Prednisolone and prednisone pharmacokinetics in renal transplant recipients

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The greater the diversity, the greater the perfection.

Thomas Berry (1914-2009)

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Abbreviations

APC	Antigen-presenting cells
AP-1	Activating protein 1
AUC	Area under the concentration-time curve
AUC_{dose}	The sum of $AUC_{0\text{-}7}$, $AUC_{0\text{-}12}$ or $AUC_{0\text{–}24}$ plus $AUC_{last\text{–}\infty}$ minus the AUC contribution from the previous dose of prednisolone (C_0/k_e), i.e. the AUC used for the determination of CL.
BW	Body weight
C_0	Pre-dose concentration, trough concentration
C_x	Concentration measured X hours post-dose
C_{max}	Maximum concentration
CBG	Corticosteroid binding globulin
CD	Cluster of differentiation
CL	Clearance
CL_{int}	Clearance intrinsic
CNI	Calcineurin inhibitor
CV	Coefficient of variation
CYP	Cytochrome P450
F	Bioavailability
GC	Glucocorticoid
GR	Glucocorticoid receptor
HLA	Human leukocyte antigens
HPA	Hypothalamus-pituitary-adrenal
HPLC-MSxMS	High-performance liquid chromatography coupled with tandem mass spectrometry

HSD	Hydroxysteroid dehydrogenase
IIV	Inter-individual variability
IL	Interleukin
K_e	The elimination rate constant
LSS	Limited sampling strategy
MPA	Mycophenolic acid
MR	Mineralocorticoid receptor
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor-κB
NAD	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate
P-gp	P-glycoprotein
PPE	Percentage prediction error
PL/PN	Prednisolone/prednisone
RTR	Renal transplant recipients
T_{max}	Time to C_{max} within dose interval
TNF	Tumor necrosis factor
TDM	Therapeutic drug monitoring
V_D	Volume of distribution

Definitions

New onset diabetes after transplantation (NODAT) was defined according to the World Health Organization/American Diabetes Association (WHO/ADA) guidelines for the testing and defining of dysglycemic states posttransplant: fasting blood glucose level ≥7.0 mmol/L or 2- hour blood glucose level ≥11.1 mmol/L (1).

Publications included

Paper I: Prednisolone and Prednisone Pharmacokinetics in Pediatric Renal Transplant Recipients—A Prospective Study.

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Ther Drug Monit. 2017; 39 (5): 472-482.

Paper II: Prednisolone and Prednisone Pharmacokinetics in Adult Renal Transplant Recipients

Ragnhild Heier Skauby, MD; Marte Theie Gustavsen, MSc Pharm; Anders M. Andersen, BSc, Nils Tore Vethe, MSc Pharm, PhD; Anders Åsberg, MSc Pharm, PhD, Karsten Midtvedt MD, PhD, Anna Bjerre MD, PhD and Stein Bergan, MSc Pharm, PhD

Ther Drug Monit. 2021; 43 (2): 247-255.

Paper III: In vitro assessments predict that CYP3A4 contributes to a greater extent than CYP3A5 to prednisolone clearance

Ragnhild Heier Skauby, MD; Stein Bergan, MSc Pharm, PhD; Anders Andersen, BSc; Nils Tore Vethe, MSc Pharm, PhD; and Hege Christensen, MSc Pharm, PhD

Basic Clin Pharmacol Toxicol. 2021 Dec;129(6):427-436.

Abstract

Refined immunosuppressive therapy for renal transplant patients has reduced the incidence of acute rejection episodes as a short term benefit. Long term outcome, however, has not improved significantly, and patients face a significant risk of opportunistic infections, cancer and cardiovascular diseases, which all contribute to a reduced long term patient survival. Glucocorticoids remain cornerstones in immunosuppressive therapy, but are associated with a wide range of unwanted side effects, such as hyperglycemia, hypertension, hyperlipidemia and osteopenia. One strategy to improve long term outcome without jeopardizing graft function has been glucocorticoid withdrawal or substitution of glucocorticoids with other immunosuppressive agents. Nonetheless, in the attempt to minimize glucocorticoid immunosuppression to avoid adverse effects, the specific immunological efficacy of glucocorticoids is also weakened, and the risk of acute rejection increases. A safer alternative to glucocorticoid withdrawal or avoidance may be better management of prednisolone therapy. The overall aim of this thesis was therefore to investigate the pharmacokinetics of prednisolone and prednisone, and to identify the need and feasibility for individualizing the dosing of prednisolone.

First, pediatric renal transplant recipients (n=11, age 1-15 years) receiving standardized (mg/kg) doses of prednisolone were recruited at Oslo University Hospital (January 2010 - January 2012). Blood samples were collected, and a 7 or 12-hour concentration-time profile was obtained at 1, 2, 3 4, 12 and 52 weeks post-engraftment.

Then, adult renal transplant recipients (n=28, age 22-78 years) receiving fixed doses of prednisolone, tapered according to protocol (median dose 15 mg (7.5-20), were recruited at Oslo University Hospital (December 2015 – May 2017). Blood samples were collected, and a 24-hour concentration-time profile was obtained within 4-6 weeks post-engraftment.

In both populations, the blood samples were analyzed for total concentrations of prednisolone, prednisone, cortisol and cortisone, and selected gene variants with potential relevance for prednisolone pharmacokinetics were investigated.

A considerable variability in prednisolone and prednisone pharmacokinetics between patients was observed in both populations, even after adjusting for dose and body weight. A larger exposure of prednisolone and a higher ratio of prednisolone/prednisone were observed in patients with new onset diabetes after transplantation (NODAT), in patients receiving concurrent intravenous methyl-prednisolone, and in adults compared with children. A lower exposure of prednisolone in CYP3A5 expressers compared with non-expressers was noted in both the adult and pediatric populations, however, the studies were not powered to investigate this variable. In fasting adults, a strong negative correlation ($p<0.01$) between prednisolone area under the concentration versus time curve (AUC) and morning levels of cortisol was demonstrated. An algorithm based on three pharmacokinetic sampling time points: pre-dose plus two and four hours after prednisolone dosing, predicted prednisolone $AUC_{0\text{-}24}$ in adults with a low percentage prediction error (PPE=5.2±1.5%) and a good correlation of determination ($r^2=0.91$).

Finally, an *in vitro* incubation study was performed, using recombinant CYP3A4 and CYP3A5 expressed in insect microsomes. Applying a substrate depletion approach, the intrinsic clearance of prednisolone was determined in both recombinant enzymes. The results indicated that prednisolone is metabolized by rCYP3A4 rather than rCYP3A5.

In conclusion*:* the observed large inter-individual variability in prednisolone and prednisone pharmacokinetics emphasizes the need for improvements in prednisolone dosing regimens. The results also indicate that a more moderate dosing of prednisolone may be possible, particularly in special patient groups. The work presented in this thesis contributes to knowledge that can be utilized in the development of strategies for individualizing and optimizing prednisolone therapy without compromising efficacy.

1. Introduction

1.1 Renal transplantation

The greatest challenge in transplantation history has been the patient's strong immune response to the new organ (host versus graft reaction). When the first successful renal transplantation between two monozygotic twins was performed in 1954, the genetic match was an advantage (2). Perhaps an even more impressive accomplishment was the successfully conducted renal transplantation between two dizygotic twins two years later, after first suppressing the recipient's immune system utilizing total body irradiation (TBI) (3). However, neither TBI nor the early immunosuppressive mercaptopurine improved graft and patient survival. In 1963, less than 10% of several hundred allograft renal transplant recipients had survived as long as 3 months (4). The future for human transplantation looked rather grim. A new immunosuppressive protocol was then presented by Thomas Starzl. He reported that although rejection usually occurred in patients on azathioprine (a pro-drug for mercaptopurine) alone, it was usually reversible with large doses of prednisone. This was a game-changer in human transplantation with one-year renal graft survival improving to over 70% (3). Since then, renal transplantation has moved from experimental to standardized therapy.

The first allogeneic renal transplantation in Norway was performed at Rikshospitalet, Oslo in 1956. The patient survived for 30 days, which was remarkable, given the insufficient immunosuppressive treatment available at that time (TBI and cortisone) (5). Today, all renal transplantations in Norway are conducted at Oslo University Hospital, Rikshospitalet. Counting both living and deceased donors, between 250 to 300 renal transplantations have been performed per year the last 10 years, making the center the largest in northern Europe (6). The list of patients actively waiting for a kidney transplant at the end of 2020 included 411 patients. Primary renal diseases before renal transplantations in the period 2010-2020 are given in figure 1.

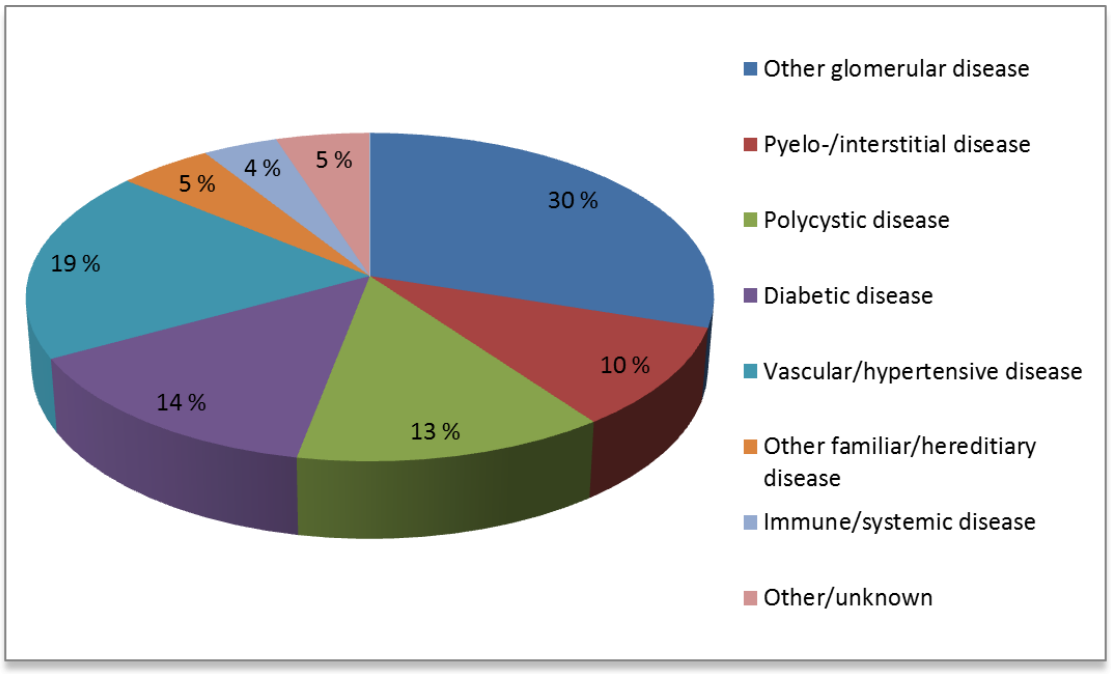

Figure 1: *Primary renal disease before renal transplantation 2010-2020 (6)*

(Morten Heier Skauby, with permission)

Due to the advances in immunosuppression and subsequent decrease in early acute rejections, one year graft survival has improved and is currently above 95% for renal transplant recipients in Norway. Long term graft survival, however, has not improved significantly over the last decades (6). Too high exposure of the immunosuppressive drug calcineurin inhibitor (CNI) causes nephrotoxicity, which is considered to be the major contributing cause to the lack of improvement in graft survival (7).

Following deceased donor transplantation, one year patient survival in Norway is today approximately 97%, whereas the 5- and 10-year survival is about 84% and 62%, respectively. Following living donor transplantation, the 1, 5- and 10-year patient survival is about 99%, 95% and 85%, respectively (figure 2) (6). One of the main challenges in transplantation is that the lifelong immunosuppressive therapy includes a number of side effects. Hence, in addition to be fundamental in renal transplant therapy, immunosuppression drugs constitute a significant risk of opportunistic infections, cancer and cardiovascular diseases, which all contribute to a reduced long term patient survival (8).

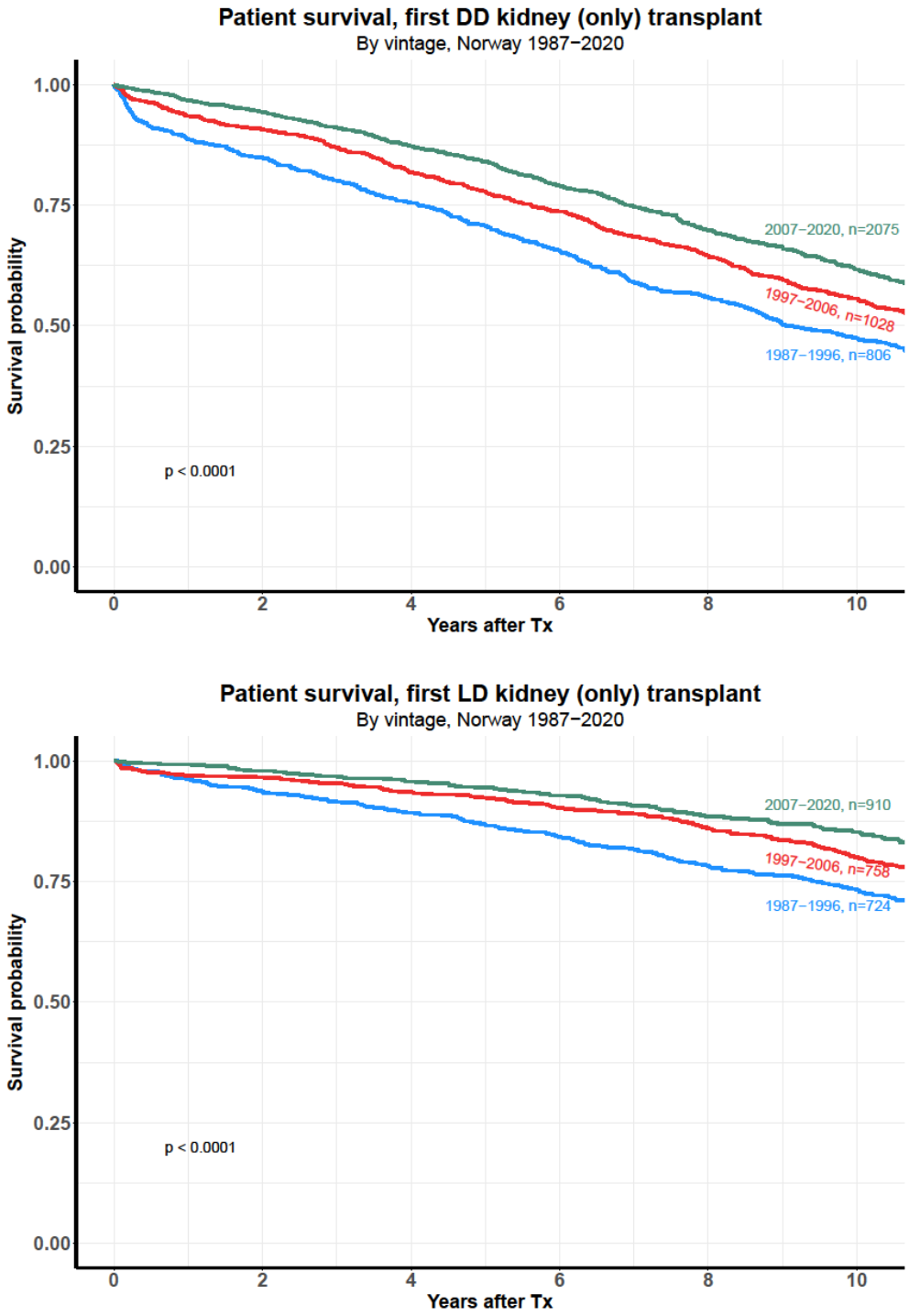

Figure 2: *patient survival for first deceased donor (top) and first living donor (bottom) renal transplantation (6). DD; deceased donor, LD; living donor; Tx; transplantation (Anders Åsberg, with permission)*

1.2 Immunology in allograft transplantation

When an allo-transplantation is performed, cells, tissues, or organs are transferred between genetically non-identical members of the same species. The transplant is called an allograft. When foreign material (e.g. virus, bacteria) enters the body, the immune system is activated and elicits an immune response that recognizes, responds and resolves infection and disease attacks. The allograft is recognized as foreign by the recipient's immune system and triggers a massive allo-immune response, which eventually leads to an acute or chronic rejection and graft loss.

This immune response (allo-reactivity) is primarily T-cell dependent. Human leukocyte antigens (HLA) from the allograft are processed and presented as peptide antigens by self-HLA molecules on the surface of antigen-presenting cells (APC), like dendritic cells or macrophages. The recipient's naïve T cell recognizes the non-self peptide antigen and triggers activation and proliferation of the T-cell through three independent signals (figure 3). Signal 1(specific antigen signal) is a stimulatory signal from the T-cell receptor, generated by the binding of the HLA complex on the surface of APC to the T-cell receptor. Signal 2 (non-specific antigen signal) is a stimulatory co-signal, initiated when CD80 and CD86 (co-stimulating molecules on the APC) bind to CD28 on the T-cell. Lack of signal 2 in presence of signal 1 has been shown to leave the T-cell in a state of anergy, without the ability to attack the allograft. Once the T cell has received a specific antigen signal 1 and a general signal 2, three signal transduction pathways are activated: the calcium-calcineurin pathway, the mitogen activated protein (MAP) kinase pathway and the nuclear factor-κB (NF-κB) pathway, which in turn activate the transcription factors: nuclear factor of activated T cells (NFAT), activating protein 1 (AP-1) and NF-κB. The transcription factors translocate to the nucleus and induce mRNA synthesis of pro-inflammatory cytokines, such as IL-2, IL-3, IL-4, IL-5, interferon gamma, tumor necrosis factor alpha (TNFα) and granulocyte colony stimulating factor (CSF). IL-2 binding to its receptor activates the mTOR ("mechanistic target of rapamycin") pathway (signal 3), resulting in T-cell clonal proliferation. The pro-inflammatory cytokines secreted from the activated T-cell

stimulate other T-cells to undergo clonal expansion, differentiate into effector T-cells, augment natural killer cell cytolytic activity and migrate into the allograft where they promote tissue destruction (8-12).

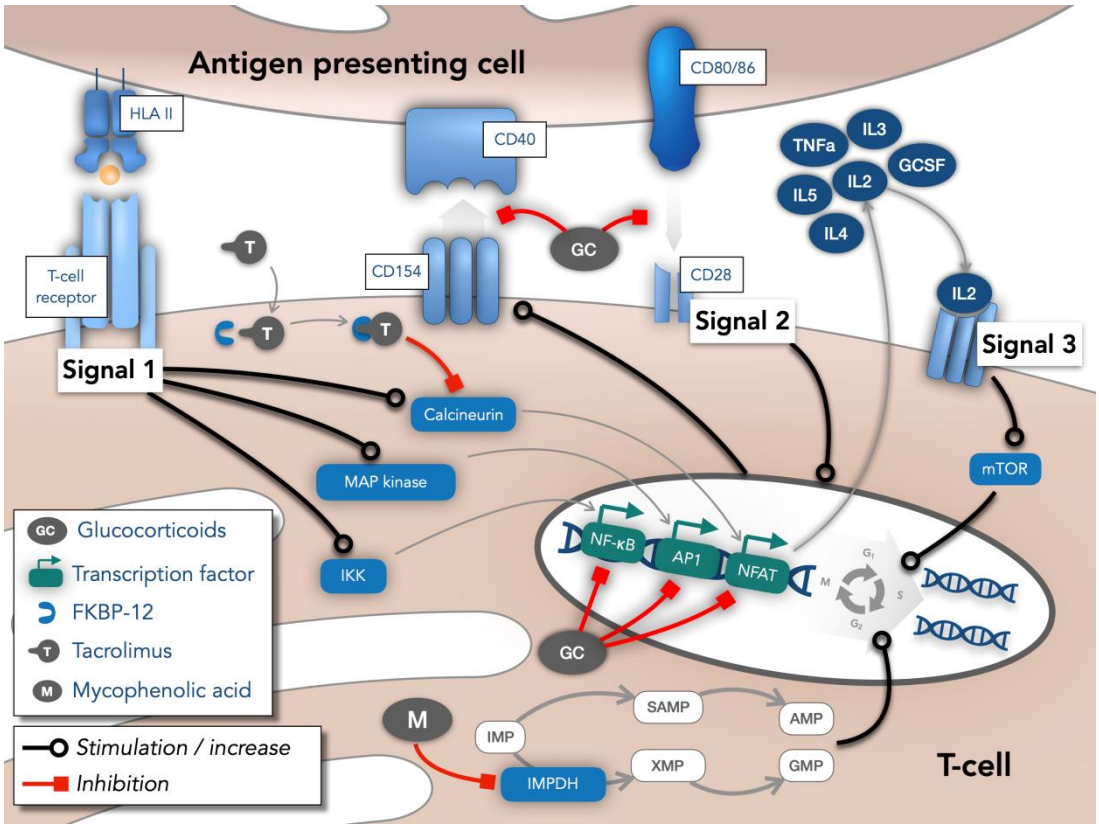

Figure 3: *T – cell activation and immunosuppressive targets (Rolf Klaasen, with permission)*

An allo-immune activation triggering cellular and antibody responses will lead to an acute or chronic rejection, and inevitably a graft loss. To prevent or to reverse this process, renal transplant recipients are in need of lifelong immunosuppressive treatment.

