Individualized dosing of calcineurin inhibitors in renal transplantation

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INHOUD

LIST OF ABBREVIATIONS

ABCB	ATP-Binding Cassette sub-family B
APC	Antigen Presenting Cell
AUC	Area under the blood concentration-over time curve
CsA	Ciclosporin A
CAN	Chronic Allograft Nephropathy
CMV	Cytomegalovirus
CNI	Calcineurin inhibitor
CYP	Cytochrome P450
Cyp	Cyclophilines
DDPR	Daily Dose Prednisolone
EBV	Epstein Bar Virus
ESRD	End Stage Renal Disease
FKBP	FK506-Binding Protein
FPIA	Fluorescence Polarization Immunoassay
GFR	Glomerular Filtration Rate
HLA	Human Leukocyte Antigen
IF/TA	Interstitial Fibrosis/Tubular Atrophy
IIV	Inter-individual variability
IOV	Inter-occasion variability
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
MEIA	Microparticle Enzyme Immunoassay
MVOF	Minimum Value of the Objective Function
NFAT	Nuclear Factor of Activated T-cells
NONMEM	Non Linear Mixed Effects Modeling
PCR	Polymerase Chain Reaction
PD	Pharmacodynamics
PK	Pharmacokinetics
PPP	Protein Phosphatase
PXR	Pregnane X receptor
SCR	Subclinical Rejection
SNP	Single Nucleotide Polymorphism
TDM	Therapeutic Drug Monitoring
TRL	Tacrolimus

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General Introduction

In The Netherlands 13,000 patients suffer from end stage renal disease (ESRD), which left untreated inevitably results in death. Every year this number increases with approximately 3% newly diagnosed patients. In order to survive, these patients require life long renal replacement therapy consisting of either dialysis or renal transplantation. The possibility of dialysis was provided by the Dutch physician Willem Kolff in 1943. Nowadays 5,800 patients with ESRD depend on dialysis in The Netherlands. But, since dialysis is associated with a high rate of cardiovascular disease and a poor quality of life, renal transplantation has become an important alternative with currently 7,300 Dutch patients carrying a transplanted kidney. The first transplantation was performed in Boston in 1954, USA, while the first Dutch (kidney) transplantation took place in Leiden in 1966. Since then over 16,000 patients received renal transplantation in The Netherlands, with yearly almost 800 newly transplanted kidneys. This number reflects around 80% of the total number of around 1000 solid organ transplantations in The Netherlands (2009) [1,2].
The prevailing therapeutic aim is to save the transplanted kidney for the recipient by preventing acute rejection episodes and chronic allograft nephropathy (CAN) from occurring. To achieve this, immunosuppressive therapy is needed to modulate the recipient's immune response, which is activated as a host defense to remove the foreign donor material from the body. With the start of this approach a cat and mouse play between immunosuppressive drugs and immune cells has been initiated, with drug toxicity as an unpleasant and complicating factor. Improvements in immunosuppressive therapy have led to acute rejection rates of 10-20% in the first year after transplantation. Although acute rejection is treated successfully in most cases, with current graft survival rates nowadays around 90-95% the first year after transplantation, graft survival declines rapidly to 75-85% at 5 years and 50% at 10 years after transplantation [3,4-8].
At this time, the survival of transplanted kidneys can be up to 40 years under the appropriate conditions. Donor and transplant procedure characteristics are very important. An

organ transplanted from a living donor, either related or unrelated, has a superior function and survival compared to an organ obtained from a deceased donor (heart beating or non-heart beating). Oxidative stress as a result of the long cold ischemic time is the most important factor responsible for decreased quality of the kidney. Other factors, such as donor age and HLA(-DR) matching, play a major role as well. Furthermore, recipient characteristics, such as immunological, cardiovascular and health status are determinants for success. The final factor that has improved the prospects for patients over the years is the development of potent immunosuppressive therapy along with strict guidance [9].

Toxic immunosuppressive regimens have increased organ survival at the expense of cardiovascular disease, nephrotoxicity (damage to the transplanted kidney), infections (polyoma viruses, cytomegalovirus), diabetes and tumors (lymphomas, skin cancer). The immunosuppressive cocktail has developed to a widely used quadruple therapy in 2009, consisting of induction therapy with an interleukin-2 blocker (basiliximab, daclizumab) and maintenance therapy using three drugs: a calcineurin inhibitor (tacrolimus, ciclosporin A), mycophenolic acid and prednisolone. These drugs are combined in relatively low doses to prevent severe toxicity but to maximize clinical efficacy. The goal is to reduce the intensity of the immunosuppression as soon as possible. This is done with tapering the doses of these drugs in the first months after transplantation. Finally, in some protocols drugs are withdrawn ending up with for instance two low dosed immunosuppressive drugs [9-15].

The calcineurin inhibitors, ciclosporin A and tacrolimus, are the mainstay of the immunsosuppressive regimen. These drugs have a narrow therapeutic window, while they are characterized by a high between patient variability in treatment effect. Especially early after transplantation, when these drugs are highly dosed to prevent acute rejection episodes, the balance between efficacy and toxicity is put to the test. Therefore, to guide calcineurin inhibitor therapy, a concentration (bio)-marker was introduced. Specifically, drug concentrations in whole blood, somewhere after administration of the drug, were measured. This could either be the pre-dose or trough level, the peak level or the total exposure in terms of the area-under-the-blood concentration *versus* time curve (AUC). With the introduction of such a therapeutic drug monitoring (TDM) strategy a tool was provided to improve the balance between acute rejection and toxicity episodes [16-32].

Although large improvements for renal transplantation are made over the years, partly as a result of strict TDM protocols, further optimization of the immunosuppressive therapy with calcineurin inhibitors is needed. At present, with the body weight based dose it could take weeks to achieve target exposure levels for calcineurin inhibitors. This indicates that other factors likely determine the dose for an individual patient. Several factors are known, but the list is incomplete and the magnitude of the contribution of each factor is known. Moreover, despite adequate blood levels still acute rejection or toxicity episodes occur. In contrast, high blood levels not necessarily relate to clinical toxicity and low blood levels do not have to cause acute rejection episodes. Clearly, the concentration biomarker is not 100% predictive, indicating between patient differences in susceptibility for CsA. Therefore it would be interesting and promising to search for other biomarkers that can be measured easily and relate to the potency or efficacy of calcineurin inhibitors [33].

Scope of this thesis

The first part of this thesis aims at identifying factors that are predictive for an individual's dose, based on current clinical practice. This comprises factors related to the pharmacokinetics ('what the body does to the drug') of calcineurin inhibitors in renal transplantation The second part aims at identifying other biomarkers that relate to drug efficacy, or otherwise stated, the pharmacodynamic level ('what the drug does to the body').
The second chapter serves as the introduction to the thesis (**Chapter 2**). Sources of variability in response to calcineurin inhibitor therapy are discussed with the focus on genetic variability in pharmacokinetics and pharmacodynamics. In the next chapters the pharmacokinetics of the calcineurin inhibitors are described aiming to explain variability in drug exposure between individuals, to improve dosing regimens and to optimize TDM strategies (**Chapters 3** and **4**). **Chapter 5** is the transition between the first and second part of the thesis. It points out where improvements in calcineurin inhibitor therapy and monitoring are likely to be made and which factors should be taken into account. The research regarding the concentration biomarker will be extended with the clinical use of a drug target related biomarker in **chapter 6**. The aim is to identify a new biomarker for monitoring purposes, specifically the inhibition of the target of the calcineurin inhibitors, the calcineurin enzyme. Finally, **chapter 7** aims at identifying pharmacological factors which are associated with (subclinical) acute rejection. In the last two chapters the summary and the general discussion are provided (**Chapters 8** and **9**).

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Individualizing Calcineurin Inhibitor Therapy in Renal Transplantation – Current Limitations and Perspectives

ABSTRACT

Patient variability in clinical response to the calcineurin inhibitors (CNIs) ciclosporin A and tacrolimus partly results from differences in CNI exposure. For tacrolimus drug interactions and genetic variability relate to tacrolimus exposure. Patients carrying the CYP3A5*1 allele have an increased tacrolimus metabolism, hence lower drug exposure. Adjusting the tacrolimus dose to this genotype is a tool to optimize therapy from a pharmacokinetic perspective. In contrast, no genetic variants have been found to clearly relate to ciclosporin A exposure. Despite therapeutic drug monitoring aimed at individualizing CNI therapy, patients still suffer from acute or chronic rejection and CNI toxicity. To further optimize CNI therapy future research may incorporate genetic polymorphisms in proteins involved in CNI pharmacodynamics (i.e. drug target). Proteins potentially relevant for drug response are calcineurin and the CNI binding proteins immunophilins. Moreover, since the expression of the nuclear factor of activated T-cells (NFAT) is reduced after calcineurin inhibition, genetic polymorphisms in the genes encoding NFAT may also be interesting candidates for studying inter-patient differences in CNI efficacy and toxicity. In addition, the existence of isoforms and differences in tissue distribution of the calcineurin protein could potentially explain variable drug response.

At present, the focus has been on the metabolism of CNIs and not on variability in the drug target. Therefore, future improvements in CNI therapy are likely to occur from a systems pharmacology approach taking into account genetic markers for both CNI pharmacokinetics and pharmacodynamics.

Introduction

The use of tacrolimus (TRL) and ciclosporin A (CsA) in renal transplantation is associated with severe toxicity, such as acute and chronic nephrotoxicity, diabetes, liver-and neurotoxicity [1,2]. Toxicity is the main reason for the current trend to minimize and even withdraw the calcineurin inhibitors (CNIs) as early as possible after renal transplantation [3]. However, CNIs remain the primary immunosuppressant in most renal transplantation protocols since immunosuppressive therapy with these drugs has led to acute rejection rates as low as 10% at 1-year after transplantation [1]. This important clinical achievement has benefitted from individualized dosing achieved by therapeutic drug monitoring (TDM), aiming at target CNI blood exposure in terms of area-under-the-blood-concentration-*versus*-time-curve (AUC), trough level or C2-level [4]. Indeed, TDM has become routine clinical practice in renal transplantation and is related to lower acute rejection rates as well as lower toxicity rates [5,6].

Dose individualization and reaching adequate drug exposure typically takes days to weeks due to factors such as co-administration of interacting drugs and changing physiology of the transplant recipient. Moreover, the genetic constitution of enzymes involved in the metabolism of CNIs, such as CYP3A5*1 allele, is associated with an increased time to achieve target exposure [7,8] and potentially impacts acute rejection rates. However, acute rejection episodes are observed in graft recipients with adequate CNI exposure and appropriate transplant related factors such as HLA-matching. In addition, patients that experience CNI toxicity do include individuals with adequate drug exposure and vice versa. Clearly, drug response to immunosuppressants is a complex trait and is determined by many factors. To optimize immunosuppressive therapy early after transplantation it is necessary to understand the factors that contribute to the variability in drug response. Pharmacokinetic information alone appears to be insufficient to explain the total variability of CNI response. To further optimize CNI therapy, clinical variability in pharmacodynamic drug targets may be interesting, but is yet marginally explored for CNIs in renal transplantation [9]. Additionally, (patient) characteristics such as demographics, co-administration of interacting drugs and diarrhea [7] may be taken into account in developing predictive models and representing a systems pharmacology approach.

This review describes the sources of variability in CNI therapy in renal transplantation with an emphasis on variability in genes encoding drug metabolizing enzymes and drug targets.

Variability in CNI pharmacokinetics

CNIs display a large variability in exposure upon standard oral dosing. Naturally, variability in drug bioavailability and clearance are the reasons for the variable exposure which originates in the intestine and the liver [7]. For TRL a range in apparent clearance (CL/F) of 15-70 L/h [10,11] has been described and for bioavailability (F) a range from 5 to 93% with a median of 25% [7,12]. For CsA (Neoral®) a median apparent clearance of 29 L/h with a range from 20-50 L/h [13-15] and a bioavailability ranging from 16 to 55% [16-18] has been reported. This variability in apparent clearance after oral administration of CNIs is

multi-causal and relates first of all to intestinal motility. An uremic intestine in dialysis patients or delayed emptying of the stomach in diabetics is likely to affect drug absorption [7,19-21]. Food is thought to reduce the uptake of CNIs, while diarrhea increases exposure to TRL [22-24]. Subsequently, metabolic or efflux proteins in cells lining the intestinal wall and/or in the liver are present to eliminate CNIs out of the body [7,25]. This *first-pass metabolism* is the main reason that CNIs have a relatively low and highly variable bioavailability. The cytochromic enzymes CYP3A4 and CYP3A5 are responsible for metabolism of CNIs in the intestine and the liver [26-28] (Figure 1). Variable metabolic activity originates from inherited differences in metabolic capacity of specific isozymes in the liver accompanied by acquired differences due to co-medication of drugs, hepatitis or other sources of liver dysfunction or failure [7,26,29-35]. Moreover, the efflux transporter P-glycoprotein (*ABCB1, MDR1*) is responsible for transporting the drug out of the intestinal cell or removing it from liver cells into the bile [26-28].

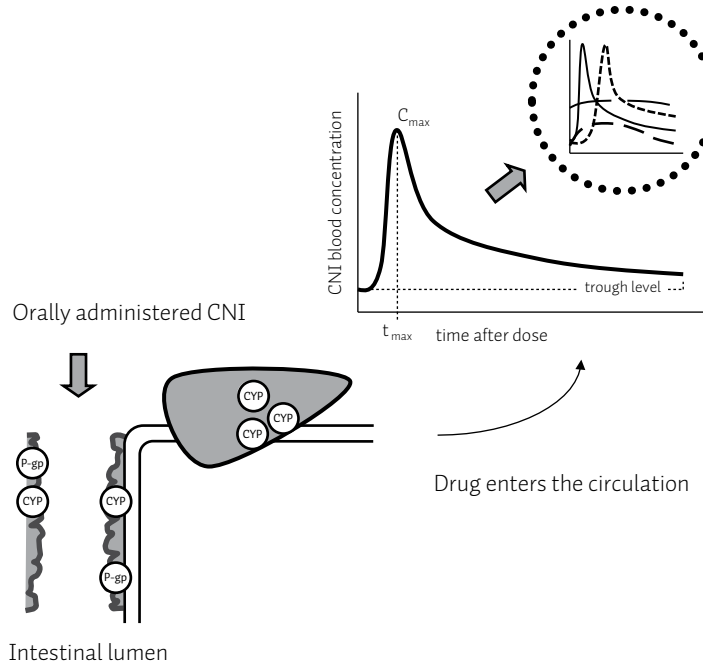

Figure 1. The variability in absorption and elimination of CNIs upon oral administration. The drug efflux pump P-glycoprotein is present in the intestine and acts together with the cytochrome enzyme subfamily CYP3A, present in the intestine and liver, to eliminate the CNIs from the body. The reflection of this process is represented by the typical CNI blood concentration *versus* time curve in the right part of the plot, with the trough level, C_{max} and t_{max} indicated. The dotted circle inlay in the upper right part depicts the variability in the absorption process and demonstrates the variability in trough level as well as C_{max} in relation to total exposure (AUC). The three curves reflect the absorption of the *typical* patient (solid line), a *slow* absorption profile (dotted line, i.e. diabetic), a *low* absorption profile (small striped line) and a *flat* absorption profile (wide striped line). P-glycoprotein (P-gp), cytochrome CYP3A-enzymes (CYP), maximum concentration after dosing (C_{max}), time after dose when maximum concentration occurs (t_{max}), calcineurin inhibitor (CNI).

As soon as CNIs have passed the metabolizing organs they distribute within the vasculature and throughout the body while bound to serum albumin, lipoproteins, α-acid-glycoprotein and binding proteins in the red blood cells [7,25,36-40]. Especially, early after transplantation these factors tend to be highly variable and are a major source of variability in CNI pharmacokinetics [41,42]. Ultimately these drugs bind in tissues to binding proteins such as FK-BP and cyclophilin [43]. Distribution throughout the body is influenced by drug transporters such as P-glycoprotein at the blood brain barrier, kidneys and T-cells. Tissue specific expression of P-glycoprotein is likely to be responsible for selective tissue distribution of CNIs [44]. This could potentially explain variability in drug effects (T-cell) and toxicity such as nephro-and neurotoxicity.

In the present situation, CNI therapy is guided by measuring drug concentrations in blood. These measurements have an emphasis on the absorption phase which typically covers the first 4-hours after dose intake. This *absorption profiling* is performed to deal with the variability as discussed above and is a reflection of the systemic exposure after oral drug administration [45-47] (Figure 1). Whole blood is selected for routine clinical practice due to the highly variable measurements in plasma [36,37,40,48-50]. The measurement of the unbound concentration of these drugs would be the preferred method as it theoretically reflects best the concentration available for the target cell, but this remains a time-consuming and difficult technique [38,40,51]. The AUC has been demonstrated to relate to clinical outcome in renal transplantation in terms of nephrotoxicity and acute rejection [5,6]. Despite the fact that it has been shown that TRL and CsA trough levels relate poorly to the AUC they are still widely used in routine clinical practice, because this is a more practical way of monitoring for most centers [45,46]. Also CsA C2 levels have been used to estimate CsA exposure. The C2 reflects the concentration 2 hours after dosing and is thought to be equal to the maximum blood-concentration (C_{max}). However, due to high variability in the time after drug administration to reach the maximum blood-concentration (t_{max}), this method is not considered first choice [52-57] (Figure 1). A *mini*-AUC obtained by a limited sampling Bayesian model is the best reflection of the total exposure, hence the preferred approach for routine TDM [15,45,46].

TDM is an adaptive strategy after administration of a starting dose based only upon the patient's bodyweight. In order to reach the CNI target level as early as possible after transplantation and to limit the number of monitoring visits, additional markers for initial CNI dosing are necessary. In the previous section it has been described that CYP3A4, CYP3A5 and P-glycoprotein have a role in the absorption and metabolism of CNIs, and therefore genetic variants in the genes encoding these enzymes may be useful in individualizing CNI therapy. Moreover, the nuclear pregnane X receptor (*PXR*) is involved in the regulation of gene expression of the CYP3A and MDR-1 genes and may thus play an indirect role in CNI disposition and metabolism [58-64]. The genes encoding these enzymes are highly polymorphic and genetic variability may therefore be the cause of inter-individual variability in pharmacokinetics.

CYP3A4 and CYP3A5

In vitro experiments have demonstrated that CYP3A5 is involved in the metabolism of TRL [65,66]. In fact, the variability in TRL exposure between renal transplant recipients

is shown to be related to a genetic polymorphism in CYP3A5. Specifically, carriers of the CYP3A5*1 allele have a lower concentration-to-dose ratio [67-71] or a lower AUC [71-73] as compared to CYP3A5*3 allele carriers. However, whether this affects clinical outcome, such as acute rejection, remains uncertain since no conclusive study has been presented so far mainly due to intensive TDM protocols, limited sample size and the absence of measurements of total exposure [74,75]. An informative study would be to identify the effect of CYP3A5 on subclinical rejection, by obtaining protocol biopsies 6 months after transplantation in a large cohort of renal transplant patients, using a sensitive measure for drug exposure (AUC) and accounting for other factors known to influence the exposure of TRL [76]. Interestingly, CYP3A5 genotype is related to the time to achieve target concentration of TRL. In clinical practice it could take days to weeks to achieve target TRL exposure in carriers of the CYP3A5*1 allele, whereas in CYP3A5*3 allele carriers target TRL exposure was observed directly or in the first days after transplantation [8,77]. In contrast to CYP3A5, contradictory reports are published with regard to CYP3A4*1B as a marker for TRL exposure [68,77]. Hesselink *et al.* reported a lower TRL dose-adjusted trough level for CYP3A4*1B carriers compared to the wild type (*1/*1) genotype [68], while Roy *et al.* did not find a relationship between CYP3A4 polymorphisms and TRL pharmacokinetics [77]. These findings indicate that the CYP3A5 marker for TRL exposure has greater potential clinical relevance than the CYP3A4 genotype.

Despite the fact that CsA is mainly metabolized by CYP3A4 in the liver [27], genetic variants in the *CYP3A4* gene were found not associated with CsA exposure in kidney transplant recipients [68]. The CYP3A4*1B genotype overall was not related to a pharmacokinetic parameter for CsA [78]. However, a small effect was reported which consisted of a 9% higher apparent clearance in carriers of the CYP3A4*1B allele [14]. This study was performed in a mixed transplant population (heart, kidney) without incorporation of the effect of a concomitant prednisolone dose in the model. In addition, no association was found of the CYP3A5*1 allele with CsA exposure, which is in line with the observation that CYP3A5 is thought to play only a minor role in CsA metabolism [78-81].

P-glycoprotein

The drug transporter P-glycoprotein is encoded by the ATP-binding cassette B1 gene (*ABCB1*). Several genetic variants in the *ABCB1* gene, such as C3435T, C1236T, G2677T, T-129C have been described in relation to TRL exposure [68,70,72,73,81-84]. Recent reports suggest that these genetic variants of *ABCB1* are not related to TRL exposure [70-73,85], while Roy *et al.* showed that less than three copies or SNPs of *ABCB1* polymorphisms (T-129C, C3435T and G2677T) are associated with lower TRL blood levels [77], hence requiring higher TRL doses. In contrast, a study by Anglicheau *et al.* identified the SNP *ABCB1* G2677T to be related to higher TRL concentration/dose ratio, hence lower dose requirement [86]. In conclusion, genetic markers in the ABCB1 gene do not seem to relate (clinically relevant) to TRL exposure.

P-glycoprotein in the small intestine plays a role in the absorption of CsA [27]. However, several studies have shown no relationship between genetic polymorphisms in *ABCB1* and CsA exposure in kidney transplant recipients [16,81,84]. However, in a recent study by Fanta *et al.* a higher pre-hepatic extraction ratio for carriers of the *ABCB1* 2677 G-allele

was found in pre-transplant pediatric patients on dialysis. This study used a population pharmacokinetic approach and identified factors relevant for the variability in drug exposure, while accounting for other factors such as bodyweight. An explanation for the disconcordant findings is that in the study of Fanta *et al.*, the analysis was performed in a different population including steroid free pediatric patients waiting for dialysis and is therefore not comparable to the adult kidney transplant population [87].

pregnane X receptor

The pregnane X receptor (PXR), coded by the *NR1I2* gene is a nuclear factor receptor involved in the induction of various genes, such as *CYP3A4* and *ABCB1* [88]. Rifampicin and prednisolone are ligands for this receptor and can stimulate indirectly the expression of *CYP3A4* and *ABCB1* [58]. Especially, prednisolone is of interest since the majority of regimens in renal transplantation include this immunosuppressant. Prednisolone therapy is often started in high doses as part of the induction therapy and gradually tapered towards low maintenance doses. Steroids are finally withdrawn in a limited number of centers [89]. High levels of cortisol or exogenous steroids saturate the glucocorticoid receptor and these steroids then activate PXR as a low affinity high capacity receptor in order to induce its own metabolism [58]. At that point, the use of prednisolone may affect the pharmacokinetics of other substrates for *CYP3A4* or *ABCB1*, such as the CNIs. This could potentially be the mechanism behind the interactions between prednisolone and the CNIs [90-92]. Specifically, inter-individual variability in this interaction could be explained by genetic variability in the gene encoding this receptor. To date, no genetic variants in *NR1I2* have been identified that influence CsA or TRL pharmacokinetics. However, a recent study by Miura *et al.* demonstrated a significantly lower prednisolone AUC in PXR 7635 G-allele carriers [93]. The A+7635G SNP as well as other SNPs in the gene encoding PXR could be of interest regarding a relationship with TRL clearance.

Intracellular CNI exposure

Clinical impact of pharmacogenetics in genes encoding metabolic enzymes related to CNIs may be limited due to the fact that whole blood concentrations are not very representative for intracellular drug concentrations in the target T-cell. In fact, it has been reported that intracellular CsA concentrations correlate poorly to blood concentrations [94]. The concentrations in the target T-cell can differ from the concentration in blood due to the presence of transporters such as P-glycoprotein (ABCB1) on the cell surface of T-cells [95] (Figure 2). Efficacy of CNIs, especially CsA, could be lowered by an active P-glycoprotein pump eliminating CNIs out of the cell [96]. Falck *et al.* demonstrated declined CsA T-lymphocyte concentrations 3 days prior to a rejection episode, while genetic variants of *ABCB1* could not be related to this decline [96]. However, Crettol *et al.* reported a 1.7 fold increased intracellular concentrations for patients carrying the *ABCB1* 3435T-allele [94].
Similar to variable drug concentrations in the target cell is the potential variable drug exposure in organ tissue prone to toxicity. Nephrotoxicity is an important and major clinical problem during CNI treatment after renal transplantation [1,2]. Nephrotoxicity could potentially be related to polymorphisms in ABCB1 as P-glycoprotein is expressed in the kidney and is likely to be involved in transporting CsA into renal tubules [97,98].

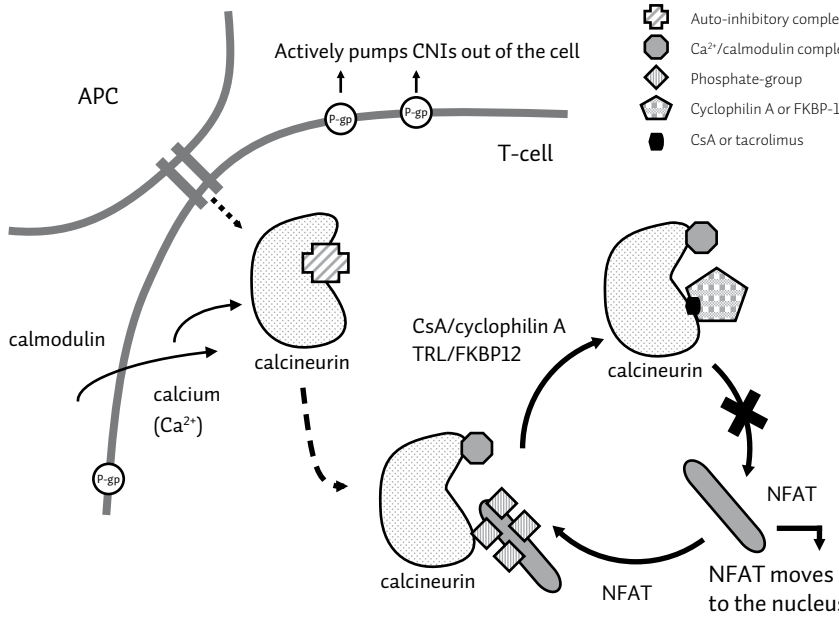

Figure 2. Calcineurin-inhibition pathway. An organ recipient's T-cell is activated by donor antigen presented by an APC to the T-cell. Upon activation of the T-cell calcium enters into the cell. Calcium mobilizes calmodulin to form a complex leading to activation of calcineurin by initiating the removal of the autoinhibitory complex of calcineurin. Calcineurin is able to de-phosphorylate NFAT which translocates to the nucleus of the cell to initiate gene transcription. The calcineurin inhibitors CsA and tacrolimus are able to inhibit calcineurin a-specifically. The drugs bind to the immunophilins cyclophilin A and FKBP-12 respectively forming a complex which is able to sterically hindering the active site of calcineurin.

Hauser *et al.* published a study relating a polymorphism in *ABCB1* to nephrotoxicity [99]. An important conclusion from this study was that the donor's *ABCB1* 3435 TT genotype appeared to be strongly associated with CsA related nephrotoxicity as demonstrated by an odds ratio of 13 (CI: 1.2 to 148). Moreover, tissue specificity of CYP3A enzymes has also been described with a specific role for CYP3A5 in the kidney and the absence of CYP3A enzyme expression in peripheral blood lymphocytes [100]. Finally, a study by Kuypers *et al.* demonstrated the development of biopsy proven TRL related nephrotoxicity in renal transplant recipients carrying CYP3A4*1 or *1B and CYP3A5*1 alleles [101].

Variability in CNI pharmacodynamics

Recent years focused on biomarkers originating from the pharmacokinetic pathway in order to predict exposure, as was described in the previous section. The second part of this review will elaborate on the (future) identification of markers derived from the pharmacodynamic pathway.

In transplant immunology a T-cell encounters donor-antigen presented on a HLA molecule by antigen presenting cells (APC) originating from the donor or the recipient (Figure 2).

The HLA/antigen domain interacts with T-cells through the T-cell receptor (CD3) upon which it activates the T-cell and initializes a calcium influx into the cell [102,103]. Intracellular calcium then forms a complex with the calcium binding protein calmodulin. This calcium/calmodulin complex is able to bind to the enzyme calcineurin A (CNA), facilitates a conformational change and causes the autoinhibitory domain of CNA to free the catalytic site upon which CNA is activated [104,105]. Activated calcineurin *de*-phosphorylates members of the transcription factor family of nuclear factor of activated T-cells (NF-AT) within the cell cytoplasm. *De*-phosphorylated NFAT transports to the nucleus of the cell and act as a transcription factor initiating gene expression of cytokines and receptors (i.e. expression of interleukin-2) [104,106].
This pathway can pharmacologically be disrupted by the use of CNIs [107,108] after the drug has entered the target T-cell. TRL and CsA bind to FK506-bindingprotein (FK-BP) and cyclophilin A respectively, which are intracellular immunophilins acting as binding proteins [109]. The immunophilin/CNI complex binds to calcineurin with the CNI side sterically hindering the active site of calcineurin with the large immunophilin tale [105,110]. NFAT can then no longer be *de*-phosphorylated and gene expression is reduced with immunosuppression as the clinical result (Figure 2).
Measurement of calcineurin activity and calcineurin inhibition upon CNI administration is interesting as a potential biomarker for CNI therapy [111-114]. Measuring calcineurin activity after renal transplantation could add to the current CNI monitoring strategy which mainly concerns whole blood level monitoring [115-118]. However, the role of measuring calcineurin inhibition has to be determined.
Genetic variants in proteins involved in the calcineurin inhibition pathway are of potential interest for understanding variable CNI drug response [119]. The calcineurin inhibition pathway provides three main protein groups: calcineurin, immunophilins and NFAT.

Calcineurin

Calcineurin, a serine/threonine phosphatase also known as protein phosphatase 2B (PP2B) or protein phosphatase 3 (PPP3), is a heterodimer of the catalytic subunit A and the regulatory subunit B [104,107,120]. Its structure is highly conserved from yeast to man and essential for activity [121]. Calcineurin has a relatively narrow substrate specificity including phosphoproteins such as DARP32 and inhibitor-1 and for this paper more importantly NFATs [121,122].
Calcineurin A (CNA) is composed of a catalytic domain and three regulatory domains: a CNB binding domain, a calmodulin binding domain and an auto-inhibitory domain [105,123]. The C-terminus of calcineurin contains the auto-inhibitory domain which relieves its activity upon calmodulin binding. Adjacent to this area the calmodulin binding domain is situated followed by the CNB binding domain [122] (Figure 3).
Three genes code for three specific isoforms of CNA: CNAα (*PPP3CA*: neural, kidney), CNAβ (*PPP3CB*: widely, T-cell, B-cell), CNAγ (*PPP3CC*: testis) [120,121,124-126]. The predominant isoform contributing to calcineurin activity in most tissues is CNAα [124]. CNAα and CNAβ were localized on human chromosomes 4 and 10 respectively [127]. The isoforms have large overlap in structure. CNAα compares for 84% to CNAβ and for 81% toCNAγ with greatest similarities in the catalytic domain [125].

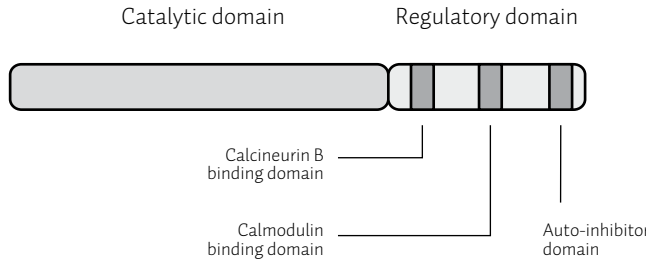

Figure 3. The structural domains of calcineurin A. Calcineurin A consists of a catalytic domain and a regulatory domain. The latter consists of three important parts, a calcineurin B binding domain, a calmodulin binding domain and an auto-inhibitory domain.

Calcineurin B (CNB), also called PPP3R, belongs to the so called 'EF-hand calcium binding protein family' [105,121]. CNB is essential for calcineurin activity and like calmodulin it is activated by calcium. In fact it has a similar structure to calmodulin [121,128], but a different activity. The calcium/CNB complex increases affinity of calcineurin for its substrate in contrast to calcium/calmodulin which displaces the auto-inhibitory domain and increases the enzymatic rate. CNA alone has a low activity which becomes a very high specific activity upon binding to CNB [105,121,128]. Two mammalian isoforms are known, CNB1 and CNB2, of which the first is associated with CNAα and CNAβ, while CNB2 relates to the γ variant and is only expressed in testis [121]. The genes coding for CNB1 and CNB2 are *PPP3R1* and *PPP3R2* respectively [127].

The interface between the CNB binding domain in CNA and CNB itself is the target area for the immunophilin/CNI complex. A large part of the contact area for the FK-BP/tacrolimus complex origins as the 'latch region' where CNB and the cyclophilin/CsA complex binds and which consists of residues 118-125 in the loop of CNB [129]. Interestingly, this is the principle reason that these two complexes display competitive binding to calcineurin [121].

To date, limited studies are published investigating polymorphisms in the gene encoding calcineurin and no studies are known relating them to clinical effect. Potentially, three regions are of interest for explaining variability in CNI response. First, the latch region between CNA and CNB is the principle region for binding the immunophilin/CNI complex. Genetic polymorphisms resulting in aminoacid changes in this region may affect the ability of the complex to bind to calcineurin. Indeed, Wang *et al.* demonstrated that the CNB binding domain is important for the inhibition of calcineurin [123]. Substitutions in the latch region are shown to result in resistance to CNI inhibition. This is also true for aminoacid changes in the CNB binding domain (Thr352, Leu354, Lys360) [129]. As an extension to this, the so called *Loop7* has been investigated. This is a β-hairpin within CNA which contacts immunophilin/CNI complexes as well as the auto-inhibitory domain. The connections made by Loop7 are important for CNI. Inhibition by TRL appeared to be increased by single deletions of Val314 or surrounding residues, while it reduced CsA mediated inhibition [129]. Second, basal phosphatase activity of calcineurin could theoretically be altered by changes in the calmodulin binding region with a lower basal activity of calcineurin as

a result. This is likely to have its consequences for baseline calcineurin activity. Potentially this could affect the enzymatic activity of calcineurin by altering an essential factor in the motor of the system. Third, conformational changes in the catalytic site of calcineurin are likely to change the affinity of calcineurin for its substrates, the phosphorylated NFATs. One can imagine that these changes are able to lead to a different basal enzymatic activity, which could explain inter-individual changes in baseline activity and probably the potential for CNIs to inhibit calcineurin activity.

