mg*h/L (range 7.3-102) was reported in the first month post-transplant.[153] Brunet *et al.* found that the MPA exposure was relatively low in 15 liver transplant recipients during the first month after transplantation.[151] They further demonstrated variable exposure of MPA, with median AUC_{0-12h} of 17.4 mg*h/L (day 6 post-transplant, range 13.2-39.7), 16.3 mg*h/L (day 10, range 8.4-51.3), 26.3 mg*h/L (day 16, range 13.1-45.8) and 33.6 mg*h/L (month 3, range 15.1-54.6). The proportion of patients below the target range (30-60 mg*h/L) of AUC_{0-12h} was 85%, 92%, 64% and 30% at day 6, 10, 16 and month 3, respectively. Pisupati *et al.* observed large pharmacokinetic variability in ten patients at three time points during the first six weeks post-transplant, with MPA AUC_{0-12h} (mean± SD) of 50.8±42.1 mg*h/L (≤ 1week), 60.3±38.5 mg*h/L (> 1 week and ≤ 2 weeks) and 118.0±57.6 mg*h/L (≥ 1 week and ≤ 6 weeks).[154] The MPA exposure described by Pisupati *et al.* was generally higher than reported by Jain, Brunet and in our study, where the mean AUC_{0-12h} was within and above the recommended target area. However, a large proportion of the liver transplant patients were reported to reach sub-therapeutic levels of MPA short-term after transplantation.

There are several possible explanations for the observed low MPA exposure in the early phase after liver transplantation. The absorption might be reduced in the early post-operative phase. Anaesthesia and surgical trauma can cause impairment of gastric motility and the absorption of orally administered drugs.[156,157] A second explanation might be an increased clearance short-term after transplantation. An earlier study found that the oral bioavailability of MPA in the immediate phase after liver transplantation was less than 50%, and that an increase in pre-dose concentration and exposure was associated with increasing plasma albumin concentration.[158] These findings were consistent with the

findings of our study, (see section 4.2 for biochemical data). MPA is highly bound to albumin and is a low clearance drug. Thus the clearance of MPA will be affected by the degree of protein binding and plasma albumin levels. Benichou *et al*. report that the variability in free MPA exposure was much higher than that of total MPA exposure in the immediate phase after liver transplantation.[159] An increased risk of leukopenia has been reported for total MPA $AUC_{0\text{-}12h}$ above 40 mg*h/L within 2 weeks after liver transplantation.[152] Although the MPA exposure was in the recommended target range, this does not necessarily reflect the free concentration. Hence patients with impaired hepatic or renal function, liver transplant recipients or patients with hypoalbumineamia might benefit from free drug measurement.

As described in section 1.6, the *UGT1A9* sequence variants -275T>A and -2152C>T cause higher UGT1A9 expression and increased MPA glucuronidation, resulting in a lower MPA exposure. In the study population decribed in this paper one patient was heterozygous for both -275T>A and -2152C>T, the rest carried the wild-type alleles. For the sequence variants -440C>T and -331T>C seven patients were heterozygous and one homozygous for both. In this small group of recipients there were no significant associations between UGT variants and the variability in $AUC_{0–12h}$ and C_0. In another, larger study of renal transplant recipients, carriers of -275T>A and/or -2152C>T variants had lower MPA $AUC_{0\text{-}12h}$.[89] As for the *CYP3A5* genotyping in liver transplant recipients, the liver specific expression makes the interpretation of UGT variants difficult, hence both donor and recipient must be genotyped. Moreover, the *UGT1A9* variants -275T>A and -2152C>T increase the MPA glucuronidation, while the –440C>T and –331T>C variants decrease it. Measurement of the metabolite MPAG might help describing the overall effect of *UGT1A9* sequence variants.