1.3 The immunosuppressive protocol in renal transplantation

The allo-immune response is primarily T-cell dependent, hence the main target for immunosuppressive drugs are T cells and T cell signaling molecules. Their mechanisms of actions are different (figure 3), but in general aimed at preventing lymphocyte activation, proliferation or downstream effector mechanisms.

In Norway, the current standard immunosuppressive protocol after renal transplantation is a quadruple regimen consisting of intravenous induction therapy with two doses of 20 mg basiliximab plus methylprednisolone 250 mg (350 mg iv if body weight (BW)> 90 kg) and peroral maintenance therapy of low-dose CNI in combination with mycophenolic acid (MPA) and prednisolone. Per oral prednisolone is administered in the morning according to a tapering schedule starting at 20 mg/day, and tapered to a maintenance dose of 5 mg/day by day 180 (13). In cases of acute rejection episodes, intravenous methylprednisolone is administered.

Both prednisolone and methylprednisolone belong to a class of corticosteroid hormones called glucocorticoids (GC), which have served as the backbone of immunosuppressive therapy since the beginning of the transplantation era. Although new and sophisticated immunosuppressants have contributed to an improvement of clinical outcome, GCs remain cornerstones in the immunosuppressive regimen.

1.4 Glucocorticoids

GCs are used therapeutically in a variety of clinical contexts: as frontline therapy for autoimmune diseases, for preventing the rejection of solid organ transplants and in the combinatorial therapy of certain cancers. Despite the development of targeted antibody-based therapies, GCs remain a critical component in the management of such diseases. GCs are among the most widely prescribed drugs in developed countries, taken by up to 1.2% of the adult population (14, 15).

The naturally occurring glucocorticoid, cortisol, is synthesized in the adrenal gland in response to stress and is regulated via negative feedback via the hypothalamus-pituitary-adrenal (HPA)-axis (figure 4).

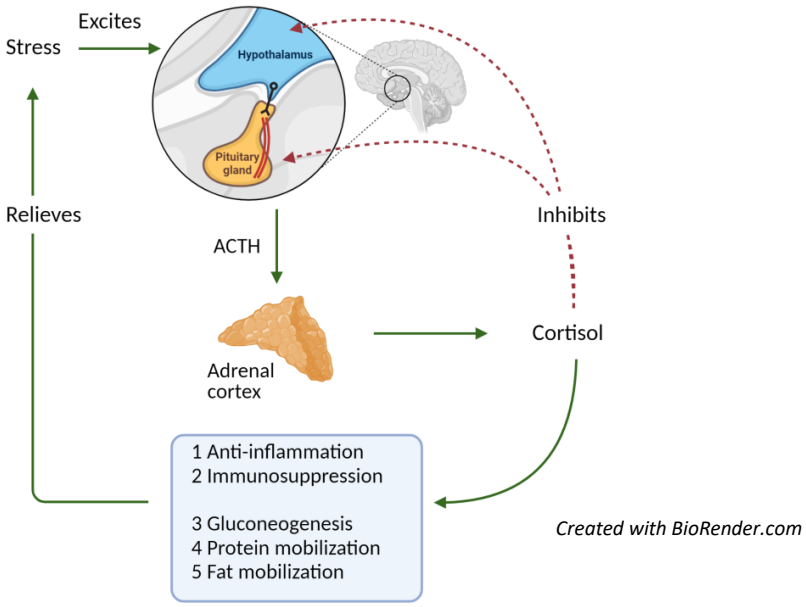

Figure 4: *Regulation of cortisol (ACTH; adrenocorticotropic hormone)*
(modified from Guyton and Hall (16)

In an unstressed adult, the release of cortisol follows a circadian rhythm, where circulating levels are high in the morning between 06:00 and 08:00 am, decline during the day and reach a nadir between 23:00 pm and 01:00 am before a slow increase again starts between 02:00 and 04:00 am. Cortisol modulates cellular and tissue functions, and efficiently suppresses the normal response of the immune and inflammatory systems when these systems are triggered by exogenous

stimuli (17). After the compound was isolated and characterized from adrenal cortex in the laboratories of Kendall and Reichstein in the late 1930s, the biochemical properties of cortisol was soon acknowledged as attractive for therapeutic use (18). Kendall's compound E (cortisone) was synthesized for the first time in 1949, and in the late 1950s, Philip Hench used synthetic glucocorticoids for the first time in patients with rheumatic arthritis and rheumatic fever (19). The patients showed remarkable improvement in what so far had been perceived as relentless diseases. Kendall, Reichstein and Hench received a Nobel Prize in 1950 for their discoveries concerning hormones of the adrenal cortex. Synthetic GCs such as prednisolone, methylprednisolone and dexamethasone are based on the cortisol (hydrocortisone) structure, with modifications that enhance selected properties, e.g. increased anti-inflammatory effect, reduction of the affinity to the mineralocorticoid receptor (MR) or increased glucocorticoid receptor (GR) binding (20). Because their hepatic metabolism is lower, synthetic GCs have longer half-lives than cortisol (half-life of about 1.8 hour). Thus, GCs of different potencies may be divided into short acting /intermediate/long acting drugs (table 1) (21-23). Prednisolone and other synthetic glucocorticoids suppress the endogenous cortisol secretion (17, 21).

Glucocorticoid	Glucocorticoid potency	Mineralocorticoid potency	Equivalent Dose (mg)	Plasma half-life (hours), oral dose
Cortisone	0.8	0.8	25	0.5
Cortisol (hydrocortisone)	1	1	20	1.8 ± 0.5
Prednisone	3.5 – 5	0.8	5	3.3 ± 1.3
Prednisolone	4	0.8	5	3.2 ± 1.0
Methylprednisolone	5-7.5	0.0	4	2.5 ± 1.2
Betamethasone	25–30	0.0	0.75	6.5
Dexamethasone	25–80	0.0	0.75	4.0 ± 0.9
Mineralocorticoid				
Aldosterone	0.3	200 - 1000		< 0.33

Table 1: *the relative potencies and equivalent doses of common glucocorticoids and one mineralocorticoid (aldosterone). Because prednisone is rapidly converted to prednisolone in vivo (see section 1.7), the drug's properties are quite similar to prednisolone. (modified from Asare et al. (24))*

1.4.1 Mechanisms of action

The effects of GCs are mediated by two main mechanisms of action: the genomic and the non-genomic effect. The genomic effects of glucocorticoids depends on initial binding to the GR, and develop slowly over a time span of 4-24 hours, as it implicates time-consuming processes like mRNA transcription and translation for protein synthesis. The non-genomic mode of action, however, do not influence gene expression and are characterized by a rapid onset (seconds to minutes) and short duration of action (60-90 min) (21, 22, 25).

The genomic effects are exerted as the lipophilic GC diffuse across the cell membrane and binds to the GR, which causes a conformational change in the receptor. The GR-GC complex translocates into the nucleus of the cell (figure 5), binds to specific glucocorticoid response elements (GREs) that are associated with genes that either suppress or stimulate transcription, and subsequently initiates gene induction or repression. Production of anti-inflammatory mediators is increased (transactivation), whereas production of pro-inflammatory mediators is decreased (transrepression) (20-22).

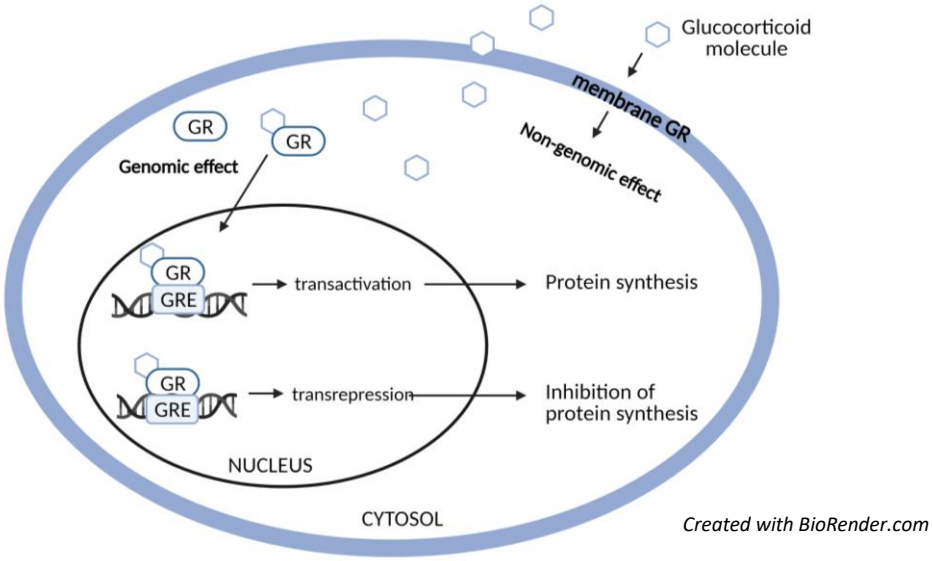

Figure 5: *Genomic and non-genomic actions of glucocorticoids.*
GR; glucocorticoid receptor, GRE; glucocorticoid response element (modified from Ponticelli et al. (22))

The non-genomic effects are mediated either through intracellular or membrane-bound GRs, or via direct interaction of GCs with cell membranes, changing the physicochemical properties of the membranes and affecting the activity of membrane associated proteins (21, 26). Non-genomic effects result in inhibition of inflammatory cell function (25), and it is believed that the rapid non-genomic effects are a way of preparing the cell for the following GC induced genomic changes, and closing the time-gap between the immediate need of GC effects and the delayed genomic response (22).

The **regulatory and metabolic effects** of GCs are mediated mainly via genomic mechanisms. The **regulatory effects** are the anti-inflammatory and immunosuppressive activities of GCs, which are exerted on different levels against a host-defense response in the body.

The anti-inflammatory effects of GCs result from induction of anti-inflammatory cytokines (such as IL10 and TGFβ), and repression of pro-inflammatory cytokines (including TNFα and various interleukins) (27, 28). Furthermore, GCs suppress the production of inflammatory enzymes (e.g. arachidonic acid and its metabolites, cyclooxygenase (COX)-2), which are important mediators of the inflammatory response (29). GCs counteract oedema induced by inflammation, by inducing the synthesis of enzymes that degrade bradykinin, a vasodilator (30), and reduce the migration of immune cells to the site of inflammation by repression of adhesion molecules (31).

The immunosuppressive effects of GCs result from inhibition of T-cell activation and subsequent B-cell activation, T-cell proliferation and T-cell differentiation. This is achieved by suppression of NFAT, AP-1 and NF-κB, thus inhibiting the production of several pro-inflammatory cytokines (including IL-2), thereby blocking signal 1 in the immune response (figure 3) (27, 32). By blocking the costimulatory CD40 ligand (i.e. CD154) on lymphocytes and the costimulatory molecules (CD40, CD80 and CD86) on dendritic cells (figure 3), GCs inhibit antigen presentation in dendritic cells and T-cell differentiation from Th0 to Th1 cells (33, 34). In addition, GCs induce apoptosis of immune cells (lymphocytes),

leading to a weakening of the immune response. Apoptosis of T-cells is indicated as a contributing mechanism to the immunosuppressive effect of intravenous methylprednisolone pulse therapy (35).

In sum, the effects of GCs result in an anti-inflammatory effect which includes vasoconstriction, a reduced capillary permeability and a decreased rate of leucocyte migration to tissues. Furthermore, and more importantly in a renal transplant setting, the immunosuppressive effects of GCs result in an inhibition of the initial immune response in addition to a suppression of activated T cells that blocks the cell-mediated immune response. GCs prevent acute rejection of the allograft by down-regulating adhesion molecules and chemokine receptors that are upgraded in acute rejection episodes, and by inducing apoptosis of T-cells.

The main outcome of the **metabolic effects** of GCs is an increase in blood glucose concentration. Through transactivation, GCs alter the balance of carbohydrates (enhance gluconeogenesis, reduce consumption of glucose in the cells, increase liver glycogen storage, reduce uptake of glucose by producing insulin resistance in both muscle and adipose tissue) and proteins (increase catabolism, reduce anabolism). In addition, GCs facilitate lipolysis and redistribution of fat (17).

1.4.2 Adverse effects

Immediately after the discovery and therapeutic use of "Kendall's compound E", cortisone was tested in numerous studies and clinical conditions (18), proving to be highly efficient in adrenal deficiency disorders, inflammatory diseases and some types of malignancies – although detrimental in infections. However, because of the wide distribution of GR, the range of GC effects is extraordinary and includes both benefits and harms. A myriad of adverse effects have become evident after long-term GC treatment, whereas less frequently after short-term treatment (36, 37).

GC treatment is a risk factor for hyperglycemia in patients without known diabetes mellitus (induction of diabetes) and aggravation of established diabetes mellitus.

Insulin resistance has been associated with prednisolone dose, both short and long term after renal transplantation (38, 39). Insulin resistance increases very low-density lipoprotein (VLDL), free fatty acid and triglyceride concentrations, and lowers high-density lipoprotein (HDL) concentrations (40). In addition, GCs induce arterial hypertension. Taken together, long term GC usage leads to an increased risk of cardiovascular disease. Bone loss from collagen catabolism leading to osteoporosis and fractures caused by long-term GC therapy are well documented (37). An impaired linear growth in children using systemic GC is commonly experienced (41, 42), and the effect seems more pronounced in pre-pubertal than pubertal patients. Furthermore, skin and muscle atrophy due to increased protein catabolism, gastric ulcer, redistribution of adipose tissue centrally to the trunk, neck, and face, increased appetite and weight gain, sleep disorders are all adverse effects associated with GC therapy (43). Modest doses of glucocorticoids can improve mood, but at therapeutic doses, they can also cause psychosis (17).

1.5 Glucocorticoid withdrawal or avoidance in renal transplantation

In pediatric renal transplant recipients, the GC induced growth retardation and development of cardiovascular diseases is of particular concern (43). Over the past two decades, there has been an increasing interest in immunosuppressive regimens where prednisolone or prednisone is minimized, eventually withdrawn or completely avoided. From 2000-2009, several randomized controlled trials were conducted, evaluating "steroid avoidance" or "withdrawal" (SAW) regimens in pediatric renal transplant recipients (42, 44-46). Death and graft loss at five years were found to be significantly lower for children withdrawn from GCs (45). Kidney function was reported not significantly different between patients receiving GCs and patients in GC withdrawal group, two years post-transplant (44). An improved longitudinal growth, a lower prevalence of the metabolic syndrome and a decrease of the prevalence and severity of arterial hypertension were among the reported benefits of the SAW regimens (42, 44, 46). Due to their low sample sizes, these studies were limited and not sufficient to draw robust conclusions (47). Nevertheless, in 2009, the Kidney Disease: Improving Global Outcomes (KDIGO) guidelines recommended that GCs could be discontinued during the first week of transplant in patients at low immunologic risk (48, 49). Appropriate identification of the low-immunologic-risk patient eligible for a SAW regimen proved to be challenging, and the practice of GC avoidance varied among transplant centers. In 2016, Zhang et al suggested that a patient eligible for a SAW regimen should have the following characteristics: prepubertal, caucasian, primary disease not related to immunological factors, *de novo* kidney transplant recipient and a low panel of reactive antibodies (47). In 2017, Nehus demonstrated in a propensity score-matched cohort analysis that GC avoidance can be safely practiced also in pediatric patients with higher risk, and that the decision to use GCs should "not be solely based on race, sensitization or other risk factors for inferior outcomes". Rather, the immunosuppression regimen should be tailored to the individual patient (48). At the Norwegian National Transplant Center, a "steroid-free protocol" (i.e. withdrawal of prednisolone maintenance dose within

1-6 months post-transplant) for pediatric patients with low immunological risk has been in use since 2018.

For adult renal transplant recipients, the results are different from the pediatric population. As reviewed by Haller et al., steroid withdrawal or avoidance in adult renal transplantation patients show little or no effect on mortality or graft loss, however, the risk of acute rejection did significantly increase by 77% after GC withdrawal and by 58% after GC avoidance, compared with GC maintenance (50). The protocol for adult renal transplantation at the Norwegian National Transplant Center includes the use of GCs, regardless of immunological risk.

Although a steroid-free regimen is successfully practiced in a selected pediatric patient group, the numerous adverse effects of GCs persist to be a clinical problem in the remaining population of renal transplant patients. To obtain an optimal balance between efficacy and toxicity for each individual patient, a further knowledge of the relationship between prednisolone exposure and efficacy and adverse effects is required.

1.6 Pharmacokinetic principles

Pharmacokinetics refers to the processes of drug absorption, distribution, metabolism and excretion, and is a quantitation of the time course of a drug and its metabolites in the body. The fraction of ingested dose reaching the systemic circulation and site of action, to what extent the drug is distributed to different tissues and how efficiently the drug is eliminated from the body is determined by numerous factors such as genetic factors, age, gender, BW, interactions with drugs given concomitantly, physiological states, pathological conditions and environmental factors. The key to determine the optimal dose regimen and drug therapy for the individual patient lies in thorough knowledge about the factors causing differences in pharmacokinetic processes and drug response (51).

1.6.1 Absorption

For a drug given as oral dosing, absorption is defined as the movement of drug across the intestinal epithelium (51). The drug encounters several barriers on its way to the systemic circulation, e.g., metabolizing enzymes in the gut wall or removal of drug in the liver, all of which may reduce the fraction of dose that reaches the circulation. Bioavailability (F) is defined as the fraction of the administered dose systemically absorbed intact (51). The rate and extent of drug absorption is primarily influenced by gastric emptying time and intestinal motility, both of which are initially reduced after surgical procedures (e.g. renal transplantation).

1.6.2 Distribution

Drug distribution refers to the reversible movement of a drug between the blood and various tissues of the body. Drugs in the body can be distributed in an unbound state or bound to plasma proteins or tissue components. Normally, only the unbound fraction of a drug is capable of diffusing through cell membranes to exert a pharmacological effect (51). Since drug binding in general is rapidly

reversible, the bound and the unbound forms of the drug can be assumed to be in a dynamic equilibrium at all times. This equilibrium will partly depend on physical (e.g. hydrophobic vs hydrophilic, degree of ionization) and physiological (e.g. protein binding, tissue uptake) properties and processes (52), which means that drug distribution is affected by factors like protein binding, pH, membrane permeability, blood flow and body composition. Volume of distribution (V_D) is an indicator of the extent of drug distribution into body fluids and tissues at equilibrium, and is defined by amount of drug in the body relative to the plasma drug concentration (51).

1.6.3 Elimination

Elimination is the irreversible removal of drug from the body, either in an unaltered form (unbound) or modified as a metabolite. Drug elimination can take place through both excretion and metabolism. Excretion is the irreversible loss of chemically unchanged drug through the kidneys (renal elimination), and sometimes via the bile or in the breath (51). A few, water-soluble, drugs are excreted directly and entirely via the kidneys in the urine. Most drugs, however, need to be chemically altered before they are eliminated through the kidneys.

Metabolism (non-renal elimination) is the major pathway for elimination of drugs. This occurs predominantly in the liver, but other organs such as the kidney, lung, blood, gastrointestinal tract, brain and placenta may also have metabolic capacity. The most common drug metabolizing reactions involves oxidation, reduction or hydrolysis (phase I) followed by conjugation (phase II) of the drug (51). The major group of phase I metabolizing enzymes is the cytochrome P450 (CYP) superfamily. Of 57 CYP isoforms identified in humans only about a dozen enzymes, belonging primarily to the CYP1, 2, and 3 families, are relevant for drug metabolism. CYP1, 2, and 3 contribute to the metabolism of 70-80 % of all drugs in clinical use. The CYP3A subfamily, mainly comprising two isoforms CYP3A4 and CYP3A5 in adults, is responsible for the oxidative metabolism of over 50% of the drugs in widespread use (53). Due to the large distribution and variability of

CYP3A enzymes in both liver and small intestine, these enzymes are considered to have a major influence on first pass metabolism and therefore the bioavailability after oral drug administration (54).

Clearance (CL) is the volume of blood completely cleared for drug per unit of time (L/h or mL/min). CL provides a measure on how efficient the drug is eliminated from the body, primarily as the sum of liver metabolism and renal excretion. The elimination rate constant (k_e) is the fraction of a drug that is eliminated from the body per unit of time. It describes the rate at which a drug is removed from the body. The rate of elimination is described as the product of clearance and drug concentration (51).

The drug concentration in the body is determined by the processes of absorption, distribution, metabolism and excretion. The time course of a drug and the systemic exposure can be illustrated by plotting a drug-concentration curve (figure 6).