Immunophilins

The immunophilins cyclophilin and FK-BP belong to the protein families collectively referred to as peptidyl-prolyl isomerases (PPI*ases*) [130-132]. Besides these proteins parvulins are considered the third member of this group [132]. PPI*ases* are *cis-trans* petidyl-prolyl isomerases (rotamases) which catalyze the *cis-trans* interconversion of peptide bonds N-terminal to proline with a physiological role in protein folding and conformational stability. But their role in inhibition of calcineurin is different from their physiological role [132,133]. The function of immunophilins in calcineurin inhibition is related to its size, as binding of cyclophilin A and CsA leads to a large complex which is able to sterically hinder the catalytic site of calcineurin to phosphorylate NFAT. CsA binds on one side to a groove on cyclophilin A, while the other side remains available for binding calcineurin [132] (Figure 2).

Immunophilins contain CNI binding properties and were thought to be abundant in cells [130]. This abundancy is currently under debate [115,134] and one can easily hypothesize that these proteins are expressed in different amounts between or even within individuals. Therefore, genetic variability in the genes encoding the immunophilin may lead to differences in response to CNIs. Other possible sources of genetic variability that could result in therapeutic differences are changes in the binding domain for CNIs. A lower or higher affinity could change the potency of CNIs to inhibit calcineurin.

Cyclophilines (Cyp) are a group of 15 different members of which cyclophilin A (CypA) is thought to be the most important in the context of calcineurin inhibition. The residues in CypA that are involved in CsA binding are highly conserved in other members of the group indicating a potential role for other Cyps (i.e. Cyp B, C and F) as a target for CNIs. *PPIA* is the gene coding for CypA [132,135] and CsA binds to CypA through contact on its hydrophobic pocket [132,136]. Only one study in renal transplant recipients is reported in literature [137] where a CypA polymorphisms in a coding region (exon: +36 G/A) and one in the promoter region (-11 G/C) were identified. The promotor polymorphism was associated with clinical nephrotoxicity. In this study DNA was not obtained from the donor but from the recipient, which makes the interpretation of this result difficult. Moreover, nephrotoxicity was not confirmed by a biopsy, but only related to an increase in serum creatinine values. In an earlier study this polymorphism was related to rapid CD4+ T-cell loss and progression in patients diagnosed with Auto-Immune Deficiency Syndrome (G *vs.* C allele) [135].

FK506-binding proteins (FKBP) constitute a group of 16 human proteins of which FKBP12 and FKBP12.6 are related to the inhibition of calcineurin phosphatase activity [138]. Immunosuppressive properties are primarily thought to be regulated by FKBP12. However,

one cannot exclude the role of other PPI*ases* with abundancy in human, such as FKBP51 [138]. The FKBP family members are structurally variable, but the PPI*ase* domains of FKBP proteins do display overlap in parts of the protein sequence. The FK506 binding site consists of a hydrophobic pocket of at least 12 amino acid residues [138]. The positions K34, H87 and I90 appeared to be the most variable positions and were thought to be important for TRL-FKBP interaction [138]. Indeed, it was shown that a change at the position of K34 from a lysine to a threonine residue lowers the affinity for calcineurin [138].

Nuclear Factor of Activated T-cells (NFAT)

NFAT is a family of transcription factors inducing gene transcription in T-cells and other immune system related cells [139,140]. NFAT proteins are present in the cell cytoplasm in the phosphorylated form (Figure 2). NFAT is a substrate for the phosphatase calcineurin which is capable of de-phosphorylating NFAT. When NFAT is *de*-phosposphorylated it translocates to the nucleus were it becomes transcriptionally active and regulates the transcription of a large number of genes [106,141]. Calcineurin binds NFAT at its regulatory domain at two specific calcineurin binding regions which are called binding region A and B. Region A is called PxIxIT or SPRIEIT found in all NFAT proteins, while region B is dedicated too NFAT 2 and 4 [106]. Polymorphisms resulting in changes in the calcineurin binding regions are likely to result in altered activity of NFAT, while changes in the DNA binding domain (*Rel homology domain*) are also likely to result in altered gene expression. Besides a potential alteration in the catalytic site of calcineurin a change in the PxIxIT recognition region of calcineurin is a potential source for differences in response [104,106,107,142,143].

The NFAT family constitutes of 5 proteins NFAT1-5 [106,140] and is constructed out of an activation domain, a regulatory domain, a DNA binding domain and the C-terminal domain [142]. A highly variable tissue distribution is observed for NFAT. Protein and mRNA of NFAT1 and 2 are present in T-cells and other immune cells, but not in kidney cells, while mRNA of NFAT3 and 4 is found in kidney cells [139].

Systems pharmacology approach

Variability in response to CNI treatment may be related to several factors originating in the pharmacokinetic and pharmacodynamic pathway [7].

The past years the focus was mainly on variability in the pharmacokinetics of CNIs and this has lead to a practice of measuring blood concentrations as a monitoring tool for CNI therapy. More recently, pharmacogenetics has been introduced and related to variable pharmacokinetics. Genetic variants were explored as potential markers to optimize CNI therapy as early as possible after transplantation. There appears to be a role for CYP3A5*1 in TRL but not in CsA dosing. However, conclusive evidence regarding the impact of genotyping in routine clinical practice is still warranted. Especially, the impact of genotype based dosing on clinical outcome remains unclear [74,144]. A limitation of pharmacogenetic studies in renal transplantation done so far is that the majority did not take other important factors contributing to CNI disposition and metabolism, such as motility problems and co-administration of interacting drugs, into account. Moreover, often an

insensitive marker for exposure, such as the dose-adjusted trough level, was used. Optimizing CNI therapy from a pharmacokinetic perspective is limited by the fact that whole blood concentrations are measured, while plasma-, free-drug-or even T-cell-concentrations would be more informative. Technical advancement is necessary to routinely estimate drug concentrations at the site of action.

Future optimization of CNI therapy could be the use of pharmacodynamic measurements. Several groups have reported assays to determine calcineurin activity and its inhibition by CNIs [111-113,145-148]. Technical difficulties with regard to defining and obtaining the intended blood fraction have been discussed as well as the preservation of the phosphatase activity and the blockade of other phosphatases. Yet, the interpretation of these findings in relation to clinical usefulness is unclear [145,149,150]. More data should be generated to establish the usefulness and feasibility of this marker in routine clinical practice. Moreover, genetic variability in the genes encoding the proteins involved in the calcineurin inhibition pathway such as immunophilins, calcineurin and NFAT are likely to be related to differences in potency, efficacy and toxicity of CNIs. Genetic variants in genes encoding these proteins could be explored as potential markers for CNI therapy. One should be aware that specific isoforms of these proteins are known to exist which are likely to result in differences in tissue sensitivity [124].

Besides pharmacogenetics in PK and PD, also non-genetic determinants are related to the response of CNIs. These factors include co-morbidity (diabetes, cardiovascular), demographics (age, sex, race and bodyweight), organ functioning (intestine, liver, kidney, cardiovascular system), the administration of interactive drugs (prednisolon, fluconazol) and food [7] and finally compliance to drug therapy [151-154].

Obviously, response to CNIs is a complex trait and the result of interactions of a complex system. Therefore, it is unlikely that variability of CNI response can be captured by the variability in a single determinant. Instead, a systems pharmacology approach should be used incorporating the most important sources of genetic and non-genetic variability in terms of pharmacokinetics and pharmacodynamics. Several population pharmacokinetic approaches are published for CNIs used in a kidney transplant population [10,11,13,14,155], but only few used an integrative genetic-non-genetic approach [14,156]. Currently, no studies are published applying a population pharmacodynamic or combined PK/PD analyses in renal transplant recipients. In liver transplant recipients two such studies have been published [157,158], but these were of limited sample size with respects to patients as well as blood sampling and therefore had low power to identify factors associated with variability in drug response. Moreover, these studies did not incorporate pharmacogenetic markers.

Interestingly, novel approaches could also include the genotype of the donor next to the genotype of the recipient. Different genetic profiles between donor and recipient can potentially explain differences in nephrotoxicity in kidney transplant recipients [99], which is in agreement with liver transplant recipients displaying different metabolic capacity between intestine and liver [69]. Besides the inherited profiles, the body could also be responsible for different activities in organs due to tissue specific gene expression [58].

Conclusion

The high variability in response to CNI therapy has a multi-factorial origin and is likely to be related to genetic variability in genes encoding proteins in the pharmacokinetic and pharmacodynamic pathways of the CNIs. The complexity of response to CNIs requires a sophisticated approach in order to individualize CNI therapy. Specifically, a systems pharmacology approach integrating genetic information with other non-genetic determinants of pharmacokinetics and pharmacodynamics is likely to result in predictive models useful for optimal CNI mediated immunosuppression, further reducing toxicity and rejection episodes.

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Explaining Variability in Tacrolimus Pharmacokinetics to Optimize Early Exposure in Adult Kidney Transplant Recipients

ABSTRACT

To prevent acute rejection episodes it is important to reach adequate tacrolimus exposure early after kidney transplantation. With a better understanding of the high variability in the pharmacokinetics of tacrolimus the starting dose can be individualized, resulting in a reduction in dose adjustments to obtain the target exposure. A population pharmacokinetic analysis was performed to estimate the effects of demographic factors, hematocrit, serum albumin concentration, prednisolone dose, tacrolimus dose interval, polymorphisms in genes coding for ABCB1, CYP3A5, CYP3A4 and the pregnane X receptor on tacrolimus pharmacokinetics. Pharmacokinetic data were prospectively obtained in 31 *de novo* kidney transplant patients randomized to receive tacrolimus once or twice daily and subsequently, the data were analyzed by means of Non-Linear-Mixed-Effects-Modelling. Tacrolimus clearance was 1.5 fold higher for patients with the CYP3A5*1/*3 genotype compare to the CYP3A5*3/*3 genotype (5.5±0.5L/h *versus* 3.7±0.3 L/h respectively). This factor explained 30% of the inter-individual variability in apparent clearance (exposure). Also, a relationship between the pregnane X receptor A+7635G genotype and tacrolimus clearance was identified with a clearance of 3.9±0.3 L/h in the A-allele carriers *versus* 5.4±0.6L/h in the GG genotype. Finally, a concomitant prednisolone dose of more than 10 mg/day increased the tacrolimus apparent clearance by 15%. In contrast body weight was not related to tacrolimus clearance in this population. As patients are typically dosed per kg body weight this might result in under- and overexposure in patients, with a low and high body weight respectively. This integrated analysis shows that adult renal transplant recipients with the CYP3A5*1/*3 genotype require a 1.5 times higher fixed starting dose compared to CYP3A5*3/*3 in order to reach the predefined target exposure early after transplantation.

Introduction

The calcineurin inhibitor (CNI) tacrolimus (TRL) is an important immunosuppressive drug commonly used in the early phases after solid organ transplantation. The current trend is to minimize exposure to CNIs, but at the same time to maintain the current low acute rejection rates [1]. This indicates that adequate early TRL exposure may become more important to prevent acute rejection episodes. Next to acute rejection, prevention of acute CNI-induced nephrotoxicity is another key objective, since both conditions were found to constitute the major risk factors for the occurrence of chronic allograft nephropathy in protocol biopsies obtained two years after renal transplantation [2].

CNIs are critical dose drugs with a large inter- and intra-individual variability in TRL pharmacokinetics (PK) and therewith systemic exposure [3,4]. In order to reach the target exposure of TRL as early as possible after transplantation it is important to have a clear understanding of the key factors (covariates) that explain variability in TRL exposure. Previous studies have identified a variety of demographic [3] and clinical factors (albumin [5,6], hematocrit [7,8]), co-medication (prednisolone [9-11], fluconazole [12]) as well as polymorphisms in genes encoding for CYP3A5 [13-17], CYP3A4 [18,19], P-glycoprotein (ABCB1/MDR1) [13,20] and the pregnane X receptor (PXR) [21-23] as relevant determinants of variability in TRL PK. The majority of these studies were limited by the fact that only a single factor was studied in relation to PK parameters such as area-under-the-concentration-over-time-curve (AUC), bioavailability or the clearance (CL/F) of TRL, while in most cases dose-adjusted trough levels were used. Recently, the importance of genotyping (CYP3A5) to reach TRL target exposure was emphasized by MacPhee *et al.* [24], but until now no population analysis has been performed in *de novo* kidney transplant recipients in which both genetic and non-genetic covariates were studied [25,26].

At present most transplant centers calculate the TRL starting dose based on body weight according to the manufacturer's instructions, which apparently is not sufficient to deal with inter-individual variability. In the present study a comprehensive analysis was performed on a rich data set to estimate the relative contribution of the factors that explain variability in early TRL exposure. With this population based PK approach an individualized TRL dosing strategy was developed in order to reach a predefined target exposure of TRL early after transplantation.

Patients and methods

Patients and immunosuppressive therapy

De novo kidney transplant recipients ($n=31$), aged between 18 and 70 years, were studied for one year following transplantation in the period September 2000 to March 2003. Recipient, donor and transplant characteristics as well as outcome parameters (acute rejection rate, patient and graft survival, renal function) are summarized in Table 1. Only recipients of a first kidney graft from a deceased or living (non HLA-identical) donor were included. Patients received quadruple immunosuppression, including induction therapy with basiliximab 20 mg before transplantation and on day 4, a fixed dose (500 mg twice daily) of mycophenolate mofetil (MMF), prednisolone (50 mg twice daily on the day of transplantation, rapidly tapered towards 5 mg once daily on day 22) and TRL. Patients re-

Table 1. Clinical characteristics.

Variable	Once daily (*n* = 16)	Twice daily (*n* = 15)	p-value
Recipient characteristics			
Age (mean ± SD)	43.9 ± 13.3	46.8 ± 12.0	0.53
Male sex (*n*)	12	12	0.74
Caucasian (*n*)	14	12	0.65
Native kidney disease			0.81
Glomerulonephritis	7	5	
Hereditary / congenital	3	5	
Hypertension	3	2	
DM	0	1	
Unknown	3	2	
Donor characteristics			
Age (mean ± SD)	47.4 ± 13.1	45.8 ± 14.3	0.75
Male sex (*n*)	13	6	0.03
DD-heart beating	6	7	0.72
DD-non heart beating	2	2	0.94
LRD	4	4	0.92
LURD	4	2	0.65
Transplant characteristics			
HLA-mismatch mean ± SD	2.81 ± 1.52	2.73 ± 2.25	
Class I	1.94 ± 1.06	1.87 ± 1.51	0.75
Class II	0.88 ± 0.72	0.87 ± 0.83	0.90
Cold ischemia time (h) DD only	18.7 ± 5.4	24.7 ± 7.8	0.09
Acute rejection 6 months (*n*)	0	2	0.14
Need for ATG (*n*)	0	2	0.14
Patient survival (*n*)			0.51
1 year	16	15	
2 years	16	15	
Death-censored Graft survival (*n*)			0.52
1 year	16	15	
2 years	15	14	
Nankivell clearance (mean ± SD)			0.39
Week 2	59 ± 30	53 ± 22	
Week 6	65 ± 15	60 ± 12	
Month 3	68 ± 11	65 ± 12	
Month 6	71 ± 12	65 ± 16	
Month 9	70 ± 12	64 ± 18	
Year 1	67 ± 14	62 ± 20	
Year 2	66 ± 14	60 ± 15	

DM, diabetes mellitus; DD, deceased donor; LRD, living related donor; LURD, living unrelated donor; HLA, human leukocyte antigen; ATG, anti-thymocyte globulin; SD, standard deviation.

ceived a TRL (Prograf®) daily dose of 0.2 mg/kg/day in either a once or twice daily regimen (Table 1). Therapeutic drug monitoring (TDM) for TRL twice daily (b.i.d.) regimen was aimed at an AUC_{0-12h} of 210 µg×h/L in the first six weeks and subsequently lowered to 125 µg×h/L. For the once daily (o.d.) regimen these values were 420 µg×h/L and 250 µg×h/L respectively. From each patient written informed consent was obtained. The study was approved by the Medical Ethics Committee of the Leiden University Medical Center.

Therapeutic drug monitoring

Therapeutic drug monitoring was performed on the basis of a limited sampling strategy (blood concentration at t=0, 2 and 3 h) and Bayesian estimation of the AUC_{0-12h} using MW/Pharm version 3.5 (Mediware, Groningen, The Netherlands) as previously described [27]. Routine TDM samples (t=0, 2 and 3 h) were taken during the mornings of weeks 4, 8, 10, 17, 21 and 39 after transplantation. Additional samples were taken during the mornings of weeks 2, 6, 12, 26 and 52 with samples at t=0, 1, 2, 3, 4, 6 up to 12 hours after administration. These weeks were clustered for presentation purposes. As a matter of fact week 2 is the first rich sampling moment performed in the first 3 weeks after transplantation with a median on day 10 after transplantation and the first day as early as day 6. In order to reach a steady state, TRL exposure was determined several days after transplantation. This did not rule out trough level monitoring in the first week after transplantation to avoid extreme low and high exposure. All patients were sampled on 11 occasions, with the exception of 5 patients who were sampled less frequently (ranging from 4 to 10 occasions). TRL blood concentrations were determined in whole blood by micro particle enzyme immunoassay (MEIA, Abbott laboratories, Abbott Park, IL, USA). Assay inter-day variation, derived from routine measurements, was 20% (5 µg/L), 15% (11 µg/L) and 12% (22 µg/L). The linear range of the assay was up to 30 µg/L. Levels higher than 25 µg/L were diluted according to the manufacturer's instructions.

Genotyping assays

DNA was isolated from EDTA-blood. Primers and probes used in the Taqman based genotyping assays, as well as primers and sequences used in the Pyrosequence assays are listed in Table 2. *ABCB1* C1236T, T3435C and G2677T were determined with TaqMan 7500 (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) with custom designed assays, according to the manufacturer's protocol. *ABCB1* T-129C, *CYP3A5* *3 and *6, *CYP3A4**1B and *PXR (NR1I2)* C-25385T, A-24381C, G-24113A, A+252G, A+7635G were determined with Pyrosequencer 96MA (Isogen, IJsselstein, The Netherlands). In short, PCR reactions contained 10 nanogram of DNA, and 5 pmol of each PCR primer (listed in table 2) in a total volume of 12 microliters. Cycle conditions were: initial denaturation for 15 minutes at 95°C, 35 cycles of 95°C-55°C-72°C each for 30 seconds, ended by 10 minutes at 72°C. The pyrosequence reactions were performed according to the manufacturer's protocol. The sequence used for analysis and the calculated dispensation order for each SNP are listed in Table 2. The nucleotides shown in lower case are negative controls, which were not incorporated in the target DNA and consequently did not appear in the pyrogram. As quality control, 5% of samples were genotyped in duplicate. In addition, negative controls (water) were used. No inconsistencies were observed. All allele frequencies were in Hardy-Weinberg equilibrium. Table 5 presents the genotype distribution in the overall genotyped population.

Table 2. Primers and Probes for TaqMan and Pyrosequence analysis.

SNP	Target [a]	Sequence 5'-3	Modification [b]
ABCB1 C1236T	PCR-f	CACCGTCTGCCCACTCT	
	PCR-r	GTGTCTGTGAATTGCCTTGAAGTTT	
	Probe-T	TTCAGGTTCAGACCCTT	VIC
	Probe-C	CAGGTTCAGGCCCTT	FAM
ABCB1 G2677T	PCR-f	CTTAGAGCATAGTAAGCAGTAGGGAGT	
	PCR-r	GAAATGAAAATGTTGTCTGGACAAGCA	
	Probe-G	TTCCCAGCACCTTC	VIC
	Probe-T	TTCCCAGAACCTTC	FAM
ABCB1 T3435C	PCR-f	ATGTATGTTGGCCTCCTTTGCT	
	PCR-r	GCCGGGTGGTGTCACA	
	Probe-T	CCCTCACAATCTCT	VIC
	Probe-C	CCCTCACGATCTCT	FAM
Pyrosequence			
ABCB1 T-129C	PCR-f	TCGAAGTTTTTATCCCA	Biotine
	PCR-r	CCTCCTGGAAATTCAACCTGTT	
	Sequence primer	TACTCCGACTTTAGTGGAAAGACC	
	Target Sequence	CTG/ACTCGAATGAG	
CYP3A5*3	PCR-f	CTGCCTTCAATTTTTCACT	
	PCR-r	TATGTTATGTAATCCATACCCC	Biotine
	Sequence primer	AGAGCTCTTTTGTCTTTCA	
	Target Sequence	A/GTATCTC	
CYP3A4*1B	PCR-f	CAGCCATAGAGACAAGGGC	
	PCR-r	GAAGAGGCTTCTCCACCTT	Biotine
	Sequence primer	CCATAGAGACAAGGGCA	
	Target Sequence	A/GGAGAGAGG	
CYP3A5*6	PCR-f	TCTTTGGGGCCTACAGCATG	
	PCR-r	AAAGAAATAATAGCCCACATACTTATTGAGAG	Biotine
	Sequence primer	AGAAACCAAATTTTAGGAA	
	Target Sequence	CTTC/TTTAG	
PXR C-25385T	PCR-f	GTGGTCATTTTTTGGCAATCCC	
	PCR-r	AGCCTCTGGCAACAGTAAAGCA	Biotine
	Sequence primer	TTGGCAATCCCAGGT	
	Target Sequence	TC/TTCTTTTCTACCTGTT	
PXR A-24381C	PCR-f	AGTGGGAATCTCGGCCTCA	
	PCR-r	CTGGGGTCCACTTTGAACAATC	Biotine
	Sequence primer	GCTAATACTCCTGTCCTGAA	
	Target Sequence	A/CAAGGCAGCGGCTCCTTG	
PXR G-24113A	PCR-f	GAATCATGTTGGCCTTGCTGC	
	PCR-r	GCATCAGTAATGGGGCTCAAC	Biotine
	Sequence primer	TCTCCTCATTTCTAGGGT	
	Target Sequence	C/TCACCCTAG	
PXR A+252G	PCR-f	TGCAAGGGCTTTTTCAGGTAGAGT	
	PCR-r	TGAACCTGGGGGATAGGTCAAG	Biotine
	Sequence primer	ACTGACCCACTGGGTAA	
	Target Sequence	CA/GTCTCAGGGC	
PXR A+7635G	PCR-f	AGCCATCCTCCCTCTTC	Biotine
	PCR-r	CAGCAGCCATCCCATAATC	
	Sequence primer	CATAATCCAGAAGTTGGG	
	Target Sequence	GGC/TGAGAGGAA	

[a] f = forward orientated, r = reverse orientated. [b] VIC and FAM are fluorescent dyes, biotine is necessary to obtain single stranded DNA. ABC, ATP-binding cassette; CYP, cytochrome P450; PXR, pregnane X receptor; PCR, polymerase chain reaction.

Population pharmacokinetic analysis

The PK of TRL were analyzed by non-linear mixed effects modelling (NONMEM) [28]. Mixed effects models consists of a structural model, describing the relationship between dose and concentration in terms of structural PK parameters (i.e. clearance (CL), volume of distribution (V)), and a stochastic model, describing the random variability in the structural model parameters. For population PK modelling these random-effects are the expression of inter-individual and inter-occasion variability. Inter-individual variability describes the random variability of structural parameters within the population, whereas inter-occasion variability describes the variability of an individual parameter value from one occasion to another. The second level of stochastic-effects, σ^2, describes the variability of the difference between observed and predicted responses. This residual error includes among other factors model misspecification, intra-individual variability and measurement error. In the mixed-effects modelling approach, structural and stochastic parameters are simultaneously estimated by fitting the model to the data. In this respect the following parameters were estimated: PK parameters, variance and covariance (ω^2) of each individual specific parameter value (η) and variance (σ^2) of the residual error. As a result, individual *post hoc* estimates of parameters associated with inter-individual variability and inter-occasion variability could be obtained.

STRUCTURAL MODEL. The PK of TRL was fitted to linear compartmental models. As in the present analysis only data after oral administration were available, the absolute oral bioavailability could not be identified. Therefore, the value for the oral bioavailability was fixed to 23%, as previously described [29] and used in the clinically applied TDM model [27].

RANDOM EFFECTS. Inter-individual variability and inter-occasion variability were described assuming a log normal distribution with the following equation:

$$PKj = TVPK \times e^{\eta jPK}$$

in which PKj is the PK parameter for the j^{th} individual and ηjPK is the difference between the individual specific parameter and the population value. TVPK is the population value of the PK parameter and the difference of the logarithm between the individual value of subject *j* and the population mean (ηjPK) is normally distributed with a mean of zero and variance ω^2_{PK}. The residual error was assumed to be proportional to the predicted concentration:

$$Cij = Cpredij \times (1 + \varepsilon ij)$$

in which Cij is the i^{th} observation for the j^{th} individual, Cpredij is the concentration of TRL in the blood predicted by the PK model, and εij (difference between Cij and Cpredij) is a normally distributed random variable with mean zero and variance σ^2.

COVARIATE ANALYSIS. The following covariates were selected on the basis of their known or theoretical relationships with TRL PK: hematocrit, serum albumin concentration, serum cholesterol (LDL, HDL) concentration, prednisolone dose, TRL dose regimen (once or twice daily dosing), polymorphisms in *ABCB1*, *CYP3A5*, *CYP3A4*, and *PXR*, and demographic factors such as body weight, age and gender. Only covariates with a clear visual

Table 3. Population pharmacokinetic parameters for TRL obtained from the bootstrap of the final model. This table shows the mean and coefficient of variation of the PK parameter estimates as well as the median and percentiles of these estimates. The variability concerns the actual random variability in the PK parameter relative to the population mean value.

PK Parameter	Mean value	variability	CV (%)	median	Percentiles 2.5-97.5 (%)
CL CYP3A5*3*3 (L/h)	3.7		8.2	3.8	3.1-4.3
CL CYP3A5*1*3 (L/h)	5.5		9.8	5.5	4.4-6.6
F (fixed)	0.23				
F (pred > 10 mg)	-15% [a]		-30		
V_c (once daily dosing) (L)	61		11	61	48-74
V_c (twice daily dosing) (L)	42		10	42	35-51
Q (L/h)	10		10	10	8-12
V_p (L)	= V_c [b]				
k_a (once daily dosing) (h^{-1})	3.7		23	3.7	2.2-5.1
k_a (twice daily dosing) (h^{-1})	1.6		14	1.6	1.2-2.1
D_{50} (mg)	25		30	24	14-42
IIV CL (ω^2)	0.038	19%	32	0.037	0.016-0.064
IIV V_c (ω^2)	0.080	28%	31	0.081	0.034-0.129
IOV F (ω^2)	0.047	22%	13	0.047	0.036-0.058
Residual var. (σ^2)	0.051	23%	6	0.051	0.044-0.057

CL, clearance; F, bioavailability; V_c, central volume of distribution; Q, inter-compartmental clearance; V_p, peripheral volume of distribution; k_a, absorption rate constant; D_{50}, dose with half minimal bioavailability; pred, prednisolone dose; CV, coefficient of variation; IOV, inter occasion variability; IIV, interindividual variability. Var, variability. [a] This means a 15% lower value for TRL bioavailability. [b] V_p is equal to V_c due to imprecise parameter estimation according to the bootstrap analysis.

relationship between the random effects in the base model (i.e. the model without covariates) and the covariate values were formally tested. Subsequently, the selected covariate relationships were evaluated by a forward inclusion and a backward deletion procedure [30]. A covariate effect was only maintained in the model if the inclusion resulted in a reduction in the random variability and in an improved model fit.

COMPUTATION. Non-Linear-Mixed-Effects-Modelling (NONMEM, version VI, Icon Development Solutions, Ellicott City, Maryland, USA) was used for modelling TRL PK. Modelling results were analyzed using the statistical software package S-Plus® for Windows (version 6.2 Professional, Insightful Corp., Seattle, USA). A convergence criterion of 3 significant digits in the parameter estimates was used. For model comparisons, the obtained minimum value of the objective function (MVOF) defined as minus twice the log-likelihood was used. First order conditional estimation (FOCE) with interaction was used throughout the modelling process. The modelling process was guided by statistical and visual checks (i.e. diagnostic 'goodness of fit' plots).

A model parameter was retained in the model when the difference in the minimum value of the objective function (minus twice the log likelihood) from the base model was at least 6.63 points. This compares to a Type I error of 1% under the assumption that this difference is χ^2 distributed with 1 degree of freedom. However, it is known that the NONMEM FOCE method produces only an approximation to the maximum likelihood assumptions and that the null hypothesis will be rejected more frequently than the nominal Type I error value [31,32]. Therefore, a randomization test (also called *re*-sampling or permutation) was performed to estimate the true distribution under the null hypothesis (i.e. the probability of observing the covariate effect by chance alone). This allows deriving the nominal difference in the objective function to reject the null hypothesis of no difference between the model with and without the covariate of interest with a specific Type I error rate. The randomization test involves fitting the model without and with the covariate effect to randomized data where the covariate was randomly allocated to subjects breaking any association between the covariate and the PK parameter of interest. This procedure was repeated 1,000 times generating a distribution of differences in the minimum value of the

Table 4. Covariate table. Listed in this table are the significant covariates improving model fit together with the effects on the observed variability. The forward inclusion and backward deletion procedure are displayed according to the procedure explained in the results section.

COVARIATE TESTED	MVOF	ΔOF[a]	IIV CL (%)	Expl var. CL (%)	IIV V_c (%)	Expl Var V_c (%)
BASE MODEL	5969		29		29	
+CYP3A5*1 (CL)	5951	-18	20	9	29	
+PXR A+7635G (CL)	5961	-8	25	4	30	
+ABCB1 T-129C (CL)	5965	-4[b]	26	3	29	
+Ht (allometric) (V_c)	5965	-4[b]	29		27	2
Forward inclusion						
BASE	5969		29		29	
BASE+CYP3A5	5951	-18	20	9	29	
Backward deletion						
FINAL MODEL	5951		20		29	
- PRED	5961	+10	19		28	
- PRED-CYP3A5	5981	+30	27		28	
- PRED-CYP3A5-GRP	6003	+52	27		30	
- PRED-CYP3A5-GRP-DDOSE	6059	+108	35		36	

CYP, cytochrome P450; PXR, pregnane X receptor; ABC, ATP-binding cassette; DDOSE, daily dose TRL; Ht, hematocrit; PRED, daily dose prednisolone; GRP, once and twice daily dose group; MVOF, minimum value of the objective function; ΔOF, change in MVOF; IIV, inter-individual variability; Expl.Var, explained variability; CL, clearance, V_c, central volume of distribution. [a]ΔOF: Decrease in the minimum value of the objective function compared to the base model or increase compared to final model. [b]not significant (P>0.01). It is significant with 95% confidence p<0.05 (ΔOF>3.84).

objective function (MVOF) between these models. The 99th percentile of this distribution reflects the difference in the MVOF for a Type I error rate of 1%.

VISUAL PREDICTIVE CHECK. The model prediction was evaluated using a Visual Predictive Check (VPC), which evaluates whether the identified model would be able to predict the observed variability for 80% of the population in the PK data that was used for model identification. Therefore, the PK of each individual using its individual specific dosing history and covariate values was simulated at least 200 times by means of a Monte Carlo simulation, in which random samples were drawn from the identified distributions for inter-individual variability, inter-occasion variability and residual variability. The distribution (median and 10th and 90th percentiles) of the simulated concentration-time courses was compared with the distribution of the observed values in the original data set. Differences and overlap of the simulated and original distributions indicated the accuracy of the identified model.

BOOTSTRAP. A bootstrap analysis was performed to assess the precision of the PK parameter estimates. The observed data set was re-sampled with replacement in order to generate a new data set with the same size and population characteristics, such as the number of patients per genotype, as the original set. This procedure was repeated 1,000 times to generate a distribution of the PK parameters with a mean and coefficient of variation as well as the median and 2.5th and 97.5th percentiles. Fitting the same re-sampled data set with the base model (i.e. model without covariate effect) and calculating the difference in MVOF between the base and covariate model allows calculating the power of the study to estimate a covariate retrospectively. The power is defined as the number of times that the difference in MVOF is greater than the difference associated with a type I error rate of 1% derived from the randomization test.

Results

Structural model

The PK of TRL was best described by a two compartment model with first-order absorption and first-order elimination from the central compartment. Random effects parameters were identified for the inter-individual variability in TRL clearance (CL) and the volume of the central compartment. Moreover, the variability between the occasions at which PK samples were collected was described with a random effect on the (fixed) bioavailability term (F). As a next step, the random effects were evaluated for structural relationships with dose, dosing frequency and time, to obtain a model with unbiased and randomly distributed random effects for the covariate analysis. A relationship between dose and clearance was observed, showing an increase in apparent clearance with increasing dose according to $F = 0.23 \times [1 - (\text{daily dose}/(D_{50} + \text{daily dose}))]$. D_{50} is the estimated dose with half minimal bioavailability (Table 3). Incorporating this relationship improved the model fit considerably in terms of objective function and goodness of fit plots. Secondly, the random effect parameters were not randomly distributed due to an overall decrease in apparent clearance (CL/F) in the first 6 weeks after transplantation. It appeared that co-administrating prednisolone in a dose over 10 mg/day was related to a 15% lower TRL bioavailability compared to lower doses. A final source of bias in the analysis arose from

the fact that once daily TRL dosing was clearly associated with an increased absorption rate as well as an increase in volume of distribution (Table 3). The model including all of the above described relationships was considered the base model for studying covariate effects.

Covariate model

The data showed considerable inter-individual variability (29%) and inter-occasion variability (22%) in CL/F with values ranging from 8-78 L/h. The variability in TRL clearance could be explained by genetic factors as two subpopulations with different values for TRL clearance were observed. These populations could be related to a genetic polymorphism (SNP) in CYP3A5 (Figure 1). Specifically, in the CYP*3/*3 genotype CL=3.7±0.3 L/h whereas in the CYP3A5*1/*3 genotype clearance was: 5.5±0.5L/h. Thus, the CYP3A5*1/*3 genotype was associated with higher TRL clearance, hence lower exposure in terms of AUC (Figure 2). This SNP accounted for 9% of the inter-individual variability in TRL clearance (Table 4). Relative to the observed variability in apparent clearance of 29% this genetic factor explained 30% of the inter-individual variability in TRL exposure. The estimated difference in clearance is significant, since the decrease in MVOF from the model without this covariate effect is 18 points (Table 4), which is considerably greater compared to the difference of 5.42 required for a significant difference with a type I error of 1%. In addition, a retrospective power of 99% was found, indicating that the study was sufficiently powered to estimate the difference in clearance.

