Optimizing **immunosuppression**

with mTOR inhibitors in renal transplant recipients

D.J.A.R. Moes

The research presented in this thesis was performed at the departments of Clinical Pharmacy and Toxicology and Nephrology of Leiden University Medical Center, Leiden, The Netherlands.

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Voor Roelof en Geke

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Learn from yesterday,
live for today, hope for tomorrow.
The important thing is to not
stop questioning

Albert Einstein
(1879-1955)

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General introduction

Introduction

Currently over one million humans are estimated to fulfill the criteria for chronic kidney damage in the Netherlands. More than 60.000 have serious kidney problems, ranging from renal failure to end stage renal disease (ESRD). Of these, 16.000 patients need lifelong renal replacement consisting of either dialysis or renal transplantation and this number is increasing every year. Dialysis treatment is associated with high mortality and reduces quality of life of the patients dramatically. One out of six patients dies every year because of this high mortality. Moreover, the health system costs associated with dialysis treatment per patient are very high. Currently over 6500 patients are treated with dialysis with an average annual cost of €75.000 per patient. The other option; renal transplantation became available in the Netherlands since 1966, but is limited by availability of donor organs. By the end of the year 2012, 855 patients were on the waiting list for a renal transplantation and every year 200 patients die because the shortage of donor organs. In 2012, 961 kidneys were transplanted and 50% of the donor organs were from a life donor [1–3]. Following transplantation immunosuppressive therapy is needed to prevent the recipient's immune system of rejecting the graft. The last two decades the rejection rates have been significantly reduced to 10–20% with the introduction of the calcineurin inhibitors regimens. However, despite the low acute rejection rates and successful treatment in the first year after transplantation long term outcome after renal transplantation remains poor [4]. Optimal survival of the transplanted kidney depends on a number of factors; the donor and transplant procedure characteristics such as living versus deceased donor, cold ischemic time, donor age, HLA matching as well as co-morbidities of the recipient and optimal immunosuppressive therapy [5–8]. Immunosuppressive agents have a small therapeutic window and have often highly variable pharmacokinetics which makes therapeutic drug monitoring (TDM) of immunosuppressive drug concentrations essential for individualizing the dose and thereby preventing serious toxicity or rejection [9–12]. Suboptimal use of immunosuppressive medication such as under-immunosuppression and calcineurin-inhibitor toxicity plays a central role in the shortened long-term graft survival. Recent studies indicate that chronic antibody-mediated rejection is another important barrier to improve long term outcome [13]. Maintaining adequate overall immunosuppression is essential for prevention of chronic antibody-mediated rejection. Currently the most used immunosuppressive regimen consist of: induction therapy with an interleukin-2 blocking agent such as basiliximab, and maintenance therapy using a

calcineurin inhibitor (tacrolimus), mycophenolic acid and corticosteroids (prednisolone) [14]. Since the introduction of mTOR inhibitors the search to find the most optimal immunosuppressive regimen has further increased and different calcineurin inhibitor sparing regimens are emerging in an attempt to further improve long term outcome [15,16]. Although TDM has proven its effectiveness, still some patients experience toxicity and or rejection, therefore further optimization is warranted. In addition finding biomarkers, such as polymorphisms in genes coding for proteins involved in metabolism and dynamics of immunosuppressive drugs, which can predict altered pharmacokinetics or dynamics could further improve outcome for renal transplant recipients. Pharmacometrics; which uses mathematical models based on physiology, pharmacology and disease for quantitative analysis of interaction between drugs and patients [17] as used throughout this thesis can be a helpful tool to find such biomarkers.

Aim and Scope

The general aim of this thesis is to optimize immunosuppressive therapy, especially everolimus therapy in renal transplantation recipients by identifying pharmacological and pharmacogenetic risk factors influencing pharmacokinetics, and dynamics such as side effects and patient outcome. **Chapter 2** describes the knowledge of clinical pharmacokinetics and dynamics of mTOR inhibitors in renal transplantation at the start of this PhD project and functions as an introduction for this thesis. TDM of oral immunosuppressive agents is essential to prevent toxicity and/or rejection. Therefore it is very important to use a reliable and accurate bioanalytical assay. In **Chapter 3** the differences between the most used analytical assays of measuring everolimus in whole blood and its effect on dosing advice are investigated. TDM is performed based on either trough or AUC monitoring and pharmacogenetics might be a valuable addition to TDM to get the drug as soon as possible on target concentration. In **Chapter 4** the population pharmacokinetics of everolimus in a calcineurin free regimen and the search for predictive factors such as pharmacogenetics as well the development of a limited sampling model is described which enables physicians to accurately predict everolimus exposure with limited patient discomfort. MTOR inhibitors are known for a variety of side effects and high discontinuation rates. **Chapter 5** evaluates potential risk factors for the most severe side effect of mTOR inhibitors, interstitial pneumonitis, in a case control

study. Furthermore **Chapter 6** describes a comprehensive analysis identifying risk factors for discontinuation and a number of side effects in a population of renal transplant patients on a regimen of everolimus and prednisolone dual therapy. In **Chapter 7** the most promising polymorphisms in renal transplantation are in investigated for influence on pharmacokinetics on the main stay immunosuppressive drugs cyclosporine, everolimus and tacrolimus. In addition **Chapter 8** reports the findings of the effect of peroxide reductase (POR) and CYP3A5 polymorphisms and their combination on everolimus pharmacokinetics. Finally **Chapter 9** aims at identifying risk factor associated with delayed graft function, acute rejection and subclinical rejection in patients on a cyclosporine based immunosuppressive regimen. This thesis ends with a general discussion in **Chapter 10** and finally this thesis is summarized in a English and Dutch summary.

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2

Clinical pharmacokinetics and pharmacodynamics of mTOR inhibitors in renal transplantation

D.J.A.R. Moes, H-J Guchelaar and J.W. de Fijter

Submitted

Abstract

The mammalian target of rapamycin (mTOR) inhibitors sirolimus and everolimus are a relatively new therapeutic group in renal transplantation and have shown their efficacy in recent trials. Their main advantage compared to the calcineurin inhibitors cyclosporine and tacrolimus are their relative lack of nephrotoxicity. Sirolimus differs from everolimus mainly in pharmacokinetic characteristics such as elimination half-life and bioavailability. The oral mTOR inhibitors exert both highly variable inter- and intra-individual pharmacokinetics. They are metabolized by CYP3A4, CYP3A5 and CYP2C8 enzymes and are substrates for P-glycoprotein and share similar pharmacodynamics. Polymorphisms in genes coding for these enzymes might be of interest for optimizing immunosuppressive therapy. The most important side effects of sirolimus and everolimus are thrombocytopenia, leukopenia, hypercholesterolemia, diarrhea and although rare but potentially life threatening interstitial pneumonia. The narrow therapeutic window of mTOR inhibitors, together with high variability in pharmacokinetics, makes therapeutic drug monitoring essential for individualizing the dose and thereby preventing toxicity or rejection. The main future challenge is to further optimize mTOR inhibitor based immunosuppressive therapy.

Introduction

In the last 30 years considerable progress has been made in the field of renal transplantation with regard to immunosuppression, since the calcineurin inhibitors (CNIs) cyclosporine and later on tacrolimus came available to the clinic. However, despite this success, calcineurin inhibitors are also associated with severe toxicity such as acute and chronic nephrotoxicity [1,2]. In an effort to find new immunosuppressive drugs without or less nephrotoxicity mTOR inhibitors were introduced in renal transplantation. The mTOR inhibitors sirolimus (Rapamune®) and everolimus (Certican®) are potent orally administered immunosuppressive agents. Both are derived from a macrocyclic lactone produced by *streptomyces hygroscopicus* recovered from Easter Island [3,4]. Similarities exist between other macrocyclic lactones such as erythromycin and tacrolimus with regard to their chemical structures. Although highly active against *Candida Albicans* sirolimus was commercially launched for its immunosuppressive potency discovered in animals [5,6] and later suggested for clinical renal transplantation [7]. Everolimus is a derivative of rapamycin (sirolimus) and was developed for prevention of acute and chronic rejection of solid organ transplants. Instead of a hydrogen atom at position 40 it has a 2-hydroxethyl chain (Figure 1a en 1b) substitution which improves the solubility and bioavailability of the drug [4].

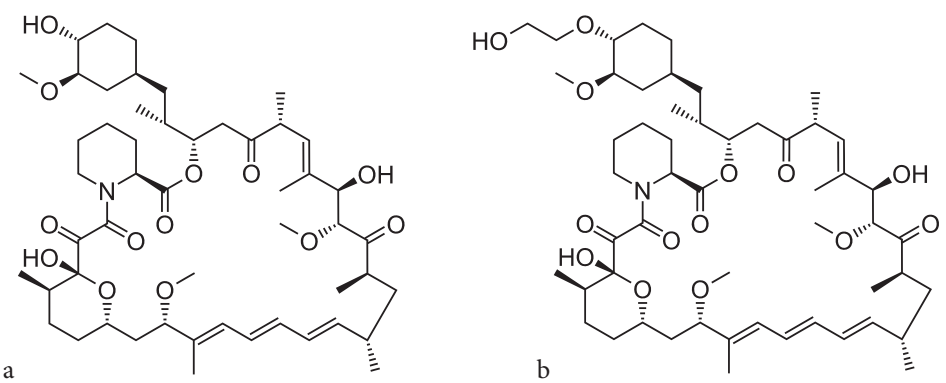

Figure 1: Chemical structure of Sirolimus and Everolimus.

In the past years mTOR inhibitors were only prescribed in combination with cyclosporine and steroids since a synergistic effect and different mechanism of action is present compared to CNIs [8,9], but as a result of the damaging effects of cyclosporine on the donor kidney everolimus is now tested in absence of cyclosporine in clinical trials [10,11].

Meanwhile a combined CNI everolimus regimen has proven its effectiveness in a number of clinical trials [12,13]. This systematic review gives an oversight on current knowledge of clinical pharmacokinetics, pharmacodynamics and pharmacogenetics of mTOR inhibitors in renal transplantation.

Literature search methods and results

An initial Pubmed search was conducted to find all available literature concerning clinical pharmacokinetics and pharmacodynamics of mTOR inhibitors using the following search criteria: {(Everolimus OR SDZ-RAD OR 40-O-(2-hydroxyethyl)-rapamycin OR "SDZ RAD" OR Certican OR "RAD 001" OR RAD001 OR Sirolimus) AND (pharmacokinetics OR pharmacokinetic* OR "Area Under Curve" OR "Biological Availability" OR "Metabolic Clearance Rate" OR "Therapeutic Equivalency" OR "Tissue Distribution" OR "Pharmacogenetics" OR "Pharmacogenetic"* OR "Pharmacodynamics" OR "Pharmacodynamic*") AND (renal transplantation OR kidney transplant) NOT oncology NOT tumors}. This resulted in 300 articles derived from Pubmed, subsequently the same search criteria was used for Web of Science (316 articles), EMBASE (102 articles) and Cochrane (2 articles). Articles were limited to those written in the English language. After removing duplicates 525 remained were reviewed for relevancy. 344 articles remained after evaluating the titles and abstracts. Focusing on pharmacokinetics, pharmacodynamics, therapeutic drug monitoring and side effects led to a total of 109 obtained full text articles which were used to summarize these findings.

Pharmacokinetics

Absorption

Sirolimus

Sirolimus is rapidly absorbed after oral administration with an average maximum blood concentration (c_{max}) (SD) of 40.5 ± 22.2 μg/L when administering a dose of 2.5 mg. The maximum concentration is reached after 2.7 ± 2.1 hours (t_{max}) and is dependent on the dose administered (0.5 - 6.5 mg) [14]. In patients receiving an immunosuppressive regimen of cyclosporine and prednisone with single or multiple doses of sirolimus, sirolimus

was absorbed rapidly with average t_{max} (%CV): 1.6 (81%) and 1.4 (85%) hours after administration respectively [14,15]. Steady state was reached within 14 days. Its steady state maximum concentration and area under the blood concentration versus time after administration curve (AUC) were dose proportional over the dose range of 0.5 – 6.5 mg/m^2 once daily [14]. The absolute bioavailability of sirolimus in humans is unknown, however is has been estimated to be around 14% and highly variable (range 10.9 – 16.9%) [16]. Results from preclinical studies also showed a low bioavailability (10%) [17]. Food intake strongly affects the bioavailiability of sirolimus; a 35% increase in AUC after a fatty meal was observed in a clinical trial, but absorption was more slowly [18]. Therefore sirolimus should be administered consistently in individual patients, either with or without meals to assure consistent exposure. In a cohort of 150 renal transplant patients, no correlation was found between sirolimus concentrations and bodyweight, gender, age or dose [19]. Currently two formulations are available in the clinic: a tablet and a non-aqueous oral solution. In a comparative study, values of c_{max} for the solution were significantly greater compared to the tablet. Moreover c_{max} for the tablet observed on day 1 was significantly greater compared with days 30 and 90. Furthermore t_{max} was significantly greater for the tablet. However average sirolimus pharmacokinetic parameters were not significantly different when comparing both formulations, only t_{max} was slower for tablet administration but no clinically relevant differences were found [20]. Similar results were found in a conversion study from one formulation to the other [21]. Intestinal CYP3A metabolism and intestinal P-glycoprotein (P-gp) counter transport, intestinal membrane permeability and hepatic first-pass affect bioavailability most likely also influence sirolimus absorption since sirolimus is a substrate for these enzymes and transporters [22] as schematically shown in Figure 2. Pharmacokinetic parameters are clearly influenced by the presence and timing of co-administration of cyclosporine [23] since both drugs are substrate and inhibitors of the same metabolizing enzymes [22,24].

Everolimus

Everolimus is rapidly absorbed after oral administration with an average c_{max} (SD) of 45 (±21) μg/L when administering a dose of 2.5 mg. The maximum concentration is reached after 1.3 ± 0.4 hours after dose administration and is dependent on the dose administered (0.25 - 25mg) [12]. In a study with patients with immunosuppressive regimen of cyclosporine and prednisone receiving multiple doses of everolimus, everolimus was absorbed rapidly (average t_{max} 2 hours), Steady state was reached within 7 days. Steady

state maximum concentration and AUC were dose proportional over the dose range of 0.5 – 2 mg twice daily [25]. The bioavailability of everolimus in animal models is low with an amount of around 16% [26,27] but slightly higher than sirolimus. Absolute bioavailability data of everolimus is not available since no intravenous formulation exists but intra- and inter-individual variability is high [25]. Currently two everolimus formulations are on the market; a solid tablet and a dispersible tablet, the latter initially developed for pediatrics. The bioavailability of everolimus from the dispersible tablet was found to be 10% lower relative to the conventional tablet [28]. As sirolimus, the relative bioavailability of everolimus is affected by food since food affects the absorption [29,30]. In healthy subjects receiving a single 2 mg dose it was found that when combining with a high-fat meal t_{max} was delayed by a median 1.25 hours. Furthermore c_{max} was reduced by 60% and reduced AUC by 16%. In renal transplant recipients, a high-fat meal delayed t_{max} by a median 1.75 hours and reduced c_{max} by 53% and AUC by 21%. Everolimus trough levels showed no food effect, while peak-trough fluctuation was lowered by 52%. [30]. Everolimus should therefore be consistently administered with or without food in individual patients. Intestinal CYP3A metabolism and intestinal P-glycoprotein activity, Intestinal membrane permeability and hepatic first-pass affect bioavailability probably play a large role in the absorption of everolimus since everolimus is also substrate for CYP3A4, CYP3A5, CYP2C8 and P-gp [31] as schematically shown in Figure 2. Co-administration of cyclosporine leads to an altered metabolism since both drugs are substrate and inhibitors of the same metabolizing enzymes [24,32].

Distribution

Sirolimus

Sirolimus is a hydrophobic compound, is extensively distributed to various organs with an steady state distribution volume (Vss) of 7-19 L/kg [15,33] and is more partitioned into red blood cells (up to 95%) than plasma (3%) and lymphocytes 1% [16,34]. Whole blood is therefore the matrix of choice for therapeutic drug monitoring. Plasma to blood ratio was found to be 35:1 in a group of 36 stable renal transplant recipients and considerable inter-individual variability (CV of 52%) was reported [15]. Sirolimus was primarily associated with non-lipoprotein fractions in plasma [34]. In studies in rats considerable accumulation of sirolimus in the heart, kidney, intestine, and testes were found [35]. Whether this is the same in humans has not been investigated.

Everolimus

The less hydrophobic compound everolimus is at therapeutic concentrations for more than 75% partitioned into red blood cells and 75% of the plasma fraction is bound to plasma proteins [25]. The estimated volume of distribution for a 71 kg patient is at steady state 110 L and is increased with 1.14 for each kilogram increase in body weight [29]. In rats the highest binding potential was observed in thymus, lungs and spleen [27]. In monkey lung transplant recipients the highest concentrations were found in gall bladder, transplant lung, cerebellum, kidneys and spleen [36]. Data in humans is not available.

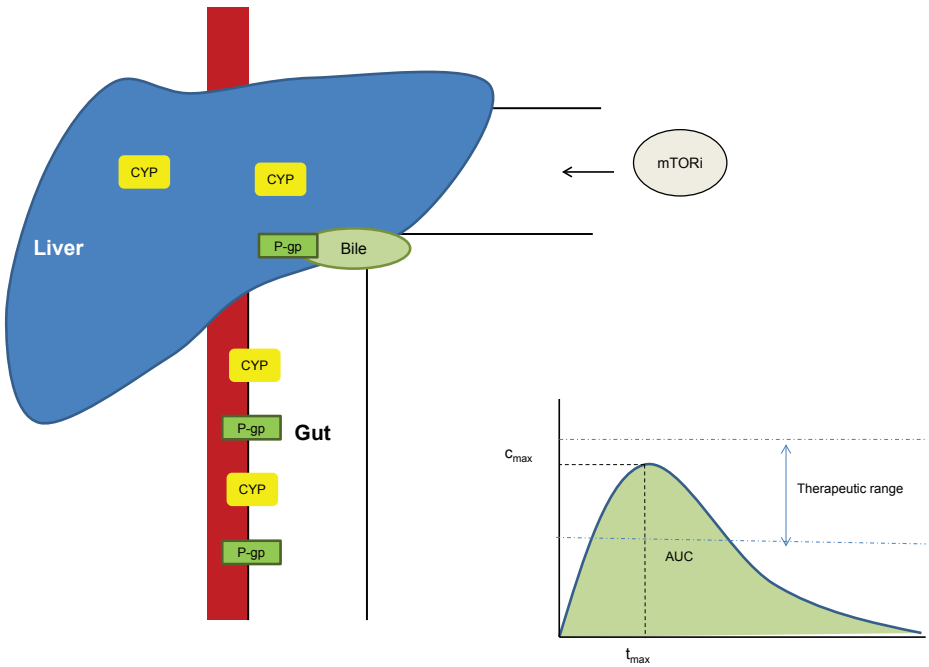

Figure 2: Schematic representation of oral administration of mTOR inhibitors, interaction with metabolic enzymes and effect on blood levels. AUC, area under the blood concentration vs time after dose administration curve; CYP, cytochrome P450 enzymes (CYP3A4, CYP3A5 and CYP2C8); C_{max}, maximum blood concentration; mTORi, mTOR inhibitor; $t_{max,}$ time to reach maximum blood concentration.

Clearance

Sirolimus

Sirolimus is primarily metabolized by CYP3A4, but also by CYP3A5 and CYP2C8 [22,37,38]. The large inter-individual variability in metabolism of sirolimus is probably

a reflection of the wide inter-individual variability in expression of these enzymes [39]. Moreover sirolimus is also a substrate for P-glycoprotein [40]. In a population pharmacokinetic analysis of 36 renal transplant patients a wide variability in clearance was found, terminal half-life was 63 hours (27.5%) and apparent oral blood clearance of 8.9 L/hr (38.2%). Elimination was not influenced by dose [15]. In another pharmacokinetic study with 40 stable renal transplant patients clearance was found to be 0,208 (45%) mL/hr/kg; terminal half-life was, 62 (±16) hours allowing a once daily regimen. Furthermore a loading dose of three times the maintenance dose was suggested to achieve therapeutic concentrations more rapidly [14]. The four main metabolites of sirolimus are 16-O-demethyl-sirolimus, 39-O-demethyl-sirolimus, 27-39-O-di-demethyl-sirolimus and di-hydroxy-sirolimus [41]. The activity of these metabolites seems to be less than 10% of the parent compound [42]. Preliminary results showed that black renal transplant patients had a higher metabolism compared to non-blacks [15]. Furthermore, another study showed significant lower trough concentrations and higher acute rejection rates for black patients [43]. In a study with 18 adult subjects with mild to moderate hepatic impairment and 18 healthy control subjects, mean whole-blood sirolimus weight-normalized and oral-dose clearances (CL/F) were significantly decreased in subjects with mild to moderate hepatic impairment by 31.8% and 36.0%, respectively, compared with controls after administration of a single 15 mg oral solution dose [44].

Everolimus

Everolimus is also metabolized by CYP3A4, CYP3A5 and CYP2C8 and is a substrate for P-gp [22,31,45]. In a "first into human" study with single everolimus doses the elimination half-life and ranged from 24 to 35 h across the doses in the range of 0.25 – 25 mg. The average AUC (μg*h/L) ranged from 171 ± 50 μg*h/L for the 0.75 mg group to 2400 ± 608 μg*h/L for the 25 mg group [46]. In a population pharmacokinetic analysis of 673 patients [29] the following pharmacokinetic parameters were found: the apparent average clearance for a 44 years Caucasian patient old weighing 71 kg was 8.8 L/h (± 27%) with a central distribution volume of 110 L (± 36%). Everolimus pharmacokinetics is greatly affected by cyclosporine which inhibits CYP3A4 [29]. In 8 healthy volunteers, everolimus apparent clearance was 19.4 L/h in absence of cyclosporine [47]. Therefore renal transplant patients probably also have a higher clearance in cyclosporine free regimens. Everolimus pharmacokinetics was not affected by age, sex and weight in adults. Asian ethnicity did not affect everolimus clearance. Patients indicated as black had a 20% higher clearance

compared to non-black patients [29,48]. Since everolimus has a rapid clearance, everolimus requires twice-daily administration in contrast to sirolimus. The four main metabolites of everolimus are: hydroxyl-everolimus, dihydroxy-everolimus, dimethyl-everolimus and a ring opened form of everolimus [37]. In a population pharmacokinetic study the inter-individual variability in clearance was reduced to 27% after accounting for the covariates [29]. The intra-individual variability and residual error was 31%. In a multicenter randomized double blind study of 101 renal transplant patients inter-individual variability in terms of AUC for everolimus was 85.4%, intra-individual, inter-occasion variability was 40.8% [49], implicating the need for therapeutic drug monitoring. In a study investigating the influence of hepatic impairment on everolimus pharmacokinetics it was found that the apparent clearance of everolimus was significantly reduced by 53% in subjects with moderate hepatic impairment compared with healthy subjects. This was reflected by a 115% higher AUC (245 +/- 91 versus 114 +/- 45 μg*h/L) and 84% prolonged half-life (79 +/- 42 versus 43 +/- 18 hours) [47]. Furthermore a significant positive correlation of the everolimus AUC with bilirubin level (r = 0.86) and a significant negative correlation with albumin concentration (r = 0.72) was found. Therefore dose reduction and close TDM may be indicated.

Excretion

Sirolimus

Sirolimus is metabolized trough the liver, 91% of sirolimus metabolites are excreted in the bile, only 1.2% is excreted trough urine [50].

Everolimus

Everolimus is also metabolized trough the liver, after metabolizing approximately 98% is excreted as metabolites in the bile [46].

Drug interactions

Sirolimus

Since sirolimus is metabolized by CYP3A4, CYP3A5 and CYP2C8 and a substrate of P-gp, inhibitors or inducers of these enzyme most likely show pharmacokinetics interactions. In vitro anti-CYP3A antibodies, as well as the specific CYP3A inhibitors troleandomycin and erythromycin, inhibited small intestinal metabolism of sirolimus [22]. In a renal transplant recipient an interaction between dronedarone and sirolimus was reported. A 3 fold

increase of sirolimus trough concentration (38.6 μg/L) was observed 3 days after initiation of dronedarone. If concurrent administration cannot be avoided, close monitoring and a 50-75% dose reduction of sirolimus prior to dronedarone initiation was recommended [51]. Trimethoprim-sulphamethoxazole combination did not affect sirolimus steady state pharmacokinetics in 15 renal transplant recipients [52]. In two case reports rifampicin significantly increased sirolimus pharmacokinetics; the dosage of sirolimus had to be increased, in one case up to six-fold and in the second case up to five-fold, to maintain serum levels after starting the rifampicin [53]. Diltiazem increased sirolimus AUC by 60%, ketoconazole increased sirolimus AUC by 990% and rifampicin reduced sirolimus AUC by 82% in a phase III trial [54]. In a pharmacokinetic analysis of 36 patients cyclosporine did not seem to affect sirolimus pharmacokinetics [15]. In contrast *Cattaneo et al.* reported that concomitant cyclosporine therapy resulted in significantly higher sirolimus trough values compared to concomitant tacrolimus or mycophenolate mofetil therapy [55]. Moreover in another study with 24 stable renal transplant recipients sirolimus AUC and trough levels were consistently and significantly higher when both cyclosporine and sirolimus were administered concomitantly, than when they were administered 4 hours apart indicating a inhibiting effect of cyclosporine on sirolimus pharmacokinetics [23]. Generic and brand name cyclosporine also seem to alter sirolimus pharmacokinetic differently as was reported by Kovarik et al [56]. Finally a twofold increase in cyclosporine AUC was associated with a 63% mean increase in sirolimus AUC in 53 stable kidney transplant recipients [57]. The combination of cyclosporine and sirolimus is synergistic as previously demonstrated in vitro and in vivo in animal transplant experiments [9]. Sirolimus not only increases cyclosporine concentrations in blood but also in the kidney. This interaction may lead to increased cyclosporine associated nephrotoxicity by a mechanism which is still not entirely understood [9]. In a pharmacokinetic study investigating the effect of tacrolimus on sirolimus pharmacokinetics neither pharmacokinetic profiles of sirolimus nor those of tacrolimus were altered by simultaneous administration [58].

Everolimus

Administration of erythromycin, azithromycin, or itraconazole in combination with everolimus (0.75 or 1.5 mg twice daily) resulted in a 22, 18 and 74% lower everolimus clearance compared to everolimus alone [29]. Calcium channel blockers, quinolones and trimethoprim-sulfamethoxazole had no effect on everolimus pharmacokinetics [29]. In 12 healthy subjects, rifampicin co-administration, a CYP3A and P-gp inducer, resulted in a

significantly increased apparent clearance of 172% on average [59]. Co-administration of atorvastatine (CYP3A4 substrate) or pravastatin (P-gp substrate) has no clinically relevant interaction with everolimus as was found in 24 healthy volunteers [60]. Everolimus trough concentrations were significantly elevated in the presence of cyclosporine [61]. In a study with 56 de novo renal transplant recipients received basiliximab, corticosteroid and either immediate or delayed initiation of cyclosporine based on renal function, trough concentrations were significantly lower (3 fold) in absence vs in presence of cyclosporine [61]. In healthy volunteers is was shown that two cyclosporine formulations; neoral® and sandimmune® had different effects on everolimus pharmacokinetics. Neoral® co-administration resulted significantly greater everolimus AUC compared to sandimmune® co-administration 168% vs 74% increase [62]. Co-administration of tacrolimus seems to have a much less pronounced effect than cyclosporine on everolimus pharmacokinetics. No clinically relevant change in everolimus exposure was found [63].

Pharmacodynamics

Mechanism of action

Sirolimus and everolimus share the same mechanism of action (Figure 3). They block Ca2+-dependent and Ca2+ -independent events during G1 phase of the cell cycle, including transduction of second signals delivered by interleukin (IL)-2, IL-3, IL-5 and IL-6. They also block, but to a lesser extent, the signals delivered by fibroblast growth factor, stem cell factor, platelet-derived growth factor, colony-stimulating factor and insulin growth factor. In in vitro experiments, sirolimus and everolimus inhibited a variety of mitogen- and antigen driven B- and T-lymphocyte proliferative responses [6,64,65].

Sirolimus and everolimus bind to FK506 (tacrolimus) binding protein (FKBP12) and subsequently it binds to a protein known as mTOR. Both have compounds have an effector domain forming a composite surface with FKBP that interacts with the mammalian target of rapamycin, mTOR, as well as a binding domain that mediates the interaction with FKBP [64,66]. mTOR is an atypical serine/threonine protein kinase that belongs to the phosphoinositide 3-kinase PI3K-related kinase family and interacts with several proteins to form two distinct complexes named mTORC1 and mTORC2. mTORC1 responds to amino acids, stress, oxygen, energy and growth factors and is directly sensitive to sirolimus and everolimus. Cell growth is promoted by induction an inhibition of anabolic and

catabolic processes. mTORC1 also drives cell-cycle progression. In contrast, mTORC2 is insensitive to acute exposure of rapamycin, but chronic exposure can disrupt its structure. Moreover mTORC2 responds to growth factors and regulates cell survival and metabolism. mTORC 2 also regulates the cytoskeleton [67]. The mTORi-FKBP12-mTOR interaction causes dephosphorylation and inactivation of p70S6 kinase and which, when activated, stimulates the production of ribosomal components necessary for protein synthesis and cell-cycle progression. Cyclin dependent kinases (CDK) and cyclins are also inhibited, which are necessary to keep the cell cycle progress running. Consequently, sirolimus and everolimus inhibit T- and B-cell proliferation and differentiation and antibody production, as well as non-immune cell (fibroblasts, endothelial cells, hepatocytes, and smooth muscle cells) proliferation [68–70]. When compared with sirolimus, the in vitro activity of everolimus is in general about two to three times lower; however, when administered orally, everolimus is at least as active in vivo as rapamycin [65].

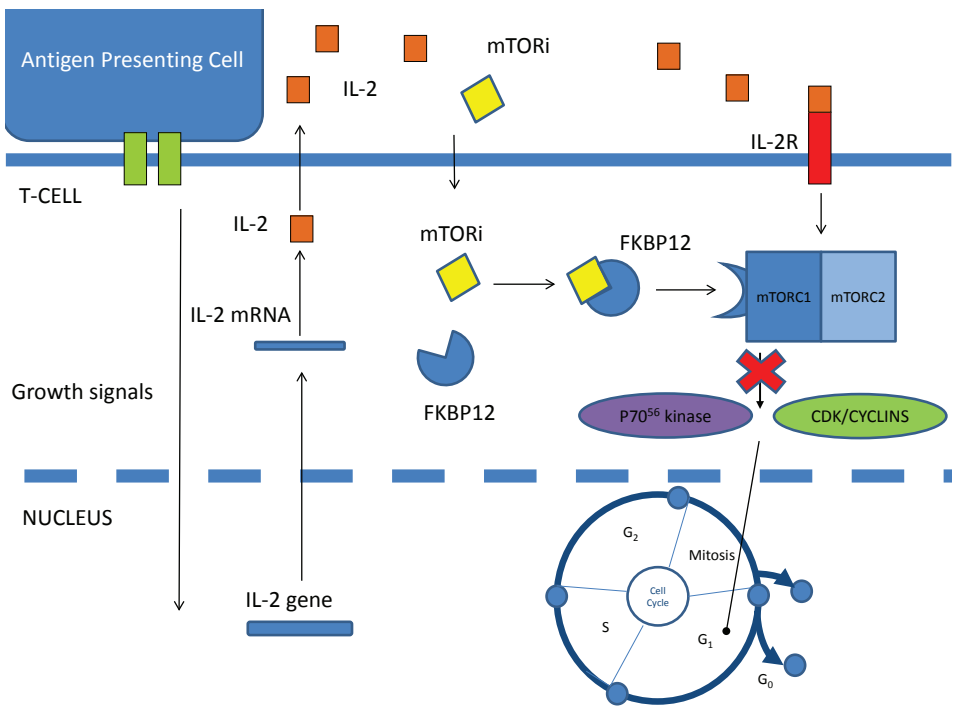

Figure 3: Simplified schematic representation of mTOR inhibitor mechanism of action. IL-2R, interleukin-2 receptor; IL-2, interleukin-2; mRNA, messenger ribonucleic acid; CDK, cyclin dependent kinase; FKBP12, FK506 (tacrolimus) binding protein; mTORi, mTOR inhibitor.

Side Effects

Sirolimus

The main and most common adverse effects attributed to sirolimus are anemia, thrombocytopenia and increase in triglyceride and cholesterol levels. Significant relationships were found between trough concentrations and the occurrence of thrombocytopenia (<100 x 10^9/L), leukopenia (<4 x 10^9/L) and hypertriglyceridemia (>750 mg/dL), but not hypercholesterolemia (>400 mg/dL). Toxic concentrations were established at >15 mg/L. Furthermore sirolimus has a narrow therapeutic window (≤ 5 µg/L) [19]. Hyperlipidemia occurs in about 40% of patients on sirolimus therapy. In a comparative study with azathioprine, increased fasting serum cholesterol and triglyceride concentration were observed, on average almost twice as high as in the azathioprine group. It is suggested that sirolimus inhibits the clearance of circulating, low, intermediate and very-low-density lipoproteins as well as their remnants [71,72]. Nevertheless only one patient discontinued the study because of hypertriglyceridemia and countermeasure therapy is often adequate [19]. Increased incidence of cardiovascular complications were not shown at phase III trials at one year after initiation [73]. Diarrhea incidence was also significantly higher than in the azathioprine group. Infections incidence including sepsis, cytomegalovirus, Epstein-Barr virus and Herpes zoster and lung infections were significantly higher in the 5 mg sirolimus group compared to the 2 mg sirolimus and the azathioprine group. The overall incidences of malignant disease besides lymphoma and lymphoproliferative disorders were similar in all treatment groups.

Everolimus

In a large (503 patients) multicenter study patients on a CNI free regimen of MPA and everolimus showed higher mean lipid concentrations, slightly increased urinary protein excretion, lower hemoglobin concentrations, also thrombocytopenia (6% vs 0%), aphthous stomatitis (15 vs 1%) and diarrhea (21 vs 8%) was reported more often compared to the CNI and MPA regimen. [11] A correlation was found between thrombocytopenia (<100 x 10^9/L) with increasing everolimus AUC [49] and trends were observed for increased incidence of hypertriglyceridemia and hypercholesterolemia with increasing everolimus AUC. The incidence of leukopenia was not related to everolimus exposure. In a multicenter double blind, placebo controlled dose escalating phase I study, also dose dependent incidence of thrombocytopenia was found [74]. Notable reversible elevations of cholesterol were also observed at the 10 mg/day dose. Other changes in laboratory

evaluations, including triglycerides, were minor, reversible and did not appear to be dose dependent.

mTOR pneumonia

The use of mTOR inhibitors in renal transplantation is associated with many side effects as mentioned above: one of the potentially most severe being interstitial pneumonitis. Non-infectious interstitial pneumonitis is characterized by non-infectious, non-malignant and non-specific inflammatory infiltrates in combination with negative bacterial tests for blood and broncho alveolar lavage (BAL) [75,76]. Non-infectious pneumonitis is a class-related adverse effect of mTOR inhibitors. At the onset of this complication, patients present themselves with cough and/or dyspnea and/or hypoxemia. Sometimes systemic symptoms such as fever and fatigue are present. Pathology reveals non-specific interstitial pneumonitis, bronchiolitis obliterans organizing pneumonia, alveolar hemorrhage, desquamative interstitial pneumonia and vasculitis. The precise mechanism is unknown but one of the suggested mechanisms is a cell mediated autoimmune response after exposure of cryptic antigens or T-cell-mediated delayed-type hypersensitivity. Inhibitors of mTOR could also exert part of their action by limiting the destructive remodeling of lung structure. Over the years a number of case report were published concerning mTOR pneumonitis in transplantation [77–79]. The Incidence of pneumonia or pneumonitis with the usage of sirolimus (SRL) is about 1-10% [80]. The introduction of sirolimus led to an increased frequency of unexplained interstitial pneumonitis in renal transplant patients, which was later also observed in liver and heart transplant patients [81]. Because of its positive effect in cancer everolimus is currently also indicated for a number of oncological indications. This inflammatory disorder was also reported in everolimus-treated non-transplanted metastatic renal cell carcinoma patients at a frequency of 8% [82]. Another study reported a frequency of 9.9% with everolimus therapy [83]. So far, no clear patient-related or context-related risk factors have been identified. Many patients are asymptomatic despite presenting signs of the complication on radiography or high resolution tomography computer tomography (HRCT)[84]. The management of this mTOR pneumonitis depends on the grade of the side effect, Grade 1 with no clinical symptoms but a positive CT up to grade 4: Life threatening complications [85]. By identifying patients at risk for mTOR pneumonia before treatment patient could be excluded from mTOR therapy and switched to another immunosuppressive drug.

Therapeutic Drug Monitoring

Sirolimus

Sirolimus blood levels show good correlation with clinical outcomes and drug related toxicity [19,55]. Trough concentration (C_{trough}) AUC correlation seems reasonable [19], however others showed worse correlation [86]. AUC monitoring on the other hand is often laborious and patient unfriendly unless limited sampling formulas and models are used. In general Bayesian limited sampling models are less rigid than limited sampling formulas and are therefore more accurate. A number of these have been published [87–89] with sampling times 0,1 and 3 hours as the most accurate and with the least discomfort for the patient in a calcineurin inhibitor based regimen [89] using Bayesian estimation. AUC better reflects true exposure but whether AUC monitoring is superior to trough monitoring with respect to firm long-term endpoints has never been investigated. Whole blood concentration can be measured with a number of analytical techniques. Toxic concentrations were established at >15 μg/L [19] and a therapeutic window has been proposed of 5-15 μg/L or 6-12 μg/L for calcineurin inhibitor included regimens and 10-20 μg/L for regimens without calcineur inhibitors [19,55,58]. Currently the most used techniques for sirolimus therapeutic drug monitoring (TDM) are liquid chromatography based techniques with or without mass spectrometry and immuno assay kits.

Everolimus

Since immunosuppression efficacy and occurrence and severity of side adverse effects are correlated with everolimus blood concentrations [25] TDM is also indicated. The recommended therapeutic range for everolimus evaluated as part of a calcineurin inhibitor regimen a number of studies is a trough of 3 to 8 μg/L in renal transplant patients [90–93]. C_{trough} AUC correlation has not been intensively investigated in renal transplant patients. Everolimus target concentrations in a regimen without calcineurin inhibitors ranges from 6-10 μg/L [10,11]. To date no limited sampling strategies have been developed for everolimus especially not in a cyclosporine free regimen. Currently the most used techniques for everolimus TDM are liquid chromatography based techniques with or without mass spectrometry and immuno assay kits.

Pharmacogenetics

A limitation of TDM is that during the critical period of the first days after transplantation the exposure cannot be influenced. Especially drugs with a long elimination half-life are at risk of under or overexposure because correcting them takes more time. For this reason pharmacogenetics could be of additional value to TDM, by differentiating in initial dose between genotype groups and subsequently decreasing the time to reach target concentration for all patients. However, whether this also leads to prolonged graft survival and lower incidence of acute rejection is not established. The mTOR inhibitors sirolimus and everolimus are metabolized by cytochrome P450 (CYP) enzymes CYP3A4, CYP3A5 and CYP2C8. Both compounds are also a substrate for the efflux pump P-glycoprotein (ABCB1). Genetic polymorphisms in genes encoding these enzymes could in theory explain a part of the variability in pharmacokinetics. Several single nucleotide polymorphisms (SNPs) have been identified in the genes encoding for CYP3A4, CYP3A5 and P-glycoprotein, including CYP3A4 -392A>G (rs2740574), CYP3A5 6986A>G (rs776746), ABCB1 3435C>T (rs1045642), ABCB1 1236C>T (rs1128503) and ABCB1 2677G>T/A (rs2032582) and some have been linked to pharmacokinetics of calcineurin inhibitors [94]. The most recognized clinically relevant single-nucleotide polymorphism (SNP) CYP3A5 A6986G has been linked in a number of studies to an increased tacrolimus clearance [95–97]. Initial dose adjustments have been proposed and are implemented in some transplantation centers. To date for CYP3A4 no conclusive results for candidate polymorphisms have been identified to optimize immunosuppressive therapy [98].

For mTOR inhibitors a limited number of pharmacogenetic studies have been published; *Le meur et al.* reported in a study of 47 patients that patients carrying at least one *CYP3A5* SNP had significantly lower AUC/dose, C_{max}/dose, C_{trough}/dose for sirolimus indicating a higher clearance [99]. In 22 renal transplant patients *Djebli et al.* found a 2 fold higher clearance for carriers of at least one *CYP3A5*1* allele [89] compared to non-carriers. In another pharmacogenetic study of 149 renal transplant recipients the effect of CYP3A4 -392A>G (rs2740574), CYP3A5 6986A>G (rs776746), ABCB1 3435C>T (rs1045642), ABCB1 1236C>T (rs1128503) and ABCB1 2677G>T/A (rs2032582), on sirolimus pharmacokinetics was evaluated. *CYP3A5* (around 1.5 fold higher compared to mutants) and *CYP3A4* (almost 2 fold higher compared to mutants) genotype correlated significantly with concentration/dose ratio but variability within the genotype groups was considerable. This genotype effect however was only found in patients without a calcineurin inhibitor

[100]. Polymorphism in *ABCB1* did not correlate to different concentration dose ratio in all populations. Furthermore *Renders et al.* found a trend (not significant) for CYP3A5 expressors toward higher (2 fold) clearance in 20 renal transplant patients and no influence for *ABCB1* and *ABCC2* genotypes [101]. In contrast to the above mentioned findings *Mourad et al.* [102] found no association between adjusted trough concentrations and dose requirements and *CYP3A5* genotype in 58 renal transplant recipients.

For everolimus *Picard et al* found no association between CYP3A5 polymorphism and everolimus pharmacokinetics in renal transplant patients [103]. Furthermore in vitro results supported this conclusion. The potential influence of polymorphisms in *CYP2C8* and *ABCB1* on everolimus pharmacokinetics is still unknown. More studies investigating the potential influence of polymorphisms in *CYP3A4*, *CYP3A5*, *CYP2C8* on pharmacokinetics and pharmacodynamics are needed to establish the potential influence and clinical relevancy.

The pregnane X receptor (PXR; NR1I2) is a member of the nuclear receptor (NR) superfamily. PXR is mainly associated with the cellular response to xenobiotics, including induction of enzymes involved in drug oxidation and conjugation, as well as induction of xenobiotic and endobiotic transporters [104]. These include the phase I enzymes cytochrome P450 (CYP) CYP2C8 and CYP3A4 and the transporters, multidrug resistance protein 1 (MDR1), MDR2, multidrug resistance-associated protein 2 (MRP2) and the organic anion transporter polypeptide 2 (OATP2) which are relevant for mTOR inhibitor metabolism, [105–107]. Polymorphism in genes coding for this receptor could be of interest for explaining variability in pharmacokinetics and dynamics [98].

Little is known about polymorphism genes coding for mTOR proteins and their effect on mTOR inhibitors pharmacodynamics. Recently *Woillard et al* [108] examined candidate polymorphisms in mTOR, Raptor and p70S6 kinase and a number of other time-constant covariates and time varying covariates. They found an significant association in decrease of haemoglobin levels and an mTOR variant haplotype. However, critical questions were asked about the matching of the two study groups [109].

Conclusions

The macrolide immunosuppressant sirolimus and everolimus form a relatively new therapeutic group in renal transplantation and have shown their efficacy in recent trials. The

advantage of these compounds is the lack of nephrotoxicity compared to the calcineurin inhibitors cyclosporine and tacrolimus. In contrast to sirolimus everolimus is dosed twice daily because of its shorter half-life and is therefore easier to manage with therapeutic drug monitoring. Both drugs are metabolized by CYP3A4, CYP3A5 and CYP2C8 enzymes and are substrates for P-glycoprotein and share the same pharmacodynamics. The most important side effects of these are thrombocytopenia, leukopenia, hypercholesterolemia, diarrhea and although rare but potentially life threatening interstitial pneumonia. The narrow therapeutic window of mTOR inhibitors, together with high variability in pharmacokinetics, makes therapeutic drug monitoring essential for individualizing the dose and thereby prevent toxicity or rejection. Pharmacogenetics might play a role in further optimization of mTOR base immunosuppressive therapy.

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3

Liquid chromatography – tandem mass spectrometry outperforms fluorescence polarization immunoassay in monitoring everolimus therapy in renal transplantation

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Abstract

There is a need to monitor everolimus blood concentrations in renal transplant recipients as a result of its high pharmacokinetic variability and narrow therapeutic window. However, analytical methods to determine blood concentrations often differ in performance. Therefore, we investigated whether two commonly used therapeutic drug monitoring methods for everolimus were in agreement and to what extent their differences could lead to differences in dosage advice. Six hundred twelve whole blood samples were obtained from 28 adult renal transplant recipients receiving everolimus and prednisolone therapy. These samples included 286 everolimus trough concentrations. The remaining samples were obtained up to 6 hours post everolimus intake and allowed calculation of 84 $AUCs_{0-12h}$. All samples were analyzed with fluorescence polarization immunoassay (FPIA) on an Abbott TDxFLx analyzer and liquid chromatography–tandem mass spectrometry (LC-MS/MS). Everolimus blood concentrations measured with FPIA and LC-MS/MS were not in agreement. Concentrations determined by FPIA were, on average, 23% higher than concentrations quantified by LC-MS/MS. Moreover, concentrations lower than 15 mg/L or AUC_{0-12h} determined with FPIA could be twofold higher than with LC-MS/MS. This variability can lead to clinically relevant differences in dose adjustment of up to 1.25 mg everolimus despite using a correction factor of 23%. Finally, when trough concentrations were measured with FPIA, higher intra-patient variability was observed compared with the use of LC-MS/MS. LC-MS/MS outperforms FPIA for clinical drug monitoring and intervention of everolimus therapy in adult renal transplant recipients on dual therapy with prednisolone. Specifically, the use of FPIA can lead to clinically relevant differences in everolimus dosage advice and higher intra-patient variability.