A close, inverse association between MPA plasma concentration and IMPDH activity after oral MMF administration has been demonstrated.[108,160-163] Glander *et al.* demonstrated large variability in pre-transplant IMPDH activity between individuals, and a poor correlation between pre-dose MPA concentration and IMPDH activity.[108] As described in section 1.6, *IMPDH* variants are associated with episodes of acute rejection and the presence of an *IMPDH2* 3757T>C allele is associated with increased IMPDH activity in renal transplant recipients.[105-107] Bremer *et al.* demonstrated that the IMPDH expression in lymphocytes increased early after transplantation, and that increased IMPDH2 expression is associated with acute rejection in renal transplant recipients. [103] High pre-transplant IMPDH activity is also associated with rejection. [108] In liver transplant

recipients, patients with MPA-related side effects tended to have a higher level of *IMPDH2* expression.[164]

Individualizing mycophenolic acid therapy

In our study material, the tacrolimus exposure is generally low in the first study period. The low exposure is followed by an increase in tacrolimus dosing in order to reach target concentrations. As described in section 1.8 the initial low tacrolimus concentrations might reflect the clinical management of renal function in the early post-operative period (period I and II). This relatively low dosing of tacrolimus immediately after transplantation is in a period where the risk of acute rejection is the greatest. Maintaining adequate immunosuppression in this period is crucial to avoid rejection episodes. The efficacy of MPA (i.e. IMPDH inhibition) might be of great importance in this period. In order to ensure adequate immunosuppression in the early-phase after transplantation, PD-monitoring of MPA in combination with PK monitoring of tacrolimus and MPA might be valuable. Several complications and serious adverse effects have been described for calcineurin inhibitors (CNI), where nephrotoxicity is of big concern.[165] As mycophenolic acid is not associated with impairment in renal function, the combination of reduced CNI dosing and concentration controlled MPA dosing is an attractive approach. Assisted by therapeutic drug monitoring, an increased dosing of MPA could provide a more adequate immunosuppression in selected patients in the early post-operative phase. As a result, a more moderate dosing of calcineurin inhibitors (e.g. tacrolimus) might be considered to avoid CNI-induced nephrotoxicity. The Opticept® trial revealed that the combination of low-dose CNI and concentration-controlled dosing of MMF was not inferior to fixed MMF and standard tacrolimus dosing in renal transplant recipients, with treatment failure as the end point.[166]

Pharmacodynamic (PD) monitoring and pharmacogenetics, might in a combination with more conventional pharmacokinetic (PK) monitoring describe the overall MPA response better than PK monitoring alone. To allow reductions of CNI and steroid dosing, PD monitoring may enable a closer follow-up of the patients, while ensuring adequate immunosuppression. However, there are some limitations in the pharmacodynamic monitoring approach. The most suitable marker (e.g. pre-dose or minimum IMPDH activity, degree of inhibition) for MPA efficacy must be further elucidated. A therapeutic range of IMPDH activity must be established. Furthermore, the time point after MPA dose for sampling must be decided. In our study, the time for maximum IMPDH inhibition

(T_{min}) ranged from 0.5 to 10 hours between individuals (medians between 1.0 and 2.0 hours in the four periods), so finding the appropriate sampling time point is challenging. The matrix for measuring the IMPDH activity must be standardized (e.g. whole blood, mononuclear cells or CD4+ cells). Available assays for quantification of IMPDH activity is quite time consuming and laborious, which limits the implementation of PD monitoring in the daily routine. Even though using IMPDH activity as a biomarker for the pharmacological effect of MPA is promising, further investigation is needed to ensure its validity. Larger, prospective studies addressing the PK-PD relationship must be performed.

Conclusion of thesis

Given the role of the liver in the pharmacokinetics of drugs and all the potential factors leading to individual variability in immunosuppressive therapy, the present thesis aimed to describe the pharmacokinetics of glucocorticoids, tacrolimus and mycophenolic acid in the first weeks after liver transplantation. Furthermore, the pharmacodynamics of mycophenolic acid was investigated in addition to relevant genetic analyses.