The second relevant genetic factor was PXR A+7635G. In A-allele carriers CL=3.9±0.3 *versus* CL=5.4±0.5L/h in the GG genotype. Thus the PXR7635 GG SNP was also associated with higher TRL clearance, but explained only 4% of the inter-individual variability

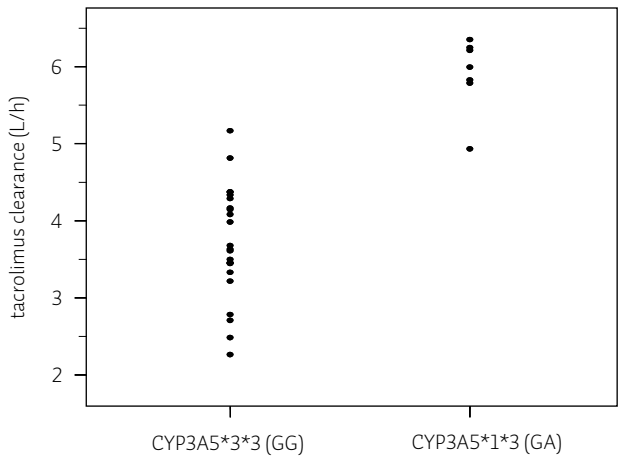

Figure 1. The relationship between CYP3A5 genotype and tacrolimus clearance.

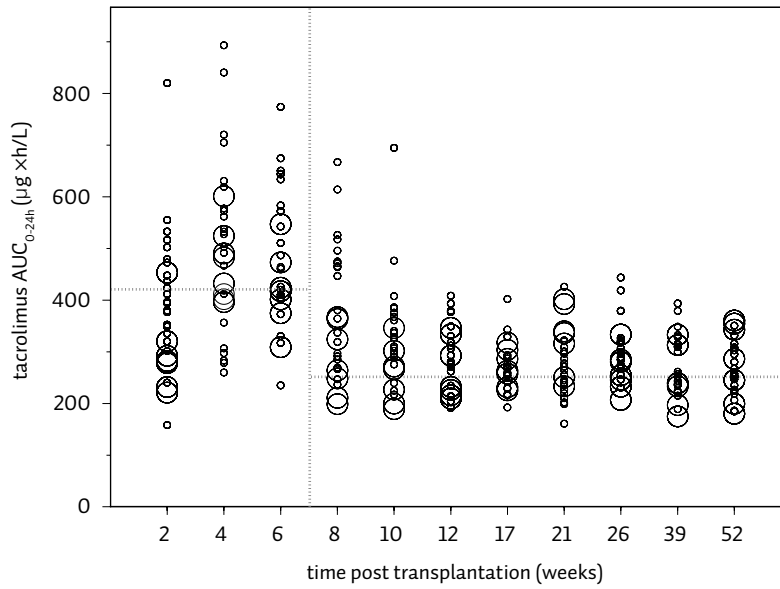

Figure 2. AUC0-24 hours *versus* time post transplantation, small circles are patients with the CYP3A5*3*3 genotype, large circles represent CYP3A5*1*3. The dotted lines are the target AUC0-24 hours in the first 6 weeks (left, top) and the target value beyond the first 6 weeks (right, bottom). Note that week 2 is a grouping category including exposure measurements as soon as day 6 after transplantation.

in TRL clearance (Table 4). Three out of five individuals with the PXR A+7635G GG genotype displayed an overlap with 3 (out of 7) individuals carrying the CYP3A5*1/*3 genotype. Moreover, an association between the CT genotype in the ABCB1 gene promoter SNP T-129C was associated with a decreased TRL clearance (Table 4). This SNP accounted for 3% of the variability in TRL clearance with this limited number of observations (Table 5). The remainder of the SNPs in the ABCB1-gene (encoding the P-glycoprotein transporter) was studied together by means of the amount of functional alleles or gene-dose effect, but a correlation with TRL clearance was not found. The other selected PXR genotypes (PXR C-25385T, PXR A-24381C, PXR G-24113A, PXR A+252G) did not have a relationship with TRL clearance either.

Another interesting factor is hematocrit which displayed an allometric relationship with central volume of distribution (V_c) (i.e. $V_c=61\times(Ht/0.37)^{-0.5}$), indicating a decreasing V_c upon rising hematocrit. This relationship was clearly present during the visual covariate analysis and caused a decrease in objective function, which was not considered strong enough to be incorporated into the model (Table 4).

Interestingly, body weight (43-109 kg, mean and median 75 kg) could not be identified as a covariate on neither CL/F nor V_c/F. The relationship between dose and weight introduced by weight-based dosing disappeared by applying TDM. Figure 3A demonstrates the absence of a relationship between body weight and TRL clearance. Yet, a relationship between body weight and the difference between observed AUC and target AUC (AUC

observed - target AUC) was observed (Figure 3B). This plot visualizes the relationship between the initial TRL dose and achieving target exposure. The left plot has been obtained from the data. The plot on the right is the same plot but scaled to a dose of 0.2 mg/kg/day. Scaling was done to correct for the fact that obese patients were dosed on lean body weight and in most cases received a lower body weight based TRL dose. Figure 3B indicates that underexposure could be related to low bodyweight and overexposure to higher bodyweight. The covariates serum albumin concentration, cholesterol (HDL, LDL, total), age and gender, were found not to have a relationship with TRL PK.
Finally, the results of the backward deletion procedure are presented in Table 4, where the effect of sequentially deleting the relevant covariates prednisolone daily dose, CYP3A5 genotype, once and twice daily TRL dosing and daily TRL dose from the model is shown. The final model was found to adequately predict the observed trend and variability in the TRL concentrations according to the visual predictive check (Figure 4).

Discussion

In the present study a comprehensive analysis was performed to estimate the contribution of a wide range of factors involved in the variability of early TRL exposure. The aim was to identify covariates relevant for individualized dosing. The three most relevant factors identified were, in rank order of importance, CYP3A5*1/*3, PXR A+7635G GG and prednisolone co-medication in a dose over 10 mg per day. The clear effect of the SNP in CYP3A5 regardless of the patient's body weight underscores the importance of an individualized initial dosing strategy in this population of adult kidney transplant recipients. In the current analysis patients with the CYP3A5*1/*3 genotype required a 1.5 times higher fixed initial dose compared to CYP3A5*3/*3 in order to reach adequate TRL target exposure early after transplantation.
In this study no subjects with CYP3A5*1/*1 genotype were found. Since the *1 allele is associated with increased CYP3A5 function, one would expect a higher TRL dose for the *1/*1 genotype as well [20]. Earlier studies have documented the impact of polymorphisms in genes encoding CYP3A4, CYP3A5 and ABCB1 (P-glycoprotein) on TRL PK, but in the large majority only in relation to (dose-corrected) trough levels [13,15,33]. Unfortunately, trough levels have repeatedly been shown to correlate poorly with the systemic exposure measured in terms of AUC [27,34]. Indeed, the use of trough levels to adjust the TRL dose in clinical practice will introduce variability in the actual TRL exposure, since we observed a trough level range of 3 to 20 µg/L when dosing twice daily aiming at a target AUC of 210 µg×h/L (range 190 to 230 µg/L).
The population analysis methodology used in the present study differentiates between structural variability (within an individual) and random variability (between individuals). In contrast to non-population based approaches this results in greater statistical power to identify a covariate effect, because both sources of information are used instead of one. When analyzing multiple observations per subject one is able to compensate for the small number of individuals. This is supported by the retrospective power analysis, which shows that the power was 99% to identify a difference in TRL clearance of 47% between carriers of the CYP3A5*1 and the CYP3A5*3 genotype.

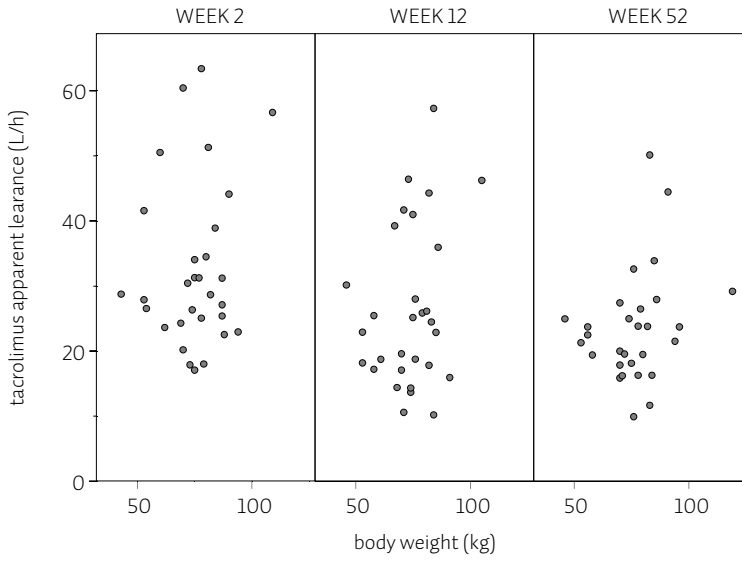

Figure 3A. The relationship of TRL apparent clearance with body weight plotted on 3 occasions (week 2, 12 and 52) after transplantation.

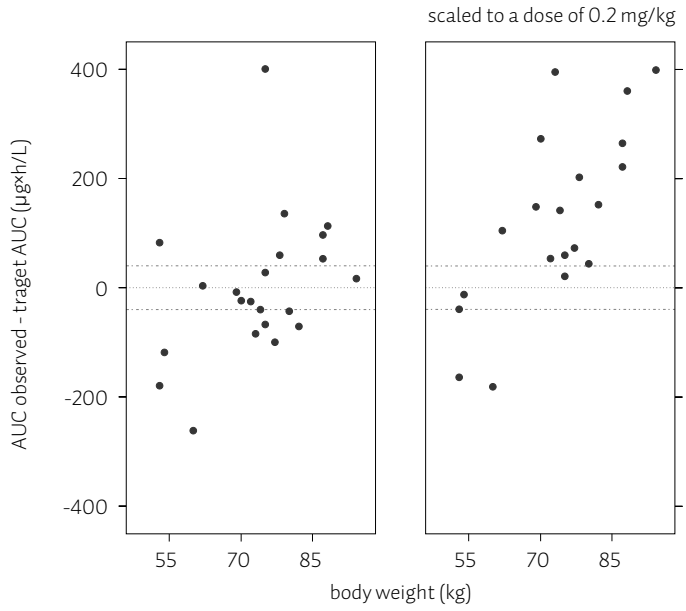

Figure 3B. $AUC_{0\text{-}24h}$ observed minus target $AUC_{0\text{-}24h}$ on the *first* exposure measurement post transplantation ($CYP3A5^*3^*3$ only) on the left. In the right plot the data are scaled to the theoretical dose of 0.2 mg/kg/day.

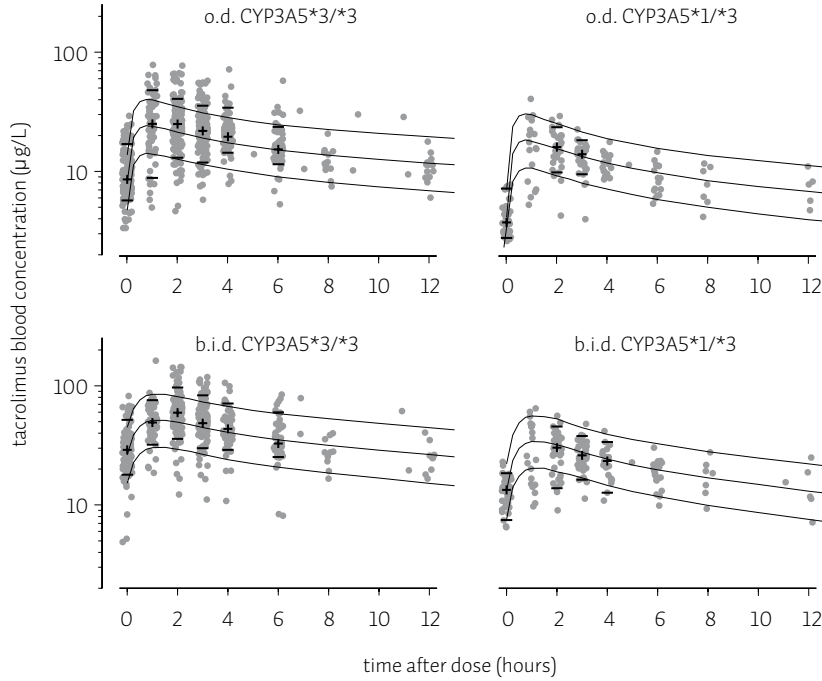

Figure 4. The visual predictive check with the 80% prediction interval (area between the outer solid lines) depicted for the genotype groups CYP3A5*1/*3 and CYP3A5*3/*3 and the once and twice daily dose groups. The middle solid line represents the median of the model prediction. The observed concentrations are shown as closed symbols, whereas the median of observed concentrations per time point are shown with the cross (+) symbol. The horizontal lines indicate the 10th and 90th percentile of the observed data. In case the PK analysis indicated linear PK, that would allow scaling the TRL doses to the median of the dose, 7 mg for the o.d. and 5 mg for the b.i.d group, as different doses are administered to patients due to TDM. o.d.: once daily dosing, b.i.d.: twice daily dosing.

There are two possible confounding factors relevant for this analysis. First, the identified CYP3A5 genotype effect could potentially be influenced by the immunoassay used to determine TRL blood concentrations. It is known that this assay displays cross-reactivity of TRL metabolites, which limits assay performance [35,36]. Patients with the CYP3A5*1 genotype have an increased TRL metabolism resulting in a higher metabolite fraction in the blood samples obtained during routine monitoring. In theory, these samples could be biased more by the cross-reactivity due to the higher metabolite fraction. However, it is not likely that this interfered with the obtained results as it has been shown by Moreton et al that CYP3A5 genotype differences do not influence the blood concentration determination [37]. And, furthermore, if this would have interfered with the obtained results, this would have inflated the result. The CYP3A5*1 group would then in fact have lower actual blood levels, hence higher clearance than already demonstrated. A second important question is whether we would have to consider a circadian variation, also called chronopharmacokinetics, in TRL metabolism. This is currently under debate in literature [38-42]. No difference in daytime versus night time AUC was observed in several studies with oral TRL

Table 5. Genotype distribution in the study population ($n = 31$).

SNP	Frequency			
ABCB1 T3435C	T/T 7 (23%)	C/T 14 (47%)	C/C 9 (30%)	1 UG
ABCB1 G2677T	G/G 10 (33%)	G/T 16 (53%)	T/T 4 (13%)	1 UG
ABCB1 C1236T	C/C 11 (37%)	C/T 14 (47%)	T/T 5 (17%)	1 UG
ABCB1 T-129C	T/T 27 (90%)	C/T 3 (10%)	C/C 0	1 UG
CYP3A4*1B	A/A 26 (87%)	G/A 4 (13%)	G/G 0	1 UG
CYP3A5*3	G/G 23 (77%)	A/G 7 (23%)	A/A 0	1 UG
CYP3A5*6	C/C 30 (100%)	C/T 0	T/T 0	1 UG
PXR C-25385T	C/C 8 (27%)	C/T 18 (60%)	T/T 4 (13%)	1 UG
PXR A-24381C	A/A 7 (23%)	A/C 19 (63%)	C/C 4 (13%)	1 UG
PXR G-24113A	G/G 8 (28%)	G/A 17 (59%)	A/A 4 (14%)	2 UG
PXR A+252G	A/A 11 (37%)	A/G 13 (43%)	G/G 6 (20%)	1 UG
PXR A+7635G	T/T 11 (38%)	C/T 13 (45%)	C/C 5 (17%)	2 UG

SNP, single nucleotide polymorphism; ABC, ATP-binding cassette; CYP, cytochrome P450; PXR, pregnane X receptor; UG, unsuccessfully genotyped (1 individual in particular). Frequency determined on successfully genotyped individuals.

[38,39,41], while a slower and delayed absorption was identified in one of these studies [41]. Interestingly, two studies pointed out that night-time TRL administration resulted in significantly lower AUC and C_{max} [42,43], while intravenous TRL did result in a slightly lower clearance and consequently higher AUC at night compared to morning administration [40]. It is thought that the absorption process in terms of gastric emptying time and perfusion possibly influenced by an evening meal is crucial in the observations [3,42]. The circadian effect itself is therefore contestable. In case the effect exists it does not seem to be a large effect. Moreover, it is unlikely that this would have influenced our findings which are based on morning administration of TRL in a once or twice daily fashion.

A new polymorphism (PXR A+7635G) in the gene encoding for the pregnane X receptor (PXR) was identified which explained 3.5% of the variability in TRL exposure. However, the impact of this polymorphism was relatively low in comparison to the already discussed polymorphism in CYP3A5 which explained 9% of the inter-individual variability in apparent clearance (CL/F) (or 30% of the inter-individual variability in CL/F). Importantly, activation of the PXR is one of the determinants involved in the (tissue specific) expression of both CYP3A and ABCB1. Therefore, the role of PXR can be especially relevant in the first weeks or months after organ transplantation, when usually high prednisolone doses are prescribed. A maintenance dose of 5-10 mg/day is still considered to be pharmacologically active resulting in a variable continuation of PXR activation. In addition, other endogenous (cortisol) or unknown factors in transplant recipients may be respon-

sible for activation of the PXR. This is supported by the observation, that a subgroup of our patients displayed a decrease in CL/F within the first 6 weeks after transplantation that could not be related to the SNPs studied in the PXR gene.
The present model was improved by including the effect of prednisolone on TRL bioavailability, which was a relative term due to the absence of intravenous TRL data. A concomitant prednisolone dose over 10 mg/day resulted in a 15% lower TRL bioavailability compared to a concomitant prednisolone dose of 10 mg/day or lower. A limitation to the present study was the absence of the effect of treating acute rejection episodes with high dose steroids (Solu-Medrol) [11]. Neither could we determine the effect of a low dose (less than 10 mg/day) or a steroid free regimen on TRL apparent clearance [10].
TRL absorption and distribution kinetics differed when considering the once daily and twice daily dosing regimens. A higher absorption rate and higher volume of distribution were observed for the once daily TRL dosing group. However, this is not clinically relevant since the TRL dosing interval did not appear to have an effect on TRL exposure. Recently a modified release formulation of tacrolimus has been introduced in order to improve patient compliance [44,45]. The clinical and pharmacokinetic data obtained in the present study indicate that once daily dosing could also be an option with the conventional formulation.
The observed relationship between TRL dose and clearance could, at least partly, be the result of an interaction between prednisolone and TRL, explained by a decreased inductive effect on TRL clearance following steroid taper. The relationship between TRL dose and clearance is however most likely the consequence of patient selection through the use of the strict adherence to the defined TDM protocol. According to the protocol, the patients with high TRL blood levels (i.e. with a lower clearance) were titrated to receive lower doses and *vice versa*. Therefore the apparent relationship between CL/F and dose reflects the study design, i.e. is the result of adjusting the dose on the basis of a predefined target. This conclusion is supported by the observation that before TDM was started, no relationship between dose and CL/F was found. A recent simulation study has documented a similar effect for carbamazepine [46].
High dose TRL in combination with a low hematocrit resulted in a non-linear PK behaviour which has been explained by saturation of binding capacity to red blood cells [7,25]. In the present analysis, with hematocrit values in the range of 0.26 to 0.52, such a relationship could not be clearly identified. It has been shown that hematocrit influences the TRL blood level determinations with an immunoassay [47-49]. However, as this especially concerns hematocrit values of 0.25 and lower this does not form a major issue in this study. The relationship between hematocrit and TRL PK has been described before [7,25]. Low hematocrit values resulted in a higher apparent volume of distribution, reflecting more unbound TRL available for distribution in peripheral tissues. The observed non-linearity may have relevant clinical implications (delayed graft function, liver- and neurotoxicity) especially in the first weeks after organ transplantation, where low hematocrit is accompanied by high TRL doses. Other potentially relevant factors that could contribute to observed variability in TRL PK, such as diarrhea [50,51] and non-compliance, could not be quantified in this study.

TRL is a low clearance drug and in adult transplant recipients, body weight was not related to either central volume of distribution or TRL clearance (Figure 3A). Our data (Figures 2 and 3B) indicate that a predefined target can be reached faster using a genotype-based instead of a bodyweight-based initial dosing regimen. In the present study the TRL dose was adjusted to lean body mass by protocol. As a result, patients with a body mass index over 25 on average received 0.15 mg/kg body weight per day. Taking this into account the relationship strengthened (Figure 3B), indicating that without this intervention these patients would have received a starting dose which was far too high. A fixed standard dose of 14 mg/day (corresponding to 0.2 mg/kg/day for a 70 kg person) to reach the defined AUC could be a better therapeutic approach for patients with the CYP3A5*3/*3 genotype compared to the currently applied body weight based regimen. Moreover, a fixed daily dose of 20 mg (10 mg b.i.d.) would be more appropriate in patients with the CYP3A5*1/*3 genotype due to the 1.5 times higher apparent clearance. Clearly, this strategy warrants validation in a prospective study which includes larger numbers of renal transplant recipients
Despite the well documented high inter patient variability in TRL pharmacokinetics, bodyweight is still the only variable that drives the TRL starting dose at the time of transplantation. The findings of the present study suggest that a genotype-based dosing strategy in combination with TDM, may be a novel and superior approach to optimize initial exposure in adult kidney transplant recipients. Justification for this approach is the current trend towards early CNI minimization protocols, since both acute rejection and acute CNI-induced nephrotoxicity have been identified as the major risk factors for chronic allograft nephropathy [2].

Conclusions

Three covariates were found to contribute to individualized TRL dosing in order to optimize early TRL exposure in kidney transplant patients. Adult CYP3A5*1 allele carriers have a higher TRL clearance compared to CYP3A5*3 allele carriers. These individuals, therefore, should receive higher TRL fixed starting doses since TRL starting doses should not be adjusted to patient's body weight. Moreover, the Pregane-X-Receptor A+7635G GG genotype as well as concomitant prednisolone administration in a dose over 10mg/day are associated with increased TRL metabolism.

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Explaining Variability in Ciclosporin Exposure in Adult Kidney Transplant Recipients

ABSTRACT

PURPOSE Optimal ciclosporin A (CsA) exposure in kidney transplant recipients is difficult to attain because of variability in CsA pharmacokinetics. A better understanding of the variability in CsA exposure could be a good means of individualizing therapy. Specifically, genetic variability in genes involved in CsA metabolism could explain exposure differences. Therefore, this study is aimed at identifying a relationship between genetic polymorphisms and the variability in CsA exposure, while accounting for non-genetic sources of variability.

METHODS *De novo* kidney transplant patients ($n=33$) were treated with CsA for 1 year and extensive blood sampling was performed on multiple occasions throughout the year. The effects of the non-genetic covariates hematocrit, serum albumin concentration, cholesterol, demographics (i.e. bodyweight), CsA dose interval, prednisolone dose and genetic polymorphisms in genes encoding ABCB1, CYP3A4, CYP3A5, and PXR on CsA pharmacokinetics were studied using non-linear mixed effect modeling.

RESULTS The pharmacokinetics of CsA were described by a two-compartment disposition model with delayed absorption. Body weight was identified as the most important covariate and explained 35% of the random inter-individual variability in CsA clearance. Moreover, concurrent prednisolone use at a dosage of 20 mg/day or higher was associated with a 22% higher clearance of CsA, hence lower CsA exposure. In contrast, no considerable genotype effects (i.e. greater than 30-50%) on CsA clearance were found for the selected genes.

CONCLUSIONS It appears that the selected genetic markers explain variability in CsA exposure insufficiently to be of clinical relevance. Therefore, therapeutic drug monitoring is still required to optimize CsA exposure after administration of individualized doses based on body weight and as this study suggests, co-administration of prednisolone.

Introduction

The trend toward lowering the exposure of the calcineurin inhibitor ciclosporin (CsA) is widely propagated [1] as CsA exposure, in terms of drug blood concentrations, relates to the clinical endpoints rejection and toxicity [2]. Since therapy with CsA is characterized by considerable *inter*- and *intra*-individual variability [3] in its pharmacokinetics (PK), it is difficult to remain within the therapeutic window. Therefore, to optimize therapy in clinical practice, therapeutic drug monitoring (TDM), aimed at individualized CsA dosing is common practice [2,4,5]. As a next step, to further optimize CsA therapy, insight into the sources of this variability in CsA exposure is necessary.

It has been demonstrated that the observed variability in CsA PK originates from non-genetic biological and lifestyle-related factors, including age, body size, gender, food intake [3], serum albumin concentration, hematocrit, lipoproteins (HDL, LDL) [6,7], and co-administration of interacting drugs [3,8-11]. Yet, even when these factors are taken into account, a considerable part of the variability remains unexplained, which could potentially be attributed to genetic differences between patients. Insight into this relationship could aid in optimizing CsA exposure early after transplantation. Indeed, CsA disposition is characterized by extensive metabolism by cytochrome P450 (CYP) enzymes [12,13]. Moreover, CsA is a substrate for the efflux pump P-glycoprotein (ABCB1) [12,13]. Recently, the pregnane X receptor (PXR) was reported to be the key nuclear receptor regulating expression of cytochrome enzymes and certain transport proteins and mediating their induction [14-16]. To date no studies have been published relating CsA PK parameters to polymorphisms in PXR. Several investigators have studied the role of genetic variants in genes encoding for the drug metabolizing enzymes CYP3A4, CYP3A5, and the multidrug resistance transporter ABCB1 [13,17]. These studies show conflicting results with regard to the contribution of single nucleotide polymorphisms (SNPs) in *ABCB1* C1236T, T3435C, G2677A and T-129C, *CYP3A4**1B, and *CYP3A5**1 [18-22].

A limitation of many studies is that often other sources of variability, which can mask the actual relationship between genetic factors and CsA exposure, have not been taken into account. This is especially the case because associations between SNPs in these enzymes are mainly related to dose corrected trough concentrations, which are not a very sensitive measure of variability in exposure. An integrated analysis on the basis of full concentration vs time profiles, accounting for the observed variability in CsA PK by including a wide range of covariates, is the approach for identifying any relationship between CsA exposure and genetic polymorphisms in genes encoding CYP3A4, CYP3A5, P-glycoprotein or the pregnane X receptor. Therefore, in this study, a population analysis of CsA PK was performed, aimed at a comprehensive exploration of the determinants for individualizing the CsA dose in kidney transplant recipients.

Materials & Methods

Patients and therapy

De novo kidney transplant patients ($n=33$) aged between 18 and 70 years were followed for 1 year after transplantation (Table 1). Recipient, donor, and transplant characteristics as well

Table 1. Clinical characteristics.

Variable	Once daily (*n* = 17)	Twice daily (*n* = 16)	p-value
Recipient characteristics			
Age (mean ± SD)	43.8 ± 14.5	48.9 ± 10.5	0.79
Male sex (*n*)	15	11	0.23
Caucasian (*n*)	14	12	0.69
Native kidney disease			0.29
Pyelonephritis	0	2	
Glomerulonephritis	6	6	
Hereditary / congenital	2	4	
Hypertension	4	0	
DM	1	2	
Other	2	1	
Unknown	2	1	
Donor characteristics			
Age (mean ± SD)	44.9 ± 16.7	43.1 ± 12.6	0.72
Male sex (*n*)	7	6	0.83
DD-heart beating	7	9	0.49
DD-non heart beating	6	1	0.09
LRD	2	4	0.40
LURD	2	2	0.95
Transplant characteristics			
HLA-mismatch mean ± SD	2.00 ± 1.50	2.38 ± 1.82	
Class I	1.47 ± 1.23	1.63 ± 1.36	0.79
Class II	0.53 ± 0.51	0.75 ± 0.68	0.40
Cold ischemia time (h) DD only	19.9 ± 4.3	23.0 ± 8.4	0.25
Acute rejection 6 months (*n*)	4	2	0.40
Need for ATG (*n*)	3	1	0.33
Patient survival (*n*)			
1 year	17	16	
2 years	17	16	
Death-censored Graft survival (*n*)			0.60
1 year	16	16	
2 years	16	16	
Nankivell clearance (mean ± SD)			0.44
Week 2	45 ± 23	48 ± 21	
Week 6	62 ± 17	61 ± 15	
Month 3	64 ± 14	61 ± 13	
Month 6	68 ± 16	64 ± 10	
Month 9	68 ± 15	63 ± 11	
Year 1	69 ± 17	64 ± 12	
Year 2	64 ± 16	60 ± 11	

DM, diabetes mellitus; DD, deceased donor; LRD, living related donor; LURD, living unrelated donor; HLA, human leukocyte antigen; ATG, anti-thymocyte globulin; SD, standard deviation.

as outcome parameters (acute rejection rate, patient and graft survival, and renal function) are summarized in Table 1. Only recipients of a first kidney graft from a deceased or living (non-HLA-identical) donor were included. From each patient written informed consent was obtained. The study was performed in accordance with the Declaration of Helsinki and was approved by the Medical Ethics Committee of the Leiden University Medical Center.
Patients received quadruple immunosuppression consisting of induction therapy with basiliximab on the day of transplantation and on day 4, a fixed dose (1,000 mg twice daily) of mycophenolate mofetil (MMF), prednisolone (50 mg twice daily on the day of transplantation, but rapidly tapered toward 10 mg once daily at day 22) and CsA (Neoral®). Patients were randomized to receive a CsA daily dose of 8 mg/kg/day in either a once or twice daily regimen (Table 1). TDM for twice daily CsA was aimed at a target AUC of 5,400 µg×h/L in the first 6 weeks and at 3,250 µg×h/L after this period. Likewise, for the once daily regimen these values were 10,800 µg×h/L and 6,500 µg×h/L respectively.

Therapeutic drug monitoring

Therapeutic drug monitoring (TDM) was performed on the basis of a limited sampling strategy (blood concentration at t=0, 2, and 3 h) and the Bayesian estimation of the $AUC_{0\text{-}12h}$ using MW/Pharm version 3.5 (Mediware, Groningen, The Netherlands), as described previously [4]. Routine TDM samples were taken during the mornings of weeks 4, 8, 10, 17, 21, and 39 after transplantation. In addition, PK was densely sampled in weeks 2, 6, 12, 26 and 52 with samples at t=0, 1, 2, 3, 4, 6, and 24 h. A majority of patients (22) was sampled on each of the eleven study occasions, while 6 patients were sampled on ten occasions and 2 patients on twelve occasions. The remaining 3 patients were sampled less frequently (ranging from three to six occasions). Furthermore, at every TDM visit CsA dosage information was recorded, which consisted of the actual time of dosing (that morning) and the time of dosing the evening before, as well as the amount and dose interval. As patients had to use the same dose for at least 3 days to reach a steady state, the start of therapy or the last date of dose change was recorded.
Ciclosporin A concentrations were determined in whole blood by fluorescence polarization immunoassay (FPIA; Abbott Laboratories, Abbott Park, IL, USA) and analyzed according to the manufacturer's instructions. Assay inter-day variation derived from routine measurements was: 10.4% (70 µg/L, low level), 7.8% (300 µg/L, medium level) and 7.5% (600 µg/L, high level). The assay was linear up to a concentration of 800 µg/L.

Genotyping assays

The DNA was isolated from EDTA-blood. Primers and probes used in the Taqman-based genotyping assays, as well as primers and sequences used in the pyrosequence assays are listed in Table 2. *ABCB1* C1236T (rs1128503), T3435C (rs1045642), and G2677T (rs2032582) were determined with TaqMan 7500 (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) with custom designed assays, according to the manufacturer's protocol. *ABCB1* T-129C (rs3213619), *CYP3A5**3/*6 (rs776746/rs10264272), *CYP3A4**1B, (rs2740574) and *NR1I2* or *PXR* SNPs C-25385T (rs3814055), A-24381C (rs1523127), G-24113A (rs2276706), A+252G (rs1464603), and A+7635G (rs6785049) [14,15] were determined with Pyrosequencer 96MA (Isogen, IJsselstein, The Netherlands). In short, PCR reactions contained

Table 2. Primers and Probes for TaqMan and Pyrosequence analysis.

SNP	Target	Sequence 5'-3'	Modification[a]
ABCB1 C1236T	PCR-f	CACCGTCTGCCCACTCT	
	PCR-r	GTGTCTGTGAATTGCCTTGAAGTTT	
	Probe-T	TTCAGGTTCAGACCCTT	VIC
	Probe-C	CAGGTTCAGGCCCTT	FAM
ABCB1 G2677T	PCR-f	CTTAGAGCATAGTAAGCAGTAGGGAGT	
	PCR-r	GAAATGAAAATGTTGTCTGGACAAGCA	
	Probe-G	TTCCCAGCACCTTC	VIC
	Probe-T	TTCCCAGAACCTTC	FAM
ABCB1 T3435C	PCR-f	ATGTATGTTGGCCTCCTTTGCT	
	PCR-r	GCCGGGTGGTGTCACA	
	Probe-T	CCCTCACAATCTCT	VIC
	Probe-C	CCCTCACGATCTCT	FAM
Pyrosequence			
ABCB1 T-129C	PCR-f	TCGAAGTTTTTATCCCA	Biotine
	PCR-r	CCTCCTGGAAATTCAACCTGTT	
	Sequence primer	TACTCCGACTTTAGTGGAAAGACC	
	Target Sequence	CTG/ACTCGAATGAG	
CYP3A5*3	PCR-f	CTGCCTTCAATTTTTCACT	
	PCR-r	TATGTTATGTAATCCATACCCC	Biotine
	Sequence primer	AGAGCTCTTTTGTCTTTCA	
	Target Sequence	A/GTATCTC	
CYP3A4*1B	PCR-f	CAGCCATAGAGACAAGGGC	
	PCR-r	GAAGAGGCTTCTCCACCTT	Biotine
	Sequence primer	CCATAGAGACAAGGGCA	
	Target Sequence	A/GGAGAGAGG	
CYP3A5*6	PCR-f	TCTTTGGGGCCTACAGCATG	
	PCR-r	AAAGAAATAATAGCCCACATACTTATTGAGAG	Biotine
	Sequence primer	AGAAACCAAATTTTAGGAA	
	Target Sequence	CTTC/TTTAG	
PXR C-25385T	PCR-f	GTGGTCATTTTTTGGCAATCCC	
	PCR-r	AGCCTCTGGCAACAGTAAAGCA	Biotine
	Sequence primer	TTGGCAATCCCAGGT	
	Target Sequence	TC/TTCTTTTCTACCTGTT	
PXR A-24381C	PCR-f	AGTGGGAATCTCGGCCTCA	
	PCR-r	CTGGGGTCCACTTTGAACAATC	Biotine
	Sequence primer	GCTAATACTCCTGTCCTGAA	
	Target Sequence	A/CAAGGCAGCGGCTCCTTG	
PXR G-24113A	PCR-f	GAATCATGTTGGCCTTGCTGC	
	PCR-r	GCATCAGTAATGGGGCTCAAC	Biotine
	Sequence primer	TCTCCTCATTTCTAGGGT	
	Target Sequence	C/TCACCCTAG	
PXR A+252G	PCR-f	TGCAAGGGCTTTTTCAGGTAGAGT	
	PCR-r	TGAACCTGGGGGATAGGTCAAG	Biotine
	Sequence primer	ACTGACCCACTGGGTAA	
	Target Sequence	CA/GTCTCAGGGC	
PXR A+7635G	PCR-f	AGCCATCCTCCCTCTTC	Biotine
	PCR-r	CAGCAGCCATCCCATAATC	
	Sequence primer	CATAATCCAGAAGTTGGG	
	Target Sequence	GGC/TGAGAGGAA	

f, forward orientated, r, reverse orientated, ABC, ATP-binding cassette, CYP, cytochrome P450, PXR, pregnane X receptor, PCR, polymerase chain reaction. [a]VIC and FAM are fluorescent dyes, biotine is necessary to obtain single stranded DNA

10 ng of DNA, and 5 pmol of each PCR primer (Table 2) in a total volume of 12 µl. Cycle conditions were: initial denaturation for 15 min at 95˚C, 35 cycles of 95˚C–55˚C–72˚C each for 30 seconds, ending with 10 minutes at 720C. The pyrosequence reactions were performed according to the manufacturer's protocol. The sequence used for analysis and the calculated dispensation order for each SNP are listed in Table 2. Note that the lower case nucleotides in the dispensation sequence are negative controls, which are not incorporated into the target DNA and consequently should not appear in the pyrogram. As quality control, 5% of samples were genotyped in duplicate. In addition, negative controls (water) were used. The allele frequencies were found to be in Hardy-Weinberg equilibrium. Table 3 presents the genotype distribution in the overall genotyped population. The haplotype analysis for ABCB1 SNPs that are in linkage disequilibrium was performed using gPLINK with haplotypes set with a certainty greater than 0.97.