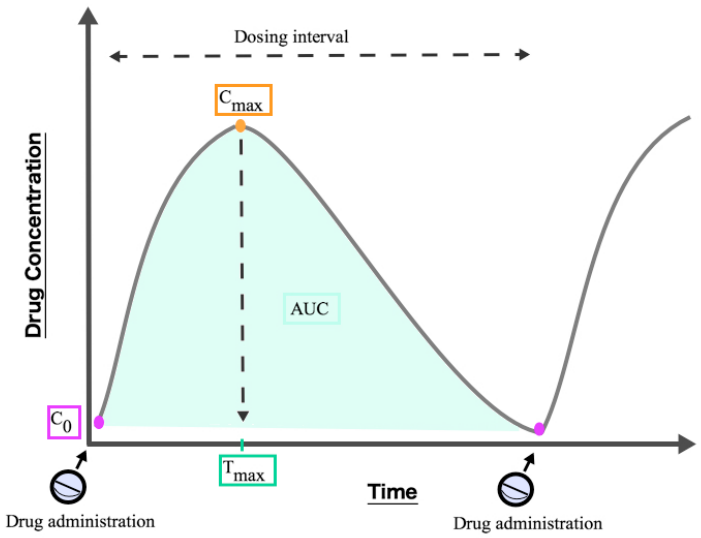

Figure 6: *C_0 is the drug concentration measured right before the next dose is administered, and is usually the lowest concentration in a dose interval. C_{max} is the maximum drug concentration at time T_{max}. The area under the concentration versus time curve (AUC) represents the total drug exposure as a function over time.* *(Marte Theie Gustavsen, with permission)*

1.7 Prednisolone and prednisone

In addition to the regulation of GCs via the HPA axis, there is also tissue specific regulation. Since only hormonally active GC is capable to elicit a response in the GR, local GC tissue concentrations can be further controlled through the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymatic system, for which both endogenous and synthetic GCs are substrates (55). The hormonally inactive prednisone must be converted to its hormonally active compound, prednisolone, by the enzyme 11β-HSD1 in order to exert its physiological effect.

1.7.1 Interconversion between active and inactive glucocorticoids

The biological activity of GCs is determined by a hydroxyl group at position C11 of the steroid structure (hydroxysteroid), whereas an oxidation of this group to an 11-keto group inactivates the GC (ketosteroid). In this way, the two isozymes of 11β-HSD (11β-HSD1 and 11β-HSD2) control the availability of GC for binding to the glucocorticoid receptor (GR) (56, 57). The 11β-HSD1acts primarily as a NADP(H) dependent reductase, converting inactive GC to active GC, whereas the function of the NAD dependent dehydrogenase 11β-HSD2 is to transform the active GC to inactive GC (figure 7). *In vitro*, however, the 11β-HSD1 has been shown to be a bi-directional enzyme, capable to carry out both reductase and dehydrogenase activity (57).

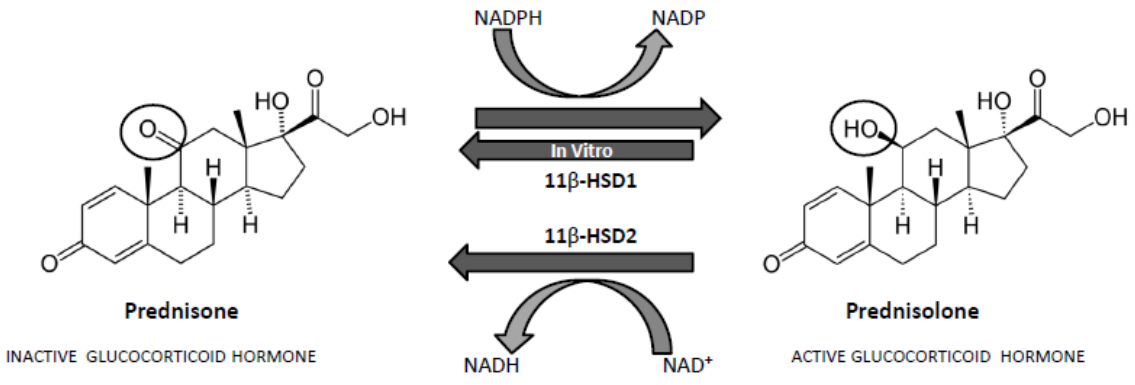

Figure 7: *Interconversion between inactive prednisone and active prednisolone by the enzymes 11β-hydroxysteroid dehydrogenase (11β-HSD) 1 and 2 (modified from Draper et al. (57))*

11β-HSD1and 2 are encoded by two different genes, *HSD11B1 (*located on chromosome 1) and *HSD11B2* (located on chromosome 16), sharing only 21% homology (58, 59). The expression of the two enzymes is tissue specific, a feature that plays a crucial role in regulating both glucocorticoid and mineralocorticoid receptor activation. Because its function is to supply tissue with active GC, the 11β-HSD1 is highly expressed in glucocorticoid target tissues, such as liver, lungs, gonads, pituitary, adrenal cortex, central nervous system and adipose tissue. In contrast, 11β-HSD2 is typically expressed in mineralocorticoid target tissues such as the kidneys, colon, salivary glands and placenta. Although the ligand for the MR is primarily aldosterone, GCs also have binding affinity for the MR, and circulating concentrations of GCs are several orders of magnitude higher than aldosterone (60). The important function of 11β-HSD2 is to inactivate GCs, thereby protecting the MR from occupation by high concentrations of cortisol and prevent undesired stimulation of the MR.

The activity of 11β-HSD1and 2 is associated with several pathological conditions. Increased adipose 11β-HSD1expression and activity is found in patients with metabolic syndrome (61, 62), but there are mixed results regarding the association between 11β-HSD1 polymorphisms and metabolic syndrome according to a recent review by Gregory et al. (63). Nevertheless, interventional studies with 11β-HSD1 inhibitors have been shown to improve insulin sensitivity, lower fasting blood glucose and reduce HbA1c, BW and high-density lipoprotein (LDL) (64, 65).

A reduced 11β-HSD1 activity due to a functional polymorphism in *HSD11B1* has been associated with polycystic ovary syndrome (PCOS) (66), however, larger studies have shown no linkage between polymorphisms in *HSD11B1* and PCOS (18, 67).

Decreased or inhibited 11β-HSD2 activity results in high concentrations of active GCs in mineralocorticoid tissues (e.g. the kidney), and subsequently hypertension and hypokalemia (18). Gene variants that significantly impair 11β-HSD2 activity have been associated with essential hypertension (68, 69), although other studies have been negative (70). Rather, loss of function of 11β-HSD2 activity may be

regarded as a contributing factor to hypertension, along with dietary salt intake and impairment of homeostatic systems controlling blood pressure. In humans carrying null mutations in the *HSD11B2* gene, however, the syndrome of apparent mineralocorticoid excess (AME), characterized by hypertension, hypokalemia and low renin levels despite normal aldosterone levels is observed (71).

1.7.2 Pharmacokinetics

Absorption

Prednisolone and prednisone are rapidly and quite extensively absorbed after oral administration with a peak plasma concentration (C_{max}) within 1-2 h after oral dosing (72). Food intake prolongs the time to C_{max} (T_{max}), but not the extent of drug absorption. The conversion of prednisone to prednisolone is usually rapid and comprehensive (73). Both drugs are substrates for the transmembrane P-glycoprotein (P-gp) encoded by the *ABCB1* gene (74, 75). This efflux transporter provides a functional barrier in a variety of tissues, especially small intestine and kidneys, and is a major determinant of how much of the drug will get into the systemic circulation after an oral dose. In renal transplant recipients, bioavailability of total prednisolone from oral prednisone and oral prednisolone has been reported as 86.1±9.1% and 93.6±9.2%, respectively (72).

Distribution

Prednisolone displays dose-dependent pharmacokinetics; as dose and drug concentrations increase, the V_D and CL increase (76). The increase in CL may be due to induction of metabolism or saturable binding to plasma proteins. Hence, a prednisolone dose of 0.15mg/kg has a V_D of 29.3L, while a 0.30 mg/kg dose has a V_D of 44.2L (73). This can be explained by non-linear protein binding for protein bound drug. 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 (73, 77). At plasma concentrations of about 200 µg/L, the protein

binding of prednisolone is approximately 95%, and decreases non-linearly down to 60-70% at plasma concentrations of 800 µg/L (21, 78). Whereas V_D and CL increase with drug concentration, the elimination half-life is relatively constant for prednisolone doses given as maintenance therapy (<60-70 mg/day) (21, 79). At very high doses prednisolone CL is reported to decrease, indicating a partial saturation of the metabolizing enzymes (21, 80).

The relationship between plasma prednisolone and prednisone concentrations changes during a dose interval, and may be explained by the concentration–dependent pharmacokinetics of prednisolone and differences in affinity for CBG for prednisolone and prednisone. Whereas albumin binds prednisolone and prednisone with equal affinity, CBG binds prednisolone with a 10 fold greater affinity compared with prednisone. In presence of prednisolone, prednisone is displaced from the CBG-binding sites and binds primarily to albumin. With increasing prednisolone concentrations, CBG capacity is exceeded, and prednisolone shifts to albumin with low affinity. This causes an increase in unbound prednisolone from about 5-10% at low concentrations up to 40% at maximum prednisolone concentrations. Due to the large binding capacity, prednisolone and prednisone do not compete for albumin binding sites. Hence, the unbound fraction of prednisone remains stable at about 45% regardless of prednisolone concentration, whereas protein binding and the unbound fraction of prednisolone are concentration dependent (73, 76, 81, 82). These differences in concentration-dependent binding patterns result in an increased clearance at high concentrations for prednisolone, but not for prednisone. Hence, as prednisolone concentrations decrease during a dose interval, the PL/PN ratio decrease correspondingly (81).

Elimination

A small fraction of an oral or intravenous dose is directly excreted as prednisolone (12-26%) and prednisone (2-4%) by the kidneys (78, 83, 84). *In vitro* studies in animals also indicate that prednisolone can be metabolized by renal tissue (85).

Interconversion between prednisolone and prednisone ensures that about 76% the drug dose is recycled. Clearance of prednisolone by inactivation to prednisone is 4-10 fold greater than vice versa, and occurs until the 11β-HSD2 enzyme is saturated at a prednisone concentration of about 60 µg/L (76, 83). The major mechanism for prednisolone and prednisone elimination, however, is through hepatic metabolism involving phase I and phase II biotransformation. The isoenzymes CYP3A4 and 5 are assumed to be involved in prednisolone metabolism, as they are reported to catalyze several other steroid hormones (86-89). However, the degree of involvement of these enzymes, especially the role of CYP3A5, in prednisolone and prednisone metabolism has not been fully elucidated. Prednisone, 20-dihydro-prednisolone and 6β-OH-prednisolone are reported to be among the major unconjugated metabolites of prednisolone, but more than 20 metabolites are described (figure 8) (90). After biotransformation in the liver, the hydrophilic metabolites are subsequently excreted in the urine.

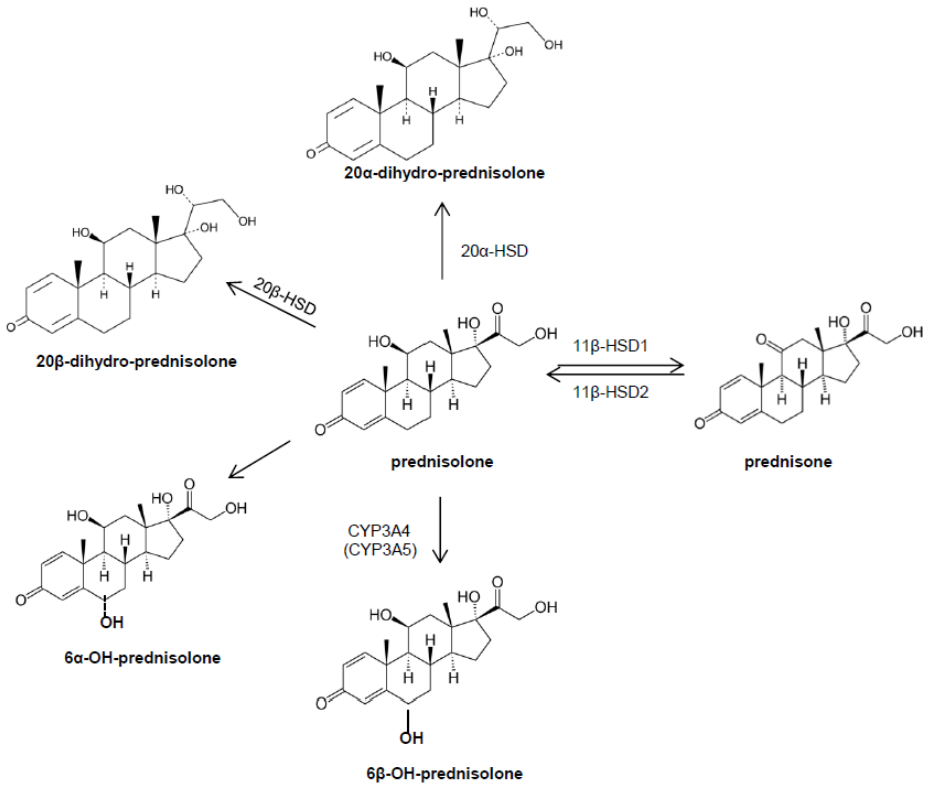

Figure 8: *Major metabolites of prednisolone.*

1.8 Therapeutic drug monitoring

The concept of therapeutic drug monitoring (TDM) is based on the assumption that clinical effects correlate better with drug concentrations than with drug dose, and the goal is to individually adjust the drug dose according to drug exposure, in order to improve patient outcome. TDM is relevant for drugs where the following criteria are met (91, 92):

- a narrow therapeutic window, meaning that small changes in dose may cause either severe consequences for the patient in terms of overexposure (toxicity) or underexposure (loss of efficacy)
- a clear relationship between drug exposure and clinical outcome
- a considerable inter-individual pharmacokinetic variability, and therefore a poor and unpredictable relationship between dose and drug concentration
- an available assay for measuring drug exposure
- if a metabolite/unbound fraction is the active compound, it should be measured.
- the drug must have a reversible action, and the concentration of the drug at the site of sampling should reflect the drug concentration at the receptor site/at target.
- a therapeutic range of the measured variable must be established, or alternatively: an individual therapeutic range, i.e. the patient serves as his/her own control over time.

Administering immunosuppression therapy is a subtle balance between the risk of underexposure (immunological failure and graft loss) versus overexposure (opportunistic infections). The large arsenal of sophisticated immunosuppressive drugs available used in different combinations for the prevention and treatment of graft rejections have reduced the incidence of acute rejection episodes to approximately 20%, and one year graft survival rates are generally over 90%. At the same time the adverse effects associated with long-term outcomes (cancer, cardiovascular morbidity and infections) are of concern (93). Using TDM to tailor

the dose regimen in order to achieve maximal efficacy with minimal toxicity for each individual is therefore essential. Furthermore, TDM may be useful to identify potential causes of therapeutic failure that may result from poor adherence, and to investigate suspected drug to drug interactions or unforeseen toxicities (94).

The most frequent form of TDM is pharmacokinetic monitoring, which is based on quantification of drug levels in a biological matrix, most commonly plasma, serum or saliva. The measurements are based on either single point concentrations or drug exposure (area under the concentration curve, AUC). A full AUC is the best marker of drug exposure. However, a full AUC requires several sampling points to cover the dose interval of 12 or 24 hours, which is inconvenient. Considering the easy access and patient convenience, single point measurements such as trough measurements (C_0), or drug concentrations at a given time after administered dose (C_1, C_2, etc.), are rather used as surrogate markers, provided that a good correlation between the surrogate marker and the AUC is established. Multiple regression-derived limited sampling strategies (LSS) combine several single point measurements in a mathematical algorithm that estimate the AUC with even better accuracy than a single point alone, while being less cumbersome than a full AUC. Population pharmacokinetic modelling incorporates environmental, demographic and drug-related factors as well as pharmacokinetic parameters in the mathematical model, and is increasingly used for tacrolimus (95, 96). Bayesian forecasting is a method for estimating individual pharmacokinetic parameters, using a combination of prior information and individual information (97). Combined with Bayesian forecasting, population pharmacokinetic modelling is able to determine individual pharmacokinetic profiles and individual dosage regimens to achieve specific target concentrations at desired times.

1.9 Therapeutic drug monitoring in renal transplantation

Due to its narrow therapeuticwindow, TDM of **tacrolimus** is almost always performed in current immunosuppressive protocols. Trough (C_0) measurements are traditionally used. In the literature, there are conflicting views regarding how well a tacrolimus trough concentration correlates with AUC (98-101). Furthermore, some studies report that the relationship between trough concentration and tacrolimus efficacy is not precise (102, 103), and that identifying the appropriate target concentration for each individual is challenging (104). New TDM approaches for tacrolimus are now developing, including not only the pharmacokinetic but also pharmacogenetic and pharmacodynamic variables, in order to provide a more personalized treatment (105). **MPA** and **basiliximab** are approved for the prevention of rejection using fixed doses, currently without monitoring. However, TDM of MPA in renal transplant recipients has been explored for some time. Based on the results from 3 large studies (APOMYGRE (106), FDCC (107) and OptiCept (108)), TDM seems to be useful, especially early post-transplant. According to the immunosuppressive protocols, **prednisolone** should be dosed as fixed doses in adults and by BW in children (13). The dose of prednisolone is solely based on a clinical assessment of the risk of rejection versus the risk of adverse effects. There is no consensus on the appropriate dosing strategy, or whether prednisolone should be given as maintenance doses at all. Whether TDM of prednisolone is able improve clinical outcomes in renal transplantation is currently unknown.

2. Aims of the study

2.1 General aim

The overall aim of this thesis was to characterize prednisolone and prednisone pharmacokinetics in renal transplant recipients and to identify the need and feasibility for individualizing the dosing of prednisolone.

2.2 Specific aims

I. To characterize the pharmacokinetics of prednisolone and prednisone in a pediatric renal transplant recipient population (Paper I)

II. To characterize the pharmacokinetics of prednisolone and prednisone in an adult renal transplant recipient population (Paper II)

III. To investigate the relative importance of CYP3A4 and CYP3A5 in the metabolism of prednisolone *in vitro* (Paper III)

IV. To examine the relationship between prednisolone AUC and pharmacokinetic parameters of prednisolone and cortisol to evaluate whether there would be a potential for individualized dosing of prednisolone in solid organ transplantation. (Paper I, II and III)

3. Materials and Methods

3.1 Materials

3.1.1 Paper I

Eleven pediatric renal transplant recipients (4 females) were included in this prospective, descriptive, non-randomized, and non-interventional study. All patients were recruited at Oslo University Hospital, Rikshospitalet, in the period between January 2010 and January 2012. The inclusion criteria were age<16 years, living donor kidney and immunosuppressive therapy according to standard protocol, consisting of prednisolone, methylprednisolone, tacrolimus, MPA and basiliximab, for pediatric kidney transplantation at this center.

Venous blood samples were collected (from a central venous catheter, vascular access port, or venipuncture fossa cubiti) pre-transplant and at six follow-up days during week 1, 2, 3, 4, 12, and 52 post-transplant (i.e.; observation periods I, II, III, IV, V, and VI). At each follow-up day, a twelve or seven hour pharmacokinetic profile was obtained, which contained a total of six samples: at pre-dose (C_0) and approximately 1, 2, 4, 6 and 12 hours after peroral prednisolone dose (hospitalized patients) or after 1, 2, 4, 6 and 7 hours (outpatients). In children with BW <10 kg, the sampling was limited to 0, 1, and 2 hours after dosing. Plasma was separated and stored at -20°C until analysis.

3.1.2 Paper II

28 adult renal transplant recipients (7 females) were included in this prospective, observational pharmacokinetic study. All patients were recruited from the National Transplant Center in Norway, Oslo University Hospital, Rikshospitalet, in the period between December 2015 and May 2017. The inclusion criteria were age ≥18 years, receiving immunosuppressive therapy according to the standard

protocol, consisting of prednisolone, methylprednisolone, tacrolimus, MPA and basiliximab, for adult kidney transplantation at this center.

Dose administration was performed either in a fasting-state (± 2 hours fasting) or under real-life non-fasting conditions (administered as in patients everyday life; no meal restrictions). From each patient, a full 24 hour pharmacokinetic profile was obtained between 13 and 54 days post-engraftment (median 27 days), and repeated within 4 weeks in 8 of the patients (from the same population). A total of 26 venous blood samples were obtained during the dose interval. Plasma was separated and stored at -80 C until analysis.

3.1.3 Paper III

Baculovirus-transfected insect cell microsomes selectively co-expressing human CYP3A4 or CYP3A5 enzymes, cytochrome P450 reductase and cytochrome b5 (Supersomes) were used as the *in vitro* model. A solution of sucrose, hepes and EGTA constituted the growth medium, into which the microsomes were diluted. Prednisolone was incubated according to incubation conditions optimized by Hermann et al. (109) at 37°C in Tris-H_2SO_4 (pH 7.4), $MgSO_4$, and NADPH, The reaction was initiated when a preheated mixture of microsomes and growth medium were suspended in the prednisolone buffer solution, and terminated after 0 to 120 min with 882 µL ice-cold precipitation solution (MeOH:$ZnSO_4$, 2:1) including internal standard (prednisolone-d8) and put on ice for 30 min. After centrifugation, a 200 µL aliquot of the supernatant was extracted and frozen at -20°C.

3.2 Laboratory methods

3.2.1 Measurements of drug concentrations

In **paper I**, the total plasma concentrations of prednisolone, prednisone, cortisol, and cortisone were determined using a previously published high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MSxMS) method (110). In brief, plasma sample (500 mL) preparation included protein precipitation with acetonitrile with isotope labelled internal standards, followed by liquid/liquid extraction with dichloromethane and evaporation under nitrogen (40°C, 15 min). The sample was then re-constituted in methanol, centrifuged and transferred to an autosampler for analysis. The analytical instrumentation used was an Alliance HT 2795 high performance chromatograph, interfaced to a Micromass Quattro micro API tandem mass spectrometer with electrospray ionization, (Waters, Manchester, United Kingdom). For all analytes, the between-runs coefficient of variation (CV) was below 15%. Tacrolimus concentrations were determined using a chemiluminescent microparticle immunoassay (analyzed on the Architect Instrument; Abbott Laboratories, Abbot Park, IL). The lower limit of quantification was 1.0 µg/L. The between series CV were 6% at 2 µg/L and 3.5% at 7.2 µg/L, respectively.