Introduction

Everolimus (Certican; Novartis, Basel, Switzerland) is an orally administered immunosuppressive agent targeting the mammalian target of rapamycin receptor and is used in the prevention of acute and chronic rejection of solid organ transplants. Its high pharmacokinetic variability together with a narrow therapeutic window makes therapeutic drug monitoring (TDM) crucial for dose individualization [1]. Everolimus concentrations are determined in whole blood because over 75% of the drug is partitioned into red blood cells [2]. Currently, immunoassays such as fluorescence polarization immunoassay (FPIA) and chromatographic methods such as high-pressure liquid chromatography (HPLC) or the more sophisticated HPLC combined with (tandem) mass spectrometry (LC-MS/MS) are the most commonly used analytical techniques for TDM of everolimus [3–7]. These methods may differ in specificity and sensitivity leading to altered accuracy and precision. Inaccuracy in dosage advice caused by these differences could impact on patient outcomes such as toxicity or increased risk for transplant rejection. In this study, two of the most applied analytical techniques for everolimus, FPIA on an Abbott TDxFLx analyzer and LC-MS/MS, were compared. A large number of blood samples were obtained from stable adult renal transplant patients receiving everolimus therapy. This comparison was aimed at identifying whether differences between the two techniques could lead to different everolimus dosages in clinical practice.

Materials and methods

Patients and Samples

Whole blood samples from 28 adult renal transplant recipients (18 male and 10 female) were obtained. Mean age was 52 years (± 10) and ranged from 35 to 69 years. Stable renal transplant recipients treated with immunosuppressive therapy consisting of everolimus (Certican; Novartis) and prednisolone [8] were studied from 6 months up to 2 years after transplantation. The study was approved by the Medical Ethics Committee of the Leiden University Medical Center and patients gave written informed consent. Everolimus therapy was started at an oral dose of 3 mg twice daily and was supported by routine TDM based on trough concentrations and AUC_{0-12h}. Routine TDM samples were obtained throughout time after starting everolimus therapy and were analyzed by FPIA. The target AUC_{0-12h} for FPIA was set at 150 μg*h/L [8], which roughly corresponds with an everolimus trough

concentration of 7 to 11 μg/L. All samples were also quantified with LC-MS/MS. Finally, TDM resulted in a between-patient everolimus range from 1 mg to 4.5 mg twice daily.
A total of 612 whole blood samples were obtained. This number corresponds to 286 trough concentration measurements and 326 samples drawn at 1, 2, 3, 4, 5, or 6 hours after dose intake. The measurements obtained up to 6 hours, representing 27 full (seven or six time points) and 57 sparsely sampled (four time points) AUCs, allowed the calculation of 84 $AUCs_{0-12h}$.
The performance of the assays was investigated by the quality control (QC) samples, which were included in each series of everolimus patient samples. Accuracy bias for FPIA and LC-MS/MS was determined by calculating the concentration of the control sample as a percentage of the nominal concentration (determined by the manufacturer) using the formula: $[(C_c - C_n)/C_n*100\%]$ with C_c as the concentration of the control sample and C_n as the nominal concentration. Assay performance, in terms of limits of quantification, was in agreement with the guidelines regarding bioanalytical method validation of *Shah et al.* [9].

Innofluor Certican Assay System

Quantification of everolimus blood concentrations was performed with FPIA (Seradyn Inc, Indianapolis, IN) on a TDxFLx instrument from Abbott Diagnostics (Abbott Park, IL). FPIA is a homogeneous fluorescence polarization assay using a polyclonal rabbit antibody directed against everolimus [10]. Routine measurements were performed according to manufacturer's guidelines [10]. Everolimus calibrators (0, 2.5, 5.0, 10.0, 20.2, and 39.1 μg/L) and controls levels 1, 2, and 3 were obtained from Seradyn (Seradyn Inc). Calibrators and controls were prepared by gravimetric addition of everolimus to a human blood hemolysate matrix. Each calibrator was value-assigned by the manufacturer's reference laboratory using a validated HPLC-MS method [11,12].
Blood samples as well as calibrator and control samples (600 μL) were pretreated with methanol (700 mL) (Merck, Darmstadt, Germany) and precipitation reagent (100 μL), vortexed for at least 10 seconds, and centrifuged for 5 minutes at 4000 rpm. Subsequently, 700 μL of each supernatant was divided into two identical samples of 350 μL and transferred into two reaction cells.
According to the manufacturer's specifications, the lower limit of quantification was 2.0 μg/L, whereas the upper limit of quantification was 40 μg/L [10]. Samples with values above 40 μg/L were diluted four times with calibrator A (everolimus-free). Lot-dependent calibrators were used. FPIA within-run accuracy and precision were determined by

analyzing two controls in duplicate. Between-run precision and accuracy were evaluated by analyzing the QC results of each determination for the duration of the study. Controls used for FPIA were: control level 1: 4.0 µg/L displayed an accuracy bias of 13.0% and an imprecision with a coefficient of variation (CV) of 19.9%; control level 2: 11.5 µg/L had an accuracy bias of 1.6% and an imprecision with a CV of 15.4%; and finally control level 3: 23.0 µg/L showed an accuracy bias of 2.2% and an imprecision with a CV of 13.5% (n = 78).

Liquid Chromatography–Tandem Mass Spectrometry Assay

Quantification of everolimus with LC-MS/MS was performed with a validated assay capable of analyzing everolimus, sirolimus, and tacrolimus simultaneously. The system consisted of an Ultimate 3000 autosampler, a thermostatted column compartment TCC 100, and a p680 HPLC dual low-pressure gradient pump (analytical). All were purchased from Dionex Benelux BV (Amsterdam, The Netherlands). The MS/MS used was a Quattro micro API Tandem Quadrupole system from Waters Corporation, Milford, MA. Two hundred microliters of blood samples, controls, or calibrators were diluted with 200 µL 0.1M $ZnSO_4$ and 500 µL internal standard solution. Internal standard solution consisted of 100 µL 16 µg/L desmethyl sirolimus in methanol and 25 mL acetonitrile (LiChrosolv; Merck KGaA, Darmstadt Germany). A 6 + 1 multilevel calibrator set (0, 2.1, 6.0, 12.3, 18.2, 25.3, 46.5 µg/L) was used, which was obtained from Chromsystems (Munich, Germany). Blood control levels 1, 2, and 3 were obtained from RECIPE (Munich, Germany). After diluting, vortex mixing for 2 minutes followed by 5 minutes of centrifugation at 13,000 rpm was conducted. After centrifugation, the supernatant was transferred into a cylindrical crimp neck autosampler vial.

A 50 µL aliquot of supernatant was injected into an online solid phase extraction column (Cartridge Hysphere 5C18 HD, 7-µm particle size 10×2 mm; Spark, Emmen, The Netherlands) for enrichment. For sample cleanup, two mobile phases were used: mobile phase A: 0.1% v/v formic acid + 2 mM ammonium acetate in water and mobile phase B: 0.1% v/v formic acid + 2 mM ammonium acetate in methanol. The elution gradient used on the solid phase extraction column was 50% A and 50% B for 2 minutes followed by 0.8 minute 100% B and 1.5 minutes 50% A and 50% B for elution of everolimus and internal standard for isocratic liquid chromatography on the precolumn (Hypersil 4×2 mm; Phenomenex, Utrecht, The Netherlands) and analytical column (Hypersil Phenyl 50×3 mm, 3-µm particle size; Thermo Scientific, Geel, Belgium). The column oven was set at 55°C. The elution gradient for chromatographic separation to the MS was 10% A

and 90% B at a flow rate of 600 µL/min. Mass spectrometric detection was in positive ion mode using selected reactant monitoring (everolimus m/z 975.7→908.3, internal standard, desmethyl sirolimus, m/z 901.7→834.3).

The lower limit of quantification for everolimus was 0.2 µg/L determined with the following criteria: accuracy limits of 80% to 120% and imprecision CV less than 20%. LC-MS/MS within-run accuracy and precision were determined by analyzing the three controls in duplicate. Interassay precision and accuracy were evaluated by analyzing the QCs of each determination, which provided data for this study. The accuracy biases of the calibrators (2.1, 6.0, 12.3, 18.2, 25.3, 46.5 µg/L) were 3.3, –1.8, –1.1, –0.9, –0.4, and 0.5%, respectively with CVs for imprecision of 6.1%, 4.0%, 3.1%, 2.8%, 2.4%, and 1.6%, respectively (n = 105), QC samples used for LC-MS/MS were: control level 1 with a theoretical value of 3.3 µg/L had an accuracy bias of –7.3% and a CV for imprecision of 7.0%; control level 2: 10.5 µg/L had an accuracy bias of –2.7% and a CV for imprecision of 5.2%. Finally, control level 3: 17.2 µg/L had an accuracy bias of –2.2% and a CV for imprecision of 5.5% (n = 115).

Statistical Analysis

Agreement between LC-MS/MS and FPIA measurements of everolimus whole blood concentrations was determined using Bland and Altman analysis [13–15]. Passing-Bablok regression analysis was used to confirm the Bland-Altman results and to check for a linear relationship between the two methods. Analysis was performed with Microsoft Office Excel (Microsoft Inc, Redmond, WA) add-in Analyse it statistics software (Analyse-it Software, Ltd, Leeds, UK). Areas under the curve (AUCs) were calculated using the linear trapezoidal rule with everolimus trough concentrations used as 12-hour values. Figures were made with S-Plus (Insightful Corporation, Seattle, WA).

As suggested by Altman et al [16], we introduced a clinical acceptance limit to be able to decide whether two methods were in agreement. We chose the clinical acceptance limit to be a 20% range around the average difference between the methods. This clinical acceptance limit was based on the lowest everolimus oral dose available of 0.25 mg and a dose change that would be clinically relevant. This can be explained by the fact that the same exposure in terms of either trough concentration or AUC could be reached for instance with 1 mg for one individual versus 3 mg in another. A 20% difference in dose would mean a 0.2 mg (in clinical practice 0.25 mg) and 0.6 mg (in clinical practice 0.5 mg) dose difference, respectively.

Results

The everolimus concentrations ranged from 2.3 to 59.2 µg/L and 2.1 to 50.0 µg/L measured by FPIA and LC-MS/MS, respectively. Everolimus trough blood concentrations in 286 samples ranged from 2.3 to 25.0 µg/L and 2.1 to 18.0 µg/L measured by LC-MS/MS and FPIA, respectively. The everolimus whole blood pharmacokinetic curves constructed from the full AUCs collected 2 weeks after conversion from patients receiving 3 mg everolimus are presented in Figure 1. The mean (n = 20) AUC_{0-12h} determined with FPIA was 166 µg*h/L (± 57) and the mean (n = 20) AUC_{0-12h} determined with LC-MS/MS was 140 µg*h/L (± 41) (P ,0.001; paired Student t test).

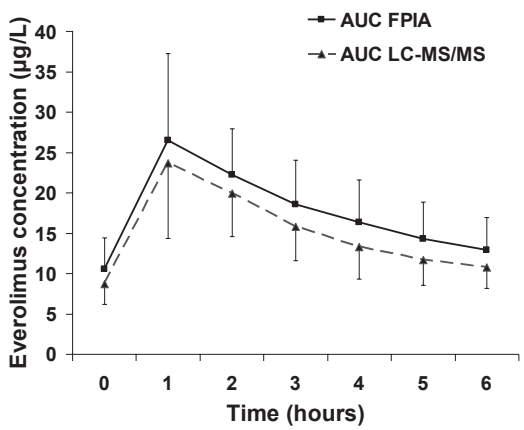

Figure 1: Mean everolimus whole blood concentration profile (0–6 hours after administration) of 3 mg everolimus start dose (n = 20) determined in renal transplant recipients using fluorescence polarization immunoassay (FPIA) and liquid chromatography–tandem mass spectrometry (LC-MS/MS). Bars represent the standard deviation. AUC, area under the curve.

First of all, a Passing-Bablok analysis was performed to give insight into the relationship between the two methods (Fig. 2A–B). In particular, linearity between the two methods and deviation from the line of identity were investigated. Figure 2A shows the relationship between trough concentrations measured with FPIA and LC-MS/MS, whereas a similar figure for all measurements is presented in Figure 2B. The slope of the regression equation for the trough concentrations was higher than the slope of the regression equation describing all concentrations. Furthermore, the regression equation for trough concentrations demonstrated a constant bias (95% confidence interval [CI]: –1.9 to –0.5) for the intercept and a proportional bias (95% CI: 1.3–1.5) for the slope. In contrast, the

regression equation for all concentrations only showed a proportional bias for the slope (95% CI: –0.2 to –0.6). Finally, when the Passing-Bablok analysis for all concentrations was tested for linearity with the cusum test, a significant deviation from linearity was observed (P< 0.01), which was not the case for the trough concentrations (P > 0.10).

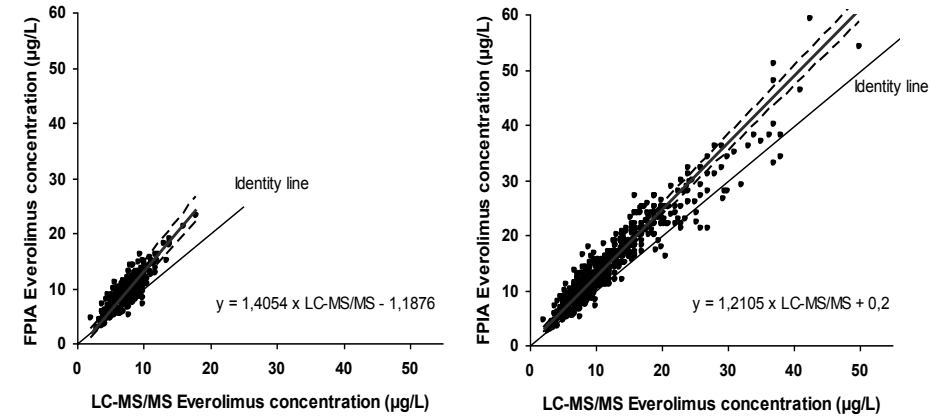

Figure 2: Passing-Bablok comparison plot of the everolimus trough concentrations of renal transplant recipients (n = 286) (A) and all measurements (n = 612) (B) obtained by liquid chromatography–tandem mass spectrometry (LC-MS/ MS) and fluorescence polarization immunoassay (FPIA) with the line of identity. Dashed lines represent the 95% confidence intervals. The solid line within the dashed lines represents the regression equation.

To test whether the two methods were in agreement, the Bland-Altman plot of the difference between the two methods against the mean of the two methods was constructed as shown in Figure 3A. This Bland-Altman plot shows that the absolute difference ranged from –6.0 to 16.7 µg/L with a mean difference of 2.5 µg/L. Differences between FPIA and LC-MS/MS increased with higher mean concentrations of both methods. As has been described previously [13,14,16], the influence of this trend should be taken into account by either a log transformation of the data or using a concentration ratio (FPIA:LC-MS/ MS). For interpretation purposes, we chose the second option [14]. The ratio was plotted against the mean concentration. The proportional trend disappeared and the range of the difference decreased with higher mean concentrations (Fig. 3B). The geometric mean and median of the concentration ratio were 1.23, but the ratio ranged from 0.65 to 2.14. Moreover, the range of the concentration ratio FPIA:LC-MS/MS below a mean concentration of 15 µg/L is much larger than above a mean concentration of 15 µg/L

(0.65–2.14 below compared with 0.78–1.69 above). At the start of this comparison, we set the clinical acceptance limits at 6 20% of the mean ratio. As can be seen from Figure 3B, this acceptance limit falls well within the 95% CI (mean ± 1.96 standard deviation). More specifically, 19% (119 of 612) of the data points exceeded the clinical acceptance limits. The majority of these data points (80%) were below a mean concentration of 15 µg/L. Trough concentrations represented 73% of the data points that were lower than 15 µg/L and were outside the upper acceptance limit (36 of 49). This indicates that concentrations in the lower range such as trough concentrations vary more between the two methods. Indeed, this is likely to be the case because the Passing-Bablok analysis also showed a difference for the two methods regarding linearity and the regression equation when comparing trough concentrations and all concentrations.

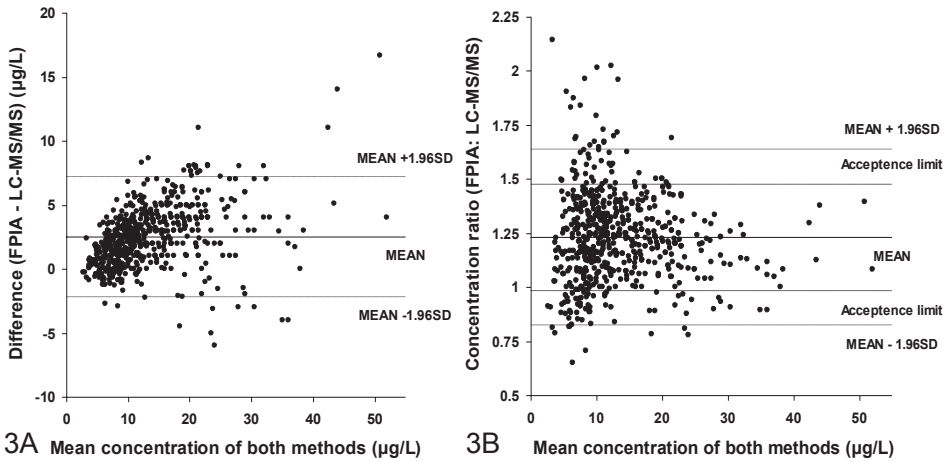

Figure 3: Bland-Altman plot of difference (A) and concentration ratio (B) against mean everolimus concentration measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS) and fluorescence polarization immunoassay (FPIA) (n = 612) showing data range, mean (2A: 2.5 µg/L, 2B: 1.23) with 95% limits of agreement and clinical acceptance limits (1.48 and 0.98).

Agreement between FPIA and LC-MS/MS was also investigated using AUC_{0-12h} values as presented in the AUC ratio plot (FPIA:LC-MS/MS) in Figure 4. The ratio plot showed a mean ratio and median of 1.24 meaning that, on average, AUC_{0-12h} measured with FPIA resulted in a 24% higher AUC_{0-12h} than with LC-MS/MS. The ratio ranged from 0.92 to 1.94 showing a large variability. All except two data points were within the 95% CIs of the mean ratio, but the acceptance limit was crossed by 10% of the data points, again confirming that the two methods are not in agreement.

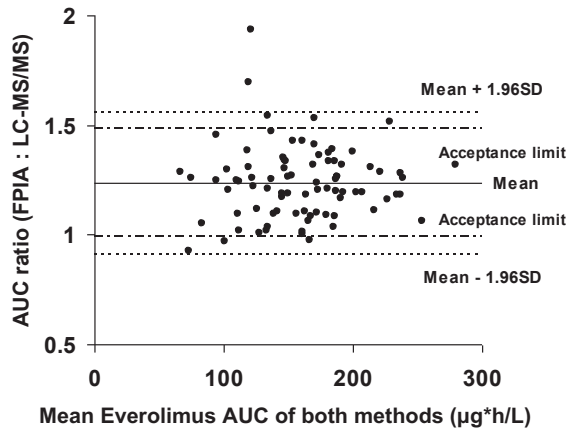

Figure 4: Bland-Altman plot of ratio against mean area under the curve (AUC) obtained by liquid chromatography–tandem mass spectrometry (LC-MS/MS) and fluorescence polarization immunoassay (FPIA) (n = 84) showing data range, mean (1.24) with 95% limits of agreement, and clinical acceptance limits (1.48 and 0.99).

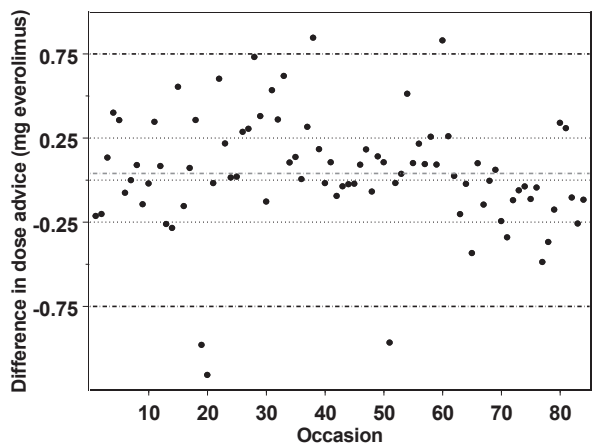

Figure 5: Difference in dosage advice (fluorescence polarization immunoassay [FPIA] versus liquid chromatography– tandem mass spectrometry [LC-MS/MS]) given in the study period showing range and mean (0.04 mg). Dose difference limits –0.25, 0.25, –0.75, and 0.75 are presented as dashed lines. Target area under the curve (AUC) FPIA = 150 µg*h/L, target AUC LC-MS/MS = 120 µg*h/L.

To investigate whether this variability would have clinical implications, dose adjustments were calculated using the 84 $AUCs_{0–12h}$ from 28 patients. The target $AUC_{0–12h}$ for FPIA was 150 µg*h/L and therefore target $AUC_{0–12h}$ for LC-MS/MS was set at 120 µg*h/L because we found that FPIA results were 24% higher than LC-MS/MS results. Figure 5 shows the

difference in individual dosage advice based on everolimus AUC_{0-12h} as determined by FPIA or LC-MS/MS. The differences ranged from –1.18 mg to 0.85 mg with an average difference of 0.04. Differences of 0.5 mg or higher would cause clinical concern. All extreme outliers (greater than 0.75 mg) were from different patients. The graph shows a large variability in everolimus doses leading to actual differences in dose adjustments up to 1.25 mg when using the average ratio (FPIA:LC-MS/MS) as a correction factor.

To investigate the variability of the methods with time during clinical monitoring of everolimus, dose-corrected trough concentrations from six different patients as determined by the two methods were plotted chronologically in Figure 6.

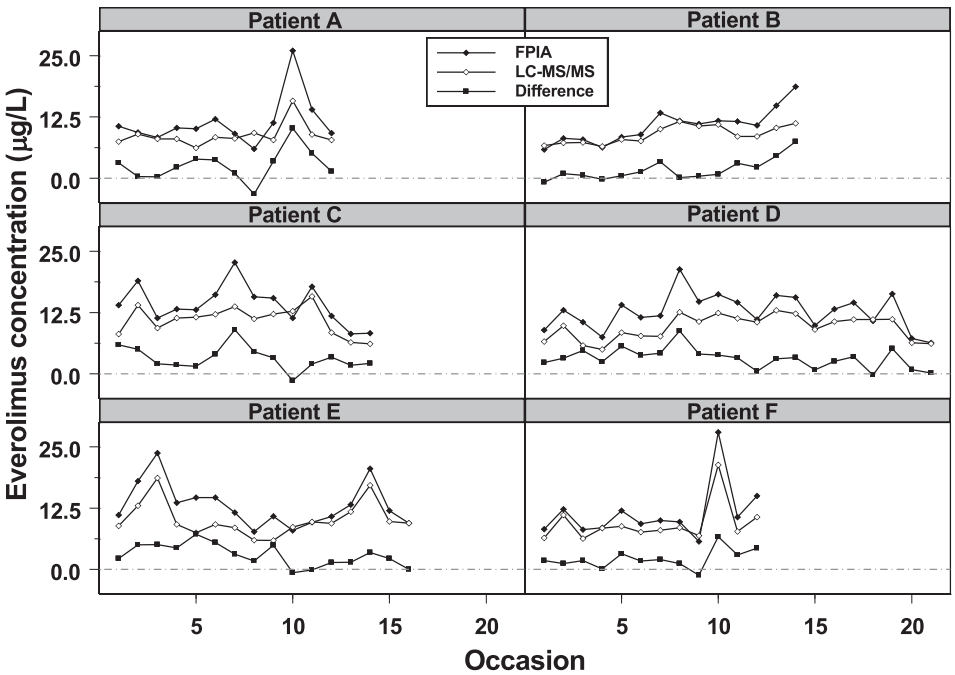

Figure 6: Dose-corrected trough concentrations at monitoring occasions measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS) and fluorescence polarization immunoassay (FPIA) showing variability in difference between FPIA and LC-MS/MS in six patients.

This means that all concentrations were scaled to an everolimus dose of 3 mg twice daily to correct for dose-related concentration differences. Next to the two lines of concentrations measured with both methods, a third line is introduced, which indicates the differences between the FPIA line and LC-MS/MS line. The horizontal axis reflects the occasion number. The FPIA line ascends and descends, whereas the LC-MS/MS line remained more

stable, resulting in pronounced fluctuations of the difference line. If both methods showed no difference at all, the difference line would be a flat line. Overall, FPIA-determined everolimus trough concentrations were higher than those determined by LC-MS/MS, but the difference between the two methods was not constant. This is illustrated by multiple peaks with the FPIA methods in this figure, whereas a straight line is observed for LC-MS/MS. For instance, this is the case for Patient B at occasion 12 or Patient C at occasion 7.

Discussion

In this study, the two widely used analytical techniques for everolimus blood concentration measurement, FPIA and LC-MS/MS, were compared using a large series of blood samples from stable adult renal transplant recipients. Overall, this study demonstrated that these two methods were not in agreement, because the preset acceptance limit was exceeded. Furthermore, this study showed that everolimus concentrations determined by FPIA are, on average, 23% higher than LC-MS/MS. However, the variability found between FPIA and LC-MS/MS could be twofold for concentrations lower than 15 µg/L or AUC_{0-12h}. This suggests a relatively large effect on variability of FPIA versus LC-MS/MS when monitoring trough concentrations. Moreover, the large variability of the everolimus concentrations determined with FPIA can lead to differences in everolimus doses of 1.25 mg compared with LC-MS/MS when applying dose adjustments based on a preset target AUC despite using a correction factor of 23%. Finally, the within-patient variability for trough concentrations appeared to be higher using the FPIA method.

Previous method comparisons for everolimus [3,4,17,18] were based mainly on trough concentration measurements and not AUCs. Differences between the methods using trough concentrations, other concentration time points, or the AUC were not investigated, and implications of the differences between the methods on dose adjustments remained unclear. Moreover, in previous studies, samples were obtained from patients on a cyclosporine A, prednisolone, and everolimus based immunosuppressive regimen. Because an interaction between cyclosporine A and everolimus has been described [19], this could affect the results.

When comparing the regression equations obtained with this study and earlier studies, the regression equation: FPIA = 1.21 x LC-MS/MS + 0.2 was comparable to those of *Salm et al*: FPIA =1.19 × HPLC-MS + 0.5118 and *Koster et al*: 1.34 × LC-MS/MS + 0.855 found

in patients on a everolimus with a calcineurin inhibitor regimen. *Dially et al.* found, with a smaller number of samples of renal and heart transplant patients; FPIA = 0.851 × LC-MS/MS + 1.773 [4], a larger difference in slope but a higher intercept, possibly caused by use of different internal standards [4,20]. *Khoschsorur et al.* [17] found in their trough concentration comparison of FPIA versus HPLC an equation of FPIA = 1.11 × HPLC + 0.378 [17]. The difference with *Khoschsorur et al.* [17] in the trough concentration equation could be caused by higher process efficiency of LC-MS/MS, population differences, and cyclosporine A use.

In the present study, different QC samples were used for determining within-day and between-day precision for the two methods. It would be best to use the same controls for both methods but this was not possible as a result of the prefabricated packages with FPIA. However, the controls used were similar to those used by other research groups [3,4,17,18]. This study used a large number of samples obtained from 28 individuals. This means that multiple samples from individual patients were used for the comparison. To exclude any bias from repeated measurements we repeated all the analyses on data sets containing only one measurement per individual. All conclusions remained the same when applying that procedure. In fact, one should not consider these as repeated measurements because different days with different clinical situations (alternating hematocrit, co-medication) and different everolimus doses within an individual were compared.

Bland-Altman analysis of all everolimus concentrations showed large variability and a lack of agreement between the two methods. The majority of the data points that exceeded the acceptance limits were below a mean concentration of 15 µg/L. The ratio between AUC_{0-12h} determined with FPIA and AUC_{0-12h} determined with LC-MS/MS had a large variability corresponding to large differences in dosage advice. The dosing differences between 0.25 mg and –0.25 mg were considered not relevant because the lowest tablet dose available is 0.25 mg. Nevertheless, a large number of data points exceeded the ± 0.25-mg line, resulting in clinically relevant dosing differences. However, a difference of 0.25 mg on a total of dose of 3 mg has less impact than a difference of 0.25 mg on a total dose of 1 mg. This clinical impact is taken into account by our clinical acceptance limit. A 20% difference on a dose of 1 mg or 3 mg leads to a maximum adjustment of the everolimus exposure of 20%. The large number of data points that exceeded the clinical acceptance limit indicated that the two methods cannot be interchanged. All data points exceeding or near the 20% clinical acceptance limits in Figure 4 correspond with large differences in dosing advice in Figure 5. Therefore, it is not desirable or acceptable to use the average AUC ratio of 1.24 or the

average concentration ratio of 1.23 as correction factors, because this could lead to clinically relevant differences in everolimus dose adjustments. The differences in dosage advice that were found could raise the question whether the target AUC of LC-MS/MS was calculated correctly. We investigated this as follows; compared with a target AUC of 115 µg*h/L or 125 µg*h/L, the chosen target AUC of 120 µg*h/L resulted in a mean difference closest to zero, which means that the average dosage advice of FPIA and LC-MS/MS is similar. With any other approach, the dose differences are higher.

The variability of the concentration ratio was higher for concentrations less than 15 µg/L. This of course could be the result of nonspecific binding of the antibodies [10]. The majority of the concentrations lower than 15 µg/L were trough concentrations. The concentrations are normally relatively low at the trough concentration (Fig. 3) and metabolites are present in relatively high concentrations before the next dose. Therefore, crossreactivity of these metabolites would probably have a greater impact on trough concentrations. For cyclosporine A, which has a similar metabolism [21], *Schütz et al.* [22] demonstrated that the relative cyclosporine A metabolite concentration was higher at trough concentration than at 2 hours after dose intake. *Johnston et al.*[23] showed higher crossreactivity at trough concentration for cyclosporine A as compared with 2 hours after dose intake. Crossreactivity of the FPIA assay was investigated by *Tobin Strom et al.* [24,25]. They identified metabolite patterns of everolimus in trough blood samples of renal transplant patients and found metabolite concentrations of the three main metabolites: 46-hydroxy 44,1% (0–784%), 24 hydroxy 7.7% (0–85.6%), and 25-hydroxy 14.4% (0– 155.4%) (25). For FPIA, they found crossreactivity of 1% or less for 46-hydroxy and 24-hydroxy everolimus and 6% or less for 25-hydroxy everolimus [24]. This suggests that at least part of the overestimation of FPIA may be caused by crossreactivity.

The variability in differences in dosage advice showed that the risk of suboptimal dosage advice is present and clinically relevant. With FPIA, the question raises if an elevated or reduced trough concentration of a patient sample result is correct or the result of the variability of the assay. In the first 6 months after transplantation (patients using cyclosporine A and everolimus), an incorrect dose adjustment of 25% (0.75 on 3 mg total) resulting in too low an exposure to everolimus increases the risk of rejection up to 10% [26]. The risk of toxicity such as trombocytopenia after a similar incorrect dose adjustment resulting in an everolimus exposure 25% too high could increase up to 7% depending on the everolimus blood concentrations reached [26]. In general, LC-MS/MS is a more specific, more stable, and more accurate method for everolimus TDM. LC-MS/

MS is able to simultaneously measure several immunosuppressive drugs in a single run and can provide high specificity and sensitivity. The limitations are high initial capital investment and highly trained analysts for operation and maintenance. Because of this mainly financial limitation, not every clinical laboratory has a LC-MS/MS at its disposal [6]. Although FPIA is easy to operate, the analysis costs are relatively high.

Conclusion

The analytical methods FPIA and LC-MS/MS are not in agreement. Everolimus blood concentration measurement using FPIA results in higher everolimus concentrations compared with LC-MS/MS. Furthermore, LC-MS/MS outperforms FPIA for clinical monitoring and intervention of everolimus therapy in adult renal transplant recipients on duo therapy with prednisolone. Specifically, the use of FPIA can lead to clinically relevant differences in everolimus dosage advice and higher intra-patient variability.

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Population pharmacokinetics and pharmacogenetics of everolimus in renal transplant patients

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Abstract

Everolimus is a novel macrolide immunosuppressant used in the prevention of acute and chronic rejection of solid organ transplants. Everolimus is actively being investigated worldwide as a non-nephrotoxic alternative for calcineurin inhibitors. Its highly variable pharmacokinetics and narrow therapeutic window makes it difficult to maintain everolimus at adequate exposure to prevent serious adverse effects. The primary objective of this study was to improve prediction of everolimus systemic exposure in renal transplant patients by describing the pharmacokinetics of everolimus and identifying the influence of demographic factors and a selection of polymorphisms in genes coding for ABCB1, CYP3A5, CYP2C8 and PXR. The secondary objective of this study was to develop a limited sampling strategy to enable prediction of everolimus exposure in an efficient way and to compare it with the widely used trough concentration (C_{trough}) monitoring. A total of 783 blood samples were obtained from 53 renal transplant patients who had been switched from a triple therapy of cyclosporine, mycophenolate mofetil and prednisolone to a calcineurin inhibitor free dual therapy of everolimus (twice daily) and prednisolone. Everolimus blood concentrations were analyzed in whole blood using liquid chromatography tandem mass spectrometry during routine therapeutic drug monitoring targeting an AUC_{12} of 120 μg*h/L. A population pharmacokinetic model was developed and demographic factors and genetic polymorphisms in genes coding for ABCB1, CYP3A5, CYP2C8, PXR were included as covariates. In addition, a limited sampling strategy was developed. Maintaining everolimus systemic exposure at an AUC_{12} of 120 μg*h/L resulted in low rejection rates but considerable numbers of adverse events and toxicity. Everolimus pharmacokinetics were best described by a two-compartment model with lag-time (CL/F = 17.9L/H, V_1/F = 148 L and k_a = 7.36 h^{-1}). Ideal Body Weight was significantly related to V_1/F. None of the selected polymorphisms in genes coding

for enzymes involved in distribution and metabolism of everolimus had a significant influence on everolimus pharmacokinetics. The pharmacokinetic limited sampling model (C_{trough} and C_2) resulted in a significant improved prediction of everolimus exposure compared to the widely used C_{trough} monitoring. A two compartment pharmacokinetic model with lag-time describing the concentration time profile of oral everolimus in renal transplant patients has been developed using pharmacokinetic modeling. Ideal Body Weight significantly influenced V_1/F of everolimus, however the selected polymorphisms in genes coding for ABCB1, CYP3A5, CYP2C8, PXR had no clinically relevant effect on everolimus pharmacokinetics. Everolimus C_{trough} and C_2 as limited sampling model can be used to accurately estimate everolimus systemic exposure, an improvement compared to the widely used C_{trough} monitoring.

Introduction

Everolimus (Certican®), is a non-nephrotoxic alternative for calcineurin inhibitors (CNIs) in renal transplantation. Its narrow therapeutic window, together with high variability in pharmacokinetics makes Therapeutic Drug Monitoring (TDM) useful to individualize the dose and thereby prevent toxicity or rejection. Everolimus is often prescribed in combination with CNIs. Due to CNI-related nephrotoxicity CNI minimizing and CNI free strategies are being actively investigated worldwide [1–6]. Everolimus is metabolized by cytochrome P450 enzymes CYP3A4, CYP3A5 and CYP2C8. Everolimus is also a substrate for the efflux pump P-Glycoprotein (ABCB1)[7,8]. The nuclear pregnane X receptor (PXR) mediates expression of CYP3A4 and could therefore also influence everolimus pharmacokinetics [9]. Differences in activity of metabolizing enzymes are likely to be responsible for a main part of the variability in pharmacokinetics [7,8]. Genetic polymorphisms in genes encoding these enzymes could explain variability in everolimus pharmacokinetics but this has only been investigated sparsely. Two studies investigated the role of genetic variants in genes encoding for CYP3A5 in everolimus pharmacokinetics but no relationship was identified [10,11]. However, these studies had the limitation that data were derived from patients on cyclosporine, prednisolone and everolimus and did not use population pharmacokinetic analysis. This approach enables to differentiate between inter-patient and intra-patient variability and results in enhanced statistical power to identify covariates. Therefore we

chose to perform a population pharmacokinetic analysis including demographic and pharmacogenetic data as potential covariates. There has been one previous population pharmacokinetic study of everolimus in renal transplant patients [12], but data were derived from patients on a combined cyclosporine and mTOR based regimen. More importantly, it lacked inclusion of genetic polymorphisms. Since cyclosporine affects everolimus pharmacokinetics, the results from this population pharmacokinetic study cannot be extrapolated to everolimus and prednisolone dual therapy. Everolimus TDM is mostly performed based on trough blood concentrations (C_{trough}) [13] but some clinics use area under the blood concentration curve (AUC) [3]. Whether everolimus therapy should be based on troughs or on AUC warrants further investigation. This choice has a practical aspect based on the fact that TDM based on trapezoidal AUC is more laborious for both patients and clinic since multiple concentration markers are needed to accurately calculate AUC. A less laborious method such as a limited sampling strategy could influence the choice of performing TDM based on C_{trough} or AUC.

The primary aim of the current study was to describe the population pharmacokinetics of everolimus in renal transplant patients following oral administration of everolimus twice daily in absence of a CNI and to identify covariates explaining variability. The secondary aim was to develop a limited sampling strategy to enable prediction of everolimus exposure in an efficient way and to compare it with the widely used C_{trough} monitoring.

Patients and Methods

Patients

Clinical data from 53 stable renal transplant recipients treated with immunosuppressive dual therapy consisting of everolimus (Certican®, Novartis, Basel, Switzerland) and prednisolone, participating in a prospective, open, randomized, multicenter study were studied from 6-24 months after transplantation [3]. During the first six months, patients were treated with a immunosuppressive regimen including cyclosporine, prednisolone and mycophenolate mofetil, Thereafter a scheduled biopsy was performed. Patients whose biopsy showed no sign of rejection were included and randomized into three groups [3]. Subsequently cyclosporine and mycophenolate mofetil were discontinued [3]. From this moment on data was collected for this pharmacokinetic study. Everolimus therapy was started at an oral dose of 3 mg twice daily and was supported by routine TDM based on

C_{trough} concentrations and AUC from time zero to 12 hours (AUC_{12}). Dose adjustments were based on AUC_{12}. TDM was aimed at a target of 120 µg*h/L. The study was approved by the Medical Ethics Committee of Leiden University Medical Center (Leiden, the Netherlands) and patients gave written informed consent.

Bioanalytics

TDM was performed on the basis of trapezoidal rule (blood concentration at t = 0,1,2,3,4,5 and 6 hours or t = 0,1,2 and 3 hours) in kinfit menu using MW/Pharm® 3.5 (Mediware, Groningen, The Netherlands)[14]. TDM samples were taken at week 6, week 36, week 62 and week 78 after conversion and determined in whole blood by a validated liquid chromatography-mass spectrometric (LC-MS) method in two laboratories [15,16]. Quantification of everolimus with liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed with a validated assay capable of analyzing everolimus, sirolimus and tacrolimus simultaneously. The system consisted of an Ultimate® 3000 autosampler, a thermostatted column compartment TCC 100 and a p680 HPLC dual low-pressure gradient pump (analytical). All were purchased from Dionex Benelux B.V (Amsterdam, The Netherlands). The MS/MS used was a Quattro micro™ API Tandem Quadrupole system from Waters corporation, Milford, U.S.A.

200 µL of blood samples, controls or calibrators were diluted with 200 µL 0.1 M $ZnSO_4$ and 500 µL internal standard solution. Internal standard solution consisted of 100 µL 16 µg/L desmethyl sirolimus in methanol and 25 mL acetonitrile (LiChrosolv, Merck KGaA, Darmstadt Germany). A 6+1 multilevel calibrator set (0, 2.1, 6.0, 12.3, 18.2, 25.3, 46.5 µg/L) was used, which was obtained from Chromsystems (Munich, Germany). Blood control levels 1, 2 and 3 were obtained from RECIPE (Munich, Germany). After diluting, vortex mixing for 2 minutes followed by 5 minutes of centrifugation at 13000 rpm was conducted. After centrifugation, the supernatant was transferred into a cylindrical crimp neck autosampler vial.

A 50 µL aliquot of supernatant was injected into an online solid phase extraction (SPE) column (Cartridge Hysphere 5C18 HD, 7µm particle size 10 x 2 mm, Spark, Emmen, The Netherlands) for enrichment. For sample clean-up two mobile phases were used: mobile phase A: 0.1% v/v formic acid + 2 mM ammonium acetate in water and mobile phase B: 0.1% v/v formic acid + 2 mM ammonium acetate in methanol. The elution gradient used on the SPE column was 50% A and 50% B for 2 minutes, followed by 0.8 minute 100% B and 1.5 minutes 50% A and 50% B for elution of everolimus and IS for isocratic liquid

chromatography on the pre-column (Hypersil 4 x 2 mm, Phenomenex) and analytical column (Hypersil Phenyl 50 x 3 mm, 3 µm particle size, Thermo Scientific). The column oven was set at 55°C. The elution gradient for chromatographic separation to the MS was 10% A and 90% B at a flow rate of 600 µL/min. Mass-spectrometric detection was in positive ion mode using selected reactant monitoring (everolimus m/z 975.7→908.3, internal standard, desmethyl sirolimus, m/z 901.7→834.3). The lower limit of quantification for everolimus was 0.2 µg/L. Supplementary Table I shows the samples distribution.

Supplementary table I: Pharmacokinetic data distribution.

Pharmacokinetic data	Mean / Number	SD	Median	Range
Concentrations (µg/L)	12.1	6.7	12.1	2.6 - 50
Samples per patient	14.8	6.9	14	7 - 29
Total Samples	783			
t=0	146			
t=1	138			
t=2	140			
t=3	148			
t=4	73			
t=5	68			
t=6	70			
Samples per occasion (official visit)				
Week 32	271			
Week 52	148			
Week 78	75			
Week 104	193			
Other	96			

Supplementary Table **II:** Primers and Probes for TaqMan and Pyrosequence Analysis.

TaqMan (SNP)	ASSAY	Target	Sequence 5'-3'	Modification	Reference
ABCB1 C1236t (rs1128503)	Predesigned	PCR-f	CACCGTCTGCCCACTCT		C_758662_10
		PCR-r	GTGTCTGTGAATTGCCTTGAAGTTT		
		PCR-G	TTCAGGTTCAGACCCTT	VIC	
		PCR-T	CAGGTTCAGGCCCTT	FAM	
ABCB1 G2677T (rs2032582)	Custom	PCR-f	CTTAGAGCATAGTAAGCAGTAGGGAGT		
		PCR-r	GAAATGAAAATGTTGTCTGGACAAGCA		
		PCR-G	TTCCCAGCACCTTC	VIC	
		PCR-T	TTCCCAGAACCTTC	FAM	
ABCB1 T3435C (rs1045642)	Predesigned	PCR-f	ATGTATGTTGGCCTCCTTTGCT		C_7586657_20
		PCR-r	GCCGGGTGGTGTCACA		
		PCR-G	CCCTCACAATCTCT	VIC	
		PCR-T	CCCTCACGATCTCT	FAM	
CYP2C8e8 (rs10509681)	Predesigned				C_25625782_20
CYP2C8e3 (rs11572080)	Predesigned				C_25625794_10
Pyrosequence (SNP)	**ASSAY**	**Target**	**Sequence 5'-3'**	**Modification**	**Reference**
ABCB1 129CT (rs3213619)		PCR-f	TCGAAGTTTTTATCCCA	Biotin	
		PCR-r	CCTCCTGGAAATTCAACCTGTT		
		Sequence primer	TACTCCGACTTTAGTGGAAAGACC		
		Target sequence	CTG/ACTCGAATGAG		
CYP3A5*3 (rs776746)		PCR-f	CTGCCTTCAATTTTTCACT	Biotin	
		PCR-r	TATGTTATGTAATCCATACCCC		
		Sequence primer	AGAGCTCTTTTGTCTTTCA		
		Target sequence	A/GTATCTC		
CYP3A5*6 (rs10264272)		PCR-f	TCTTTGGGGCCTACAGCATG	Biotin	
		PCR-r	AAAGAAATAATAGCCCACATACTTATTGAGAG		
		Sequence primer	AGAAACCAAATTTTAGGAA		
		Target sequence	CTTC/TTTAG		
PXR (NR1|2) G-24113A (rs2276706)		PCR-f	GAATCATGTTGGCCTTGCTGC	Biotin	
		PCR-r	GCATCAGTAATGGGGCTCAAC		
		Sequence primer	TCTCCTCATTTCTAGGGT		
		Target sequence	C/TCACCCTAG		
PXR(NR1|2) A+7635G (rs6785049)		PCR-f	AGCCATCCTCCCTCTTC	Biotin	
		PCR-r	CAGCAGCCATCCCATAATC		
		Sequence primer	CATAATCCAGAAGTTGGG		
		Target sequence	GGC/TGAGAGGAA		

f, forward orientated; r, reverse orientated; ABC, ATP-binding casette; CYP, cytochrome P450; PXR, pregnane X receptor; PCR, polymerase chain reaction.
Modification: VIC and FAM are fluorescent dyes, biotin is necessary to obtain single stranded DNA.