Population pharmacokinetic analysis

The PK of CsA was analyzed by non-linear mixed effects modeling. Mixed effects models consist of a structural model, describing the relationship between dose and concentration in terms of structural PK parameters (i.e. CL, V), and a stochastic model, describing the random variability in the structural model parameters. The random effects are the expression of inter-individual and inter-occasion variability. Inter-individual variability describes the random variability of structural parameters within the population, whereas

Table 3. Genotype distribution in the study population ($n = 33$). The number of individuals carrying a certain genotype are presented.

SNP	Frequency			
ABCB1 T3435C	T/T 9	C/T 12	C/C 10	2 UG
ABCB1 G2677T	G/G 10	G/T 13	T/T 7	3 UG
ABCB1 C1236T	C/C 10	C/T 14	T/T 7	2 UG
ABCB1 T-129C	T/T 29	C/T 2	C/C 0	2 UG
CYP3A4*1B	A/A 27	G/A 3	G/G 1	2 UG
CYP3A5*3	G/G 25	A/G 4	A/A 2	2 UG
CYP3A5*6	C/C 28	C/T 3	T/T 0	2 UG
PXR C-25385T	C/C 7	C/T 16	T/T 8	2 UG
PXR A-24381C	A/A 6	A/C 15	C/C 10	2 UG
PXR G-24113A	A/A 8	G/A 16	G/G 7	2 UG
PXR A+252G	A/A 10	G/A 15	G/G 6	2 UG
PXR A+7635G	A/A 6	G/A 19	G/G 6	2 UG

Frequency determined in successfully genotyped individuals. *SNP* single nucleotide polymorphism, *ABC* ATP-binding cassette, *CYP* cytochrome P450, *PXR* pregnane X receptor, *UG* unsuccessfully genotyped (2 individuals in particular)

inter-occasion variability describes the variability of an individual parameter value from one occasion to another. The second level of stochastic effects, σ2, describes the variability of the difference between observed and predicted responses. This residual error includes, among other factors, model mis-specification, intra-individual variability, and measurement error. In the mixed effects modeling approach, structural and stochastic parameters are simultaneously estimated by fitting the model to the data. In this respect the following parameters were estimated: PK parameters, variance and covariance (ω^2) of each individual specific parameter value (η) and variance (σ^2) of the residual error. As a result, individual *post hoc* estimates of parameters associated with inter-individual and inter-occasion variability could be obtained.

STRUCTURAL MODEL. The PK of CsA was fitted to linear compartmental models. The value for the oral bioavailability was fixed to 50%, as previously described [3,23] and used in the clinically applied TDM model [4].

RANDOM EFFECTS. Inter-individual variability (IIV) and inter-occasion variability (IOV) were described assuming a log normal distribution with the following equation:

$$PKj = TVPK \times e^{\eta jPK}$$

in which *PKj* is the PK parameter in the j^{th} individual and ηjPK is the difference between the individual specific parameter and the population value. TVPK is the population value of the PK parameter and the difference of the logarithm between the individual value of subject *j* and the population mean (ηjPK) is normally distributed with a mean of zero and variance ω^2_{PK}. The residual error was assumed to be additive to the predicted concentration after log-transformation:

$$log(Cij) = log(Cpredij) + \varepsilon ij$$

in which *cij* is the i^{th} observation for the j^{th} individual, *Cpredij* is the concentration of CsA in the blood predicted by the PK model, and *εij* (difference between *Cij* and *Cpredij*) is a normally distributed variable with mean zero and variance σ^2.

COVARIATE ANALYSIS. The non-genetic biologic and life-style covariates hematocrit, serum albumin concentration, prednisolone daily dose, CsA dose regimen, cholesterol (LDL, HDL), the demographic factors body weight, age, sex and body surface area (BSA) and the genetic markers for *ABCB1* (T3435C, G2677T, C1236T, T-129C), *CYP3A4* (*1B), *CYP3A5* (*3,*6), and *NR1I2/PXR* (C-25385T, A-24381C, G-24113A, A+252G, A+7635G) were selected on the basis of their known or theoretical relationships with CsA PK. Covariates with a clear visual relationship between the random effects in the model without covariates (base model) and the covariate values were formally tested with the model. When the relationship was described allometrically (i.e. in a body weight adjusted manner) it was done in the form $PK = TVPK \times (BW/meanBW)^y$, where *BW* is the individual body weight value, *meanBW* is the body weight population mean and *y* is the allometric exponent with typically a value of 0.75 for clearance and 1 for volume of distribution [24]. Subsequently, the selected covariate relationships were evaluated by a forward inclusion and a backward

deletion procedure [25]. Including a covariate effect should result in a reduction in the identified random variability and an improvement of the model fit.

COMPUTATION. Non-Linear-Mixed-Effects-Modelling (NONMEM, version VI release 1.2, Icon Development Solutions, Ellicott City, Maryland, USA) [26] was used for modelling CsA PK. Modelling results were analyzed using the statistical software package S-Plus® for Windows (version 6.2 Professional, Insightful Corp., Seattle, USA). A convergence criterion of 3 significant digits in the parameter estimates was used. For model comparisons the obtained minimum value of the objective function (MVOF) defined as minus twice the log-likelihood, was used. First order conditional estimation (FOCE) with interaction was used throughout the modelling process. The modelling process was guided by statistical and visual checks (i.e. diagnostic 'goodness of fit' plots). A model parameter or a covariate was retained in the model when including this parameter in the model resulted in a decrease of 6.63 points (χ^2-distribution, 1 degree of freedom, $P = 0.01$) in the minimum value of the objective function ($\Delta MVOF \geq 6.63$) or vice versa with backward deletion from the model. This conservative p-value (Type I error) was selected, since it is known that the NONMEM FOCE method produces only an approximation to the maximum likelihood assumptions and that the null hypothesis will be rejected more frequently than the nominal Type I error value [27].

VISUAL PREDICTIVE CHECK. The model prediction was evaluated using a Visual Predictive Check (VPC), which evaluates whether the identified model would be able to predict the observed variability for 80% of the population in the PK data that was used for model identification [28]. Therefore, the PK of each individual using its individual specific dosing history and covariate values was simulated using the individual specific values for dose and covariates. In a Monte Carlo simulation 100 data sets were simulated by drawing random samples for the PK parameters from the identified distributions for inter-individual variability, inter-occasion variability and residual variability. The distribution (median and 10^{th} and 90^{th} percentiles) of the simulated concentration-time courses was compared with the distribution of the observed values in the original data set. Differences and overlap of the simulated and original distributions indicate the accuracy of the identified model.

BOOTSTRAP. A bootstrap analysis was performed to assess the precision of the PK parameter estimates. The observed data set was re-sampled with replacement in order to generate a new data set with the same size and population characteristics, such as the number of patients per genotype, as the original set. This procedure was repeated 500 times to generate a distribution of the PK parameters with a mean and coefficient of variation as well as the median and 2.5^{th} and 97.5^{th} percentiles.

Results

Ciclosporin A PK is characterized by variable peak concentrations in the first 3 h after administration. The variability in the delayed absorption of CsA could best be described with a transit compartment, using a first-order rate constant describing the transfer from the dose compartment into the transit compartment and subsequently into the central compartment (Figure 1). Distribution and elimination of CsA could be adequately described by a two-compartment model with first-order elimination (Figure 1). Random

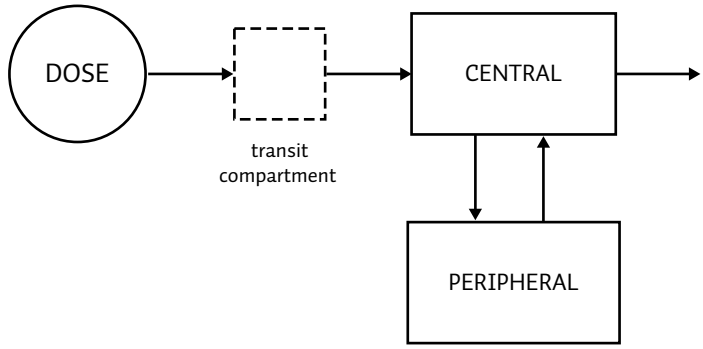

Figure 1. Linear 2-compartment model with first order absorption and elimination and a transit compartment in order to describe the variability in the absorption phase.

effects for the inter-individual variability were estimated for clearance; volume of the central compartment and the absorption rate constant.

The value of the absorption rate constant varied from 0.5 to 3.2 h^{-1}. The first-order transit rate constant is set to be equal to the absorption rate constant; thus the transit time can be calculated with $1/k_a \times (n+1)$, where n is the number of transit compartments [29]. The transit time or lag time was typically 1 h (range 0.6 to 4 hours). A concomitant prednisolone dose of 20 mg/day or higher was related to a 55% lower CsA absorption rate (ΔMVOF = +233 points, deletion from the final model, Table 4).

The range in apparent clearance (CL/F) in the population was 13-64 L/h, with a median of 32 L/h. The relationships among body weight, CsA clearance and the central volume of distribution were described allometrically and the parameters were scaled to the median body weight, i.e. $CL = 15 \times (\text{body weight}/76)^{0.75}$. The body weight range in the population was 49 to 140 kg with a median of 76 kg. Incorporating body weight into the model explained 9% of the inter-individual variability in CL/F (decrease from 26 to 17%, while it accounted for 8% (decrease from 43 to 35%) of the inter-individual variability in the volume of the central compartment (ΔMVOF = +17 when deleting the effect of body weight on both parameters from the final model). This means that, relative to the observed variability, 35% and 19% of the inter-individual variability in these parameters are explained by the covariate body weight respectively.

Inter-occasion variability was estimated for the fixed bioavailability term (Table 4) and not for the clearance because of a better model fit. The model clearly improved when accounting for a 22% lower bioavailability if a prednisolone dose of 20 mg/day or higher was co-administered (ΔMVOF = +51, deletion from final model). This accounted for 20% of the inter-occasion variability as the variability value decreased to 14%.

The model including CL/F and V/F scaled to individual body weight and the effect of concomitant prednisolone administration adequately described the CsA concentrations in time as shown by the results of the Visual Predictive Check (Figure 2), which displays the observed and predicted variability in the concentration measurements. The medians of

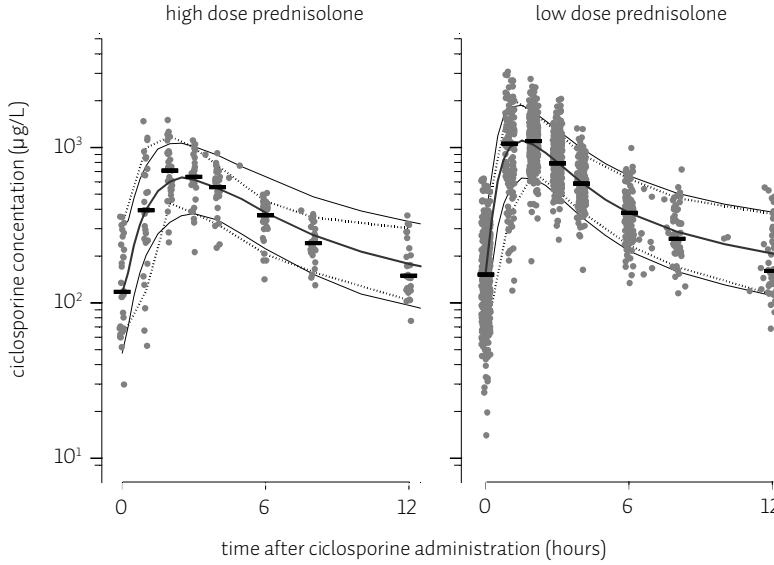

Figure 2. The visual predictive check with the 80% prediction interval (area between the *outer solid lines*). The *middle solid line* represents the median of the model prediction. The observed concentrations are shown as *closed symbols*, whereas the median of the observed concentrations per time point are shown with the *stripe* (–) symbol. The *dotted lines* indicate the 10th and 90th percentile of the observed data.

the observed and simulated CsA concentrations are similar (Figure 2); the same holds for the 80% prediction interval compared with the 10^{th} and 90^{th} percentiles of the observed data. This model was therefore considered the base model for further covariate analysis.
Besides body weight and prednisolone use in a daily dose of 20 mg or higher, none of the selected demographic, clinical chemistry or other non-genetic covariates displayed a significant relationship with apparent clearance, apparent central volume of distribution or absorption rate (ka). In addition, none of the selected genetic polymorphisms had a significant relationship with CL/F when tested on the base model. Furthermore, no relationship between haplotypes for ABCB1 genotypes and apparent clearance was found. In Table 5 the haplotype combinations and frequencies are presented.
Finally, the AUC monitoring strategy over time in this population of kidney transplant patients is depicted in Figure 3. This illustrates the procedure of adjusting the daily dose to a preset target in terms of $AUC_{0\text{-}24h}$.

Discussion

Explaining variability in CsA pharmacokinetics is important to reach target exposure early after transplantation. The current trend toward minimizing exposure to calcineurin inhibitors [1] requires insight into the sources of variability in CsA pharmacokinetics, because of an increased risk of acute rejection episodes. A multitude of factors can be responsible for the variability in the pharmacokinetics. From the literature, an array of

Table 4. Population pharmacokinetic parameters for ciclosporin A (CsA) obtained from the bootstrap of the final model. This table shows the mean and coefficient of variation of the pharmacokinetic (PK) parameter estimates as well as the median and percentiles of these estimates. The variability concerns the actual random variability in the PK parameter relative to the population mean value.

PK parameter	Mean value	Variability	CV (%)	Median	Percentiles 2.5-97.5 (%)
Absorption rate constant (k_a, h^{-1})[a]	2.0		11	2.0	1.6-2.5
DDPR ≥ 20 mg	-55%[b]		-10	-56%	-66- -42
Number of transit compartments	1				
Transit time or lag time (h)[a]	1				
CsA Clearance (L/h)	15		4	15	14-16
Central volume of distribution (V_c) (L)	56		7	57	49-64
Peripheral volume of distribution (V_p) (L)	125		10	125	100-149
Intercompartmental clearance (Q) (L/h)	14		9	14	12-16
Bioavailibility (F)	0.5				
DDPR ≥ 20 mg	-22%[b]		-13	-22%	-27- -16
IIV absorption rate	0.09	30%	31	0.09	0.04-0.16
IIV clearance	0.03	17%	24	0.03	0.02-0.05
IIV central volume of distribution	0.12	35%	40	0.11	0.05-0.24
IOV bioavailability	0.02	14%	17	0.02	0.01-0.02
Residual variability	0.07	26%	10	0.07	0.06-0.09

DDPR, daily dose prednisolone, SE, standard error, CV, coefficient of variation, IOV, inter-occasion variability, IIV, inter-individual variability. [a]Transit time with 1 transit compartment is equal to: $1/k_a \times 2$. [b]These numbers mean a 55% lower value for the absorption rate constant and a 22% lower value for CsA bioavailability.

Table 5. ABCB1 haplotype table, which contains respectively the SNPs ABCB1 T3435C (rs1045642), C1236T (rs1128503), and G2677T (rs2032582). This table shows the frequencies of the haplotype combinations on the left side, while on the right side the frequency of the individual triplets among the total amount of 60 triplets, 2 loci (of 3 alleles) × 30 individuals, is presented.

Haplotype (*n* = 30)			Total triplet (*n* = 60)	
HAP1	HAP2	*n* per group	Haplotype frequency (%)	
TTT	CCG	10	CCG	0.45
CCG	CCG	7	TTT	0.38
TTT	TTT	6	TCG	0.10
TTT	TCG	2	CTT	0.03
CTT	CCG	1	CTG	0.03
CCG	CTG	1	CCT	0.01
CTT	TTT	1		
TCG	TCG	1		
CCG	TCG	1		

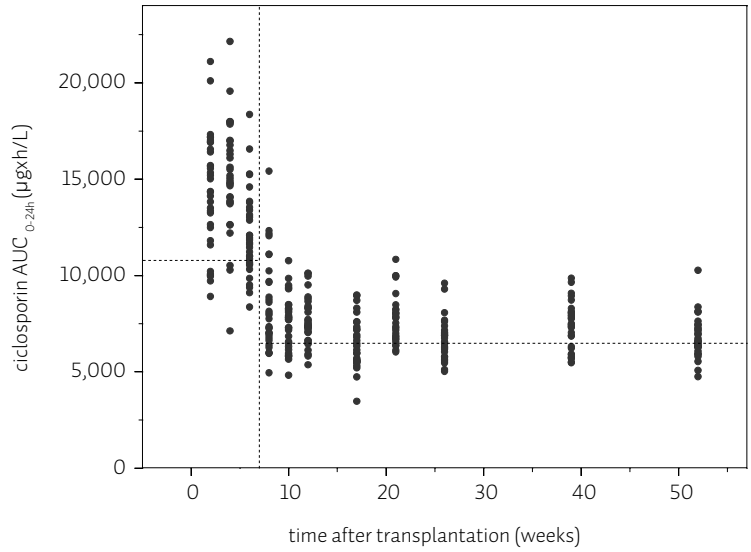

Figure 3. $AUC_{0\text{-}24h}$ vs time post-transplantation for every patient included in the analysis. Target exposure is represented by the *dotted lines*. After week 6 target exposure of ciclosporin A was minimized from $AUC_{0\text{-}24h}$ 10,800 to 6,500 µg × h/L. Week 2 should be regarded as a grouping variable with the first AUC measurement as early as 5 days post-transplantation and a median of 9 days post-transplantation. *AUC* area under the blood concentration *versus* time curve.

non-genetic biological and lifestyle-related factors were selected, including age, body size, gender, food intake [3], serum albumin concentration, hematocrit and lipoproteins (HDL, LDL) [6,7], and co-administration of interacting drugs [3,8-11] known to affect CsA PK. Moreover, a number of genetic variants in genes encoding for the involved drug-metabolizing enzymes CYP3A4 and CYP3A5 and the multidrug resistance transporter ABCB1 [13,17] were selected. Studies associating single genetic variants in these genes, not considering other non-genetic factors, have been performed, but show conflicting results [22,30]. With respect to PXR, there are no studies published that explore the relationship of genetic variants in this gene with CsA PK parameters in adult renal transplant recipients.

This leads to an integrated or population analysis combining genetic and non-genetic factors. A total of seven genetic polymorphisms in *CYP3A4*, *CYP3A5* and *ABCB1* enzymes were included in the analysis. In addition, a relationship between the five selected genetic polymorphisms in the pregnane X receptor could not be related to CsA PK. Interestingly, two covariates did appear to be relevant for individualizing therapy, body weight, and prednisolone dose. In our analysis body weight explained 35% of the variability in CsA clearance between patients, while a prednisolone dose of over 20 mg/day explained 20% of the within-patient variability in apparent clearance. After taking the relevant covariates into account, 17% of inter-individual variability in clearance remained unexplained (Table 4).

Earlier studies have been inconclusive with regard to the relationship between CsA exposure and genetic polymorphisms in CYP3A4 and P-glycoprotein (*ABCB1/MDR1*) [18-21,31].

In contrast to the current study, these studies mostly used dose-adjusted trough concentrations as a measure of drug exposure. It is known that trough concentrations correlate poorly with exposure in terms of AUC [4]. In our study full PK profiles were obtained, which made it possible to accurately estimate AUC and hence apparent clearance. In addition, the PK parameter clearance obtained with a population model is very sensitive to detecting a covariate effect (i.e. genotype effect) as one can account for the contributions of other covariates in the analysis. The population analysis methodology used in the present study differentiates between structural variability (within an individual) and random variability (between individuals). In contrast to non-population-based approaches this results in greater statistical power to identify a covariate effect, because two sources of information are used instead of one. When analyzing multiple observations per subject one is able to compensate for the small number of individuals. Still, this study was not designed primarily to identify genotype effects. Therefore, we performed a posterior power calculation to estimate the minimum genotype effect that could be identified with a power of 80% and 95% confidence [32]. This was done for genotypes with a frequency of 10, 20 and 30% based on Table 3. For a genotype frequency of 10, 20 or 30% the minimum genotype effect that could be detected with a power of 80% was 55%, 35% or 32% respectively. This raises the question whether the genotype effects that could not be identified with a power of 80% (e.g., the effect of 30% with a genotype frequency of 20%) would be clinically relevant early after transplantation. Therefore, one should consider the dose reduction necessary to reach the target AUC, in this example within 6 weeks after transplantation. The median starting dose was 300 mg, while a median dose decrease of 58% or 125 mg CsA b.i.d. was necessary to obtain a median AUC decrease of around 2,000 μg×h/L. This demonstrates that relatively large dose steps are clinically necessary early post-transplantation, while the genotype effects appear to be small. In addition, small genotype effects will display overlapping distributions, harming the specificity of this approach when applying clinically.

In the literature, only one study was found that was set up using comparable design and data analysis; in this study, an age-related effect of *ABCB1* polymorphisms on CsA oral bioavailability was found [33]. This study was performed in 104 pediatric dialysis patients who received a single pre-transplant intravenous CsA dose and a subgroup also received an oral dose at least 1 day later. This design has the advantage that CsA bioavailability could be estimated, since intravenous and oral data were analyzed simultaneously. Both this study and the present study were comparable with regard to the amount of data analyzed as well as inclusion of body weight into the model. The discrepancy with regard to the role of *ABCB1* genetic polymorphisms may be explained by differences in study populations and co-medication. Fanta *et al.* [33] analyzed data from pediatric pre-transplant patients on dialysis who received a single CsA dose without co-administration of prednisolone. Specifically, dialysis patients have typical clinical characteristics such as the presence of a uremic intestine, which could have a marked effect on absorption and perhaps on the activity of the intestinal enzymes. Indeed, adult pre-transplant dialysis patients display highly variable oral bioavailability [34]. Moreover, co-administration of the enzyme inducer prednisolone could potentially mask a relationship between *ABCB1* and CsA clearance. Finally, the population presented in our study is a reflection of a

typical clinical transplantation cohort with patients for whom therapy with the immunosuppressant CsA is applied. Thus, from our study we can conclude that the model we presented including body weight and prednisolone dose, but without (*ABCB1*) genotype is adequate for application in an adult kidney transplant population on triple therapy including prednisolone, within the first year after transplantation.

The SNPs in the gene encoding the pregnane X receptor (PXR) were not found to be relevant for explaining variability in CsA PK. We hypothesize that the nuclear receptor could be responsible for increased CsA clearance because of induction of *CYP3A4* and *ABCB1* [35,36]. Indeed, prednisolone is able to activate *PXR,* as described previously [14], and could in theory be responsible for increased CsA clearance as observed early after transplantation. Inter-individual variability in this drug-drug interaction could potentially be related to polymorphisms in the gene coding for PXR, as hypothesized previously for tacrolimus [37]. Yet, the present analysis revealed that all patients were affected in the same manner by a decrease in CsA apparent clearance early after transplantation, which limited the possibility of finding a relationship between *PXR* genotype and CsA clearance. Therefore, the covariate prednisolone dose over 20 mg/day appeared to be sufficient to explain the decrease in apparent CsA clearance in time. In addition, the prednisolone dose was rapidly tapered from 100 mg on the day of transplantation to 10 mg once daily in the first 3 weeks post-transplantation and therefore was correlated with time post-transplantation. Specifically, in the first 2 weeks after transplantation a significantly lower bioavailability was estimated. Furthermore, this could also be the result of the improved health status of the patients shortly after transplantation attendant with an increase in CsA binding factors, such as serum albumin concentration and hematocrit. However, this analysis did not reveal a covariate effect of these markers. Although unambiguous evidence for the interaction between prednisolone and CsA is lacking [38-40], the effect on bioavailability was attributed to a prednisolone dose in this study and not to time post-transplantation. Yet, to draw conclusions on this matter, CsA PK in the absence of prednisolone should be compared. The effect of prednisolone, or the time post-transplantation effect, on CsA exposure hinders TDM, as can be seen from Figure 3. After the first AUC visit grouped as week 2, the CsA dose was reduced in the majority of patients. Interestingly, this dose reduction did not result in a lower CsA AUC at the next visit. Because the prednisolone dose was tapered at the same time, CsA clearance decreased and the AUC remained at the same level. Hence, one should account for co-administration of prednisolone when applying a TDM strategy and adjusting the dose.

As described in the materials and methods section, steady-state PK was assumed for the analysis of these data. Three factors could obscure this assumption, namely dietary fluctuations, variability in dosing intervals, and compliance issues. Patients were allowed to take a light breakfast just prior to their TDM visit in the outpatient clinic. However, most likely the evening before these patients had taken a heavy meal. This dietary variability could cause altered CsA concentrations. The same holds true for daily fluctuations in dosing interval or accidentally missing a dose. These factors cause fluctuations in PK parameters over time and were accounted for with intra-occasion variability.

This population analysis demonstrates that body weight is an important covariate, while the selected genetic polymorphisms appear to have, if any, only a non-clinically relevant

effect on CsA exposure. In contrast, for tacrolimus genetic factors were relevant for individualized dosing, whereas body weight appeared irrelevant [37]. The two calcineurin inhibitors are often bracketed together because of their grouping and interchangeable use, which suggests that they are comparable drugs. However, one should be careful when comparing these two calcineurin inhibitors as they are chemically and thus pharmacokinetically very different. Both display characteristic absorption profiles, since CsA is mainly absorbed in the upper intestine (duodenum and proximal jejunum) [41], while tacrolimus is absorbed throughout a larger part of the intestine (duodenum down to the ileum) [42]. In addition, these drugs distribute differently throughout the body and both bind to different immunophilins, FK-BP and cyclophilin [43]. In blood both drugs bind to red blood cells, albumin, and α-acid protein, but in fairly different ways and to different extents [6,44,45]. Finally, they are both metabolized in a unique pattern [3,46].
Both CsA and tacrolimus are subject to dose reduction protocols [1] which inevitably makes variability in exposure an issue for both drugs. CsA displays relatively low inter-individual variability, at least compared with TRL, in which genetic markers have been shown to be relevant for individualized dosing [18,37]. In clinical practice the variability for CsA is handled with TDM. The unexplained inter-occasion variability (14%) in apparent clearance is lower than the unexplained inter-individual variability (17%) which supports the role of TDM. The low intra-individual variability post-transplantation while on a stable prednisolone dose indicates that as soon as the patient's blood concentrations are adjusted to the target level the frequency in monitoring visits could be reduced. From that moment onward monitoring is necessary mostly during conditions, such as infection and diarrhea, and at times when potentially interactive co-medication is started.

Conclusion

Individualizing CsA treatment in adult kidney transplant recipients can be achieved by a body weight-based dosage followed by a TDM strategy. The CsA dosage should be adjusted to the decrease in apparent CsA clearance in the first weeks after transplantation, possibly as a result of tapering the concomitant prednisolone dose. It appears that the selected genetic polymorphisms in *CYP3A4*, *CYP3A5*, *ABCB1,* and *PXR* explain variability in CsA exposure insufficiently to be of clinical relevance. Genotyping for these polymorphisms will probably not lead to an improved dosing strategy for optimizing exposure early after transplantation.

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A call for advanced pharmacokinetic and pharmacodynamic monitoring to guide calcineurin inhibitor dosing in renal transplant recipients

The calcineurin inhibitors (CNIs) ciclosporin A, and later, tacrolimus, have revolutionized the results of organ transplantation, leading to current acute-rejection rates between 10-20% [1]. Despite substantial reductions in acute-rejection rates, however, late graft loss still remains a critical issue after renal transplantation, with a progressive decline in long-term graft survival [2]. The 2 major causes of graft loss are death with a functioning graft and chronic allograft nephropathy. The latter has long been considered a late-onset condition, but recent studies have shown a high prevalence of interstitial fibrosis and tubular atrophy at a median of 3 months after transplantation [3]. Protocol biopsies obtained 2 years after transplantation from grafts with stable renal function have identified previous acute-rejection episodes and acute CNI-related nephrotoxicity as the most important predictors of chronic allograft nephropathy [4]. Donor and recipient characteristics, as well as subclinical rejection (SCR), also play a role in disease progression.

SCR is found on protocol biopsy and is defined as tubulointerstitial infiltrates of the renal allograft without functional deterioration. In daily practice, renal function is evaluated by measuring the serum creatinine concentration, but for several reasons this variable is not an adequate marker of the glomerular filtration rate (GFR) [5]. The relationship between the serum creatinine concentration and the GFR is not linear, and at a certain part of the curve only a small increment in the creatinine concentration is associated with a marked decrease in the GFR. Moreover, SCR is a patchy process in which uninvolved nephrons can hyperfiltrate and thereby maintain a typical serum creatinine concentration.

Although the causal relationship and clinical consequences of SCR are still under debate, prevention of late acute rejection after empirical reduction in the CNI dose appears to be critical [6]. Recently CNI minimization has been advocated as the preferred approach early after renal transplantation [7]. Of note is that inadequate (minimal) initial dosing and

inappropriate empirical tapering increase the risk of (subclinical) acute-rejection episodes and late rejection, respectively. Early minimization strategies would benefit the most from a predictive strategy instead of the current reactive strategy of monitoring drug concentration. CNI exposure could be determined during the pre-transplantation workup, but a patient undergoing maintenance dialysis is different in many aspects from the early post-transplantation phase, including differences in serum albumin, hematocrit, and comedications. Genotyping patients on the renal transplantation waiting list has the potential to improve initial CNI dosing. A potential marker for tacrolimus exposure is a polymorphism in the gene (CYP3A5, cytochrome P450, family 3, subfamily A, polypeptide 5) that encodes the metabolic enzyme cytochrome P450 3A5. One of alleles (*1) has been associated with increased tacrolimus clearance. Additional genetic variability could originate in the *ABCB1* (ATP-binding cassette, sub-family B (MDR/TAP), member 1) gene, which encodes the efflux transporter P-glycoprotein and occurs on membranes of intestine, liver, kidney, and T cells. Variation in genes coding for the target protein calcineurin or the immunophilins may further explain the observed differences in susceptibility to CNIs [8].

We assume that drug-induced nephrotoxicity correlates best with the exposure of the amount of drug to the kidney, which in turn is determined by the area under the concentration-over-time curve (AUC) for the CNI concentration in blood. Both ciclosporin A and tacrolimus are known to have a poor and variable absorption and elimination. These characteristics, in conjunction with the narrow therapeutic window for these drugs, necessitate therapeutic drug monitoring (TDM). Although the correlation between the trough concentration (C_0) and its efficacy in preventing acute rejection or chronic nephrotoxicity is poor or nonexistent, this parameter is still used almost universally to guide CNI dosing [9]. Even when CNI C_0 concentrations are maintained within the 'therapeutic range', a large group of patients still experience either acute rejection episodes, SCR, and/or nephrotoxicity, plus BK virus-associated nephropathy [5].

In general, a considerable range in $AUC_{0\text{-}12h}$ values can be expected at a single trough concentration (Figure 1), which of course is augmented by the range of trough concentrations thought to be acceptable in clinical practice. Figure 1A shows data, generated with the AxSYM fluorescence polarization immunoassay (Abbott Laboratories), for 128 renal transplant recipients in the first year after transplantation, with multiple comparisons per patient. The correlation coefficient is 0.87, but at a target trough concentration interval of 150-250 µg/L the AUC(0-12 h) ranges from 3000 to 9000 µg×h/L, a 3-fold difference. Figure 1B shows data, generated with Abbott Laboratories' IMx microparticle enzyme immunoassay tacrolimus II (MEIA II) assay, for 33 renal transplant recipients on tacrolimus therapy. The correlation coefficient is 0.90 this time, but at a target interval of 5-10 µg/L, the AUC(0-12 h) values again shows a 3-fold range (75-225 µg×h/L). Stated another way, at an AUC(0-12 h) target of 210 µg×h/L with a 10% range, the tacrolimus trough concentration ranges from 4 to 20 µg/L, a 5-fold difference. Patients with low systemic CNI exposure compared with their trough concentrations could be expected to have an increased risk of developing acute, subclinical, and/or chronic rejection, whereas patients with a high AUC/C_0 ratio are likely to be overdosed, with an enhanced risk of opportunistic infections, lymphoproliferative disorders, and cancer [1]. Several studies have conclusively documented that estimates of systemic drug exposure in terms of the AUC, and

the absorption profile in particular, correlate better with clinical events (acute rejections, nephrotoxicity) than do trough concentrations [9]. Remarkably, AUC monitoring has not gained much popularity, largely because of inconvenience and cost considerations.