In **paper II**, total plasma concentrations of prednisolone, prednisone, cortisol and cortisone were determined using a newer in-house developed HPLC-MSxMS method. In brief, plasma sample (125 mL) preparation included protein precipitation with 400 mL of cold (MeOH:$ZnSO_4$, 2:1) with isotope labelled internal standards, pipetted to a deep-well plate. The plate was sealed and shaken for 4 min, then centrifuged for 10 min and transferred to an autosampler for analysis. The analytical instrumentation used was a Transcend II LX-2 ultra-HPLC-system coupled to a TSQ Quantiva mass spectrometer (Thermo Fisher Scientific, Waltham, MA). For all analytes, the between runs CV was below 10%, except for the lower limit of quantification for prednisone, cortisol and cortisone, where the CV was below 13%. Details regarding the specified conditions for

reagents, assays, sample preparations and analysis are provided in **paper II**, supplemental data (111).

In **paper III**, prednisolone concentrations were determined using the in-house HPLC-MSxMS performed as described in **Paper II** (111). Within- and between runs CV were below 7%. The same HPLC-MSxMS assay, with some minor modifications, was used for identification and semi-quantification of the prednisolone metabolite 6β-OH-prednisolone. Absolute quantitation of 6β-OH-prednisolone was not performed due to lack of a suitable isotope labelled internal standard, and also apparent impurities in the reference compound.

3.2.2 Genetic markers in whole blood

DNA was exctracted from whole-blood samples using MagNA Pure 96 or MagNA Pure LC instrument (both: Roche Diagnostics, Mannheim and Penzberg, Germany). Genetic variants in *CYP3A4*, *CYP3A5*, and *ABCB1* were investigated using real time polymerase chain reaction and melting curve analysis with allele-specific hybridization probes on the LightCycler 480 instrument (Roche) (112),

The following sequence variants were determined:

*CYP3A5*3* (NM_000777.3:c.219-237A>G), A=*CYP3A5*1* and G=*CYP3A5*3*

*CYP3A4*22* (NM_001202855.2:c.522-191C>T)

ABCB1 NM_000927.4:c.1236T>C

ABCB1 NM_000927.4:c.2677T>G/A

ABCB1 NM_000927.4:c.3435T>C

Genetic variants in *HSD11B2* (exons 2, 3, and 5 and flanking intron sequences) were investigated using Sanger sequencing on ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA).

3.3 Pharmacokinetic analysis

In **paper I and II**, the pharmacokinetic variables and parameters were derived from the plasma concentration data as follows: pre-dose (trough) concentration (C_0), maximum concentration (C_{max}), and time to reach C_{max} (T_{max}) were read directly from the concentration vs time curves. In **paper II**, the morning cortisol level was defined as cortisol C_0. The evening cortisol level was defined as the mean of the measured cortisol levels at 7 and 9 p.m. The ratio of the evening-to-morning cortisol was stated as a percentage. The elimination rate constant (k_e) was calculated by log-linear regression of the terminal part of the concentration versus time curve. In **paper I**, the elimination phase was defined by two data points in 9 of 47 k_e calculations, otherwise by three data points. In **paper II**, the elimination phase was defined by four data points in all 28 k_e calculations. The elimination half-life ($t_{1/2}$) was calculated from k_e ($t_{1/2}$=LN(2)/k_e).

The area under the concentration curve (AUC) was calculated using the linear trapezoidal rule. In **paper I**, the C_{12} was missing in 7 of the 47 dose intervals, hence the AUCs from 0 to 7 hours ($AUC_{0–7}$) were calculated in these cases, whereas for the remaining dose intervals $AUC_{0–12}$ were calculated. In **paper II**, $AUC_{0–24}$ was calculated. The AUCs from the last measurement to infinity ($AUC_{last–\infty}$) were calculated as C_7, C_{12} or C_{24} divided by k_e. The AUCs from time zero to infinity ($AUC_{0-\infty}$) were calculated as AUC_{0-7}, AUC_{0-12} or AUC_{0-24} + $AUC_{last-\infty}$.

The AUCs derived from the dose investigated (AUC_{dose}, i.e. the AUC used for the determination of CL) were calculated as the sum of AUC_{0-7}, AUC_{0-12} or $AUC_{0–24}$ plus $AUC_{last–\infty}$ minus the AUC contribution from the previous dose of prednisolone (C_0/k_e). The AUC was not calculated for children with BW <10 kg.

The apparent total clearance from plasma after an oral dose (CL/F, where F=bioavailability) was determined from the dose divided by the AUC_{dose}. The apparent volume of distribution (V_D/F) was calculated as (CL/F)/k_e.

The pharmacokinetic parameters and variables were calculated relative to the prednisolone dose (mg) per kg BW (dose-BW-adjusted). The ratios of active and inactive forms of glucocorticoids were calculated with respect to AUC_{dose} and C_{max}. The pharmacokinetic parameters of prednisone were calculated based on doses given as prednisolone and assuming single compartment pharmacokinetics. Body mass index (BMI) was defined as BW/(height^2) and expressed as kg/m^2.

In **paper III**, the velocity constant k (min^{-1}) for the substrate depletion was estimated in each experiment using the equation $C_t = C_0 e^{-kt}$, and the intrinsic clearance (CL_{int}) was calculated as $CL_{int} = kV$, where V is the incubation volume.

3.4 Statistics and data analysis

Statistical analysis and calculation were performed using SPSS (version 25.0), Microsoft Excel (version 2010) and Graph Pad (version 8.0.1). All continuous variables were reported as median and range unless otherwise stated. A two-tailed p-value of 0.05 or less was considered statistically significant.

In **paper I**, to determine the intra-individual variability (IIV), the individual relative deviation from the mean of the 6 periods was calculated in each patient for each period, and then the median for each period was reported. In **paper II**, the percentage prediction errors (PPE) were reported as the root mean square ± 95% confidence interval (CI). The Wilcoxon signed-rank test was used to compare the paired pharmacokinetic variables in fasting conditions *vs.* non-fasting conditions. The Mann-Whitney U test was performed for data comparison between males and females. The Spearman rank correlation coefficient was used for investigating statistical bivariate correlation between covariates contributing to prednisolone variability and bioavailability of prednisolone. For correlation of pharmacokinetic variables with measured prednisolone AUC, the coefficient of determination was analyzed by linear univariate regression analysis. Forward stepwise regression analysis was used to identify independent predictors of estimated prednisolone AUC. The PPE was calculated for each equation as (estimated $AUC_{0\text{-}24}$ - measured

AUC_{dose})/measured AUC_{dose}) × 100 for comparison of the limited-sampling strategies (LSS).

In **paper III**, substrate depletion data of prednisolone for the incubation period of 0-45 min were fitted to a mono-exponential decay model ($C_t = C_0 e^{-kt}$) with a 1/Y weighting. Relative depletion of substrate concentration was determined by calculating (prednisolone concentration at 45 min – prednisolone concentration at 0 min) / (prednisolone concentration at 0 min)* 100%. To determine any statistical significant difference in prednisolone concentration at 0 min vs 45 min, for both CYP3A4 and CYP3A5, a paired t-test was used. The extent of formation of 6β-OH-prednisolone by CYP3A4 and CYP3A5 was compared by assessment of the formation of 6β-OH-prednisolone after 45 min incubation in CYP3A5 microsomes relative to CYP3A4 microsomes.

3.4.1 Missing data/secondary exclusions

Paper I: one patient withdrew from the study after period IV. Due to acute rejection and subsequent plasmapheresis therapy on the day of sampling, samples from another patient were missed for periods II and III. Samples from a third patient were missed for period II due to illness on the sampling day. In period I, the AUC calculated was excluded for two patients, as the plasma concentration versus time curve did not show a distinct elimination phase.

Paper II: one patient missed four (C_3, C_4, C_{11} and C_{12}), and three patients missed each one of a total of 26 venous samples during the dose interval.

Paper III: one of the duplicate time points at 90 min, in one of the three rCYP3A5 experiments was identified as an outlier (Thompsons Tau test) and removed.

3.5 Ethical considerations

The studies were performed in accordance with the Declaration of Helsinki and guidelines for Good Clinical Practice. In **paper I**, the study was approved by the Regional Committee for Medical Research Ethics. Informed written consent from the participants (if age 12–16 years) and their parents was obtained. In **paper II**, the study was approved by the Norwegian Medicine Agency (EudraCT number: 2015-004734-10) and the local ethics committee (reference number 2015/2098). All patients received verbal and written information and signed an informed consent form before study participation.

4. Summary of results

4.1 Paper I

Despite standardized (mg/kg) peroral dosing of prednisolone, a considerable IIV in prednisolone and prednisone plasma concentrations, the AUC_{dose} and CL/F for prednisolone, and prednisolone/prednisone (PL/PN) AUC was demonstrated in pediatric renal transplant recipients. The first week post-transplant, a 5 fold difference in AUC_{dose} between patients was observed, increasing to 7 fold difference when adjusted for dose and BW. The lowest dose-BW adjusted AUC_{dose} was observed in a patient identified as *CYP3A5*1*3* genotype. PL/PN AUC_{dose} ratios during the first 4 weeks post-transplant were high (median=11), indicating a high exposure of prednisolone, and tended to drop at 3 and 12 months post-transplant (median=7), coinciding with dose reduction. PL/PN AUC_{dose} ratios of 50 and 57 were observed in patients with concurrent intravenous methylprednisolone therapy. Throughout the study, 3 patients experienced insulin-dependent NODAT. When dose intervals with concurrent methylprednisolone therapy were omitted, higher PL/PN AUC_{dose} ratios (>12) were observed in these patients compared with the remaining population.

This pilot study suggests that there may be a potential for improvement of prednisolone therapy in children by individualization of dose, and even more so in the event that methylprednisolone is introduced.

4.2 Paper II

In **paper II**, prednisolone plasma concentrations of 28 adult renal transplant recipients receiving fixed doses of prednisolone, but no concurrent methylprednisolone therapy at the time of sample collection, showed a high prednisolone exposure (median prednisolone AUC_{dose} adjusted for dose-BW was 22379 [μg•h/L]/[mg/kg]), and a high PL/PN AUC_{dose} ratio (median =21). The CYP3A5 expressers (n=3) displayed a 25% lower prednisolone AUC_{dose} adjusted for dose-BW compared with CYP3A5 non-expressers (n=22). A large IIV was

observed, ranging from 3 fold for C_{max} and AUC_{dose} and up to 10 fold for T_{max}. The very low observed cortisol evening-to-morning ratio (median value of 0.1 as opposed to a normal value of 0.5) demonstrated a pronounced suppression of endogenous cortisol production. In fasting patients (n=8), a strong correlation between prednisolone AUC_{dose} and morning levels of cortisol ($p<0.01$, Spearman's Rho –0.833) was observed. A limited sample strategy (LSS) based on 3 pharmacokinetic sampling time points: 0, 2, and 4 hours after prednisolone dosing, predicted prednisolone $AUC_{0\text{-}24}$ well with a low percentage prediction error (PPE=5.2 ± 1.5%) and a good correlation of determination ($r^2 = 0.91$).

The results indicate that a more moderate dosing of prednisolone without affecting therapeutic efficacy may be possible, and emphasize the need for individualized prednisolone dosing. Utilizing the LSS described in **paper II** as a tool to provide reliable estimates of AUCs can potentially reduce variability and side effects while preserving protection against rejection.

4.3 Paper III

In **paper I** and **II**, the metabolic enzyme CYP3A5 was discussed as a potential contributor to the observed variability in prednisolone pharmacokinetics. In the literature, the role of CYP3A5 in the metabolism of prednisolone has not been fully elucidated. Therefore, the metabolism of prednisolone by CYP3A4 and CYP3A5 was investigated in **paper III**. Through *in vitro* incubations using recombinant CYP3A4 and CYP3A5 insect cell microsomes, intrinsic clearance (CL_{int}) of prednisolone was determined by the substrate depletion approach. CL_{int} for prednisolone by rCYP3A5 was found to be less than 26% relative to rCYP3A4. Formation of 6β -OH-prednisolone by rCYP3A5 was less than 10% relative to rCYP3A4. The study indicates that 6β-hydroxylation of prednisolone assessed *in vitro* in microsomes depends on rCYP3A4 rather than rCYP3A5, and that CYP3A5 may be responsible for the formation of other prednisolone metabolite(s) in addition to 6β-OH-prednisolone.

5. General discussion

5.1 Methodological considerations

Study design:

The main strength in **paper I** was the follow up period of 12 months, allowing repeated pharmacokinetic investigations on a total of six occasions per patient. All samples were analyzed for cortisol and cortisone, as well as prednisolone and prednisone, which enabled us to study the impact of prednisolone therapy on the endogenous GC production. Due to the young age of the participants in this study, the number of blood samples drawn during a dose interval was limited to six. In **paper II,** the main strength was the rich sampling obtained following a morning dose of prednisolone. A total of 26 samples per 24-hour pharmacokinetic investigation were analyzed. This ensured detailed individual description of prednisolone, prednisone, cortisol, and cortisone pharmacokinetics during the full 24-hour interval.

The study designs in **paper I** and **II** were not powered for and designed to establish a significant association between genetic variants in *CYP3A* or *ABCB1* and prednisolone exposure. Additional pharmacogenetic analyses might further explain the observed variability. However, a larger population would be needed because of the rather low frequency of variants in relevant genes such as *HSD11B2*, *CYP3A*, and *ABCB1*.

Bioanalytical:

Total concentrations of prednisolone were measured because a HPLC-MSxMS method for quantification of total drug concentration was the only method available at the time. Changes in prednisolone binding proteins (CBG, albumin) may significantly alter total prednisolone levels whereas levels of unbound prednisolone are relatively preserved (section 1.7.2). A limitation in both **paper I** and **II** is the lack of data on the unbound fraction of prednisolone, which could have contributed to a better understanding of the pharmacokinetics of prednisolone and prednisone. As the drug response correlates with the amount of unbound

prednisolone that binds to the GR, measuring the unbound fraction of prednisolone concentration might be the more useful way of monitoring drug exposure. On the other hand, measurement of unbound prednisolone requires separation of the unbound from the bound fraction, which in a routine setting is both time and labor-consuming. For this to be of value, the correlation between unbound prednisolone concentrations and clinical outcome should be higher compared with the correlation between total prednisolone measurements and clinical outcome.

The sample preparation in HPLC-MSxMS method used in **paper I** (110) was quite laborious and time consuming and the sample volume required for analysis was relatively large (500 µL). In addition, the analysis run time for each sample was 12 minutes. A newer in-house HPLC-MSxMS method was therefore developed, and used in **Paper II** and **III** (111). This method required a smaller sample volume (125 µL), sample preparation was less cumbersome as the liquid/liquid extraction step was eliminated, and analysis run time was reduced to approximately 3.5 minutes. The between-runs coefficient of variation (CV) for all analytes were <15%, <10% and <7% in **paper I, II** and **III**, respectively, indicating an improvement in imprecision for the in-house method, although satisfactorily for both methods. The measurement range of the assay in **paper I** was 4.0-1000 µg/L for prednisolone, 3.0-200 µg/L for prednisone, 3.0-400 µg/L for cortisol and 3.0-200 µg/L for cortisone, whereas the measurement range of the assay in **paper II** was 1.7-1016 µg/L for prednisolone, 0.4-242 µg/L for prednisone, 0.7-400 µg/L for cortisol and 0.3-160 µg/L for cortisone. The use of two different HPLC-MSxMS assays may have contributed to a bias in measured concentrations. In both assays, however, reagents used for the preparation of calibrators and quality controls were provided from the same manufacturer, and the sample preparations and analysis were performed in the same laboratory by the same personnel. To the best of our knowledge, there is no external quality assessment program for prednisolone, which could have controlled the accuracy of prednisolone measurements.

Ideally, each measured analyte should have its respective isotope labelled internal standard, for optimal quantification. However, in order to optimize experimental conditions, and due to observed interferences from some internal standards being tested, compounds with similar retention times and molecular structure were assigned a common internal standard (in **paper I**: prednisolone - cortisol, prednisone-cortisone, in **paper II**: prednisone - cortisone, in **paper III**: prednisolone - 6β-OH-prednisolone).

Pharmacokinetic:

Pharmacokinetic analyses were performed on a median dose of 15 mg prednisolone, which is higher than that in the long-term maintenance regimens (5–10 mg/day). Any potential association between pharmacokinetics and response should be evaluated over a wider range of dosages. Linear kinetics was assumed, which may be controversial at high prednisolone doses. Two-compartment models are sometimes used in prednisolone pharmacokinetic studies, and always after intravenous administration, but a one-compartment model as used in **paper I** and **II** is sufficient after oral administration (21).

Experimental:

In **paper III**, the substrate depletion method was used to assess the *in vitro* intrinsic clearance (CL_{int}) of prednisolone by rCYP3A4 and rCYP3A5. Traditionally, in vitro measurements of CL_{int} in microsomes or hepatocytes are performed using the metabolite formation method. In such studies, the initial rate of metabolite production is measured over a range of substrate concentrations, under linear conditions with respect to protein concentration and time. The short incubation times (< 20 min) and low enzyme concentrations (0.01-0.2 mg/mL) (113) required in this method avoid the pitfalls of enzyme instability (due to long incubation time), non-specific binding (due to high enzyme concentrations) and end product-inhibition (due to phase I metabolite accumulation). On the flip side, the metabolite formation method assumes a prior knowledge of the particular metabolic pathways of the substrate studied, and their impact on the total metabolism of the drug, in order to provide a proper estimation of clearance (114).

This information was not available for prednisolone. In addition, a method for a precise quantification of the metabolite 6β-OH-prednisolone was not readily at hand, and a suitable isotope labelled internal standard for 6β-OH-prednisolone was lacking. The substrate depletion method, which does not require formal kinetic characterization or metabolite quantification, was therefore chosen. Compared with the metabolite formation method (113), this method uses longer incubation times and higher enzyme concentrations (up to 90 min(115) and 10 mg/ml (116) have been reported, respectively), and the aforementioned pitfalls related to these aspects needed to be addressed. Evaluating the substrate depletion approach and its limitations, Jones and Houston (114) established a set of recommended incubation conditions for this method. In line with this, the enzyme concentrations were kept below 0.5 mg/mL, the prednisolone concentration of 0.5 µM was well below the apparent value of Km for rCYP3A4-mediated prednisolone metabolism, reported to be 40-166 µM (117) and incubation time was restricted to 45 minutes. Linearity studies were performed, to ensure correct use of a mono-exponential model when calculating the CL_{int} of prednisolone by rCYP3A4 and rCYP3A5.

The experiments in **paper III** were conducted with recombinant CYP3A enzymes, which are artificial systems that need to be related to a tissue derived enzyme source before a comparison with human liver microsome data is possible. To convert the current results into *in vivo* relevance, scaling factors (e.g. a relative activity factor or an intersystem extrapolation factor) must be applied to the recombinant data. The assessment of such scaling factors was beyond the scope of this thesis.

The substrate depletion approach is associated with an increased imprecision, as measuring a depletion involves subtracting two large numbers (118). In addition, compared with microsomes, hepatocytes are reported to produce more accurate and precise predictions of CL_{int} (119). The quality control results indicate, however, that the measurements are precise, with an imprecision well below the observed changes in prednisolone concentrations.

5.2 Discussion of the results

5.2.1 Prednisolone and prednisone pharmacokinetics in a pediatric renal transplant recipient population (Paper I)

In a cohort of pediatric renal transplant recipients, up to six 7- or 12-hour concentration-time profiles were obtained from eleven patients receiving per oral prednisolone over a period of 12 months (period I-VI). Data from patients with BW below 10 kg (n=2) deviated distinctly from the remaining population, and are commented separately in section 5.2.4.

A considerable IIV in prednisolone and prednisone plasma concentrations, the AUC_{dose} and CL/F for prednisolone was demonstrated. The pharmacokinetic profiles (period I, IV, V and VI) and parameters of prednisolone and prednisone (period I-VI) are given in **paper I**, figure 2 and table 3, respectively. The first week post-engraftment, the prednisolone dose-BW adjusted C_{max} was 658(257-1427) ng/mL/(mg/kg) and AUC_{dose} was 3818(1315-9647) ng*h/mL/(mg/kg), whereas CL/F was 8.4(3.7-16.5) L/h. Time to reach C_{max} (T_{max}) was 65(60-257) min, and the elimination half-life ($T_{1/2}$) was 3.3(2.6-6.7) hours. All values are given as median (range).

Studies on prednisolone and prednisone pharmacokinetics in pediatric renal transplant recipients are limited. Results between studies may be challenging to compare, as there are differences in study approaches, patient diagnosis, dose regimens, assay methods and parameters assessed. The time interval over which the AUC of prednisolone is estimated varies, and the estimated AUC value is rarely dose-BW adjusted. Sagcal-Gironella et al. (120) obtained and analyzed full 9-hour pharmacokinetic profiles in eight patients (12-28 years) with systemic lupus erythematosus receiving 20(5-40) mg of prednisolone. The total prednisolone levels adjusted for dose and BW observed in this study (C_{max} 1097(301-2211) ng/mL/(mg/kg), AUC_{0-9} 4361(1136-9580) ng*h/mL/(mg/kg), and $T_{1/2}$ 2.6 hours) corresponded well with our observations in period I-IV, whereas CL/F was reported slightly higher (11.4(6.7-13.7) L/h). Conversely, in children with nephrotic syndrome in active phase, Gatti et al. (121) reported a dose-BW

adjusted AUC_{0-24} of 1393(1054-1821) ng*h/mL/(mg/kg), and $T_{1/2}$ of 2.6 hours) whereas CL/F was (22.4(15.3-42.1) L/h. Median prednisolone dose was 60 mg, median albumin level was 13 (10-28) g/L. Both high dosing and hypoalbuminemia increase CL/F of prednisolone, which most likely caused the low AUC observed in Gatti's study. The elimination half-life of prednisolone however, is fairly stable regardless of dose. In the present study, the $T_{1/2}$ remained constant throughout the follow-up period, consistent with findings in other studies (122-124).