An LC-MS/MS method was developed for quantification of prednisolone, prednisone, cortisol, cortisone, methylprednisolone and dexamethasone in human plasma, which is applicable for pharmacokinetic studies. The assay was validated and was in accordance with the bioanalytical guidelines from U.S. Food and Drug Administration (FDA). In addition, matrix effects were validated qualitatively and quantitatively, and were satisfactory.

Large intra- and inter-individual variability was observed in the pharmacokinetics of prednisolone and prednisone in adult liver transplant recipients. Dose per body weight adjusted exposure of prednisolone increased significantly during the follow-up periods. A significant decrease in apparent clearance (CL/F) combined with a reduction in the apparent volume of distribution (V_D/F) indicate that the bioavailability (F) increased with time after transplantation. The ratio between pharmacologically active prednisolone and inactive prednisone were generally stable throughout the study periods, but one patient had markedly elevated ratios in all periods, compared to the population medians. In patients receiving methylprednisolone intravenously, the prednisone concentrations in plasma decreased with a subsequent increase in this ratio. The mechanism behind this finding (e.g. sequence variants in *HSD11B1* and *HSD11B2*) contributing to this elevated ratio should be further investigated.

In parallel to the findings for prednisolone and prednisone, the intra- and inter-individual variability of tacrolimus pharmacokinetics was large in the same study population. The tacrolimus exposure was relative low the first week after transplantation, with significant increases in dose per bodyweight adjusted $AUC_{0\text{-}12h}$ from period I to the following periods. In order to attain the target C_0-concentrations the median tacrolimus dose was doubled from period I to period III-IV.

A trend towards a lower degree of IMPDH inhibition was observed in the patient with episodes of acute rejection, compared to the patients without rejection episodes. More than 50% of the patients fell below the suggested target range for MPA exposure (30-60 mg*h/L) in all four follow-up periods, suggesting that the initial dose might be too low the first days post-transplant in some of the patients. Pharmacodynamic monitoring of MPA, by measuring IMPDH activity, may assist in identifying patients with a suboptimal effect of MPA.

The overall conclusion of this thesis is that in the adult liver transplant population, short-term after transplantation, the intra- and inter-individual variability in the pharmacokinetics of immunosuppressive drugs is large. As a consequence, a significant proportion of patients may be at sub-therapeutic immunosuppression in a period when the risk of acute rejection episodes is highest. The factors responsible for this variability must be further addressed and taken into account in order to further individualize the dosing of these immunosuppressive drugs.

Future perspectives

To add further knowledge to individualization of glucocorticoid therapy, future investigations should include studies in other populations (i.e. other patients and healthy volunteers). Studies investigating the glucocorticoid pharmacokinetics in healthy volunteers and in children with acute lymphatic leukaemia and in paediatric transplanted recipients have been initiated by our group. Further research is needed to determine if there is a rationale for therapeutic drug monitoring of glucocorticoids. A possible association between the prednisolone/prednisone ratio and risk of adverse effects should be investigated, whether the ratio can predict the risk of glucocorticoid related side effects or not. Future studies must address the relationship between appropriately timed concentration measurements, and the effects of glucocorticoid treatment of organ transplant recipients. Rejection, graft loss, patient survival and adverse effects should be addressed as end-points in such a prospective study, due to the serious side effect profile of glucocorticoids. Measuring the unbound concentrations of glucocorticoids should be explored, due to the dose-dependent non-linear protein binding in plasma, which affects the biological active fraction available. With regards to the pre-receptor metabolism of glucocorticoids, sequence variants of 11β-HSD should be determined to see whether there is an association between these variants and the pharmacokinetics. The future of pharmacodynamic monitoring of mycophenolic acid relies on larger prospective studies to find monitoring strategies with regards to suitable time points for sampling, sampling material and therapeutic ranges for IMPDH activity. To further investigate the effect of the *UGT1A9* sequence variants on MPA pharmacokinetics, quantification of the metabolite MPAG should be performed.

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