A first prerequisite to overcome the reluctance towards AUC monitoring is a simple and flexible strategy to estimate systemic drug exposure, because 'full' 12-h AUC sampling is not a realistic option in daily practice. Given that the highest between-patient variability can be identified in the first 4-h postdose, a limited sampling strategy that includes the C_0 and 1 or 2 additional samples colected within the first 4-h postdose can be used to adequately estimate systemic exposure. This so-called mini-AUC (C_0-C_2-C_3) also allows for the identification of patients with typical, low, or slow absorption profiles [10,11]. The major disadvantage of a limited sampling strategy that uses a regression equation is the imperative of accurately timing blood samples. When a sample is collected 15 min later than the predefined time point, the regression equation is no longer valid. On the other hand, population pharmacokinetics including Bayesian forecasting is a TDM tool that allows a more-variable timing of blood sampling. Prior information consisting of disease population-specific pharmacokinetic parameters (such as drug clearance and volume of distribution) and their variation is combined with patient-specific variables (e.g., body weight, hematocrit. and serum albumin concentration) to increase the likelihood of adequately predicting an individual's drug clearance--and hence exposure--achieved with a specific dose. Optimally, these techniques also inform the clinician of the next appropriate dose to maintain or reach the desired drug exposure. This approach has been validated and successfully applied in a prospective clinical trial [12]. A subsequent step to reduce costs could then be to determine the AUC on 1 or 2 occasions after transplantation and to identify the patient-specific trough concentration for follow-up in the outpatient setting. Within- and between-patient variation can be further reduced by applying superior laboratory methodologies, such as liquid chromatography-tandem mass spectroscopy (LC-MS/MS), instead of the nonspecific immunoassays [13]. AUC and patient-specific trough

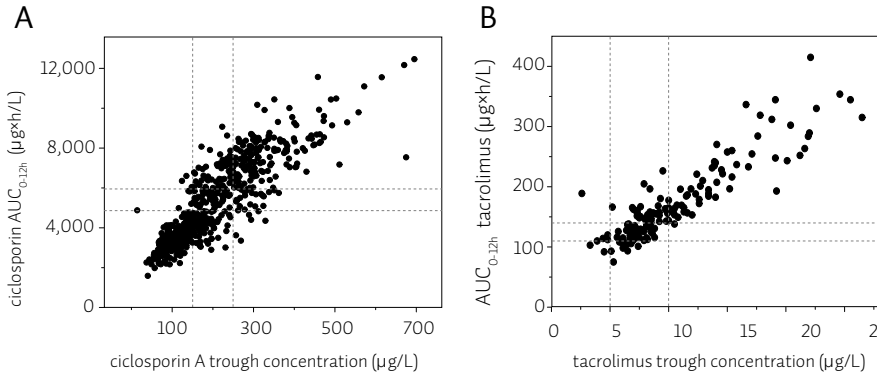

Figure 1. AUC versus trough concentration for (A) ciclosporin A and (B) tacrolimus.

concentration-guided protocols based on LC-MS/MS drug analyses could be an approach that embraces the best of both worlds, but it still needs to be validated.

In our view, population pharmacokinetics, mini-AUCs, and the Bayesian estimator constitute the preferred CNI-monitoring strategy after transplantation. Rethinking this concept, however, also requires acknowledging that it remains a rather crude way to monitor the effect of CNIs. In whole blood, the CNIs partition predominantly into erythrocytes, with the majority of the remaining molecules staying in the plasma bound to lipoproteins. Consequently, variation in these parameters markedly influences cellular distribution [14]. A straightforward approach to avoid this methodological issue could be intracellular measurement of CNI concentrations in T cells; however, for identifying patient-specific CNI susceptibility, a pharmacodynamic parameter or a response biomarker is, theoretically at least, the preferred and most accurate approach. It would bring TDM one step closer to the intracellular site of action and the corresponding individual pharmacological sensitivity at this concentration. In addition, such a marker allows a higher-level detection of pharmacologic or immunologic drug-drug interactions [15].

Calcineurin activity assays have the important advantage that they directly measure the effect of CNIs on their target enzyme. As an alternative, downstream immunologic markers, including cytokine concentrations, production of surface activation proteins, and transcripts after lymphocyte or T cell specific stimulation, have been investigated, but these markers lack selectivity for CNIs [14]. For calcineurin activity, clinical proof of concept has been demonstrated in liver and hematologic transplantation, but convincing data in renal transplantation are not yet available. A comprehensive validation is required before calcineurin activity can be used as pharmacodynamic marker in renal transplant recipients [14]. To date, these data regarding calcineurin activity are lacking, despite the fact that the first report on calcineurin activity was published more than 15 years ago.

The CNI trough concentration and serum creatinine monitoring are the current standard biomarkers to assess systemic drug exposure and renal function, respectively. Serum creatinine is a notoriously unreliable marker for GFR; changes in creatinine concentration occur late in disease progression and do not accurately represent the ongoing underlying renal damage [5]. Our point is that monitoring the trough concentration without information on the patient's absorption profile or the related systemic drug exposure is equally unreliable for guiding initial CNI dosing or for controlling systemic drug exposure while tapering. Until more sophisticated pharmacodynamic tools become available, advanced TDM with population pharmacokinetics constitutes the preferred CNI intervention strategy to optimize the long-term graft survival of the scarce organs available for transplantation.

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Is Calcineurin Activity a Useful Biomarker to Optimize Ciclosporin A Therapy in Renal Transplant Recipients?

ABSTRACT

Despite therapeutic drug monitoring of ciclosporin A (CsA) blood concentrations, renal transplant recipients still suffer from acute rejection episodes and nephrotoxicity. Insight into the individual susceptibility for CsA therapy is warranted to further individualize therapy. A biomarker such as the activity of calcineurin, the target enzyme of CsA, could potentially reflect the between patient variability in treatment response. Therefore, the pharmacokinetic-pharmacodynamic (PK-PD) relationship between CsA blood concentration and calcineurin activity was evaluated.

Renal transplant recipients ($n = 98$) were treated with CsA for 6 months after transplantation. CsA blood concentrations and calcineurin phosphatase activity in leukocytes were measured frequently and analyzed using a population PK-PD analysis.

The PK of CsA was found to be linear with delayed absorption. The change in calcineurin activity was directly related to the CsA blood concentration and the PK-PD relationship was best described with a sigmoid maximum-effect (E_{max}) model. The baseline activity (E_0) with a median value of 10 pmol/min/mg protein showed considerable within subject variability of 28%, which could be partly explained by differences in intra-cellular protein amount and assay-variability. The E_{max} was 48% of the baseline activity and the CsA potency (IC_{50}) was found to be 223 µg/L, with only a small between subject variability in E_{max} of 13%. Although a clear relationship between CsA blood concentration and calcineurin activity in leukocytes was observed in the population, differences in individual susceptibility for CsA, in terms of efficacy and potency, could not be identified, limiting the usefulness of this biomarker for the individualization of CsA dosing.

Introduction

Renal transplant recipients on ciclosporin A (CsA) therapy show high variability in treatment response reflected by the occurrence of either rejection episodes or toxicity episodes. At least part of this variability in clinical response can be related to inter-individual variability in CsA pharmacokinetics. Indeed, a relationship has been shown between CsA exposure, nephrotoxicity and acute organ rejection [1,2]. Therefore, therapeutic drug monitoring (TDM) has become essential upon renal transplantation [3]. Despite the application of this monitoring strategy on basis of blood CsA concentrations, still acute and chronic(nephro-)toxicity, acute rejection and subclinical acute rejection constitute major clinical problems. A biomarker other than drug concentration (i.e. an effect biomarker) could be useful to explain differences in clinical response between patients [4]. Specifically, the measurement of the phosphatase-activity of calcineurin, the target enzyme of CsA, has potential to serve as a basis to further individualize CsA therapy [5], as this may reflect differences in sensitivity at the pharmacodynamic level. Several methods to measure the inhibition of calcineurin have been described [6-16]. Subsequently, the pharmacodynamic relationship between CsA concentration and calcineurin activity has been evaluated [7,13,16-22]. Despite the demonstration of the relationship between CsA concentration and calcineurin activity, no population analyses describing the within- and between-patient variability in this relationship in renal transplant recipients have been published to date. The few population analyses that have been performed concerned liver transplant recipients [23-25]. These studies primarily used tacrolimus as an immunosuppressant and did neither provide information regarding baseline calcineurin activity nor convincing information on the relationship of calcineurin activity with clinical outcome. Therefore, in the present study the pharmacokinetic-pharmacodynamic (PK-PD) relationship between CsA exposure and the activity of the calcineurin enzyme was evaluated in renal transplant recipients using a population approach. The ultimate aim of the study was to determine whether this biomarker complies with the conditions for use in clinical practice. In this regard, between patient variability in the biomarker should be larger than any within patient variability. Hence, there should be clear between patient variability in PD parameters such as potency (IC_{50}) or efficacy (E_{max}) to be able to identify a relationship with clinical outcome, such as rejection episodes or nephrotoxicity.

Methods

Patients & Therapy

Renal transplant recipients ($n=98$), aged between 19 and 73 years (median age 52 years) were studied for 6 months after transplantation. Included were 61 male and 37 female recipients with a body weight range of 43 to 105 kg (median 74 kg). Only recipients of a kidney graft (first or second) from a deceased donor or a living non-HLA identical donor were included. From each patient written informed consent was obtained. The study was approved by the Medical Ethics Committee of the Leiden University Medical Center.

Patients received quadruple immunosuppressive therapy consisting of basiliximab 20 mg on the day of transplantation (day 0) and day 4, mycophenolate-sodium 720 mg twice

daily (b.i.d.), prednisolone (50 mg b.i.d. on day 0, rapidly tapered towards 10 mg daily at 3 weeks) and CsA. The initial oral CsA dose of 4 mg/kg b.i.d. was adjusted to a target $AUC_{0\text{-}12h}$ of 5400 µg×h/L in the first 6 weeks after transplantation and 3250 µg×h/L thereafter as part of a strict TDM strategy. In this context CsA concentrations were measured together with calcineurin activity in the mornings of weeks 1, 4, 8, 16 and 26. During weeks 1 and 26, a 6-hour profile of both CsA concentrations and calcineurin phosphatase activity after dose intake was obtained. On the other occasions a limited sampling strategy was applied, routinely measuring on t=0, 2 and 3 h to estimate the AUC of CsA as described by Cremers *et al.* [26] Furthermore, baseline calcineurin activity was determined prior to the transplantation.

Assays

Calcineurin phosphatase activity was determined in the leukocyte cell fraction, obtained from peripheral whole blood, with a spectrophotometric-assay based on phosphate quantification as described by Sellar *et al.* [12]. In short, erythrocyte lysis was performed by incubation of 2.5 mL of whole blood with 37.5 mL of lysis buffer (8.4 g/L NH4Cl and 1.0 g/L KHCO3, pH 7.3) for 10 minutes on ice. Leukocytes were washed twice with 10 ml Hepes-buffered saline (HBS, 9.0 g/L NaCl and 10 mM Hepes, pH 7.5) before resuspendation in 1.5 mL of HBS, cell counting and aliquotting per 2 million cells. Cell lysis was performed by addition of 200 µL of leukocyte lysis buffer (50 mM Tris-HCl pH 7.7, 1.0 mM DTT, 5.0 mM ascorbic acid, 0.02% v/v NP40 (nonionic detergent), 50 mg/L soybean trypsin inhibitor, 50 mg/L phenylmethylsulfonyl fluoride, 5.0 mg/L leupeptin, and 5.0 mg/L aprotinin) and three freeze-thaw cycles (liquid N_2/ 30°C).

The calcineurin activity assay was performed twice, one in the presence and one in the absence of phosphopeptide substrate to correct for background absorbance. The assay buffer consisted of 40 µl of 0.375 mM RII phosphopeptide substrate, 75 mM Tris (pH 7.5), 150 mM NaCl, 9 mM MgCl2, 0.75 mM DTT, 0.0375% NP40, 0.625 µM okadaic acid, 5.0 mM ascorbic acid, 0.313 µM calmodulin and 0.75 mM CaCl2. Enzymatic reactions were started by addition of 15 µl of sample lysate and run for 30 minutes. Reactions were stopped by addition of 100 µl of malachite Green reagent and color was allowed to develop for 50 min. Phosphate was quantified out of a 0-5 nmol phosphate calibration curve. A pool of recombinant calcineurin was used as assay quality control, which displayed an inter-assay variability of 10%. Calcineurin activity was expressed in two ways, as enzyme activity per million white blood cells and per mg protein. Protein concentrations of sample lysate were determined using the Coomassie Plus protein assay (Thermo Scientific, Rockford, IL, USA) CsA concentrations were determined in whole blood with a fluorescence polarization immunoassay (Abbott Laboratories, Abbott Park, IL). Assay between-day variation derived from routine measurements, were (CV): 10% (low QC, 70 mg/L), 9% (medium QC, 300 mg/L) and 10% (high QC, 600 mg/L).

Population pharmacokinetic-pharmacodynamic analysis

The PK and PD of CsA were analyzed by non-linear-mixed-effects-modelling. Mixed effects models consist of a structural model, describing the relationship between dose, concentration and effect in terms of structural parameters (i.e. *CL, V,* E_{max}, IC_{50}), and a

stochastic model, describing the random variability in the structural model parameters. As a result, individual *post hoc* estimates of parameters associated with inter-individual and inter-occasion variability could be obtained.

STRUCTURAL MODEL. The PK of CsA was fitted to linear compartmental models. The delayed absorption was described with a transit compartment, using a first-order rate constant describing the transfer from the dose compartment into the transit compartment and subsequently into the central compartment (Figure 1). Distribution and elimination were described by a two compartment model with first-order elimination. In fact, this model was used in a population PK analysis described previously by Press *et al.* [27] The data of the present study ($n=98$) were analyzed together with the rich data of the previous study ($n=33$) to obtain the most accurate PK parameter estimates in a data set containing a total of 131 individuals. In a sequential approach, the individual PK post hoc estimates were used to describe the relationship between CsA concentration and calcineurin activity in the study population ($n=98$).

The relationship between calcineurin activity and CsA blood concentration was explored for possible hysteresis. Therefore, effect compartment and turnover models were tested in case a time delay between the occurrence of the maximum concentration and the minimal calcineurin activity after dosing was identified. As such a time delay was not identi-

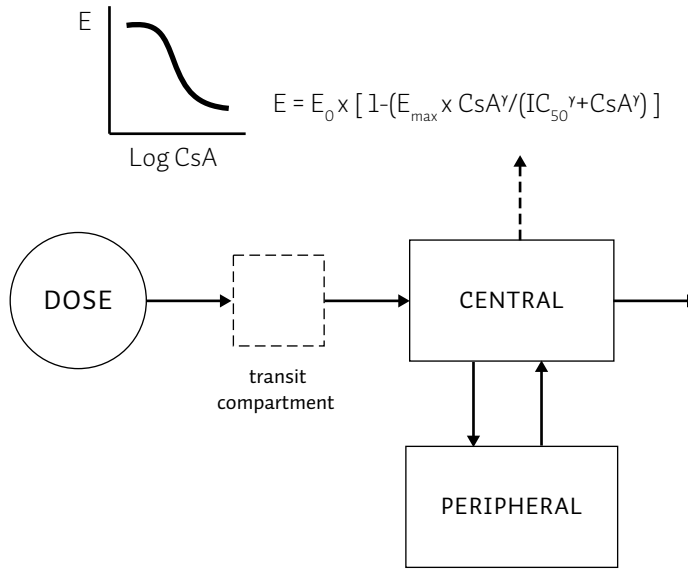

Figure 1. Pharmacokinetic-pharmacodynamic (PK-PD) model for ciclosporin A (CsA). In the model first-order rate constants describe the transfer of CsA from the dose compartment into the transit compartment and subsequently into the central compartment. Distribution and elimination were described by a two compartment model with first-order elimination. A direct effect model describes the relationship between CsA concentration and calcineurin activity in the central compartment.

fied, calcineurin activity was directly correlated to CsA blood concentrations with linear and (sigmoid) maximum effect (E_{max})-models. The sigmoid E_{max}-model is described by:

$$E = E_o \times [1 - (E_{max} \times CsA^{\gamma}) / (IC_{50}{}^{\gamma} + CsA^{\gamma})]$$

with E_o defined as the baseline activity, E_{max} as the maximum effect, IC_{50} as the concentration at the half-maximal effect, g as the Hill factor and finally CsA as the concentration of ciclosporin A in whole blood. Yet, the PD relationship was studied using absolute inhibition of calcineurin activity as a pharmacodynamic endpoint. Absolute inhibition was defined as the absolute activity obtained directly from the calcineurin activity measurements at every time point.

RANDOM EFFECTS. Inter-individual variability (IIV) and inter-occasion variability (IOV) were described assuming a log normal distribution with the following equation:

$$PPj = TVPP \times e^{\eta jPP}$$

in which *PPj* is the PK-PD or population parameter in the j^{th} individual and *ηjPP* is the difference between the individual specific parameter and the population value. TVPP is the population value of the PK or PD parameter and the difference of the logarithm between the individual value of subject *j* and the population mean (ηjPK) is normally distributed with a mean of zero and variance ω^2_{PK}. A different approach was used for E_{max} and E_o, in which case a linear term was used: $PPj = TVPP \times (1 + \eta jPP)$. The residual error was assumed to be additive to the predicted concentration:

$$Cij = Cpredij + \varepsilon ij$$

in which *cij* is the i^{th} observation for the j^{th} individual, *Cpredij* is the concentration of CsA in the blood predicted by the PK model, and *εij* (difference between *Cij* and *Cpredij*) is a normally distributed variable with mean zero and variance σ^2.

COVARIATE MODEL. A series of covariates were collected to evaluate their effect on the PK and PD. These covariates included demographics (age, bodyweight, sex) and prednisolone dose. Specifically, covariates potentially responsible for within patient variability in pharmacodynamic response were included to increase statistical power to identify between patient variability. The covariates were white blood cell fraction differentiation (monocytes, lymphocytes, granulocytes (basophil, neutrophil, eosinophil)) and intracellular protein concentration which is a reflection of these cell subsets. These covariates were selected since variability in the composition of the white blood cell sample has been shown to relate to different levels of calcineurin activity [28,29]. Moreover, covariates showing a clear visual relationship with the model parameters or random effects of these parameters were formally tested with the model. When the relationship was described allometrically (i.e. in a body weight adjusted manner) it was done in the form $PP = TVPP \times (BW / meanBW)^y$, where *BW* is the individual body weight value, *meanBW* is the body weight population mean and *y* is the allometric exponent with typically a value of 0.75 for clearance and 1 for volume of distribution [30]. Subsequently, the selected covariate relationships were evaluated by a forward

inclusion and a backward deletion procedure [31]. Including a covariate effect should result in a decrease of the minimum value of the objective function with 6.63 points, a reduction in the identified random variability and therewith in an improvement of the model fit.

COMPUTATION. NONMEM (version VI release 1.2, Icon Development Solutions, Ellicott City, Maryland, USA) [32] was used for modelling. Modelling results were analyzed using the statistical software package S-Plus® for Windows (version 6.2 Professional, Insightful Corp., Seattle, USA). A convergence criterion of 3 significant digits in the parameter estimates was used. First-order conditional estimation with interaction was used. The modelling process was guided by statistical and visual checks (i.e. diagnostic 'goodness of fit' plots). A model parameter was retained in the model when including this parameter in the model resulted in a decrease of 6.63 points in the minimum value of the objective function. Visual predictive check The model prediction was evaluated using a Visual Predictive Check (VPC), which evaluates whether the identified model would be able to predict the observed variability for 80% of the population in the PK data that was used for model identification [33]. Therefore, the PK of each individual using its individual specific dosing history and covariate values were simulated. In a Monte Carlo simulation 100 data

Table 1. Population pharmacokinetic (PK) parameters for ciclosporin A in renal transplant recipients. This table shows the mean values of the PK parameters for the present study. These parameters are identified from data of 131 renal transplant recipients, consisting of 98 individuals from the present study and 33 individuals of a previous study. The data from the previous study are presented separately for comparison.

PK parameter	Mean value this study	Variability	Mean value previous study [a]	CV (%)
absorption rate constant (k_a, h^{-1})	2.4		2	11
DDPR ≥ 20 mg [b]	-48%		-55%	-10
CsA Clearance (L/h)	15		15	4
Central volume of distribution (V_c) (L)	49		56	7
Peripheral volume of distribution (V_p) (L)	124		125	10
Intercompartmental clearance (Q) (L/h)	13		14	9
bioavailibility (F):	0.5		0.5	
DDPR ≥ 20 mg [c]	-15%		-22%	-13
IIV absorption rate	0.148	38%	0.09	31
IIV clearance	0.038	20%	0.03	24
IIV central volume of distribution	0.137	37%	0.12	40
IOV bioavailability	0.022	15%	0.02	17
residual variability	0.075	27%	0.07	10

[a] Data (n = 33) presented by Press *et al*. EJCP2010
[b] These numbers mean a 48 and 55% lower value for k_a when 20 mg prednisolon is co-administered
[c] These numbers mean a 15 and 22% lower value for F when 20 mg prednisolon is co-administered
CV = coefficient of variation, DDPR = daily dose prednisolone, IIV = inter-individual variability, IOV = inter-occasion variability

sets were simulated by drawing random samples for the PK parameters from the identified distributions for inter-individual variability, inter-occasion variability and residual variability. The distribution (median and 10th and 90th percentiles) of the simulated concentration-time courses was compared with the distribution of the observed values in the original data set. Differences and overlap of the simulated and original distributions indicate the accuracy of the identified model.

Results

CsA showed variable and delayed absorption which could be described with a transit compartment (Figure 1). Distribution and elimination were described by a two compartment model with first-order elimination. Both the structure and parameter estimates were similar to the model that has been published recently by Press *et al.* [27] (Table 1). In the analysis body weight was included as a covariate. To this end the PK parameters were allometrically scaled to bodyweight to the power of 0.75 and 1 for clearance and volume of distribution respectively. Furthermore, CsA disposition was affected by concomitant prednisolone administration, with a prednisolone dose over 20 mg/day resulting in a 15% higher apparent clearance and a 48% lower absorption rate constant. The PK data obtained from the 98 individuals from the present study are displayed in Figure 2. The final model was able to adequately predict the observed trend and variability in CsA concentrations according to the visual predictive check.
A clear concentration *versus* effect relationship was observed as shown in Figures 3 and 4. In Figure 3A the CsA concentration and calcineurin activity are displayed *versus* time after dose for the only patient that was sampled for up to 11 hours. Both the maximum

Table 2 Pharmacodynamic parameters for the relationship between CsA concentration in whole blood and calcineurin activity in leukocytes.

Parameter	Calcineurin activity	Variability	CV (%)
baseline - E_0 (pmol/min/mg protein)	10		4
> E_0 covariate - mg intracellular protein [a]	-0.0023		-16
potency - EC_{50} (µg/L)	223		10
efficacy - E_{max}	0.48		6
Hill factor	1.7		10
E_0 within patient variability	0.08	*28%*	10
E_{max} between patient variability	0.017	*13%*	42
residual variability (pmol/min/mg protein) [b]	1.18	*1.1*	11
residual variability (pmol/min/mg protein)	5.94	*2.4*	30

[a] Covariate relationship incorporated as follows: $E_0 = 10 \times [1 - 0.0023 \times (\text{protein amount} - 194)]$. [b] Residual variability adjusted. First experiments with different batch of reagents. CV = coefficient of variation.

CsA concentration and the minimum calcineurin activity are reached at approximately 1-2 hours after dosing. This indicates a direct effect of CsA, which is also demonstrated by Figure 3B which does not indicate hysteresis. Indeed, the concentration-effect relationship for the absolute inhibition could best be described with a direct-effect and a sigmoid E_{max}-model, using the CsA blood concentration. All observed calcineurin activity and CsA concentrations are presented in Figure 4 together with the profile for the typical patient in this population. Clearly, the model describes the trend in the data nicely but also a high variability in the data is observed, since for every concentration value approximately a 6-fold range in calcineurin activity can be observed.

The baseline activity (E_o), the calcineurin activity in the absence of CsA, has a median value of 10 pmol/min/mg protein (or 210 pmol/min/10^6 leukocytes), but showed considerable within subject variability of 28% (Table 2). Figure 5 shows the values of the baseline activity. These baseline values were estimated from the PK-PD analysis for each individual on every occasion. This variability in the estimated baseline value reflects the observed variability in the concentration-effect relationship within an individual. Specifically, the calcineurin activity *versus* CsA concentration curve varies randomly in time after transplantation within a patient. Unfortunately, this within patient variability in calcineurin activity was much greater than the between patient variability, which is reflected by a value of 28% for the within patient variability in baseline (Table 2). Furthermore, a maximum inhibition

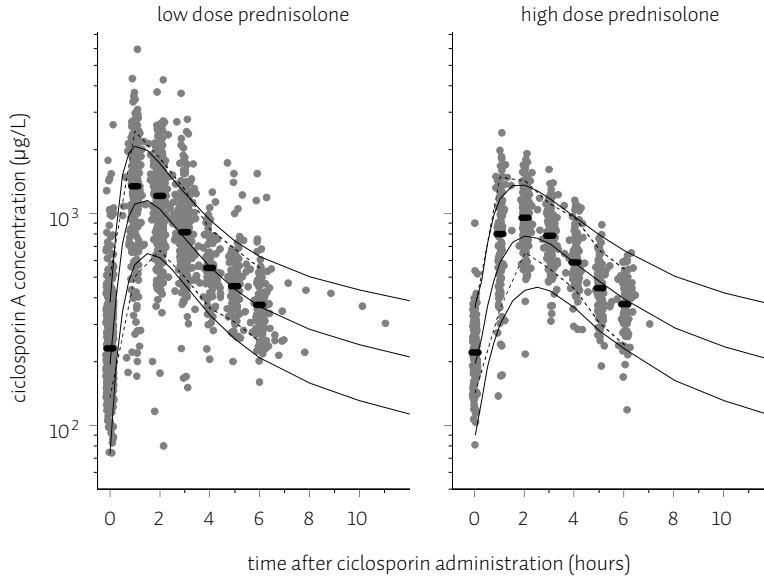

Figure 2. The visual predictive check of the pharmacokinetic model showing the 80% prediction interval (area between the outer solid lines) for the 98 included patients. The middle solid line represents the median of the model prediction. The observed concentrations are shown as closed symbols, whereas the median of observed concentrations per time point are shown with the stripe (-) symbol. The dotted lines indicate the 10th and 90th percentile of the observed data.

(E_{max}) of 48% of the baseline activity with low between patient variability of 13% was identified and an IC_{50} of 223 µg/L was estimated. These parameters remained the same when expressing activity per number of white blood cells (pmol/min/10^6 leukocytes).
The biomarker was measured in a white blood cell sample consisting of lymphocytes, granulocytes and monocytes. Each of these subsets of white blood cells is likely to have a different activity of calcineurin [29]. Furthermore, each of these cell types contains different protein amounts in the cell. The amount of intracellular protein ranged from 93 mg to 407 mg with a median of 194 mg. As described in the methods section, the covariate intracellular protein was used to explain part of the within subject variability as it reflects cell subset fluctuations. Indeed, intracellular protein was structurally related to the baseline activity (E_0). The relationship was linear with a baseline decrease of 2.3% upon a 10 mg increase in amount of protein (Table 2).

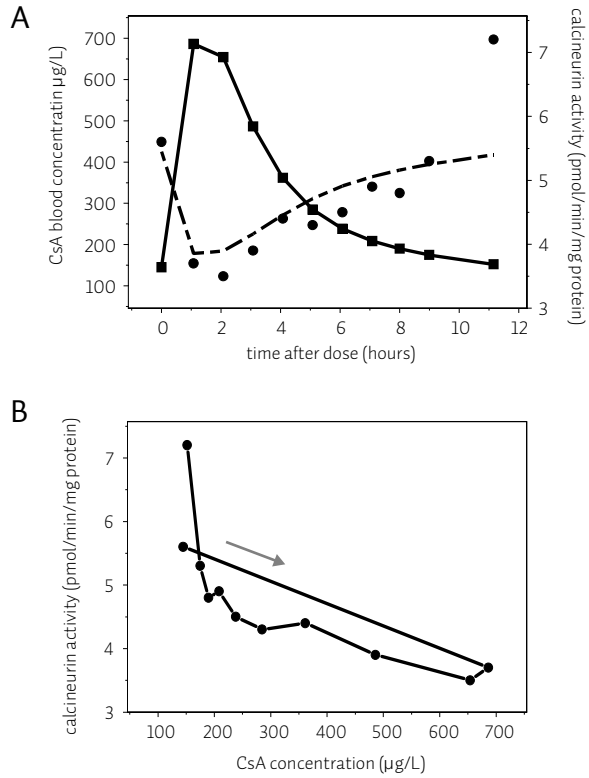

Figure 3. (**A**) CsA blood concentration and calcineurin activity profile of a single subject sampled up to 11 hours at 6 months post transplantation. The striped line represents the time course of the calcineurin activity obtained with the PK-PD model, while the dots are the actually measured calcineurin activities. The solid line represents the actual measured CsA concentrations. **B** represents the concentration *versus* calcineurin activity curve for this individual, showing that there is no significant hysteresis between blood CsA concentration and calcineurin activity in leukocytes.

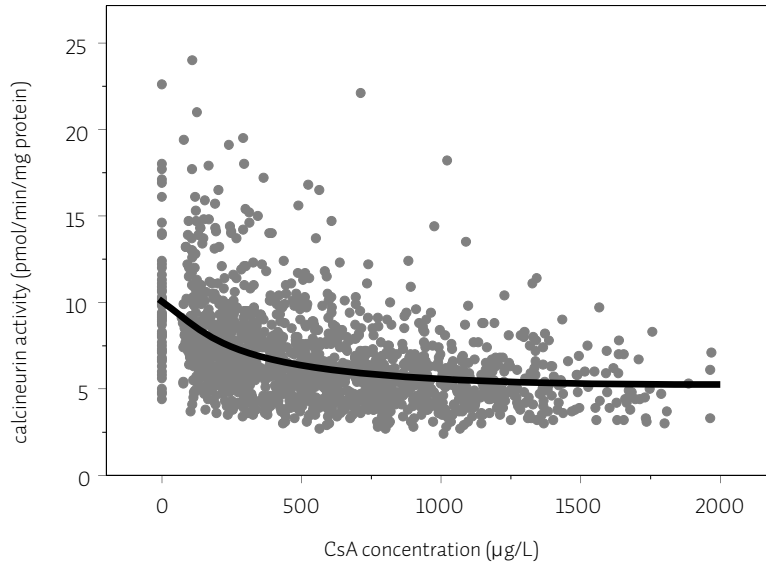

Figure 4. The relationship between calcineurin activity in leukocytes and ciclosporin A (CsA) concentration in whole blood in this study population. The closed circles are all observed CsA blood concentrations and calcineurin activity measurements of each individual on a certain time after administration and after transplantation. The black solid line reflects the calcineurin activity *versus* CsA concentration profile of the typical patient.

The large effect of the inter-occasion variability in baseline calcineurin activity could be the result of using the absolute value of the calcineurin activity in the analysis. As an approach to circumvent the difficulties resulting from the large inter-occasion variability, one could analyze the effect of the calcineurin activity relative to a baseline. Therefore, next the concentration versus effect relationship between CsA concentration and the relative calcineurin activity as well as the absolute difference in calcineurin activity was studied using the relationship: $E = E_{max} \times CsA^{\gamma} / (IC_{50}{}^{\gamma} + CsA^{\gamma})$. In these specific situations the percentage decrease or the absolute decrease in calcineurin activity is calculated at each time point after dosing relative to the calcineurin activity pre dose (trough level sample) on every occasion. However, as a considerable amount of information is lost in this manner, the parameter values could not be estimated precisely.
Finally, in this AUC-controlled population 15 acute rejection episodes were observed, while 11 biopsies demonstrated signs of subclinical acute rejection at 6 months after transplantation. Unfortunately, the low event rate in combination with the high variability in calcineurin activity within patients precluded an association analysis of calcineurin activity and clinical outcome.

Discussion

Despite therapeutic drug monitoring of ciclosporin A (CsA) blood concentrations, renal transplant recipients still suffer from acute rejection episodes and nephrotoxicity. Insight

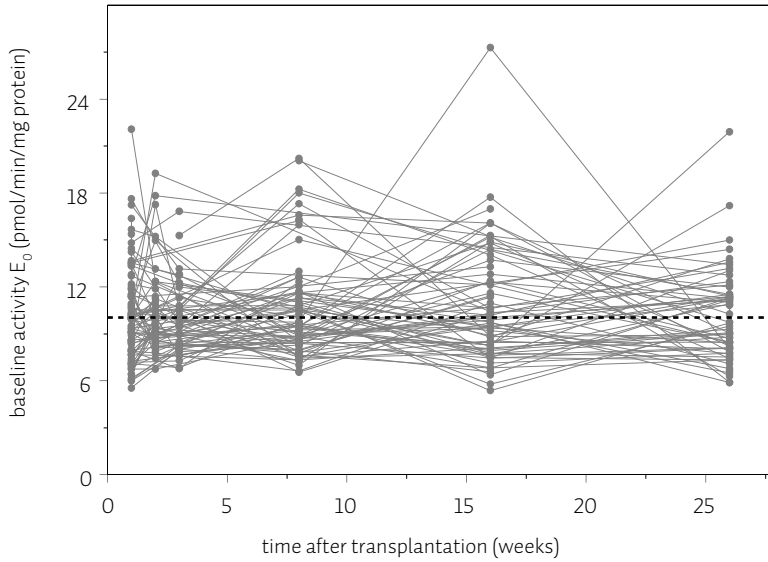

Figure 5. Baseline activity (E_0) estimated on every occasion for each individual up to 26 weeks after transplantation. Each line reflects the baseline activity pattern of a single patient. The dotted line at the baseline activity of 10 pmol/min/mg protein reflects the typical or median baseline activity in the population.

into the individual susceptibility for CsA therapy is warranted to further individualize therapy. In the present study the pharmacokinetic-pharmacodynamic (PK-PD) relationship between CsA and the activity of the calcineurin enzyme, the target enzyme of CsA, was evaluated. Therefore, in a large cohort of 98 renal transplant recipients calcineurin activity in leukocytes was determined on multiple time points after CsA administration and on multiple occasions in the first 6 months after transplantation.

In this study renal transplant recipients were treated with quadruple immunosuppression which generally leads to low acute rejection rates. The 98 patients displayed a number of 15 acute rejection episodes and 11 biopsies with signs of subclinical acute rejection. Unfortunately, this low incidence of rejections precludes an association analysis with calcineurin phosphatase activity. Such an analysis would have limited power to identify a relationship. In addition, a relatively high clinical variability of the calcineurin phosphatase assay further reduces the power to detect a relationship. Finally, conceptually it seems unreasonable to correlate the clinical effect of the quadruple therapy only to one of the immunosuppressive drugs. Therefore, we aimed to study the calcineurin activity assay prospectively on multiple occasions after transplantation with a population approach. This first step would give insight whether the biomarker complies with the conditions for use in clinical practice.