The variability in total prednisolone AUC_{dose} between patients in the current study was 5 fold, increasing to 7 fold when adjusted for dose and BW. An even larger (8 fold) IIV in dose-BW adjusted AUC was observed in the study of Sagcal-Gironella et al. (120). A 3-4 fold IIV in prednisolone AUC_{0-8} were also reported in pediatric patients with nephrotic syndrome (122) and in children with acute lymphoblastic leukemia (125), whereas Petersen et al. (123) observed a prednisolone AUC_{0-8} and estimated CL that differed less than 2 fold among individuals in a population of children with acute lymphoblastic leukemia. His findings were supported by Teeninga et al., reporting a modest IIV (CV=44.7%) in CL among children with nephrotic syndrome (126). In the last two studies, unbound prednisolone was measured, which may in part explain the lower IIV compared with the current study.

During the first 4 weeks post-engraftment, the PL/PN AUC_{dose} ratios were high and displayed a considerable IIV (median 11, range 7-56) (**Paper I**, table 5), both of which may be caused by the high prednisolone dosage early post-transplant and the dose-dependent pharmacokinetics of prednisolone. However, the high exposure of prednisolone and larger PL/PN AUC_{dose} ratio can also be explained by an increased expression or activity of 11β-HSD1 (converting prednisone to prednisolone) or a reduced activity of 11β-HSD2 (inactivating prednisolone). GC themselves induce expression and activity of the11β-HSD1 enzyme. Hence, prednisolone therapy contributed to a positive feed-back loop that may have enhanced prednisolone exposure in this study (18). Expression of 11β-HSD2 is reduced in patients with renal failure (127, 128), which may have influenced the

prednisolone exposure in patient 1, 2 and 5 (**Paper I**, table 5), as their creatinine levels were 5-9 fold the upper reference limit. Gene variants that significantly impair 11β-HSD2 function have been reported in *HSD11B2* exons 2, 3 and 5 (57, 68). Sequencing *HSD11B2* exons 2, 3 and 5 in patient samples from the current study did not reveal any gene variants with functional impact on 11β-HSD2, however. At high GC concentrations, the capacity of 11β-HSD2 is challenged, prednisone levels stagnate, and the PL/PN AUC ratio promptly escalates, as reported by Saeves et al. (129). In our study, the two PL/PN AUC_{dose} ratios of 50 and 56 (**Paper I**, table 5) were observed in patients with concurrent intravenous methylprednisolone therapy, and a saturation of 11β-HSD2 may be the most likely explanation to the high levels.

Throughout the study, the three patients with the highest PL/PN AUC_{dose} ratios experienced insulin-dependent NODAT. When dose intervals with concurrent methylprednisolone therapy were omitted, the individual mean PL/PN AUC_{dose} ratios observed in these patients stayed high (range 12-15) compared with the remaining population (range 7-11) (**Paper I**, table 5). One of the three patients did not receive methylprednisolone therapy. In the literature, there is strong evidence to support that high prednisolone exposure causes diabetes or hyperglycemia (130, 131), supporting a theory that a high PL/PN AUC ratio may be a risk factor for NODAT. Tacrolimus is also a well-known risk factor for diabetes. In our study, trough concentrations of tacrolimus were within target. However, the risk of NODAT may have been increased by the combination of tacrolimus and prednisolone.

As prednisone is not biologically active, it may seem that monitoring of prednisolone concentrations should suffice as a tool to individualize dosing of prednisolone. But knowing that prednisolone concentrations not only vary with dose but also with the expression and activity of 11β-HSD isoenzymes, the prednisone concentrations should be taken into account. Exceptionally high and/or consistent PL/PN AUC ratios may provide valuable information about the causes of the increased prednisolone exposure, and may be signaling a possible drug-drug

interaction or an impairment involving 11β-HSD isoenzymes that should be further investigated. The use of a PL/PN AUC ratio as a marker of 11β-HSD activity may, however, be limited by the fact that the plasma ratio does not necessarily reflect the local prednisolone and prednisone concentrations in different tissues. As described in 1.7.1, the distribution of the isoenzymes 11β-HSD 1 and 2 is tissue specific. A plasma PL/PN ratio may rather be interpreted as an indirect measure of the mean ratio of 11β-HSD1 and 2 expression and activity in the body, where the hepatic contribution is dominant.

Altogether, a large intra-individual variability in prednisolone exposure in pediatric renal transplant patients receiving BW adjusted prednisolone dosing was demonstrated in this study. The findings indicate that there may be a potential for improvement of prednisolone therapy in children by individualization of dose, and even more so in the event that methylprednisolone is introduced.

5.2.2 Prednisolone and prednisone pharmacokinetics in an adult renal transplant recipient population (Paper II)

In a population of 28 adult renal transplant recipients receiving fixed doses of prednisolone, a 24-hour concentration-time profile was obtained within 4-6 weeks post-engraftment. The main findings in the study were a relatively high exposure of prednisolone, a high plasma PL/PN AUC_{dose} ratio and a large (3-4 fold) inter-individual variability (IIV) in pharmacokinetic parameters, even after adjusting for dose and BW. The pharmacokinetic profiles and parameters of prednisolone and prednisone are given in **paper II**, figure 1 and table 2, respectively. The prednisolone C_{max} was 482 (271-912) ng/mL, AUC_{dose} was 3821 (2232-5382) ng*h/mL, whereas CL/F was 3.8(2.5-6.7) L/h. Time to reach C_{max} (T_{max}) was 155(31-299) min, and the elimination half-life ($T_{1/2}$) was 3.8(2.8-5.3) hours. All values are given as median (range).

The median peak prednisolone concentration level (C_{max}) and $T_{1/2}$ value observed in our study concurred with previous prednisolone pharmacokinetic studies in

solid organ transplant patients, whereas the prednisolone AUC_{dose} values observed were higher (129, 130, 132-134). Studies using a lower prednisolone dose than in the current study observed lower prednisolone AUCs (130, 135). In some studies, adjusting the AUC values by dose or by dose and BW settled the differences in AUC levels (131), in other studies it did not (129, 132). In several of the prednisolone pharmacokinetic studies in the literature, the prednisolone AUC values were based on a shorter time interval than in our study (e.g. AUC_{0-8} as opposed to AUC_{0-24}), leading to a smaller AUC (134, 136, 137). In line with this, the prednisolone AUC_{0-24} value of 2900 (1900-4000) ng*h/mL reported in a study by Öst et al. (138) was closer to the prednisolone AUC_{dose} value observed in the current study. The number of samples obtained in the study by Öst, however, were fewer (n=9). When the number of samples during a dose interval is sparse, the true C_{max} may pass undetected, and the AUC may be underestimated. As many as 26 venous samples were obtained during a 24-h dose interval in the current study, and this rich data set may have contributed to a larger AUC value. Accordingly, Barraclough et al. (139) reported a dose-adjusted prednisolone AUC_{0-12} (based on 13 venous samples) of 225±54 (ng*h/mL)/mg (mean±1SD) in a cohort of adult renal transplant recipients. This finding corresponds well with the dose-adjusted AUC_{dose} of 261 (149-402) (ng*h/mL)/mg (median, range) observed in our study.

In contrast to tacrolimus pharmacokinetic reports (140), fasting status presented little influence on the resulting prednisolone AUC_{dose} (**paper II**, table 3). Additional factors that may have contributed to influence the level of and IIV in prednisolone AUC_{dose} in this study are discussed in section 5.2.4.

The high prednisolone exposure observed was paralleled by strong suppression of the endogenous cortisol and cortisone profiles (**Paper II**, figure 1), with a considerable IIV in total cortisol AUC_{0-24} and morning cortisol levels (**Paper II**, table 2). The strong suppression of the HPA-axis was demonstrated by a very low cortisol evening-to-morning ratio when compared with a normal cortisol reference interval (0.1 versus 0.5). Among the morning cortisol profiles obtained, 50% were below the normal morning reference interval for adults > 16 years (50-230 µg/L).

A strong correlation was observed between prednisolone AUC_{0-24} and cortisol morning levels ($p<0.01$, Spearman's Rho –0.833) in fasting patients (n=8).

The results indicate that renal transplant recipients (RTR) treated with prednisolone according to standard protocol experience a considerable suppression of endogenous cortisol production which (under fasting conditions) increases with increasing prednisolone exposure. The findings are in line with previous studies demonstrating suppression of endogenous GC production in solid organ recipients receiving chronic prednisolone treatment (129, 141, 142). Our study was not designed to investigate a possible relationship between the degree of suppression of the endogenous cortisol production and pharmacological effects of prednisolone, for which a follow-up study over several years is needed. This is, however, evaluated in a study by de Vries et al. (143), investigating stable RTR chronically treated with prednisolone (n= 563) at median 6 years post-transplant. Using 24-h urinary cortisol excretion as measures for HPA-axis activity, de Vries et al. found that a higher degree of HPA-axis suppression is associated with higher prevalence of metabolic syndrome. Patients with the lowest amount of endogenous cortisol production displayed significantly higher body weight, higher BMI, higher waist circumference, higher fasting triglycerides and a higher prevalence of NODAT. Importantly, an association between daily prednisolone dose and any of the metabolic parameters was not found. The hypothesis that endogenous cortisol production is associated with the pharmacological effects of prednisolone is also supported in a recent study by Vulto et al. (144) They investigated whether there were differences in endogenous GC production and 11βHSD1 activity in RTR treated with prednisolone (n=693) versus healthy controls (n=275), and whether there were implications for long-term survival. Vulto et al. found that urinary excretion of cortisol and its metabolites were decreased in RTR compared with healthy controls, and that this was associated with an increased risk of mortality long-term after renal transplantation. Although a causal relationship is not established, these results taken together indicate that assessment of the endogenous cortisol production may be a useful tool in monitoring pharmacological effects of prednisolone.

The plasma cortisol /cortisone AUC_{0-24} ratio of 8 (6-15) observed in the current study was high compared with healthy controls (145). Correspondingly, Vulto et al. reported increased urinary ratios of cortisol/cortisone and their corresponding metabolites in the RTR group compared with healthy controls. Furthermore, they found a significant correlation between high ratios and an increased risk of mortality long-term after renal transplantation. Gant et al. observed that both urinary ratios of cortisol/cortisone and their corresponding metabolites in patients with type 2 diabetes were higher compared with healthy controls, and that ratios were highest in patients with concurrent lower renal function (146). A higher ratio of active vs inactive GCs in plasma or urine indicates that the peripheral GC balance as maintained by 11β-HSD enzymes has shifted toward an increase in 11β-HSD1 activity and/or a reduction in 11β-HSD2 activity. This highlights the importance of measuring the inactive compound when evaluating GC pharmacokinetics, as it provides important information about GC metabolism. Possible mechanisms underlying altered 11β-HSD enzyme activities are further discussed in section 5.2.1 and 5.2.4.

In conclusion, the observed IIV in prednisolone pharmacokinetics in this study confirms reports in the literature of high inter-individual variability in systemic prednisolone exposure (43, 120, 130, 132, 147), and emphasizes the need for improvements in prednisolone dosing regimens. Assessment of endogenous GC production and their metabolites in plasma or urine may be a useful tool in monitoring pharmacological effects of prednisolone. To further investigate the potential for individualized dosing of prednisolone, utilizing AUC has been suggested. Based on the findings in paper II, the relationship between prednisolone exposure and prednisolone pharmacokinetic parameters for the purpose of TDM are further discussed in section 5.2.5

5.2.3 The relative importance of CYP3A4 and CYP3A5 in the metabolism of prednisolone *in vitro* (Paper III)

In **paper I**, we found that the patient with the lowest dose-BW adjusted prednisolone AUC_{dose} and the highest prednisolone CL/F was a CYP3A5 expresser (*1/*3) (**paper I**, figure 3, patient 4). Furthermore, in **paper II** we noticed that three CYP3A5 expressers (*1/*3) presented a mean dose-BW adjusted prednisolone AUC_{dose} which was 25% lower compared with CYP3A5 non-expressers (n=25), (16411 vs 21984 µg*h/L/(mg/kg)). (111). Inspired by these findings, we sought to further investigate the degree of involvement of the CYP3A4 and 5 isoenzymes in the metabolism of prednisolone. Therefore, in **paper III**, the relative contribution of rCYP3A4 and rCYP3A5 in the metabolism of prednisolone was examined *in vitro* by way of a substrate depletion study.

The results showed that the CL_{int} for prednisolone by rCYP3A5 was less than 26% relative to rCYP3A4 in microsomes, whereas formation of 6β-OH-prednisolone by rCYP3A5 was less than 10% relative to rCYP3A4. These results suggest that prednisolone is metabolized by rCYP3A4, but do not support rCYP3A5 to be of major importance. In addition, the observed discrepancy between CL_{int} for prednisolone and metabolite formation by rCYP3A5 relative to rCYP3A4 (26% and 10%, respectively) indicate that rCYP3A5 may be responsible for the formation of one or more prednisolone metabolite(s) in addition to the 6β-OH-prednisolone detected.

Previous *in vitro* studies evaluating the role of rCYP3A4 and rCYP3A5 in the metabolism of prednisolone in particular are scant (117, 148), but similar studies on the metabolism of steroids other than prednisolone are available (87-89, 149). Taken together, these reports seem to concur regarding rCYP3A4 being the preferred rCYP3A enzyme responsible for the 6β-hydroxylation activity in prednisolone metabolism.

Pharmacokinetic drug-drug interaction studies have demonstrated that co-administration of CYP3A inducers may cause an increase in clearance and decreased half-life of prednisolone (21). Conversely, CYP3A inhibitors have been

shown to increase prednisolone exposure (150, 151). A major challenge in interpreting the data from these studies is the well-documented phenomenon of auto-induction of CYP3A enzymes by GCs themselves (152-154). Fluticasone has been shown to have the potential to inhibit CYP3A5, but not CYP3A4, in a time dependent manner (155), but to our best knowledge, this is not demonstrated for prednisolone.

CYP3A5 and CYP3A4 have a wide range of overlapping substrate specificities, due to the high (84%) homology between the enzymes (156). However, despite largely sharing the same set of substrates, there are differences between the two isoenzymes regarding catalytic efficiency, and susceptibility to inhibitors. Results from studies using molecular docking simulations indicate that altered accessibility of substrates and inhibitors to the heme moiety of CYP3A molecules, which is a preferred location for oxidation at C6β position, may be a possible explanation for the difference in affinity between CYP3A4 and CYP3A5 (157, 158).

Based on reports that 6β-hydroxylation of cortisol is catalyzed by the isoenzymes CYP3A4 (86) and CYP3A5 (87), these enzymes have also been assumed to be partially involved in prednisolone metabolism. Due to differences in physicochemical properties, the various GCs differ in how they are influenced by other drugs (21). The pharmacokinetic characteristics of one glucocorticoid cannot be simply replaced by another. When also the (small) difference in CYP3A4 and CYP3A5 substrate specificity is taken into account, assumptions regarding the metabolism and pharmacokinetic properties of prednisolone based on pharmacokinetic studies of other GCs seem unfortunate. Direct studies on the metabolism of prednisolone by either CYP3A4 and/or CYP3A5 should be further investigated.

Microsomal systems are known to underestimate CL_{int} in vivo, since they lack certain enzyme and transporter activities which are present in the human hepatocyte (113, 114). The results from this study should therefore be corroborated in human liver microsomes. Additionally, clinical studies allowing a

comparison of prednisolone metabolism between CYP3A5 expressers and non-expressers, where also a potential effect of a CYP3A inhibitor is evaluated, are necessary to quantify the eventual contribution by CYP3A5-mediated metabolism of prednisolone in a clinical setting.

5.2.4 Variability in prednisolone and prednisone pharmacokinetics

The concentration-dependent kinetics of prednisolone, causing increased total body clearance with increasing plasma concentrations, is a major contributor to the IIV in the pharmacokinetics of prednisolone (76). Indeed, dose has been found to account for up to 42% of the variability in prednisolone pharmacokinetics (159). In **paper I**, the patient receiving the highest mg/BW dosage, also displayed the largest BW adjusted CL/F and a correspondingly low dose-BW adjusted AUC_{dose} (**paper I**, figure 3, patient 4). Furthermore, the decline in IIV throughout one year of follow-up coincided with dose reduction (**paper I**, figure 2), although this may also reflect normalization of organ function. Yet, in both **paper I** and **II**, a considerable IIV in prednisolone and prednisone pharmacokinetic parameters remained after adjusting for dose and BW, indicating that additional factors influenced the pharmacokinetics of prednisolone and prednisone.

Protein-binding

With reference to the protein-binding profiles of prednisolone and prednisone (section 1.7.2), clinical conditions causing changes in plasma-protein levels may alter the pharmacokinetics of these drugs. Hypoalbuminemia is associated with a decreased protein binding of prednisolone and prednisone. The initial increase in unbound prednisolone leads to a more rapid drug elimination, and a decrease in total prednisolone exposure (73, 122, 160-162). Consistent with this, one patient in **paper I** who experienced a relapse of focal segmental glomerulosclerosis presented a distinct increase in CL/F paralleled by a reduction in adjusted AUC_{dose} in period VI (**paper I**, figure 3, patient 9). Conversely, the use of estrogen therapy or oral contraceptives has been shown to increase CBG levels and plasma protein-binding, leading to a decrease in CL of prednisolone and higher total prednisolone

exposure (76, 137, 163, 164). In **paper II**, median albumin levels were within normal range (40(37-45) g/L). Of the seven female participants in **paper II**, five were between 22 and 49 years, two were 70 and 71. Data regarding estrogen therapy or oral contraceptives was not obtained and CBG levels were not monitored, both of which could have added further information.

Renal and hepatic impairment

There is strong evidence that uremic toxins reduce CYP-mediated metabolism through direct inhibition or by transcriptional downregulation, as reviewed by Ladda and Goralski (165). The effect of uremia on CYP metabolism is initially reversed by renal transplantation, but due to relapse of original disease or use of nephrotoxic drugs, renal function is impaired to varying degrees in many transplant recipients. In **paper II**, a negative correlation between measured glomerular filtration rate (mGFR) and dose-BW-adjusted AUC_{dose} of prednisolone was noticed ($p<0.044$, Spearman's Rho -0,383). In patients with mGFR≤45 mL/min (n=10), the median dose-BW-adjusted AUC_{dose} was 22% higher (24026 vs 19691 µg*h/L/(mg/kg)) when compared with patients with mGFR>45 ml/min (n=18). A study by Bergrem et al. supports this observation, reporting that uremic patients have a longer elimination half-time, a larger AUC and a lower renal clearance of total and unbound prednisolone compared with healthy controls (166).

The impact of hepatic dysfunction on changes in prednisolone exposure is unresolved, and concurrent hypoalbuminemia may confound results (43, 167). A reduced hepatic 11β-HSD1activity has been suggested to cause low plasma concentrations of prednisolone (168). However, in non-transplant patients with various liver diseases, Renner et al. (169) demonstrated increased concentrations of both total and unbound prednisolone with decreasing liver function. The possible impact of an impaired 11β-HSD1activity was explained balanced by the reduced metabolic clearance of prednisolone. In line with this, the metabolism of

prednisolone has been shown to be slower in renal and liver transplant recipients, yielding a higher prednisolone AUC compared with healthy volunteers (136).

Diabetes

Diabetes has been associated with a higher dose-adjusted AUC of prednisolone (131), possibly explained by a shift in the balance between 11β-HSD1 vs 2 (as discussed in section 5.2.1) or due to a reduction of CYP3A metabolism (170). In **paper I**, the three patients experiencing insulin-dependent NODAT also presented the highest prednisolone exposure in the group. In **paper II**, however, three patients with long term diabetes presented prednisolone AUC_{dose} levels close to median values.

CYP3A, P-glycoprotein and the pregnane X receptor

Gene variants and drug interactions that influence the expression or activity of the CYP3A enzymes and P-glycoprotein **(**P-gp) may also account for variability in prednisolone concentrations or exposure.

In **paper I** and **II**, a lower dose-BW adjusted prednisolone AUC_{dose} was noticed in patients with a functional *CYP3A5*1* allele. There are conflicting opinions regarding the influence of CYP3A activity on prednisolone metabolism (21, 43, 126, 162) (see section 5.2.3). Direct studies on the influence of CYP3A enzymes on prednisolone metabolism *in vivo* are sparse. Miura et al. compared CYP3A5 genotypes among renal transplant recipients and found no significant differences in prednisolone pharmacokinetics (135), whereas in a cohort of children with nephrotic syndrome, Chiou et al. found a trend of association between CYP3A5 expressers and sensitivity to prednisolone (171). The role of CYP3A enzymes, and in particular CYP3A5, in prednisolone metabolism *in vivo* remains unclear.