Genotyping Assays

DNA was isolated from EDTA blood collected from patients. ABCB1-1236C>T, ABCB1-3435C>T, ABCB1-2677T>G, CYP2C8-467603213T>C and CYP2C8-47631494C>T were determined with TaqMan 7500 (Applied Biosystems, Nieuwerkerk a.d. IJssel, The Netherlands) with custom designed assays, according to manufacturers' protocol. ABCB1-129T>C, CYP3A5-6986A>G, CYP3A5-7225G>A, PXR-G-24113A, PXR-A+7635G were determined with Pyrosequencer 96MA (Isogen, IJsselstein, The Netherlands). PCRs contained 10 ng of DNA and 5 pmol of each PCR primer in a total volume of 12 mL. Cycle conditions were initial denaturation for 15 minutes at 95°C, 35 cycles of 95°C, 55°C, and 72°C each for 30 seconds, ended by 10 minutes at 72°C. The pyrosequence reactions were performed according to manufacturers' protocol. Primers and probes used in the TaqMan-based genotyping assays and primers and sequences used in the pyrosequence assays for each SNP are listed in Supplementary Table II. 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. Haplotype analysis for ABCB1 and CYP2C8 SNPs was performed using gPLINK with haplotypes set with a certainty greater than 0.97.

Pharmacokinetic modeling

Nonlinear mixed effect modeling was used to estimate everolimus pharmacokinetic parameters from blood concentration-time data. NONMEM® (v7.1.2, Icon Development Solutions, Ellicott City, MD, USA) was used for modeling everolimus pharmacokinetics, using PsN toolkit [17], and Piranã version 2.3.0 [18] as modeling environment. Results were analyzed using statistical software package R (v2.11.0). First order conditional estimation method with interaction (FOCE-I) was used throughout the analysis. Model selection was based on statistical significance, goodness of fit and stability. Throughout the model building process, an altered model was chosen over a precursor model if a difference in the objective function values (OFVs) (-2 log likelyhood) was >6.63 ($P<0.01$, with 1 degree of freedom, assuming χ^2 distribution).

Base model

Initially, the model was developed strictly pharmacokinetic without covariates. Plots

of observed concentration-time data of everolimus were examined. One and two compartmental pharmacokinetic models with first-order elimination were compared to find the best fit of the concentration-time data. The use of transit compartments and a lag time for drug absorption were explored.

Covariate analysis

Diagnostic plots were constructed of the random effects of apparent oral clearance (CL/F), apparent volume of distribution of the central compartment after oral administration (V_1/F) and first order absorption rate constant (k_a) versus the demographic (age, total body weight (TBW), sex, ethnicity, length, Ideal Body Weight (IBW), Body Surface Area (BSA), Body Mass Index (BMI), Lean Body Weight (LBW), hematocrit, albumin, underlying disease and co-medications (also weighted residual vs co-medications plots) and pharmacogenetic *(ABCB1*, *CYP3A5*, *CYP2C8* and *PXR* polymorphisms) characteristics. Polymorphisms were selected based on theoretical relationship and minimal allele frequency (>0.06) to assure detection of clinically relevant effects on everolimus PK. Based on these plots further testing in the pharmacostatistical model was performed. Subsequently, selected covariate relationships were evaluated by forward inclusion and backward deletion procedure. A covariate effect was only maintained in the model if the inclusion resulted in a reduction in random variability and improved model fit. Evaluation of the precision of the pharmacokinetic parameters was performed with 1000 bootstrap replicates.

Visual predictive check with prediction-correction

Performance of candidate and final models for the everolimus pharmacokinetic model was evaluated using prediction corrected visual predictive checks (predVPC), by simulation of 500 simulated datasets [19,20]. Since observations were spread around nominal time points, bin separators in the VPC were set at the lowest densities of sample points over time, i.e. this positions the bins such that the periods with densest sampling were in the middle of the bins. Shrinkage in interindividual variability and residual errors was automatically calculated by NONMEM® v7.1.2. to assess the informativeness of the data for using individual predictions in the evaluation of model fit. The distribution (median, 10th and 90th percentiles) of the simulated concentration–time courses was compared with the distribution of the observed values in the original dataset. Differences and overlap of the simulated and original distributions indicated the accuracy of the identified model.

Limited sampling strategy

Patients and data collection

Fifty two full AUCs from 52 different patients were used for development of a limited sampling strategy (1 patient had one missing sample and was therefore excluded). Pharmacokinetic profiles consisted out of 7 blood samples collected over 6 hours (0 [predose] and 1,2,3,4,5 and 6 hours postdose).

Pharmacokinetic and statistical analysis

Trapezoidal AUC_{12} (full AUC) was calculated from all measured concentration-time points using trapezoidal rule using T=0 hours as T=12 hours. Limited sampling model (LSM) AUC_{12} (predicted AUC) was calculated by selecting several concentration-time points and combinations of time points. Bias and imprecision were calculated to assess the performance of the different LSM's according to the guidelines proposed by Sheiner and Beal [21]. A Pearson correlation coefficient test was performed to determine the correlation between trapezoidal AUC_{12} and LSM AUC_{12}. The formulas of the predictive performance measures used are presented in Supplementary Table III.

Supplementary Table III: Formulas.

Demographic Covariate Formulas	
IBW calculated as:	
Males: 52 kg + 1.9 kg for every inch over 5 feet (33)	
Females: 49 kg + 1.7 kg for every inch over 5 feet (33)	
BMI calculated as:	$BMI\left(\frac{kg}{m^2}\right)=\frac{Weight(kg)}{[Height(m)]^2}$
LBW calculated as:	
males:	$LBW=1.10\times(Weight(kg))-\frac{128(Weight(kg))^2}{[100(Height(m))]^2}$
females:	$LBW=1.07\times(Weight(kg))-\frac{148(Weight(kg))^2}{[100(Height(m))]^2}$

BSA (Mosteller(1987)) $BSA(m^2) = \sqrt{\frac{(Height(cm) \times (Weight(kg)}{3600}}$

Limited Sampling Strategy Statistical analysis Formulas
Bias Mean prediction error = mean (AUCpred – AUCfull) Mean percentage prediction error (MPPE) = mean [100% * (AUCpred-AUCfull)/AUCfull]
Imprecision Root mean squared prediction error (√mean (AUCpred-AUCfull)^2) Mean absolute percentage prediction error (MAPE) = mean [100% * |AUCpred-AUCfull|/AUCfull]
The percentage of AUCpred within a 15% radius of AUCfull is decreased by both greater bias and worse precision and is therefore a usefull measure and overall predictive ability.
Discordance Discordance between AUCpred and AUCfull (The percentage of times that AUCpred failed to indicate a everolimus dosage adjustment of 20% when AUCfull was out of the target range, or indicated a dosage adjustment of 20% or higher when AUCfull was within the target range).

Results

Patients and Pharmacokinetic data

Fifty-three adult renal transplant recipients, 35 men and 18 women were included in this study. The majority of patients (81%) were Caucasian, 4% of the patients were Black and 15% belonged to other ethnicities. Mean age was 52 ± 12 years (range: 23-71 years), mean TBW was 80.7 ± 16.2 kg (52-128.8 kg). The dataset consisted out of 783 samples. Everolimus concentrations were obtained at steady state at clinical visits which were planned at 32, 52, 78 and 104 weeks after transplantation. The concentration-time data were reviewed for completeness and consistency of sampling and dosing times. Clinical characteristics and details about exposure and efficacy and safety response are listed in Table I. Genotype distribution for *CYP3A5*, *CYP2C8*, *ABCB1* and *PXR* are listed in Table II. Haplotype distribution for *ABCB1* and *CYP2C8* are listed in Table III.

Table I: Clinical characteristics.

Recipient characteristics	Number (%)	Mean ± SD	Median (Range)
Male	35 (66%)		
Female	18 (44%)		
Age (y) [a]		52 ± 12	55 (23 - 71)
Caucasian (%)	43 (81%)		
Total Body Weight (kg) [a]		80.7 ± 16.2	77.5 (52 - 128.8)
Body surface Area (m^2) [b]		1.96 ± 0.23	1.93 (1.51 - 2.52)
Lean Body Mass (kg) [b]		60.5 ± 8.6	59.4 (43.2 - 79.9)
Ideal Body Weight (kg) [b]		68 ± 7.5	68.3 (52 - 83.1)
Height (m) [b]		174 ± 10	174 (152 – 194)
Creatinine (µmol/L) [a]		116 ± 34	116 (59 – 226)
Albumin (g/L)		42 ± 3.6	43 (25 – 49)
Hematocrit (L/L)		0.38 ± 0.04	0.38 (0.26 - 0.48)
Underlying disease			
Polycystic kidney disease	12 (22.6%)		
Glomerulonephritis	11 (20.8%)		
Diabetes mellitus	4 (7.5%)		
Hypertension	8 (15.1%)		
Focal segmental Glomerulosclerosis	2 (3.8%)		
Unknown	2 (3.8%)		
Interstitial nephritis	1 (1.9%)		
Urological	6(11.3%)		
Other	7(13.2%)		
Exposure			
Everolimus Dose (mg) [a]		2.44 ± 0.75	2.25 (0.75 - 4.50)
Everolimus area under the curve (AUC) (µg*hour/L)		130 ± 39	127 (55 – 260)
Concentrations (µg/L)		12.1 ± 6.7	12.1 (2.6 – 50)

Renal function			
Creatinine clearance (mL/min) [c]		70 ± 27	58 (26-120)
Week 32		67 ± 27	57 (28-120)
Week 52		67± 24	58 (28-120)
Week 78		76 ± 27	68 (37-120)
Week 104		72 ± 27	57 (30-120)
Efficacy and safety responses			
Freedom from acute rejection	53 (100%)		
Hypercholesterolemia (> 6.5 mmol/L)	33 (62.3%)		
Hypertriglyceridemia (> 2.9 mmol/L)	27 (50.1%)		
Thrombocytopenia (< 75 *10^9/L)	0 (0.0%)		
Leukopenia (< 4 *10^9/L)	5 (9.4%)		
Diarrhea	6 (11.3%)		
Dyspnea	4 (7.6%)		
BK nephropathy	1 (1.9%)		
PCP infection	2 (3.8%)		
Pneumonia	3 (5.7%)		
Edema	4 (7.6%)		
Infections (airway, gastro-intestinal, urinary tract)	6 (11.3%)		
Cardiovascular events	2 (3.8%)		
Patients who did not complete the entire study	16 (30.2%)		

[a], During trial; [b], At first TDM moment; [c], Modification of Diet in Renal Disease (MDRD) when <60ml/min and Cockcroft-Gault when > 60 ml/min (cut-off 120 ml/min); AUC, area under the blood concentration vs time curve.

Table II: Genotype frequencies in studied population (n = 53).

Gene	SNP(s)	Nucleotide position and alleles	Genotype	Frequency [N (%)]
ABCB1				
	rs1128503	1236C>T	C/C	20 (38)
			C/T	25 (47)
			T/T	8 (15)
	rs2032582	2677T>G	T/T	22 (42)
			G/T	25 (47)
			G/G	6 (11)
	rs1045642	3435C>T	C/C	16 (30)
			C/T	30 (57)
			T/T	7 (13)
	rs3213619	-129T>C	T/T	50 (94)
			C/T	3 (6)
			C/C	0 (0)
CYP2C8				
	rs10509681	47603213T>C	T/T	47 (89)
			C/T	6 (11)
	rs11572080	47631494C>T	C/C	47 (89)
			C/T	6 (11)
CYP3A5				
	rs776746	6986A>G	A/A	2 (4)
			G/A	4 (7)
			G/G	47 (89)
	rs10264272	7225G>A	G/G	53 (100)
			A/G	0 (0)
			A/A	0 (0)
PXR (NR1|2)				
	rs2276706	*-24113G>A*	G/G	23 (43.4)
			G/A	23 (43.4)
			A/A	7 (13.4)
	rs6785049	7635A>G	G/G	7 (13)
			A/G	20 (38)
			A/A	26 (49)

CYP, cytochrome p450; PXR, pregnane X receptor; SNP, single nucleotide polymorphism.

Table III: *ABCB1* and *CYP2C8* haplotype table. This table shows the frequencies of the haplotype combinations on the left, and the frequency of the individual triplets/duplets on the total amount of 60 triplets/duplets on the right side (2 loci per allele, n= 53).

Gene	SNP(s) in haplotype	Haplotype Block 1	Haplotype Block 2	Frequency [N (%)]	Haplotype	Frequency [N (%)]
ABCB1						
	rs1128503	CCG	CCG	13 (24)	CCG	55 (52)
	rs2032582	CCG	CTG	2 (4)	TTT	35 (33)
	rs1045642	CCG	TTT	19 (36)	TCG	9 (8)
		CCT	TTT	1 (2)	CTG	4 (4)
		CTG	TTT	2 (4)	CTT	2 (2)
		CTT	CCG	1 (2)	CCT	1 (1)
		TCG	CCG	7 (13)		
		TTT	CTT	1 (2)		
		TTT	TCG	2 (4)		
		TTT	TTT	5 (10)		
CYP2C8						
	rs10509681	CT	CT	47 (89)	CT	100 (94)
	rs11572080	TC	CT	6 (11)	TC	6 (6)

CYP, cytochrome P450; SNP, single nucleotide polymorphism.

Structural model development

The pharmacokinetic data of everolimus was best described by a two-compartmental model with first order absorption with lag time and first order elimination from the central compartment. Random effect parameters for inter-individual variability in clearance (CL), distribution volume of central compartment (V_1) and rate of absorption (k_a) were identified. Variability between occasions was best described with a random effect on k_a and (fixed) bioavailability (F). Thereafter the random effects were tested for structural relationship with dose and time to create a model with unbiased and randomly distributed random effects for covariate analysis. The structural pharmacokinetic model indicated a CL/F of 18 L/h, an V_1/F of 153 L and an apparent peripheral distribution volume (V_2/F) of 495 L (Table IV). The absorption rate constant was 7.36 h^{-1}. Inter-compartmental clearance was 56.1 L/h and lag time was 0.714 h. Inter-occasion variability was estimated

for the fixed bioavailability term and not for clearance because of a better model fit. The pharmacokinetic data showed inter-individual variability in CL/F of 26.4% and inter-occasion variability (25.9%) with a range of 6-37 L/h. A dose clearance relationship was observed showing an increase in apparent clearance with increasing dose according to TVCL={[dose/2.25]**0.5}. This relationship improved the model fit in terms of objective function. After testing the dose clearance relation according to *Jae Eun Ahn et al.* [22] the effect appeared to be caused by strict TDM. Patients with a high everolimus clearance will change over time to a higher daily dosage and vice versa. As a results; an apparent concentration-clearance relationship emerges.

The base model was used in the search for demographic and genetic covariates. Diagnostic plots of random effect from CL, V_1 and k_a in the initial model against age, TBW, sex, hematocrit, albumin, Length, IBW, BSA, BMI, LBW, co-medication, underlying disease and ethnicity were constructed. Plots of weighted residuals versus co-medications were made in case co-medications were not constantly administered. The co-medications that were evaluated are found in Supplementary Table IV. Criteria for evaluation were probability of interaction based on literature and high enough frequency of administration.

The plots of random effects of distribution volume against sex, length and IBW indicated a relationship between V_1/F and these covariates. The addition of IBW centered on the population median as exponential function on V_1/F improved the model (ΔOFV = -15.3, $P<0.001$), explaining 21% of the random variability between individuals in V_1/F. In the backward elimination step, removal of the covariate resulted in an increase of the OFV of 15.3. Sex, length, and IBW were also related to V_1/F but IBW had the highest objective function decrease and showed a better prediction corrected Visual Predictive Check. TBW, age, LBW, BSA, BMI, albumin, hematocrit, co-medication and underlying disease were not significant covariates on CL/F, V_1/F or k_a.

Diagnostic plots of random effects of CL/F, V_1/F and k_a against genetic polymorphisms in *CYP3A5*, *CYP2C8*, *ABCB1* or *PXR* were created. The inclusion of a genetic polymorphism in *CYP3A5*, *CYP2C8*, *ABCB1* or *PXR* as covariate on CL/F, V_1/F or k_a of the base model did not result in a significant OFV drop ($P>0.05$ or $P>0.01$). The population pharmacokinetic parameters obtained with the base and final model are presented in Table V.

Table IV: Model building process.

Model number	Covariate	Relationship of covariates with pharmacokinetic parameters	Compared with model number	OFV	ΔOFV	p-Value
One compartment						
1		$CL = \theta_1 \times EXP(IIV) \times (DOSE/2.25)^{\theta_6}$ $V = \theta_2 \times EXP(IIV)$ $k_a = \theta_3 \times EXP(IIV+IOV)$ $F = \theta_4 \times EXP(IOV_2)$ $ALAG = \theta_5$		2760.111		
Two compartment						
2	Base	$CL = \theta_1 \times EXP(IIV) \times (DOSE/2.25)^{\theta_6}$ $V_1 = \theta_2 \times EXP(IIV)$ $Q = \theta_3$ $V_2 = \theta_4$ $k_a = \theta_5 \times EXP(IIV+IOV)$ $F = \theta_7 \times EXP(IOV_2)$ $ALAG = \theta_8$	1	2371.205	-382.929	<0.001
3	IBW on V_1/F	$CL = \theta_1 \times EXP(IIV) \times (DOSE/2.25)^{\theta_6}$ $V_1 = \theta_2 \times EXP(IIV) \times (65.75/IBW)^{\theta_9}$ [a] $Q = \theta_3$ $V_2 = \theta_4$ $k_a = \theta_5 \times EXP(IIV+IOV)$ $F = \theta_7 \times EXP(IOV_2)$ $ALAG = \theta_8$	2	2354.671	-15.318	<0.001

a, The population V1/F term was standardized to 65.75, which represents the median value of IBW in the dataset used.

ALAG, lag-time; CL/F, apparent oral clearance; F, bioavailability; IBW, ideal body weight; IIV, inter-individual variability; IOV, inter-occasion variability; ka, absorption rate constant; OFV, objective function value derived from NONMEM® (nonlinear mixed-effects modelling); Q, inter-compartmental clearance; V1/F, volume of distribution of central compartment after oral administration; V2/F, volume of distribution of peripheral compartment after oral administration.

Supplementary Table **IV:** Comedications of interest for covariate analysis an number of patients using them.

Immunosuppression
Prednisolone (=53)
Statins (n=33) *
Atorvastatin (n=24)
Pravastatin (n=33)
Simvastatin (n=6)
Antibiotics
Sulfamethoxazole / Trimethoprim (n=11)
Hypertension
Nifedipine (n=39)
Proton Pump inhibitors (24)
Pantoprazole (n=6)
Omeprazole (n=16)
Esomeprazole (n=2)

* In some cases statins were switched for another statin.

Evaluation of the precision of the pharmacokinetic parameters was performed with 1000 bootstrap replicates. The mean values for all fixed effect parameters were within 15% of those obtained by the final model, indicating good reliability (Table V). Since different dosages were used during the study the performance of the model was evaluated with a predictive corrected visual predictive check (19)(Figure 1). Predicted and observed intervals (median, 10th and, 90th percentiles) are almost identical showing good predictive performance of the final model. Figure 2a shows the variable pharmacokinetics of everolimus two weeks after conversion (3 mg twice daily). The median trapezoidal everolimus AUC_{12} was 155 µg*h/L (range: 81-178 µg*h/L) while the target AUC_{12} was 120 µg*h/L [3,15]. Figure 2b shows trough and trapezoidal AUC_{12} correlation with the trough and corresponding trapezoidal AUC_{12} target range. Although a good correlation is found between C_{trough} and $AUC_{12,}$ some outliers remain at risk. The trough target range (6-8 µg/L) used in this study [3,15] corresponds with a relatively wide or at least twofold AUC_{12} range. When aiming at a target AUC_{12} of 120 µg*h/L using this trough range the actual exposure will be in 13% of the patients more than 20% lower or higher than intended. This could result in incorrect dose adjustments leading to increased risk of toxicity or rejection [15,23,24]. However, using a full AUC based on trapezoidal rule remains an invasive and intensive way of performing TDM since a large number of samples are required. A limiting sampling model (LSM) with good predictive performance could help solve this problem.

Table V: Summary of model parameter estimates from base and final model with relative standard error (RSE%) and parameter estimates from 1000 bootstrap replicates with 95 % confidence interval (CI).

	Base Model		Final Model		1000 bootstrap runs	
PK Parameter	Mean Value	RSE(%)	Mean Value	RSE(%)	Median Value	95% CI
CL	18	4.5	17.9	4.5	18.0	16.4 to 19.7
F (fixed)	1	0	1	0	1.0	1 to 1
V_1 (L)	153	5.7	148	6.2	146.7	130.0 to 166.4
Q (L/h)	56.1	6	55.7	6.8	55.7	49.1 to 64.1
V_2 (L)	495	9.7	498	13.8	491.9	325.1 to 1209.3
k_a (h-1)	7.36	8.8	7.55	14.2	8.0	5.1 to 15.1
t_{lag}	0.714	3.3	0.709	2.1	0.714	0.67 to 0.80
Dose CL (TDM effect)	0.505	16.8	0.532	15.9	0.545	0.3 to 0.7
θ_{IBW} on V_1/F	NA	NA	-1.41	27.1	-1.4	-2.30 to -0.56
Interindividual variability						
IIV CL (CV%)	26.4	18.8	26.2	18.7	24.9	13.5 to 35.0
IIV V_1 (CV%)	35.2	20.2	27.7	14.7	26.6	16.3 to 35.7
IIV K_a (CV%)	110.5	17.6	108.6	20.2	104.9	45.9 to 152.0
Interoccasion variability						
IOV K_a (CV%)	131.1	13.3	135.6	14.7	140.8	99.5 to 189.3
IOV F (CV%)	25.9	7.2	25.9	6.9	25.9	22.2 to 29.4
Random residual variability						
σ^1 (proportional error)	14.0	NA	13.9	NA	13.9	11.8 to 16.2

CL/F, apparent oral clearance; CV, coefficient of variation; F, bioavailability; IBW, ideal body weight; ka, absorption rate constant; NA, not applicable; Q, inter-compartmental clearance; RSE, relative standard error; TDM, therapeutic drug monitoring; V_1/F, volume of distribution of central compartment after oral administration; V_2/F, volume of distribution of peripheral compartment after oral administration; t_{lag}, lagtime.

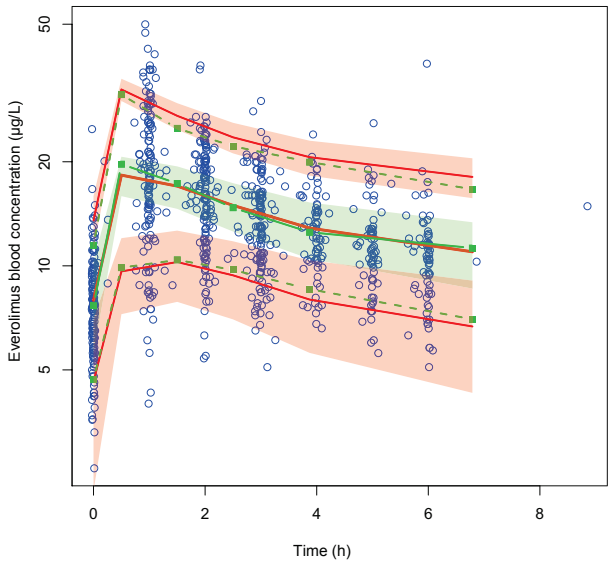


Figure 1: Prediction corrected visual predictive check with median, 10th and 90th prediction and observed percentile. The observed everolimus blood concentrations are shown as open circles. The dashed lines with square symbols represent the observed median and 10th and 90th percentile. The solid lines represent the prediction median and 10th and 90th percentile. The shaded areas around the prediction intervals represent the 95% CI around each of the prediction percentiles.

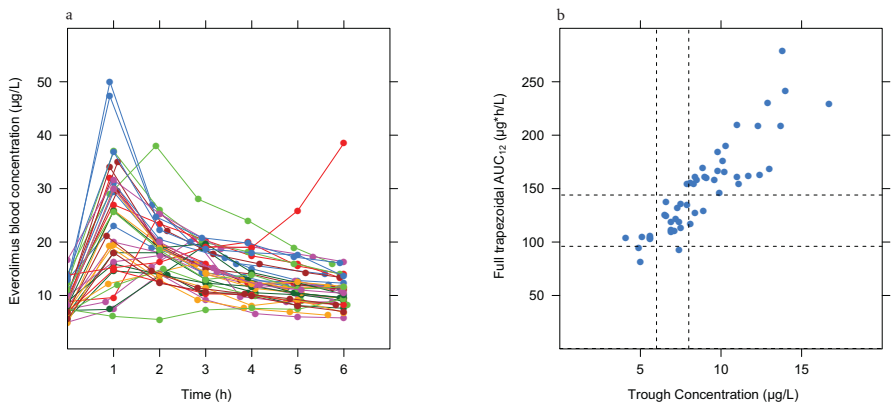


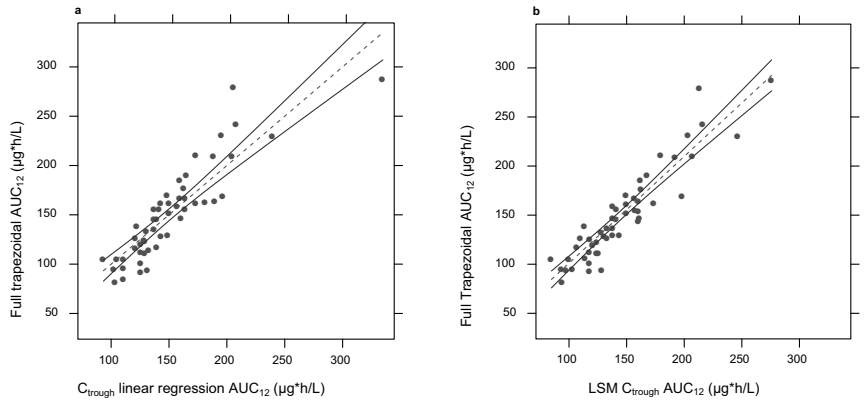
Figure 2: (a) Full concentration-time profiles of everolimus in 37 renal transplant patients exactly 2 weeks after conversion receiving everolimus 3 mg twice daily. (b) Trough blood concentration and trapezoidal AUC_{12} correlation of 52 different patients (based on full AUC_{12} curves) 32–104 weeks after transplantation (dose range 1 – 4.5 mg twice daily). Dotted lines crossing the x-axis represent the trough blood concentration target area used in this trial. Dotted lines crossing the y-axis represent the 20% deviation area from the target AUC_{12} of 120µg*h/L. AUC_{12}, area under the blood concentration-time curve from time zero to 12 hours.

With the development of a LSM we calculated the predictive performance of different LSMs and since trough concentrations are widely used in TDM also a limited sampling formula (LSF) of C_{trough} to show the difference between the two methods. Using NONMEM® *post hoc* estimation on the final model, LSMs were calculated using 52 curves based on one or a combination of measured blood concentrations at different time points. For the LSF the linear regression equation was calculated. Results of the development of a LSM and LSF of C_{trough} are shown in Figure 3 and Supplementary Table V. Predictive performance measurements are; the percentage of predicted AUCs within a 15% range of the AUC calculated with the "trapezoidal rule"; discordance (%) [meaning a predicted AUC leading to incorrect dose change] and different ways of describing bias and imprecision: mean prediction error (MPE), mean percentage prediction error (MPPE), mean absolute percentage error (MAPE), Root Mean Square prediction Error (RMSE)) and correlation. Figure 3 shows results of four LSMs, with both regression lines with 95% CI as measurements of predictive performance; The limited sampling formula of C_{trough} ($11.605 \times C_{trough}$+45.774) for everolimus in predicting systemic exposure had a reasonable correlation with full trapezoidal AUC_{12} (Discordance=5.77%, MAPE=10.66% and R^2=0.87). The best single point marker was C_{trough} (Discordance=3.85%,MAPE=8.48%; R^2=0.87). The best two points marker was C_{trough} and C_2 (Discordance=1.92%; MAPE=7.10%; R^2=0.90). The best 3 points marker was C_{trough}, C_1 and C_3 (Discordance=1.92%, MAPE=5.31% and R^2=0.92). The widely used C_{trough} showed poorer performance with LSF and LSM compared to the two point marker C_{trough} and C_2. When taking predictive performance, intensity of the sampling for patient and clinic into account, using the two point markers C_{trough} and C_2 is the best option.

Supplementary Table V: Limited Sampling Methods based on one or multiple time points.

Time points Blood Sampling	R^2 pearson	Percentage of AUCpred within 15% radius of AUCfull	Discordance (%)	MPE (µg*h/L)	MPPE (%)	RMSE (µg*h/L)	MAPE (%)
Without model							
T = 0	0.78						
Limited Sampling Formula = (11.605 * C_0 + 45.774)							
T = 0	0.78	76.92	5.77	-0.003	1.96	20.96	10.66
Individualized POP-PK model							
T = 6,5,4,3,2,1,0	0.99	100.00	0.00	-7.58	-4.79	9.07	4.84
T = 0	0.87	88.46	3.85	-5.24	-2.07	17.26	8.48
T = 1	0.18	51.92	38.46	-11.09	-2.73	44.16	17.41
T = 2	0.52	67.31	21.15	-17.05	-8.59	35.65	14.06
T = 3	0.70	73.08	15.38	-11.98	-5.71	28.03	12.08
T = 4	0.56	78.85	7.69	-10.43	-4.93	31.93	10.79
T = 5	0.62	82.69	5.77	-10.67	-5.53	30.31	8.97
T = 6	0.52	84.62	5.77	-8.68	-4.74	38.24	10.28
T = 5,4,3,2,1,0	0.96	98.08	1.92	-8.20	-4.87	13.39	5.07
T = 4,3,2,1,0	0.93	98.08	1.92	-8.26	-4.67	15.47	5.31
T = 3,2,1,0	0.92	98.08	1.92	-8.42	-4.56	16.32	6.04
T = 2,1,0	0.91	96.15	1.92	-7.79	-4.04	16.55	5.91
T = 1,0	0.88	94.23	3.85	-4.79	-1.99	16.64	6.65
T = 0,2	0.90	92.31	1.92	-8.57	-4.30	18.02	7.10
T = 0,3	0.90	90.38	3.85	-7.05	-3.43	16.55	7.79
T = 1,2	0.53	75.00	17.31	-14.33	-7.21	34.16	12.78
T = 2,3	0.68	71.15	11.54	-13.13	-6.68	28.97	12.25
T = 1,3	0.75	82.69	9.62	-10.92	-5.53	25.55	9.65
T = 1,4	0.58	88.46	5.77	-10.88	-5.44	31.46	8.33
T = 3,4	0.79	75.00	7.69	-9.04	-4.64	22.97	10.18
T = 4,6	0.59	84.62	7.69	-8.27	-4.62	33.46	9.80
T = 0,1,2	0.91	96.15	1.92	-7.79	-4.04	16.55	5.91
T = 0,1,3	0.92	96.15	1.92	-6.92	-3.63	15.26	5.31
T = 0,2,3	0.90	94.23	1.92	-8.82	-4.56	18.05	7.43
T = 1,3,6	0.92	98.08	1.92	-8.86	-5.30	15.74	6.61
T = 1,4,6	0.70	98.08	1.92	-10.07	-5.75	27.25	6.50
T = 1,2,3	0.74	86.54	7.69	-12.38	-6.54	26.52	9.86

Bias is the systematic error, the tendency of consitently over- or under- estimating the parameter precision is the random error, and reflects the magnitude of variation in the prediction.



Plot	R^2 pearson	AUC_{pred} within 15% AUC_{full} (%)	Discordance (%)
a	0.78	76.92	5.77
b	0.87	88.46	3.85
c	0.90	92.31	1.92
d	0.92	96.15	1.92

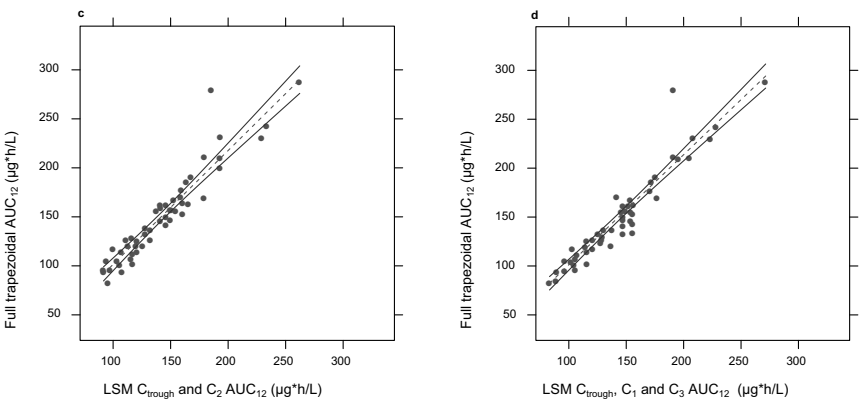


Figure 3: Regression line (dotted lines) plots comparing the different limited sampling methods with 95% confidence intervals (solid lines) and predictive performance data. The full trapezoidal AUC_{12} (using eight timepoints) is presented on the y-axis. The predicted AUC_{12} of the different limited sampling models (b, c and d) and a linear regression limited sampling formula (a) are shown on the x-axis. (a) Shows the predictive performance of C_{trough} used in a limited sampling formula without the use of the pharmacokinetic model (linear regression = $11.605 \times C_{trough} + 45.774$); (b) shows the predictive performance of C_{trough} as limited sampling pharmacokinetic model; (c) shows the predictive performance of C_{trough} and C_2 as limited sampling pharmacokinetic model; (d) shows the predictive performance of C_{trough}, C_1 and C_3 as limited sampling pharmacokinetic model. The table shows the corresponding measurements of predicting performance of each of the graphs. AUC_{12} = area under the blood concentration-time curve from time zero to 12 hours; AUC_{full}, trapezoidal AUC_{12}; AUC_{pred}, limited sampling model AUC_{12}; C_x, blood drug concentration at x hour(s) postdose; C_{trough}, trough blood concentration; LSM, limited sampling model.

Discussion

The pharmacokinetics of everolimus in renal transplant patients using everolimus and prednisolone was best described by a two-compartmental model with first order absorption and lag time. This study shows for the first time that everolimus pharmacokinetics is not significantly influenced by genetic polymorphisms in coding genes for the metabolizing enzymes CYP3A5, CYP2C8, ABCB1 and PXR or drug transporter ABCB1. In addition, demographic covariates TBW, age, sex, hematocrit, albumin, length, BMI, BSA, LBW, underlying disease, co-medication and ethnicity did not significantly influence everolimus pharmacokinetics. In contrast, IBW did significantly correlate with the variability in V_1/F. The development of a LSM resulted in identification of a two point concentration marker for accurately predicting everolimus systemic exposure and can be used to optimize therapy in renal transplant patients. In this study, the mean CL/F and V_1/F was 17.9 L/h and 148L respectively. The pharmacokinetic parameter estimates were in agreement with those (CL/F = 8.8 L/h) found by *Kovarik et al.* when taking the effect of cyclosporine on everolimus pharmacokinetics, differences in patient population, and differences in modeling into account. Cyclosporine is known to influence everolimus pharmacokinetics leading to an increase of everolimus C_{max} and AUC by 82% and 168% respectively [25].
In this analysis we report for the first time that IBW significantly correlates with V_1/F. Since everolimus is for more than 75% partitioned into red blood cells and 75% of the plasma fraction is bound to plasma proteins this relationship can be physiologically explained since length and sex are incorporated in the IBW formula [26,27]. In contrast, *Kovarik et al.* [12] found a significant influence of TBW on clearance. The small influence of TBW could be explained by the presence of cyclosporine in the immunosuppressive regimen in this study. TBW has been reported earlier to be a covariate on cyclosporine clearance [28]. The relationship between ethnicity and clearance as was found by *Kovarik et al.* could not be identified in our study [12]. This difference can be explained by the small amount of black patients in our cohort. The fact that we found no effect for concomitant medications is probably caused by the fact that none of the medications that were previously found to be of influence on everolimus clearance were administered to our patient population for safety reasons [12]. Although theoretically plausible, we neither found an effect for drugs such as statins, nifedipine and sulfamethoxazole/trimethoprim, This is in accordance with the results of Kovarik et al [12]. Noncompliance, diarrhea and fatty food intake could not be quantified in this study, although these factors could contribute to the observed

variability as previously published studies reported [29,23].

This is the first comprehensive study investigating the influence of pharmacogenetics on everolimus pharmacokinetics. More specifically, polymorphisms in genes encoding CYP3A5, CYP2C8 and PXR, and the multidrug-resistance transporter ABCB1 were investigated for possible relationship with everolimus pharmacokinetics. At the time the study was conducted CYP3A4 lacked a reliable genetic marker for prediction of CYP3A4 expression [30–32], therefore SNPs in *CYP3A4* were not included. However recent data suggests that *CYP3A4*22* may influence CNI pharmacokinetics [33]. These polymorphisms were studied before in relation to pharmacokinetics of sirolimus, tacrolimus and cyclosporine [34–37]. For sirolimus and tacrolimus clearance a relationship to *CYP3A5* genotype was found [38,39,40]. Since everolimus is primarily eliminated by CYP3A enzymes [7,8] and PXR is related to CYP3A4 expression [31], *CYP3A5*, *CYP2C8* and *ABCB1* and *PXR* genotypes were evaluated. Allele frequencies found in our dataset corresponded with those published previously [41]. We found subjects with *CYP3A5 *3/*3*, **1/*3* and **1/*1* genotype. However, *CYP3A5* as covariate on apparent clearance did not explain variability in pharmacokinetics [12]. Previously, no relationship was found between dose requirement, C_{trough}, C_{max} or AUC_{12} and *CYP3A5* polymorphisms [10,11]. Although it is known from in *vitro* and *in vivo* studies that CYP3A5, CYP2C8 enzymes and ABCB1 are involved in everolimus pharmacokinetics [7], no relationship was found between the genetic polymorphisms in *ABCB1*, *CYP3A5*, *CYP2C8* and *PXR* and everolimus pharmacokinetics in our study. In contrast to nonpopulation- based approaches a population model has greater statistical power to identify a covariate effect on PK parameters, since analyzing multiple observations per subject one is able to compensate for the small number of individuals. We performed a posterior power calculation to determine the power (95% convidence) of our study to find a minimum clinically relevant genotype effect (i.e. 20%) on everolimus PK [42,43]. With the most unfavorable genotype distribution (Table II and III) we found a power of 79% in detecting a clinically relevant genotype effect. Therefore it is unlikely that our analysis missed a clinically relevant effect of genotypes. However for *CYP3A5*6* which is only found in individuals who are genetically sub-Saharan African, only one subgroup was identified, therefore a comparison could not be made. The remaining variability of our final being model was 26.2% and could reflect the wide inter-individual variability in CYP3A4 expression [44]. The novel *CYP3A4*22* polymorphism [33] is suggested to predict CYP3A4 activity. Analysis of this SNP and everolimus pharmacokinetics warrants investigation. Furthermore, phenotyping CYP3A4

by use of a midazolam probe could help to explain the remaining variability in everolimus pharmacokinetics [45,46].

Our study has some limitations. This study was performed with patients on a cyclosporine free regimen; therefore the model cannot be used for patients using normal dose cyclosporine and everolimus simultaneously, since cyclosporine has a significant effect on everolimus pharmacokinetics [25]. However, although contradictory results were found [47], the majority of research suggests that tacrolimus does not influence everolimus pharmacokinetics. As a consequence, the applicability of the presented model on tacrolimus + everolimus regimens warrants to be investigated. Since CNI minimizing and CNI free strategies are being actively investigated worldwide [1–6] there could be an increasing demand for implementing the developed model in clinical practice. Mostly everolimus trough concentrations are monitored. Besides the higher impact of assay variability [15] when using one marker to predict everolimus systemic exposure, the correlation between C_{trough} and AUC is not optimal and could theoretically lead to therapy failure when exposure is 20% higher or lower as intended. Maintaining everolimus exposure above the target range in a regimen of everolimus with reduced-dose cyclosporine increases the rate of thrombocytopenia by 6% and hypertriglyceridemia by 16% [24,26]. Exposure below the target range increases the rate of acute rejection with 49% [24,26]. Worse predictive performance of a TDM marker can lead to incorrect dose adjustments resulting in exposure outside the target range. Maintaining a target AUC_{12} of 120 μg*h/L in the current study resulted in low acute rejection rates and renal function was preserved. Moreover low rates of thrombocytopenia and leukopenia were reported. On the other hand higher rates of hypercholesterolemia and adverse events were found. Adverse events and side effects were the main reasons of stopping everolimus therapy before the trial ended. In our study C_{trough} monitoring had a worse performance in estimating AUC_{12} when using LSF and LSM compared with C_{trough} and C_2 in LSM, especially the LSF which resulted in a 15.5% higher percentage of patients outside of the 15% range of the full trapezoidal AUC and a 3.8% increase in discordance. The presented pharmacokinetic model and limited sampling method is a clear improvement in terms of inconvenience for patient and clinic and predictive performance. C_{trough} and C_2 monitoring based on the presented PK model results in an improved predictive performance compared to C_{trough} monitoring. Clinicians should decide whether this improved performance as shown in figure 3 is worth the effort. Whether TDM based on trough or AUC_{12} does lead to differences the occurrence of hazardous side effects in clinical in side effects warrants to be investigated more

thoroughly. The limited sampling models were developed using the most densely sampled AUC_{12} from the same dataset used for population pharmacokinetic model development. Therefore we recommend external validation to evaluate the limited sampling models.

Conclusion

A wide range of factors possibly contributing to variability of everolimus pharmacokinetics were investigated in this study. The population pharmacokinetics of everolimus in renal transplant patients is described by a two compartment pharmacokinetic model with lag-time. IBW significantly contributes to the pharmacokinetics of everolimus, by explaining variability in apparent volume of distribution. Polymorphisms in genes coding for ABCB1, CYP3A5, CYP2C8 and PXR with an allele frequency >6% do not clinically relevant influence everolimus pharmacokinetics and are therefore not suitable to improve prediction of everolimus exposure. However using the pharmacokinetic model and limited sampling model as presented here can be further tested in clinical practice to predict systemic exposure in an efficient and less invasive way for both patient and clinic. Everolimus C_{trough} and C_2 can be used to accurately estimate everolimus systemic exposure, an improvement compared to the widely used C_{trough} monitoring.

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5

Interstitial pneumonitis caused by everolimus: a case-cohort study in renal transplant recipients

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Abstract

The use of inhibitors of the mammalian target of rapamycin (mTORi) in renal transplantation is associated with many side effects, the potentially most severe being interstitial pneumonitis. Several papers have reported on sirolimus-induced pneumonitis, but less is published on everolimus-induced pneumonitis (EIP). Data on risk factors for contracting EIP are even more scarce. In the present casecohort study in renal transplant recipients (RTR), we aimed to assess the incidence and risk factors of EIP after renal transplantation. This study is a retrospective substudy of a multi-center randomized controlled trial. All patients included in the original trial and treated with prednisolone/everolimus were included in this substudy. RTR who developed EIP, were identified as cases. RTR without pulmonary symptoms served as controls. Thirteen out of 102 patients (12.7%) developed EIP. We did not find any predisposing factors, especially no correlation with everolimus concentration. On pulmonary CT scan, EIP presented with an organizing pneumonia-like pattern, a non-specific interstitial pneumonitis-like pattern or both Median time (range) to the development of EIP after start of everolimus was 162 (38-407) days. In conclusion, EIP is common in RTR, presenting with an organizing pneumonia, a non-specific interstitial pneumonitis-like pattern or both. No predisposing factors could be identified.

Introduction

Inhibitors of the mammalian target of rapamycin (mTORi), sirolimus and everolimus, are potent immunosuppressive drugs widely used after organ transplantation. They have been introduced in renal transplantation because of their supposed lack of nephrotoxicity and potential anti-oncogenic and anti-atherosclerotic effects [1-5]. Unfortunately, the use of mTORi is associated with many side effects like edema, impaired wound healing, mouth ulcers, anemia, proteinuria, development of lymphoceles, hyperlipidemia and hypertriglyceridemia [6]. Also interstitial pneumonitis may complicate treatment with an mTOR inhibitor. There are many reports of sirolimus-induced pneumonitis (SIP) [7]. Estimates of the incidence of SIP vary between 5 and 15% in solid organ transplant recipients. Clinical presentation ranges from asymptomatic to respiratory failure, but published reports suggest that SIP generally has a mild course and resolution of symptoms usually occurs after dose reduction or discontinuation of sirolimus. Far less is known on everolimus-induced pneumonitis (EIP), case reports of EIP do exist in solid organ transplantation and oncology, but systematic case-control studies have not been performed in renal transplant recipients (RTR).

The mechanism responsible for pulmonary toxicity by mTORi is not completely understood. Some suggest a dose-dependent risk [8-10], but there are also reports of cases with low mTORi trough levels [11,12]. Apart from the dose of mTORi, other possible risk factors have been identified in patients with non-small cell lung cancer, like smoking and pre-existing pulmonary disease [13]. Other studies found plasma creatinine and glomerular filtration rate (GFR) to be risk factors for development of EIP [14], indicating that the tolerance to mTORi may be altered in the presence of severe renal insufficiency. The presence of lymphocytes and eosinophils in broncho-alveolar lavage fluid suggests an immune mediated reaction [7,10,15]. It has been hypothesized that sirolimus binds to plasma proteins and that this complex is processed by antigen presenting cells in the lungs with consecutive T-cell recognition and recruitment of inflammatory cells like macrophages [7]. Others suggested that sirolimus exposes cryptic alveolar antigens evoking an ongoing cellular immune response [10]. Both mTOR inhibitors, despite inhibiting the adaptive immune response, enhance innate immunity [16,17], thereby possibly contributing to the development of pulmonary inflammation. Histopathological patterns include bronchiolitis obliterans organizing pneumonia, lymphocytic interstitial pneumonia, non-necrotizing granulomatous inflammation and vasculitis that support

the immune mediated hypothesis [7,8,10,18,19]. The mechanisms involved in EIP are speculative due to the lack of detailed studies. However, a recent study suggests a similar immunological mechanism for EIP [12], although there are also reports of resolution of SIP after conversion to everolimus [20-22]. In conclusion, ongoing exposure to mTORi may lead to a persistent inflammatory response in the lungs presenting clinically as pneumonitis. With the present case-cohort study we aimed to describe the incidence, clinical presentation, radiologic findings and predisposing factors of EIP in RTR.