A clear concentration *versus* effect relationship between CsA and calcineurin activity was observed. In fact, the estimated IC_{50} of 223 µg/L is in agreement with previous publications [7,24], while this is only roughly the case for the E_{max}. However, the variability in

baseline calcineurin activity between occasions was greater than the inter-individual variability in efficacy (E_{max}) of CsA, while between patient variability in potency (IC_{50}) of CsA to inhibit calcineurin could not be identified. The low between patient variability in the efficacy and potency parameters hindered any correlation between these susceptibility parameters for CsA and the variability in treatment response between individuals.
The variability within individuals could in theory be the result of biological variability or analytical variability. Furthermore, the source of biological variability could originate from variability in the subset of cells present in the sample that was drawn, as has been demonstrated previously [12,28]. Indeed, this indirectly has been confirmed in the present study with intracellular protein amount as a covariate for calcineurin baseline activity. The amount of intracellular protein changes between cell types. Large cells have high protein content, whereas small cells have low protein content. Consequently, large cells (i.e. monocytes) have higher calcineurin activity than small cells. This relationship appeared small, with the largest fluctuations early after transplantation, and was therefore not sufficient to explain variability in calcineurin activity within transplant recipients. Moreover, basal activity of the calcineurin enzyme could change due to changing physiology of the transplant patient or the progression of immunological processes after transplantation, while CsA therapy itself could also be responsible for adjustments in the system. Despite these possibilities, the fact that the random within-patient variability exceeded the inter-patient variability extensively, did urge us to focus on analytical issues. Although, a recombinant calcineurin protein with a 10% inter-day variability was used as a control sample for the activity measurement in this study, a patient control sample was

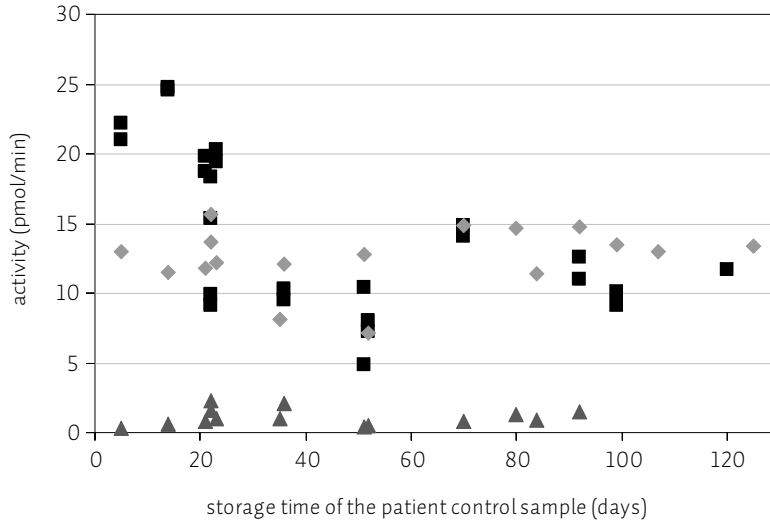

Figure 6. This figure shows duplicate calcineurin activity measurements from a *control sample*, obtained from an aliquot of *human whole blood*, plotted against the storage time in days at -20°C (closed squares). The closed diamonds represent the *recombinant calcineurin standard* used as a quality control sample in different assays. The closed triangles are *negative control samples* which consisted only of lysis buffer without enzymatic activity.

applied in the most recent experiments (Figure 6). This control was an aliquot of a leukocyte lysate sample derived from human whole blood, which was stored at -20°C prior to calcineurin measurement. This control sample revealed a large variability between assays in time (days), which was not observed during the validation of the assay as described by Sellar *et al.* [12]. Specifically, the calcineurin activity between days could vary up to 5-fold (Figure 6). Besides this variability, a decrease in calcineurin activity with storage time can be seen within the same sample, despite frozen storage. Preservation of calcineurin activity during storage was the primary reason for using a recombinant protein control sample and not a sample derived from human blood. But, when accounting for a structural decrease the activity still could vary 3-fold (Figure 6, day 30 and thereafter). Clearly, the recombinant protein standard appeared to be inadequate for quality control purpose. The source of variability is likely to be related to the presence of other constituents of the white blood cells and/or other phosphatases than the calcium/calmodulin dependent calcineurin, since that constitutes the primary difference between patient and recombinant control sample. In theory, in the assay the presence of other phosphatases was accounted for by including okadaic acid in the reaction mixture and by using chelators as EGTA to distinguish calcium-dependent from calcium-independent phosphatase activity [12]. Maybe this was not an efficient approach or other phosphatases were interfering, which could have caused the variable measurements. Another explanation could relate to the current approach which is sensitive to stability or batch differences of reagents, laboratory conditions such as temperature, preparation of the reaction mixture, etc.

To date, calcineurin activity assays do not report the use of control samples originating from patients or healthy volunteers in most cases [6,8-11,13] Studies that reported the use of some sort of control sample originating from patients or healthy volunteers [7,14,16,28,34] did not report structural use of a quality control sample. In addition, two of these reports used some kind of standardization on basis of healthy subjects or alkaline phosphatase to correct for the large variation in analytical results. Moreover, Caruso *et al.* reported the use of whole blood samples due to large intra-individual variability in calcineurin activity [7]. The applications of these assays were mostly on basis of limited data which consisted mainly of trough level measurements on a single occasion. One exception is the study by Blanchet *et al.* in liver transplant recipients [23]. The authors provide calcineurin activity data on 3 occasions post transplantation with measurements of calcineurin activity up to 9 hours after drug administration. Unfortunately, no information is provided on the CsA concentration *versus* response relationship within individuals. One can speculate from the figures in the article that they also experienced high inter-occasion variability. Moreover, only few thorough PK-PD analyses have been performed, while none of these were based on a rich data set in renal transplant recipients on CsA therapy [23-25]. The current analysis has shown that it is essential to apply a population approach on repeated measurements early in assay development to obtain a clear view on the variability in the data. In addition, the use of a patient control sample is indispensable, despite the complicating stability issues.

This population analysis confirms that calcineurin can only be inhibited for 50% and not for 100%. This is independent of the cell type, as this seems to be the case in a isolated lymphocyte subsets as well [6]. This suggests a highly complex system in which we are able

to effectively modify the immunological process with most physiological functions being unaffected. However, it is difficult to obtain the right conditions *in vitro* and to optimize all relevant factors for this biochemical pathway in a laboratory setting. This study suggests that we should thoroughly review the calcineurin assays for reproducibility and ask ourselves: what are we measuring? These assays are designed to measure calcineurin capacity rather than activity. In order to perform these measurements cell contents or enzymes have to be brought in optimal conditions and have to be stimulated to perform maximally. This may not reflect the patient condition, may not be the physiological right choice and could be difficult to reproduce. Moreover, all reported assays try to minimize the effect of other phosphatases, which may not be done successfully.
In this study the biomarker could not be used to explain differences in susceptibility for CsA between patients. Still, the measurement of calcineurin inhibition as a biomarker seems relevant from a mechanistic point of view. Therefore, further optimization of this biomarker resulting in a reduction in the within subject variability might result in a clinically relevant biomarker. This could be achieved by improving the measurement itself, the sample preparation procedure or by measuring calcineurin activity at the target site, i.e. calcineurin activity in T-cells.
Indeed, such a biomarker can be used to identify which patients are more or less susceptible to CsA therapy compared to others.

Conclusions

A clear relationship between CsA blood concentration and calcineurin activity in leukocytes was observed. However, within patient variability for calcineurin activity was higher than between patient variability. Therefore, differences in individual susceptibility for CsA, in terms of efficacy (E_{max}) and potency (IC_{50}), could not be identified, limiting the usefulness of this biomarker for the individualization of CsA dosing.

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Identifying Pharmacological Risk Factors for Subclinical Rejection in Renal Transplant Recipients on Controlled Ciclosporin Exposure

ABSTRACT

Subclinical Rejection (SCR) has been associated with interstitial fibrosis and tubular atrophy, which predisposes for renal function deterioration. Pharmacological factors (i.e. AUC, pharmacogenetics), besides immunological risk factors (i.e. a previous acute rejection), may be predictive for SCR in patients on calcineurin inhibitor based immunosuppression. Specifically, genetic variability in the genes encoding calcineurin (*PPP3CA*/*PPP3CB*) was of interest. Adult renal transplant recipients ($n=361$), receiving quadruple immunosuppression consisting of basiliximab, prednisolone, mycophenolate sodium and ciclosporin A (CsA), were followed for 6 months as part of a multicenter study. At 6 months after transplantation a scheduled biopsy was obtained and reviewed for SCR. Together with demographic and transplant related factors, CsA exposure data ($AUC_{0\text{-}12h}$) and pharmacogenetic data (variability in the genes *ABCB1*, *CYP3A5*, *CYP2C8*, *NR1I2, PPP3CA* and *PPP3CB*) were analyzed. Biopsies were obtained for 275 transplant recipients, of which 18% ($n=50$) displayed SCR. A previous acute rejection episode and a deceased donor kidney were the most important determinants for SCR, leading to a risk of 52% of SCR at 6 months (*versus* 11% average). In addition, these factors, along with female sex and carrying *ABCB1* TTT-haplotype, were related to a higher drop-out frequency (overall drop-out 24%). Genetic variability in the genes (*PPP3CA*/*PPP3CB*) coding for calcineurin were not significantly related tot SCR. It is concluded that transplant related factors are the most important determinants of SCR for this AUC-controlled population based on CsA therapy.

Introduction

Despite low acute rejection rates of 10-20% in the first year after transplantation long term outcome after renal transplantation remains poor [1]. Protocol biopsies two years after transplantation have shown high prevalence of chronic allograft nephropathy (CAN), defined by renal interstitial fibrosis and tubular atrophy (IF/TA), in calcineurin inhibitor (CNI) treated patients [2]. The causes of IF/TA are multi-factorial and determined by transplantation related factors including donor organ quality, ischemic/reperfusion injury, acute rejection, calcineurin inhibitor toxicity, and polyoma viruses (BK-virus nephropathy). Subclinical Rejection (SCR) has been associated with IF/TA in subsequent biopsies and inadequate immune suppression and/or tapering may turn out to be a key factor in the persistent or recurrent (chronic) cellular rejection, finally leading to IF/TA and progressive loss of renal function [3-5].

SCR is defined by a renal allograft biopsy with (cortical) tubulo-interstitial mononuclear cell infiltration without detectable functional renal deterioration and for the diagnosis it requires per protocol biopsies at a fixed time after transplantation. If graded according to Banff approximately two-third can be identified as borderline and the remainder as grade I rejection. The prevalence of SCR decreases over time after transplantation [4]. An important factor in the prevention of SCR is the amount of administered immunosuppression. This is illustrated by a decrease in SCR at 3 months post-transplantation from 63% in the era of ciclosporin A (CsA)/azathioprine, towards 5% with tacrolimus/mycophenolic acid for comparable groups of transplant recipients [5]. In general, immunosuppressive therapy with ciclosporin A (CsA) is associated with a higher frequency of SCR compared with the other calcineurin inhibitor tacrolimus [6-9]. Furthermore, the use and type of induction therapy also determines the risk for SCR [10,11].

Besides the immunosuppressive therapy, a prior acute rejection episode, histocompatibility, sensitization and donor age have been reported as risk factors for SCR [6,7,12,13]. The role of pharmacological factors, such as drug exposure and pharmacogenetic effects, for the occurrence of SCR is still unclear. It has previously been suggested that optimal CNI exposure may prevent SCR and progressive renal dysfunction [8]. In this context variability in the genes coding for the metabolic cytochrome enzymes (i.e. *CYP3A5)*, transporter proteins (i.e. *ABCB1),* and the nuclear factor pregnane X receptor (*NR1I2)* may be of interest. While there are no clear relationships between single nucleotide polymorphisms (SNPs) in *ABCB1* and CsA exposure [14], associations between genetic variants in *ABCB1* and graft function and graft survival have been described [15-18]. Transplant recipients on CsA therapy carrying T-allelic variants in *ABCB1* C3435T or G2677T had a 3-fold higher risk for delayed graft function and a lower glomerular filtration rate at study end [16], while *ABCB1* 2677T allele carriers had a 3-fold higher odds of developing acute rejection [17]. Furthermore, graft survival was not altered in renal transplant recipients on CsA therapy, when either these recipients or their donors were carriers of the *CYP3A5*1* allele [19], but these recipients were found to have a survival benefit [20]. A metabolic enzyme of potential relevance to CsA therapy could be CYP2C8. The *CYP2C8*3* allele was related to a higher risk of developing renal toxicity in liver transplant recipients on CNIs, predominantly

tacrolimus [21]. But, to our knowledge no pharmacogenetic risk factors for SCR have been reported for renal transplant recipients on CsA therapy.

We hypothesize that genetic variability in genes coding for calcineurin, the target enzyme of CsA, alters the susceptibility for CsA. Hence, polymorphisms in these genes could potentially be related to SCR [14]. Yet, different calcineurin isoforms exist with the alpha-subtype predominantly expressed in the kidney and the beta-subtype by immune cells (lymphocytes). These calcineurin isoforms are encoded by two different genes, *PPP3CA* and *PPP3CB* respectively. Therefore, we hypothesize that variability in these genes, and especially the *PPP3CB* gene of the recipient, may be related to SCR.

A group of 361 transplant recipients entered a run-in phase of a well defined multicenter, prospective study. These patients were on quadruple immunosuppressive therapy (basiliximab, CsA, mycophenolic acid and prednisolone) for six months and a scheduled biopsy was performed at six months after transplantation to review for SCR. For the purpose of identifying risk factors for SCR, relevant transplant related and generally accepted risk factors, such as an acute rejection episode or HLA-DR matching were combined with demographic factors and pharmacological factors. The principal aim was to identify the contribution of CsA exposure and genetic variability in the genes coding for *PPP3CA*, *PPP3CB*, *ABCB1*, *CYP3A5*, *CYP2C8* and *NR1I2* to the risk for SCR.

Patients & Methods

Study design and patient population

Renal transplant recipients (n=361) participating in a run-in phase for a multicenter, randomized prospective trial aiming to minimize immunosuppression 6 months after transplantation [22]. Patients were treated in the Academic Medical Center Amsterdam (n=137), the University Medical Center Groningen (n=126) and the Leiden University Medical Center (n=98). Patients were aged between 18 and 70 years, either received a first or second kidney graft from either a deceased or living kidney donor. The following exclusion criteria were applied: HLA-identical sibling donor, third or fourth transplant, current or historical panel reactive antibodies (> 50%), female patients unwilling to use adequate contraception during the study and a cholesterol level higher than 8.5 mmol/L despite the use of lipid lowering drugs. Medical-ethical approval was provided by the review board of all participating centers and written informed consent was obtained from each patient before study entry.

The immunosuppressive regimen up to 6 months after transplantation consisted of induction therapy with 2 doses of 20 mg basiliximab (Simulect®) intravenously before transplantation and on day 4, rapidly tapered prednisolone dose (50 mg b.i.d. intravenous tapered to daily 10 mg oral prednisolone), twice daily 720 mg mycophenolate sodium (Myfortic®) and twice daily CsA (Neoral®). CsA was initially dosed 4 mg/kg b.i.d. The dose was adjusted to a preset whole blood target Area under the blood-concentration *versus* time curve ($AUC_{0\text{-}12h}$) of 5400 µg×h/L the first 6 weeks and 3250 µg×h/L thereafter.

Therapeutic drug monitoring was performed on four visits, starting the first week after transplantation, followed by 6 weeks and 3 months after transplantation with the last one at 6 months performed at the time of biopsy, just prior to entering the randomization phase of the study. Patients were seen in the outpatient clinic in between these study visits.

To guide safe reduction of immunosuppressive drugs a protocol biopsy was performed at 6 months after transplantation which was examined for histological signs of acute rejection according to the Banff 2005 grading system. The biopsy scores used in this study were not divided into borderline changes or at least grade IA rejection. We considered this justified by the fact that these criteria are based on *for-cause* biopsies and not protocol biopsies In addition, especially for borderline changes, there are issues related to sampling error and inter-observer variability [23-25]. Furthermore creatinine is not only a poor marker for changes in renal function [26], also the definition for stable renal function in different studies was not strict and ranged from 10 to 25% difference in creatinine relative to baseline.
Patient characteristics (Table 1) considered relevant for this study were: demographics (age, body weight, length, sex), underlying disease, transplantation characteristics (donor type, deceased donation type, donor age, HLA-matching (class I-A,-B, class II-DR), cold-ischemic time, end of study reason, acute rejection episode and the time of this event after transplantation, information of the scheduled biopsy at 6 months after transplantation, serum creatinin concentration, CsA exposure and pharmacokinetic parameters and finally pharmacogenetic information.

Therapeutic drug monitoring & Pharmacokinetic modeling
To determine CsA exposure ($AUC_{0\text{-}12h}$) routine whole blood samples (EDTA-blood) were obtained from transplant candidates just before (trough) and 1, 2, 3, 4, 5 and 6 hours after drug administration on official study visits. In case the exposure was determined on other visits to the outpatient clinic, samples were drawn just before and 2 and 3 hours after drug administration. Whole blood concentrations were determined with fluorescence polarization immunoassay (Axsym®, Abbott Laboratories) in the laboratories of the three participating centers. Pharmacokinetic parameters of interest were $AUC_{0\text{-}12h}$, CsA clearance and CsA dose. Post-hoc pharmacokinetic parameters (AUC, apparent clearance) were derived using a previously published population pharmacokinetic model for CsA [27].

Pharmacogenetics
Renal transplant recipients ($n=294$) were genotyped for genetic variants in the relevant genes *PPP3CA* and *PPP3CB* and in the genes *ABCB1*, *CYP3A5*, *CYP2C8* and *NR1I2*. Primarily due to early drop-out of patients or low quality of the collected material, genetic information could not be obtained from all participants.
PPP3CA and *PPP3CB* SNPs were selected based on tagging SNPs for *PPP3CA* and *PPP3CB* haplotypes. Haploblocks in *PPP3CA* and *PPP3CB* were set using HAPMAP CEU population data covering variability in the gene area, without extra basepairs, with the haploblock definition of Gabriel *et al.* in Haploview [28]. Haploblocks were constructed for the CEU population since 86% of our population of transplant recipients is Caucausian, (Table 1). Next, it was verified whether the selected SNPs were able to reflect haplotype variability in other populations (Japanese, African-American). The SNPs that best reflected genetic variability among all ethnic groups consisted of 5 SNPs for the *PPP3CA* gene (rs13146281, rs7665292, rs2201677, rs10031159, rs13117493) and 3 SNPs for the gene *PPP3CB* (rs12644, rs12775630, rs3763679).

Table 1. Patient characteristics.

Characteristic	
number of included patients (*n*)	361
recipient age (yr)	51±13
recipient gender (% male)	63%
race (% Caucasian)	86%
diabetes at baseline (%)	42 (12%)
primary kidney disease (*n*):	
- polycystic kidney disease	78
- glomerulonephritis	62
- diabetic nephropathy	18
- hypertension	60
- focal glomerulosclerosis	15
- etiology uncertain (e.c.i.)	14
- interstitial disease	11
- urological origin	26
- other	77
cold ischemia (h) (living donor excl.)	17±5
donor age (yr)	49±13
donor type (*n*):	
- living donor, related	76
- living donor, unrelated	93
- deceased donor, heart beating	121
- deceased donor, non-heart beating	68
HLA-mismatches (*n*):	
- class 1 mismatches	1.94±1.15
- class 2 mismatches	0.84±0.63
Delayed graft function (%; living donor excl.)	28%
% patients with at least 1 AR episode	13.3
serum creatinine conc. at baseline (μmol/L)	770±277
serum creatinine conc. at week 2 (μmol/L)	246±244
serum creatinine conc. at week 6 (μmol/L)	145±62
serum creatinine conc. at month 3 (μmol/L)	138±70
serum creatinine conc. at month 6 (μmol/L)	129±39
End of study reasons (*n*):	
- patients reaching 6 month endpoint	275
- patient withdrawal	55
- Graft loss, dialysis or low renal function	19
- death	7
- other	5

In addition, four single SNPs in *ABCB1* were determined, in the promoter region T-129C (rs3213619) and a haplotype consisting of T3435C (rs1045642), C1236T (rs1128503) and G2677T (rs2032582). For the CsA metabolic pathway were determined: CYP3A5*1 (rs776746), CYP3A5*6 (rs10264272) and CYP2C8*3, the latter using 2 SNPs (rs10509681, rs11572080). Genetic variability in the nuclear factor pregnane X receptor (*NR1I2*) was based on 2 SNPs, A+7635G (rs6785049) and G-24113A (rs2276706).

DNA was isolated from EDTA-blood samples with MagNA Pure Compact DNA Isolation kit (Roche Diagnostics, Almere, The Netherlands). DNA concentrations were quantified on the nanodrop (Isogen, IJsselstein, The Netherlands). Taqman assays were obtained from Applied Biosystems (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). SNP genotyping was performed with the LightCycler 480 II Real-Time PCR System (Roche Diagnostics, Almere, The Netherlands). All assays were performed according to protocols provided by the manufacturer. Genotyping was performed in a blinded fashion, without knowledge of the clinical data. As a quality control, 10% of the patient samples and genotype assays were analyzed in duplicate. As negative controls water was used. Overall, no inconsistencies in genotypes were observed.

Genotype distributions are presented in Table 2. The success rates for all genotyping analyses were higher than 97%. Genotype frequencies for 14 of 18 SNPs were in Hardy-Weinberg equilibrium ($P > 0.05$), while *CYP3A5* rs776746, *NR1I2* rs2276706 and *PPP3CA* rs7665292, *PPP3CB* rs12644 were not in Hardy-Weinberg equilibrium. However, these genotype frequencies are similar to frequencies for Caucasians in previous reports and similar to the reported frequencies in the NCBI dbSNP database. Therefore, data were allowed for analysis.

Haplotypes in our population were set with gPLINK (http://pngu.mgh.harvard.edu/purcell/plink/), whereas no phase uncertainty in the defined haploblocks and haplotypes ($Rh^2 > 0.98$) was seen. The haploblock definition for *ABCB1* included 1236C>T, 2677G>A/T, and 3435C>T (Table 2), for *PPP3CA* gene it included rs13146281, rs7665292, rs2201677, rs10031159, rs13117493 and for *PPP3CB* rs12644, rs12775630, rs3763679 were used.

Statistical Analysis

The binary endpoint for subclinical rejection (yes/no) was analyzed with a proportional odds model. Patients that dropped-out during the first half year were included in the analysis to avoid over-prediction of subclinical rejection. The base model for patients with a biopsy at 6 months was defined by:

$$Y=(1\text{-}Pdo)\times(PSCR\times DV+(1\text{-}PSCR)\times(1\text{-}DV))$$

with *Y* being the likelihood of the model, *Pdo* the probability of dropping out and *PSCR* as the probability of SCR. The variable *DV* is a binary outcome with DV=1 if SCR is present and DV=0 otherwise. For individuals with a premature study-end (drop-out), the likelihood of the model is Y=Pdo. The model parameters were estimated by maximizing its likelihood using the Laplacian method in the nonlinear mixed effect modelling package NONMEM (version 6.2; Icon Development Solutions, Ellicott City, Maryland, USA). The

results of the model were analysed using S-Plus® for Windows (version 8.0 Professional, release 1, Insightful Corp., Seattle, USA).

Covariates may increase or decrease the occurrence of SCR. A covariate model was developed for the likelihood of SCR, using a three-step analysis. A covariate was selected on the basis of the difference in objective function (MVOF) between the base and covariate model, which approximates the χ^2-distribution with one degree of freedom. A type-I error of 5% was selected and was reflected by a difference in objective function of 3.84 points. All pre-selected covariates were evaluated one by one in the base model. The covariates with a significant decrease in MVOF were added sequentially to the base model starting with the covariate that generated the largest drop in the MVOF. Added covariates were retained in the model when the decrease in the MVOF was larger than 3.84 points.

Table 2. Haplotype and genotype frequencies in renal transplant recipients for the genes coding for calcineurin alpha (*PPP3CA*) and beta (*PPP3CB*), CYP2C8, P-glycoprotein (*ABCB1*), CYP3A5 and pregnane X receptor *(NR1I2*).

Haplotype & genotype frequency table							
PPP3CA haplotype *n* = 283		PPP3CB haplotype *n* = 289		CYP2C8 haplotype *3 *n* = 288		ABCB1 haplotype *n* = 283	
CTCCT	0.53	CAC	0.8	CT	0.89	CCG	0.43
CCACT	0.18	TAT	0.08	TC	0.1	TTT	0.37
ACACT	0.11	CTC	0.06			TCG	0.14
ACCTG	0.11	TAC	0.05			CTG	0.02
ACCTT	0.04					CTT	0.02
CTCCG	0.02						

Haplotype & genotype frequency table									
ABCB1 T-129C *n* = 292		CYP3A5 *1 *n* = 293		CYP3A5 *6 *n* = 291		NR1I2 A+7635G *n* = 294		NR1I2 G-24113A *n* = 294	
TT	0.91	GG	0.81	GG	0.99	AA	0.36	GG	0.31
TC	0.09	GA	0.16	GA	0.01	AG	0.47	GA	0.54
		AA	0.03			GG	0.17	AA	0.14

Table 3 Demographic and transplant related factors within the groups with and without biopsies displaying subclinical acute rejection (SCR).

Characteristics at 6 months	SCR Absent (n = 225)	SCR Present (n = 50)
recipient age (yr)	51±13	49±12
recipient gender (% male)	64%	78%
recipient race Caucasian (%)	87%	86%
donor age (yr)	49±13	48±14
donor > 60 yr (%)	17%	19%
HLA mismatches (n)		
class I mismatches	2.03±1.14	1.67±1.20
class II mismatches	0.84±0.65	0.86±0.58
delayed graft function (%, living donor excluded)	30%	25%
Recipients with a previous AR episode (%)	7%	18%
serum creatinin concentration (mean±sd) at baseline	754±252	865±364
serum creatinin concentration (mean±sd) at week 2	219±214	312±320
serum creatinin concentration (mean±sd) at week 6	136±53	160±44
serum creatinin concentration (mean±sd) at month 3	127±41	173±111
serum creatinin concentration (mean±sd) at month 6	118±36	149±45

Results

Patient characteristics are provided in Table 1. Initially 361 renal transplant recipients were included in the study. A biopsy could be obtained from 275 transplant recipients at 6 months after transplantation, which provides a drop-out percentage of 24%. Subclinical acute rejection was observed in 18% (n=50) of the biopsies. When considering the demographic and transplant related factors, occurrence of SCR was likely to differ with male sex and the experience of a previous acute rejection episode (Table 2).

Patients were genotyped for the polymorphisms in genes encoding the cytochrome P450 3A5 and 2C8 enzymes, P-glycoprotein and the calcineurin protein. Haplotypes and genotypes are provided in Table 3. Besides pharmacogenetic factors, exposure is a potential important pharmacological risk factor for SCR as well. CsA exposure was monitored throughout the study period and the change in AUC over time after transplantation is presented in Figure 1. Exposure was found to be higher than the target value in the first 6 weeks for the majority of the transplant recipients. After 6 weeks, when exposure to CsA was minimized, the CsA AUC was kept within a range of roughly 2000-4500 µg×h/L (target 3250 µg×h/L) for most patients.

The statistical model was used to quantify the results. Transplantation, immunological, demographic and pharmacological covariates were tested. Covariates univariately related

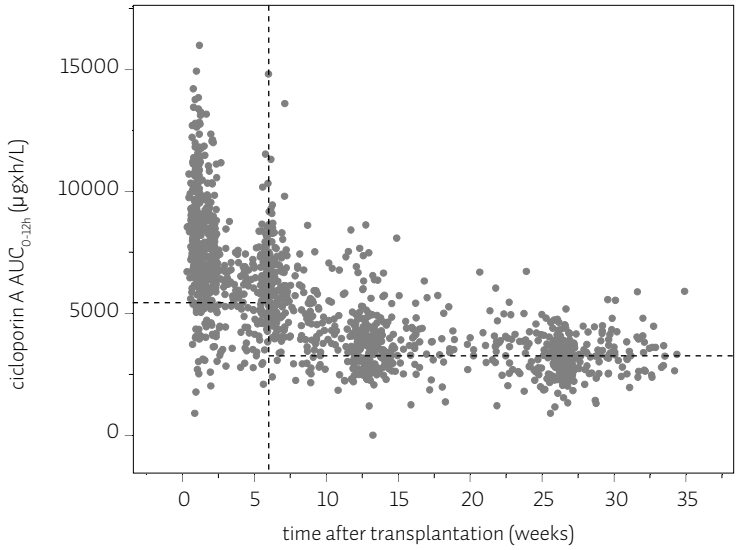

Figure 1 $AUC_{0\text{-}12h}$ in time after transplantation. Target AUC (horizontal striped lines) was 5400 µg × h/L up to 6 weeks after transplantation and 3250 µg × h/L thereafter.

to an increased incidence of SCR were identified (Table 4). No pharmacological factors were found to be related to SCR. In order of magnitude, with the most significant covariate first: a previous acute rejection episode and receiving a deceased donor. The experience of a previous acute rejection episode increased the incidence of SCR to 42% *versus* 16% in case the patient did not suffer from a previous acute rejection episode. Receiving a deceased donor kidney was associated with an incidence of SCR of 24%, while other living donation types displayed an incidence of 13%.

When including drop-out information, in the context that every patient was intended to be treated, factors could be identified related to an increased risk of dropping-out of the study. The covariates that were found to be related to an increased risk of dropping-out were a previous acute rejection episode, a deceased donor, female sex and the *ABCB1* TTT-haplotype (Table 4). In case patients did not carry a TTT-haplotype they had the lowest percentage of drop-outs with 11% and 21% of patients dropping-out without or with TTT-alleles respectively.

In a next step, these factors were combined in a multivariate approach (Table 5). The highest risk category for SCR was identified with the final model, constituting a SCR-incidence of 52% for a deceased donor kidney for whom the recipients were treated for an acute rejection episode previously. In contrast, living donation types in the absence of a previous acute rejection showed a SCR incidence of only 11%.

These results hold true for the situation that a transplant recipient reached the 6 month biopsy. However during the study period patients dropped-out and transplant recipients with a previous acute rejection episode, which received a deceased donor kidney had the highest percentage of drop-outs of 70%, while 22% of patient dropped-out with living do-

nated kidneys of which the recipient did not experience a rejection episode. When splitting this last result for sex as a risk factor for dropping-out of the study, female recipients displayed a drop-out rate of 70% instead of 48% for males in case of a deceased donation and a previous acute rejection. For the living donations, without a previous acute rejection, that would be a drop-out rate of 10% (males) versus 22% (females).
Of note, in the multivariate analysis the *ABCB1* TTT-haplotype was deliberately left out due to the small effect (P = 0.04, Table 4) on drop-out, together with the fact that the genotypes were not present for all individuals.
Kidneys originating from deceased and living donors differ in quality and considering the higher SCR risk profile for deceased donations, one could argue to analyze these populations separately. In that case the outcome as presented above remains the same, considering the fact that no other covariates could be identified. However, factors that have already been identified displayed a different effect between the groups. Only female sex was related to a higher drop-out (40% *versus* 22%) for deceased donations. In contrast, a previous acute rejection only was a significant risk factor (incidence 38 *versus* 10%) in living donations and was found to be related to a higher drop-out rate (48 *versus* 12%) in that group.

Table 4. Factors with significant effects on either the incidence of subclinical acute rejection or the incidence of drop-outs.

Covariaat	Incidence of SCR	Δ OF/LL	P-value	Drop-out Frequency	Δ OF/LL [a]	P-value
BASE-model	18%			24%		
Previous acute rejection episode		-9.1	0.0026		-11.6	0.0007
if yes	42%			45%		
if no	16%			21%		
Type of Donation		-5.5	0.019		-6.4	0.0114
if deceased	24%			29%		
If living	13%			18%		
sex, *male*		N.S.		18%	-9.5	0.0021
female				33%		
ABCB1, *no copy of TTT-haplotype* [b]		N.S.		11%	-4.2	0.0404
carriers of TTT				21%		

[a] ΔOF/LL -3.84 (P = 0.05, *chi*-square test). [b] Based on a smaller data set due to missing data. N.S., not significant.

Table 5. Multivariate analysis: forward inclusion/backward deletion.

MODEL	absolute MVOF	ΔMVOF	P-value
forward inclusion			
BASE-model	652.4		
AND effect previous acute rejection on drop out	640.8	-11.6	0.0007
AND effect recipient sex on drop-out	628.7	-12.1	0.0005
AND effect previous acute rejection on SCR	619.7	-9	0.0027
AND effect donation type on drop-out	612.1	-7.6	0.0058
AND effect donation type on SCR	606.5	-5.6	0.018
backward deletion			
FINAL-model	606.5		
MINUS effect donation type on SCR	612.1	5.6	0.018
MINUS effect donation type on drop-out	619.7	7.6	0.0058
MINUS effect previous acute rejection on SCR	628.7	9	0.0027
MINUS effect previous acute rejection on drop out	642.9	14.2	0.0002
MINUS effect recipient sex on drop-out	652.4	9.5	0.0021

MVOF, minimum value of the objective function; SCR, subclinical rejection.

Discussion

This analysis on a large group of transplant recipients participating in a run-in phase of a multicenter, randomized clinical trial on quadruple therapy with basiliximab, prednisolone, mycophenolate sodium and CsA, aimed to identify pharmacological risk factors for SCR 6 months after renal transplantation. Especially, variability in CsA exposure and/or genetic variability in genes encoding calcineurin, P-glycoprotein and CYP3A5 were of interest. The incidence of SCR at 6 months was found to be 18%. Pharmacological factors, such as exposure and genetic variability in the selected genes, were not found to be related to the risk for SCR. The most important risk factors were a previous acute rejection episode, and receiving a deceased donor kidney. These factors were associated with a high rate of dropping-out of the study as well, with the overall percentage of dropping-out being 24%. Other factors related to dropping-out were female sex and carrying a copy of the *ABCB1* TTT-haplotype. The incidence of drop-out was the lowest for patients who did not carry a copy of the *ABCB1* haplotype.

The results of this study confirm the seminal findings of Nankivell *et al.* [5], since previous acute rejection episodes were found to constitute the dominant risk factor for SCR in the present study. Incidence of SCR depends on time after transplantation and the immunosuppressive regimen [29], which complicates comparison of studies due to the different immunosuppressive regimens and transplant characteristics. But, SCR in early protocol

biopsies were previously found to be associated with HLA-matching [7,30,31], prior acute rejection episode [31], donor age [7] and donor source [13,30]. Although CsA exposure was not related to the incidence of SCR at 6 months, it is relevant to note that the present analysis was performed on a CsA exposure (AUC) controlled population of transplant recipients. Exposure was high short after transplantation (>5400 µg×h/L) and maintained between 2000 and 4500 µg×h/L after 3 months. This tight exposure control prevents low exposure, which may bias the relationship between exposure and SCR in this study.