The P-gp efflux pump works in synergy with CYP3A enzymes and plays an important role in absorption and metabolism of prednisolone and prednisone. In **paper I**, two patients were homozygous for all three of the most common genetic variants in the gene encoding the P-gp (*ABCB1* (C1236T, *G*2677T/A, *C*3435T)). Interestingly, these two patients presented the largest and the lowest dose-BW

adjusted AUC_{dose}, respectively (figure 3, patient 3 and 4). A positive correlation between P-gp expression and total prednisone dose in children with nephrotic syndrome was demonstrated in a study by Wasilewska et al. (172). However, a confounding factor is that glucocorticoids themselves induce P-gp expression (173, 174). The association of P-gp polymorphisms with responsiveness to GCs has been evaluated in several studies, but the results have been conflicting (28, 43, 162).

The pregnane X receptor (PXR), encoded by the *NR1I2* gene, regulates the expression of both *CYP3A* and *ABCB1* genes. Activation of PXR, i.e. by steroids, leads to upregulation of an array of drug detoxification genes, including *CYP3A* and *ABCB1* (175). Miura et al. (135) evaluated the influence of *CYP3A5*, *ABCB1* and *NR1I2* polymorphisms on prednisolone plasma concentrations in renal transplant recipients, 28 days post-engraftment. In this study population, there was no significant difference in prednisolone pharmacokinetics between groups having the *CYP3A5* (A6986G) or *ABCB1* (C1236T, *G*2677T/A, *C*3435T) genotypes. Rather, a polymorphism in *NR1I2* (A7635G) was associated with a high metabolic activity of prednisolone with subsequent reduction in prednisolone plasma concentrations (135).

Prednisolone exposure is dependent on the interplay between drug transporters and enzymes in the prednisolone and prednisone metabolic pathways. An altered exposure of prednisolone exposure due to genetics may be the result of a combination of polymorphisms in more than one of the relevant genes, and should be investigated as such. To date, the number of studies investigating an effect of genetic variants on prednisolone pharmacokinetics is small, and although there may be a potential association, a conclusion is not possible.

Age, sex and body composition

Comparing adults (**paper II**) with children (**paper I**) at four weeks post-transplant, there were distinct differences in prednisolone pharmacokinetic parameters (table 2). The median prednisolone AUC_{dose} in adults, was about 1.6 fold higher compared with the median prednisolone AUC_{dose} observed in children,

increasing to 6 fold when adjusting for dose and BW. In addition to a longer dose-interval and richer sampling in **paper II**, physiological age differences in clearance may explain the larger prednisolone AUC_{dose} in adults. **Renal** clearance per kg BW is higher and may lead to a lower prednisolone exposure in children vs adults, and also in younger compared with older children (123, 125, 147, 176). The two patients with BW<10 kg in **paper I** presented a 25% lower mean prednisolone C_2 and a 40% higher prednisone C_2 in periods I–IV, hence their PL/PN C_2 ratios were low (4-8) compared with the main group (11-13). Although these two patients received higher mg per BW dosage than the main group, the higher clearance in younger children may have led to lower concentrations, leaving the 11β-HSD2 enzyme unsaturated. **Metabolic** clearance per kg BW is also lower in adults compared with children and decreases with age (176). Hence, saturation of enzymatic capacity may occur at lower prednisolone concentrations in adults compared with children, which in case will lead to a lower CL and larger AUC in adults. In children receiving a single dose of prednisolone (177), Green et al reported a shorter $T_{1/2}$ of total prednisolone compared with adult reference values, supporting our own observations (table 2). The 2-3 fold difference in T_{max} between children and adults is explained by fasting vs non-fasting conditions. Within the adult population in **paper II**, however, age did not correlate with total AUC_{dose} of prednisolone. In line with this, Miura et al. (178) found no influence of aging on the pharmacokinetics of prednisolone in renal transplant recipients one month post-transplant. Conversely, Stuck et al. (179) found a lower non-renal and renal clearance of prednisolone in elderly (65-89 years) compared with young (23-34 years) individuals.

		Prednisolone		Prednisone	
		Adults	Children	Adults	Children
Prednisolone dose		15 (7.5 – 20) mg	0.53 mg/kg		
C_{max}	[µg/L]	482 (271-912)	450 (300-615)	18.4 (10-31)	35 (18-53)
C_{max} /(dose/BW)	[µg/L]/[mg/kg]	2728 (1514-4190)	932 (587-1618)	97.3 (48-186)	65 (33–102)
AUC_{0-24}	[µg*h/L]	3821 (2232-5382)	2422 (1769-3284)	171 (100-237)	233 (125-419)
AUC_{0-24} /(dose/BW)	[µg*h/L]/[mg/kg]	22379 (12932-31611)	3868 (3612-12561)	892 (607-1650)	430 (232-888)
T_{max}	(min)	155 (31-299)	65 (60-125)	179 (150-541)	71 (60-265)
$T_{1/2}$	(h)	3.8 (2.8-5.3)	2.5 (1.8-4.5)	3.3 (2.4-5.8)	3 (1.6-5.8)
CL/F	(L/h)	3.8 (2.5-6.7)	7.3 (4.2-10.9)		
C_{max} ratio		27 (16-46)	14.5 (9-25)		
AUC ratio		21 (15-37)	8.6 (7-21)		

Table 2: *Pharmacokinetics of total prednisone and prednisolone, 4 weeks post-transplant: adults (**paper II**) versus children (**paper I**)*

In **paper II**, females presented significant higher dose-BW adjusted prednisolone C_{max} compared with males (3009 vs 2676 µg/L/(mg/kg), whereas a corresponding sex difference in dose-BW adjusted prednisolone AUC_{dose} was not observed. Studies in solid organ transplant recipients as well as healthy volunteers have reported that female sex is associated with a lower CL/F and/or a higher exposure of total and unbound prednisolone (137, 180). Investigating prednisolone pharmacokinetics in healthy volunteers, Suarez-Kurz et al. (181) revealed no effect of sex in C_{max} or total AUC_{0-24}, whereas the mean CL/F was observed higher (26%) in males than in females. Morton et al. (159) found a higher total prednisolone AUC_{0-6} in female compared with male lung transplant recipients (456 vs 304 µg/L/mg, $p<0.02$), but implied that a reduced body surface area in females accounted for the difference observed.

Investigating the influence of body composition on prednisolone pharmacokinetics in solid organ transplantation, Milsap et al. (182) demonstrated a considerable higher CL/F of prednisolone and a lower total and unbound prednisolone AUC in obese vs normal weight individuals. They hypothesized that due to the presence of

11β-HSD enzymes in abdominal adipose tissue, it may represent a peripheral site for distribution and clearance of prednisolone. Adipose tissue depots in obese subjects do express 11β-HSD1 (but not 11β–HSD2), and perhaps to a larger extent in human visceral than subcutaneous fat (18). An increased 11β-HSD1 activity is observed in human adipose tissue (183), possibly due to enhanced glucose availability (184), favoring the conversion from inactive to active GC (57, 185). Thus, active GC induces expression and activity of the 11β -HSD1 enzyme, providing a positive feed-back loop for increased levels of active GC. This fat-specific 11β -HSD1 excess in central obesity, named "Cushing's disease of the omentum" is associated with insulin resistance and metabolic syndrome, but the impact on prednisolone plasma levels is not unraveled. Notably, in the small single study by Milsap et al., the PL/PN ratio was reported similar in both obese and non-obese individuals. In **paper I**, one single patient presented a BMI of 36, whereas in the remaining patients the BMI ranged from 14-19, with a median value of 16. In **paper II**, the BMI ranged from 18-30, with a median value of 26. Given the narrow range of BMI values observed, an investigation of the effect of obesity on prednisolone pharmacokinetics in this population was not considered as informative.

5.2.5 Is there a potential for individualized dosing of prednisolone in solid organ transplantation?

In the meta-analysis by Haller et al. (50) it was also noted that since long-term studies on GC avoidance and withdrawal are lacking, the consequences of these regimens are unknown. In addition, studies comparing steroid vs. non-steroid regimens often discuss clinical outcomes without reference to GC dose, drug-drug interactions or individual variability in metabolism, which makes interpretation of the data difficult (186). A standardized evaluation of GCs is necessary if the side effects of a chronic low-dose of prednisolone should be weighed against the risk of reduced kidney function caused by the nephrotoxic CNIs or graft rejection.

To perform responsible drug tapering, a marker or panel of markers to predict and monitor immunological risk is necessary (186), yet there are no such monitoring available for GCs. In both **paper I and II**, a relatively high prednisolone exposure was observed, indicating that a more moderate dosing of prednisolone may be possible. TDM of prednisolone can be used as a guide for the rate of drug reduction. Aided by monitoring of prednisolone AUC_{0-6}, Morton et al. proved a substantial reduction of prednisolone dose in lung transplant recipients, without any short term adverse effects (159). Conversely, many patients that were initially off prednisolone experience a return to prednisolone therapy due to increased risk of rejection. In these cases, monitoring of prednisolone concentrations may contribute to identify the lowest dose providing sufficient efficacy for each individual. Lastly, TDM of any drug, including prednisolone, is useful for investigating suspected drug to drug interactions or unforeseen toxicities. In **paper I**, altered prednisolone and prednisone exposure was observed in patients receiving intravenous methylprednisolone, indicating that prednisolone dosing can be reduced with concurrent methylprednisolone therapy.

With reference to the requirements for TDM to be of value (see section 1.8), prednisolone is generally considered to have a wide therapeutic window, i.e. the difference between therapeutic and toxic concentrations is perceived as wide and TDM has been regarded as unnecessary (137, 187). However, although prednisolone therapy is associated with a plethora of adverse effects, toxicity is usually reported on *doses*, whereas the drug *concentration* is usually not measured. The relationship between prednisolone dose and concentration is poor and unpredictable, due to the large inter-individual pharmacokinetic variability well documented in **paper I and II** and other studies (120, 130, 132, 147, 159). Several studies have reported an association between prednisolone exposure and clinical outcome: between unbound prednisolone and post-challenge hyperglycemia (130), total or unbound prednisolone and cushingoid features (120, 133, 134, 137, 188, 189), total or unbound prednisolone and hypertension or diabetes (131, 159) and between glucocorticoid exposure and growth inhibition (42, 190, 191), all suggesting a potential role for prednisolone TDM. Conversely,

there are also studies that have failed to find such an association, questioning the value of pharmacokinetic monitoring alone (79, 122, 126, 192). Importantly, all the studies referred to above differ with regard to study population, type of assay used for analysis, whether unbound versus total prednisolone was measured, and number of samples within a dose interval. The calculated AUCs span from 0 to 5, 6, 8, 9, 12 or 24 hours, which cause variability in the estimations. In addition, there are wide differences in prednisolone doses given and length of follow-up. This lack of standardization may have contributed to the ambiguity in the results. But evidently, variations in the pharmacodynamics of prednisolone, notably polymorphisms in the GR, also contribute to the variability in clinical response to prednisolone therapy, and need attention (21, 43). Another prerequisite for pharmacokinetic TDM is an assay for determination of drug concentrations. Earlier, the most commonly used methodology used for measuring GCs were immunoassays, which were hampered with cross-reactivity of prednisolone and cortisol. This made interpretation of the data difficult. The advent of chromatography combined with tandem mass spectrometry has ensured accurate methods for measuring both total and unbound prednisolone. Finally, a therapeutic range is called for. This requires a better knowledge of the prednisolone pharmacokinetics within different subpopulations. Alternatively: the patient may serve as his/her own control over time, i.e. an individual therapeutic range. In both situations, TDM would be a valuable tool.

A full AUC is the best marker of drug exposure. Single point measures are frequently used as surrogate markers, but do not always correlate well with the AUC. Another option is the LSSs, using an equation derived from multiple linear regression analysis to estimate exposure based on one or a few concentrations collected during the dose interval. In **paper II**, the correlation between prednisolone AUC_{dose} and prednisolone concentrations were investigated in adult renal transplant recipients. The best single point prednisolone concentration to predict prednisolone $AUC_{0\text{-}24}$ was C_6 (r^2=0.82; mean PPE 7.4 ±2.0%), whereas an LSS incorporating C_0, C_2 and C_4 estimated the prednisolone $AUC_{0\text{-}24}$ with an even better accuracy (r^2=0.91; mean PPE 5.2 ±1.5%). When tested on three renal

transplant recipients outside of the study population, the LSS showed acceptable performance as their predicted AUC_{0-24} deviated from their actual AUC_{0-24} by solely 7, -4 and 2%. If these results can be validated in an independent population, this LSS offers a clinical practical sample strategy to estimate total prednisolone exposure in a similar population.

In the literature, there are limited reports regarding the use of TDM in prednisolone therapy. The study of Suarez-Kurz et al. explored LSS strategies for predicting total prednisolone $AUC_{0-\infty}$ in a population of 24 healthy subjects (12 females), ingesting a prednisone dose of 20 mg. The results showed that single point regression equations ($C_{1.5}$ or C_7) provided precise estimates of total prednisolone $AUC_{0-\infty}$, whereas combining single point measurements into a two point LSS marginally improved the performance (181). The inter-individual variability in healthy individuals is markedly lower than the inter-individual variability observed in renal transplant recipients, which may partly explain the higher correlation coefficient between $AUC_{0-\infty}$ estimated with $AUC_{0-\infty}$ measured (r^2=0.98) achieved in the study of Suarez-Kurz compared with the corresponding correlation in **paper II**. This is demonstrated in a study by Potter et al., presenting a stronger correlation between prednisolone plasma concentration/mg 2- hours post-dose (C_2/mg) with AUC_{0-6}/mg for healthy controls (r^2=0.994, $p<0.001$) compared with renal transplant recipients (r^2=0.682, $p<0.001$) (137). In a population of lung transplant recipients, Morton et al. examined the predictive value of prednisolone single point measures on the measured prednisolone AUC_{0-6} (both parameters dose-adjusted), finding C_2/mg to be the most useful single time point (r^2=0.948). The best LSS equation to predict total prednisolone AUC_{0-6} /mg included C_1, C_2 and C_4/mg (r^2=0.992), which also gave acceptable predictions when later tested on follow-up AUCs (100% of values within 10% of the measured AUC_{0-6}/mg) (159).

A major limitation to the LSS is that its application should be restricted to the patient population and the dosage regimen used when the LSS was developed (193). The predictive power of the LSS cannot be guaranteed in patient

subpopulations or where the dose regimen is changed, and possibly subject to drug-drug interactions. Nonetheless, evaluating the LSS strategies earlier reported (159, 181), Barraclough et al. found an acceptable predictive power of prednisolone $AUC_{0\text{-}12}$ in their cohort of renal transplant recipients, reporting a bias and imprecision below 15% (139). Furthermore, Barraclough et al. observed only moderate correlation between dose-adjusted unbound and total prednisolone $AUC_{0\text{-}12}$ values in patients > 3 months post-transplant (r^2=0.79, p<0.0001). An even weaker correlation (r^2=0.42, p<0.04) was observed in the early post-transplant period, when prednisolone doses were higher. The poor correlation early post-transplant was explained by the non-linear increase in unbound fraction seen as total prednisolone concentrations increase (73, 167). Barraclough et al. then evaluated the unbound drug concentrations for TDM, reporting C_6 to be best single point measure to predict unbound prednisolone $AUC_{0\text{-}12}$ (r^2=0.87, p<0.001), and that an LSSs incorporating $C_{0.25}$, C_2 and C_4 (r^2=0.98, median PPE 4.6%) showed the highest power for predicting unbound prednisolone $AUC_{0\text{-}12}$. These results are strikingly similar to the results observed in **paper II**. Validating the unbound prednisolone LSSs developed by Barraclough et al. in a cohort of renal transplant recipients 3 to 4 weeks post-transplant with a dosing regimen similar to Barraclough's study population, Yates et al. found that the LSS including $C_{1.25}$ and C_3 demonstrated the greatest predictive power for unbound prednisolone $AUC_{0\text{-}12}$ (r^2=0.97, median PPE 1.2%) (194).

Salivary concentrations of prednisolone is reported to correlate well with unbound prednisolone serum levels (r^2= 0.867, p<0.001) in healthy individuals (195, 196), and in a cohort of renal transplant recipients (r^2= 0.88), when measured as a single concentration 4 hours post-dose. Correlation between unbound prednisolone $AUC_{0\text{-}12}$ and saliva $AUC_{0\text{-}12}$ however, is reported to be poor (r^2= 0.07) (132). A population pharmacokinetic model able to predict unbound serum prednisolone levels from salivary levels has been developed by Teeninga et al. (196). Saliva samples are easily obtained, in a non-invasive manner and may be the preferred matrix, especially in children. If validated in an independent population, this offers new possibilities for sampling outside a clinical setting.

In conclusion: although prednisolone is generally considered to have a wide therapeutic window, the inter-patient variabilities in pharmacokinetic parameters are large, prednisolone therapy results in considerable toxicities even at low doses, and prednisolone exposure has been associated with adverse clinical outcomes in several studies. As the technical difficulties in separating steroid compounds are overcome by the advent of chromatography combined with tandem mass spectrometry, accurate methods for measuring both total and unbound prednisolone are established. Measurement of unbound as opposed to total prednisolone concentrations has been discussed as an alternative method, given the dose-dependent kinetics of total prednisolone. While a single point measurement may be insufficient, the LSSs described in the literature have demonstrated an ability to predict both total and unbound prednisolone AUC with an acceptable accuracy. The time points suggested to be incorporated in the LSS models from different studies are quite similar and consistent with the findings in **paper II**. Regarding patient convenience, the LSS should be well tolerated. Taken together, this demonstrates both the need for and feasibility of TDM of prednisolone.

6. Conclusions

The present thesis describes the pharmacokinetics of prednisolone and prednisone in renal transplant recipients, and aimed to identify the need and feasibility for individualizing the dosing of prednisolone. The following conclusions have been made:

I. In both adult and pediatric renal transplant recipients, there was a large inter-individual variability in prednisolone and prednisone pharmacokinetics, which persisted after adjusting for dose and BW.

II. A higher prednisolone exposure and a higher prednisolone/prednisone AUC_{dose} ratio was observed in patients with (as compared to without) concurrent methylprednisolone, in adults compared with children, and in three patients with (as compared to without) NODAT.

III. Prednisolone clearance, as assessed in an *in vitro* study using microsomes, depends on rCYP3A4 rather than rCYP3A5.

IV. A strong negative correlation between prednisolone AUC_{dose} and morning cortisol levels was observed in fasting adult renal transplant recipients. An LSS incorporating C_0, C_2 and C_4 estimated the prednisolone $AUC_{0\text{-}24}$ with a good accuracy (r^2=0.91; mean PPE 5.2 ±1.5%). When tested on three renal transplant recipients outside of the study population, the LSS showed acceptable performance.

The knowledge generated in the included studies has emphasized the need for improvements in prednisolone dosing regimens. The relatively high exposure of prednisolone observed indicates that a more moderate dosing of prednisolone may be possible. TDM can be a valuable tool for individualizing and optimizing prednisolone therapy, with the aim of reducing prednisolone doses without compromising efficacy. This may contribute to ameliorate immediate and long-term adverse effects of prednisolone therapy while preserving protection against rejection.

7. Future perspectives:

- A comprehensive validation of the LSS described in **paper II** should be performed in an independent population of renal transplant recipients with a sufficient number of patients included. Due to its dose-dependent, non-linear protein binding in plasma, it would be of interest to also measure the unbound (biologically active) fraction of prednisolone, and investigate whether the LSS predicts a full AUC better using unbound fraction of prednisolone concentrations than with total prednisolone concentrations.

- Additionally, the prednisolone pharmacokinetic data from **paper I** and **II** could potentially be applied in further studies, such as for developing the LSS in combination with population pharmacokinetic model derived Bayesian estimators to accurately predict individual AUC of prednisolone in kidney transplant recipients. This methodology may include covariates that are known to influence the pharmacokinetic parameters, such as various genotypes of metabolizing enzymes and drug transporters, or endogenous GCs and their metabolites as measured in plasma or urine, and could allow for more individualized dosing recommendations.

- In the light of the results from paper III, an *in vitro* study of the relative contribution of rCYP3A4 and rCYP3A5 in the metabolism of prednisolone in human liver microsomes should be performed, with assessment of scaling factors to convert the results into *in vivo* relevance. Furthermore, a clinical study comparing CYP3A5 expressers with CYP3A5 non-expressers with and without a CYP3A inhibitor such as ketoconazole, and also a CYP3A4 specific inactivator such as CYP3cide, could provide additional insights into the relative role of CYP3A4 and CYP3A5 in prednisolone metabolism.

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III

In vitro assessments predict that CYP3A4 contributes to a greater extent than CYP3A5 to prednisolone clearance

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ABSTRACT

Because several steroid hormones are metabolized to their respective 6β-hydroxy forms by CYP3A4 and CYP3A5, these isoenzymes have also been assumed to metabolize the immunosuppressive drug prednisolone, with conflicting results in the literature with respect to their relative importance. A direct study of the metabolism of prednisolone by microsomal CYP3A4 and CYP3A5 is missing. The aim of this *in vitro* study was to investigate the relative importance of rCYP3A4 and rCYP3A5 in the metabolism of prednisolone and to compare the extent of formation of 6β-OH-prednisolone by the two enzymes. Through *in vitro* incubations using rCYP3A4 and rCYP3A5 insect cell microsomes, intrinsic clearance (CL_{int}) of prednisolone was determined by the substrate depletion approach. Formation of the metabolite 6β-OH-prednisolone by rCYP3A4 and rCYP3A5, respectively, were compared. Prednisolone concentrations were measured and its metabolite 6β-OH-prednisolone was identified using a HPLC-MS/MS in-house method. CL_{int} for prednisolone by rCYP3A5 was less than 26% relative to rCYP3A4. Formation of 6β -OH-prednisolone by rCYP3A5 was less than 10% relative to rCYP3A4. The study indicates that 6β-hydroxylation of prednisolone assessed *in vitro* in microsomes depends on rCYP3A4 rather than rCYP3A5, and that CYP3A5 may be responsible for the formation of other prednisolone metabolite(s) in addition to 6β-OH-prednisolone.