Patients and Methods

Patients

This study was conducted as part of a larger prospective, multicenter randomized trial studying the effects of withdrawal of cyclosporin A (CsA) from an immunosuppressive regimen containing an IL-2 antagonist (basiliximab), CsA, prednisolone (P) and mycophenolate sodium (MPS) early after transplantation. Three university hospitals in the Netherlands participated in this trial from January 2005 until December 2009: the Academic Medical Center in Amsterdam (AMC), the Leiden University Medical Center (LUMC) and the University Medical Center in Groningen (UMCG). Institutional review board approval has been obtained. The study was conducted in accordance with the 2000 Declaration of Helsinki and the Declaration of Istanbul 2008. Informed consent was obtained from every patient. The details and results of an interim analysis of this trial have previously been published (trial registration number: NTR567 (Dutch trial registry), ISRCTN69188731, www.trialregister.nl) [23].

In short, RTR, receiving their first or second renal transplant, were treated with quadruple immunosuppressive therapy consisting of P, CsA, MPS and basiliximab. After 6 months, RTR were (in the absence of rejection, proven by renal biopsy) randomized to one of three immunosuppressive regimens: P/CsA, P/MPS and P/everolimus. Drug exposure of CsA and everolimus was monitored by AUCs at fixed moments. The target value of the AUC for CsA was 5400 μg*h/L in the first 6 weeks and 3250 μg*h/L thereafter. The target AUC for everolimus was 150 μg*h/L for Fluorescence Polarization Immunoassay (FPIA) and 120 μg*h/L for Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS), corresponding to the average 23% overestimation of FPIA [24]. The primary outcome was interstitial graft fibrosis and hyalinosis. Secondary outcome was, among others,

graft rejection. Patients who received a third or fourth transplant were excluded, as were patients with >50% panel reactive antibodies.

Case definition

For this retrospective sub-study, all RTR who were randomized to treatment with P/everolimus and/or effectively switched to treatment with P/everolimus during the study were included. Pulmonary problems in patients using everolimus were detected by the trial reports of (serious) adverse events and review of the charts of all included patients. Charts were analyzed for clinical signs (for example dypnea, cough or fever) and radiological signs of pulmonary involvement (abnormal chest X-ray and pulmonary CT scans). RTR, who developed symptoms of an EIP, were identified as cases. We used the following criteria for EIP [10]: (1) exposure to everolimus before the onset of pulmonary symptoms, (2) exclusion of other pulmonary disease, especially infection, (3) radiographic findings on CT of the chest not compatible with other diagnoses and (4) resolution of pulmonary symptoms after discontinuation of everolimus. When available, histopathological diagnosis consistent with drug-induced lung-toxicity was considered gold standard.

RTR who were treated with P/everolimus, but did not develop pulmonary symptoms, served as control patients. Patients in whom everolimus was discontinued because of pulmonary symptoms, but in whom no CT imaging was performed were excluded from the analysis. These patients were classified as possible EIP. The following data were retrospectively collected from medical records: sex, age, race, original renal disease, organ origin (living related or deceased), data on rejection episodes and CMV infection, analysis of BAL fluid, dialysis mode, history of pulmonary disease, smoking, everolimus AUCs and trough levels. Chest X-rays and (HR)CT of the chest from possible cases were re-analyzed by two independent reviewers (radiologist (IB) and pulmonologist (RJ)), who were blinded to the clinical information of patients. New abnormalities (compared to a pre-transplantation chest X-ray) were scored. Pulmonary function tests (when performed) were also recorded. The course of the EIP was analyzed and time to clinical recovery was noted.

Radiologic classification

Imaging findings on chest CT scan were classified into three distinct patterns (a simplified version of the approach by *Endo et al.* [25]): 1) multifocal areas of airspace consolidation with a predominantly peribronchial and/or sub-pleural distribution and bronchial wall thickening, compatible with OP, 2) extensive bilateral ground-glass attenuation or airspace

consolidation with traction bronchiectasis, compatible with a NSIP, or 3) a combination of OP and NSIP.

Measurements

Plasma creatinine was measured with an enzymatic PAP+ (phenol /4-aminoantipyrine) assay on a Roche Modular analyser (Roche, Almere, the Netherlands). Estimated GFR was calculated using the abbreviated MDRD formula: GFR = 175 x (Pcr ÷ 88.4)-1.154 x age-0.203 (female: multiply result by 0.742, black: multiply result by 1.210). Cytology, Ziehl-Neelsen staining, bacterial, viral and fungal cultures were routinely performed on all BAL fluid specimens. $AUCs_{0\text{-}12h}$ for everolimus were calculated from blood samples drawn at T=0, 1, 2, 3, 4, 5 and 6 hours after administration. The everolimus $AUCs_{0\text{-}12h}$ consisted of full AUCs (seven or six time points) and sparsely sampled AUCs (four time points), calculated using linear trapezoidal rule. Everolimus levels were determined by immunoassay (Innofluor® Certican® Assay System) according to manufacturers' instructions (Seradyn Inc, IN, USA) or by a validated LC-MS/MS method [24]. Since there is an average overestimation of 23 % by FPIA [24], the average $AUC_{0\text{-}12h}$ measured with LC-MS/MS was corrected by this 23 % to eliminate the differences between both methods. Pulmonary function (VC and DCLO) was measured using standard testing procedures.

Statistical analysis

All statistical analyses were performed using SPSS statistical software, version 16.0 (SPSS Inc., Chicago, Illinois, USA). Univariate analysis was performed to identify risk factors associated with EIP. Associations of discrete variables with EIP are expressed in terms of exact odds-ratios with their 95% confidence interval and analyzed with a chi-square test. Associations of continuous variables were analyzed with a Mann-Whitney U test. A p-value < 0.05 was considered statistically significant. Areas under the curve ($AUCs_{0\text{-}12h}$) were calculated using the linear trapezoidal rule with everolimus trough concentrations used as 12-hour values. AUCs were grouped into three different time periods (range): 1 month (0.2-3.5), six months (4.0-8.1) and 12 (9.4-14.5) months after start of everolimus. If one patient had multiple AUC measurements within one time period, the average AUC was calculated and used in the analysis.

Results

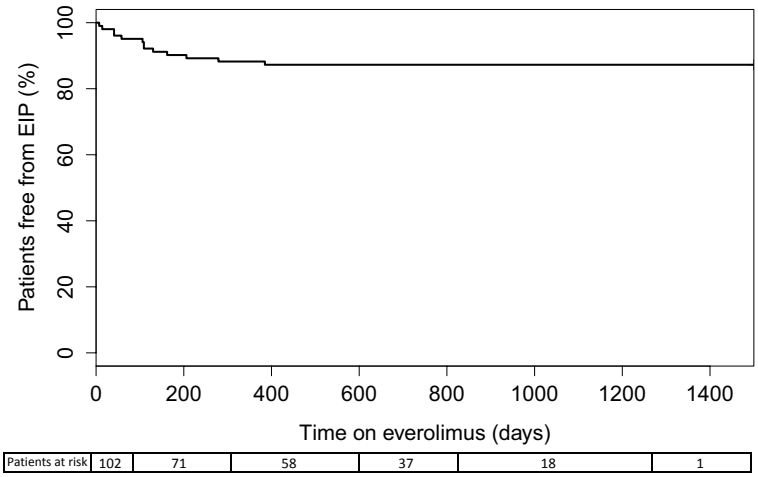

Figure 1: Kaplan Meyer curve demonstrating the time to development of everolimus induced interstitial pneumonitis (EIP) in 13/102 (12.7%) renal transplant recipients treated with everolimus.

Presentation of EIP

102 RTR were treated with prednisolone (P) and everolimus during the study period. At 6 months, 96 patients were randomized to P/everolimus [Bemelman et al, Transplantation 2009]. Six additional patients who switched to P/everolimus for various reasons outside the study protocol, were also included in this case-cohort study. We identified 13 cases, corresponding with an incidence of 12.7% (i.e. 13/102). Seven cases were classified as 'possible cases' and were excluded from the definite analysis. A detailed description of these patients can be found as supplementary data (supplementary table S1). Eighty-two RTR who did not develop pulmonary symptoms, served as control patients. Table 1 shows the demographic data of cases and control patients. The characteristics of the 13 patients who developed an EIP are listed in table 2. The median (range) time on everolimus of all patients was 752 (32-1502) days. In the cases, the median time (range) on P/everolimus until confirmation of EIP by computed tomography (CT) was 162 (38-407) days. Beyond 407 days, no more EIP occurred (figure 1). The most common presenting symptoms were dyspnea and cough (10/13 cases). Fever was present in 8/13 cases. One patient was asymptomatic, however 2-deoxy-2-(18F) fluoro-D-glucose (FDG) positive pulmonary infiltrates were discovered on a PET scan performed because of multiple unexplained bone fractures. A consecutive HRCT scan showed an image compatible with drug induced pneumonitis.

Table 1: Univariate analysis of risk factors for everolimus-induced pneumonitis among renal transplant recipients.

	Cases (n=13)	Control patients (n=82)	Odds ratio (CI)	*P*-value
Male gender n (%)	9 (69.2)	50 (61.0)	0.694 (0.20-2.45)	0.57
Recipient age, median (range)	50.0 (32-71)	53.5 (22-70)	-	0.37
Caucasian n (%)	11 (84.6)	70 (85.4)	1.061 (0.21-5.39)	0.94
Underlying renal disease				0.25
vascular	3 (23.1%)	15 (18.3%)	1.00	
immunological	4 (30.8%)	22 (26.8%)	0.91 (0.2-4.7)	
urological	-	10 (12.2%)	0.00	
other	3 (23.1%)	28 (34.1%)	0.54 (0.1-3.0)	
eci	3 (23.1%)	7 (8.5%)	2.14 (0.3-13.4)	
Renal transplant type (living) n (%)	6 (46.2)	43 (52.4)	1.286 (0.40-4.16)	0.67
Smoking				0.52
yes	1 (7.7)	17 (22.1)	0.2 (0.04-2.6)	
stopped prior to Tx	4 (30.8)	19 (24.7)	1.1 (1.3-4.0)	
no	8 (61.5)	41 (53.2)	1.0	
Pulmonary history n (%)	4 (30.8)	14 (17.1)	0.463 (0.13-1.72)	0.25
Rejection episode n (%)	1 (7.7)	16 (19.5)	2.909 (0.35-24.04)	0.32
Time on RRT (months)	48.1 (0-277)	28.8 (0-344)	-	0.23
Dialysis mode n (%)				0.34
pre-emptive	1 (7.7)	13 (15.9)	1.0	
HD	7 (53.8)	23 (28.0)	4.0 (0.4-35.8)	
PD	3 (23.1)	31 (37.8)	1.3 (0.1-13.2)	
HD & PD	2 (15.4)	15 (18.3)	1.7 (0.1-21.4)	
GFR* (ml/min)				
6 months after Tx	59.1 (30.8-87.8)	52.4 (17.4-110.2)	-	0.10
9 months after Tx	54.5 (35.8-79.5)	52.8 (20.6-102.8)	-	0.53
12 months after Tx	50.4 (35.5-75.4)	51.2 (11.7-96.8)	-	0.65
18 months after Tx	54.2 (37.0-93.3)	50.1 (14.3-101.6)	-	0.84
24 months after Tx	58.8 (22.6-97.8)	47.0 (10.1-104.6)	-	0.45

Time on EVL (days)	157.5 (32-485)	864.5 (69-1502)	-	<0.001
AUC EVL 1 month after start (µg*h/L)	173 (65-447)	169.5 (77-439)	-	0.972
AUC EVL 6 months after start (µg*h/L)	172 (164-238)	171 (98-356)	-	0.403
AUC EVL 12 months after start (µg*h/L)	237	169 (89-261)	-	NA
Trough level EVL 1 month after start (µg/L)	9.2 (3.8-25.4)	9.1 (4.0-28.1)	-	0.982
Trough level EVL 6 months after start (µg/L)	10.8 (8.0-14.0)	9.4 (2.9-22.0)	-	0.438
Trough level EVL 12 months after start (µg/L)	14.5	8.9 (4.5-14.7)	-	NA
CMV-infection n (%)				
- primary infection	1 (7.7)	7 (8.5)	1.120 (0.13-9.93)	0.92
- reactivation	3 (23.1)	28 (34.1)	1.728 (0.44-6.79)	0.43

AUC, Area Under the Curve; CI, confidence interval; CMV, cytomegalovirus; EVL, everolimus; HD, haemodialysis; NA, not available; PD, peritoneal dialysis; RRT, renal replacement therapy; Tx, transplantation. *GFR estimated by the abbreviated MDRD. Associations of discrete variables with everolimus-associated pneumonitis are expressed in terms of exact odds-ratios with their 95% confidence interval and analyzed with a chi-square test. Associations of continuous variables are analyzed with Mann-Whitney U test.

In all identified cases, the pulmonary CT scan revealed consolidations matching an organizing pneumonia (OP), a non-specific interstitial pneumonitis (NSIP)-like pattern or a combination of the two (figure 2). In one patient, no CT scan could be retrieved, but EIP was confirmed with pulmonary biopsy. Eight cases underwent a bronchoalveolar lavage (BAL). No pathogenic micro-organisms could be detected. In all cases everolimus was discontinued. In 6/13 cases everolimus was only discontinued when antibiotic therapy did not result in improvement. The absence of any microorganisms in the BAL fluid and the failure of empirical antibiotic treatment ruled out infection in these patients. Corticosteroids were administered in three cases. Pulmonary function tests were performed just after the onset of symptoms in 6/13 cases, showing normal to mildly lowered VC 90.2% (range 68-112), normal Forced Expiratory Volume in 1 second (FEV1) 84.8% (70-100) with a decreased single-breath diffusion capacity for carbon monoxide (DCLO) in all, 56% (range 38-75).

Table 2: Characteristics of renal transplant recipients with an everolimus-induced pneumonitis.

Patient	Age	Gender	Time on EVL until symptoms (days)	Symptoms	Radiologic findings on pulmonary CT	BAL	Treatment	Time to recovery
1	66	Male	109	dyspnea, coughing	OP/NSIP	NA	discontinue EVL	< 3 months
2	49	Male	206	dyspnea, coughing, fever	NSIP	NA	discontinue EVL	< 12 months
3	61	Male	162	dyspnea	NSIP/OP	negative	discontinue EVL	< 6 months
4	48	Female	279	coughing, fever	OP	NA	[4]AB + discontinue EVL	< 3 months
5	32	Female	385	none	OP	NA	discontinue EVL	
6	49	Male	130	dyspnea, coughing, fever	OP with GG	negative	discontinue EVL	< 1 month
7	71	female	14	dyspnea	OP/NSIP	NA	discontinue EVL	< 12 months
8	50	male	58	coughing, fever	OP	negative	discontinue EVL	Unknown
9	70	male	41	dyspnea, coughing, fever	VATS: OP	negative	[9]AB + discontinue EVL + corticosteroids	< 3 months
10	38	female	106	dyspnea, coughing, fever	OP	negative	[10]discontinue EVL + AB + corticosteroids	< 1 month
11	60	male	41	dyspnea, coughing, fever	OP	negative	[11]AB + discontinue EVL + corticosteroids	< 3 months
12	64	male	109	coughing, fever	OP/NSIP	negative	[12]AB + discontinue EVL	< 3 months
13	48	male	7	dyspnea, coughing	OP	negative	[13]discontinue EVL	Unknown

AB, antibiotics; OP, organizing pneumonia; CT, computed tomography; EVL, everolimus; NA, not available; NSIP, non-specific interstitial pneumonia; VATS, video assisted thoracoscopy; BAL, Bronco-alveolar lavage.

[4] First AB (ceftriaxone) was given, which did not improve the pulmonary symptoms. Hereafter ceftriaxone was stopped and everolimus was discontinued.

[9] First AB (amoxicilline/clavulanic acid) was given which did not improve the pulmonary symptoms and AB was discontinued. After histopathologic prove of organizing pneumonia, everolimus was discontinued and 60 mg prednisolone was started.

[10] Everolimus was discontinued and AB (ciprofloxacine and co-trimoxazole) together with 40 mg prednisolone were given. Sputum cultures revealed no bacteria, some candida species. After one day oseltamivir was added and three days later voriconazol.

[11] First AB (doxycycline) was given which did not improve the pulmonary symptoms and AB was discontinued. Then everolimus was discontinued, 30 mg of prednisolone was administered and pulmonary symptoms resolved.

[12] AB (cefuroxime) was given due to 10-100 colonies of *Escherichia coli* in sputum, because of lack of improvement, everolimus was discontinued and pulmonary symptoms resolved.

[13] One month before pulmonary CT, patient was admitted with suspected pneumonia. AB were given. BAL cultures remained negative, everolimus was discontinued. Because of continuing pulmonary symptoms, patient was readmitted one month later (while on prednisolone and tacrolimus). CT revealed OP and pulmonary embolism, anticoagulation was started.

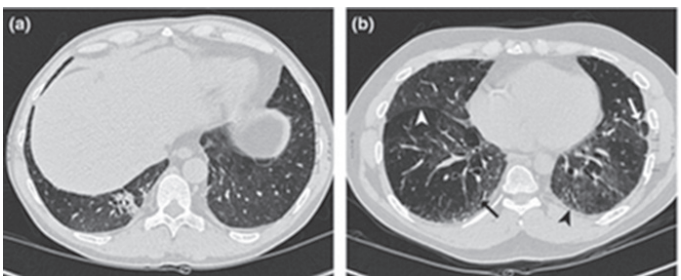

Figure 2: (a) Organizing pneumonia: sharply demarcated consolidation, with a peribronchial and subpleural localization in the right-sided dorsal pleural sinus. Both lungs reveal a mosaic pattern. (b) Nonspecific interstitial pneumonitis: subpleural and peribronchovascular ground-glass opacities (white arrow head). Bronchodilation (black arrow) and thickened interlobular septa (black arrow head) within these ground-glass opacities. Furthermore, perilobular septal thickening (white arrow) compatible with a component of organizing pneumonia.

Follow-up after EIP

All patients had a full clinical recovery within one year. In nine cases this was a subjective recovery because of the absence of follow-up with CT-scan or pulmonary function tests. Only in one case pulmonary function tests were performed after discontinuation of everolimus, showing an improvement of pulmonary function (data not shown). In three cases follow-up CT-scans were made after the diagnosis of EIP, which showed complete resolution of pulmonary abnormalities compatible with pneumonitis seen on earlier CT-scans. None of the patients were re-challenged with everolimus.

Follow-up data on renal outcome were available for 12/13 patients. Of those 12 patients, 7/12 switched to P/CsA, 2/12 switched to P/tacrolimus, 2/12 switched to P/CsA/MPS (of those one continued later on P/MPS) and 1/12 switched to P/MPS. None of these patients developed a rejection after conversion. The median time from the switch from everolimus to another immunosuppressive regimen and last follow-up was 658 (0 – 1217) days. In that period, eGFR declined with a median (range) of 4.5 (-14.1 to 24.2) ml/min, corresponding with a median decline of 2.8 (range -5.1 to 18.3) ml/year. Kidney function in the patients on everolimus who did not develop an EIP, remained stable after switch from P/CsA/MMF at 6 months until 2 years after transplantation (median (range) GFR change + 1.3 (– 24.2 to 13.4) ml/min/year).

Risk analysis for EIP development

We could not identify any predisposing factors to EIP, for example a known prior pulmonary history or smoking, nor was there a difference in renal function between cases and controls. Exposition to everolimus, expressed as Area Under the Curve (AUC) or trough levels, was similar in cases and control patients (table 1). According to the study protocol, everolimus exposure was monitored by AUCs one month, six months, 12 months and 18 months after the initiation of everolimus. Additional everolimus AUC or trough level measurements were only performed when asked for by the treating nephrologist. In cases, median time between confirmation of EIP by CT scan and most recent AUC was 69 (6-318) days. In case of patient compliance the AUC is expected to be stable. The (median) AUC of everolimus was 207 (108 -266) μg*h/L, corresponding with trough levels of 10.7 (6.6 -15.2) μg/L. During follow-up, 68.4% and 50% of the AUCs measured in the cases were > 150 and > 200 μg*h/L, respectively, versus 69.0% and 32.2% in the control patients (NS). 73.7% and 38.9% of the trough levels measured in cases versus 69.4% and 23.1% in control patients, respectively, were > 8 and > 12 μg/L.

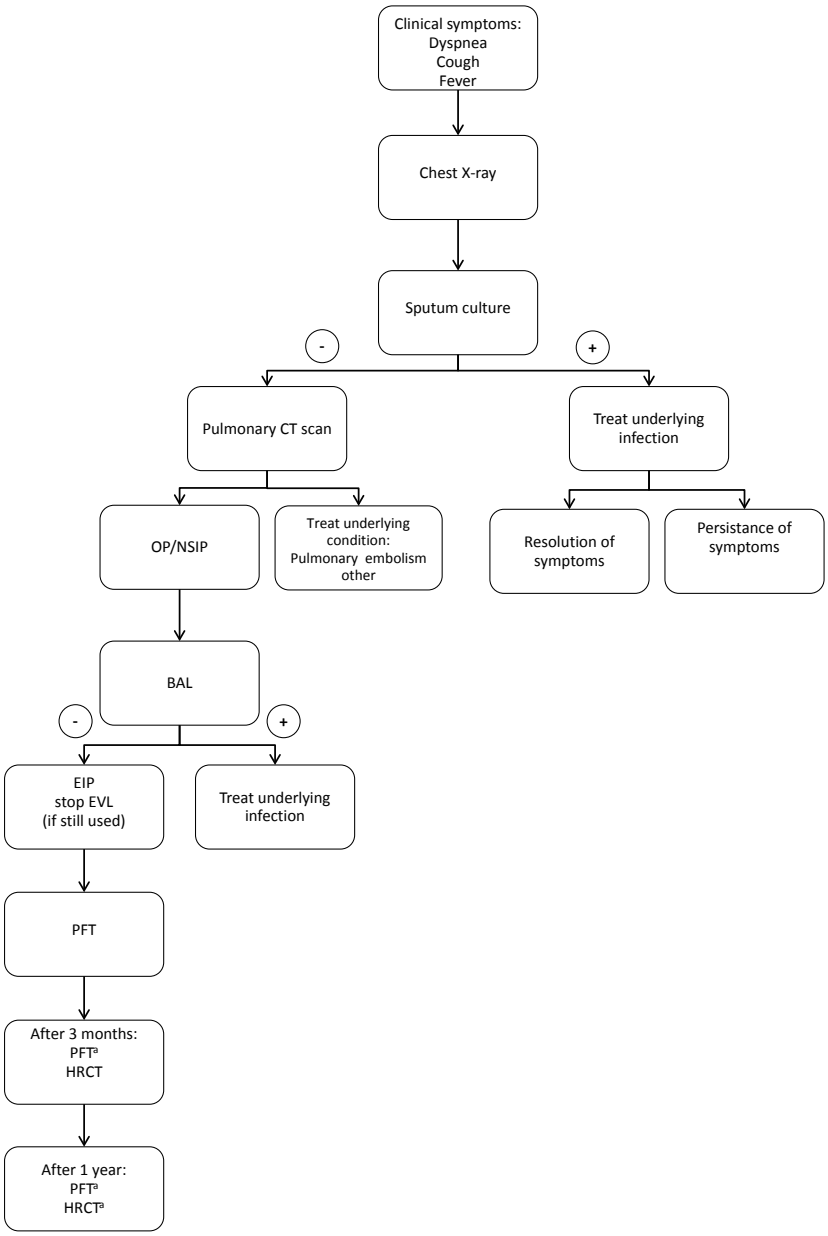

Figure 3: Algorithm for the diagnosis everolimus-induced pneumonitis in patients using everolimus. OP, organizing pneumonia; NSIP, nonspecific interstitial pneumonitis; BAL, broncho-alveolar lavage; PFT, pulmonary function test; EIP, everolimus-induced pneumonitis; EVL, everolimus; HRCT, high resolution CT. a If abnormal in previous test.

Discussion

Our study is the largest case series of everolimus induced pulmonary disease in solid organ transplantation. Pneumonitis appears a common adverse event complicating the use of everolimus after renal transplantation, with an incidence of 12.7%. No clear predisposing factors are identified in our case-cohort study. Pulmonary CT scans reveal an OP or NSIP-like pattern. The course seems benign with disappearance of symptoms within one year after discontinuation of the drug. The incidence of EIP (12.7%) reported in our study is higher than previously reported in RTR on mTORi, varying between 4 and 6.8% [26-28]. The true incidence of EIP in our cohort might even be higher because possible cases in which pulmonary imaging with CT scan was lacking, were excluded from analysis (table A, supplementary data). Furthermore, the reported incidence in our study is an underestimation of the true incidence of EIP, since EIP can be present on pulmonary CT scan without causing symptoms as demonstrated by White et al, who routinely performed pulmonary CT scans in patients with advanced non-small cell lung cancer treated with everolimus [13]. We identified one asymptomatic case in our cohort.

In patients treated with everolimus for renal cell carcinoma the incidence of EIP has been reported to be around 25% [13,29,30]. This high incidence of EIP has been attributed to higher dosage of everolimus in these patients in combination with a higher detection level of EIP due to routinely performed pulmonary CT scans. In our study, drug exposure was relatively high with an AUC around 170 μg*h/L and trough levels around 10 μg/ml since everolimus was prescribed as part of a double immunosuppressive regimen. However, everolimus exposure was not higher in the cases compared to controls. Remarkably, all patients developed EIP within 407 days; hereafter no EIP occurred. When reviewing the literature, we found only two cases of EIP occurring beyond 407 days'. Much debate exists on the etiology of mTOR-induced pneumonitis. White et al. [13] showed that patients with interstitial lung disease on baseline CT scans, whether focal or diffuse, had a higher incidence of all types of pneumonitis. This may reflect the tendency of patients with underlying lung disease to develop more serious toxicity. Therefore, we hypothesized that previous pulmonary disorders (reported in the medical charts) could be a predisposing factor to the development of EIP in our patient cohort. The incidence of an underlying pulmonary disease was 30.8 and 17.1% in cases and controls, respectively. This difference was not significant (p=0.25), nor was the difference in smoking. Furthermore, we found no difference in GFR which has also been suggested as a potential risk factor [14]. Therapeutic

drug monitoring (TDM) of everolimus is essential due to the narrow therapeutic window in combination with highly variable pharmacokinetics. Moreover, direct toxicity of everolimus in the etiology of EIP is suggested [8]. Since systematic everolimus AUCs and trough levels were determined in our study, we were able to accurately assess the exposure to everolimus in the cases and controls. Comparable exposure to everolimus in cases and controls makes toxicity simply based on higher exposure unlikely. We were not able to confirm the immune mediated hypothesis, due to lack of flowcytometric analysis of BAL fluid.

Our study confirms the previous findings of EIP presenting radiographically with an OP-like pattern, NSIP-like pattern, or a combination of both, making CT imaging a valuable tool to discriminate infection from a direct everolimus effect. Limitations of this study are its retrospective design and the lack of a standardized follow-up of the patients. Although this is a large cohort of patients and we found an incidence of EIP of 12.7%, the absolute number of cases is still limited, which might have masked significant risk factors. Another limitation is that in some patients a BAL to rule out pathogenic micro-organisms, was not performed and that previous use of antibiotics could have masked underlying infection in those patients who underwent a BAL. However, antibiotic treatment did not result in clinical improvement and recovery only occurred when treatment with everolimus was stopped. Three patients received additional corticosteroids. The effect of corticosteroids, administered at the same time as withdrawal of everolimus, on the disappearance of symptoms is unclear. Some found that inhibition of mTOR blocks the anti-inflammatory effects of glucocorticoids in myeloid immune cells [31], suggesting that corticosteroids might not be beneficial in mTOR-induced pneumonitis. All patients subjectively recovered within one year. The long-term outcome after EIP is unclear since NSIP is known to potentially result in pulmonary fibrosis.

Table S1: Characteristics of 7 renal transplant recipients with pulmonary symptoms not surely attributable to everolimus.

Patient	Age	Gender	Time on EVL until symptoms	Symptoms	Radiologic findings on pulmonary CT	Broncheo-alveolar lavage	Treatment	Time to recovery
1	58	Male	221	dyspnea, coughing	bronchopneumonia	NA	[1]AB + discontinue EVL + corticosteroids	< 3 months
2	64	Male	186	dyspnea, coughing, fever	NA	negative	discontinue EVL	< 1 month
3	60	Female	14	Itch, dyspnea	NA	NA	discontinue EVL	unknown
4	42	Male	12	coughing, fever	NA	CMV	[4]discontinue EVL + AB + ganciclovir	< 1 month
5	70	Male	81	dyspnea	NA	NA	[5]NA	died
6	58	Male	1	dyspnea, coughing	NA	NA	[6]discontinue EVL	< 12 months
7	56	Male	6	Dyspnoe, coughing, rash	NA	NA	[7]discontinue EVL	< 3 months

AB, antibiotics; CT, computer tomography; EVL, everolimus; NA, not available.

[1] Patient was admitted with suspected pneumonia, AB (erythromycin/amoxicilline) were given. Since no improvement occurred everolimus was discontinued and prednisolone was increased to 30 mg. Cardiac ultrasound revealed a LVEF of 13%, later improving to 35%.

[4] Ganciclovir was started intravenously, cefuroxim was given and everolimus was discontinued. Patient recovered.

[5] Patient presented with dyspnoe with unknown cause, symptoms resolved spontaneously after 1 week. One month later symptoms re-occurred accompanied by chest pain. Patient died in his sleep one month hereafter.

[6] Patient presented with dyspnoe directly after start of everolimus. Chest X-ray revealed cardiomegaly. After one month everolimus was discontinued. Dyspnoe improved. Symptoms completely resolved after a percutaneous coronary angiography with stent placement one year later.

[7] Chest X-ray more compatible with COP than another diagnosis.

Conclusion

EIP is a common side-effect of everolimus in RTR presenting radiographically with consolidations matching an organizing pneumonia, a non-specific interstitial pneumonitis like pattern or a combination of both. No clear predisposing factors could be identified. Since the presentation of EIP can be insidious or even asymptomatic, we recommend to perform radiographic imaging of the lungs when patients present with dyspnea, cough or fever while on treatment with this drug according to the algorithm shown in figure 3. Moreover, since we did not find a correlation with exposure to everolimus between cases and controls, we advise to halt everolimus instead of reducing the dosage following EIP.

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6

Exploring risk factors for everolimus discontinuation and serious side effects in renal transplant recipients on everolimus and prednisolone dual therapy

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In preparation for submission

Abstract

The mTOR inhibitor everolimus is an emergent non-nephrotoxic alternative for calcineurin inhibitors (CNIs) with substantial potential non-renal benefits in renal transplantation which slowly finds its way into new immunosuppressive regimens. Despite its proven efficacy and close therapeutic drug monitoring everolimus is also known for relative high discontinuation rates and some serious side effects. The aim of this study was to find risk factors for discontinuation and serious side effect to further optimize everolimus immunosuppressive therapy and to improve patient outcome. An extensive dataset consisting out of demographic, transplant related and pharmacogenetic data of 99 stable adult renal transplant recipients was used for a systematic analysis using a parametric survival model to describe the time to discontinuation and the most hazardous side effects including pneumonitis, (opportunistic) infection and new onset diabetes mellitus by means of nonlinear mixed-effects modelling. The baseline hazard of discontinuation, pneumonitis and infection data was best described by a Gompertz function and an exponential hazard function was used to the baseline hazard new onset diabetes mellitus. Risk factors for everolimus discontinuation of renal transplant recipients on a regimen of everolimus and prednisone duo therapy were constant too high everolimus (> 120 μg*h/L) exposure and increasing age. Furthermore, risk factors for the hazardous side-effect non-infectious interstitial pneumonitis were constant too high everolimus exposure and PXR (NR1|2)(-24113G>A): AA genotype. For infection and new onset diabetes mellitus no significant covariates could be detected. The current findings indicate that discontinuation rates and non-infectious pneumonitis in renal transplant recipients on everolimus can be prevented by avoiding excess initial and/or prolonged excess maintenance everolimus exposure.

Introduction

Improving long term survival remains currently the key challenge in renal transplantation. Although calcineurin inhibitors (CNIs) have lowered the incidence of acute rejection dramatically, especially in the higher dosing ranges and vulnerable kidney grafts they are associated with allograft nephropathy and calcineurin toxicity [1,2]. The mTOR inhibitor everolimus is an emerging in essence non-nephrotoxic alternative for either mycophenolate or CNIs in renal transplantation which slowly finds its way into new immunosuppressive regimens. Everolimus is registered for maintenance immunosuppressive combination therapy with CNIs. Due to CNI-related nephrotoxicity and possible synergism between mTOR inhibitors and CNIs, CNI-minimizing and CNI free strategies combined with mTOR inhibitors are being actively investigated worldwide [3–7]. The narrow therapeutic window and high variable pharmacokinetics of everolimus, makes therapeutic drug monitoring (TDM) essential for prevention of toxicity or rejection [8]. Despite its proven efficacy and close TDM, everolimus is also known for high discontinuation rates and some serious side effects. Discontinuation is often directly side effect related [9,10]. Leukopenia, thrombocytopenia, hypertriglyceridemia and hypercholesterolemia are the most common side effects of mTOR inhibitors [5] and can often be managed with counteracting medication and/or dose reduction [9,11]. A potentially life threatening side effect of everolimus is non-infectious interstitial pneumonitis. This pneumonitis is characterized by the non-infectious, non-malignant infiltration of the lungs that presents as ground-glass opacities and focal consolidations on CT-scan [12]. It typically presents itself within 2 to 6 months after initiation of mTOR inhibitor therapy [13]. The exact mechanism of mTOR inhibitor-induced pneumonitis is still unknown. Direct damage to alveolar structures, formation of immunogenic molecules that react with specific antibodies, and direct immunologic drug responses have been suggested as possible mechanisms [14]. A dose relationship may be present and a higher incidence was found in males versus females on sirolimus therapy [15]. Infectious diseases are an important cause of death in transplant recipients [16,17] and strongly related to excessive and/or long-term clinical immunosuppression [18]. Everolimus is associated with a relatively low incidence of viral infections as compared to other immunosuppressive groups [19–21]. Everolimus is also associated with a higher incidence of new onset diabetes mellitus (NODM) which subsequently is associated with increased graft failure and mortality due to cardiovascular events [22]. Patients with diabetes mellitus or NODM have lower long term survival compared with non-diabetic patients [23].

NODM is therefore a serious complication of immunosuppressive therapy in transplant recipients. It occurs in around 4-5 % of renal transplant patients treated with everolimus and low dose cyclosporine [24,25]. In a non-CNI based regimen study it was 1% [26]. Tacrolimus based regimens are currently associated with the highest incidence of NODM [17,27]. Finding risk factors for discontinuation and the most severe side effects could help improve immunosuppressive therapy with mTOR inhibitors by monitoring them more closely or excluding patients with excessive risk from everolimus therapy. The cytochrome P450 (CYP) enzymes CYP3A4, CYP3A5 and CYP2C8 are involved in the metabolism of everolimus and everolimus is also a substrate for the efflux pump P-glycoprotein (ABCB1) [28,29]. The nuclear pregnane X receptor (PXR) mediates expression of CYP3A4 and multi drug resistance proteins (MDR1 and MDR2) and could therefore also influence everolimus pharmacokinetics [30–32]. Although polymorphisms in genes coding for these metabolizing enzymes do not seem to affect pharmacokinetics [33,34], tissue and immune cell concentrations and metabolite patterns might be affected resulting in differences in susceptibility for certain side effects. In the present study we performed a systematic analysis using a parametric survival model to describe the time to everolimus discontinuation and the most hazardous side effects data in renal transplant recipients by to explore potential risk factors for everolimus discontinuation and the most common and severe side effects. Such an approach has advantages compared to non-parametric and semi parametric analyses, because it enables inclusion of time-varying covariates and allows simulation based on the final model. A wide range of demographic, transplantation related, drug exposure as well as pharmacogenetic parameters were available for the analysis. The primary aim of this study was to develop time-to-event models for the time to drug discontinuation and the key side effect (i.e pneumonitis, infection and new onset diabetes mellitus) to identify risk factors that may determine therapy outcome.

Methods

Patients

Clinical data from 99 stable renal transplant recipients treated with immunosuppressive dual therapy consisting of everolimus (Certican®, Novartis, Basel, Switzerland) and prednisolone, who participated in a prospective, open, randomized, multicenter study were studied from 6 to 24 months after transplantation. During the first 6 months

after transplantation, patients received induction therapy with basiliximab (20 mg days 0 and 4; Simulect Novartis, Basel, Switzerland), prednisolone dose (50 mg twice daily intravenously), rapidly tapered to daily 10 mg oral at day 4. Additional maintenance immunosuppressive therapy consisted of cyclosporine, prednisolone and mycophenolate mofetil. At 6 months after transplantation a scheduled biopsy was performed. Patients without inflammation were included and randomized in three groups [4] and cyclosporine and mycophenolate mofetil were subsequently discontinued [4]. From this point onwards data of the everolimus group were collected for the present study. The study was approved by the Medical Ethics Committee of Leiden University Medical Center (Leiden, the Netherlands) and patients gave written informed consent.

Bioanalytics

Everolimus therapy was started at an oral dose of 3 mg twice daily and was supported by routine TDM. TDM was aimed at a target $AUC_{0\text{-}12h}$ of 120 μ*h/L for LC-MS/MS and 150 μg*h/L for FPIA. During the trial $AUC_{0\text{-}12h}$ were calculated using the trapezoidal rule using blood concentrations drawn at t = 0, 1, 2, 3, 4, 5, and 6 h after transplantation using the KinFit tool of MW/Pharm 3.5 (Mediware, Groningen, The Netherlands). Determined in whole blood by a validated liquid chromatography tandem mass spectrometric (LC-MS/MS) and previously published method [35,36] or fluorescent polarization assay FPIA [37] in three laboratories. Individual pharmacokinetic data for the current study such as AUC, C_{max} and C_{min} were derived using the previously published population pharmacokinetic model by *Moes et al.* [34] which accounts for the differences between the analytical methods and inter-occasion variability. For patients (n=3) without blood samples, the population mode prediction based on dose and demographic properties was used to estimate exposure.

Genotyping Assays

DNA was isolated from EDTA blood collected from patients. ABCB1-1236C>T, ABCB1-3435C>T, ABCB1-2677T>G and CYP3A4-15389C>T were determined with TaqMan 7500 (Applied Biosystems, Nieuwerkerk a.d. IJssel, the Netherlands) with predesigned or custom designed assays, according to manufacturers' protocol. ABCB1-129T>C, CYP3A5-6986A>G, CYP3A5-7225G>A, PXR-G-24113A and PXR-A+ 7635G were determined with Pyrosequencer 96MA (Isogen, IJsselstein, the Netherlands). Polymerase chain reactions (PCRs) contained 10 ng of DNA and 5 pmol of each PCR primer in a total volume of 12mL. Cycle conditions were initial denaturation for 15 minutes at 95°C, 35 cycles of 95°C, 55°C

and 72°C each for 30 seconds, ended by 10 minutes at 72°C. The pyrosequence reactions were performed according to manufacturers' protocol. Primers and probes used in the TaqMan based genotyping assays and primers and sequences used in the pyrosequence assays for each single-nucleotide polymorphism (SNP) are listed Supplementary Table I. 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. Haplotype analysis for ABCB1 was performed using gPLINK with haplotypes set with a certainty greater than 0.97. To investigate the combined effect of CYP3A5*3 and CYP3A4*22, genotype clusters were made as follows: Slow metabolizers (C1): No CYP3A5 activity (*CYP3A5*3/*3)* and at least one decreased activity allele in *CYP3A4 (CYP3A4*22/*22* or *CYP3A4*1/*22*), Intermediate metabolizers group 1 (C2): No CYP3A5 activity (*CYP3A5*3/*3)* and no decreased activity allele in *CYP3A4* (*CYP3A4*1/*1*), Intermediate metabolizers group 2 (C3): Carriers of at least one increased activity allele in *CYP3A5* (*CYP3A5*1/*1* or *CYP3A5*1/*3)* and at least one decreased activity allele in *CYP3A4* (*CYP3A4*22/*22* or *CYP3A4*1/*22*) and extensive metabolizers (C4): Carriers of at least one increased activity allele in *CYP3A5 (CYP3A5*1/*1* or *CYP3A5*1/*3)* and no decreased activity allele in *CYP3A4 (CYP3A4*1/*1).*

Discontinuation

Discontinuation was recorded on the exact date therefore the exact time to discontinuation could be calculated. Patients who did not discontinue therapy were censored at study end (550 days).

Side effects

For side effects the exact date of the start of symptoms were recorded, as well as the date that symptoms disappeared. For the analysis the date of the start was used to calculate the time to side effect. Patients who did not have a side effect during everolimus therapy were censored at discontinuation or at study end (550 days).

Non-infectious Interstitial Pneumonitis

Pulmonary problems in patients using everolimus were detected by the trial reports of serious adverse events and adverse events and review of the patient charts of all included

patients [38]. Charts were analyzed for clinical signs (for example dyspnea, cough or fever) and radiological signs of pulmonary involvement (abnormal chest X-ray and pulmonary CT scans). Patients, who developed symptoms of a non-infectious pneumonitis, were identified as cases. For the time to pneumonitis the date of the first symptoms were marked as the start date. The following criteria for pneumonitis were used [39]: (1) exposure to everolimus before the onset of pulmonary symptoms, (2) exclusion of other pulmonary disease, especially infection, (3) radiographic findings on CT of the chest not compatible with other diagnoses and (4) resolution of pulmonary symptoms after discontinuation of everolimus. Whenever available, a histopathological diagnosis consistent with drug-induced lung-toxicity was considered gold standard [38]. Patients classified with non-infectious pneumonitis were identified as cases. For the time-to-pneumonitis the date of the first symptoms were marked as the start date. Any infection (viral, fungal or bacterial) reported as (serious) adverse event requiring treatment was used for the time-event-analysis to identify risk factors for infection. New onset diabetes mellitus was diagnosed according to world health organization the (WHO) guidelines: – fasting plasma glucose ≥ 7.0 mmol/l (126 mg/dl) or 2–h plasma glucose ≥ 11.1 mmol/l (200 mg/dl) after everolimus initiation with the oral glucose tolerance test (OGTT) as the diagnostic test.

Model Development

The development of the different time-to-event models was a two steps approach: first a base model without any explanatory factors was constructed; thereafter the base model was used to explore potential covariates. In order to describe the time-to-first event a parametric survival function was used as presented below:

$$S(t) = e^{-\int_0^t h(t)dt}$$

The hazard is h(t), and the 'survival' S(t) is a function of the cumulative hazard within the time interval between start of the study (time zero) and the time t of the event describing the probability of not experiencing an event within this interval. Since time to first event only happens once per individual, random effect of the baseline hazard could not be estimated, therefor the same baseline hazard was assumed for all subjects. The base models were developed by exploring different function for the hazard h(t): Weibull, exponential, Gompertz, log-logistic and log-normal distributions [40].

Supplementary Table I: Primers and Probes for TaqMan and Pyrosequence Analysis

TaqMan (SNP)	ASSAY	Target	Sequence 5'-3'	Modification	Reference
ABCB1 C1236t (rs1128503)	Predesigned	PCR-f	CACCGTCTGCCCACTCT		C_758662_10
		PCR-r	GTGTCTGTGAATTGCCTTGAAGTTT		
		PCR-G	TTCAGGTTCAGACCCTT	VIC	
		PCR-T	CAGGTTCAGGCCCTT	FAM	
ABCB1 G2677T (rs2032582)	Custom	PCR-f	CTTAGAGCATAGTAAGCAGTAGGGAGT		
		PCR-r	GAAATGAAAATGTTGTCTGGACAAGCA		
		PCR-G	TTCCCAGCACCTTC	VIC	
		PCR-T	TTCCCAGAACCTTC	FAM	
ABCB1 T3435C (rs1045642)	Predesigned	PCR-f	ATGTATGTTGGCCTCCTTTGCT		C_7586657_20
		PCR-r	GCCGGGTGGTGTCACA		
		PCR-G	CCCTCACAATCTCT	VIC	
		PCR-T	CCCTCACGATCTCT	FAM	
CYP3A4*22 (rs35599367)	Predesigned				C_59013445_10
Pyrosequence (SNP)	**ASSAY**	**Target**	**Sequence 5'-3'**	**Modification**	**Reference**
ABCB1 129CT (rs3213619)		PCR-f	TCGAAGTTTTTATCCCA	Biotin	
		PCR-r	CCTCCTGGAAATTCAACCTGTT		
		Sequence primer	TACTCCGACTTTAGTGGAAAGACC		
		Target sequence	CTG/ACTCGAATGAG		
CYP3A5*3 (rs776746)		PCR-f	CTGCCTTCAATTTTTCACT	Biotin	
		PCR-r	TATGTTATGTAATCCATACCCC		
		Sequence primer	AGAGCTCTTTTGTCTTTCA		
		Target sequence	A/GTATCTC		
CYP3A5*6 (rs10264272)		PCR-f	TCTTTGGGGCCTACAGCATG	Biotin	
		PCR-r	AAAGAAATAATAGCCCACATACTTATTGAGAG		
		Sequence primer	AGAAACCAAATTTTAGGAA		
		Target sequence	CTTC/TTTAG		
PXR (NR1|2) G-24113A (rs2276706)		PCR-f	GAATCATGTTGGCCTTGCTGC	Biotin	
		PCR-r	GCATCAGTAATGGGGCTCAAC		
		Sequence primer	TCTCCTCATTTCTAGGGT		
		Target sequence	C/TCACCCTAG		
PXR(NR1|2) A+7635G (rs6785049)		PCR-f	AGCCATCCTCCCTCTTC	Biotin	
		PCR-r	CAGCAGCCATCCCATAATC		
		Sequence primer	CATAATCCAGAAGTTGGG		
		Target sequence	GGC/TGAGAGGAA		

f, forward orientated; r, reverse orientated; ABC, ATP-binding casette; CYP, cytochrome P450; PXR, pregnane X receptor; PCR, polymerase chain reaction.