This is the first report on the genetic variability in the genes coding for calcineurin isoforms, *PPP3CA* and *PPP3CB*. No relationship between the genetic variability in these genes and the incidence of SCR was found. In this study we determined genetic variability in two genes coding for calcineurin, the target protein of CNIs. *PPP3CB* could be primarily of relevance since this gene principally encodes calcineurin in cells of the immune system, whereas *PPP3CA* is thought to be more relevant in other tissue including renal tubular epithelial cells. Genetic variability in the gene *PPP3CA* within kidney donors would be more relevant for renal toxicity. To be able to confirm this theory we determined haploblocks in both genes, but in the present study genetic variability in *PPP3CB* was not related to SCR. The selected haplotype combination reflects the overall variability in the calcineurin gene, but may not specifically represent variability in the structure of the actual calmodulin and calcineurin binding parts, responsible for the susceptibility for CsA as hypothesized previously [14]. In addition, expression of this protein may be regulated by other (nuclear) factors.

No relationship could be identified between any of the selected genes in drug transport (*ABCB1*), metabolism (*CYP3A5, CYP2C8*) and regulation of these genes (PXR-*NR1I2*). However, carrying at least one copy of the *ABCB1* TTT-haplotype was found to be related to a 2-fold higher risk of a premature study end. This may be seen in line with previous reports [16,17] in which transplant recipients with the *ABCB1* 2677T allele triple the odds of developing BPAR and T-allelic variants predispose for a higher risk of delayed graft function and decreased renal function at study end. Possibly, the patients with a premature study-end experienced a higher frequency of adverse events (including acute rejection) due to the TTT-haplotype which is associated with low P-glycoprotein activity. But, likely this is independent from kidney survival, where donor *ABCB1* genotype may be of higher relevance [15,18]. Although a combined donor-recipient homozygosity for the C3435T variant in *ABCB1* was associated with chronic allograft damage [32], also for tacrolimus no relationship has been found between carrying the CYP3A5*1 allele and SCR, acute rejection, graft function or graft survival [33,34]. The fact that a previous acute rejection episode predisposes for SCR and a high drop-out rate suggests that, besides immunological factors,, pharmacological factors are relevant for the incidence of acute rejection. A next step should be a time-to-event analysis to identify whether the selected genetic variants and/or whole blood exposure are predictive for the 13% acute rejections in this study.

Early minimization of CsA or tacrolimus is increasingly applied an attempt to reduce toxicity and to improve long term outcome [35-37]. While it is still debated whether SCR should be treated as acute rejection episode, it is accepted that SCR constitutes a potential threat to the longevity of the transplanted kidney [6,8,12,38]. To safely taper CNI therapy within the immunosuppressive regimen after renal transplantation the risk of

acute rejection should be minimized. It is generally assumed that CNI minimization or withdrawal is safest if a protocol biopsy shows no subclinical rejection [8,12,36] and exposure to the remaining drug(s) is adequate. Rush *et al.* demonstrated less early as well as late acute rejection episodes after treating SCR in early protocol biopsies with high dose steroids [39]. Hazzan *et al.* showed that inadequate MPA exposure and SCR were independent risk factors for subsequent acute rejection after early CNI withdrawal [40]. Maintaining adequate controlled CsA exposure in a triple immunosuppressive regimen may be as effective in the prevention of late acute rejection. In this context, despite SCR in a relevant proportion of 6 month biopsies, no significant differences for renal function or severity of fibrosis in sequential biopsies were observed [8].

The integrated approach used in this study, combining demographic, transplantation information together with detailed exposure and genetic information in genes related to pharmacokinetics as well as pharmacodynamics, is very powerful to detect relationships with clinical events and identified a previous acute rejection and a kidney transplant from a deceased donor as the dominant risk factors for inflammation in 6 month protocol biopsies in patients with controlled systemic drug exposure. Although, effects of exposure and genetics could not be identified in this analysis, likely this approach can be successful identifying risks of early acute rejection or nephrotoxicity, or other forms of drug-related toxicity, in transplant recipients. Indeed, kidneys from donors carrying the *ABCB1* variant haplotype 1236T/2677T/3435T have been associated with inferior graft survival (hazard ratio 9.3) and renal function [18], while donors carrying the 3435TT genotype were associated with nephrotoxicity (odds ratio 13.4) [15]. Such a conclusive analysis should include genetic variability in the genes *ABCB1, CYP3A5, PPP3CA* of the donor.

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8

Individualized Dosing of Calcineurin Inhibitors in Renal Transplantation – General Discussion and Perspectives

Immunosuppressive therapy to prevent kidney rejection is build around calcineurin inhibitors (CNIs). As a drawback to this therapy, patients receiving CNIs have a high risk of encountering clinical toxicity. Especially acute liver-, kidney- and neuro-toxicity are complicating factors early after transplantation. On the long term chronic damage to heart and vasculature is the primary cause of patient death. Complicating factors are glucose intolerance or diabetes and dyslipidemia [1]. Overall, the primary outcome for renal transplantation remains kidney survival, which is highly influenced by chronic damage to the kidney. This has been shown to be the result of several factors of which CNI toxicity is a principal factor [2]. At this point, it is likely the leading factor for the lack of improvement in kidney survival [3-5].

Only one aspect of immunosuppressive therapy offers a promising perspective to improve graft survival, the withdrawal of calcineurin inhibitor therapy. But, CNI therapy is a prerequisite for the transplanted kidney to survive the first weeks after transplantation, which only allows withdrawal in the context of slightly lower immunological risk, thus several weeks or months post transplantation. That must be the starting point from where non-nephrotoxic alternatives should be employed [6,7].

Calcineurin inhibitor therapy has become crucial for a successful transplantation. Considering the low frequency of acute rejection episodes, apparently the risk for rejection has become less important. This is the direct result of optimal immune-modulation with triple or quadruple therapy. The quadruple approach typically consists of induction with an IL2-blocker (i.e. basiliximab) and maintenance therapy with prednisolone, mycophenolic acid and a CNI. The decrease of the risk for toxicity and over-immunosuppression has become the most crucial aim in the first period after transplantation. Specifically, opportunistic infections exploit conditions with too much immunosuppression, with a

major role for cytomegalovirus (CMV), Epstein-Bar virus (EBV) and polyoma viruses [8]. The emphasis should be placed on the latter, which typically constitutes of BK-virus and culminates into a kidney deteriorating BK-nephropathy [9,10].
To battle the consequences of toxicity or over-immunosuppression, currently low dose CNI regimens have found to be of use. Specifically, the use of low dose tacrolimus has proven to be advantageous [6]. However, the average dose itself is not the only variable that drives patient or graft outcome. Patients display large between patient variability in response to CNI administration, partly resulting from variability in pharmacokinetics [11,12]. To circumvent pharmacokinetic variability, exposure of these drugs is routinely measured in whole blood [13]. In case the exposure deviates from the target exposure the dose of the drug is adjusted accordingly. Indeed, CNI whole blood exposure has found to be related to the risk of acute rejection and the risk for nephrotoxicity [14-16]. Although improvements in clinical transplantation have been achieved with individualization of the CNI dose using exposure measurements, it remains a fairly reactive approach which can result in a long interval between start of therapy and achieving target exposure [17]. This raises the important question how CNI dosing can be optimally individualized. To answer this question this thesis is build up with a tight structure, starting with the analysis of the pharmacokinetics of ciclosporin A and tacrolimus. In a next step an effect biomarker is developed and finally a study with a clinical endpoint has been performed.

Variability in pharmacokinetics of calcineurin inhibitors

The pharmacokinetics of the CNIs are very complex and characterized by enormous variability. Upon oral administration these highly lipophilic drugs are absorbed to different extents down to the ileum. As soon as they reach intestinal mucosa and the absorption process starts, they are metabolized by the cytochrome P450 enzyme system in the intestine as well as the liver. Specifically the CYP3A4 and CYP3A5 enzymes are involved, which act together with the efflux pump P-glycoprotein that actively transports CNIs out of the cell. The highly variable fraction of the drug that reaches the blood circulation, the bio-available fraction, distributes within the blood primarily to the red blood cell, while the remainder binds to albumin, α-acid protein and lipoproteins. Only a very small fraction is unbound in blood. When CNIs distribute to cells within (organ) tissue, they bind to immunophillins to be able to a-specifically inhibit the target enzyme calcineurin by sterical hindrance of the active site. Finally, CNIs are metabolized to an array of metabolites which are principally eliminated with bile and only a small fraction with urine [11,12]. Since large part of CNIs distribute to the red blood cell, within the blood fraction, either the plasma/serum concentration or the unbound concentration would be of interest for pharmacokinetic or exposure analysis. Technical difficulties prevented the measurement of these fractions. Therefore the whole blood concentration was chosen as a concentration biomarker to reflect pharmacokinetic variability for CNIs. Despite the fact, that it may be a poor reflection of the drug at the site of action [18,19].
To achieve target whole blood exposure early after transplantation, factors or covariates should be identified that explain variability between or within transplant recipients. With the identification of factors that can be obtained before transplantation, these could be used to predict an individual's dose prior to transplantation. This was the focus of the

research described in the Chapters 3 and 4 of this thesis. To be able to discriminate between effects of multiple covariates on the pharmacokinetics of CNIs, a population analyses is essential. Such an analysis is typically performed with *non-linear mixed effects modeling* (NONMEM) [20]. This approach distinguishes between structural and random effects. With the structural model describing the time course of the drug's concentration using parameters such as absorption rate constant, volume of distribution and clearance. The random effects parameters are used to describe variability in these parameters between and within individuals. When variability in the pharmacokinetic parameters is adequately described, a covariate model can be applied to explain the identified variability and to distinguish the effects of several covariates. An advantage of this powerful technique is the possibility to analyze rich and sparse data together, which provides a possibility to use all available data.

The absorption, distribution and elimination of tacrolimus were mathematically described with a 2-compartment model with linear first-order absorption and first-order elimination. Variability in tacrolimus clearance between patients was explained by a polymorphism in the gene encoding the cytochrome enzyme CYP3A5. Roughly 20% of the Dutch renal transplant population carries one *1 allele coding for this metabolizing enzyme which leads to a 50% higher tacrolimus clearance (Chapter 3) [21-23]. In the current clinical practice they have a 50% lower exposure in terms of area-under-the-blood-concentration *versus* time curve (AUC). Depending on the number of *1 alleles present, recipients should be dosed 50% (one allele) to 100% (2 alleles) higher compared to carriers of the *3 allele, to minimize the time to target exposure or to prevent rejection [21,24]. African-Americans have been shown to carry the CYP3A5*1 allele in over 75% of transplant recipients, while this is the case for only 10-20% of Caucasians [25,26]. Indeed, the rejection rate is higher for African-Americans [27], which may be attributed to the *CYP3A5* genotype. However, other factors (waiting time on dialysis, socio-economic status, non-compliance and co-morbidity (diabetes, hypertension)) seem to play a (more important) role as well [27,28].

Although initially tacrolimus is dosed based on a persons body weight, as advised in the package insert of tacrolimus (Prograft®), body weight was not found to be related to drug clearance in chapter 3. When a strict body weight based dosing regimen would be applied this could lead to severe tacrolimus under- and overexposure for patients with low and high body weight, respectively. Furthermore, co-administration of prednisolone in a dose of 10 mg or higher was found to increase apparent tacrolimus clearance with 15%, explaining variability in tacrolimus exposure within patients (Chapter 3).

Similar to the model for tacrolimus, ciclosporin A disposition was described with a 2 compartment distribution and elimination model. But, this time a delayed absorption was identified which was described with a transit compartment between the dose compartment and the central compartment. In contrast to tacrolimus, ciclosporin A clearance did depend on a patient's body weight. Also ciclosporin A apparent clearance was 22% higher with a concomitant prednisolone dose greater than 20 mg, which explained variability within the transplant recipient (Chapter 4).

Drug interactions are responsible in large part for patient variability in drug exposure and/or response to CNIs [12,29-31]. Prednisolone is often administered concomitantly in

varying doses in transplant medicine. In most cases high intravenous induction doses are used around transplantation, which are rapidly converted or tapered to low dose oral regimens. Prednisolone is known to induce the metabolism of tacrolimus, while it is less obvious for ciclosporin A [32-34]. Rapid tapering of prednisolone early after transplantation could decrease the inductive effect of prednisolone on tacrolimus metabolism with increasing exposure as a result. Often researchers have dedicated this to an independent factor 'time after transplantation' [12,35-37]. This factor comprises all physiological changes in a transplant recipient early after transplantation and not solely the decrease in steroids. Typically, it is assumed that an interaction works the same way for everyone within a patient group. Generally, the concept of drug-interaction was thought to be solely a drug-drug effect, disregarding the role of the host. Naturally, patients do differ in their susceptibility for an interaction. For prednisolone it was hypothesized that the interaction was the result of activating the nuclear factor pregnane X receptor (PXR). Normally, glucocorticoids regulate gene expression by activating the high affinity, low capacity glucocorticoid receptor. In the situation of high endogenous cortisol (i.e. stress) this receptor is saturated and glucocorticoids bind to the low affinity, high capacity PXR to induce its own metabolism and transport which at that time is not sufficient. Subsequently, CYP3A enzymes are induced to increase the metabolism of glucocorticoids [38]. The same is likely to occur for administration of exogenous prednisolone, which at the same time would increase the metabolism of CNIs as well. Genetic variability in *NR1I2*, the gene coding for the pregnane X receptor could then be responsible for differences in susceptibility for this inductive effect between transplant recipients. These differences could result in variability in exposure to CNIs during concomitant administration of prednisolone. A polymorphism in *NR1I2* was associated with increased tacrolimus clearance, but did not explain differences in susceptibility for the interaction between tacrolimus and steroids. Neither was a relationship found between ciclosporin pharmacokinetics and genetic variability in *NR1I2* (Chapter 3 and 4).

With this in mind, the best tacrolimus dosing strategy in current clinical practice would be a genotype based fixed dose, for instance 5 mg for carriers of CYP3A5 *3/*3 genotype and 8 mg for patients carrying a single *1 allele. In case a transplant recipient is a homozygous CYP3A5*1 carrier even higher doses would be necessary. Yet, one should be cautious with these high tacrolimus doses for two reasons. First, whole blood target exposure of the parent drug is likely to be attained early with this approach, but this coincides with a relatively high exposure to tacrolimus metabolites. At least 8 metabolites have been identified of which one has demonstrated pharmacological activity *in vitro* [39-43]. Although no relationships of these metabolites with clinical toxicity have been reported, it is advised that clinicians carefully observe these CYP3A5*1 carriers during clinical follow-up. An alternative approach could be switching homozygous patients with CYP3A5*1/*1 genotype, or possibly *1 allele carriers in general, to ciclosporin A. An additional third, but non clinical, reason is related to (lower) costs, with the higher costs of a double tacrolimus dose being replaced by a standard ciclosporin dose.

Whereas CYP3A5*1 is related to high tacrolimus clearance, predictive factors for low tacrolimus clearance, hence high drug exposure, early after transplantation remain unidentified. A single nucleotide polymorphism in the promoter region of *ABCB1*, T-129C was only weakly

associated with low tacrolimus clearance (Chapter 3). With the absence of strong predictors for overexposure one should use an alternative approach to prevent this from occurring. Using tacrolimus as an example, excessive exposure such as can be seen in Figure 1 should be prevented. To prevent tacrolimus overexposure shortly after transplantation currently in clinical practice, an early trough concentration measurement should be performed at day 2, after 3 or 4 tacrolimus administrations. When considering a target tacrolimus exposure of 160 µg×h/L the first 6 weeks after transplantation, which is about to be used in our center, trough concentrations greater than 15 µg/L should result in pre-emptive dose reduction (Figure 2). As can be derived from the figure 2, this trough concentration reflects an $AUC_{0\text{-}12h}$ of 175-300 µg×h/L. Subsequently, AUC-monitoring should be performed just before discharge from the hospital, which nowadays occurs 1 week after transplantation. In the weeks thereafter any gradual decrease in CNI dose should be corrected for tapering the concomitant prednisolone dose, which causes the inductive effect to fade away. Therefore, a relatively larger dose reduction is necessary to obtain target CNI exposure. This early trough concentration approach could also be useful for ciclosporin which is reasonably dosed on body weight in most cases. To detect extreme low or high exposure an early trough concentration measurement is the only marker available at this point.

In conclusion, to obtain whole blood target exposure early after transplantation a strict TDM strategy is not sufficient. The CNI starting dose should be individualized using other factors besides body weight, such as genotyping for the presence of a CYP3A5*1 allele for tacrolimus or accounting for co-administration of prednisolone.

As has been described in chapter 5 AUC-monitoring has a clear advantage over trough concentration monitoring. This is supported by the 3-5 fold difference in trough concentration at a certain AUC target value. Preferably, the monitoring approach should be kept as practical as possible. Therefore, with chronic CNI use (arbitrarily longer than 2 months, monitoring could potentially be reduced by introducing a patient specific trough concentration. In case two or more AUCs of a renal transplant recipient are obtained during follow up and when there is an acceptable relationship between trough concentration and AUC for an individual, a patient specific trough concentration can be defined. Despite large variability between AUC and trough concentration more specific analytical techniques have come available, such as LC-MS/MS [44]. With this method the accuracy and precision of the concentration measurements have increased. The combination of these factors with already a relatively low within patient variability in pharmacokinetics for CNIs, provides a promising tool for developing the concept of a patient specific trough concentration (Chapter 5).

Although the use of the concentration biomarker in whole blood has improved therapy with CNIs in renal transplantation, more precise biomarkers are required to further optimize CNI therapy. First of all a more precise concentration measurement is desired, which is able to quantify CNIs closer to the site of action, for instance within the T-lymphocyte. When concentrations are more precisely measured in for instance plasma or the T-lymphocyte it remains unclear to what extent the concentration reflects the actual unbound CNI concentration or the CNI-immunophilin complex at the site of action, the calcineurin enzyme. Besides, variability on the pharmacodynamic level may have consequences for the response to CNIs, which will be discussed in the next section.

Variability in pharmacodynamics of calcineurin inhibitors

Unfortunately, concentration measurement in whole blood is not the Holy Grail for CNI dose optimization. Blood concentration does not entail an individual's drug response solely. Variability in the exposure *versus* response relationship for CNIs (i.e. differences in potency and maximum effect) determine susceptibility as well. This currently manifests clinically when patients, at target whole blood exposure, still encounter toxicity or rejection episodes. In contrast, patients at very high or very low exposure not necessarily develop toxicity or a rejection event, respectively.

The reason for this is twofold. First, CNI concentration measurements are performed on whole blood. This has evolved over the years because CNI measurements in the routinely used plasma samples were highly variable. CNIs are primarily bound within erythrocytes and to plasma proteins. To overcome technical issues the whole blood matrix was introduced [18,19,45-47]. The whole blood concentration may be a poor reflection of the concentration at the site of action, the donor specific T-cell. In pharmacology it is believed that the free or unbound drug concentration in blood would reflect the concentration at the site of action. Probably, in most instances this is the case, but for CNIs transport enzymes on the cell membranes of lymphocytes, such as P-glycoprotein (*ABCB1*), are likely to disturb this relationship [48,49]. Therefore, attempts have been made to measure CNIs in leukocytes or T-cells using LC-MS/MS [45,50]. Concentration measurements at the site of action may be of great benefit to optimization of CNI therapy. At least it would be a more sophisticated way of defining a patient's drug exposure than the present use of measurements in whole blood. With the current approach ciclosporin and tacrolimus concentrations actually reflect the amount in the red blood cell, with around 60% and 80% being bound in these cells respectively [11,12]. Besides, the effect of CNI-metabolites should be taken into account as well. Especially, since immunoassay and LC-MS/MS techniques are both used extensively at this time and as described above they differ in metabolite interference.

The second reason constitutes the target enzyme of CNIs, calcineurin. Ciclosporin A and tacrolimus exert their drug effect by inhibition of the calcineurin activity in T-cells. Dephosporylation of the nuclear factor of activated T-cells (NFAT) is inhibited resulting in decreased gene transcription of pro-inflammatory mediators or cytokines. CNIs inhibit calcineurin by a-specific binding of CNIs next to the active site of calcineurin. Since CNIs bind to immunophilins they are able to sterically hinder the active site of calcineurin. This non-competitive way of enzyme inhibition could be variable since the susceptibility of this system is likely to vary among transplant recipients. Patients could differ in the maximum effect or E_{max}, which provides variability among transplant recipients in the maximum inhibition of calcineurin. Another parameter which could be relevant is the potency or IC_{50}. Although CNIs act by a non-competitive way of inhibition of calcineurin, patients still could differ in the potency, due to genetic variability in the genes coding for calcineurin, leading to (conformational) changes in the structure of the enzyme and possibly altered affinity of the CNI for calcineurin. In addition, differences in the activity of P-glycoprotein on T-lymphocytes could cause variability in the concentration that reaches the active [51-53].

Variability in the susceptibility for ciclosporin A between patients was tested by developing a biomarker based on inhibition of calcineurin activity (Chapter 6). A clear concentra-

tion *versus* effect relationship was observed between ciclosporin A concentration in whole blood and calcineurin activity in leukocytes obtained from 98 renal transplant recipients followed for 6 months after transplantation. However, between patient variability in the biomarker was too small (13% *inter*-individual variability in E_{max}) to explain differences in susceptibility for ciclosporin A. Interestingly, within patient variability was high, 28%, which raised concerns regarding the methodology of calcineurin activity measurements in leukocytes. Clearly, the development of a clinical useful biomarker is complex. To be able to quantify calcineurin activity *in vitro*, concessions are being made. First of all the concentration is whole blood is correlated to calcineurin activity in leukocytes. The concentration in whole blood may not represent the concentration within the leukocytes and the active site of the enzyme. Furthermore, leukocytes consist of granulocytes, monocytes and lymphocytes with different calcineurin activity, which urges more specific measurement in for instance T-lymphocytes [54]. To be able to measure calcineurin phosphatase activity, the activity of other phosphatases has to be eliminated by using okadaic acid and EGTA. There is no guarantee that this is a successful approach and that all disturbing phosphatases are ruled out. Moreover, the calcineurin activity actually is a capacity measurement where the enzyme is maximally stimulated *in vitro* to attain maximum activity. This may not be a very reproducible approach. These assumptions may not be a problem if an adequate quality control exists for these measurements. In contrast to concentration measurements where the use of a quality control sample is common and essential, this is still in development for enzyme activity measurements. Of course it is much more complex to develop an adequate quality control since frozen storage is detrimental to the activity of a control sample. Whereas researchers worldwide are working already for over 15 years on the development of calcineurin activity as a biomarker, still no breakthrough in terms of clinical relationships has been reported. The study presented in Chapter 6 was the first to report on calcineurin activity in a population approach using data from multiple occasions. This provided insight in the behavior of this biomarker in transplant recipients in time, and explains the current lack of information on the association between *in vitro* enzyme activity and acute rejection or other clinical outcome measures. In fact, the high within patient variability presented in chapter 6 allows questioning previous reports on the relationship between calcineurin activity on a single time point with either nephrotoxicity/acute rejection after liver transplantation [55] or graft versus host disease after bone marrow transplantation [56,57]. Despite the high importance of demonstrating clinical relationships, chapter 6 illustrates the complexity of (immunological) biomarker development and underlines the importance to analyze repeated measurements of the biomarker in human material and to apply a population approach. A more precise and accurate technique for calcineurin activity measurement is necessary and the development of alternative biomarkers should be explored.

(Sub-)clinical relationships with CNI exposure and pharmacogenetics

The quadruple immunosuppressive regimen that currently is used throughout the world is capable of decreasing the occurrence of acute rejection episodes to around 10% (Chapter 7). CNIs are part of this regimen and are the first choice to taper or withdraw as soon as possible after transplantation. To be able to do this safely, information should

be obtained on the risk of rejection after decreasing the level of immunosuppression. In fact individualized tapering regimes are required. In this respect adequate biomarkers are important [58]. By means of biomarkers the choice of the most effective and least toxic combination of immunosuppressive drugs, and their doses, could be determined. Cur-

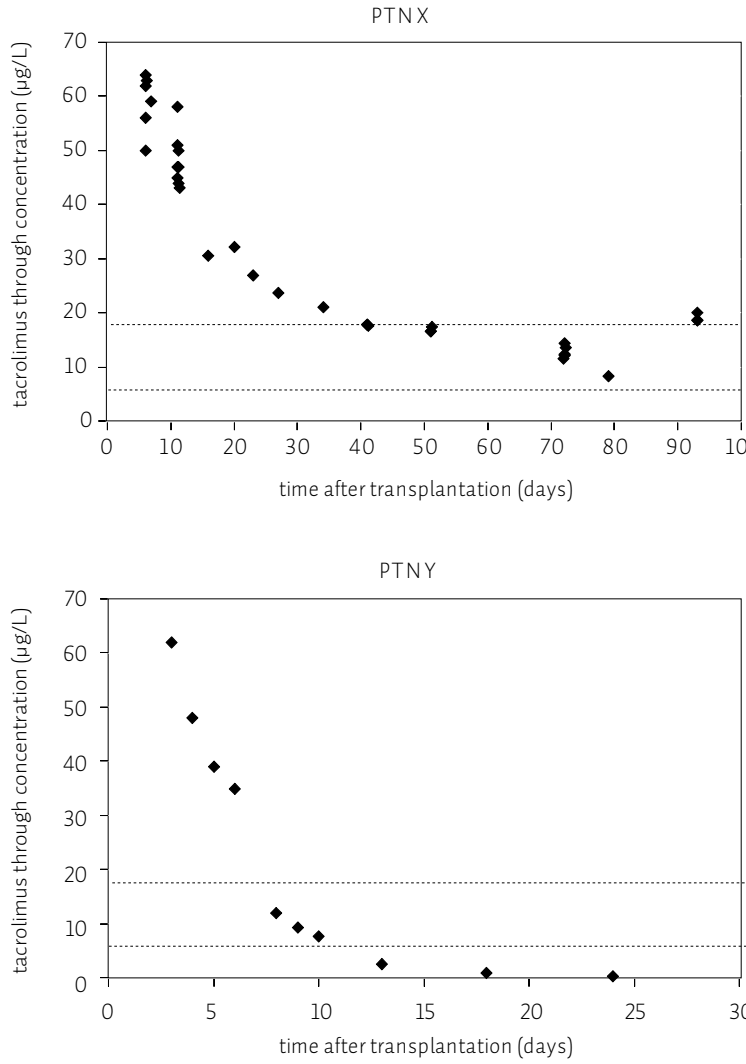

Figure 1. Tacrolimus concentration *versus* time after transplantation, for two renal transplant recipients. These figures illustrate excessive exposure for transplant recipients in the first weeks after transplantation. No interactions or other conditions were present that could explain the high exposure after an initial (lean) body weight based tacrolimus doses of 8 and 5 mg b.i.d. respectively. Target trough exposure is indicated with the solid line (between 5 and 18 µg/L). Patient X was a 34 years old male and weighed 100 kg when transplanted in 2005 and patient Y was a 67 years old female renal transplant recipient and weighed 55 kg when transplanted in 2010.

rently, for this purpose non-invasive biomarkers are not available, the only reliable marker is a renal biopsy. To safely withdraw immunosuppressive drugs a biopsy should show no signs of acute rejection, also not in the absence of functional kidney deterioration, so called subclinical rejection (SCR). But, more importantly SCR may be related to chronic damage to the kidney, so called interstitial fibrosis/tubular atrophy. Therefore insight into the factors determining SCR should be obtained and was the object of study in Chapter 7. In a multicenter study, 361 renal transplant recipients were followed for 6 months after transplantation and a renal biopsy was obtained at 6 months. Covariates were selected that could theoretically be related to this outcome measure and concerned besides demographic (age) and transplant related factors (donor information, HLA-matching, transplant type), exposure data ($AUC_{0\text{-}12h}$) and pharmacogenetic information. Of interest were variability in genes (possibly) related to metabolism and transport of ciclosporin A: *ABCB1*, *CYP3A5*, *CYP2C8*, *NR1I2*.

Besides, genetic variability in the genes encoding the target protein calcineurin were of interest as well. Three isoforms for calcineurin have been described: alpha, beta en gamma [59,60]. There is evidence that the calcineurin alpha form, coded for by the *PPP3CA* gene, is highly expressed in renal tubular cells, while the beta form coded for by *PPP3CB* is primarily expressed in immune cells (lymphocytes). *PPP3CC,* coding for the gamma variant is predominantly in the testis. The clinical relevance of these different isoforms as determinants of inter-individual variation in immune suppression has not been demonstrated yet, but is illustrated by genetic differences between renal transplant recipient and donor. Whereas the kidney originates from the donor with its genetic constitution of the *PPP3CA* gene, the immune system consequences are related to the recipient with is genetic code for the *PPP3CB* gene. To test this hypothesis genetic variability in the *PPP3CB* gene in renal transplant recipients was studied in Chapter 7 by selecting polymorphisms to create a haploblock. A haploblock consisting of 3 polymorphisms was found to reflect genetic variability in the *PPP3CB* gene. To check the assumption regarding the isoforms and variable tissue distribution, a haploblock for the larger *PPP3CA* gene was identified as well and consisted of 5 polymorphisms.

The binary outcome measure SCR was analyzed with an integrated approach, including the number of patients that drop-out during the study and including all covariate information. A biopsy was obtained from 275 patients, of which 18% contained signs of SCR. However, only the experience of a previous acute rejection episode and receiving a cadaveric donor were related to a SCR incidence of 52% versus an incidence of 11% for living donations in the absence of an acute rejection episode. This powerful approach on an AUC targeted population did not identify genetic factors as relevant covariates for SCR. Despite the absence of relationships between the selected genetic factors and SCR, a powerful analysis tool was used. It would be too simple to relate the susceptibility for CNIs solely to genetic variability in calcineurin isoforms, what is often done for other genetic association studies, disregarding the effect of other important factors. Therefore, as reported in Chapter 7 an array of demographic, transplantation related factors and exposure measurements should be taken into account as well, a more systems pharmacology approach. But, as discussed in Chapter 2 one should also study genetic variability in immunophillins and in the nuclear factor of activated T-cells (NFAT) itself. This could be the focus in future projects.

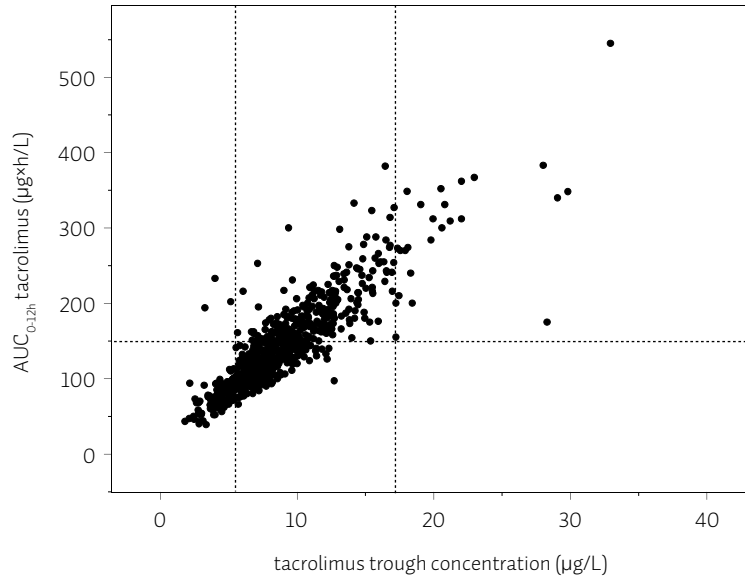

Figure 2. AUC_{0-12h} *versus* trough concentration (n = 734 data couples) for tacrolimus obtained from 343 transplant recipients. Tacrolimus concentrations were determined with LC-MS/MS in the LUMC in the period March 2009 to May 2010.

Perspectives

The one thing we need to achieve is getting the right dose of CNIs, to the right patient, at the right time. In this sentence lies the entire foundation for this thesis. It is not a new approach, but as old as Paracelsus (1493-1541) [61,62]. The attempts made and described in this thesis aimed to achieve this in renal transplantation. Especially since transplantation medicine is pre-eminently the specialism to optimize drug treatment. Balancing between the risk for acute or subclinical rejection on one side and acute toxicity, infection, malignancies, vascular damage and chronic allograft nephropathy (CAN) on the other side, is a great challenge in which important progress has been made in recent years. The biggest challenge in renal transplantation will be to prolong graft survival along with low comorbidity for the recipient.

The key to such improved outcome from a pharmacological perspective will be determined by three factors: the development of biomarkers for immunological risk and biomarkers that reflect the response to the combination of the various immunosuppressive drugs, pharmacometric analysis of the available data and enlargement of the amount of data by co-operations of transplant centers.

The initial immunological risk depends highly on an individuals transplantation characteristics and the extent to which one's immunosuppressive therapy is individualized [63,64]. Immunological risk results from transplant characteristics, such as type of transplantation, HLA-DR mismatch, cold-ischemic time, donor age etc. To attain the optimal

level of immunosuppression, therapy should be adjusted to the level of his or her individual immunologic risk. In clinical practice this is not applied universally, due to the absence of appropriate biomarkers [64]. But, in case organs are transplanted between twins or donor-acceptor couples without HLA-mismatches, immunosuppression will generally be lower compared to patients with higher immunologic risk. Besides the initial immunological risk one should also take the dynamic interplay between the immune system of the transplant recipient, the donor organ and the immunosuppressive drugs into account that develops after transplantation. A distinction between high and low risk patients may be made, ultimately resulting in chronic rejection and tolerance respectively [65]. Generally immunosuppression is stepwise reduced after transplantation towards minimal immunosuppression with two drugs or in certain instances even one immunosuppressant. In the latter cases an almost tolerant state is achieved. The ultimate goal is operational tolerance, where transplant recipients maintain graft function without using immunosuppressive drugs. Currently this is primarily observed in non-compliant patients and is still exceptional in renal transplantation [64,66-68]. In clinical practice the biomarker used to determine the possibility of minimizing immunosuppression is an invasive one, the kidney biopsy. In case no immune cell infiltrates are observed, SCR is absent, and kidney function is stable, one could decide to further reduce maintenance immunosuppression several months after transplantation. Clearly, the need for adequate biomarkers to give insight in the activity of the host's immune response against the foreign organ is warranted.

Besides tailoring the number of immunosuppressants to ones immunological risk the immunosuppressive effect is not solely determined by the dose of the different drugs. As previously discussed, between patient variability in pharmacokinetics and pharmacodynamics are responsible for this. In this respect we now placed biomarkers and finally clinical outcome central in this thesis to elucidate which dose should be administrated to which patient. Individualization of the CNI dose clearly is irrefutable, but has some big challenges to overcome. Obvious it is a complex trait to optimize treatment with 3 or 4 drugs with a different mechanism of action. A combination of biomarkers, each reflecting a drug's action, could assist clinicians. Considering the low number of rejection events with the quadruple therapy, biomarkers could be used to further reduce or individualize immunosuppression.