INTRODUCTION AND BACKGROUND

Prednisolone is a synthetic glucocorticoid administered in a wide range of conditions that require anti-inflammatory or immunosuppressive treatment, such as autoimmune diseases, malignancies and after organ transplantation. Glucocorticoid therapy is, however, associated with a considerable variability in both desired and unwanted effects and even at relatively low comparable bodyweight (BW)-adjusted doses, a wide range of unwanted side effects including hyperglycemia, hypertension, hyperlipidemia and osteopenia are common.[1]

The main metabolic pathways of prednisolone are through oxidation of the hydroxyl group in C11 (creating equilibrium between the biologically inactive 11-hydroxy and biologically active 11-keto forms via the 11-β-hydroxysteroid dehydrogenase 1 and 2 enzyme), and through reduction of ketone in C20 (forming the 20 alpha and beta-dihydro-prednisolone/prednisone metabolites via the

20-hydroxysteroid dehydrogenase enzyme). In addition, both prednisolone and prednisone are hydroxylated in C6, forming the alpha and beta isomers of 6β-OH-prednisolone (Figure 1).[2] Based on reports that 6β-hydroxylation of cortisol is catalyzed by the isoenzymes CYP3A4[3] and CYP3A5[4], these enzymes have also been assumed to be partially involved in prednisolone metabolism[5,6], with conflicting results in the literature with respect to the degree of involvement.[7,8] In particular, the role of CYP3A5 in the metabolism of prednisolone has not been fully elucidated.

Cytochrome P450s (CYP) is a superfamily of heme-containing enzymes, capable of catalyzing the oxidative metabolism of numerous substrates.[9] The CYP3A subfamily, comprising two isoforms, CYP3A4 and CYP3A5, is quantitatively the most abundant CYP enzymes in the human liver and small intestine in adults.[10] Together, CYP3A4 and CYP3A5 account for approximately 30% of hepatic cytochrome P450[11], and are responsible for the oxidative metabolism of over 50% of the drugs in widespread use.[12] Genetic polymorphisms have been identified in both enzymes. For CYP3A5, this has a functional relevance in that the *CYP3A5*3* allele encodes for a nonfunctional enzyme, whereas *CYP3A5*1* is associated with CYP3A5 expression.[10] CYP3A5 is expressed in more than 50% of African Americans, in approximately 30% of Japanese and in 15-25% of Caucasians.[10,13] Individuals carrying the *CYP3A5*1* allele experience a higher clearance and lower bioavailability of CYP3A5 substrates compared with individuals not carrying the *CYP3A5*1* allele, as has been demonstrated for

the immunosuppressive drug tacrolimus.[14,15] Genetic variants in CYP3A4 that have a functional relevance are infrequent (https://www.pharmvar.org/gene/CYP3A4). However, it has been found that the *CYP3A4*22* allele is linked to reduced CYP3A4 mRNA expression and hepatic CYP3A4 activity[16], and is associated with reduced tacrolimus and cyclosporine A clearance.[17,18] Nevertheless, the general relevance of *CYP3A4*22* on CYP3A4 phenotype is unclear, as reviewed by Werk and Cascorbi.[19] Although the inducible CYP3A4 is generally recognized as the predominant CYP3A enzyme; in some individuals CYP3A5 can constitute more than 50% of the total hepatic CYP3A[4,10,20], and may in such cases represent a considerable share of total CYP3A activity. As CYP3A4 and CYP3A5 are 83% homologous in terms of amino acid sequences[21], CYP3A4 and CYP3A5 were thought to be similar in substrate specificity. However, differences in catalytic capability and regioselectivity towards some substrates have been identified, and the relative importance of CYP3A4 and CYP3A5 in overall CYP3A mediated metabolism differs between substrates.[10,22]

In an earlier study investigating adult renal transplant recipients, we observed three CYP3A5 expressors (*1/*3) presenting a mean dose/BW adjusted prednisolone AUC_{0-24} which was 25% lower compared with CYP3A5 non-expressors (n=25).[23] Moreover, in a population of nine pediatric renal transplant recipients, we found that the patient with the lowest prednisolone- AUC_{0-12} and the highest prednisolone-CL/F was a CYP3A5 expressor (*1/*3).[24] Hence, the aim of this *in vitro* study was to examine the relative contribution by rCYP3A4 and rCYP3A5 in the metabolism of prednisolone in microsomes, and to compare the extent of formation of 6β-OH-prednisolone by the two enzymes.

MATERIALS AND METHODS

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[25]

Reagents

Prednisolone, HPLC-grade water and zinc sulfate 0.10 mol/L were purchased from Sigma-Aldrich (St.Louis, MO). Prednisolone-d8 and 6β-OH-prednisolone were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Baculovirus-transfected insect cell microsomes selectively co-expressing human CYP3A enzymes, cytochrome P450 reductase and cytochrome b5 (Supersomes) were purchased from Corning Inc (NY, 14831 USA). Stock solutions of prednisolone calibrators (1 mg/mL) and deuterated internal standard (1 mg/mL) were prepared in MeOH. Analyte working solutions were prepared by diluting the stock solutions in MeOH. A stock solution of 6β-OH-prednisolone (1 mg/mL) was prepared in MeOH. This stock was diluted 1:1000 in MeOH to prepare a 1 mg/L tune solution for MS-optimization, and diluted in stripped fetal bovine serum from Sigma-Aldrich (St.Louis, MO) to relevant concentrations for the verification of chromatographic peaks. For the preparation of calibrators and quality controls, the analyte working solution was also diluted in stripped fetal bovine serum. The internal standard working solution was diluted 2000-fold in the precipitation solution, which consisted of MeOH and $ZnSO_4$ (2:1). NADPH, EGTA, $MgSO_4$, hepes and sucrose were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), and Tris-H_2SO_4 was purchased from VWR International (Radnor, PA). HPLC-grade acetonitrile and methanol were obtained from Rathburn Chemicals (Walkerburn, Scotland). Mobile phases were prepared with ultra-HPLC/MS–grade water and methanol with 0.10% formic acid from Honeywell (Maurice, NJ) and ammonium acetate, Sigma-Aldrich (St.Louis, MO).

Experimental Conditions and Sample Preparation

The metabolism of prednisolone was examined using the substrate depletion approach. Preliminary studies showed that the measured concentration of prednisolone in the buffer solution was only approximately 70% of the nominal concentration, which was perceived as a solubility problem. To increase the solubility of prednisolone, the diluting agent was changed from Tris-H_2SO_4 to organic solvent (methanol), producing a 7 µM prednisolone solution in 25% MeOH. Based on earlier studies[26],

a final enzyme concentration of 4 nM was used in the preliminary studies. Due to slow metabolization and substrate depletion, the final enzyme concentration for both rCYP3A4 and rCYP3A5 was increased to 45 nM in the total incubation volume of 220 µL (0.4 mg/mL for rCYP3A4 and 0.3 mg/mL for rCYP3A5), which was consistent with the recommended 100 pmol/mL in the literature[27], and the incubation time (initially set to 30 min) was extended to 120 min.

Incubation assays contained either rCYP3A4 or rCYP3A5, with co-expressed cytochrome b5. Microsomes were diluted in a solution (pH 7.4) consisting of 0.25 M sucrose, 10 mM Hepes, and 2 mM EGTA. Prednisolone (approximately 0.5 µM for both rCYP3A4 and rCYP3A5), exact concentration assessed at start of the experiment) was incubated at 37°C in 200 mM Tris-H_2SO_4 (pH 7.4), 20 mM $MgSO_4$, and 10 mM NADPH, according to incubation conditions optimized by Hermann et al.[28] The reaction was initiated by adding preheated microsomes, samples were then removed from the incubation assay at 0, 10, 20, 30, 45, 60, 90 and 120 min and quenched with 882 µL ice-cold precipitation solution (MeOH:$ZnSO_4$, 2:1) containing prednisolone-d8 (0.8 µM) as an internal standard, and put on ice for 30 min. The samples were then centrifuged at 1600*g* for 5 min at 4°C (Heraeus Megafuge 16R, Thermo Fisher Scientific, Waltham, MA), and 200 µL of the supernatant plus 300 µL of a diluent (mobile phase A: water with 0.10 formic acid and 2.0 mmol/L ammonium acetate) were pipetted to a deep-well plate (2.2-mL 96 deepwell polypropylene plate with square wells; Hamilton, Bonaduz, Switzerland). The plate was then sealed (Slit Seal, BioChromato, Kanagawa-ken, Japan) and shaken for 4 min (1400 rpm, 3-mm orbit; High-Speed Multi-Plate Shaker, BioSan, Riga, Latvia). The plate was centrifuged for 10 min (2000g, 4°C; Rotanta, Hettich, Tuttlingen, Germany) and transferred to an autosampler with temperature kept at 10°C. Three separate experiments were performed for each of the two microsomal preparations studied. All of the incubations in each experiment were performed in duplicate. One of the duplicate time points at 90 min, in one of the three rCYP3A5 experiments was identified as an outlier (Thompsons Tau test) and removed.

Determination of prednisolone and 6β-OH-prednisolone

All samples were analyzed for concentrations of prednisolone and 6β-OH-prednisolone using protein precipitation, followed by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS), performed as described in a previous article[23] with some minor modifications to include 6β-OH-prednisolone in the assay (Table 1, Figure 2). Stock solutions of

calibrators (1 mg/mL) and internal standards (IS; 1 mg/mL) were prepared in MeOH. Analyte working solutions were prepared by diluting the stock solutions in MeOH. The IS stock solutions were diluted in MeOH to prepare a common IS working solution. For the preparation of calibrators and quality controls, the analyte working solution was diluted in Charcoal Stripped Fetal Bovine Serum glucocorticoids from Sigma-Aldrich (St. Louis, MO). The range of the assay detection method for prednisolone was 20-1016 µg/L. Within- and between runs coefficients of variation of quality controls were <7% with three replicates at four different concentrations being used. Bias was <4% in all levels. Absolute quantitation of 6β-OH-prednisolone was not performed due to lack of a suitable isotope labelled internal standard, and also apparent impurities in the reference compound. The metabolite levels were estimated using the signal from the mass transition 377.2>341.1, quantitated by the prednisolone calibration curve. Qualitatively, presence of the metabolite in the enzyme reactions were confirmed by chromatographic retention time and by using the mass transition, 377.2>147.1 as a qualifier.

Data analysis

A logarithmic (log) transformation of the substrate depletion curves for prednisolone was performed, in order to graphically verify mono-exponential decay, revealing linear substrate depletion curves for rCYP3A4 as well as for rCYP3A5 microsomes for the time range 0-45 min (data not shown). Substrate depletion data of prednisolone from 0-45 min were therefore fitted to a mono-exponential decay model (eq. 1), with a 1/Y weighting using GraphPad Prism version 8.0.1 (GraphPad Software Inc., San Diego, CA).

$$C_t = C_0 e^{-kt} \quad (1)$$

The velocity constant k (min^{-1}) for the substrate depletion was estimated in each experiment, and consequently the intrinsic clearance (CL_{int}) was calculated (eq. 2).

$$CL_{int} = kV\ (\mu L/min), \quad (2)$$

where V is the incubation volume.

Relative depletion of substrate concentration was determined by calculating (prednisolone concentration at 45 min – prednisolone concentration at 0 min) / (prednisolone concentration at 0

min)* 100%. A paired t-test was used to determine any statistical significant difference in prednisolone concentration at 0 min vs 45 min, for both rCYP3A4 and rCYP3A5. A value of $p < 0.05$ was considered statistically significant.

The extent of formation of 6β-OH-prednisolone by rCYP3A4 and rCYP3A5 was compared by assessment of the formation of 6β-OH-prednisolone after 45 min incubation in rCYP3A5 microsomes relative to rCYP3A4 microsomes.

RESULTS

The prednisolone concentration of 0.5 µM in rCYP3A4 and rCYP3A5 microsomes was well below the apparent value of Km for rCYP3A4-mediated prednisolone metabolism, which is reported to be 40-166 µM.[29] This concentration resulted in linear formation of the metabolite and provided measurable metabolite amounts.

Determination of CL_{int}

Substrate depletion data for prednisolone using a mono-exponential decay model from 0 to 45 min was studied in both microsomal preparations, showing a monoexponential decay ($r^2 > 0.94$ for rCYP3A4 and $r^2 > 0.45$ for rCYP3A5) (Figure 3). CL_{int} for rCYP3A4 and rCYP3A5 were 0.25 and 0.07 µL/min/pmol enzyme, respectively (Table 2). CL_{int} of prednisolone by rCYP3A5 was 26% (S.E.M. 10%) relative to rCYP3A4. Relative depletion of substrate concentration after 45 min was -38% for rCYP3A4 ($p=0.008$) and -16% for rCYP3A5 ($p=0.094$).

For the time range 0-120 min, the log-transformed substrate depletion curves were non-linear (data not shown), and showed two-phase exponential decay (Figure 4).

The formation of 6β-OH-prednisolone

The formation of 6β-OH-prednisolone by rCYP3A5 was different from that of rCYP3A4. After 45 min incubation, the formation rate of 6β-OH-prednisolone by rCYP3A5 still showed a linear trend, whereas formation rate of 6β-OH-prednisolone by rCYP3A4 showed a tendency towards plateau phase. These tendencies were accentuated towards 120 min of incubation. The formation of 6β-OH-prednisolone by rCYP3A5 was less than 10% relative to rCYP3A4 (Figure 5).

DISCUSSION

In this *in vitro* substrate depletion study, investigating the relative contribution of rCYP3A4 and rCYP3A5 in the metabolism of prednisolone, we found that prednisolone is metabolized by rCYP3A5, but to a lesser degree than by rCYP3A4. CL_{int} for prednisolone by rCYP3A5 was less than 26% relative to rCYP3A4 in microsomes. Formation of 6β-OH-prednisolone by rCYP3A5 was less than 10% relative to rCYP3A4. These results suggest that the prednisolone 6β-OH-hydroxylation activity in microsomes is dependent on the rCYP3A4 activity rather than rCYP3A5.

In the literature, substrate depletion studies and metabolite formation studies on the role of CYP3A4 and CYP3A5 in prednisolone metabolism are sparse. One study by Zhang et al[29], investigating prednisolone metabolism and responsible CYP enzymes revealed results consistent with the current study. Using cDNA-expressed human cytochrome P450 enzymes, Zhang et al. reported that among the CYP enzymes investigated within phase 1 biotransformation, CYP3A4 was the principal enzyme responsible for prednisolone metabolism, although not concluding regarding CYP3A5. However, an *in vitro* study investigating epithelial toad kidney cells, not expressing CYP3A4, did indicate a role for CYP3A5 in 6β-OH-steroid formation.[30] Although reports on prednisolone and CYP3A pharmacogenetics are few, *in vitro* studies on the metabolism of steroids other than prednisolone are available. In human liver microsomes, CYP3A4 has been demonstrated to be responsible for cortisol 6β-hydroxylase activity[31], whereas both testosterone and cortisol are shown to be metabolized by CYP3A5, though at a slower rate than by CYP3A4.[4] In microsomes prepared from Hep G2 cells transfected with CYP3A enzymes, three steroids (testosterone, androstenedione and progesterone) were metabolized to their respective 6β-OH-metabolite by both CYP3A4 and CYP3A5; nonetheless, the CYP3A5 enzyme exhibited a distinctly lower (less than 25%) activity compared with CYP3A4.[32] Comparing the CYP3A4 and CYP3A5-mediated 6β-hydroxylation of cortisol in E. coli expressed recombinant enzymes, Niwa et al. found that the V_{max} and V_{cat}/K_m for CYP3A5 was less than 30% relative to CYP3A4 in the formation of the 6β-OH-cortisol, and the K_m value for CYP3A5 was 15% higher than that of CYP3A4.[33] The kinetic parameters for testosterone and progesterone showed the same tendency, although more pronounced. Pharmacokinetic drug-drug interaction studies have also demonstrated that co-administration of CYP3A inducers cause an increase in clearance and decreased half-life of prednisolone.[34,35] In line with these observations, a strong CYP3A inhibitor such

as ketoconazole is reported to increase the total and unbound prednisolone concentrations in plasma by about 50%, due to a reduced clearance.[8] A major challenge in interpreting the data from these studies is that glucocorticoids, including PL, themselves induce CYP3A enzymes.[36,37] In addition, glucocorticoids have been shown to have the potential to inhibit CYP3A5, but not CYP3A4, in a time dependent manner.[38] Altogether, studies on the metabolism of steroids seem to concur regarding CYP3A4 being the preferred CYP3A enzyme responsible for the 6β-hydroxylation activity, nevertheless, direct *in vivo* studies of the metabolism of prednisolone by CYP3A4 and CYP3A5 are lacking. The results of the present *in vitro* study indicate however, that prednisolone is metabolized by rCYP3A4, but do not support rCYP3A5 to be of major importance.

Prednisolone metabolite pattern has been characterized in several studies, identifying up to 20 different metabolites. In addition to prednisone and 20-dihydro-prednisolone, 6β-OH-prednisolone has been shown to be among the major unconjugated metabolites of prednisolone in human urine, although the corresponding metabolizing enzymes are not identified.[2,39,40] The current assay was set up to identify only one metabolite, 6β-OH-prednisolone. In our study, we observed that the formation of 6β-OH-prednisolone by rCYP3A5 was different from that of rCYP3A4. Both isoenzymes contributed to the overall metabolism, but formation of 6β-OH-prednisolone by rCYP3A5 was less than 10% relative to rCYP3A4, whereas the CL_{int} of prednisolone by rCYP3A5 was less than 26% relative to rCYP3A4. This indicates that CYP3A5 may be responsible for the formation of one or more prednisolone metabolite(s) in addition to the 6β-OH-prednisolone detected.

In this substrate depletion study, only 16% of total prednisolone was metabolized by rCYP3A5, whereas 38% of total prednisolone was metabolized by rCYP3A4 in the time span of 45 min and under the conditions as specified. Since the homology between CYP3A5 and CYP3A4 is as high as 84%, the enzymes have a wide range of overlapping substrate specificities, and there is no substrate known that is exclusively metabolized by either CYP3A4 or CYP3A5.[41] However, results from studies using molecular docking simulations indicate that there are differences in the binding affinity of certain substrates to the CYP3A4 and CYP3A5 enzymes, due to conformational changes in their respective binding sites.[42,43] Niwa et al. showed that a typical CYP3A5 substrate such as vincristine was able to dock closer to the heme region of CYP3A5 compared with CYP3A4, whereas fluconazole, a strong inhibitor of CYP3A4, could not dock effectively with the active site of CYP3A5.[44] Further studies on

steroids by Niwa et al. indicate that an altered accessibility of substrates and inhibitors to the heme moiety of CYP3A molecules, which is a preferred location for oxidation at C6β position, may be a possible explanation for the difference in affinity between CYP3A4 and CYP3A5.[43] This may in part explain the observed differences between the two isoenzymes regarding catalytic efficiency, and susceptibility to inhibitors, despite largely sharing the same set of substrates.

As demonstrated in earlier studies[23,24], the inter-individual variability in serum concentrations of prednisolone is considerable. Although experimental conditions, inter-individual variability, extrahepatic metabolism, or nonlinear kinetics may cause discrepancies between *in vitro* and *in vivo* data[45,46], the observations from this study may imply that other factors than genetic polymorphisms in CYP3A5 determine the inter-individual variability of prednisolone. The levels of expression and activity of CYP3A4 may be more relevant, however. The large inter-individual variability in CYP3A4 expression and activity documented in the literature[47,48], can in part be attributed to genetic polymorphisms, but drug to drug interactions, age, sex, comorbidity and diet may also influence the pharmacokinetics of CYP3A4 substrates, as reviewed by Zanger and Schwab.[12]

The substrate depletion method was used in this study instead of the metabolite formation method because prior knowledge of the particular metabolic pathways under study and their importance to the overall metabolic fate of prednisolone is not well described. In addition, a method for an absolute quantification of the metabolite 6β-OH-prednisolone was not available, and a suitable isotope labelled internal standard was lacking. The enzyme concentrations were kept below 0.5 mg/mL to reduce the risk of non-specific binding, in line with suggestions on standardization of substrate depletion studies in microsomes.[49] In contrast to this, Walsky and Obach found an enzyme concentration of approximately 6 nM to be appropriate when investigating testosterone 6β-hydroxylation by rCYP3A enzymes *in vitro.*[50] However, when similar concentrations were used in our preliminary studies, we experienced slow metabolization and a lack of substrate depletion.