Modification: VIC and FAM are fluorescent dyes, biotin is necessary to obtain single stranded DNA.

Development of covariate model

Of the available potential covariates different covariates were selected for each endpoint based on theoretical probability. The selected covariates for each endpoint are reported in the results. All preselected covariates were included in the stepwise covariate modelling (SCM). Relationships of the selected continuous covariates were also explored using KMMC plots [41]. Available covariates were:

Continuous: Age, Weight, AUC, C_{min}, C_{max}, AUC_{min}, Leukocytes, Thrombocytes, Cholesterol, Triglycerides. AUC_{min} is the minimal everolimus exposure during the trial of each individual. If this value was above the preset target value exposure has been too high during the entire study.

Dichotomous: Race (Caucasian vs non Caucasian); diabetes mellitus at start (yes vs no); Sex (male vs female); donor Type (deceased vs living); mean daily cyclosporine AUC until week 6 after transplantation (above target vs on/below target); mean daily cyclosporine AUC from week 6 until conversion to everolimus (above target vs on/below target). CYP3ACOMBINED (C1 vs C2,C3 and C4); PXR (NR1|2) -24113G>A (AA vs AG and GG); PXR (NR1|2) 7635A>G (GG vs AG and AA); ABCB1 3435C>T (CC vs TC and TT); ABCB1 1236C>T (TT vs CT and CC); ABCB1 2677T>G (GG vs GT and TT); ABCB1 -129T>C (TT vs TC); ABCB1 haplo CCG (2 copies vs 0 or 1 copy); ABCB1 haplo TTT (2 copies vs 0 or 1 copy); ABCB1 haplo TCG (2 copies vs 0 or 1 copy)

The hazard (h), including covariates was modeled over time (t) as:

$$h(t) = h_0(t) \cdot e^{\beta_1 \cdot X_1 + \beta_2 \cdot X_2 + \ldots + \beta_i \cdot X_i}$$

where $h_0(t)$ is the base hazard without covariates included. β_i is the coefficient describing the effect of covariate X_i. The covariate coefficient (β_i) was modeled for dichotomous covariates as $\beta_i \cdot X_i$ and for continuous covariates as a change from the median covariate: $\beta_i \cdot (X_i - X_{i\ median})$.

Dichotomous covariates were coded as 0, where for the most frequent category, otherwise 1, so that the covariate parameter is estimated for the less frequent category. All continuous covariates were tested for both linear and bi-linear relationship. All preselected covariate relationships were used for a systematic stepwise covariate modelling (SCM), with

stepwise forward inclusion and backward deletion [42]. Forward inclusion and backward deletion the level of statistical significance was set to $P < 0.05$ and $P < 0.01$, respectively, corresponding to differences in the NONMEM objective function value (OFV) of 3.84 and 6.64, respectively (1 degree of freedom).

Software: NONMEM (v7.3.0, Icon Development Solutions, Ellicott City, MD) was used for modeling, using PsN toolkit 3.4.2 [44]and Pirana version 2.8.0 [45] as modeling environment. Results were analyzed using statistical software package R (v2.15.2) and RStudio (v0.97.248; Boston, MA).

Model evaluation: Selection between hierarchical models was based on the likelihood ratio test using OFV. The OFV is proportional to -2 log likelihood and the difference in OFV for the two hierarchical models is approximately $\chi 2$ distributed. Models were also selected based on scientific plausibility and precision in parameter estimates. In addition, the TTE models were evaluated by means of visual predictive check (VPC), which is the Kaplan-Meier (KM) curve of the observed data together with a 95% prediction interval based on data simulated from the TTE model (200 replicates).

Results

Clinical details

A total of 99 adult stable renal transplant recipients were included in this study with patients followed up to 18 months after conversion to everolimus and prednisolone dual therapy. Mean age was 52 ±13 years (range: 22-71 years). Mean weight was 79 ±15 kg (range: 50 -129 kg). Rejection rate after conversion was very low (3%), however finally only 58 patients remained on therapy the entire study due to discontinuation, which was primarily caused by side effects. The majority of the patients (85%) were Caucasian. Further demographic, transplant related and exposure details and as well as efficacy and safety response data, are summarized in Table 1.

Table 1: Demographic and transplantation characteristics

Recipient characteristics	Number (%)	Mean ± SD	Median (Range)
Demographical			
Male	65 (66)		
Female	34 (34)		
Caucasian	84 (85)		
Age (years)		52 (13)	54 (22-71)
Weight (kg)		79 (15)	78 (50 -129)
Ideal Body Weight (kg)		67 (8)	67 (49-83)
Height (cm)		174 (10)	174 (152 -194)
Creatinine (µmol/L)		118 (34)	113 (61 - 251)
Diabetes Mellitus at start	11 (11)		
Hematocrit (L/L)		0.38 (0.04)	0.37 (0.29-0.5)
Underlying disease (%):			
polycystic kidney disease	23 (23.2)		
glomerulonephritis	17 (17.2)		
diabetic nephropathy	4 (4.0)		
hypertension	15 (15.2)		
focal glomerulosclerosis	4 (4.0)		
etiology uncertain (e.c.i.)	5 (5.1)		
interstitial disease	3 (3.0)		
urological origin	10 (10.1)		
other	18 (18.2)		
Transplantation related			
Rank of kidney transplantation			
1	93 (94)		
2	6 (6)		
Delayed graft function	15 (15.2)		
Cold Ischemic Time (h)		9.4 (7.8)	8.5 (2 -28)
Donor Age		49 (13)	51 (18 - 72)
Donor Type			
Living	52		
Deceased	47		
HLA-mismatches:			
HLA-AB [0/1/2/3/4]	12/16/40/18/13		
HLA-DR [0/1/2]	27/72/0		
Exposure			
Everolimus dose (mg)		2.5 (0.8)	3 (0.75 -5.25)
Everolimus AUC (µg*h/L)		156 (60)	148 (54 - 488)
Everolimus Cmax (µg/L)		24 (9.5)	23.5 (6.2 - 66.6)
Everolimus Cmin (µg/L)		9.5 (4.3)	8.5 (3.3 - 32.2)
Everolimus AUCmin (µg*h/L)		122 (37)	118 (54 -228)

Efficacy and safety responses			
Freedom from acute rejection	96 (97)		
Completion of study	58 (59)		
Hypercholesterolemia	55 (56)		
mild (>6.5 mmol/L)	37 (37)		
severe (>8 mmol/L)	18 (18)		
Hypertriglyceridemia (>2.9 mmol/L)	45 (45)		
mild (>2.9 mmol/L)	43 (43)		
severe (>11.3 mmol/L)	2 (2)		
Thrombocytopenia (<75 · 10^9/L)	2 (2)		
Leukopenia (<4 · 10^9/L)	12 (12)		
Diarrhea	7 (7)		
Dyspnea	6 (6)		
Serious infections (airway, gastrointestinal, urinary tract)	35 (35)		
Interstitial Pneumonia	11 (11)		
Edema	1 (1)		
Cardiovascular events	10 (10)		
New onset Diabetes Mellitus	14 (14)		
Malignancy	4 (4)		
Renal Function			
Week 32 (ml/min)		70 (25)	71 (23 - 120)
Week 52 (ml/min)		71 (24)	70 (23-120)
Week 104 (ml/min)		71 (25)	76 (23 -120)
Discontinuation reasons			
Pneumonia	11		
Withdrawn consent *	9		
Died	4		
Intolerability	4		
Rejection	2		
Diarrhea	2		
Dyspnea	2		
Edema	2		
BK virus nephropathy	1		
Chronic allograft nephropathy	1		
Cardiac problems	1		
Hypercholesterolemia	1		

* 6 out of 9 patients withdrew consent because of side effects and comorbidities.

Discontinuation data

Of the 99 patients, 58 patients complete the study, discontinuation reasons are presented Table 1. The majority of the discontinuations were related to side effects. The median

time to discontinuation in the group of patients who discontinued therapy was 130 days. Patients who did not discontinue therapy were censored at study end (550 days).

Side effects data

For the current analysis only the most severe side effects, being interstitial pneumonitis, infection and new onset diabetes mellitus, were analyzed separately for risk factors. Patients who did not have a side effect during everolimus therapy were censored at discontinuation or at study end (550 days).

Non-infectious interstitial pneumonitis

Of the 99 patients in this dataset, 11 patients experienced non-infectious interstitial pneumonitis and everolimus therapy had to be stopped to assure recovery. The median time to pneumonitis was 106 days.

Infection

In the period from everolimus initiation to end of study (550 days) 35 patients of the 99 total patients had an infection requiring treatment. The median time to first infection was 76 days.

New onset Diabetes Mellitus

For the new onset diabetes mellitus time to event analysis 11 patients were excluded because they were already diagnosed with diabetes mellitus. Of the 88 included patients 14 developed new onset diabetes mellitus during the study. The median time to new onset diabetes mellitus diagnosis was 254 days.

Genotyping

The distributions of all single-nucleotide polymorphisms were in Hardy–Weinberg equilibrium ($P>0.05$). The distributions of the investigated CYP3A4, CYP3A5, the combined CYP3A4 and CYP3A5 genotype and P-gp polymorphisms are listed in Table 2. Allele frequencies found in our data set corresponded with those published previously. Haplotype distributions are listed in table 3. The combined CYP3A4 and CYP3A5 genotype (CYP3ACOMBINED), in theory reflecting the largest differences in metabolite patterns [33] and was therefore chosen as the only CYP3A genotype covariate for the current analysis.

Table 2: Genotype frequencies in studied population (n = 99).

Gene	SNPs	Nucleotide position and alleles	Genotype	Frequency	[n (%)]
CYP3A4	rs35599367	15389C>T	C/C	87	[88]
			C/T	10	[10]
			T/T	1	[1]
			N.G.	1	[1]
CYP3A5	rs776746	6986A>G	A/A	83	[84]
			G/A	12	[12]
			G/G	3	[3]
			N.G.	1	[1]
	rs10264272	7225G>A	G/G	97	[98]
			A/G	1	[1]
			A/A	0	[0]
			N.G.	1	[1]
CYP3ACOMBINED			C1	11	[11]
			C2	72	[73]
			C3	0	[0]
			C4	15	[15]
			N.G.	1	[1]
ABCB1	rs1128503	1236C>T	C/C	36	[36]
			C/T	46	[47]
			T/T	16	[16]
			N.G.	1	[1]
	rs2032582	2677T>G	T/T	38	[38.5]
			G/T	42	[42.5]
			G/G	18	[18]
			N.G	1	[1]
	rs1045642	3435C>T	C/C	27	[27.5]
			C/T	47	[47.5]
			T/T	24	[24]
			N.G.	1	[1]
	rs3213619	-129T>C	T/T	89	[90]
			C/T	9	[9]
			C/C	0	[0]
			N.G.	1	[1]
PXR (NR1|2)	rs2276706	-24113G>A	G/G	14	[14]
			G/A	50	[50.5]
			A/A	34	[34.5]
			N.G.	1	[1]
	rs6785049	7635A>G	G/G	13	[13]
			A/G	47	[47.5]
			A/A	38	[38.5]
			N.G.	1	[1]

CYP, cytochrome p450; PXR, pregnane X receptor; SNP, single nucleotide polymorphism.

Table 3: Haplotype frequencies in studied population (n = 99).

Gene	SNPs in haplotype	Haplotype block 1	Haplotype block 2	Frequency	[N (%)]	Haplotype	Frequency	[N (%)]
ABCB1	rs1128503	TTT	CCG	29	29.3	CCG	87	44
	rs2032582	CCG	CCG	20	20.2	TTT	72	36.4
	rs1045642	TTT	TTT	14	14.1	TCG	27	13.6
		CCG	TCG	13	13.1	CTG	6	3
		TTT	TCG	10	10.1	CTT	2	1
		CCG	CTG	3	3.0	CCT	1	0.5
		CCG	CTT	1	1.0	TCT	1	0.5
		TTT	CTG	2	2.0			
		TCG	TCG	2	2.0			
		TTT	CCT	1	1.0			
		TTT	CTT	1	1.0			
		CCG	TCT	1	1.0			
		TCG	CTG	1	1.0			

Base model

The time to everolimus-discontinuation, non-infectious interstitial pneumonitis and infection was best described using the Gompertz function with regard to objective function value (OFV) and Kaplan-Meier plots:

$$h_0(t) = \lambda \cdot e^{\gamma \cdot t}$$

$$S(t) = e^{\frac{\lambda}{\gamma} \cdot (1 - e^{\gamma \cdot t})}$$

The formula contains two parameters: λ describing the scale and γ describing the shape of the survival curve. New onset diabetes mellitus was equally well described by an exponential, Weibull and Gompertz hazard function. The exponential was chosen over the Weibull and Gompertz because the exponential function contains only 1 parameter: λ describing the scale of the survival curve.

$$h_0(t) = \lambda$$

$$S(t) = e^{-\lambda t}$$

Table 4 shows the OFV of the different explored survival distributions for each endpoint. Figure 1 presents the VPCs of the different endpoints from 200 simulations showing that the model adequately describes the data for the different endpoints discontinuation, non-infectious interstitial pneumonitis, infection and new onset diabetes mellitus.

Covariate Model

Covariates were selected based on theoretical plausibility. All selected covariates were included in the SCM. Results of the univariate testing of the selected covariate relationships (the first step in the SCM) are presented in Table 5. Results of the full SCM analysis are presented in Table 6.

Table 4: Objective function value and number of parameters of different tested survival distribution functions.

Survival function	Number of parameters	OFV
Discontinuation		
Weibull	2	632.9
Exponential	1	642.0
Gompertz	**2**	**623.3**
Log logistic	2	630.2
Log normal	2	627.2
Pneumonia		
Weibull	2	197.6
Exponential	1	200.7
Gompertz	**2**	**194.0**
Log logistic	2	198.0
Log normal	2	196.0
Infection		
Weibull	2	545.7
Exponential	1	555.0
Gompertz	**2**	**540.3**
Log logistic	2	543.5
Log normal	2	540.3
New onset DM		
Weibull	2	243.9
Exponential	**1**	**244.0**
Gompertz	2	244.0
Log logistic	2	245.0
Log normal	2	244.0

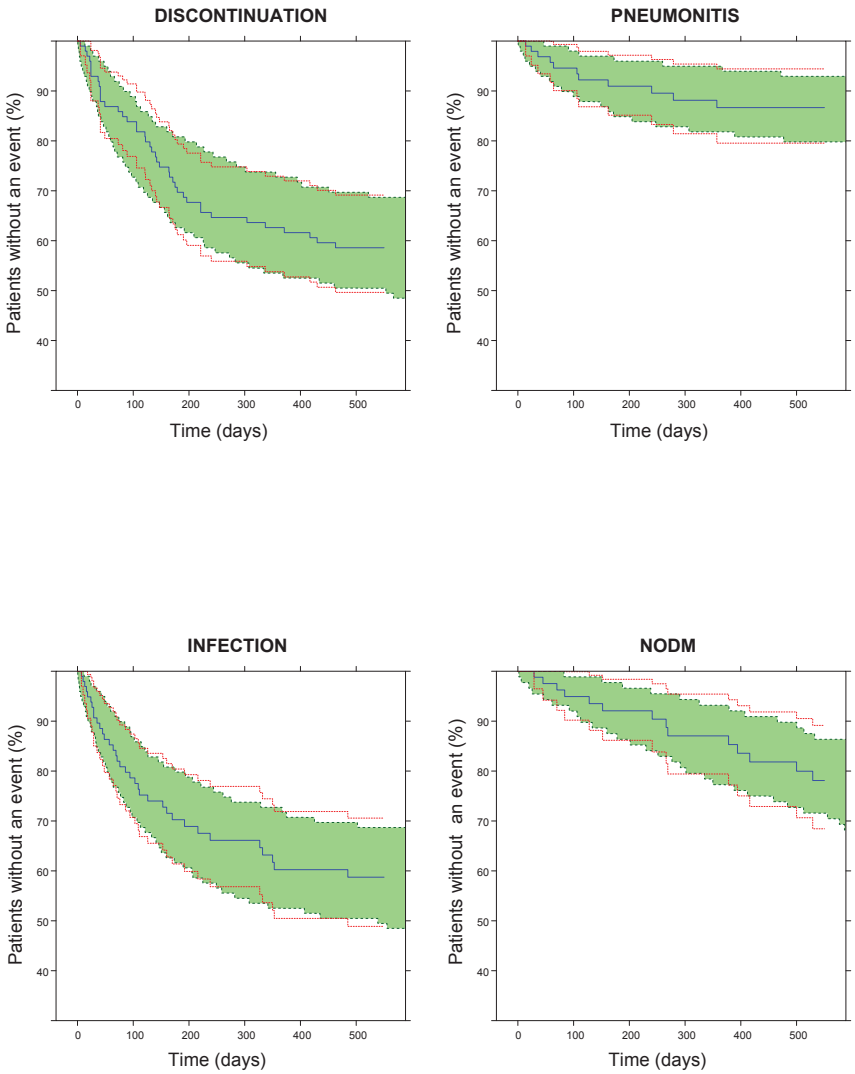

Figure 1: Kaplan-Meier Plots of the percentage of patients without an event (Discontinuation, Pneumonitis, Infection, NODM) vs Time after everolimus initiation. The shaded area represents the 95% prediction intervals for the simulated data. The continuous line represents the real data, the dashed red lines represent the 90% confidence interval of the real data.

Table 5: Results of the univariate covariate analysis for each endpoint.

	Discontinuation		Pneumonitis		Infection		New Onset DM	
Model	Δ OFV	P value	Δ OFV	P value	Δ OFV	P value	Δ OFV	P value
Base model	0	-	0	-	0	-	0	-
Continuous								
Age	**8.484**	**0.0036**	3.172	0.0749	1.200	0.2732	**6.544**	**0.0105**
Weight	**4.684**	**0.0304**	0.572	0.4494	0.001	0.9791	2.537	0.1112
AUC	0.017	0.8975	3.489	0.0618	0.763	0.3822	0.308	0.5788
C_{min}	1.469	0.2255	3.056	0.0805	1.430	0.2317	0.444	0.5050
C_{max}	0.804	0.3698	3.098	0.0784	1.338	0.2473	0.358	0.5496
AUC_{min}	**19.432**	**0.0000**	**13.663**	**0.0002**	2.620	0.1055	1.401	0.2365
Leukocytes	-	-	-	-	**4.143**	**0.0418**	-	-
Cholesterol	-	-	-	-	0.393	0.5310	-	-
Triglycerides	-	-	-	-	0.716	0.3976	-	-
Categorical								
Recipient Race	3.666	0.0555	0.005	0.9421	2.911	0.0880	0.012	0.9112
Diabetes Mellitus at start	0.789	0.3744	2.716	0.0993	0.444	0.5052	-	-
Sex	1.398	0.2371	0.355	0.5513	1.003	0.3167	1.116	0.2908

CYP3ACOMBINED	1.012	0.3144	0.704	0.4014	-	-	-	-
PXR (NR1|2) -24113G>A	**6.513**	**0.0107**	**5.612**	**0.0178**	2.028	0.1545	**4.377**	**0.0364**
PXR (NR1|2) 7635A>G	0.149	0.6996	0.291	0.5894	1.202	0.2729	0.874	0.3498
ABCB1 3435C>T	**5.604**	**0.0179**	0.520	0.4707	0.033	0.8562	2.799	0.0943
ABCB1 1236C>T	2.173	0.1405	1.406	0.2358	0.023	0.8788	0.549	0.4587
ABCB1 2677T>G	0.002	0.9633	0.214	0.6438	0.345	0.5568	1.687	0.1940
ABCB1 -129T>C	0.036	0.8495	0.951	0.3294	2.588	0.1077	0.017	0.8961
ABCB1 haplo CCG	**5.053**	**0.0246**	0.171	0.6789	0.008	0.9272	2.181	0.1398
ABCB1 haplo TTT	1.918	0.1661	0.328	0.5666	0.122	0.7264	0.294	0.5877
ABCB1 haplo TCG	1.980	1.9800	0.366	0.5450	1.341	0.2469	0.295	0.5869
Donor Type	-	-	-	-	-	-	**5.822**	**0.0158**
Avg daily CsA AUC till week 6 after Tx	-	-	-	-	-	-	3.286	0.0699
Avg daily CsA AUC from week 6 till conversion to EVR	-	-	-	-	-	-	0.187	0.6654
# AUC (AUC above 156 µg*hr/L (30% above target)	**4.147**	**0.0417**	**6.063**	**0.0138**	-	-	-	-
# AUC (AUC above 180 µg*hr/L (50% above target)	0.189	0.6637	**13.084**	**0.0003**	-	-	-	-

Δ OFV, difference in objective function value between covariate and base model #, not used for the SCM.

Discontinuation

For discontinuation the following covariates were selected: Age, Weight, AUC, C_{min}, C_{max}, and AUC_{min} as continuous covariates and Race, Diabetes Mellitus at start, Sex and a number of pharmacogenetic covariates: CYP3A combined (CYP3A5*1 and CYP3A4*22) genotype, PXR (NR1|2) -24113G>A, PXR (NR1|2) 7635A>G, ABCB1 3435C>T, ABCB1 1236C>T, ABCB1 2677T>G, ABCB1 -129T>C, ABCB1 haplo CCG, ABCB1 haplo TTT and ABCB1 haplo TCG. For discontinuation significant relationships were found in the univariate analysis for Age, Weight, AUC_{min}, PXR (NR1|2) -24113G>A (AA vs GA and GG), ABCB1 3435C>T (CC versus CT and TT) and ABCB1 haplo CCG (2 copies vs 1 or zero copies). Patients with a constant excess exposure (high AUC_{min}) had a higher hazard of drug-discontinuation as graphically shown in Figure 2. Higher age also increased the risk of discontinuation as shown in Figure 3.

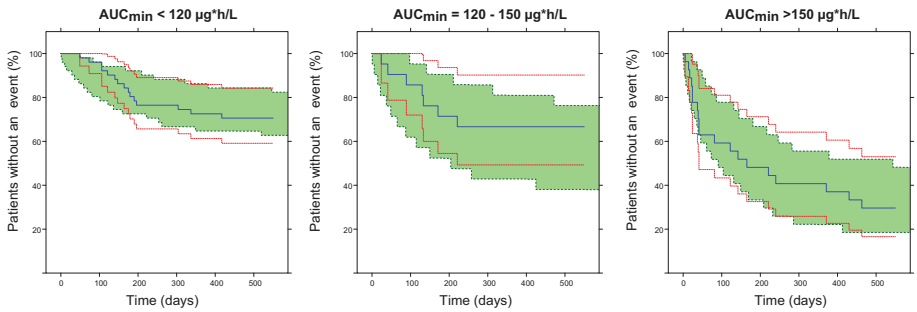

Figure 2: Kaplan-Meier VPC of final time to event model stratified by AUC_{min} groups. The shaded area represents the 95% prediction intervals for the simulated data. The continuous line represents the real data, the dashed red lines represent the 90% confidence interval of the real data.

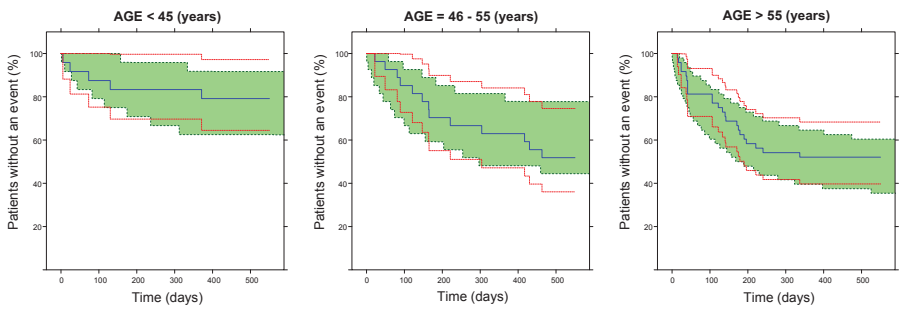

Figure 3: Kaplan-Meier VPC of final time to event model stratified by age group. The shaded area represents the 95% prediction intervals for the simulated data. The continuous line represents the real data, the dashed red lines represent the 90% confidence interval of the real data.

Table 6: Results of the multivariate covariate analysis for each endpoint.

Model	absolute OF	ΔOFV	P-value
Discontinuation			
forward inclusion			
BASE-model	623.276		
Step 1: AND effect AUCmin	603.844	-19.432	0.00001
Step 2: AND effect Age	595.580	-8.264	0.00404
Step 3: AND effect Race	589.757	-5.823	0.01581
Step 4: AND effect PXR (NR1|2) -24113G>A	584.079	-5.678	0.01718
backward deletion			
FULL-model	584.079		
Step 1: MINUS effect PXR (NR1|2) -24113G>A	589.757	5.678	0.01718
Step 2: MINUS effect Race	595.580	5.823	0.01581
Step 3: MINUS effect Age	603.844	8.264	0.00404
Step 3: MINUS effect AUCmin	614.792	19.212	0.00001
Pneumonitis			
forward inclusion			
BASE-model	194.028		
Step 1: AND effect AUCmin non-linear	173.901	-20.127	0.00001
Step 2: AND effect PXR (NR1|2) -24113G>A	165.396	-8.505	0.00354
backward deletion			
FULL-model	165.396		
Step 1: MINUS effect P227	173.901	7.406	0.00650
Step 1: MINUS effect AUCmin non-linear	194.028	20.127	0.00454
Infection			
forward inclusion			
BASE-model	521.936		
Step 1: AND effect Leukocytes	517.793	-4.143	0.04181
backward deletion			
FULL-model	517.793		
Step 1: MINUS effect Leukocytes	521.936	4.143	0.04181
New Onset Diabetes Mellitus			
forward inclusion			
BASE-model	244.012		
Step 1: AND effect Age	237.468	-6.54369	0.01053
backward deletion			
FULL-model	237.468		
Step 1: MINUS effect Age	244.012	6.54369	0.01053

ΔOFV >3.84 (P<0.05) forward inclusion and >6.64 (P<0.01) for backward deletion.* left out of the final model.
ΔOFV difference of objective function value.

ABCB1 3435C>T: CC genotype and ABCB1 CCG haplotype were associated with lower risk of discontinuation compared to the other variants. However, only AUC_{min} and Age remained significant after the forward inclusion and backward deletion step as showed in Table 6. These two covariates were included in the final time-to-event model.

Non-infectious interstitial pneumonitis

The selected covariates to investigate for non-infectious interstitial pneumonitis were: Age, Weight, AUC, C_{min}, C_{max}, and AUC_{min} as continuous covariates and Race, Diabetes Mellitus at start, Sex and the following pharmacogenetic covariates: CYP3A combined (CYP3A5*1 and CYP3A4*22) genotype, PXR (NR1|2) -24113G>A, PXR (NR1|2) 7635A>G, ABCB1 3435C>T, ABCB1 1236C>T, ABCB1 2677T>G, ABCB1 -129T>C, ABCB1 haplo CCG, ABCB1 haplo TTT and ABCB1 haplo TCG. For non-infectious interstitial pneumonitis, AUC_{min} and PXR (NR1|2) -24113G>A (AA vs GA and GG) were significant covariates on the hazard for pneumonitis. Both covariates remained significant in the multivariate analysis. Figure 4 shows graphically that having an AUC_{min} above 150 µg*hr/L clearly increased the risk of experiencing non-infectious interstitial pneumonitis. Figure 5 shows that patients with a PXR (NR1|2) (-24113G>A) AA genotype had a slight increase of the risk of experiencing pneumonitis.

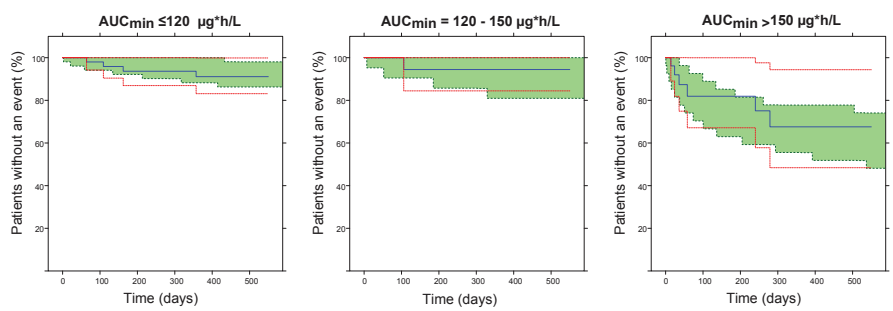

Figure 4: Kaplan-Meier VPC of final time to event model stratified by AUC_{min} groups. The shaded area represents the 95% prediction intervals for the simulated data. The continuous line represents the real data, the dashed red lines represent the 90% confidence interval of the real data.

Infection

The selected covariates to investigate for Infection were: Age, Weight, AUC, C_{min}, C_{max}, and AUC_{min}, Leukocyte count, Cholesterol and Triglycerides levels as continuous covariates and Race, Diabetes Mellitus at start, Sex and a number of pharmacogenetic covariates:

PXR (NR1|2) -24113G>A, PXR (NR1|2) 7635A>G, ABCB1 3435C>T, ABCB1 1236C>T, ABCB1 2677T>G, ABCB1 -129T>C, ABCB1 haplo CCG, ABCB1 haplo TTT and ABCB1 haplo TCG. For infection only leukocyte count had a significant effect in the univariate analysis, but was dropped after backward elimination. No significant risk factors were determined for the occurrence of infection and were not included in the final model.

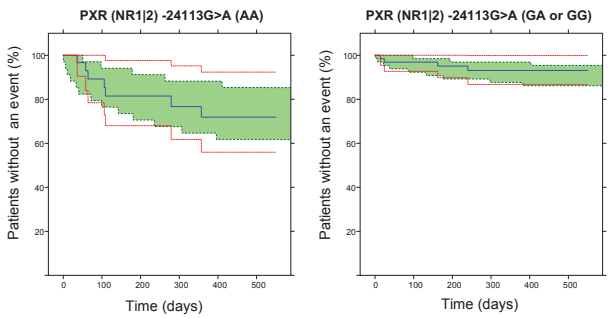

Figure 5: Kaplan-Meier VPC of final time to event model stratified by PXR (NR1|2) -24113G>A groups. The shaded area represents the 95% prediction intervals for the simulated data. The continuous line represents the real data, the dashed red lines represent the 90% confidence interval of the real data.

New Onset Diabetes Mellitus

The selected covariates to investigate for New Onset Diabetes Mellitus: Age, Weight, AUC, C_{min}, C_{max}, and AUC_{min}, as continuous covariates and Race, Sex, Donor Type, Average daily Cyclosporine AUC till week 5 after Tx (Target value was 5400 (µg*hr/L)), Average daily Cyclosporine AUC from week 6 till conversion to Everolimus (Target value was 3250 (µg*hr/L)) and a number of pharmacogenetic covariates: PXR (NR1|2) -24113G>A, PXR (NR1|2) 7635A>G, ABCB1 3435C>T, ABCB1 1236C>T, ABCB1 2677T>G, ABCB1 -129T>C, ABCB1 haplo CCG, ABCB1 haplo TTT and ABCB1 haplo TCG. For new onset diabetes mellitus, Age, PXR (NR1|2) -24113G>A and Donor type had a significant effect on the hazard in the univariate analysis. Higher age, PXR (NR1|2) -24113G>A (AA) genotype and having a kidney of a deceased donor were associated with higher risk of developing NODM. However, after the forward inclusion step only Age remained significant but was left out of the final model after the backward elimination step and therefore no covariate relationships were included in the final time-to-event model.

Discussion

In the current analysis the time from everolimus initiation to discontinuation, time to non-infectious interstitial pneumonitis and time to first infection were adequately described in a time-to-event model with the Gompertz distribution function. Time to new onset diabetes mellitus was best described by a time-to-event model with the exponential distribution function. This study shows for the first time that excess exposure during the study period and older age were risk factors for everolimus-discontinuation. The risk of experiencing non-infectious pneumonitis was also increased by prolonged excess exposure. Renal transplant recipients with a PXR (NR1|2)(-24113G>A): AA genotype had a higher risk of developing pneumonitis compared to those carrying the AG or GG genotype. In addition no significant covariates were included in the final time to event model for infection and new onset diabetes mellitus.

To our knowledge this is the first study exploring risk factors for discontinuation and severe side effects in renal transplant recipients on an everolimus and prednisolone regimen using a sophisticated time-to-event analysis with inclusion of demographic, transplant related and pharmacogenetic covariates. The effect of continuous high exposure (high AUC_{min}) on the risk of discontinuation everolimus can be explained by the fact that, in the majority of discontinuation was side effect related and certain side effects have previously shown to be dependent on exposure [46,47]. Furthermore patients experiencing non-infectious pneumonitis always discontinued everolimus therapy. As can be concluded from our results, clinicians should prevent renal transplant recipients from reaching excess everolimus exposure (i.e. AUC_{12}> 120 -150 μg*h/L), therefore close TDM remains warranted. Looking at the high discontinuation rates and low rejection risk we can extrapolate an initial target trough level between 6 μg/L and 8 μg/L from this study and an initial dose of 2 mg b.i.d. The lower exposure threshold cannot be established from this study since only 3 patients experienced an acute rejection episode. Moreover only a prospective trial should establish the lower exposure threshold for everolimus and prednisolone dual therapy.

The effect of age on discontinuation, meaning a higher age results in a higher risk of discontinuation can be explained be fact that often patients with higher age have more comorbidities and have a weaker immune system such as changes in T-cell function[48] where the effect of the same immunosuppression exposure might be higher. Furthermore older patients with more comorbidities are less able to cope with side effects compared

to young patients with no comorbidities. In addition the other investigated covariates for discontinuation that were significant in the univariate analysis were Weight, ABCB1 3435C>T and ABCB1 haplo CCG. Higher weight increased the risk of discontinuation, presumably because high weight is correlated with more comorbities and worse outcome [49]. ABCB1 3435C>T (CC vs CT and TT) and ABCB1 haplo CCG (2 copies vs 0 and 1 copy) decreased the risk of discontinuation and these genotypes are both associated with higher P-gp activity [50,51]. Since everolimus is also a substrate for the efflux pump P-glycoprotein (ABCB1) [28,29] this higher P-gp activity for patients with CC genotype and CCG haplotype could reflect lower intracellular everolimus concentrations in T-cells. P-gp is highly polymorphic and for this study four most relevant SNP's were selected. These ABCB1 polymorphism have previously also been linked to altered drug transporter activity leading to differences in peripheral blood mononuclear cell tacrolimus concentrations [52]. Concluding, the discovered effect of the ABCB1 polymorphism disappeared in the multivariate analysis, most likely by the fact that the AUC_{min} covariate effect neutralizes the effect of the polymorphisms. The polymorphism however could become more relevant when maintaining a lower target AUC than was used in the current study.

The incidence of non-infectious interstitial pneumonitis was relatively high 11% in this study. In other studies the incidence of pneumonia or pneumonitis with the usage of sirolimus (SRL) was about 1-10% [53], for everolimus 0-7% [11] and in oncology where higher daily dosage (10 mg) are used even higher (14%) incidence has been reported [13]. The precise mechanism is still unclear but a cell mediated autoimmune response after exposure of cryptic antigens or T-cell-mediated delayed-type hypersensitivity is one of the suggested mechanisms. Over the years a number of case report were published concerning mTOR pneumonitis in transplantation [39,54,55]. So far, no clear patient-related or context-related risk factors had been identified. In the current analysis we found a bi-linear relation for AUC_{min}, with increasing risk of pneumonitis starting to increase above an AUC_{min} above 120 μg*h/ L. In an earlier analysis the average AUC was not significantly related to the incidence of non-infectious pneumonitis, however in contrast to the current analysis time to event was not taken into account and this type of relationship was not investigated [38]. One could argue that AUC_{min} is not a very accurate measure for exposure during the trial, however at worst it is underestimation of the true exposure and according to the found relationship an exposure above the target value increases even further with an rising exposure. While the time varying AUC as a continuous covariate was not significant when tested linear or as a bi-linear relation, AUC was significant when

tested categorically: patients with an exposure of > 180 µg*h/L had a significant higher risk of developing pneumonitis. The measure AUC_{min} which represent the lowest measured AUC of the patient however, had a larger impact on the objective function drop and was therefore chosen over the other for inclusion in the SCM. Since all pneumonitis cases also discontinued everolimus therapy a large part of the effect of AUCmin on discontinuation is caused by the pneumonitis cases. The increase in risk of patient with that was found for patients with PXR (NR1|2) (-24113G>A) AA genotype might be related to an increased accumulation of everolimus in the lungs. In animal experiment high affinity for lungs and kidney were found for everolimus [56] and could this could also take place in humans. PXR is a nuclear receptor whose primary function is to sense the presence of foreign toxic substances and in response up regulate the expression of proteins involved in the detoxification and clearance of these substances from the body. PXR polymorphism could therefore also have an effect on drug transporter activity since PXR is able to influence enzyme activity and multi drug transporter proteins [30–32]. The effect seems to be limited as shown in figure but warrants further investigation in another dataset.

Infections continue to be an important feature in the first year following both renal and heart transplant and occur in around 50% of patients [16]. The incidence has previously been related to the intensity and type of immunosuppression [17]. For example, in a year-long comparison of everolimus versus traditional immunosuppressant treatment, viral infections occurred in 31 % of subjects receiving azathioprine versus 15% and 17% for everolimus 1.5 mg/day and 3.0 mg/day groups, respectively. The use of everolimus reduced the risk of CMV infection by almost one-third as compared with azathioprine-based therapy [18]. In the current analysis no significant factors were found of infections presumably because all patients received the same immunosuppressive regimen and exposure was aimed at a preset target value. In another study comparing two dose regimens 1,5 mg vs 3 mg everolimus (daily dose with CsA) no differences were found in the occurrence of infection [57].

New onset diabetes mellitus is a serious side effect which decreases long term survival of renal transplant recipients [23]. Although known from literature, important risk factor for the development of NODM include African ethnicity, increased age, obesity, increased number of transplants, donor type, a family history of diabetes and the use of prednisolone [27] none of these relationships remained significant in our multivariate analysis. Increased age, PXR (NR1|2) -24113G>A (AA genotype) and a having a donor kidney from a deceased donor increased the risk of NODM. The effect however appeared

to be too small to be included in the final model. The analysis for NODM has some specific limitations; the dataset lacked a significant number of patients from African ethnicity, Family history of diabetes was not available in the dataset en could therefore not be included in the covariate analysis. The number of renal transplants could not be tested since only 5 patients in the dataset had a second kidney transplant, all other patients received a first transplant. Furthermore none of the patients who had a second transplant was diagnosed with NODM during everolimus therapy. Exposure did not seem to affect the occurrence of NODM. This is in accordance with a study by *Shihab et al.* who also found no clear correlation between everolimus exposure and NODM, and the differences in exposure between patients in that particular study were even much larger [47].

Our study had some limitations, the dataset was relatively small and therefore not all side effects could be explored. However, the current study is relatively small, it is currently the largest dataset available with this specific immunosuppressive regimen and the extensive dataset is comprehensive which has advantages for identifying possible risk factors for the investigated endpoints. Data on exposure, demographic, transplant related factors as well as pharmacogenetic factor were available for analysis. Since all included patients were included in the same clinical trial and the majority of patients were of Caucasian origin the dataset was very homogeneous. The previously published pharmacokinetic model [33] was developed on the same data set, which included rich pharmacokinetic sampling thereby enabling precise estimation of the everolimus exposure.

In conclusion, risk factors of everolimus discontinuation of renal transplant recipients on a regimen of everolimus and prednisone duo therapy were constant too high everolimus exposure and increasing age. This study shows that the initial dose of 3 mg b.i.d might be too high given the high discontinuation rate and low acute rejection rate. Furthermore, risk factor for the hazardous side-effect non-infectious interstitial pneumonitis were constant too high everolimus exposure and PXR (NR1|2)(-24113G>A): AA genotype. For infection and new onset diabetes mellitus no significant covariates could be detected. The current findings can be used to further optimize everolimus based immunosuppressive therapy by preventing too high exposure by strict therapeutic drug monitoring.

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7

Effect of CYP3A4*22, CYP3A5*3 and CYP3A combined genotypes on cyclosporine, everolimus and tacrolimus pharmacokinetics in renal transplantation

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Abstract

Cyclosporine, everolimus and tacrolimus are the cornerstone of immunosuppressive therapy in renal transplantation. These drugs are characterized by narrow therapeutic windows, highly variable pharmacokinetics and metabolism by CYP3A enzymes. Recently the decreased activity allele *CYP3A4*22,* was described as a potential predictive marker for CYP3A4 activity. This study investigated the effect of *CYP3A4*22*, *CYP3A5*3* and *CYP3A* combined genotypes on cyclosporine, everolimus and tacrolimus pharmacokinetics in renal transplant patients. *CYP3A4*22* carriers showed a significant lower clearance for cyclosporine (-15%) and a trend was observed for everolimus (-7%) and tacrolimus (-16%). Patients carrying at least one *CYP3A5*1* allele had 1.5 fold higher tacrolimus clearance compared to non-carriers, however *CYP3A5*3* appeared not predictive for everolimus and cyclosporine. *CYP3A* combined genotype did not significantly improve prediction of clearance compared to *CYP3A5*3* or *CYP3A4*22* alone. These data suggest that dose individualization of cyclosporine, everolimus or tacrolimus therapy based on *CYP3A4*22* is not indicated.

Introduction

Cyclosporine, everolimus and tacrolimus are the cornerstone of maintenance immunosuppressive therapy in renal transplantation. These drugs are characterized by a small therapeutic window and highly variable pharmacokinetics (PK) which makes therapeutic drug monitoring (TDM) essential for maintaining adequate exposure and preventing serious drug-related toxicities [1–4].

Cyclosporine, everolimus and tacrolimus are primarily metabolized by cytochrome P450 enzymes CYP3A4 and CYP3A5 [5–8]. Differences in activity of these metabolizing enzymes are likely to be responsible for a significant part of the inter-individual variability in pharmacokinetics [9,10]. Genetic polymorphisms in genes encoding these metabolizing enzymes have previously been found to explain a part of the variability in pharmacokinetics of these immunosuppressive drugs [1,11–15]. Recently the decreased activity allele *CYP3A4*22* was identified as a novel predictive marker for tacrolimus pharmacokinetics [16,17], however these findings have not been successfully reproduced [12]. *CYP3A4*22* has also been investigated to a less extend in cyclosporine pharmacokinetics but its effect on everolimus pharmacokinetics is still unknown [16–18]. *CYP3A5*3* was studied before in relation to pharmacokinetics of everolimus, tacrolimus and cyclosporine [11,19–21] but the *CYP3A* combined genotype (*CYP3A4* and *CYP3A5*), which most likely better reflect CYP3A activity, has only been evaluated for tacrolimus [16].

The studies investigating the effect of *CYP3A4*22* on tacrolimus pharmacokinetics were limited by the use of trough concentrations, lack of data on co-medications and did not use population pharmacokinetic analysis. Such an approach enables to differentiate between inter-patient and intra-patient variability which results in enhanced statistical power to identify factors influencing pharmacokinetics. Therefore we investigated the effect of *CYP3A4*22*, *CYP3A5*3* and *CYP3A* combined genotype on cyclosporine, everolimus and tacrolimus pharmacokinetics using a population pharmacokinetic analysis.

Methods

Patients

Cyclosporine

Clinical data from 298 renal transplant recipients treated with a immunosuppressive regimen cyclosporine (Neoral®, Novartis, Basel, Switserland), prednisolone and

mycophenolate sodium participating in a run in phase of a prospective, open, randomized, multicenter study were studied up to 6 months after transplantation [22]. Induction therapy consisted of 2 doses of 20 mg Basiliximab (Simulect®, Novartis, Basel, Switzerland) before transplantation and on day 4, rapidly tapered prednisolone dose (50 mg b.i.d intravenously tapered to daily 10 mg oral prednisolone). Cyclosporine therapy was started at an oral dose of 4 mg/kg twice daily and was supported by routine TDM based on $AUC_{0\text{-}12h}$. TDM was aimed at a target of 5400 µg*h/L the first 6 weeks and 3250 µg*h/L thereafter. Cyclosporine concentrations were obtained at steady state at clinical visits which were scheduled at 1, 5, 12 and 24 weeks post transplantation.

Everolimus

Clinical data from 97 stable renal transplant recipients treated with immunosuppressive duotherapy consisting of everolimus (Certican®, Novartis, Basel, Switzerland) and prednisolone, participating in a prospective, open, randomized, multicenter study were studied from 6 to 24 months after transplantation [22]. During the first six months, patients were treated with an immunosuppressive regimen cyclosporine, prednisolone and mycophenolate, Thereafter a scheduled biopsy was performed. Patients whose biopsy showed no sign of rejection were included. Subsequently cyclosporine and mycophenolate were discontinued. Everolimus therapy was started at an oral dose of 3 mg twice daily and was supported by routine TDM based on $AUC_{0\text{-}12h}$. TDM was aimed at a target of 120 µg*h/L. Everolimus concentrations were obtained at steady state at regular clinical visits scheduled at 32, 52, 78 and 104 weeks after transplantation.