But, major efforts are expected from employing biomarkers in toxicity control. As soon as susceptibility differences between patients for acute and chronic CNI toxicity are explained, individualization of CNI therapy can truly be applied. With this in mind, the next year's great effort should be put into the development of biomarkers, either related to concentration of the parent compound and its metabolites (at the site of action), target enzyme activity and/or pharmacogenetics (of the target proteins) [58,69]. Emphasis should be placed on methodology of measurements in cells, especially on quality control samples. Furthermore, biomarker research in transplantation should increasingly focus on obtaining more data within the same individual. Until now often limited data obtained from single study visits were obtained.

Important development should be made in the type of analysis and patient numbers. Up to this moment in most cases renal transplant populations of up to roughly 100 patients are studied, mostly aiming at identifying a single relationship. In case multi-factorial

analyses were performed these were highly simplified. The next step would be to study the whole system defined as the donor-recipient combination with its specific treatment, a *systems pharmacology approach*. That would be a more integrative and quantitative analysis, including pharmacokinetic, pharmacodynamic or biomarker and clinical information, such as performed in Chapter 7. Here pharmacokinetic information and clinical outcome are analyzed together to identify relationships with a series of genetic and non-genetic covariates that have a relationship with either pharmacokinetics or pharmacodynamics of a single drug. Between patient and within patient variability can be described and systematically explained by covariate relationships. Including biomarkers that indicate specific activity of the immune system or the kidney as well as biomarkers reflecting the response to the different immunosuppressive drugs in the analysis, would improve this approach even more. The emphasis should be on the development of non-invasive biomarkers, observed in easily obtainable body fluids, such as blood and urine. Especially the latter could cover new ways to identify renal damage or alterations. The response to a combination of immunosuppressive drugs and/or their metabolites should be analyzed together using 3-dimensional response surfaces as has been demonstrated for anesthetic and antiviral drugs [70,71]. The theoretical concepts of the modeling of pharmacodynamic drug-drug interactions have recently been reviewed [72]. Furthermore, after transplantation the immune response alters, it adapts to the altered situation as a result of drug action. One could view that as a form of disease-progression as has been studied in for instance Alzheimer's disease [73,74]. A systems pharmacology approach [75], which should use pharmacometrics to its full extent, should be employed. Pharmacometrics concerns the comprehensive mathematical-statistical analysis of drug action. This thesis embodies this approach with the application of population analysis of pharmacokinetics, pharmacodynamics and clinical events. In future analysis these models should be extended incorporating changes in the immune system by including an immune system ('disease') progression model and 3-dimensional response surface analysis to account for interaction between the 2 or more immunosuppressive drugs that are typically used. Herewith, therapy with multiple drugs could be optimized, especially when appropriate biomarkers have become available.

Large collaborations of nephrologists should work together with clinical pharmacologists or hospital pharmacists to create large patient cohorts adding up to large databases, which can be employed to quantitatively analyze the data of multiple drugs simultaneously with a pharmacometric approach.

Clearly data collection, the development of biomarkers and models reflect an enormous amount of work and a lot of effort will have to put in to it, as we are only at the very beginning of understanding the immune system and the intervention of immunosuppressive drugs. Yet, the rate limiting step will be the development of adequate biomarkers. Although it is a magnificent challenge to describe such a complex system and to explain variability in treatment response with success not being guaranteed, this is the only successful approach to really individualize immunosuppressive therapy in renal transplantation.

Conclusions

Therapy should be optimized for drugs with a small therapeutic window and high between-patient variability in drug response, such as the calcineurin inhibitors (CNI). The initial body weight based dosed must be adjusted in renal transplant recipients to a predefined target blood-exposure to balance between acute rejection and (nephro)toxicity. With this so called therapeutic drug monitoring approach a beginning has been made to individualize CNI therapy. This thesis demonstrates that pharmacometric approaches allow us to identify factors responsible for variability in exposure and response to CNIs and to discriminate between these effects and their weight. The CYP3A5*1 allele is predictive for at least a 50% higher clearance of tacrolimus. This factor can be used to individualize the tacrolimus starting dose. Besides, the interactive effect of co-administered prednisolone should be accounted for as well. To date, AUC-monitoring combined with an early trough concentration measurement remains state of the art monitoring for CNIs in transplant medicine. This thesis demonstrates that effect biomarkers such as calcineurin activity are still in their infancy due to technical and methodological issues. Optimization and validation of these assays with human material and developing an appropriate quality control sample are essential. Furthermore, repeated measurements should be analyzed early in assay development, preferably in combination with a population approach. Finally, clinical events should be analyzed in an integrative approach as performed in the final chapter of this thesis. Especially when large data sets are analyzed with a sophisticated pharmacometric approach major developments are expected. In future analysis response biomarkers should be included that reflect the action of the combination of drugs used. When this information is combined with markers that reflect the activity of the immune system against the transplanted organ understanding of the pharmacological approach will improve substantially with true individualization of immunosuppressive therapy as a result.

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9

Summary

The research presented in this thesis aims to individualize calcineurin inhibitor (CNI) therapy in renal transplantation. Therapy with CNIs, to prevent kidney rejection, has (renal-) tissue deteriorating toxicity as a serious downside. Until other drugs are developed that prevent the use of CNIs or that allow early withdrawal, factors should be identified that provide a way to individualize the dose of the CNIs tacrolimus and ciclosporin A (CsA) in order to maintain the CNI dose as low as possible (**Chapter 1**).

Patient variability in clinical response to CsA and tacrolimus partly results from differences in CNI exposure. For tacrolimus drug interactions and genetic variability relate to tacrolimus exposure. Patients carrying the CYP3A5*1 allele have an increased tacrolimus metabolism, hence lower drug exposure. Adjusting the tacrolimus dose to this genotype is a tool to optimize therapy from a pharmacokinetic perspective. In contrast, no genetic variants have been found to clearly relate to CsA exposure. Despite therapeutic drug monitoring aimed at individualizing CNI therapy, patients still suffer from acute or chronic rejection and CNI toxicity. To further optimize CNI therapy future research may incorporate genetic polymorphisms in proteins involved in CNI pharmacodynamics (i.e. drug target). Proteins potentially relevant for drug response are calcineurin and the CNI binding proteins immunophilins. Moreover, since the expression of the nuclear factor of activated T-cells (NFAT) is reduced after calcineurin inhibition, genetic polymorphisms in the genes encoding NFAT may also be interesting candidates for studying inter-patient differences in CNI efficacy and toxicity. In addition, the existence of isoforms and differences in tissue distribution of the calcineurin protein could potentially explain variable drug response.

At present, the focus has been on the metabolism of CNIs and not on variability in the drug target. Therefore, future improvements in CNI therapy are likely to occur from a systems pharmacology approach taking into account genetic markers for both CNI pharmacokinetics and pharmacodynamics (**Chapter 2**).

To prevent acute rejection episodes it is important to reach adequate tacrolimus exposure early after kidney transplantation. With a better understanding of the high variability in the pharmacokinetics of tacrolimus the starting dose can be individualized, resulting in a reduction in dose adjustments to obtain the target exposure. A population pharmacokinetic analysis was performed to estimate the effects of demographic factors, hematocrit, serum albumin concentration, prednisolone dose, tacrolimus dose interval, polymorphisms in genes coding for ABCB1, CYP3A5, CYP3A4 and the pregnane X receptor on tacrolimus pharmacokinetics. Pharmacokinetic data were prospectively obtained in 31 *de novo* kidney transplant patients randomized to receive tacrolimus once or twice daily and subsequently, the data were analyzed by means of Non-Linear-Mixed-Effects-Modelling. Tacrolimus clearance was 1.5 fold higher for patients with the CYP3A5*1/*3 genotype compared to the CYP3A5*3/*3 genotype (5.5±0.5L/h *versus* 3.7±0.3 L/h respectively). This factor explained 30% of the inter-individual variability in apparent clearance and thus drug exposure. Also, a relationship between the pregnane X receptor A+7635G genotype and tacrolimus clearance was identified with a clearance of 3.9±0.3 L/h in the A-allele carriers *versus* 5.4±0.6L/h in the GG genotype. Finally, a concomitant prednisolone dose of more than 10 mg/day increased the tacrolimus apparent clearance by 15%. In contrast body weight was not related to tacrolimus clearance in this population. As patients are typically dosed per kg body weight this might result in under- and overexposure in patients, with a low and high body weight respectively. This integrated analysis shows that adult renal transplant recipients with the CYP3A5*1/*3 genotype require a 1.5 times higher fixed starting dose compared to CYP3A5*3/*3 in order to reach the predefined target exposure early after transplantation (**Chapter 3**).

In agreement with tacrolimus, optimal CsA exposure in kidney transplant recipients is difficult to attain because of variability in CsA pharmacokinetics. A better understanding of the variability in CsA exposure could be a good means of individualizing therapy. Specifically, genetic variability in genes involved in CsA metabolism could explain exposure differences. Therefore, chapter 4 aimed at identifying a relationship between genetic polymorphisms and the variability in CsA exposure, while accounting for non-genetic sources of variability as well. *De novo* kidney transplant patients ($n=33$) were treated with CsA for 1 year and extensive blood sampling was performed on multiple occasions throughout the year. The effects of the non-genetic covariates hematocrit, serum albumin concentration, cholesterol, demographics (i.e. bodyweight), CsA dose interval, prednisolone dose and genetic polymorphisms in genes encoding ABCB1, CYP3A4, CYP3A5, and PXR on CsA pharmacokinetics were studied using non-linear mixed effect modeling. The pharmacokinetics of CsA was described by a two-compartment disposition model with delayed absorption. Body weight was identified as the most important covariate and explained 35% of the random inter-individual variability in CsA clearance. Moreover, concurrent prednisolone use at a dosage of 20 mg/day or higher was associated with a 22% higher clearance of CsA, hence lower CsA exposure. In contrast, no considerable genotype effects (i.e. greater than 30-50%) on CsA clearance were found for the selected genes. It appears that the selected genetic markers explain variability in CsA exposure insufficiently to be of clinical relevance. Therefore, therapeutic drug monitoring is still required to op-

timize CsA exposure after administration of individualized doses based on body weight and as this study suggest, co-administration of prednisolone (**Chapter 4**).
The CNI trough concentration and serum creatinine monitoring are the current standard biomarkers to assess systemic drug exposure and renal function, respectively. Serum creatinine is a notoriously unreliable marker for GFR; changes in creatinine concentration occur late in disease progression and do not accurately represent the ongoing underlying renal damage (5). Our point is that monitoring the trough concentration without information on the patient's absorption profile or the related systemic drug exposure is equally unreliable for guiding initial CNI dosing or for controlling systemic drug exposure while tapering. Until more sophisticated pharmacodynamic tools become available, advanced TDM with population pharmacokinetics constitutes the preferred CNI intervention strategy to optimize the long-term graft survival of the scarce organs available for transplantation (**Chapter 5**).
Despite therapeutic drug monitoring of ciclosporin A (CsA) blood concentrations, renal transplant recipients still suffer from acute rejection episodes and nephrotoxicity. Insight into the individual susceptibility for CsA therapy is warranted to further individualize therapy. A biomarker such as the activity of calcineurin, the target enzyme of CsA, could potentially reflect the between patient variability in treatment response. Therefore, the pharmacokinetic-pharmacodynamic (PK-PD) relationship between CsA blood concentration and calcineurin activity was evaluated. Renal transplant recipients ($n=98$) were treated with CsA for 6 months after transplantation. CsA blood concentrations and calcineurin phosphatase activity in leukocytes were measured frequently and analyzed using a population PK-PD analysis. The PK of CsA was found to be linear with delayed absorption. The change in calcineurin activity was directly related to the CsA blood concentration and the PK-PD relationship was best described with a sigmoid maximum-effect (E_{max}) model. The baseline activity (E_0) with a median value of 10 pmol/min/mg protein showed considerable within subject variability of 28%, which could be partly explained by differences in intra-cellular protein amount and assay-variability. The E_{max} was 48% of the baseline activity and the CsA potency (IC_{50}) was found to be 223 µg/L, with only a small between subject variability in E_{max} of 13%. Although a clear relationship between CsA blood concentration and calcineurin activity in leukocytes was observed in the population, differences in individual susceptibility for CsA, in terms of efficacy and potency, could not be identified, limiting the usefulness of this biomarker for the individualization of CsA dosing (**Chapter 6**).
Subclinical acute rejection (SCR) in the first year after renal transplantation is associated with early graft loss. Besides, presence of SCR may prevent reduction of immunosuppressive therapy. Therefore, we aimed to identify which factors are predictive for SCR. Specifically, genetic variability in the genes encoding calcineurin (*PPP3CA*/*PPP3CB*) was of interest. Adult renal transplant recipients ($n=361$), receiving quadruple immunosuppression consisting of basiliximab, prednisolone, mycophenolate sodium and ciclosporin A (CsA), were followed for 6 months as part of a multicenter study. At 6 months after transplantation a scheduled biopsy was obtained and reviewed for signs of SCR. Together with demographic and transplant related factors, CsA exposure data ($AUC_{0\text{-}12h}$) and pharmacogenetic data (variability in the genes *ABCB1*, *CYP3A5*, *CYP2C8*, *NR1I2*, *PPP3CA* and *PPP3CB*)

were analyzed with S-Plus/NONMEM. Biopsies were obtained for 275 transplant recipients, of which 18% ($n=50$) displayed SCR. A previous acute rejection episode and a cadaveric donation were the most important predictors for SCR, leading to a risk of 52% of SCR at 6 months (*versus* 11% average), while these factors, along with female sex and carrying ABCB1 TTT-haplotype, were related to a premature end of study (overall drop-out 24%). Genetic variability in the genes (*PPP3CA*/*PPP3CB*) coding for calcineurin were not significantly related tot SCR. Transplant related factors were found to be the most important predictors for SCR in this AUC-controlled population on CsA (**Chapter 7**).

More insight into CNI therapy has been developed. Individualized dosing on basis of CYP3A5*1 genotype is a possible approach for tacrolimus, while body weight is an adequate predictor for ciclosporin exposure. However in both cases concentration monitoring is still necessary to further individualize therapy and to prevent overexposure early after renal transplantation. Major clinical developments in optimizing calcineurin therapy will be related to finding the lowest dose that prevents kidney rejection, but substantially reduces toxicity. With respect to renal toxicity, the main focus should be on genetic factors to be identified in the donor kidney.

Progress in optimizing the pharmacological intervention after renal transplantation is expected to come from three approaches. First of all it embraces the development of biomarkers indicative for the immunological risk as well as drug response biomarkers indicative for the action of the various immunosuppressive drugs together. The latter will include pharmacogenetic markers in the pharmacodynamic pathways of the various drugs (i.e. the *PPP3CA* and *PPP3CB* gene encoding calcineurin). Furthermore, the analysis type should be brought to higher level using pharmacometrics to its full extent. This thesis served to introduce population approaches with non-linear mixed effects modeling in renal transplantation. This should further be exploited by a more comprehensive systems pharmacology approach including biomarkers that reflect adaptation of the immune system to the new situation (transplanted organ, drugs etc.), a derivative of disease-progression models and 3-dimensional response surface analysis to account for the effect of interacting immunosuppressive drugs. Finally, large collaborations should join data to maximize success of the pharmacometric approaches (**Chapter 8**).

Nederlandse Samenvatting

Inleiding

In Nederland zijn 13.000 mensen met eindstadium nierfalen die nierfunctievervangende therapie nodig hebben. Dit aantal neemt jaarlijks toe. Deze patiënten hebben twee levensreddende therapeutische opties: *dialyse* en *nier-transplantatie*. Aangezien dialyse een slechte prognose heeft door complicerende hart- en vaatziekten en door lage kwaliteit van leven, heeft transplantatie een vlucht genomen. Vanaf de eerste Nederlandse transplantatie in 1966 tot nu hebben in ons land inmiddels meer dan 16.000 niertransplantaties plaatsgevonden. Het succes van een niertransplantatie wordt in hoge mate bepaald door de behandeling met geneesmiddelen die het immuunsysteem onderdrukken. Het onderzoek in dit proefschrift heeft betrekking op het optimaliseren van de behandeling met afweeronderdrukkende geneesmiddelen bij niertransplantatie.
Een niertransplantatie houdt in dat een nier van een persoon, de donor, wordt overgeplaatst (getransplanteerd) naar een ontvanger, de nierpatiënt. De donornier kan afkomstig zijn van een overleden persoon (overleden of hersendood) of van een levend verwante (broer/zus/vader/moeder) of onverwante donor (echtgenoot/vriend/onbekende). Het ontvangen orgaan, wordt door het afweersysteem van de ontvanger als lichaamsvreemd gezien, tenzij het afkomstig is van een eeneiige tweeling. De eerste reactie van het afweersysteem van de ontvanger is het openen van de aanval op het vreemde materiaal, de net getransplanteerde nier. Indien we niet zouden ingrijpen zou deze reactie leiden tot afstoting van de gedoneerde nier door de ontvanger.
Afweeronderdukkende geneesmiddelen (immuunsuppressiva) zijn nodig om een afweerreactie zodanig te onderdrukken dat deze reactie in toom wordt gehouden, maar dat de normale afweer tegen virussen, bacteriën en schimmels zo veel mogelijk in tact blijft. Daarnaast mogen de geneesmiddelen geen of zo min mogelijk schade aan het nieuwe orgaan toebrengen. Deze balans luistert heel nauw en dient zoveel mogelijk op een in-

dividu te worden afgestemd. Ieder mens is namelijk uniek en iedere transplantatie ook. Dit betekent dat het risico van een patiënt op afstoting van het orgaan door vele factoren wordt gekenmerkt. Allereerst is het type donatie van belang. Een orgaan van een overleden donor heeft bijvoorbeeld vaak al enige schade opgelopen doordat het een lange tijd (tot 24 uur) buiten het lichaam, op ijs, zonder zuurstof heeft doorgebracht. Verder dienen de immuunsytemen van donor en ontvanger zoveel mogelijk in overeenstemming te zijn. Dit wordt vastgesteld door genetische overeenkomst in het zogenaamde *humane leukocyt antigeen systeem* (HLA-matching). Daarnaast zijn er nog vele factoren die het risico op schade aan de nier of afstoting bepalen.

Afweeronderdrukking geschiedt doorgaans met een cocktail van geneesmiddelen. In vele behandelcentra wordt een combinatie van 4 middelen gebruikt. Het doel is meerdere middelen in een relatief lage dosering te geven om de schadelijkheid van ieder middel afzonderlijk te beperken, maar wel een optimale afweeronderdrukking te bewerkstelligen. Allereerst wordt er rondom de transplantatie zogenaamde inductietherapie gegeven waarbij snelle en effectieve afweeronderdrukking wordt bereikt. Daartoe worden blokkers ingezet die de signalen van boodschapperstoffen, zogenaamde cytokines, aan afweercellen tegenhouden. Ook worden hoge doseringen van corticosteroïden gebruikt, veelal prednisolon. In de fase kort na transplantatie voor zolang als nodig, wordt er onderhoudstherapie met afweeronderdrukkende medicatie gegeven. Doorgaans wordt ook hier prednisolon gegeven in lage doseringen, daarnaast een remmer van de deling van afweercellen, mycofenolzuur. Als hoeksteen van de behandeling worden zogenaamde calcineurineremmers gebruikt. Net als steroïden zorgen deze middelen voor een uitgebreide afweeronderdrukking.

Er zijn twee calcineurineremmers, ciclosporine A en tacrolimus. Dit zijn middelen met een erg nauwe marge tussen werkzaamheid en schadelijkheid. Indien er te laag gedoseerd wordt is er een groot risico op afstoting van de net getransplanteerde nier. Echter, in het geval van een te hoge dosering zijn deze middelen erg schadelijk, waarbij met name schade aan de getransplanteerde nier een probleem is. Ook vertonen zowel ciclosporine A als tacrolimus grote verschillen in blootstelling en daarmee werkzaamheid bij patiënten, bij eenzelfde dosering. Indien de concentratie in het lichaam wordt gemeten van een geneesmiddel gebeurt dit meestal in het gemakkelijk af te nemen bloed. Dit is een weerspiegeling van de blootstelling aan een middel. De calcineurineremmers worden dermate verschillend opgenomen door de darm en verschillend afgebroken door de lever van een persoon, dat iemand die tweemaal daags 1 mg tacrolimus slikt dezelfde blootstelling (concentratie in bloed) in het lichaam kan hebben als bijvoorbeeld iemand die tweemaal daags 15 mg slikt.

Het routinematig meten van de concentratie van deze geneesmiddelen in bloed en het aanpassen van de dosering op basis van streefconcentratie is standaard zorg in de klinische praktijk van de transplantatie-nefroloog en ziekenhuisapotheker. Echter, het kan enige tijd duren voordat een patiënt de streefconcentratie bereikt. Het heeft daarom een belangrijke toegevoegde waarde om te kunnen voorspellen waardoor patiënten verschillende doseringen nodig hebben en daaraan gekoppeld hoe de dosering kan worden aangepast aan de individuele patiënt. Het eerste deel van het onderzoek is er daarom op gericht om te identificeren welke genetische en omgevingsfactoren dit bepalen.

Het tweede deel slaat een andere weg in. Ondanks goede blootstelling ofwel geneesmiddelconcentratie in het lichaam is het voor een groep patiënten nog steeds zo dat er afstotingsreacties optreden of dat er schadelijke bijwerkingen van ciclosporine en tacrolimus zijn. Dit suggereert dat patiënten kunnen verschillen in gevoeligheid voor deze middelen, ofwel een verschil in potentie of effectiviteit van het middel tussen personen. Het zou daarom nuttig zijn om een andere 'marker' dan geneesmiddelconcentratie te kunnen meten in bloed, die dit verschil in gevoeligheid weergeeft. Dit kan bijvoorbeeld het aangrijpingspunt van ciclosporine en tacrolimus zijn, het eiwit calcineurine.

Dit proefschrift

Het inleidende **tweede hoofdstuk** geeft een beschrijving van de oorzaken van variabiliteit in de blootstelling aan de calcineurineremmers. De nadruk ligt op genetische factoren die (mogelijk) een rol spelen bij de opname en verwerking van ciclosporine en tacrolimus door het menselijk lichaam. Tevens wordt een overzicht gegeven van te onderzoeken genen die een rol spelen bij het aangrijpingspunt van deze middelen in het lichaam. Door verschillen in erfelijk materiaal tussen nier-ontvangers of hun bijbehorende donoren kan het zo zijn dat een persoon minder gevoelig of juist extreem gevoelig is voor een bepaald geneesmiddel.
De **hoofdstukken 3** en **4** richten zich, voor respectievelijk tacrolimus en ciclosporine A, op het vinden van factoren die een rol spelen bij verschillen tussen personen in de mate van opname in de darm en de afbraak door de lever. Deze factoren kunnen voorspellend zijn voor de startdosering van deze middelen ten tijde van de transplantatie. Hiertoe zijn populatie farmacokinetische modellen ontwikkeld voor ciclosporine A en tacrolimus. Deze wiskundige beschrijving van de farmacologische processen wordt wel aangemerkt met de term farmacometrie en het betreft analyses met zogenaamde niet-lineaire gemengde effecten modellen. Dit betreft een mix van een structureel en een statistisch model. Hiermee wordt structureel beschreven hoe het gedrag van een geneesmiddel is in termen van opname, verdeling en uitscheiding, gebruik makend van parameters zoals verdelingsvolume en geneesmiddel klaring. Daarnaast wordt de variabiliteit in deze parameters binnen en tussen personen in kaart gebracht. In een volgende stap wordt getracht met behulp van een zogenaamd covariaat (voorspellende of verklarende variabele) model deze variabiliteit te verklaren. Met deze modellen voor de calcineurine remmers valt te concluderen dat ciclosporine op basis van lichaamsgewicht gedoseerd kan worden. Daarbij dient wel rekening gehouden te worden met gelijktijdig gebruik van prednisolon in een dosering hoger dan 20 mg, dat de afbraak van ciclosporine met 22% verhoogt en daarmee een hogere dosering vereist. Daar staat tegenover dat de tacrolimus dosering beter niet op lichaamsgewicht kan geschieden om snel de juiste blootstelling in bloed te bereiken. CYP3A5*1, een genetische marker die geassocieerd is met verhoogde leverafbraak van tacrolimus is wel voorspellend voor de startdosering. Nierontvangers met minimaal 1 allel van CYP3A5*1 (een van beide DNA strengen) dienen een 50% hogere tacrolimus dosering te krijgen. Ook voor tacrolimus is een interactie met tacrolimus geïdentificeerd. Een gelijktijdige dosering prednisolon hoger dan 10 mg leidt tot een 15% hogere klaring van tacrolimus.

Een interactie tussen geneesmiddelen is niet voor ieder individu gelijk. De interactie tussen prednisolon en de calcineurineremmers zou kunnen afhangen van de Pregnaan-X-receptor (PXR). Dit eiwit induceert metabolisme eiwitten op het moment van hoge corticosteroid hoeveelheden in het lichaam, bijvoorbeeld tijdens stress. Hiermee wordt de afbraak van overmatige corticosteroïden versneld. Tegelijkertijd kan de afbraak van andere geneesmiddelen daarmee versneld worden, zoals van ciclosporine en tacrolimus tijdens prednisolon therapie. Het valt te verwachten dat verschillen in gevoeligheid voor deze interactie tussen individuen bestaan met een mogelijke genetische basis in het gen *NR1I2* dat codeert voor PXR. Hoewel in de populatie een 38% hogere tacrolimus klaring gevonden werd voor dragers van het *NR1I2*+7635GG genotype verklaarde dit genotype geen verschillen in gevoeligheid voor de interactie met prednisolon.
Vervolgens wordt een oproep gedaan voor een verbeterde en meer geraffineerde manier van therapiebegeleiding bij gebruik van calcineurineremmers. Er moet gedacht worden aan verbeterde meetmethoden en strategieën en metingen dichter bij de plek waar de middelen werken. De huidige biomarkers zijn kreatinine voor de nierfunctie en de calcineurineremmerconcentratie voor geneesmiddel effect en toxiciteit. Andere biomarkers gerelateerd aan het immuunsysteem of het effect van geneesmiddelen zijn mogelijk van meerwaarde (**Hoofdstuk 5**).
In de laatste twee hoofdstukken wordt onderzocht of de gevoeligheid voor ciclosporine A verschilt tussen patiënten. Dit wordt gedaan door de remming van het aangrijpingspunt van ciclosporine A te meten, het eiwit calcineurine (**Hoofdstuk 6**). Na bestudering van 98 niertransplantaat ontvangers gedurende 6 maanden blijkt dit (nog) geen bruikbare aanpak. Er werd een duidelijke relatie gezien tussen de ciclosporine concentratie in volbloed en de calcineurine activiteit in leukocyten met een basale calcineurine activiteit van 10 pmol/min/mg, een potentie van 223 µg/L en een maximale calcineurine remming van 48% van de basale calcineurine activiteit. Deze zogenaamde response biomarker fluctueert teveel binnen personen, met 38% *intra*-patiënt variatie in de basale calcineurine activiteit in de eerste 6 maanden na transplantatie. Daar tegenover staat maar een geringe variatie tussen personen van 13% in de maximale remming van calcineurine. Het blijkt dat de meetmethode nog niet toereikend is door een slechte reproduceerbaarheid. Dit vormt vooralsnog een belemmering om de dosering per individu op basis van deze marker af te stemmen.
In een volgende onderzoek (**Hoofdstuk 7**) worden de resultaten beschreven van een multi-center studie met 361 transplantatie patiënten op ciclosporine A therapie naar voorspellers van subklinische rejectie (SKR). SKR is gedefinieerd als tekenen van acute rejectie in een routine nierbiopt zonder de aanwezigheid van klinische verschijnselen van nierfunctieachteruitgang. De aanwezigheid van SKR is een belangrijke maat om de afweeronderdrukking af te kunnen bouwen. Bij afwezigheid van SKR zijn er geen belemmeringen om bijvoorbeeld op 6 maanden de hoeveelheid immuunsuppressiva van drie naar twee af te bouwen. Het routine biopt is tot op heden de enige biomarker die inzicht geeft in het immunologische proces ofwel infiltratie van afweercellen in het lichaamsvreemde orgaan. Aangezien het verkrijgen van een biopt met een naald invasief is, is het belangrijk te weten wat nu de factoren zijn die het voorkomen van of het risico op SKR bepalen. Naast demografische en transplantatie gerelateerde factoren werden genetische

en *niet*-genetische factoren onderzocht die gerelateerd zijn aan de kinetiek en dynamiek van ciclosporine A. Genetische variatie in transplantaat ontvangers voor de genen coderend voor metabolisme en transport enzymen werden onderzocht, zoals cytochroom P450 3A5 (metabolisme) en P-glycoproteïne (*ABCB1*). Tevens werd genetisch variatie in de genen coderend voor het eiwit calcineurine onderzocht. Het calcineurine eiwit wordt door 3 genen gecodeerd, *PPP3CA, PPP3CB en PPP3CC*. Deze genen verzorgen de expressie van calcineurine in verschillende weefsels, grofweg respectievelijk voor calcineurine in niertubulus, afweercellen (lymfocyten) en testis. De hypothese is dat genetische variatie in het gen *PPP3CB* gerelateerd zou kunnen zijn aan het voorkomen van SKR. Daartoe is deze variatie in kaart gebracht met behulp van een zogenaamd haploblok wat in dit geval bestaat uit 3 polymorfismen in het *PPP3CB* gen. De subklinische acute rejectie kwam voor in 18% van de patiënten waarvan een nierbiopt was verkregen ($n=275$) en werd vastgesteld in een routine biopt genomen 6 maanden na transplantatie. Er werd geen relatie gevonden tussen de incidentie van SKR en de geselecteerde genetische factoren. Wel waren een eerder doorgemaakte periode van acute afstoting en het ontvangen van een nier van een overleden donor voorspellend voor SKR. Deze individuen hadden een risico van 52% op SKR op 6 maanden tegenover 11% van de mensen die geen acute afstoting hadden doorgemaakt en een nier van een levende donor hebben ontvangen.

Dit proefschrift laat zien hoe krachtig de gebruikte populatie analysemethode is. Ondanks dat enkele factoren geïdentificeerd zijn die de therapie verder kunnen individualiseren, zoals dragerschap van CYP3A5*1, liggen er nog veel onontgonnen gebieden. De toekomst ligt in de ontwikkeling van biomarkers die enerzijds het immunologisch risico van een individu bepalen en anderzijds inzage geven in de toxiciteit van de therapie. Met name voor schade aan de nier dienen ook donorkenmerken meegenomen te worden. Daarnaast zouden biomarkers kunnen dienen om de dosering van de geneesmiddelen te individualiseren. Verder dient de farmacometrische analyse techniek binnen het transplantatie onderzoek uitgebouwd te worden met een aanpak die nog meer factoren meeneemt, zoals veranderingen in het immuunsysteem en de combinatie van meerdere geneesmiddelen. Tot slot zijn nauwe samenwerkingen tussen de verschillende transplantatie centra gewenst om tot combinatie van grote hoeveelheden data te komen, die deze analyse techniek nog beter tot zijn recht laten komen.

Curriculum vitae

Rogier Raphael Press werd geboren op 5 april 1978 te Rotterdam. In 1996 heeft hij het atheneum (VWO) aan het Dalton Scholengemeenschap te Voorburg afgerond. In hetzelfde jaar begon hij de studie Farmacie aan de Universiteit Utrecht. Tijdens de studie heeft hij basaal onderzoek gedaan naar geneesmiddeltransport door darmweefsel bij Pfizer Central Research te Sandwich, Verenigd Koninkrijk en de Universiteit Utrecht (dr. C. Kohl / dr. J.J. Tukker). Tevens heeft hij een onderzoek gedaan naar invloed van transport en metabolisme eiwitten op de opname en uitscheiding van het anti kanker geneesmiddel vinorelbine aan het Antoni van Leeuwenhoekziekenhuis te Amsterdam (dr. O. van Tellingen / Prof. dr. J.H. Beijnen). In 2001 werd de doctoraal graad in de Farmacie behaald en in februari 2003 werd te Utrecht het apothekersexamen afgelegd, waarna in hetzelfde jaar werd gestart met een baan als apotheker klinisch geneesmiddelenonderzoek binnen de afdeling Klinische Farmacie & Toxicologie (Apotheek) van het Leids Universitair Medisch Centrum (LUMC). Vervolgens verruilde Rogier deze werkzaamheden in 2005 voor een gecombineerd traject, bestaande uit de specialisatie tot ziekenhuisapotheker, apotheker voor een klinische studie (MECANO) naar immuunsuppressie bij niertransplantatiepatienten en een promotieonderzoek op het gebied van immuunsuppressieve geneesmiddelen bij niertransplantatie. In dit onderzoek ging zijn aandacht in het bijzonder uit naar de optimalisatie van calcineurineremmer therapie met behulp van populatiekinetiek en -dynamiek, zogenaamde farmacometrische analyse. Het betrof een samenwerking tussen de afdelingen Klinische Farmacie & Toxicologie (Prof. dr. H.J. Guchelaar) en de afdeling Nierziekten (Prof. dr. J.W. de Fijter) van het LUMC met de Universiteit Leiden, vakgroep Farmacologie en het Leidse LAP&P Consultants BV (dr. B.A. Ploeger / Prof. dr. M. Danhof). Per 1 maart 2011 zijn de specialisatie tot ziekenhuisapotheker en het promotieonderzoek afgerond.
Rogier Press woont samen met Judith Wessels in Amsterdam en samen hebben zij een zoon Otto.

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Met veel plezier heb ik de afgelopen jaren aan dit proefschrift gewerkt. Het onderzoek hierin weergegeven is het resultaat van een plezierige, intensieve en zeker ook vruchtbare samenwerking van de afdelingen Klinische Farmacie & Toxicologie, Nierziekten en Klinische Chemie van het Leids Universitair Medisch Centrum met de afdeling Farmacologie van het Leiden/Amsterdam Center for Drug Research (LACDR) en LAP&P Consultans BV. Daarnaast is er voor een deel van het gepresenteerde onderzoek samengewerkt met de afdelingen Nierziekten en de afdelingen Klinische Farmacie van het Academisch Medisch Centrum van de Universiteit van Amsterdam (AMC) en van het Universitair Medisch Centrum Groningen. De omvang van deze samenwerking geeft aan dat veel personen aan dit proefschrift hebben bijgedragen. Graag wil ik iedereen die hier bij betrokken is geweest, van inclusie van patiënten tot monsterverzameling en analyse en van dataverwerking tot interpretatie van en discussie over resultaten, bedanken. In het bijzonder bedank ik de niertransplantatie patiënten die bereid zijn geweest mee te werken aan de verschillende klinische onderzoeken.

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