Linearity studies were also performed. The log-transformed substrate depletion curves for rCYP3A4 and rCYP3A5 were linear in the time range 0-45 min (figure 3), whereas extending the time range to 0-120 min produced non-linear depletion curves (figure 4) , indicating a two-phase exponential decay. This biphasic effect was also recognized and explained by Jones & Houston[49] as caused by a limited

oxygen availability followed by enzyme degradation and loss of (enzyme) activity. Another suggested explanation was the end-product inhibition phenomenon: as microsomes have no functioning phase II conjugation enzymes, phase I metabolites accumulate and may compete with the parent drug for binding to the enzyme that catalyzes the first reaction, thereby inhibiting the parent metabolism.[49]

Long incubation times may be detrimental to the CYP3As, CL_{int} for prednisolone was therefore determined for the time range 0-45 min in the present study. To distinguish metabolism from baseline variability, preferably a 20% of the substrate should be metabolized within the incubation period.[49] The prednisolone depletion by rCYP3A5 after 45 min in this study was 16%. Considering the 6β-OH-prednisolone metabolite formation illustrated in figure 5, however, metabolism of prednisolone by rCYP3A5 seems to take place to a certain extent.

There are some limitations to this study. Due to different protein content in the microsomes, an enzyme concentration of 45 nM in both rCYP3A4 and rCYP3A5 resulted in a slight difference in protein/mL for rCYP3A4 and rCYP3A5 (0.4 mg/mL vs 0.3 mg/mL). The substrate depletion method is regarded as an imperfect approach compared to the metabolite formation method, because measuring depletion involves subtracting two large numbers, which increases the imprecision.[27] In addition, hepatocytes are reported to produce more accurate and precise predictions of CL_{int} than those obtained using microsomes.[51] On the other hand, the quality control results indicate that the measurements are precise, with an imprecision well below the observed changes in prednisolone concentrations.

The current study demonstrates that 6β-hydroxylation of prednisolone, assessed *in vitro* in microsomes, can be catalyzed both by rCYP3A4 and rCYP3A5. The results indicated that compared with rCYP3A5, the rCYP3A4 mediated pathway is more relevant in prednisolone metabolism, when studied *in vitro*. A further corroboration of these results in human liver microsomes is warranted. Clinical studies allowing for a comparison of PL metabolism between CYP3A5 expressers and non-expressers, where also a potential effect of a CYP3A inhibitor is evaluated, are necessary to quantify the eventual contribution by CYP3A5 mediated metabolism of prednisolone in a clinical setting.

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Authorship Contributions

Participated in research design: Christensen, Bergan, Heier Skauby

Conducted experiments: Heier Skauby, Christensen

Conducted the LC-MS/MS analysis: Heier Skauby, Andersen, Vethe

Performed data analysis: Heier Skauby, Vethe

Wrote or contributed to the writing of the manuscript: Heier Skauby, Christensen, Bergan, Andersen, Vethe

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Table 1: Conditions for Chromatography

Start Time (s)	Duration (s)	Gradient Type	Flow Rate (mL/min)	Mobile Phase B (%)
0	20	step	0.5	25
20	130	linear ramp	0.5	70
150	30	step	0.5	99
180	40	step	0.5	25

The mobile phase was directed to the mass spectrometer between 42 and 132 s. Mobile phases were (A) water and (B) methanol, both with 0.10 formic acid and 2.0-mmol/L ammonium acetate. Chromatographic separation was performed at 50°C.

Selective Reaction Monitoring

Analyte and Corresponding Internal Standard	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
Prednisolone	361.3	147.1	25	39
Prednisolone-d8	369.3	150.1	25	39
6β-OH-prednisolone-341	377.2 (Quantifier)	341.1	10	32
6β-OH-prednisolone-147	377.2 (Qualifier)	147.1	25	39

Dwell time 50 ms. Argon collision gas 1.5 mTorr. Resolution 0.7 Da (full width at half maximum).

Table 2: CL_{int}[a] for substrate depletion of prednisolone in rCYP3A4 and rCYP3A5 microsomes. Data are presented as mean ± SD from three independent experiments.

Microsomal preparation	CL_{int}
	µL/min/pmol
rCYP 3A4	0.25 ± 0.03
rCYP 3A5	0.07 ± 0.04

[a]CL_{int}; intrinsic clearance

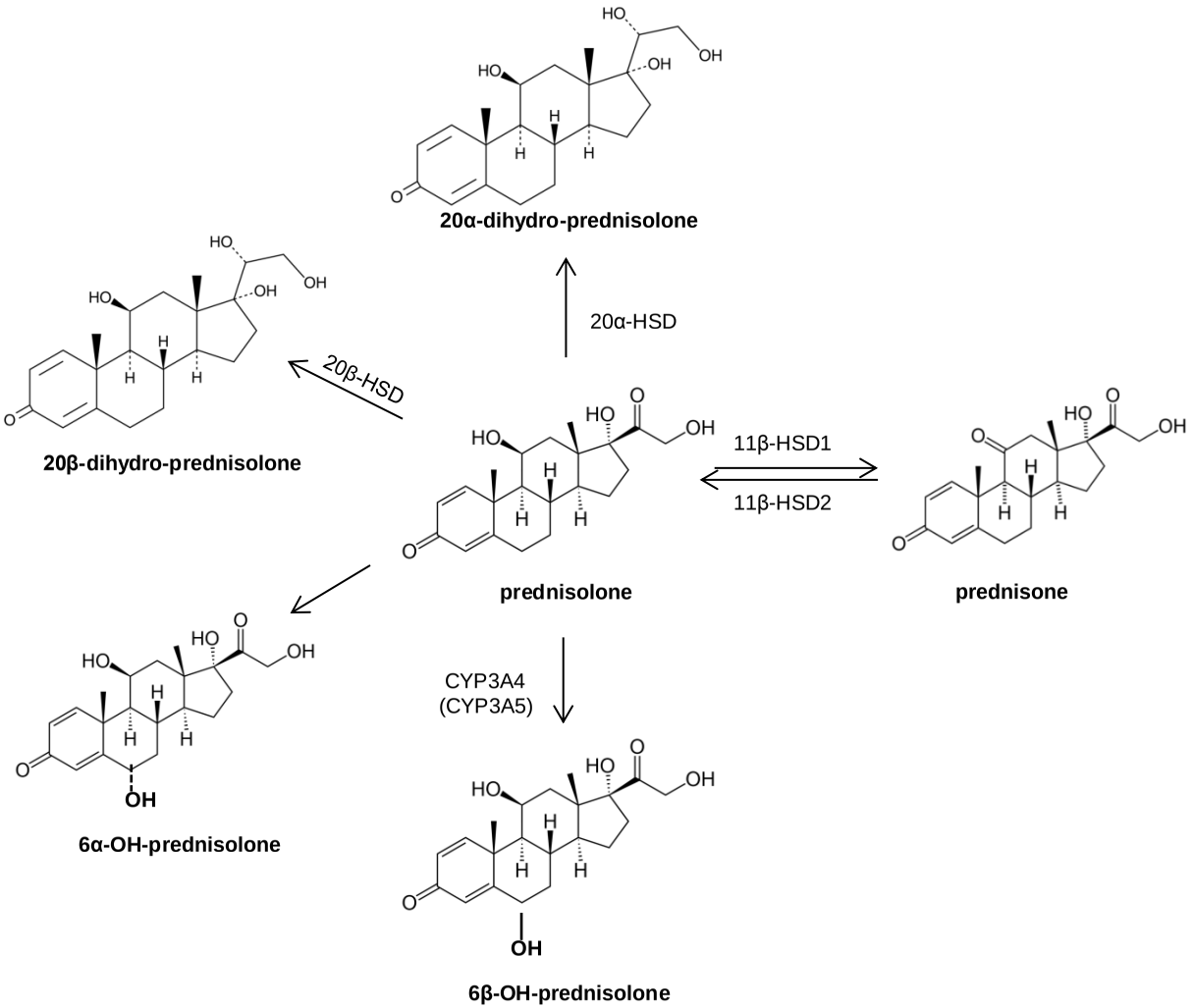

Figure 1: major metabolites of prednisolone

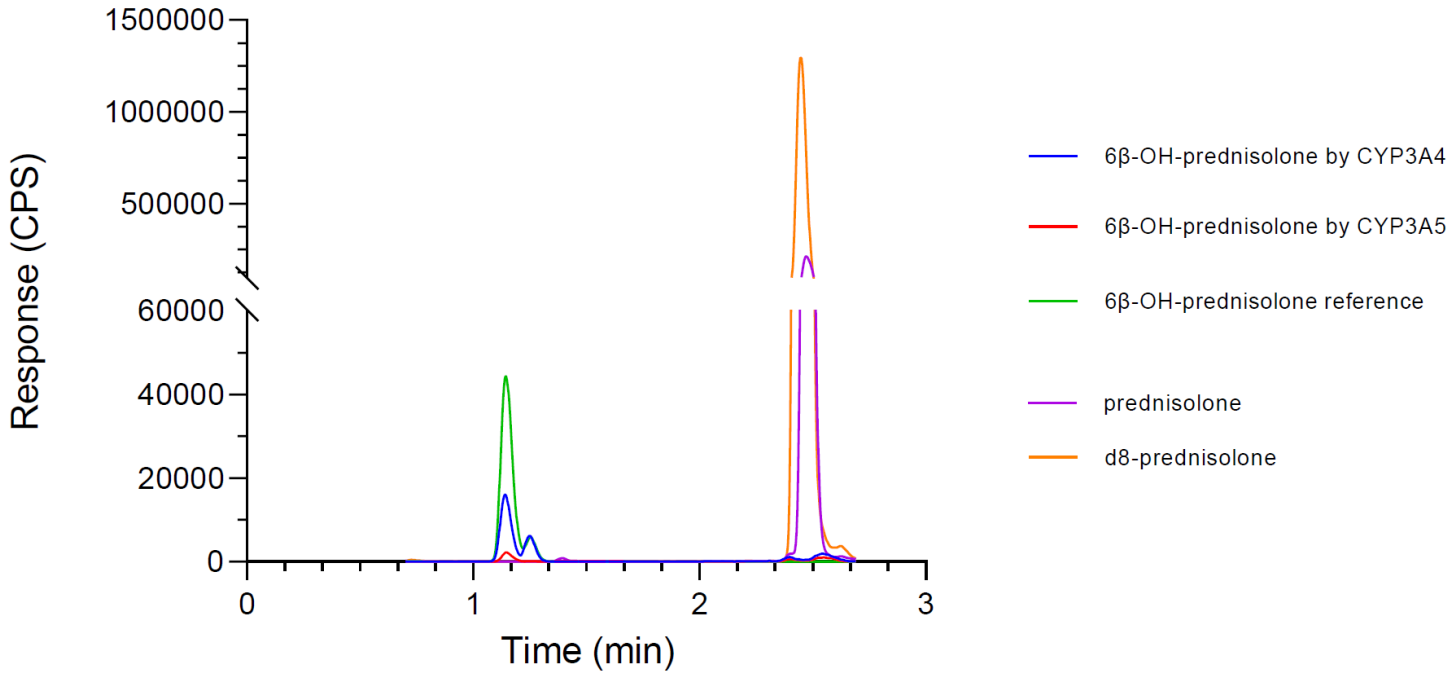

Figure 2: Ion chromatograms of prednisolone and its 6β-hydroxylated metabolites by rCYP3A4 and rCYP3A5, plus d-8 prednisolone and spiked 6β-OH-prednisolone reference. Vertical axis: instrument response (counts per second, CPS). Horizontal axis: elution time (min).

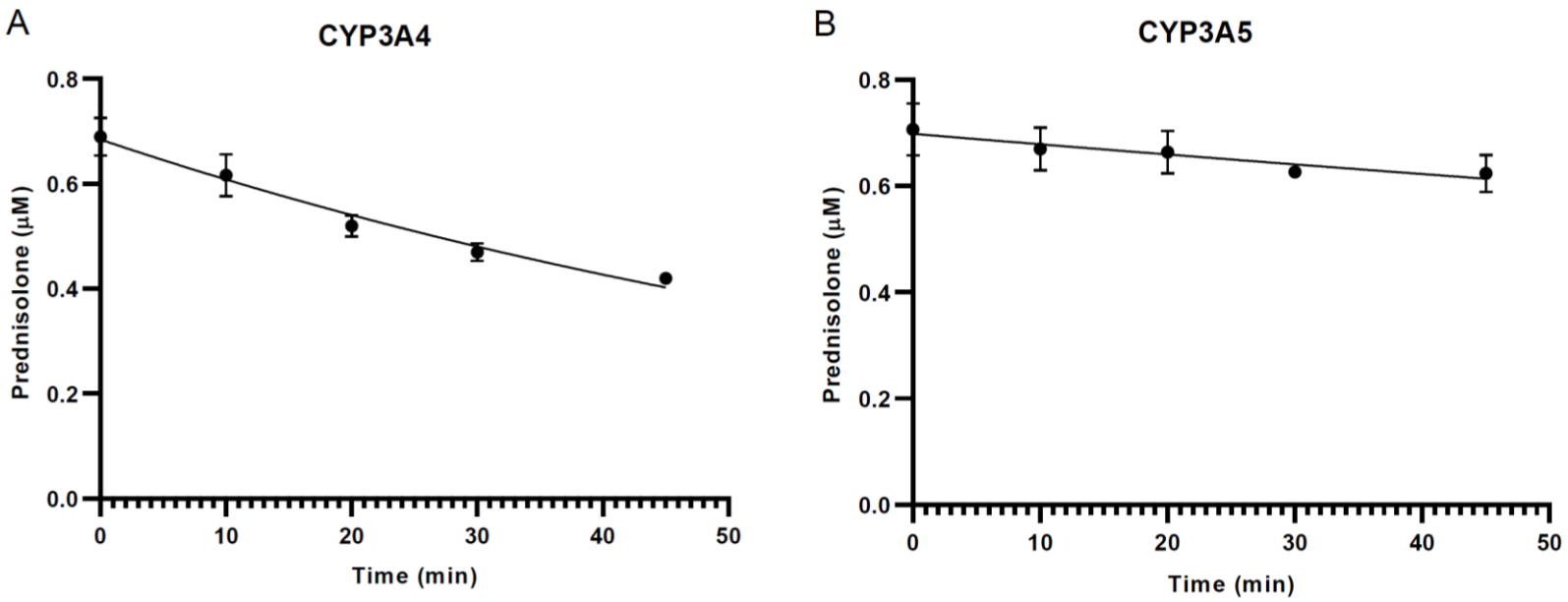

Figure 3: Substrate depletion curves for prednisolone in A) rCYP3A4 and B) rCYP3A5 microsomes for the time range 0-45 min. The initial concentration of prednisolone was 0.7 µM in both microsomal preparations. The data of prednisolone are fitted to a mono-exponential decay model ($C_t = C_0 e^{-kt}$), with a 1/Y weighting using GraphPad Prism version 8.0.1 (GraphPad Software Inc., San Diego, CA), and presented as mean ± SD from three independent experiments.

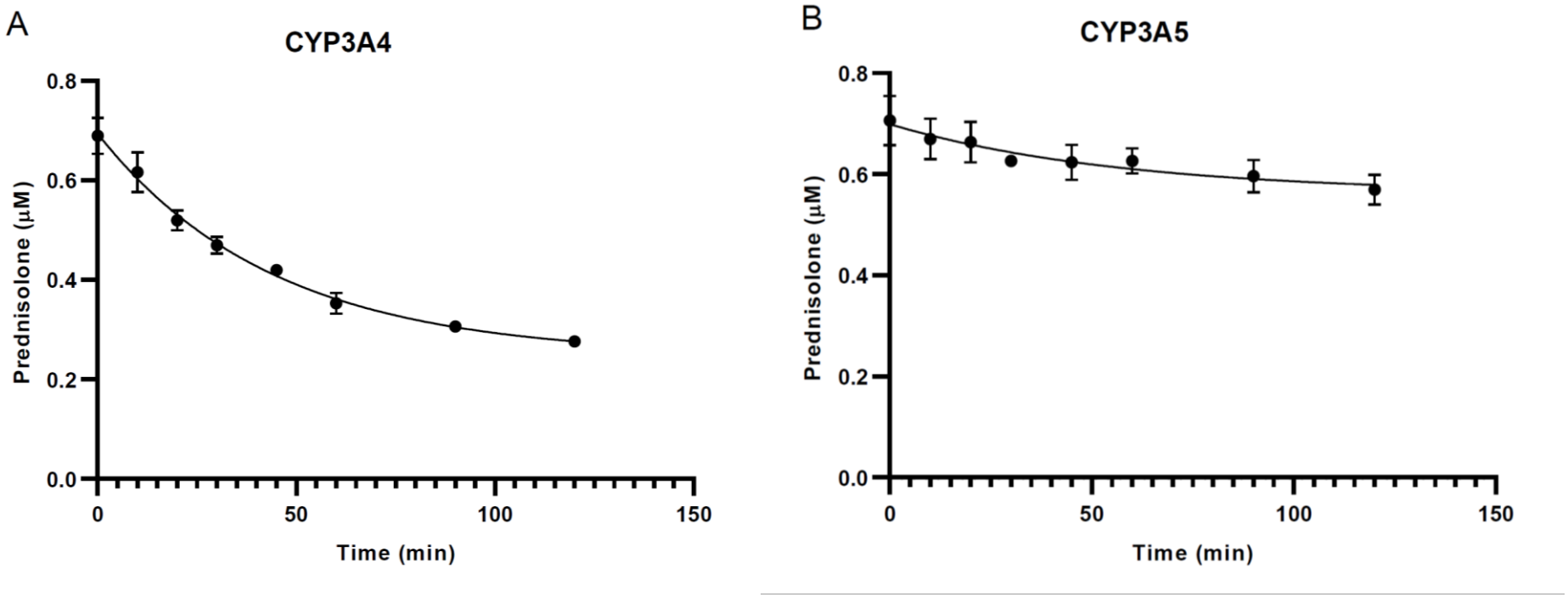

Figure 4: Substrate depletion curves for prednisolone in A) rCYP3A4 and B) rCYP3A5 microsomes for the time range 0-120 min. The initial concentration of prednisolone was 0.7 µM in both microsomal preparations. The data of prednisolone are fitted to a double exponential decay model, using GraphPad Prism version 8.0.1 (GraphPad Software Inc., San Diego, CA), and presented as mean ± SD from three independent experiments.

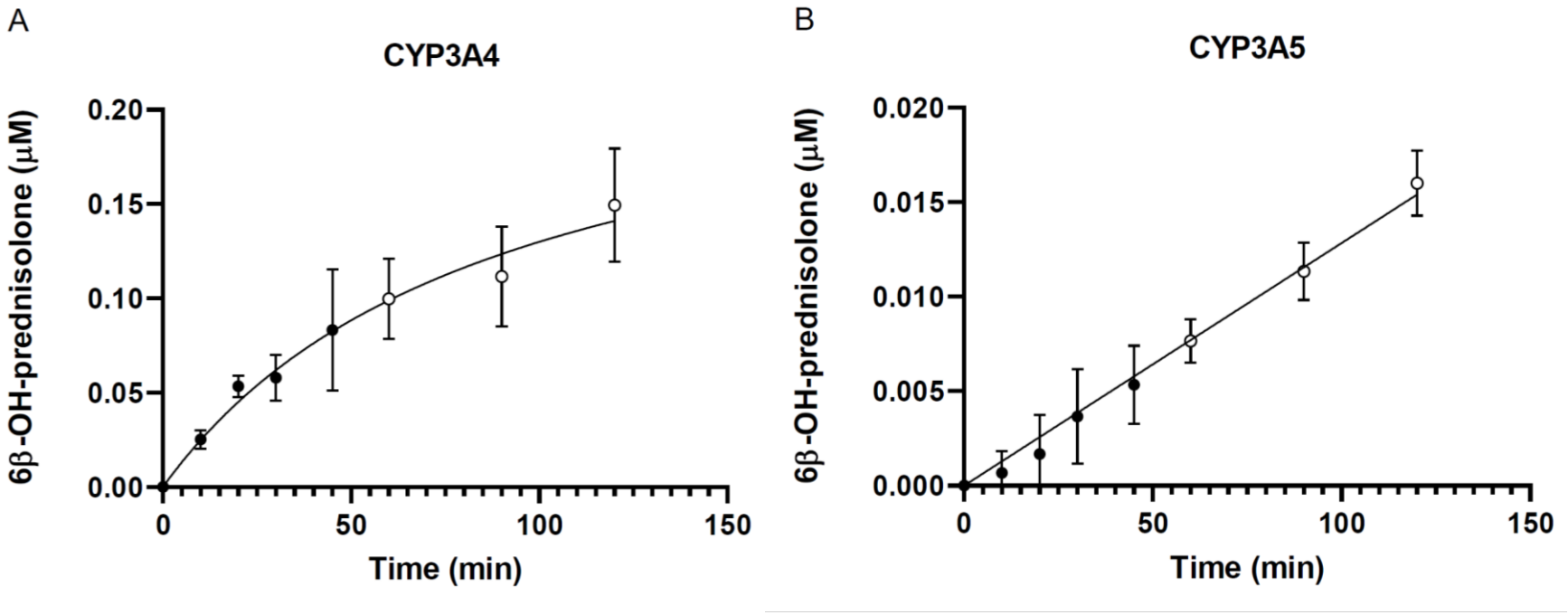

Figure 5: Formation of 6β-OH-prednisolone in A) rCYP3A4 and B) rCYP3A5 microsomes, after 0-45 min (black circles) and 60-120 min (white circles). The rCYP3A4 data are fitted to a nonlinear regression (second order polynomial) model, while the rCYP3A5 data are fitted to a linear regression model. Values are given in prednisolone units (μM), and presented as mean metabolite formation ± SD from three independent experiments.