Tacrolimus

Clinical data from 101 renal transplant patients on an immunosuppressive regimen of tacrolimus (Prograft®, Astellas, Leiden, The Netherlands), prednisolone and mycophenolate mofetil studied for first two TDM moments after transplantation. Induction therapy consisted of 2 doses of 20 mg Basiliximab (Simulect®) before transplantation and on day 4, rapidly tapered prednisolone dose (50 mg b.i.d intravenously tapered to daily 10 mg oral prednisolone). Tacrolimus therapy was started at a fixed oral dose of 5 mg twice daily and was supported by routine TDM based on $AUC_{0\text{-}12h}$. TDM was aimed at a target of 160 µg*h/L the first 6 weeks and 120 µg*h/L thereafter. Tacrolimus concentrations were obtained at steady state at ranging from 1 week to 66 weeks after transplantation with a median of 2 weeks.

The study was approved by the Medical Ethics Committee of Leiden University Medical Center and patients gave written informed consent.

Bioanalytics

TDM was performed on the basis Bayesian estimation (cyclosporine [23] and tacrolimus [24]) or trapezoidal rule (everolimus) (blood concentration at t = 0,1,2,3,4,5 and 6 (everolimus and tacrolimus) up to 12 h for some patients (cyclosporine) or t = 0,1,2,3,4 hours in a small number of visits in the everolimus dataset) using MW/Pharm 3.5 (Mediware, Groningen, The Netherlands)[25]. TDM samples were determined in whole blood by a validated liquid chromatography-mass spectrometric method in two laboratories [26,27] or by Fluorescent Polarization Immunoassay (FPIA; Abbott Laboratories, Abbott Park, IL, USA). Tacrolimus blood concentrations were all determined with LC-MS/MS, Everolimus with LC-MS/MS and FPIA and cyclosporine with FPIA alone. Table 1 shows the samples distribution of the blood concentrations used in this study.

Genotyping Assays

DNA was isolated from EDTA blood collected from patients. *CYP3A4*22* was determined with TaqMan 7500 (Applied Biosystems, Nieuwerkerk a.d. IJssel, The Netherlands) with predesigned assays, according to manufacturers' protocol. *CYP3A5*3* was determined with Pyrosequencer 96MA (Isogen, IJsselstein, The Netherlands). Further Details with regard to the genotyping protocol are provided in Supplementary Table I. No inconsistencies were observed. All allele frequencies were in Hardy–Weinberg equilibrium.

Supplementary Table I: Primers and Probes for TaqMan and Pyrosequence Analysis.

TaqMan (SNP)	Target	Sequence 5'-3'	Modification	Reference
CYP3A4*22 (rs35599367)	Predesigned			C_59013445_10

Pyrosequence (SNP)	Target	Sequence 5'-3'	Modification	Reference
CYP3A5*3 (rs776746)	PCR-f	CTGCCTTCAATTTTTCACT	Biotin	
	PCR-r	TATGTTATGTAATCCATACCCC		
	Sequence primer	AGAGCTCTTTTGTCTTTCA		
	Target sequence	A/GTATCTC		

f, forward orientated; r, reverse orientated; ABC, ATP-binding cassette; CYP, cytochrome P450; PCR, polymerase chain reaction.

Modification: VIC and FAM are fluorescent dyes, biotin is necessary to obtain single stranded DNA

Assay Protocol

PCRs contained 10ng of DNA and 5pmol of each PCR primer in a total volume of 12mL. Cycle conditions were initial denaturation for 15 minutes at 95°C, 35 cycles of 95°C, 55°C, and 72°C each for 30 seconds, ended by 10 minutes at 72°C. The pyrosequence reactions were performed according to manufacturers' protocol. Primers and probes used in the TaqMan-based genotyping assays and primers and sequences used in the pyrosequence assays for each SNP are listed in. 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.

Pharmacokinetic modeling

Nonlinear mixed effect modeling was used to estimate pharmacokinetic parameters from blood concentration-time data. NONMEM (v7.2.1, Icon Development Solutions, Ellicott City, MD) was used for modeling, using PsN toolkit 3.4.2, and Piranã version 2.8.0 [28] as modeling environment. Results were analyzed using statistical software package R (v2.15.2) and RStudio (v0.97.248). First order conditional estimation method with interaction (FOCE-I) was used throughout the analysis. Model selection was based on statistical significance, goodness of fit and stability. Throughout the model building process, an altered model was chosen over a precursor model if a difference in the objective functions (-2 log likelihood) was >6.63 ($P<0.01$, with 1 degree of freedom, assuming X^2 distribution).

Base model

The model was initially developed strictly pharmacokinetic without covariates. Since only data after oral and not after intravenous administration was available the absolute oral bioavailability could not be determined. Therefore the value for bioavailability was fixed. Plots of observed concentration-time data were examined. One and two compartmental pharmacokinetic models with first-order elimination were compared to find the best fit of the concentration-time data. The use of transit compartments and a lag time for drug absorption were explored. After building the base model, demographic and genetic covariates were explored.

Covariate analysis

Diagnostic plots were constructed of the random effects of Clearance, Volume, K_a and F versus the demographic (age, bodyweight, sex, ethnicity, length, LBW, IBW, BSA, BMI (Formulas in supplementary table II), hematocrit, underlying disease, co-medications (also weighted residuals vs co-medication plots) and pharmacogenetic *(CYP3A4*22* and *CYP3A5*3*) characteristics. Polymorphisms were selected based on theoretical relationship and minimal allele frequency (>6%) to assure detection of clinically relevant effect on pharmacokinetics. Based on these diagnostic plots further testing in the pharmacostatistical model was performed. Subsequently, selected covariate relationships were evaluated by forward inclusion and backward deletion procedure. A covariate effect was only maintained in the model if the inclusion resulted in a reduction in random variability and improved model fit.

Supplementary Table II: Formulas.

Demographic Covariate Formulas	
IBW calculated as:	
Males: 52 kg + 1.9 kg for every inch over 5 feet	
Females: 49 kg + 1.7 kg for every inch over 5 feet	
BMI calculated as:	$BMI\left(\frac{kg}{m^2}\right) = \frac{Weight(kg)}{[Height(m)]^2}$
LBW calculated as:	
Males:	$LBW = 1.10 \times (Weight(kg)) - \frac{128(Weight(kg))^2}{[100(Height(m))]^2}$
Females:	$LBW = 1.07 \times (Weight(kg)) - \frac{148(Weight(kg))^2}{[100(Height(m))]^2}$
BSA (Mosteller (1987))	$BSA(m^2) = \sqrt{\frac{(Height(cm) \times (Weight(kg)}{3600}}$

Visual predictive check with prediction-correction

Performance of candidate and final models for cyclosporine, everolimus and tacrolimus pharmacokinetic models was evaluated using prediction corrected visual predictive checks (predVPC), by simulation of 500 simulated datasets. A prediction corrected VPC differ from a traditional VPC in that both observations and the model predictions are normalized for the typical model prediction in each bin of independent variables [29].

Results

Clinical details

Cyclosporine

The cyclosporine dataset consisted of 298 adult renal transplant recipients, 187 men and 111 women. The majority of patients (88%) were of Caucasian origin. Mean age was 51

± 13 years (range 18-73), mean weight was 77±15 kg (41-141 kg). A total of 6,800 blood samples were collected.

Everolimus

97 adult renal transplant recipients, 62 men and 35 women were included. The majority of patients (86%) were of Caucasian origin. Mean age was 51±13 years (range 22-71), mean weight was 79 ± 15 kg (50-129 kg). The dataset consisted out of 1,807 blood samples.

Tacrolimus

101 adult renal transplant recipients, 56 men and 45 women were included in this analysis. The majority of patients (77%) were of Caucasian origin. Mean age was 51±14 years (range 15-77), mean weight was 76±9 kg (40-114 kg). The dataset consisted out of 921 blood samples. The concentration-time data were reviewed for completeness and consistency of sampling and dosing times. All measured concentrations were above the lower limit of quantification. Baseline characteristics of the included patients are presented in Table 1.

Genotyping

The distributions of all SNPs were in Hardy-Weinberg equilibrium. The distribution of the investigated *CYP3A5* and *CYP3A4* polymorphisms are listed in Table 2. Allele frequencies found in our dataset corresponded with those published previously [16,30–32]. To investigate the combined effect of *CYP3A4*22* and *CYP3A5*3*, genotype clusters were made:

Slow metabolizers (C1): No CYP3A5 activity (*CYP3A5*3/*3)* and at least one decreased activity allele in *CYP3A4 (CYP3A4*22/*22* or *CYP3A4*1/*22*), Intermediate metabolizers group 1 (C2): No CYP3A5 activity (*CYP3A5*3/*3)* and no decreased activity allele in *CYP3A4* (*CYP3A4*1/*1*), Intermediate metabolizers group 2 (C3): Carriers of at least one increased activity allele in *CYP3A5* (*CYP3A5*1/*1* or *CYP3A5*1/*3)* and at least one decreased activity allele in *CYP3A4* (*CYP3A4*22/*22* or *CYP3A4*1/*22*) and extensive metabolizers (C4): Carriers of at least one increased activity allele in *CYP3A5 (CYP3A5*1/*1* or *CYP3A5*1/*3)* and no decreased activity allele in *CYP3A4 (CYP3A4*1/*1).*

Table 1: Baseline characteristics of the patients included in the population PK/PG analyses.

	Ciclosporine	Everolimus	Tacrolimus
Male	187	62	56
Female	111	35	45
Age (yrs)	51 ± 13	51 ± 13	50 ± 14
Weight (kg)	77 ± 15	79 ± 15	76 ± 14
Body surface Area (m^2)	1.93 ± 0.22	1.94 ± 0.22	1.90 ± 0.22
Lean Body Mass (kg)	57 ± 10	58 ± 10	55 ± 10
Ideal BW (kg)	67 ± 9	67 ± 8	65 ± 9
Height (cm)	174 ± 10	174 ± 10	172 ± 11
Creatinine clearance (ml/min)	46 ± 30	70 ± 25	56 ± 35
Exposure			
Dose (mg)	177 ± 78 (50-500)	2.49 ± 0.79 (0.75-5.25)	4.2 ± 1.7 (0.5-12)
AUC_{0-12} (µg*hr/L)	5648 ± 2574 (702-16499)	150 ± 42 (56-336)	170 ± 81 (49-462)
Trough concentration	219 ± 131 (25-1209)	9.3 ± 4.2 (2.6-32)	10.8 ± 5.5 (3.3-33.6)
Ethicity (%)			
Caucasion	88	86	77
Mediterranean	3	5	13
Asian	6	7	9
Black	2	2	1
Other	1		
Hematocit (l/l)	0.36 ± 0.05	0.38 ± 0.04	0.34 ± 0.04
Underlying disease (n)			
Polycystic kidney disease	63	22	16
Glomerulonephritis	50	15	7
Diabetes mellitus	12	4	22
Hypertension	50	15	15
Focal segmental glomerulosclerosis	13	4	8
E.c.i.	13	5	5
Interstitial nephritis	11	3	3
Urological	23	10	3
Other	63	19	23
PK data			
Concentrations (µg/l)	591 ± 434 (25-2615)	15.8 ± 8.1 (2.6-59)	16.8 ± 10 (3.3-96)
Samples per patient	23 ± 6 (3-37)	19 ± 8 (7-36)	9 ± 2 (3-14)
Total Samples	6800	1807	921

AUC, area under the curve; BW, body weight; E.c.i., e causa ignota (cause unknown); PG, pharmacogenetic; PK, pharmacokinetic.

Table 2: Genotype distribution in study population.

SNP	Frequency and Genotype									
Cyclosporine (n=298)										
CYP3A4*22 (rs35599367)	264	***1/*1**	32	***1/*22**	2	***22/*22**			0	**NG**
CYP3A5*3 (rs776746)	239	***3/*3**	48	***1/*3**	9	***1/*1**			2	**NG**
CYP3A4/CYP3A5 cluster	29	**C1**	210	**C2**	5	**C3**	52	**C4**	2	**NG**
Everolimus (n=97)										
CYP3A4*22 (rs35599367)	87	***1/*1**	8	***1/*22**	1	***22/*22**			1	**NG**
CYP3A5*3 (rs776746)	81	***3/*3**	12	***1/*3**	3	***1/*1**			1	**NG**
CYP3A4/CYP3A5 cluster	9	**C1**	72	**C2**	0	**C3**	15	**C4**	1	**NG**
Tacrolimus (n=101)										
CYP3A4*22 (rs35599367)	92	***1/*1**	7	***1/*22**	2	***22/*22**			0	**NG**
CYP3A5*3 (rs776746)	79	***3/*3**	18	***1/*3**	4	***1/*1**			0	**NG**
CYP3A4/CYP3A5 cluster	7	**C1**	72	**C2**	2	**C3**	20	**C4**	0	**NG**

C1, CYP3A5*1 noncarriers and CYP3A4*22 carriers; C2, CYP3A5*1 noncarriers and CYP3A4*22 non-carriers; C3, CYP3A5*1 carriers and CYP3A4*22 carriers; C4, CYP3A5*1 carriers and CYP3A4*22 noncarriers; NG, not genotyped.

Concomitant medication

An overview of concomitant immunosuppressive and non-immunosuppressive medication with possible interaction of pharmacokinetics in the different groups is presented in Supplementary Table III.

Supplementary Table III: Comedications of interest for covariate analysis and number of patients using them.

	Cyclosporine (N=298)	Tacrolimus (N=101)	Everolimus (N=97)
Immunosuppression			
Corticosteroids (mainly prednisolon)	298	101	97
Statins *			
Atorvastatin	47	2	28
Pravastatin	26	5	20
Simvastatin	25	3	7
Rosuvastatin	0	0	1
Antibiotics			
Sulfamethoxazole / Trimethoprim	35	80	17
Hypertension			
Calciumantagonist	134	40	45
Proton Pump inhibitors			
Pantoprazole	28	9	7
Omeprazole	33	6	16
Esomeprazole	22	1	23
Antifungants			
Fluconazole	0	1	0

* In some cases statins were switched for another statins.

Population pharmacokinetic modeling

The pharmacokinetic data of cyclosporine, everolimus and tacrolimus was best described by a two-compartmental model with first order absorption and first order elimination from the central compartment. The delayed absorption of everolimus and tacrolimus was best described with a lag time and the delayed absorption of cyclosporine was best 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).

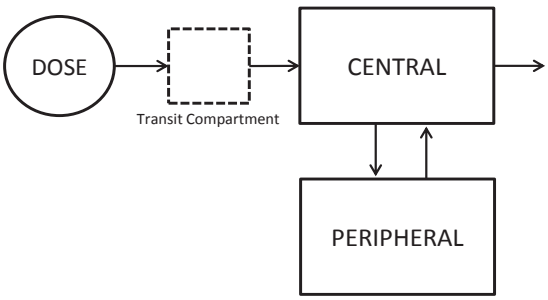

Figure 1: Schematic representation of the linear two-compartment model with first-order absorption and elimination of cyclosporine, including the transit compartment to describe the absorption phase.

Random effect parameters for inter-individual variability in clearance (CL), volume of central compartment (V_c) and were identified for all three drugs. Random effect parameters for inter-individual variability in the rate of absorption (K_a) were identified for cyclosporine and everolimus. For tacrolimus a random effect parameter for inter-individual variability was identified for bioavailability. Variability between occasions (IOV) was best described with a random effect on (fixed) bioavailability (F) for cyclosporine, everolimus and tacrolimus. For everolimus also IOV on K_a was identified. The random effects were tested for structural relationship with dose and time to create a model with unbiased and randomly distributed random effects for covariate analysis.

The structural pharmacokinetic model of cyclosporine indicated an apparent clearance (CL/F) of 15.9 L/h, with the bioavailability term fixed to 0.5, an apparent central distribution volume (V_c/F) of 59.6 L and an apparent peripheral distribution volume of 99.7 L. The absorption rate constant of was 2.1 h^{-1}. Inter-compartmental clearance was 13.1 L/h. Inter-occasion variability was estimated for the fixed bioavailability term and not for clearance because of a better model fit.

The structural pharmacokinetic model of everolimus indicated an apparent clearance (CL/F) of 16.7 L/h, with the bioavailability term fixed to 1, an apparent central distribution volume (V_c/F) of 144 L and an apparent peripheral distribution volume of 348 L. The absorption rate constant was 7.36 h^{-1}. Inter-compartmental clearance was 42.7 L/h and lag time was 0.71 h. Inter-occasion variability was estimated for the fixed bioavailability term and not for clearance because of a better model fit.

A dose clearance relationship was observed showing an increase in apparent clearance with increasing dose according to TVCL = $\{[dose/2.5]^{*0.34}\}$. This relationship improved

the model fit in terms of objective function. The effect appeared to be caused by strict TDM. Patients with high everolimus blood levels (i.e. with a lower clearance) were titrated to receive lower doses and vice versa to reach the stable target $AUC_{0\text{-}12h}$ of 120 μg*h/L. Subsequently an apparent dose clearance relationship emerges. Additional test described by Ahn et al [33] were performed and confirmed that this effect was caused by strict TDM. Since two different assays were used for the determination of everolimus blood concentrations (LC-MS/MS and FPIA) a residual error for each assay was incorporated in the model. The model improved by adding an additive error to the FPIA data. This overestimation of FPIA was expected as investigated previously [27].

The structural pharmacokinetic model of tacrolimus indicated an apparent clearance (CL/F) of 5.7 L/h, with the bioavailability term fixed to 0.23, an apparent central distribution volume (V_c/F) of 20.5 L and an apparent peripheral distribution volume of which was fixed to 500 L. The absorption rate constant was 0.55 h^{-1}. Inter-compartmental clearance was 17.2 L/h and lag time was 0.809 h. Inter-occasion variability was estimated for the fixed bioavailability term. The pharmacokinetic data of cyclosporine showed inter-individual variability in CL/F of 23.5% and inter-occasion variability (22.7%). Everolimus data revealed an inter-individual variability in CL/F of 28.8% and inter-occasion variability (26.4%). Tacrolimus showed considerably higher inter-individual variability in CL/F of 42.2% and inter-occasion variability (35.5%).

Covariate analysis

Pharmacogenetics

In table 3 the summary of the univariate pharmacogenetic covariate analysis is presented. *CYP3A4*22* was significantly associated with cyclosporine CL/F and patients who carried at least one decreased activity allele in *CYP3A4*22* had a 15% lower clearance compared to non-carriers. *CYP3A* combination showed a significant effect; C1, C2 and C3 showed lower clearance compared to C4 (-16%, -2% and -12% respectively.

Everolimus pharmacokinetics did not reveal a significant relation with *CYP3A5*3*, *CYP3A4*22* nor the CYP3A genotype combination. For tacrolimus *CYP3A5*3* was significantly associated with tacrolimus CL/F. Carriers of at least one *CYP3A5*1* allele had 53% higher clearance compared to non-carriers. In contrast, *CYP3A4*22* as covariate on CL/F did not result in a significant objective function drop (P=0.218). Although not significant a trend of 16% lower tacrolimus clearance was observed for *CYP3A4*22* allele carriers. CYP3A combination showed a significant effect on tacrolimus clearance.

Table 3: Summary of CYP3A4 and CYP3A5 covariate analysis.

Covariate Tested	MVOF	ΔOF	P value	Var. CL (%)	Expl.Var. (%)		Mean Value (%)	95% CI (%)
Cyclosporine base	-6750.443			23.5				
+ CYP3A5*3	-6751.429	0.986	0.32072	23.5	0		3	-2 to 8
+ CYP3A4*22	-6767.684	17.241	0.00003	22.7	3.4		-14.5	-20 to -8
+ CYP3A combination	-6768.565	18.123	0.00041	22.7	3.4	C1	-16	-23 to -9
						C2	-2	-7 to 3
						C3	-12	-27 to 4
						C4	0	-5 to 5
Everolimus base	5446.987			28.8				
+ CYP3A5*3	5444.175	2.059	0.15131	28.4	1.4		12	-0.3 to 24
+ CYP3A4*22	5446.234	0.753	0.38553	28.7	0.3		-7	-23 to 9
+ CYP3A combination	5443.734	3.253	0.19662	28.4	1.4	C1	-15	-29 to -1
						C2	-10	-19 to -2
						C3	NA	NA
						C4	0	-3 to 9
Tacrolimus base	3549.937			42.2				
+ CYP3A5*3	3530.215	19.722	0.00001	39.9	5.5		53	25 to 80
+ CYP3A4*22	3548.418	1.519	0.21777	41.7	1.2		-16	-47 to 14
+ CYP3A combination	3527.993	21.717	0.00007	36.6	13.3	C1	-47	-69 to -24
						C2	-33	-46 to -20
						C3	-3	-48 to 41
						C4	0	-18 to 18

ΔOF, Δ objective function; C1, CYP3A5*1 noncarriers and CYP3A4*22 carriers; C2, CYP3A5*1 noncarriers and CYP3A4*22 noncarriers; C3, CYP3A5*1 carriers and CYP3A4*22 carriers; C4, CYP3A5*1 carriers and CYP3A4*22 noncarriers; Expl. Var. (%), explained variability in percentage of total; MVOF, minimal value of objective function; NA, not applicable; P value, χ^2 distribution P value; RSE, relative standard error; Var. CL (%), remaining variability in clearance. Mean value (%) represents the difference in CL/F compared with the reference group, which is 0.

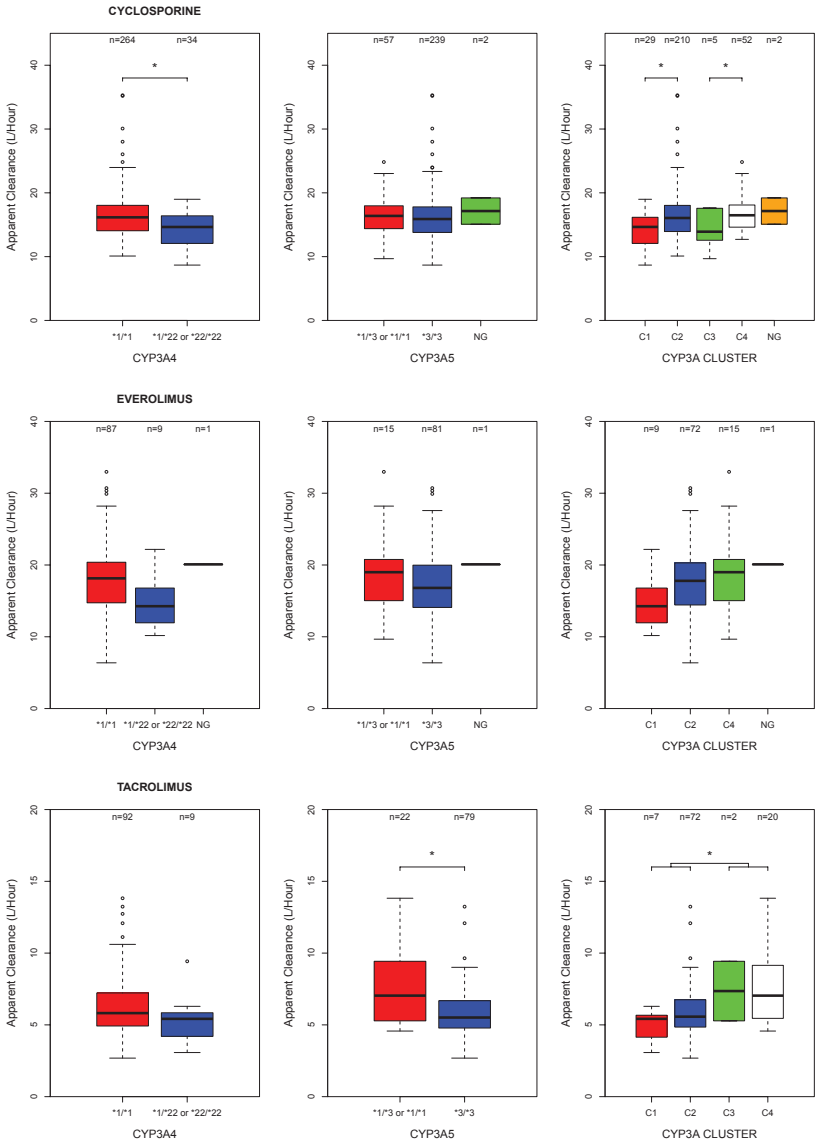

Figure 2: Box plots representing the average cyclosporine, everolimus, and tacrolimus apparent clearance (l/h) of the different genotype groups with error bars and the number of patients in each group. *CYP3A4* (*1/*1 = *CYP3A4**22 noncarriers, *1/*22 or *22/*22 = *CYP3A4**22 carriers, NG = not genotyped), *CYP3A5* (*1/*3 or *1/*1 = *CYP3A5**1 carriers, *3/*3 = *CYP3A5**1 noncarriers, NG = not genotyped), and *CYP3A* cluster: (C1: *CYP3A5**3/*3 and *CYP3A4**22/*22 or *CYP3A4**1/*22, C2: *CYP3A5**3/*3 and *CYP3A4**1/*1, C3: *CYP3A5**1/*1 or *CYP3A5**1/*3 and *CYP3A4**22/*22 or *CYP3A4**1/*22, and C4: *CYP3A5**1/*1 or *CYP3A5**1/*3 and *CYP3A4**1/*1, NG = not genotyped). *$P < 0.01$. Apparent clearance was calculated using the base model.

C1 showed C2 and C3 showed lower clearance compared to C4 (-47%, -33% and -3% respectively). Although significant, the genetic covariates explained variability in clearance to a limited degree. In figure 2 boxplots of clearance vs genotype are presented for cyclosporine, everolimus and tacrolimus and also show the significant variability within the genotype groups.

Demographics

The demographic covariates that showed a possible relation with the pharmacokinetics of the drugs in the diagnostic plots were evaluated in the covariate analysis. Univariate analysis (P<0.05) on cyclosporine showed significant associations for the following demographic covariates: Bodyweight (BW) on CL/F and Vc/F, prednisolon dose ≥ 20 mg on Ka and F for cyclosporine, ideal body weight (IBW) on Vc/F and hematocrit on Cl/F for everolimus. Significant demographic covariates for tacrolimus were prednisolone dose ≥ 25 mg on F and hematocrit on CL/F. The remaining demographic covariates such as ethnicity and other co-medication that were evaluated in this study were not significant on CL/F, V_c/F nor K_a.

After the forward inclusion and backward elimination step the following covariates remained significant ($P < 0.01$): Cyclosporine: BW on CL/F and Vc/F, prednisolon dose ≥ 20 mg on Ka and F (better model fit and objective function drop compared to prednisolon dose on CL/F) and *CYP3A4*22* on CL/F. Inter-individual variability of CL/F decreased from 23.5% to 22.6%. In Supplementary Table IV, all significant covariates improving model fit together with their effects on observed variability are presented for cyclosporine, everolimus and tacrolimus. Everolimus: IBW centered on the population median as exponential function on Vc/F improved the model reduced the random variability between individuals in Vc/F by 12%. Hematocrit was lost in de forward elimination step (P>0.01) and was therefore not incorporated in the final model. Significant covariates for tacrolimus were found in prednisolone dose ≥ 25 on F (higher objective function drop compared to prednisolon dose on CL/F), *CYP3A5*3* and hematocrit on tacrolimus CL/F. Incorporation of these covariates decreased the inter-individual variability of CL/F from 42.2% to 39.1% and the inter-occasion variability was reduced from 35.5% to 29.3%.

Supplementary Table IV: Covariate table. Significant covariates improving model fit together with the effects on observed variability.

COVARIATE TESTED	MVOF	ΔOF	IIV CL (%)	IIV Vc (%)	IIV Ka (%)	IIV F (%)	IOV F (%)
Cyclosporine (n=298)							
BASE MODEL	-6750.443		23.5	41.6	48.6	-	22.7
+ DDPR ≥ 20 mg (Ka)	-6868.306	-117.863	23.4	45.2	47.4	-	23
+ DDPR ≥ 20 mg (F1)	-6812.983	-62.54	23.7	41.6	49.5	-	21.7
+ BW (CL/F) and (Vc/F)	-6778.49	-28.047	23.5	38.5	49.5	-	22.3
+ CYP3A4*22 (CL/F)	-6767.684	-17.241	22.7	41.5	48.6	-	22.8
Forward inclusion							
BASE	-6750.443		23.5	41.6	48.6	-	22.7
BASE + DDPR (Ka)	-6868.306	-117.863	23.4	45.2	47.4	-	23
BASE + DDPR (Ka) and (F1)	-6936.034	-185.591	23.7	45.6	48.3	-	21.9
BASE + DDPR (Ka) and (F1) + BW (CL/F) and (Vc/F)	-6954.69	-204.247	23.5	42.4	48.9	-	21.7
BASE + DDPR (Ka) and (F1) + BW (CL/F) and (Vc/F) + CYP3A4*22 (CL/F)	-6972.836	-222.393	22.6	42.3	49	-	21.7
Backward deletion							
FINAL MODEL	-6972.836		22.6	42.3	49	-	21.7
- CYP3A4*22 (CL/F)	-6954.69	+ 18.146	23.5	42.4	48.9	-	21.7
- CYP3A4*22 (CL/F) - BW (CL/F) and (Vc/F)	-6936.034	+ 36.802	23.7	45.6	48.3	-	21.9
- CYP3A4*22 (CL/F) - BW (CL/F) and (Vc/F) - DDPR (F1)	-6868.306	+ 104.53	23.4	45.2	47.4	-	23
- CYP3A4*22 (CL/F) - BW (CL/F) and (Vc/F) - DDPR (F1) and DDPR (Ka)	-6750.443	+ 222.393	23.5	41.5	48.6	-	22.7
Everolimus (n=97)							
BASE MODEL	5446.987		28.8	35.1	115.8	-	26.4
+ IWT (Vc/F)	5431.226	-15.761	28.8	31.2	110.9	-	26.3
+ HTC (CL/F)	5440.565	-6.422 **	28.9	34.8	109.5	-	26.6

Forward inclusion							
BASE	5446.987		28.8	35.1	115.8	-	26.4
BASE + IBW (Vc/F)	5431.226	-15.761	28.8	31.2	110.9	-	26.3
Backward deletion							
FINAL MODEL	5431.226		28.8	31.2	110.9	-	26.3
- IWT (Vc/F)	5446.987	+ 15.761	28.8	35.1	115.8	-	26.4
Tacrolimus (n=101)							
BASE MODEL	3549.937		42.2	124.1	-	38.1	35.5
+ DDPR ≥ 20 mg (F1)	3529.91	-20.027	42.3	124.5	-	38.3	31.1
+ CYP3A5*3 (CL/F)	3530.215	-19.722	39.9	124.1	-	38.3	35.5
+ HTC (CL/F)	3540.088	-9.849	40.9	125.7	-	39.6	32.6
Forward inclusion							
BASE	3549.937		42.2	124.1	-	38.1	35.5
BASE + DDPR (F1)	3530.215	-20.027	42.3	124.5	-	38.3	31.1
BASE + DDPR (F1) + CYP3A5*3 (CL/F)	3509.351	-40.586	40	124.5	-	38.7	31.1
BASE + DDPR (F1) + CYP3A5*3 (CL/F) + HTC (CL/F)	3501.709	-48.228	39.1	124.9	-	39.2	29.3
Backward deletion							
FINAL MODEL	3501.709		39.1	124.9	-	39.2	29.3
- HTC (CL/F)	3509.351	7.642	40	124.5	-	38.7	31.1
- HTC (CL/F) - CYP3A5*3 (CL/F)	3529.91	28.201	42.3	124.5	-	38.3	31.1
- HTC (CL/F) - CYP3A5*3 (CL/F) - DDPR (F1)	3549.937	48.228	42.2	124.1	-	38.1	35.5

IBW, Ideal Body Weight ; HTC, hematocrit; CYP, cytochrome P450; DDPR, daily dose prednisolon; MVOF,minimum value of the objective function; IIV, inter-individual variability; Ka, Absorption rate constant; F1, Bioavailability; Cl, clearance; Vc, distribution volume of the central compartment. ΔOF: Decrease in the minimum objective function value compared to the base model or increase compared to the final model. ** not significant (P>0.01), but significant with P<0.05 (ΔOF>3.84).

Table 4: Summary of model parameter estimates Cyclosporine, Everolimus and Tacrolimus.

PK Parameter	Base Model			Final Model				1000 bootstrap runs	
	Mean Value	RSE (%)	Shr. (%)	Mean Value	RSE (%)	Shr. (%)	Expl. Var.(%)	Median Value	95% CI
Cyclosporine									
CL/F	15.9	2	—	15.6	1.8	—	—	15.6	15.1 to 16.1
BW on CL/F	—	—	—	0.3	32.7	—	2	0.3	0.09 to 0.48
CYP3A4*22 on CL/F	—	—	—	-0.15	21	—	4	-0.15	-0.20 to -0.08
F (fixed)	0.5	—	—	0.5	—	—	—	0.5	—
DDPR ≥ 20 mg	—	—	—	-0.12	17.3	—	4	-0.12	-0.15 to -0.08
Vc/F (L)	59.6	4	—	56	5	—	—	55.8	50.3 to 61.2
BW on Vc/F	—	—	—	0.61	36	—	7.5	0.61	0.16 to 1.04
Q/F (L/h)	13.1	5	—	13	7	—	—	13	11.5 to 15.3
Vp/F (L)	99.7	8	—	90.4	9	—	—	90.5	80.2 to 110.7
ka (h-1)	2.1	6	—	2.16	9	—	—	2.2	1.9 to 2.5
DDPR ≥ 20 mg	—	—	—	-0.45	11	—	2.5	-0.45	-0.53 to -0.34
Interindividual variability									
IIV CL/F (CV%)	23.5	8	10	22.6	9.6	10	—	22.2	18.5 to 26.7
IIV Vc/F (CV%)	41.5	8	19	42.3	10.4	19	—	42.1	32.8 to 50.8
IIV Ka (CV%)	48.6	9	21	49	10.6	23	—	49.1	38.5 to 59.0
Interoccasion variability									
IOV F (CV%)	22.7	7	26	21.7	9	29	—	21.7	18.4 to 25.0
Random residual variability									
σ1 (additive error)	0.301	6	10	0.297	12.6	10	—	0.293	0.268 to 0.335
Everolimus									
CL/F	16.7	4	—	16.7	4	—	—	16.7	15.4 to 17.8
F (fixed)	1	—	—	1	—	—	—	1	—
Vc/F (L)	144	5	—	140	5	—	—	143	131 to 156
IBW on Vc/F	—	—	—	-0.96	28	—	12	-0.95	-1.55 to -0.39
Q/F (L/h)	42.7	6	—	43.1	6	—	—	43.4	38.7 to 49.5
Vp/F (L)	348	22	—	343	20	—	—	336	247 to 585
ka (h-1)	7.3	20	—	7	16.3	—	—	7.1	4.7 to 11.1
Lagtime	0.71	3	—	0.71	3	—	—	0.71	0.65 to 0.74

Dose CL/F (TDM effect)	0.34	31	—	0.34	28	—	—	0.35	0.16 to 0.49
Interindividual variability									
IIV CL/F (CV%)	28.8	48	9	28.9	13	9	—	28.7	21.7 to 34.6
IIV Vc/F (CV%)	35.1	26	12	30.6	10	14	—	30.4	25.3 to 37.0
IIV Ka (CV%)	115.8	16	35	111	13	35	—	108.1	84.9 to 136.9
Interoccasion variability									
IOV Ka (CV%)	127.3	11	39	127.3	11	38	—	127.3	102.0 to 160.9
IOV F (CV%)	26.4	6	7	26.3	5.7	6	—	26.2	23.3 to 29.5
Random residual variability									
σ1 (proportional error) LCMS	14.5	7.7	16	14.5	7.6	15	—	14.3	11.8 to 16.7
σ2 (proportional error) FPIA	6.6	14.3	15	6.6	14.1	15	—	6.7	4.1 to 8.2
σ3 (additive error) FPIA	1.06	14	15	1.06	14	15	—	1.08	0.8 to 1.44
Tacrolimus									
CL/F	5.7	5	—	6.27	6	—	—	6.27	5.59 to 7.01
CYP3A5*3 on CL/F	—	—	—	0.52	26	—	5.5	0.52	0.25 to 0.82
HTC on CL/F + IOV F	—	—	—	-0.587	35	—	3 / 8	-0.56	-0.96 to -0.23
F (fixed)	0.23	—	—	0.23	—	—	—	0.23	—
DDPR ≤ 20 mg (IOV F)	—	—	—	0.314	27	—	12.4	0.32	0.15 to 0.49
Vc/F (L)	20.5	22	—	25.5	20	—	—	25.2	16.4 to 38.5
Q/F (L/h)	17.2	9	—	21	11	—	—	21	17 to 25.4
Vp/F (L) (fixed)	500	—	—	500	—	—	—	500	—
ka (h-1)	0.55	10	—	0.55	8	—	—	0.55	0.47 to 0.69
Lagtime	0.81	7	—	0.81	7	—	—	0.81	0.67 to 0.91
Interindividual variability									
IIV CL/F (CV%)	42.2	15	26	39.1	16	27	—	38.5	25.9 to 50.3
IIV Vc/F (CV%)	124.1	13	18	124.9	14	18	—	125.8	95.7 to 162.5
IIV F (CV%)	38.1	21	39	39.2	17	35	—	39	23.1 to 51.6
Interoccasion variability									
IOV F (CV%)	35.5	12	26	29.3	14	25	—	28.8	21.1 to 36.2
Random residual variability									
σ1 (proportional error)	17.3	5	16	17.4	5	16	—	17.3	15.7 to 19.0

IBW, Ideal Body Weight ; HTC, hematocrit; CYP, cytochrome P450; DDPR, daily dose prednisolone; IIV, inter-individual variability; IOV, inter-occasion variability; Ka, Absorption rate constant; F1, Bioavailability; CL, clearance; Vc, distribution volume of the central compartment; Q, intercompartmental clearance; Lagtime, lagtime of absorption. Shr. (%), shrinkage (%). Expl Var (%); Percentage explained of total variability.

The population pharmacokinetic parameters obtained with the base and final models are presented in Table 4. Evaluation of the precision of the pharmacokinetic parameters of all three models was performed with 1000 bootstrap replications. The percentage of successful runs was 99% for cyclosporine, 82% for everolimus and 96% for tacrolimus. Moreover, the parameter estimates of the non-successful runs were analysed and did not deviate from the parameter estimates of the successful runs. The mean values for all fixed effect parameters were within 15% of those obtained by the final model, indicating good reliability. Since different dosages were used during the study the performance of the model was evaluated with a predictive corrected visual predictive check [34]. Predictive and observed intervals (10%, 90% and median) are almost identical showing good predictive performance of the final models (Figure 3).

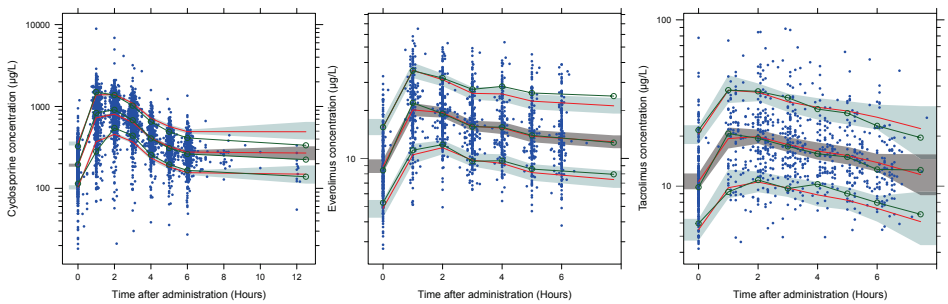

Figure 3: Prediction-corrected visual predictive checks with 80% prediction interval of cyclosporine, everolimus, and tacrolimus. The observed concentrations are shown as solid circles. The solid lines with open circles represent the observation intervals. The solid lines represent the prediction interval. The shaded areas around the prediction intervals represent the 95% confidence interval around each of the prediction interval.

Discussion

This is the first comprehensive study investigating the influence of *CYP3A4*22*, *CYP3A5*3* variant alleles and its combined clusters on the pharmacokinetics of the three main kidney transplant immunosuppressive drugs cyclosporine, everolimus and tacrolimus. This study demonstrates that carriership of the *CYP3A4*22* allele is significantly associated with a decreased cyclosporine clearance. Carriers of the *CYP3A4*22* allele showed 15% lower

cyclosporine clearance as compared to non-carriers. Moreover *CYP3A* genotype clusters were significantly associated with cyclosporine and tacrolimus clearance but not with everolimus clearance. Finally this study also demonstrates that patients carrying at least one *CYP3A5*1* allele have on average 53% higher tacrolimus clearance compared to noncarriers. Cyclosporine, everolimus and tacrolimus are primarily eliminated by CYP3A enzymes [5–7,35] and as shown before in in-vitro and in-vivo studies, CYP3A4 is involved in their pharmacokinetics [6,36,37]. CYP3A4 is most likely predominant in cyclosporine and everolimus metabolic clearance and CYP3A5 contributes more significantly to tacrolimus metabolic clearance compared with CYP3A4 [6,7]. In contrast to CYP3A5, CYP3A4 lacked a reliable genetic marker for prediction of CYP3A4 expression which was suitable for dosing adjustments [38,39], however *CYP3A4*22* was recently marked as a potential reliable marker [16,17]. In contrast, as part of our analysis only a significant influence of *CYP3A4*22* on cyclosporine pharmacokinetics was found, but a trend was also seen in tacrolimus (16% lower clearance (95%CI: -47 to 14%)) and everolimus pharmacokinetics (7% lower clearance (95%CI: -23 to 9%)). This effect is not high enough to justify dose modification based on CYP3A4*22. In clinical practice only an effect of at least 20% on clearance will lead to dose adjustments, since these drugs also possess a considerable degree of intra-individual variability. Since the clinical studies from which all data was derived were not primarily designed to identify a genotype effect and the fact that we found no clinically relevant genotype effect for *CYP3A4*22* we had to confirm afterwards that our study had enough power. Therefore we performed a posterior power calculation using the stochastic simulation and estimation tool of the PsN toolkit to determine the power (95% and 99% confidence) of our study to find a clinically relevant genotype effect (at least 20%) on cyclosporine, everolimus and tacrolimus pharmacokinetics [40,41]. With the most unfavorable genotype distribution (*CYP3A4*22*) and the least amount of data (tacrolimus) we found a power of 95% (α=0.05) and 91% (α=0.01) in detecting a clinically relevant genotype (at least 20%) effect. It is therefore highly unlikely that our analysis was underpowered and missed a clinically relevant effect of the investigated genotypes due to limited sample size.

In contrast to our findings the studies of *Elens et al.* and *Gijsen et al.* [16,17,42] showed that *CYP4A4*22* allele carriers required up to 30% lower tacrolimus doses compared to CYP3A4*1/*1 to reach target trough concentration. However these exploratory findings have not been confirmed by another research group. Moreover, more recently, *Santoro et al.* [12] presented a study in 140 renal transplant patients showing that independent effects of *CYP3A4*22* on tacrolimus dose requirements could not be verified. The studies of *Elens*

et al. [16,42] had some limitations: the data were not corrected for corticosteroid use or hematocrit levels. Corticosteroid and hematocrit levels are known to influence tacrolimus exposure [13,43] and could therefore have influenced their results. The study of *Gijsen et al.* [17] performed on a small dataset has the limitation that they could not correct their results for co-medication. Both studies [16,17] only used trough levels in their analysis, which do not give a full insight in pharmacokinetics. The more recent study of *Elens et al.* [42] in contrast used an additional 59 whole PK curves to support their conclusion, however since they were collected only on one occasion, intra-individual variability could not be assessed. To investigate whether shrinkage could have been the cause of the lack of significance of the CYP3A4*22 effect in this study, we also performed the univariate genetic covariate analysis with only the first PK profiles to be able to compare the results in more details with *Elens et al.* (Supplementary Table V).

The results were the same as with the complete dataset, so therefore the results found in the study of *Elens et al.* [42] could not be replicated in our study. In another study by *Elens et al.* [18] no significant effect was found for cyclosporine trough concentrations and *CYP3A4*22* carriership. Our analysis was based on an extensive amount of data consisting of area-under-the-curves (AUCs). Moreover a wide range of factors possibly influencing pharmacokinetics including demographic factors and co-medication was also investigated.

The difference in tacrolimus clearance between CYP3A5*1 carriers and non-carriers found in the current analysis was similar to what was published previously [11,13]. We confirmed with our study that dosing adjustments based on *CYP3A5*3* could be indicated to quickly reach target exposure, however the variability explained by *CYP3A5*3* is limited and the variability within the CYP3A5 genotype groups remains significant and therefore close TDM remains essential. The absence of a clinically relevant influence of *CYP3A5*3* on cyclosporine and everolimus pharmacokinetics is in line with previous studies [39,44,45]. Using *CYP3A* combined genotype of CYP3A4 and CYP3A5 as a predictor for cyclosporine, everolimus or tacrolimus clearance does not seem to be an improvement compared to the individual polymorphisms. As shown in the results, the combined analysis did not further improve identification groups of slow metabolizers, intermediate metabolizers and extensive metabolizers. For cyclosporine the differences in average clearance between the groups remain less than 16%. For tacrolimus a difference of 14% is introduced for non-carriers of the *CYP3A5*1* allele by the effect of *CYP3A4*22* carriership which makes a further differentiation unnecessary.

Supplementary Table V: Model parameter estimates and genetic covariate testing using only the first PK profiles.

Tacrolimus	Base Model			Univariate covariate testing			
PK Parameter	Mean Value	RSE (%)	Shr. (%)	Mean Value	95% CI	ΔOF	P value
CL/F	6.08	5					
*CYP3A5*3 on CL/F*				56%	19 to 93%	-14.847	<0.01
*CYP3A4*22 on CL/F*				-13%	-53 to 27%	-0.611	0.43
CYP3ACOMBI on CL/F						-16.059	<0.01
C1				-46%	-77 to -15%		
C2				-34%	-51 to -18%		
C3				7%	-42 to 56%		
C4				0%	-19 to 14 %		
F (fixed)	0.23						
Vc/F (L)	28.5						
Q/F (L/h)	19.6	9					
Vp/F (L) (fixed)	500	25					
k_a (h-1)	0.621	15					
Lagtime							
Interindividual variability							
IIV CL/F (CV%)	49	7	1				
IIV Vc/F (CV%)	142.8	11	18				
Random residual variability							
σ1 (proportional error)	0.0301	7	13				

CYP, cytochrome P450; IIV, inter-individual variability; IOV, inter-occasion variability; Ka, Absorption rate constant; F1, Bioavailability; CL, clearance; Vc, distribution volume of the central compartment; Q, intercompartmental clearance; Lagtime, lagtime of absorption, Shr. (%), shrinkage (%),95% CI; 95% Confidence Interval.

Up to now the only suggested clinically relevant polymorphism in *CYP3A* enzymes relevant for kidney transplantation are *CYP3A5*3* and *CYP3A5*6* for tacrolimus which are primarily found in Africans and have low allelic frequencies in the Caucasian population. CYP3A5*6 was left out of this analysis because of too low allele frequency (<6%). *CYP3A4*22* is able to predict CYP3A4 activity however the clinical relevancy seems to be limited. The search for a reliable and clinically relevant predictive biomarker for CYP3A4 is still open although CYP3A4 phenotyping shows more promising results as recently published by de *Jonge et al.* [15].

The demographic covariates that were identified in this study have been reported in previous studies [11,20,46,47]. The clinical relevancy of the different identified covariates is limited since the explained variability by the individual covariates did not exceed 12%.

The effect of prednisolone dose on cyclosporine and tacrolimus bioavailability (high dose, lower bioavailability) can be explained by CYP3A induction in the intestine and has been reported before [11,47,48]. The cut off values were chosen based on literature [11,47,48] and highest objective function drop. The pharmacokinetic parameter estimates of the three models were in agreement with those found in previous studies [20,46,49] when taking the effect of differences in fixed bioavailability terms, patient population and TDM assays into account. In contrast to a number of other studies we fixed the bioavailability term to 0.5 for cyclosporine and 0.23 for tacrolimus instead of 1, which leads to an apparent clearance twice lower for cyclosporine and 4.3 times lower for tacrolimus. The variability in pharmacokinetics was high in tacrolimus, although as known from literature [1] around 20% of this could be explained by the fact that the majority of the data used in the current analysis was collected within two weeks after transplantation. Unstable renal transplant patient show much higher variability in pharmacokinetics [1].

Cyclosporine absorption was best described with a transit compartment as we previously described [47]. As found in our smaller study Ideal Body Weight significantly correlates with V_c/F of everolimus [20]. Since everolimus is primarily partitioned into red blood cells and 75% of the plasma fraction is bound to plasma proteins this relationship can be physiologically explained since length and sex are incorporated in the ideal weight formula [3,50]. The significant effect of hematocrit on everolimus clearance in the univariate covariate analysis could also be explained by same mechanism. Ethnicity could not be identified as a covariate on clearance of everolimus or cyclosporine as was found previously by *Kovarik et al.* [51] and *Hesselink et al.* [46]. This difference could be explained by the lack of black patients in our cohort. Although theoretically plausible we did not find an effect of concomitant medication such as statins, calcium antagonists, sulfamethoxazole/trimethoprim or proton pump inhibitors on CL/F. This is in accordance with what has been previously been described in literature [9,51]. Co-medications known to have an potent effect on the pharmacokinetics of the drugs were avoided for safety reasons [22]. The remaining variability in clearance between patients of our final model was 22.6% for cyclosporine, 28.8% for everolimus and 38.9% for tacrolimus which could reflect the wide inter-individual variability in CYP3A4 expression [52].

Our study has some limitations: Fatty food intake, non-adherence or diarrhea could not be quantified, although these factors could contribute to the observed variability since previously published studies reported food interactions with the investigated drugs [9,10,53]. Furthermore, K_a of everolimus was difficult to estimate since the dataset had low

number blood samples collected between 0 and 1 hour after dose intake, but is unlikely this would have influenced the genotype covariate analysis on clearance.

In conclusion, *CYP3A4*22* does not influence cyclosporine, everolimus or tacrolimus pharmacokinetics to a clinically relevant extend. This study confirmed that *CYP3A5*3* is only suitable as a predictive marker for tacrolimus clearance but close TDM remains essential due to the remaining variability between patients with the same genotype. The *CYP3A4* and *CYP3A5* combined genotypes do not further improve the predictive performance compared to the predictive performance of the polymorphisms alone. Therefore the newly discovered *CYP3A4*22* or *CYP3A* combined genotypes are not indicative to be used for dose adjustments in clinical practice to further improve immunosuppressive therapy of cyclosporine, tacrolimus or everolimus in the investigated patient population.

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8

The effect of POR*28, CYP3A5*3 and their combined genotypes on everolimus pharmacokinetics in renal transplant recipients

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Submitted

In our recent article "Effect of CYP3A4*22, CYP3A5*3, and CYP3A Combined Genotypes on Cyclosporine, Everolimus, and Tacrolimus Pharmacokinetics in Renal Transplantation" published in this journal [1] we reported that there is no clinically relevant effect of *CYP3A5*3*, and CYP3A combined genotypes on everolimus pharmacokinetics. Recently, relationships between POR polymorphisms and tacrolimus pharmacokinetics in renal transplantation have been reported [2,3]. These publications showed that the *POR*28* allele was associated with increased in vivo CYP3A5 activity for tacrolimus metabolism in *CYP3A5**1 allele carriers. To investigate whether the effect of *POR*28* and the combined effect of *POR*28* and *CYP3A5*3* had a clinically relevant effect on everolimus pharmacokinetics we genotyped all patients for *POR*28*. Hepatic microsomal P450 enzymes require P450 oxidoreductase (POR). Polymorphisms in the gene encoding POR have been linked to altered CYP activity and they appear to be substrate specific [4], however this relationship was absent for sirolimus pharmacokinetics [5]. Our stable renal transplant recipient cohort were genotyped for *CYP3A5*3* (rs776746) and *POR*28* (rs1057868) with Pyrosequencer 96MA (Isogen, IJsselstein, The Netherlands). All allele frequencies were in Hardy–Weinberg equilibrium and distribution corresponded with previous findings [2,3,5]. Univariate covariate analysis using population pharmacokinetic methodology showed no significant association between apparent everolimus clearance and *POR*28*, *CYP3A5*3* nor POR*28 & *CYP3A5*3* combined. Clinically irrelevant trends were observed for *POR*28* (-4% for *28 allele carriers vs non-carriers), *CYP3A5*3* (+12% for *1 allele carriers vs non-carriers) and their combination (+11% for **1* allele carriers of *CYP3A5*3* with at least one *28 allele of POR vs non-carriers). Moreover ,high variability was seen within the genotype groups as shown in Figure 1. In contrast to what was found for tacrolimus by *Elens et al.* and *de Jonge et al.* [2,3] *CYP3A5*1* allele carriers that were carriers of at least 1 *POR*28* allele showed no clinically relevant effect on everolimus pharmacokinetics. Our results are similar to what was found for sirolimus by *Woillard et al.* [5]. In summary these data show that in contrast to tacrolimus but just like sirolimus, *POR*28*, or the combination of combination of *POR*28* & *CYP3A5*3* appears not to be suitable as a biomarker to improve prediction of everolimus exposure in renal transplantation recipients on everolimus and prednisolone duo therapy.

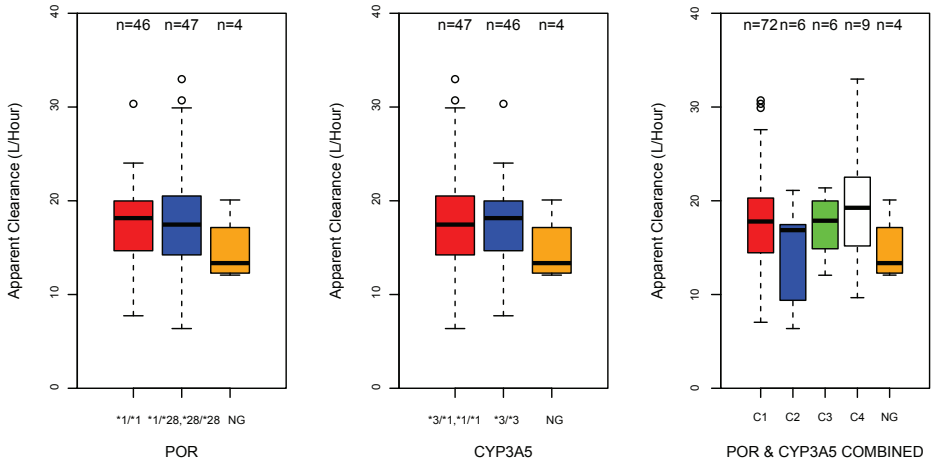

Figure 1: Box plots representing the average everolimus apparent clearance (L/hour) of the different genotype groups with error bars and the number of patients in each group. *POR* (*1/*1 = POR*28 non-carriers, *1/*28 or *28/*28 = *POR*28 carriers, NG = not genotyped), *CYP3A5* (*1/*3 or *1/*1 = *CYP3A5*1 carriers, *3/*3 = *CYP3A5*1 non-carriers, NG = not genotyped), and *POR & CYP3A5* combined: (C1: *CYP3A5*3/*3 and POR*1/*1 or POR*1/*28, C2: *CYP3A5*3/*3 and *POR*28/*28, C3: *CYP3A5*1/*1 or *CYP3A5*1/*3 and POR*1/1, and C4: *CYP3A5*1/*1 or *CYP3A5*1/*3 and *POR*1/*28 or *POR*28/*28, NG = not genotyped). Apparent clearance was calculated using the base model.

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9

Identifying risk factors for delayed graft function, acute rejection and subclinical acute rejection in renal transplant recipients on controlled cyclosporine exposure

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Abstract

Besides traditional immunological risk factors, pharmacological factors such as pharmacogenetics and drug exposure may serve as predictive biomarkers for delayed graft function (DGF), acute rejection (AR) and/or subclinical rejection (SCR) in renal transplant recipients on calcineurin-inhibitor based immunosuppression. Adult renal transplant recipients (*n*=361), receiving basiliximab prophylaxis and triple therapy including concentration controlled cyclosporine A (CsA), mycophenolate and prednisolone were followed until the first 6 months after transplantation. During this period the incidence of DGF and AR episodes were documented as well as the prevalence of SCR in and at 6 months in surveillance biopsies. Demographic, transplant related factors, and pharmacological factors, including systemic drug exposure and pharmacogenetic data (*ABCB1*, *CYP3A5*, *CYP2C8*, *NR1I2, PPP3CA* and *PPP3CB* polymorphisms) were analyzed in relation to the occurrence of DGF, time to first AR and prevalence of SCR at month 6. Fourteen percent of the patients experienced at least one clinical rejection episode and only DGF showed an significant effect on the time to AR. As expected the incidence of DGF correlated with a deceased donor kidney transplant (27% vs 0.6% of living donors). 6 month protocol biopsies biopsies were obtained for 275 transplant recipients and 50 (18%) showed SCR. A deceased donor kidney and an acute rejection history were the most important determinants for SCR, resulting in a 52% risk of SCR at 6 months (*versus* 11% on average). Along with female sex and carrying *ABCB1* TTT-haplotype, these two factors were also related to a higher drop-out (i.e. no protocol biopsy) frequency with an overall drop-out of 24%. In a subanalysis of the patients with AR, those treated with rejection treatment including antithymocyte globulin (ATG) significantly less frequent SCR was found in the 6-month biopsy (13% vs 50%). Transplant related factors were the most important determinants of DGF, AR and SCR within this AUC-controlled population on CsA-based therapy and rejection treatment with depleting antibodies effectively prevented SCR in 6 month surveillance biopsies.

Introduction

Over the past decades acute rejection (AR) rates have decreased dramatically, mainly due to calcineurin inhibitor (CNI) based immunosuppressive regimens. One of the dominant risk factors , previously identified for AR is delayed graft function (DGF) which is highly related to transplant related factors such as vulnerability of the allograft and/or prolonged preservation times [1]. Clinical episodes of AR have previously been identified as a risk factor for subclinical rejection (SCR) [2]. SCR is by definition histologically defined acute rejection and has been associated with interstitial fibrosis and tubular atrophy and with time progressive deterioration of renal function and inferior graft survival. Despite the current standard relative low acute rejection rates in the first year after transplantation with current standards for immunosuppressive therapy, long-term outcome after renal transplantation has not improved accordingly [3]. 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 CNI treated patients [4]. The causes of IF/TA are multi-factorial and determined by transplantation related factors including donor organ quality, ischemic/reperfusion injury, acute rejection and/or CNI toxicity. Subclinical rejection 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 persistent or recurrent (chronic) cellular rejection and/or humoral rejection, finally leading to IF/TA and progressive loss of renal function [5–7].

SCR is defined by (cortical) tubulo-interstitial mononuclear cell infiltration without detectable functional renal deterioration. If graded according to Banff, approximately two-third can be graded as borderline and the remainder as grade-I rejection and vascular rejection is seen in only a few cases. The prevalence of SCR decreases over time after transplantation [6] largely depending on the intensity of clinical immunosuppression [8–11] and the use and type of induction therapy [12,13]. This is illustrated by a decrease in SCR at 3 months post-transplantation from 63% in the era of cyclosporine A (CsA)/azathioprine, towards only 5% with tacrolimus/mycophenolate in otherwise comparable groups of transplant recipients [7].

Besides the choice in immunosuppressive therapy, a prior acute rejection episode, histoincompatibility, degree of sensitization and donor age have been reported as risk factors for SCR [8,9,14,15]. The role of pharmacological factors, such as drug exposure and pharmacogenetic parameters for the occurrence of SCR is still unclear. It has

previously been suggested that optimal CNI-exposure may prevent SCR and progressive renal dysfunction [11]. In this context variability in the genes coding for the metabolic cytochrome enzymes (i.e. *CYP3A5* and *CYP3A4)*, 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 [16], associations between genetic variants in *ABCB1* and graft function and graft survival have been described [17–20]. 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 [18], while *ABCB1* 2677T allele carriers had a 3-fold higher odds of developing acute rejection [19]. 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 [21], but these recipients were found to have a survival benefit [22]. 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 [23]. But, to the best of our knowledge no pharmacogenetic risk factors for SCR have been reported for renal transplant recipients on CsA therapy.

Genetic variability in genes coding for calcineurin, the target enzyme of CsA, in theory could alter the susceptibility for CsA. Polymorphisms in these genes could potentially be related to AR and/or SCR [16]. Two 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 *PPP3CB* gene of the recipient, may be related to AR and/or SCR.

For the current evaluation we selected a cohort of 361 transplant recipients, who entered the run-in phase of a multicenter, prospective study. These patients received quadruple immunosuppressive therapy with basiliximab induction and concentration-controlled CsA, mycophenolic acid and prednisolone maintenance therapy. A scheduled biopsy was performed at six months after transplantation to exclude SCR before patients were allowed to enter their assigned treatment arm during the second phase of the study. To identify risk factors for DGF, AR and SCR generally accepted risk factors were combined with demographic factors and pharmacological parameters. The principal aim was to identify the contribution of CsA exposure and/or genetic variability in the genes coding for PPP3CA, PPP3CB, ABCB1, CYP3A5, CYP2C8 and NR1I2 to the risk for DGF, AR and ultimately SCR.

Patients & Methods

Study design and patient population

Renal transplant recipients (n=361) participating in the run-in phase for a multicenter, randomized prospective trial aiming to minimize immunosuppression starting 6 months after transplantation [24]. 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 receiving 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 ethics approval was provided by the review boards 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 10 mg once daily oral prednisolone at day 4), twice daily 720 mg mycophenolate sodium (Myfortic®) and twice daily CsA (Neoral®). CsA was initially dosed 4 mg/kg b.i.d and subsequently adjusted to reach a predefined 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 time points, the first visit one week after transplantation, followed by 6 weeks, 3 months and 6 months at the time of the protocol biopsy, just prior to entering the second 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 and 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 may be issues related to sampling error and inter-observer variability [25–27]. Furthermore serum creatinine is not only a poor

marker for changes in renal function [28], 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 and age category (only for time to event analysis (1:<46 year, 2: 46 – 64 years and 3:>64 years) 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. These pharmacokinetic parameters were derived using a previously published population pharmacokinetic model for CsA [29].

Pharmacogenetics

Renal transplant recipients (n=302) were genotyped for genetic variants in the relevant genes *PPP3CA* and *PPP3CB* and in the genes *ABCB1*, *CYP3A5*, *CYP3A4, 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 [24]. 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).

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) CYP3A4*22 (35599367) 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 en 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 3. The success rates for all genotyping analyses were higher than 97%. Genotype frequencies for 15 of 19 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 (Rh2> 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

Delayed Graft function

The binary endpoint for delayed graft function (yes/no) was analyzed with a proportional odd model. Dropout was not included in the analysis since DGF only occurred directly after transplantation.

Subclinical rejection

The binary endpoint for subclinical rejection (yes/no) was analyzed with a proportional odd model. Patients that dropped-out during the first 6 months 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:

$$Y = (1\text{-}P_{do})^{*}(P_{SCR}{}^{*}SCR + (1\text{-}P_{SCR})^{*}(1\text{-}SCR))$$

With Y the likelihood of the model, P_{do} the probability of dropping out and P_{SCR} as the probability of SCR. The variable SCR is a binary outcome with SCR=1 if SCR is present and SCR=0 if SCR is absent. For individuals with a premature study-end (drop-out), the likelihood of the model is $Y = P_{do}$. For the analysis of rejection treatment on the development of SCR a sub analysis was performed on the 50 patients who had experienced an acute rejection in the first 6 months. The model parameters for the analysis of DGF and SCR were estimated by maximizing its likelihood using the Laplacian method. Throughout the model building process, an altered model was chosen over a precursor model if the difference in the objective function (OF), defined as -2 times the log-likelihood, was >3.84 ($P < 0.05$, with 1 degree of freedom, assuming χ^2 distribution) . All pre-selected covariates were evaluated one by one in the base model. Subsequently, selected covariate relationships were evaluated by forward inclusion and backward deletion procedure.

Acute Rejection – Time To Event

The time at which first acute allograft rejection occurred was recorded and for the patients who did not experience an acute rejection the time to dropout or end of study was recorded and treated as a censored observation. The time to the acute rejection was analyzed using a parametric survival model. The model was developed in two steps: First a base model was built to describe the time to first acute rejection with taking the drop out into account (right-censoring). Secondly, it was investigated if covariates could influence the time to

first acute rejection. To describe the time to the first acute allograft rejection, a parametric survival function according to the following equation (equation 1) was used:

$$S(t) = e^{-\int_0^t h(t)dt} \quad (1)$$

With h(t) as the hazard, and S(t) as the survival, which is a function of the cumulative hazard within the time interval between time zero and time t describing the probability of not experiencing an acute rejection ("surviving") within this interval. The base model was developed by exploring different functions for the hazard h(t), varying from time independent constant hazard functions (e.g. exponential) to more complex functions such as Weibull, Gompertz and log-logistic distributions. Of the preselected covariates potential covariates were selected after a stepwise approach: In a first step, a graphical analysis was performed to select potential covariates that could be investigated in a full covariate analysis. To this end for each covariate Kaplan-Meier plots, stratified per group, were inspected visually. In case of continuous covariates, data was divided in quartiles, resulting in equal number of subjects in each quartile. In a second step, based on the graphical analysis, covariates were selected to be carried forward to the single addition step. The selected covariates were added to the model one-by-one, and were retained in the model if the drop in OF > 6.63 ($p<0.01$, assuming χ^2-distribution). In a third step, forward inclusion, the covariates that were selected were added one after each other in ranking order of significance. The covariates were retained in the model if drop in OF was larger than 6.63 ($p<0.01$). In a fourth step, backward deletion, each covariate that was included in the full model, based on the forward inclusion step, was removed. This step was repeated until each remaining covariate caused an increase of at least 10.8 points in OF. The covariates were retained in the model if increase in OF >10.8 ($p<0.001$) to correct for multiple testing. The nonlinear mixed effect modelling package NONMEM (v7.2.1, Icon Development Solutions, Ellicott City, MD) was used for modelling, using PsN toolkit 3.4.2 and Piranã version 2.8.0 as modelling environment [30]. Results were analysed using statistical software package R (v2.15.2) and RStudio (v0.97.248; Boston, MA, USA).

Results

Patient characteristics are provided in Table 1. At the time of transplantation 361 renal transplant recipients were included and a 6-month protocol biopsy was obtained from 275 (76%) patients. Reasons were mainly non-medical (withdrawal of consent), return to dialysis, insufficient graft function or patient death. There were no relevant differences in the relevant demographic or transplant characteristics. Overall DGF was observed in 14% of the patients (28% in case of a deceased donor kidney, including those after cardiac death) and subclinical acute rejection was observed in 18% (n = 50) of protocol biopsies. The prevalence of SCR was higher in male recipients and patients with a history of acute rejection (Table 2).

Patients were genotyped for the polymorphisms in genes encoding the cytochrome P450 3A5 3A4 and 2C8 enzymes, P-glycoprotein and the calcineurin protein. Haplotypes and genotypes are summarized in Table 3. Besides these pharmacogenetic factors, inadequate systemic drug exposure is a potential important pharmacological risk factor for SCR as well. CsA exposure was monitored throughout the study period and the change in AUCs over time after transplantation is presented in Figure 1.

Exposure was found to be higher than the predefined target value in the first 6 weeks in the majority of the transplant recipients. After 6 weeks, when exposure to CsA was reduced, the CsA-AUC was kept within a range of roughly 2000-4500 μg*h/L (target 3250 μg*h/L) for most patients.

In univariate analysis the covariates related to the incidence of DGF (Table 4) and SCR were identified (Table 5).

Table 1: Clinical characteristics.

Characteristic	Inclusion at the time of transplantation
Inclusion at transplantation (n)	361
Recipient age (yr)	51 ± 13
Recipient gender (% male)	63
Race (% Caucasian)	86
Diabetes at baseline (%)	42 (12%)
Primary kidney disease	
Polycystic kidney disease	78
Glomerulonephritis,-sclerosis	77
Hypertension	60
Urological origin	26
Diabetic nephropathy	18
interstitial disease	11
etiology uncertain (e.c.i.)	14
other	77
cold ischemia (h) of cad donor	17
donor age (yr)	49 ± 13
donor type:	
living donor, related	76
living donor, unrelated	93
deceased donor, heart beating	121
deceased donor, non-heart beating	70
HLA-mismatches:	
Class 1 mismatces	1.94 ± 1.15
Class 2 mismatches	0.84 ± 0.63
Delayed graft function (%; living donor excl.)	28
Patients with at least 1 BPAR (%)	13.85
Patients treated with ATG (%)	34
serum creatinine at baseline (μmol/L)	770 ± 277
serum creatinine at week 2 (μmol/L)	246 ± 244
serum creatinine at week 4 (μmol/L)	145 ± 62
serum creatinine at month 2 (μmol/L)	138 ± 70
serum creatinine at month 6 (μmol/L)	129 ± 39
Patients with a 6 months biopsy	276
Drop-out (no biopsy) reasons:	
Withdrawal of consent	55
Graft loss, dialysis or eGFR < 15 ml/min	18
Patient death	7
Infection	2
Intolerability to immunosuppressive drugs	1
Other	2

Table 2: 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)	Present (n=50)
Recipient age (yr)	51 ± 13	49 ± 12
gender (male, %)	64	78
race (Caucasian ,%)	87	86
Donor age (yr)	51 ± 12	48 ± 14
age > 60 yrs (%)	14	18
HLA class I mismatches	1.98 ± 1.16	1.70 ± 1.20
HLA class II mismatches	0.80 ± 0.64	0.84 ± 0.58
Delayed graft function (living donor excluded,%)	27	25
Recipients with a previous BPAR (%)	8	16%
Renal function (mean ± SD)		
serum creatinine at baseline	754 ±257	865 ± 364
serum creatinine at week 2	234 ± 227	312 ± 320
serum creatinine at week 4	142 ± 65	160 ± 44
serum creatinine at month 2	132 ± 58	173 ± 111
serum creatinine at month 6	125 ± 37	149 ± 45

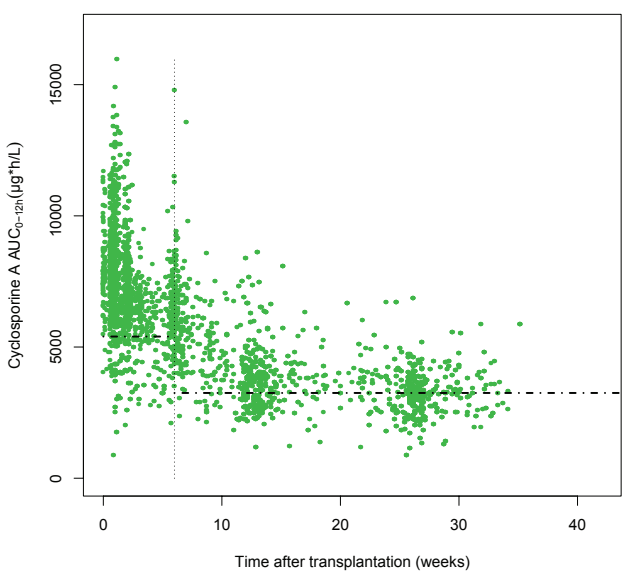

Figure 1: $AUC_{0\text{-}12h}$ in time after transplantation. Target AUC (horizontal striped lines) was 5400 µg*h/L up to 6 weeks after transplantation and 3250 µg*h/L thereafter.

Table 3: 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*).

PPP3CA haplotype		PPP3CB haplotype		CYP2C8 haplo *3		ABCB1 haplotype		ABCB1 T-129C	
n=282		n=288		n=295		n=290		n=300	
CTCCT	0.52	CAC	0.8	CT	0.81	CCG	0.44	TT	0.91
CCACT	0.18	TAT	0.08	TC	0.18	TTT	0.38	TC	0.09
ACACT	0.12	CTC	0.06			TCG	0.13		
ACCTG	0.11	TAC	0.05			CTG	0.02		
ACCTT	0.04					CTT	0.02		
CTCCG	0.02								

CYP3A5 *1		CYP3A5 *6		CYP3A4 *22		NR1I2 A+7635G		NR1I2 G-24113A	
n=300		n=300		n=302		n=302		n=301	
GG	0.81	GG	0.99	CC	0.88	AA	0.35	GG	0.32
GA	0.16	GA	0.01	CT	0.11	AG	0.48	GA	0.54
AA	0.03			TT	0.01	GG	0.17	AA	0.14

Table 4: Factors with significant effect on the incidence of delayed graft function.

Covariaat		Incidence of DGF	Δ OF/LL	P-value
BASE-model*		14%		
Deceased donor			-63.408	<0.00001
	if yes	27%		
	if no	0.6%		
Cold ischemic time > 12 hr #			-36.515	<0.00001
	if yes	26%		
	If no	7%		
PPP3CB -genotype #,	*no TAC block*	15%	-5.142	0.0234
	carriers of TAC	35%		

* ΔOF/LL >3.84 (P<0.05, chi-square test), # Based on a smaller dataset due to missing data. N.S. not significant.

Of the pharmacological factors, only PPP3B was related to the occurrence of DGF. Carriers of a at least one TAC block had a higher incidence of DGF (35% vs 15%). The only other covariate related to DGF was a deceased kidney donor (27% versus 0.6% of living donors) and a cold ischemic time over 12 hours (26% versus 7% if not).

No pharmacological factors were related to SCR. In order of relevance, the most significant covariates related to the prevalence of SCR were: a previous acute rejection episode and recipient of a kidney from a deceased donor. A history of acute rejection increased the incidence of SCR to 38% *versus* 16% without acute rejection. Receiving a deceased donor kidney was associated with an SCR prevalence of 24% versus 13% in recipients with a living donor kidney. After including the information on patients without a protocol biopsy (the context that every patient was intended-to-be-biopsied) factors could be identified related to not having a protocol biopsy performed at 6 months ("dropping-out of the study"). Covariates related to an increased risk of dropping-out were a previous acute rejection episode, a deceased donor kidney, female sex and the *ABCB1* TTT-haplotype (Table 5). In case patients did not carry a TTT-haplotype drop-out was 10%, otherwise 19%.

Table 5: 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 *	P-value
BASE-model*	18%			24%		
Previous acute rejection episode		-6.645	0.0099		-16.829	0.0000
if yes	38%			48%		
if no	16%			20%		
Type of Donation		-5.489	0.0191		-7.473	0.0063
if deceased	24%			29%		
If living	13%			17%		
Gender		-3.814	0.0508		-6.926	0.0085
male	21%			19%		
female	12%			31%		
					-4.388	0.0362
ABCB1 TTT-genotype#						
no TTT block		N.S.		10%		
carriers of TTT		N.S.		19%		
Previous rejection treatment		-7.811	0.0052			
if yes	34%				N.S.	
if no	15%				N.S.	

ΔOF >3.84 (P<0.05, chi-square test), # Based on a smaller dataset due to missing data. N.S. not significant.

In the next step, these above mentioned factors were combined in a multivariable approach (Tables 6 and 7). For DGF only a deceased kidney donor remained significantly related in the multivariate analysis with an incidence of 28%. The highest risk category for SCR was identified with the final model, identifying SCR prevalence at 6 months of 47% in case of a deceased donor kidney and a history of (treated) acute rejection. In contrast, living donation without acute rejection resulted in a SCR prevalence of 11%.

Table 6: Multivariate analysis of DGF: forward inclusion/backward deletion.

Model	absolute OF	ΔOF	P-value
forward inclusion			
BASE-model	297.324		
AND effect deceased donor on DGF	233.916	- 63.408	<0.00001
AND effect cold ischemic time on DGF *	231.415	- 2.501	0.1338
backward deletion			
FINAL-model	233.916		
MINUS effect deceased donor on DGF	297.324	63.408	<0.00001

ΔOF/LL >6.64 (P<0.01, chi-square test), * Dropped from the final model.

Table 7: Multivariate analysis of SCR: forward inclusion/backward deletion.

Model	absolute OF	ΔOF	P-value
forward inclusion			
BASE-model	654.297		
AND effect previous acute rejection on drop out	637.468	-16.829	0.0000
AND effect recipient gender on drop-out	628.991	- 8.477	0.0036
AND effect donation type on drop-out	620.677	- 8.314	0.0039
AND effect previous acute rejection on SCR	614.032	- 6.645	0.0099
AND effect donation type on SCR	608.458	- 5.574	0.0182
backward deletion			
FINAL-model	608.458		
MINUS effect donation type on SCR	614.032	5.574	0.0182
MINUS effect previous acute rejection on SCR	620.032	6	0.0143
MINUS effect donation type on drop-out	628.991	8.959	0.0028
MINUS effect recipient gender on drop-out	637.468	8.477	0.0036
MINUS effect previous acute rejection on drop out	654.297	16.829	0.0000

* ΔOF >3.84 (P<0.05, chi-square test).

During the study period patients dropped-out and recipients with a deceased donor kidney, also had the highest drop-out rate, 60% versus 27% with or without acute rejection, respectively. After splitting the results according to gender as additional risk factor for dropping-out, female recipients displayed a drop-out rate of 70% and males 51% in case of a deceased donor kidney and previous acute rejection. For comparison after living donations, without a previous acute rejection, drop-out rate were 19% (females) versus 10% (males), respectively. 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, as well as the fact that the genotypes were not available for all individuals.

Finally in a sub analysis with patients that experienced at least one acute rejection episode (n=50) the effect of the type of rejection treatment on prevention of subsequent SCR was investigated. These results are presented in Table 8. Rejection treatment that included ATG resulted in a significantly (P<0.05) lower prevalence of SCR (13% *versus* 50%).

Table 8: Sub analysis of rejection treatment and incidence of SCR in patients experiencing acute rejection.

Model	Incidence of SCR	ΔOF	P-value
BASE-model	38%		
Rejection treatment with least MPNS and ATG		-4.052	0.0441
if yes	13%		
if no	50%		

* ΔOF >3.84 (P<0.05, chi-square test).

In the time to first acute rejection analysis 3 patients had to be excluded because they dropped out of the study before start of the six month observation period. In some of the patients, more than one acute rejection was observed; Due to the limited number of these observations the analysis took only occurrence of a first acute rejection episode into account. Based on the objective function and the simulated Kaplan-Meier plots, a Gompertz model described the time to first acute rejection most adequately. The equations used for the hazard function (Equation 2) for the survival function (Equation 3) are shown below:

$$Hazard = \lambda . e^{\gamma . time} \qquad (2)$$

$$Survival = e^{\frac{\lambda}{\gamma} \cdot (1 - e^{\gamma . time})} \qquad (3)$$

With λ describing the scale and γ describing the shape of the survival curve. The Kaplan Meier plots showed adequate agreement between the observed and the simulated time to rejection. Furthermore the drop-out was adequately described by a log-logistic model.
Based on graphical non-parametric Kaplan Meier plot analysis of the different covariates 11 covariates were selected for parametric covariate analysis with the model; body mass index and CSA exposure ($AUC_{0\text{-}12h}$) as continuous covariates and DGF, PPP3CB variant TAT, Pre-existing diabetes mellitus (DM), *CYP3A5*1* genotype, 5 different HLA-mismatch categories (HLAMISS defined as 2 or more HLA DR mismatches or at least 1 HLA-B and 1 HLA DR mismatch), age category and underlying disease as categorical covariates (immunological vs non immunological). These covariates were tested for influence on the scale and the shape parameters of the survival curve. The results of the univariate analysis are presented in Table 9.
Delayed graft function and *CYP3A5*1* had a significant effect on the scale parameter of the survival curve. Age category had a significant effect on the shape parameter of the survival curve, older patients had a decreased risk of developing early acute rejection. After forward inclusion and backward deletion only DGF remained a significant risk factor for the time to acute rejection (Table 10). In Figure 2 the Kaplan Meier plot shows the difference in survival (freedom of acute rejection) in the two groups: with or without DGF. The survival model prediction shows adequate match with the observed data. Patients experiencing DGF after transplantation had an increased risk of developing early acute rejection.

Table 9: Univariate analysis of time to first AR.

Model	ΔOF	P value
Continuous covariates		
BMI on λ (linear) *	-1.06	0.3032
Cyclosporine $AUC_{0\text{-}12h}$		
AUC on λ	-0.642	0.4230
AUC on γ	-0.823	0.3643
Categorical covariates		
DGF on λ #	-12.33	0.0004
DGF on γ	-1.34	0.2470
DM on λ	-0.71	0.3994
DM on γ	-0.12	0.7290
PPP3CB - TAT on λ	-4.65	0.0311
PPP3CB - TAT on γ	-3.8	0.0513
CYP3A5*1 on λ #	-8.36	0.0038
CYP3A5*1 on γ #	-7.04	0.0080
HLA mismatch CLASS II on λ	-4.57	0.0325
HLAMISS on λ	-3.67	0.0554
HLAMISS on γ	-2.72	0.0991
HLA mismatch-A on λ	-1.74	0.1871
HLA mismatch-A on γ	-3.53	0.0603
HLA mismatch-B on λ	-3.93	0.0474
HLA mismatch-B on γ	-3.84	0.0500
HLA mismatch-DR on λ	-4.57	0.0325
HLA mismatch-DR on γ	-3.46	0.0629
Age category on λ #	-8.16	0.0028
Age category on γ #	-8.95	0.0028
Underlying disease on λ	-0.72	0.3961
Underlying disease on γ	-5.24	0.0221

* Continuous covariates were tested for linear, loglinear, allometric an Emax relationship, the relationship with the largest ΔOF is shown. # selected for multivariate analysis ΔOF >6.64 (P<0.01) N.S. not significant.

Table 10: Multivariate analysis of time to first AR.

Model	absolute OF	ΔOF	P-value
forward inclusion			
Basic survival	745.62		
AND effect DGF on λ	733.293	-12.327	0.0004
AND effect AGEcat on γ	724.646	-8.647	0.0033
AND effect CYP3A5*1 on λ	718.273	-6.373	0.0116
AND effect AGEcat on λ	722.759	-1.887	0.1695
AND effect CYP3A5.1 on γ	722.92	-1.726	0.1889
backward deletion			
Final survival model	724.646		
MINUS effect AGECAT as covariate on γ	733.293	8.647	0.0033
MINUS effect DGF as covariate on λ	736.7	12.054	0.0005

* ΔOF >6.64 (P<0.01) forward inclusion, ΔOF >10.8 (P<0.001) backward deletion (chi-quare test).

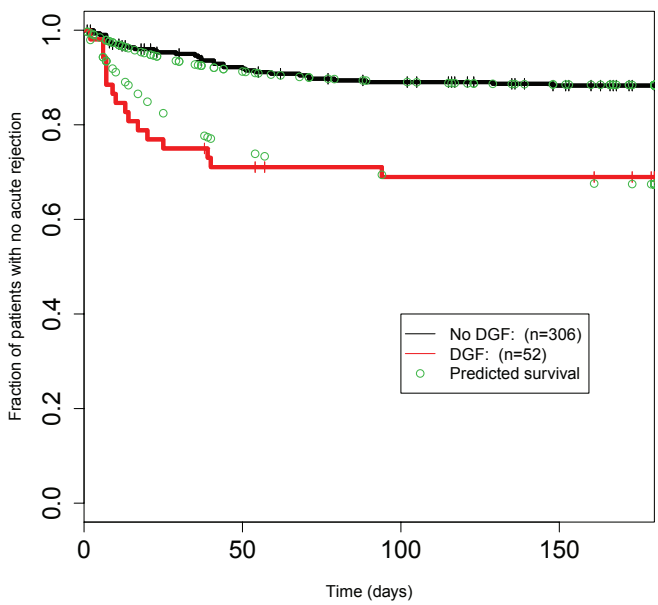

Figure 2: Survival plot of fraction of patients without acute rejection during the study showing a clear difference between patients with (red line) and without DGF (black line).The predicted survival according to the developed survival model is showed by the green open circles.

Discussion

This analysis on a relatively large homogenous group of standard to low risk transplant recipients participating in the run-in phase of a multicenter, randomized clinical trial on quadruple therapy with basiliximab, prednisolone, mycophenolate sodium and CsA with controlled systemic drug exposure, aimed to identify pharmacological risk factors for DGF, AR and SCR 6 months after renal transplantation. Especially, the variability in CsA exposure and/or genetic variability in genes encoding calcineurin, P-glycoprotein and CYP3A5 were of interest. The incidence of AR and SCR with controlled and early reduced systemic CsA-exposure within 6 months was found to be 14% and 18%, respectively. In this context pharmacological factors, including exposure and genetic variability in the selected genes, were not found to be related to the risk for DGF, AR or SCR. Receiving a kidney from a deceased donor was the dominant risk factor for DGF, with DGF being the primary risk factor for time to first AR. For SCR the most important risk factors were a previous acute rejection episode, and being recipient of a deceased donor kidney. These factors were also associated with a lower 6-months protocol biopsy rate (overall reduction of 24%). Other factors related to "dropping-out" were female sex and carrying a copy of the *ABCB1* TTT-haplotype. The incidence of biopsy drop-out was the lowest for patients without a copy of the *ABCB1* haplotype. Finally a significant relationship ($P<0.05$) was found between rejection treatment including ATG and a lower subsequent prevalence of SCR.

The results of this study confirm previous findings and of *Nankivell et al.* [7,31], in that DGF was associated with an increased risk of early acute rejection and patients with acute rejection constituted the dominant risk factor for subsequent SCR. The prevalence of SCR depends on time after transplantation and the center policy on the use/type of induction/maintenance immunosuppressive therapy and the immunologic risk profile of the recipients [32], complicating comparison of different studies. SCR in early protocol biopsies was found to be associated with HLA-matching [9,31,33], prior acute rejection episode [31], donor age [9] and donor source [15,33]. 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 ($AUC_{0\text{-}12h}$) controlled population of standard to low risk kidney transplant recipients. Exposure was relatively high the first 6 weeks after transplantation (generally over 5400 μg*h/L) and, after early reduction maintained between 2000 and 4500 μg*h/L thereafter.

This is the first report on the genetic variability in the genes coding for calcineurin isoforms, *PPP3C*A and *PPP3CB*. No relationship between the genetic variability in these genes and the time to AR or the prevalence 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 the calcineurin present in cells of the immune system, whereas *PPP3CA* is thought to be more relevant in other tissues including renal tubular epithelial cells. Variability in the *PPP3CA* gene within kidney donors would be more relevant for renal toxicity and perhaps DGF. To investigate these theoretical genetic risk factors we determined haploblocks in both genes, but in the current cohort genetic variability in *PPP3CB* was not related to time to first AR, DGF or the prevalence of 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 previously hypothesized [16]. 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, CYP3A4, CYP2C8*) and the regulation of these genes (PXR - *NR1I2*). Carrying at least one copy of the *ABCB1* TTT-haplotype, however, was related to an almost 2-fold higher drop-out rate for a 6-month protocol biopsy. At least theoretically, these patients may be prone to a higher frequency of adverse events, since the TTT-haplotype is associated with lower P-glycoprotein activity. This is independent from kidney survival, where the *ABCB1* genotype of the donor may be of higher relevance [17,20]. A combined donor-recipient homozygosity for the C3435T variant in *ABCB1* was associated with chronic allograft damage [34]. In accordance with our results no relation has been found between tacrolimus, carrying the CYP3A5*1 allele and AR or SCR, acute rejection [35,36].

The findings of the sub analysis of rejection treatment on the prevalence of subsequent SCR suggest confirms the previously reported low prevalences observed with induction therapy with depleting antibodies in patients cohort dominated by living donor kidney transplant recipients.

Early minimization of CsA or tacrolimus is increasingly applied an attempt to reduce toxicity and to improve long term outcome [37–39]. While there is still debate whether SCR should be treated as acute rejection episode, it is generally accepted that persistent or recurrent SCR constitutes a potential threat to (functional) survival of the transplanted

kidney [8,10,15,40]. 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 [14,38,41] and exposure to the remaining drug(s) is individualized and adequate. There are several lines of evidences to support this notion. Inadequate MPA exposure and SCR were independent risk factors for subsequent acute rejection after early CNI withdrawal [42]. Less early as well as late acute rejection episodes occurred after treating SCR in early protocol biopsies with high dose steroids [43]. Maintaining adequate controlled CNI exposure in a triple immunosuppressive regimen was as effective in preventing late acute rejection [10]. Despite (predominantly borderline) SCR in a relevant proportion of 6 month biopsies, no significant differences for renal function or severity of fibrosis in sequential biopsies were observed [10].

The integrated approach used in this study, combining demographic characteristics, transplant-related factors together with detailed drug-exposure and variability in genetic parameters in genes related to pharmacokinetics as well as pharmacodynamics, is very powerful to detect relationships with clinical events and identified DGF as a risk factor for early acute rejection. Moreover, a history of acute rejection recipients of kidneys from a deceased donor were identified as the dominant risk factors for inflammation in 6-month protocol biopsies despite controlled systemic drug exposure. Although, effects of exposure and genetics could not be identified in this analysis, likely this approach can be successful in identifying risks of late acute (cellular or humoral) rejection, chronic 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 [20], while donors carrying the 3435TT genotype were associated with nephrotoxicity (odds ratio 13.4) [17]. Such a conclusive analysis should at least include genetic variability in the genes *ABCB1, CYP3A5, PPP3CA* of the donor.

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10

General discussion and future perspectives

Introduction

With acute rejection rates lowered to 10-20% but limited progress with regard to long-term survival a new challenge lies ahead in optimizing immunosuppression in renal transplantation [1]. Individualizing and fine-tuning current immunosuppressive regimens is now the most promising strategy to improve long term graft survival for renal transplant recipients. The current maintenance immunosuppressive drugs, calcineurin inhibitors (CNIs), are known for their efficacy but also for their toxicity such as new onset diabetes mellitus, neurotoxicity and renal toxicity especially in the higher dose ranges [2]. Efficacy of CNI minimizing or even CNI free strategies shortly after transplantation are currently widely investigated [3–5]. Less nephrotoxic regimens including mTOR inhibitors have been developed during the last decade, but on the other hand new serious side effects, relative high discontinuation rates and/or intolerability postpone wide implementation [4,6]. Although strict therapeutic drug monitoring is implemented some patients remain at risk for serious side effects and rejection. Identifying these patients before initiation of therapy could help prevent therapy failure. The main challenge is to find the right immunosuppressive regimen and exposure at the right time for individual patients. This thesis is constructed out of a number of different analyses to further optimize maintenance immunosuppressive therapy for renal transplant recipients to prolong long-term graft survival, starting with a comparison of the most used analytical methods for therapeutic drug monitoring of everolimus, followed by evaluations of potential predictive biomarkers for everolimus pharmacokinetics and pharmacodynamics and finally also potential predictive biomarkers for calcineurin inhibitor pharmacokinetics and dynamics are explored.

Therapeutic drug monitoring techniques

Because of its highly variable pharmacokinetics and narrow therapeutic window everolimus therapeutic drug monitoring (TDM) is essential for preventing serious side effects and rejection [7]. Currently a variety of analytical methods to perform TDM are available [8–10], and methods may differ in accuracy and specificity. Whether these differences are clinically relevant is an important question. Because of high protein binding and to distribution into erythrocytes whole blood is the matrix of choice for everolimus

TDM [11]. The two widely used analytical techniques for everolimus blood concentration measurement, fluorescence polarization immuno assay (FPIA) and liquid chromatography tandem mass spectrometry (LC-MS/MS), were compared in chapter 3 of this thesis. The findings showed that these two methods are not in agreement. Everolimus concentrations determined by FPIA are, on average, 23% higher than LC-MS/MS. However, the variability found between FPIA and LC-MS/MS could be twofold for concentrations lower than 15 μg/L or AUC_{0-12h}. This suggests a relatively large effect on variability of FPIA versus LC-MS/MS when monitoring everolimus trough concentrations. The large variability in concentrations determined with FPIA can lead to clinically relevant differences in dosing advice compared with LC-MS/MS despite using a correction factor of 23%. The within-patient variability for trough concentrations appeared to be higher using the FPIA method [12], most likely caused by nonspecific binding of the antibodies [13] and crossreactivity of metabolites, which are actually present in relatively high concentrations before the next dose [14,15]. The variability in differences in dosage advice showed that the risk of suboptimal dosage advice is present and clinically relevant. In general LC-MS/MS is a more specific, more stable, and more accurate method for everolimus TDM compared to FPIA and is able to simultaneously measure several immunosuppressive drugs in a single run. However the most important limitations for broad introduction of LC-MS/MS for everolimus TDM are the need for a high initial capital investment and highly trained technicians for operation and maintenance. Centralization of sample measurements in combination with dried blood spot methodology might be a solution to this problem. While pharmacodynamic monitoring instead of pharmacokinetic monitoring in theory should give a more accurate insight on the mTOR inhibition and clinical effects a suitable method has not yet been found and implemented. Other innovative methods of measuring concentrations at the site of action like PBMCs could potentially give a more precise view at the level of immunosuppression but are currently under development and not yet accepted in clinical practice. Therefore TDM of everolimus whole blood concentrations using LC-MS/MS currently is still the method of choice.

Variability in pharmacokinetics of everolimus

Everolimus is metabolized by CYP3A4, CYP3A5, CYP2C8 and is a substrate for P-glycoprotein (P-gp), and is characterized by its high inter patient variability. The nuclear

pregnane X receptor (PXR) mediates expression of CYP3A4 and multi drug resistance proteins (MDR1 and MDR2) and could therefore potentially influence everolimus pharmacokinetics [16–18]. Monitoring area under the blood concentration versus time curve (AUC) instead of trough concentration is often more informative. However, AUC monitoring when using trapezoidal calculations remains laborious for both patients and the clinic. Limiting sampling strategies based on Bayesian estimation could be solution to this problem. A limitation of TDM is that during the critical period of the first days after transplantation or conversion to another immunosuppressive regimen the exposure cannot be influenced. Getting the initial dose right is therefore very important. Especially drugs with a long elimination half-life such as everolimus are at risk of under or overexposure because correcting them takes more time. Reaching target exposure is as soon as possible after drug initiation is essential, however currently no factors for everolimus initial dose differentiation have been identified. Pharmacogenetics, when looking at polymorphisms coding for metabolizing enzymes which lead to altered drug metabolism could be a potential factor as previously shown for tacrolimus [19]. These factors could potentially shorten the time to reach target exposure. To address the above mentioned problems the research described in chapter 4 was performed. Pharmacometrics, which uses mathematical models based on physiology, pharmacology and disease for quantitative analysis of interaction between drugs and patients was used to build a population pharmacokinetic model, a limited sampling model and evaluate potential factors influencing pharmacokinetics (covariates). The pharmacokinetics of everolimus of (primarily Caucasian) renal transplant patients using everolimus and prednisolone was best described by a two-compartmental model with first order absorption and lag time. Everolimus pharmacokinetics was not significantly influenced by genetic polymorphisms in coding genes for the metabolizing enzymes CYP3A5, CYP2C8, ABCB1 and PXR or drug transporter ABCB1. Therefore, the currently known single nucleotide polymorphisms (SNPs) are not able to predict everolimus systemic exposure to a clinically relevant extent and shorten the time to reach target exposure. In addition, demographic covariates such as total body weight, age, sex, hematocrit, albumin, length, body mass index, body surface area, lean body weight, underlying disease, co-medication and ethnicity did not significantly influence everolimus pharmacokinetics [20]. Ideal body weight did significantly correlate with the variability in apparent distribution volume of the central compartment and can be physiologically explained by the fact that everolimus is for more than 75% partitioned into red blood cells and 75% of the plasma fraction is

bound to plasma proteins since length and sex are incorporated in the ideal body weight formula [11,21]. In conclusion, no factors for initial dose differentiation of everolimus were identified. Weak CYP3A inhibitors such as statins, nifedipine and sulfamethoxazole/trimethoprim did not have a clinically relevant effects on pharmacokinetics, which was in accordance with previous findings [22] although strong CYP3A inhibitors and inducers are known to strongly influence everolimus pharmacokinetics [23]. Monitoring everolimus during initiation and discontinuation of such drugs is therefore essential.

Therapeutic drug monitoring of everolimus

The most common way to perform everolimus therapeutic drug monitoring is monitoring based on trough concentrations. However, besides the higher impact of assay variability [12] when using one marker to predict everolimus systemic exposure, the correlation between C_{trough} and AUC is not optimal and could in theory lead to therapy failure and side effects [24] . Worse predictive performance of a TDM marker can lead to incorrect dose adjustments resulting in exposure outside the target range. The developed limited sampling model (Chapter 4) is an improvement in terms of inconvenience for patient and clinic and predictive performance. C_{trough} and C_2 monitoring based on the population pharmacokinetic model resulted in an improved predictive performance compared to C_{trough} monitoring. Whether TDM based on trough or AUC_{0-12h} does lead to differences the occurrence of hazardous side effects and clinical benefit in long term warrants to be investigated more thoroughly before clinicians can be convinced to use AUC monitoring instead of trough monitoring. Since the majority of research suggests that tacrolimus does not influence everolimus pharmacokinetics, the applicability of the developed model might include on tacrolimus + everolimus regimens. Since CNI minimizing and CNI free strategies are being actively investigated worldwide [4,5,25–28] there could be an increasing interest for implementation of the developed model in clinical practice.

Pharmacodynamics: side effects and everolimus discontinuation

Despite its proven efficacy and close TDM, everolimus is also known for some serious side effects with relative high discontinuation rates [6,29]. Leukopenia, thrombocytopenia,

hypertriglyceridemia and hypercholesterolemia are the most common side effects of mTOR inhibitors [4] and can often be managed with counteracting medication or dose reduction [6,30]. Although less common but a potentially life threatening side effect of everolimus is non-infectious interstitial pneumonitis. It typically presents itself within 2 to 6 months after start of therapy [31,32]. The exact mechanism of mTOR inhibitor-induced pneumonitis is still unknown, but direct damage to alveolar structures, formation of immunogenic molecules that react with specific antibodies, and direct immunologic drug responses are suggested as possible mechanisms [33]. A dose relationship may be present and is supported findings of by higher incidence in oncology where higher daily doses are prescribed [34,35]. Moreover a higher incidence was found in males on sirolimus therapy compared to females [36]. Infectious diseases are an important cause of death in renal transplant recipients [37,38] and strongly related to excessive and/or long-term clinical immunosuppression [39]. Everolimus is associated with a relatively low incidence of viral infections as compared to other immunosuppressive groups [40–42]. Everolimus is also associated with an increased incidence of new onset diabetes mellitus (NODM) which subsequently is associated with increased graft failure and mortality due to cardiovascular events [43]. NODM is therefore a serious complication of immunosuppressive therapy in transplant recipients which shortens long term survival [44]. Finding risk factors for everolimus discontinuation and the mentioned severe side effect could help further improve individualized immunosuppressive therapy by excluding patients at high risk from everolimus therapy or monitor them more intensively. In chapter 5 and 6 risk factors were explored for everolimus-discontinuation and serious side effects in renal transplant recipients on dual therapy.

In the case-cohort study (Chapter 5) no clear predisposing factors were identified for non-infectious interstitial pneumonitis. Pulmonary CT scans revealed an organizing or non-specific interstitial pneumonitis-like pattern. The course seems benign with disappearance of symptoms within one year after discontinuation of the drug. The incidence (12.7%) reported was higher than previously reported in renal transplant recipients on mTOR-inhibitors, varying between 4 and 6.8% [45–47]. In patients treated with everolimus for renal cell carcinoma the incidence of non-infectious interstitial pneumonitis has been reported to be around 25% [34,35]. This high incidence of non-infectious interstitial pneumonitis has been attributed to higher dosage of everolimus in these patients in combination with a higher detection level of pneumonitis due to routinely performed pulmonary CT scans. In the case cohort study, drug exposure was relatively high with an AUC around 170 μg*h/L

and trough levels around 10 μg/ml since everolimus was prescribed as part of a double immunosuppressive regimen. However, average everolimus exposure was not higher in the cases compared to controls. All patients subjectively recovered within one year, however long-term outcome after non-infectious pneumonitis remains unclear since at least theoretically non-infectious pneumonitis may result in pulmonary fibrosis. Since the presentation of non-infectious pneumonitis can be insidious or even asymptomatic, performing radiographic imaging of the lungs when patients present with dyspnea, cough or fever while on treatment with this drug according to the algorithm shown in Chapter 5 is recommended.

A more sophisticated time to event analysis was used to investigate risk factors for everolimus discontinuation and the serious side effects non-infectious interstitial pneumonitis, infection and NODM (Chapter 6). Such an approach has advantages compared to non-parametric and semi parametric analyses, because it enables inclusion of time-varying covariates and allows simulation based on the final model. Results showed that excess exposure during the study period and older age were risk factors for everolimus-discontinuation. Since the majority of discontinuation was side effect related this is in line with earlier finding that certain side effects have previously shown to be dependent on exposure [48,49]. As can be concluded from our results, clinicians should prevent renal transplant recipients from reaching excess everolimus exposure (i.e. AUC_{12}> 120 -150 μg*h/L), therefore close TDM remains warranted. Looking at the high discontinuation rates and low rejection risk we can extrapolate an initial target trough level between 6 μg/L and 8 μg/L from this study and an initial dose of 2 mg b.i.d. This initial dose might lower the rate of overexposure compared to 3 mg which was used in the study. Higher age resulted in a higher risk of everolimus-discontinuation probably due to fact that often patients with higher age have more comorbidities and senescence of their immune system with changes in T-cell function [50] where the immunosuppressive effect of the same immunosuppression exposure might be higher. Older patients with more comorbidities also have more difficulty to cope or accept additional side effects compared to young patients with no comorbidities.

The risk of experiencing non-infectious pneumonitis was increased by prolonged excess exposure. Furthermore renal transplant recipients with a PXR (NR1|2)(-24113G>A): AA genotype had a higher risk of developing pneumonitis compared to those carrying the AG or GG genotype although the effect seemed to be limited. The increase in risk of patient with that was found for patients with PXR (NR1|2) (-24113G>A) AA genotype might be related to an increased accumulation of everolimus in the lungs. In experimental animals high affinity for lungs and kidney were found for everolimus [51] and could this could

be the case in humans. PXR is a nuclear receptor whose primary function is to sense the presence of foreign toxic substances and in response up regulate the expression of proteins involved in the detoxification and clearance of these substances from the body. PXR polymorphism could subsequently have an effect on drug transporter activity since PXR is able to influence enzyme activity and multi drug transporter proteins [16–18]. Infections continue to be an important feature in the first year following both renal and heart transplant and occur in around 50% of patients [37]. The incidence of (opportunistic) infections is related to the intensity and type of immunosuppression [38]. No significant relationships for infection could be identified in the current analysis, but in general differences are more pronounced when two different immunosuppressive regimens are compared [39,52].

Although known from literature, important risk factor for the development of NODM include African ethnicity, increased age, obesity, increased number of transplants, donor type, a family history of diabetes and the use of prednisolone [53], but none of these relationships were strong enough to be detected in this patient cohort. The analysis for NODM had some specific limitations; the dataset lacked a significant number of patients from African ethnicity, Family history of diabetes was not available in the dataset en could therefore not be included in the covariate analysis. Exposure did not seem to affect the occurrence of NODM which was in accordance with previous studies [49]. In conclusion, the current findings can be used to further optimize everolimus based immunosuppressive therapy by preventing excessive drug exposure by strict therapeutic drug monitoring and restrict the initial dosing to a maximum of 2 mg b.i.d.

Influence of the most promising single nucleotide polymorphisms on maintenance immunosuppressant pharmacokinetics

Pharmacogenetics has only been adopted to a small extent in clinical practice for renal transplant recipients. In chapter 7 the influence of the most promising single nucleotide polymorphism: *CYP3A4*22*, *CYP3A5*3* variant alleles and its combined clusters on the pharmacokinetics of the three main kidney transplant immunosuppressive drugs cyclosporine, everolimus and tacrolimus was investigated. Cyclosporine, everolimus and tacrolimus are primarily eliminated by CYP3A enzymes [7,54–56] and as shown before in in-vitro and in-vivo studies, CYP3A4 is involved in their pharmacokinetics [55,57,58].

CYP3A4 is most likely predominant in cyclosporine and everolimus metabolic clearance and CYP3A5 contributes more significantly to tacrolimus metabolic clearance compared with CYP3A4 [55,56]. In contrast to CYP3A5, CYP3A4 lacked a reliable genetic marker for prediction of CYP3A4 expression which was suitable for dosing adjustments [59,60], however *CYP3A4*22* was previously marked as a potential reliable marker [61,62]. The results presented in chapter 7 demonstrated that carriership of the *CYP3A4*22* allele is significantly associated with a decreased cyclosporine clearance. Carriers of the *CYP3A4*22* allele showed 15% lower cyclosporine clearance as compared to non-carriers. In clinical practice this effect is not high enough to justify dose modification based on *CYP3A4*22*, since only an effect of at least 20% on clearance would lead to dose adjustments due to considerable degree of intra-individual variability in pharmacokinetics. Combining individual SNPs in theory would increase the predictive power of the single polymorphisms. However using *CYP3A* combined genotype of *CYP3A4* and *CYP3A5* as a predictor for cyclosporine, everolimus or tacrolimus clearance does not seem to be an improvement compared to the individual polymorphisms. Finally it was also demonstrated that patients carrying at least one *CYP3A5*1* allele have on average 53% higher tacrolimus clearance compared to non-carriers. The difference in tacrolimus clearance between *CYP3A5*1* carriers and non-carriers found was similar to what was published previously [19,63]. Dosing adjustments based on *CYP3A5*3* could be indicated to quickly reach target exposure, however the variability explained by *CYP3A5*3* is limited and the variability within the *CYP3A5* genotype groups remains significant and therefore close TDM remains essential. The absence of a clinically relevant influence of *CYP3A5*3* on cyclosporine and everolimus pharmacokinetics was in line with previous studies [60,64,65]. In conclusion, *CYP3A4*22* does not influence cyclosporine, everolimus or tacrolimus pharmacokinetics to a clinically relevant extent. Therefore the newly discovered *CYP3A4*22* or *CYP3A* combined genotypes are not indicative to be used for dose adjustments in clinical practice to further improve immunosuppressive therapy of cyclosporine, tacrolimus or everolimus in the investigated patient population. Hepatic microsomal P450 enzymes require P450 oxidoreductase (POR). Polymorphisms in the gene encoding POR have been linked to altered CYP activity [66]. In an additional analysis for everolimus (Chapter 8) the effect of *POR*28*, *CYP3A5*3* and their combined genotypes were explored. In contrast to what was previously found for tacrolimus [67,68] and in accordance to what was found for sirolimus [69] *POR*28*, or the combination of combination of *POR*28* & *CYP3A5*3* appeared not to be suitable as a biomarker to improve prediction of everolimus exposure.

Risk factors for delayed graft function, acute rejection and sub clinical rejection in a CNI based regimen

Over the past decades acute rejection (AR) rates have decreased dramatically, mainly due to calcineurin inhibitor (CNI) based immunosuppressive regimens. One of the dominant risk factors , previously identified for AR is delayed graft function (DGF) which is highly related to transplant related factors such as vulnerability of the allograft and/or prolonged preservation times [70]. Clinical episodes of AR have previously been identified as a risk factor for subclinical rejection (SCR) [71]. SCR is by definition histologically defined acute rejection and, as such, has been associated with subsequent interstitial fibrosis and tubular atrophy and with time progressive deterioration of renal function and inferior graft survival. Despite low acute rejection rates in the first year after transplantation with current standards for immunosuppressive therapy, long-term outcome after renal transplantation has not improved accordingly [72]. In chapter 9, a relatively large homogenous group of standard to low risk transplant recipients participating in the run-in phase of a multicenter, randomized clinical trial on quadruple therapy with basiliximab, prednisolone, mycophenolate sodium and CsA with controlled systemic drug exposure was analysed, aimed to identify pharmacological risk factors for DGF, AR and SCR 6 months after renal transplantation. Especially, the variability in CsA exposure and/or genetic variability in genes encoding calcineurin, P-glycoprotein and CYP3A5 were of interest. The incidence of AR and prevalence of SCR with controlled and early reduced systemic CsA-exposure at 6 months was found to be 14 and 18%, respectively. In this context pharmacological factors, including exposure and genetic variability in the selected genes, were not found to be related to the risk for DGF, AR or SCR. Receiving a kidney from a deceased donor was the dominant risk factor for DGF, with DGF being the primary risk factor for time to first AR. For SCR the most important risk factors were previous acute rejection, and being recipient of a deceased donor kidney. These factors were also associated with a lower 6-months protocol biopsy rate (overall reduction of 24%). Other factors related to "dropping-out" were female sex and carrying a copy of the *ABCB1* TTT-haplotype. The incidence of biopsy "drop-out" was the lowest for patients without a copy of the *ABCB1* haplotype. Finally a significant relationship ($P<0.05$) was found between rejection treatment including ATG and a lower subsequent prevalence of SCR. Three isoforms for calcineurin have been described: alpha, beta en gamma. Genetic variability in two genes coding for calcineurin, the target protein of CNIs were determined. The

gene coding for calcineurin beta (*PPP3CB)* could be primarily of relevance since this gene principally encodes the calcineurin present in cells of the immune system, whereas the gene coding for calcineurin alfa (*PPP3CA)* is thought to be more relevant in other tissues including renal tubular epithelial cells. Variability in the *PPP3CA* gene within kidney donors would be more relevant for renal toxicity and perhaps DGF. To investigate these theoretical genetic risk factors we determined haploblocks in both genes, but in the current cohort genetic variability in *PPP3CB* was not related to time to first AR, DGF or the prevalence of 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 previously hypothesized [73]. 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, CYP3A4, CYP2C8*) and the regulation of these genes (PXR - *NR1I2*). Carrying at least one copy of the *ABCB1* TTT-haplotype, however, was related to an almost 2-fold higher "drop-out" rate for a 6-month protocol biopsy. At least theoretically, these patients may be prone to a higher frequency of adverse events, since the TTT-haplotype is associated with lower P-glycoprotein activity. This is independent from kidney survival, where the *ABCB1* genotype of the donor may be of higher relevance [74,75]. A combined donor-recipient homozygosity for the C3435T variant in *ABCB1* was associated with chronic allograft damage [76]. In accordance with our results no relation has been found between tacrolimus, carrying the CYP3A5*1 allele and AR or SCR [77,78].

The findings of the sub analysis of rejection treatment on the prevalence of subsequent SCR confirms the previously reported low prevalence observed with induction therapy with depleting antibodies in patients cohort dominated by living donor kidney transplant recipients. Early minimization of CsA or tacrolimus is increasingly applied an attempt to reduce toxicity and to improve long term outcome [3,79,80]. While there is still debate whether SCR should be treated as acute rejection episode, it is generally accepted that persistent or recurrent SCR constitutes a potential threat to (functional) survival of the transplanted kidney [81–84]. 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 [79,82,85] and exposure to the remaining drug(s) is individualized and adequate.

The integrated approach used in this last chapter combining demographic characteristics, transplant-related factors together with detailed drug-exposure and variability in genetic parameters in genes related to pharmacokinetics as well as pharmacodynamics, is very powerful to detect relationships with clinical events and identified DGF as a risk factor for early acute rejection. Moreover, a history of acute rejection recipients of kidneys from a deceased donor were identified as the dominant risk factors for inflammation in 6-month protocol biopsies despite controlled systemic drug exposure. Although, effects of exposure and genetics could not be identified in this analysis, likely this approach can be successful in identifying risks of late acute (cellular or humoral) rejection and calcineurin toxicity, in transplant recipients when using genetic information of the donors. Kidneys from donors carrying the *ABCB1* variant haplotype 1236T/2677T/3435T have previously been associated with inferior graft survival and renal function [75], while donors carrying the 3435TT genotype were associated with nephrotoxicity [74]. Such a conclusive analysis should include genetic variability in the genes *ABCB1, CYP3A5, PPP3CA* of the donor.

Future research perspectives

The balance between high efficacy and a minimum of side effects of immunosuppressive treatment is fragile, especially in transplantation were the main immunosuppressive drugs have a low bioavailability, a narrow therapeutic index and high inter-patient variability. Finding the right immunosuppressive regimen and exposure for the right patient at the right time is the main challenge for the future. This thesis aimed to fulfill a part of this challenge, however, only small steps forward were made and much more research is needed to find the optimal immunosuppressive treatment for the individual patient. Finding factors that are predictive for short term (clinical and subclinical rejection) and subsequently long term outcome (graft survival) are essential to achieve an increase in survival for renal transplant recipients. Transplant characteristics such as donor type, HLA-DR mismatch, cold ischemic time and donor age are currently still the most predictive factors for the initial immunological risk. Although strict therapeutic drug monitoring is performed for most drugs still some patients are at risk for rejection or toxicity, therefore better biomarkers are needed to guide adequate clinical immunosuppression. Ideally a biological marker reflecting the immunological status of an individual should be used for monitoring immunosuppressive treatment. Unfortunately pharmacodynamic

markers are still not suitable for clinical practice or not available. Currently attempts are made to measure immunosuppressive drug concentrations at the site of action like PBMCs [86] but research is still in its early stages. Especially drugs that are substrates of P-glycoprotein (P-gp) like CNIs and mTOR inhibitors are of interest since P-gp could potentially have a large impact on drug disposition in PBMC resulting in differences in immunosuppressive effect. On the other hand less invasive biomarkers for early prediction of acute rejection as free circulating DNA and donor-specific antibodies are currently also under investigation [87,88]. Also promising biomarkers for nephrotoxiticy are under development [89]. Combining such biomarkers with a pharmacokinetic model might help to find an individual's unique target concentration range. Pharmacogenetics on pharmacokinetic parameters has been of great interest during the past decade in the field of renal transplantation, however only a few suitable pharmacogenetic markers predicting exposure have been found. Furthermore the additional value of initial dosing based on pharmacogenetic markers with respect to long term outcome has not yet been established. The focus of pharmacogenetics should be expanded to pharmacodynamics parameters like polymorphisms in the mTOR gene or calcineurin gene to identify patients at risk for certain side effects or under immunosuppression. All efforts should be pointed at finding the optimal immunosuppressive treatment for the individual patient. To make this possible more effort should be made for collaboration between research groups. Especially in Europe the need for collaboration between clinicians and scientists is essential to gather and analyze large datasets needed to evaluate the effect of future biomarkers on patient outcome in large patient populations. Subsequently a systems pharmacology approach should be used incorporating the most important sources of variability in terms of pharmacokinetics and pharmacodynamics.

Conclusions

mTOR inhibitors form a promising new class of immunosuppressive drugs for maintenance immunosuppression in renal transplantation. The available evidence demonstrates that IL-2RA induction with an mTOR inhibitor can successfully reduce CNI exposure by at least half without a penalty in terms of rejection in low- or moderate-risk de novo transplant recipients and may offer renal and antiviral benefits [90]. Besides these advantages, high drug-discontinuation rates and some serious side effects have been limiting for broad

introduction of mTOR inhibitors into the field of kidney transplantation. Therapy should be further optimized be means of finding the right exposure at the right time. With this in mind AUC monitoring can become more and more important, especially in the Netherlands where active patient participation and at home monitoring with dried blood spot technology are important aspects of how transplantation care will be organized in the future. This can only be made possible with wide adaptation of pharmacometric tools to assure the optimal balance between minimal patient inconvenience and accurate monitoring of immunosuppressive therapy. Few single nucleotide polymorphisms have been identified to predict exposure of maintenance immunosuppressive drug, with CYP3A5*1 allele as the only undisputed and widely adopted predictive marker for tacrolimus clearance. Although an increasing amount of transplantation centers currently use this marker for initial dose differentiation, long term benefit has not yet been established. Therapeutic drug monitoring of immunosuppressive whole blood concentrations is still common practice, although more advanced variants such as monitoring intracellular (PBMC) drug concentrations slowly emerge, while pharmacodynamic monitoring is still not possible but promising new biomarkers are emerging. Pharmacometrics is the ideal tool to correlate clinical events to possible predictive factors as shown in chapter six and nine of this thesis. These types of analyses should become more widely adapted to the transplantation field. Combining available data in the renal transplantation research society and searching collaboration with pharmacometricians can assure optimal use of the available research data and will increase the chance of improvement of long term outcome of the renal transplant recipient population.

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10

Summary

Summary

Renal transplantation is currently the best option for patients with end stage renal disease. Despite the low acute rejection rates and successful treatment in the first year after renal transplantation, long term outcome after renal transplantation remains poor. An important factor influencing survival is optimal immunosuppressive treatment. The work presented in thesis aimed at optimizing immunosuppressive therapy in renal transplant recipients and especially therapy consisting of the mTOR inhibitor everolimus by identifying pharmacological and pharmacogenetic factors influencing pharmacokinetics, and pharmcodynamics such as side effects and patient outcome.

The mTOR inhibitors are a relatively new therapeutic group in renal transplantation and have shown their efficacy in recent trials. Their main advantage compared to calcineurin inhibitors cyclosporine and tacrolimus are their relative lack of nephrotoxicity. In **Chapter 2**, a systematic review describes the knowledge of clinical pharmacokinetics and pharmacodynamics of mTOR inhibitors in renal transplantation at the start of this PhD project. The narrow therapeutic window of mTOR inhibitors, together with high variability in pharmacokinetics, makes therapeutic drug monitoring essential for individualizing the dose and thereby preventing toxicity or rejection. For these reasons it is very important to use a reliable and accurate bioanalytical assay. In **Chapter 3** the differences between the mostly used analytical assays of measuring everolimus in whole blood and its effect on dosing advice are investigated. Results showed that the analytical methods Fluorescent Polarization Immuno Assay (FPIA) and Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) are not in agreement and everolimus blood concentration measurement using FPIA results in systematically higher (23% on average) everolimus concentrations compared with LC-MS/MS. Furthermore the use of FPIA can lead to clinically relevant differences in everolimus dosage advice and higher intra-patient variability. Therefore LC-MS/MS outperforms FPIA for clinical monitoring and intervention of everolimus therapy in adult renal transplant recipients on dual therapy with prednisolone. Therapeutic drug monitoring (TDM) of everolimus is performed based on either trough or AUC monitoring and pharmacogenetics might be a valuable addition to TDM in order to reach the target drug concentration as soon as possible by individualizing the initial dose. Especially drugs with a long elimination half-life such as everolimus are at risk of under or overexposure because it takes more time to reach steady state target concentration. Polymorphisms in genes coding for metabolizing enzymes

might therefore be of interest for optimizing immunosuppressive therapy. In **Chapter 4**, a population pharmacokinetic model of everolimus in a calcineurin free regimen was developed and predictive factors for pharmacokinetics such as pharmacogenetics were explored. Everolimus pharmacokinetics was not significantly influenced by genetic polymorphisms in coding genes for the metabolizing enzymes CYP3A5, CYP2C8, PXR or drug transporter ABCB1 (also known as P-glycoprotein) and these polymorphisms are therefore not suitable as a marker for initial dose individualization. Finally a limited sampling model was developed which enables physicians to accurately predict everolimus exposure with limited patient discomfort and burden. Using C_{trough} and C_2 as limited sampling markers resulted in an improved predictive performance compared to C_{trough} monitoring.

Despite its proven efficacy and close TDM, everolimus is also known for some serious side effects with relative high discontinuation rates. In **Chapter 5** potential risk factors such as demographics, underlying disease, transplant related factors, renal function and average everolimus exposure for the most severe side effect of mTOR inhibitors, interstitial pneumonitis, were evaluated in a case control study. No risk factors could be identified. In a more sophisticated time to event analysis (**Chapter 6**), risk factors for discontinuation and the side effects interstitial pneumonitis, infection and new onset diabetes mellitus were explored in a population of renal transplant patients on a regimen of everolimus and prednisolone dual therapy. Risk factors of everolimus discontinuation of renal transplant recipients on a regimen of everolimus and prednisolone dual therapy were constant too high everolimus exposure and high age. The initial dose of 3 mg b.i.d used in this study might be too high given the high initial exposure, the high discontinuation rate and low acute rejection rate. Furthermore, risk factor for the hazardous side effect non-infectious interstitial pneumonitis were a too high everolimus exposure and PXR (NR1|2) (-24113G>A): AA genotype. For infection and new onset diabetes mellitus no significant covariates were detected. Lower initial dosing and prevention of too high everolimus exposure by strict TDM might decrease the high everolimus discontinuation rates and the incidence of interstitial pneumonitis. Pharmacogenetics has only been adopted in clinical practice to a small extent for renal transplant recipients. In **Chapter 7** the most promising polymorphisms (*CYP3A5*3* and *CYP3A4*22*) in renal transplantation were evaluated for influence on the pharmacokinetics on the maintenance immunosuppressive drugs cyclosporine, everolimus and tacrolimus. Results showed that *CYP3A4*22* does not influence cyclosporine, everolimus or tacrolimus pharmacokinetics to a clinically

relevant extent. Furthermore this study confirmed that *CYP3A5*3* is only suitable as a predictive marker for tacrolimus clearance but close TDM remains essential due to the remaining variability between patients with the same genotype. The *CYP3A4* and *CYP3A5* combined genotypes do not further improve the predictive performance compared to the predictive performance of the polymorphisms alone. Based on our study the newly discovered *CYP3A4*22* or *CYP3A* combined genotypes cannot be advised to be used for dose adjustments in clinical practice to further improve immunosuppressive therapy of cyclosporine, tacrolimus or everolimus. In addition **Chapter 8** reports the findings of the effect of peroxide reductase (*POR*) and *CYP3A5* polymorphisms and their combination on everolimus pharmacokinetics. In contrast to what was found for tacrolimus but in accordance with the findings for sirolimus *POR*28* polymorphism or the combination with the *CYP3A5* polymorphism did not have a significant and clinical relevant impact on everolimus pharmacokinetics. Despite low acute rejection rates in the first year after transplantation with current standards for immunosuppressive therapy, long-term outcome after renal transplantation has not improved accordingly. Acute rejection has been previously found to be a risk factor for subclinical rejection. Subclinical rejection (SCR) is by definition histologically defined acute rejection and, as such, has been associated with subsequent interstitial fibrosis and tubular atrophy and with time progressive deterioration of renal function and inferior graft survival. In **Chapter 9** risk factor were identified for delayed graft function, acute rejection and subclinical rejection in patients on a cyclosporine based immunosuppressive regimen. The incidence of acute rejection (AR) and prevalence of SCR with controlled and early reduced systemic cyclosporine exposure within 6 months was found to be 14% and 18%, respectively. Pharmacological factors, including exposure and genetic variability in the genes coding for relevant pharmacokinetic and pharmacodynamics proteins, were not found to be related to the risk for delayed graft function (DGF), AR or SCR. Receiving a kidney from a deceased donor was the dominant risk factor for DGF, with DGF being the primary risk factor for AR. For SCR the most important risk factors were a previous acute rejection episode, and being recipient of a deceased donor kidney. Finally a significant relationship was identified between rejection treatment including ATG and a lower subsequent prevalence of SCR. Finally in **Chapter 10** the results from the performed research are discussed and future perspectives are presented. MTOR inhibitors form a promising new class of immunosuppressive drugs for maintenance immunosuppression in the field of kidney

transplantation and may offer renal and antiviral benefits without increasing the risk of acute rejection. Despite these advantages and TDM, mTOR inhibitors are also known for high discontinuation rates and some serious side effects. Even with all current options of immunosuppression long term outcome for renal transplant recipients is still poor. Immunosuppressive therapy should therefore by further optimized by means of finding the amount of immunosuppression at the right time. Finding new biomarkers for early detection of (subclinical) rejection and toxicity are therefore essential. Pharmacometrics is the ideal science for reaching this goal. Research collaborations of pharmacometricians and nephrologists should be formed to assure optimal use of the available clinical data to eventually improve long term outcome of renal transplant recipients.

Nederlandse samenvatting

(Dutch summary)

Nederlandse samenvatting

Momenteel is niertransplantatie de aangewezen behandeling voor patiënten met eindstadium nierfalen. Ondanks de lage incidentie van acute rejectie en succesvolle rejectie behandeling in het eerste jaar na niertransplantatie laten de lange termijn uitkomsten na niertransplantatie nog te wensen over. Een van de factoren die overleving beïnvloed is optimale immunosuppressieve behandeling. Het onderzoek dat in dit proefschrift gepresenteerd wordt is gericht op het optimaliseren van immunosuppressieve therapie bij niertransplantatiepatiënten en in het bijzonder de immunosuppressieve therapie met de mTOR-remmer everolimus. Dit is gedaan door het identificeren van farmacologische en farmacogenetische factoren die de farmacokinetiek en dynamiek beïnvloeden, zoals bijwerkingen en prognose van de patiënt.

De mTOR-remmers zijn een relatief nieuwe therapeutische groep in het veld van niertransplantatie en hebben in recente studies hun effectiviteit aangetoond. Het belangrijkste voordeel ten opzichte van de calcineurine remmers ciclosporine en tacrolimus is het relatieve gebrek aan nefrotoxiciteit. In **Hoofdstuk 2** wordt in een systematische review de kennis van de klinische farmacokinetiek en dynamiek van de mTOR-remmers bij niertransplantatie aan het begin van dit promotie project beschreven. De smalle therapeutische breedte van de mTOR-remmers, in combinatie met een hoge variabiliteit in farmacokinetiek, maakt therapeutische geneesmiddel monitoring (TDM) van essentieel belang voor het individualiseren van de dosering en daarmee het voorkomen van toxiciteit of rejectie. Om deze reden is het zeer belangrijk om een betrouwbare en nauwkeurige bioanalytische bepalingsmethode te gebruiken. In **Hoofdstuk 3** zijn de verschillen tussen de meest gebruikte analytische bepalingsmethodes voor het meten van everolimus concentratie in volbloed en het effect daarvan op het dosering advies onderzocht. De resultaten toonden aan dat de analysemethoden Fluorescent Polarization Immuno Assay (FPIA) en Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) niet één op één uitwisselbaar waren en dat bepaling van everolimus bloedconcentraties met behulp FPIA resulteert in systematisch hogere (23% gemiddeld) everolimus concentraties in vergelijking met LC-MS/MS. Het gebruik van FPIA als analysemethode kan bovendien leiden tot klinisch relevante verschillen in het everolimus dosering advies en introduceert tevens hogere intra-patiënt variabiliteit. Om deze redenen is LC-MS/MS een geschiktere methode dan de FPIA methode voor de controle van everolimus therapie bij niertransplantatie patiënten.

TDM van everolimus wordt uitgevoerd op basis van ofwel dalspiegels of monitoring van de zogenaamde oppervlakte onder concentratie tijd curve (AUC). Farmacogenetica kan een waardevolle aanvulling zijn op TDM om de gewenste geneesmiddelblootstelling zo snel mogelijk te realiseren door de initiële dosering te individualiseren. Vooral geneesmiddelen met een lange halfwaardetijd zoals everolimus lopen het risico om op te hoge of te lage blootstelling uit te komen omdat het bereiken van de stabiele doelconcentratie meer tijd kost. Polymorfismen in genen die coderen voor metaboliserende enzymen kunnen daarom nuttig zijn voor het optimaliseren van immunosuppressieve therapie. In **Hoofdstuk 4** wordt een populatie farmacokinetisch model van everolimus in een immunosuppressief regime zonder calcineurine-remmer ontwikkeld en worden potentieel voorspellende factoren voor de farmacokinetiek zoals farmacogenetica verkend. De farmacokinetiek van everolimus werd niet significant beïnvloed door genetische polymorfismen in coderende genen voor de enzymen CYP3A5, CYP2C8, PXR of de geneesmiddel transporter ABCB1 (ook bekend als P-glycoproteïne). Deze polymorfismen kunnen daarom niet worden gebruikt voor de individualisering van de initiële everolimus dosis. Ten slotte is een zogenaamd limited sampling model ontwikkeld dat artsen in staat stelt om de everolimus blootstelling met zo weinig mogelijk ongemak voor de patiënt toch nauwkeurig te voorspellen. Door de dalspiegel en een spiegel 2 uur na de gift te gebruiken in het ontwikkelde limited sampling model wordt een klinische relevante verbetering van de voorspellende prestaties ten opzichte van de dalspiegel monitoring behaald. Ondanks de bewezen werkzaamheid en nauwkeurige monitoring van de therapie is everolimus ook geassocieerd met een aantal ernstige bijwerkingen en een relatief hoog aantal patiënten dat stopt met de therapie. In **Hoofdstuk 5** worden potentiële risicofactoren zoals demografische factoren, onderliggende ziekte, transplantatie gerelateerde factoren, nierfunctie en gemiddelde everolimus blootstelling voor de meest ernstige bijwerking van mTOR-remmers, niet-infectieuze interstitiële pneumonitis, geëvalueerd in een case-control studie. Er werden geen risicofactoren geïdentificeerd in deze analyse. In een meer geavanceerde time-to-event analyse (**Hoofdstuk 6**), werden risicofactoren voor het staken van de therapie en de bijwerkingen; niet-infectieuze interstitiële pneumonie, infectie en diabetes mellitus onderzocht in een groep niertransplantatiepatiënten die werden behandeld met een immunosuppressief regime van everolimus en prednisolon. Risicofactoren voor het moeten staken van de everolimus therapie waren een te hoge everolimus blootstelling en hoge leeftijd. De initiële dosis van 3 mg tweemaal daags is vermoedelijk te hoog gezien de hoge initiële blootstelling, de hoge frequentie van het

staken van de therapie en lage incidentie van acute rejectie. Als risicofactoren voor de gevaarlijke bijwerking niet-infectieuze interstitiële pneumonitis werd te hoge everolimus blootstelling en het PXR (NR1 | 2) (-24113G> A): AA genotype gevonden. Voor het optreden van een infectie en het ontstaan van Diabetes Mellitus werden geen significante risicofactoren gevonden. Een lagere initiële dosering en het voorkomen van een te hoge everolimus blootstelling door strikte TDM zal de frequentie van het staken van de therapie en de incidentie van interstitiële pneumonitis waarschijnlijk verlagen. Farmacogenetica wordt momenteel slechts in beperkte mate toegepast in de klinische praktijk bij niertransplantatiepatiënten. In **Hoofdstuk 7** zijn de meest veelbelovende polymorfismen (*CYP3A5*3* en *CYP3A4*22*) die gebruikt zouden kunnen worden bij niertransplantatie onderzocht op hun invloed op de farmacokinetiek ciclosporine, everolimus en tacrolimus. De resultaten toonden aan dat *CYP3A4*22* geen klinisch relevante invloed op heeft op de farmacokinetiek van ciclosporine, everolimus of tacrolimus. Verder bevestigde deze studie dat *CYP3A5*3* alleen voor tacrolimus geschikt is als voorspellende marker voor blootstelling. Intensieve TDM blijft echter essentieel vanwege de relatief grote variabiliteit tussen patiënten met hetzelfde genotype. Het *CYP3A4* en *CYP3A5* gecombineerd genotype leidt niet tot een relevante verbetering van het voorspellend vermogen in vergelijking met het voorspellend vermogen van de individuele polymorfismen. Het nieuw ontdekte *CYP3A4*22* of *CYP3A* gecombineerd genotype zijn daarom niet geschikt om te worden gebruikt voor de aanpassing van de initiële dosering van ciclosporine, tacrolimus of everolimus. Daarnaast worden in **Hoofdstuk 8** de bevindingen van de analyse naar het effect van peroxide reductase (*POR*) en *CYP3A5*-polymorfismen en hun combinatie op de farmacokinetiek van everolimus gerapporteerd. In tegenstelling tot wat werd gevonden voor tacrolimus, maar in overeenstemming met de bevindingen voor sirolimus blijkt het P*OR*28* polymorfisme of de combinatie met het *CYP3A5* polymorfisme niet een significante en klinisch relevante invloed op de farmacokinetiek van everolimus hebben. Ondanks de lage incidentie van acute rejectie in het eerste jaar na transplantatie die zijn behaald met de huidige immunosuppressieve therapie, is de lange-termijn uitkomst niet op vergelijkbare wijze verbeterd. Acute rejectie is al eerder geïdentificeerd als een risicofactor voor subklinische rejectie (SCR). SCR is histologisch gedefinieerde acute rejectie en is als zodanig geassocieerd met opeenvolgende interstitiële fibrose en tubulaire atrofie. Dit leidt vervolgens tot progressieve verslechtering van de nierfunctie en een slechtere transplantaatoverleving. In **Hoofdstuk 9** zijn risicofactoren geïdentificeerd voor

het vertraagd op gang komen van het transplantaat (delayed graft function (DGF)), acute rejectie (AR) en SCR bij patiënten die werden behandeld met een immunosuppressief regime met als basis ciclosporine. De incidentie van AR en SCR binnen 6 maanden onder gecontroleerde en vroeg verminderde systemische ciclosporine blootstelling bleek 14% en 18%, respectievelijk te zijn. Farmacologische factoren zoals ciclosporine blootstelling en genetische variabiliteit in de genen die coderen voor relevante farmacokinetische en farmacodynamische eiwitten bleken niet gerelateerd te zijn aan het risico op DGF, AR of SCR. Het ontvangen van een nier van een overleden donor was de dominante risicofactor voor DGF. DGF was vervolgens de primaire risicofactor voor het optreden van AR. Voor SCR bleken de belangrijkste risicofactoren een eerdere doorgemaakt acute rejectie-episode en het ontvangen van donor nier van een overleden donor te zijn. Tenslotte werd een significante relatie gevonden tussen de rejectiebehandeling met Anti-Thymocyten Globuline (ATG) en een daaropvolgende lagere prevalentie van SCR. Tot slot worden (**Hoofdstuk 10**) de resultaten van al het uitgevoerde onderzoek bediscussieerd en vervolgens toekomstperspectieven voor het optimaliseren van immunosuppressieve therapie geschetst. mTOR-remmers vormen een veelbelovende nieuwe groep van immunosuppressieve geneesmiddelen voor onderhoud immunosuppressie op het gebied van niertransplantatie. Ze bieden waarschijnlijk antivirale en nierfunctie sparende voordelen, zonder een verhoging van het risico op acute rejectie. Ondanks deze voordelen en intensieve monitoring van de therapie, zijn mTOR-remmers ook bekend om het hoge aantal patiënten wat deze middelen slecht verdraagt en een aantal ernstige bijwerkingen. Zelfs met alle huidige opties van immunosuppressie is de lange termijn uitkomst bij niertransplantatiepatiënten is nog steeds matig. Immunosuppressiva dient derhalve verder geoptimaliseerd te worden door middel van het vinden van de juiste hoeveelheid immuunsuppressie op het juiste moment. Het vinden van nieuwe biomarkers waarmee (subklinische) rejectie en toxiciteit vroeg gedetecteerd kan worden zijn daarvoor essentieel. Farmacometrie is de ideale wetenschap voor het bereiken van dit doel. Samenwerkingsverbanden van specialisten in de farmacometrie en nefrologen moeten worden gevormd om een optimaal gebruik van de beschikbare klinische onderzoeksgegevens om uiteindelijk de lange termijn uitkomsten van niertransplantatiepatiënten te kunnen verbeteren.

Curriculum Vitae

Curriculum Vitae

Dirk Jan Alie Roelof Moes was born in Nijeveen, The Netherlands on October 12th 1981. He finished secondary school (VWO) in 2000 at C.S.G. Dingstede in Meppel. Subsequently he started his study Pharmacy at the University of Groningen. He performed his Master research project at the department of Molecular Pharmacology of the Faculty of Mathematics and Natural Sciences. The research was aimed at evaluating the effects of extracellular matrix proteins on smooth muscle contractility in allergic asthma (supervisors Prof. dr. H. Meurs and dr. B.G. Dekkers). In 2008, Dirk Jan finished his Master's degree in Pharmacy and started working as a GMP project pharmacist at the department of Clinical Pharmacology and Pharmacy of the VU University Medical Center in Amsterdam (supervisors dr. E.L. Swart, dr. E. Bakker and dr. M.W.J. den Brok). In 2009 he made a switch to the department of Clinical Pharmacy and Toxicology of the Leiden University Medical Center for a PhD project on the optimization of immunosuppressive therapy in renal transplant recipients. This PhD project was a collaboration between the department of Clinical Pharmacy and Toxicology (supervisor Prof. dr. H-J. Guchelaar) and the department of Nephrology (supervisor Prof. dr. J.W. de Fijter) of the Leiden University Medical Center. In January 2011 he also started his hospital pharmacy residency and since then he combined these two tasks. As part of the PhD project he visited the Uppsala University Pharmacometrics Group, Uppsala, Sweden, for a period of three months (supervisors prof. dr. M.O. Karlsson and dr. S. Jönsson). After completion of his PhD project and hospital pharmacy residency he will continue his career as a clinical researcher / hospital pharmacist in the field of pharmacometrics in oncology and renal transplantation at the Leiden University Medical Center. Dirk Jan Moes lives with his wife Judith and three children Lena, Jelle and Matthijs in Nieuw-Vennep, the Netherlands.

List of publications

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Baas MC, Struijk GH, Moes DJAR, van den Berk I a H, Jonkers RE, de Fijter JW, et al. Interstitial pneumonitis caused by everolimus: a case-cohort study in renal transplant recipients. Transpl Int. 2014 May;27(5):428–36.

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Dankwoord

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Dankwoord

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