INDIVIDUALIZATION OF IMMUNOSUPPRESSIVE THERAPY AFTER SOLID ORGAN TRANSPLANTATION

Individualisering van immunosuppressieve therapie
na solide orgaantransplantatie

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De Profeet (vrede en zegeningen zij met hem) zei:

"Wanneer de mens sterft, houden zijn daden op, behalve drie zaken:
een doorlopende liefdadigheid, kennis waar anderen profijt van hebben
of een oprechte zoon of dochter die smeekbeden voor hem verricht."

Overgeleverd door Muslim

Voor Abderrahman, mijn vader
Voor Abdelbasit, mijn man
Voor Obaida, mijn zoon

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Contents

Chapter 1

General introduction

Based on: Tacrolimus: Pharmacokinetics and pharmacogenetics; Optimized use of immunosuppressive drugs in transplantation;

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HISTORICAL OVERVIEW

When an individual's kidneys fail, there are three treatment options: hemodialysis, peritoneal dialysis or kidney transplantation. A successful kidney transplantation results in the best patient survival and a better quality of life compared to the two other treatment modalities.[1] Kidney transplantation is therefore the preferred therapy for renal failure. The first deceased donor kidney transplantation in the United States was performed in 1950 by Lawler and colleagues on Ruth Tucker, a 44-year-old woman with polycystic kidney disease.[2] Although the kidney transplant was rejected ten months later because no immunosuppressive therapy was available at the time, the intervening time allowed Tucker's remaining native kidney (it was an orthotopic transplantation) to recover and she lived for another five years.[2] At the same time in France, Küss, Hamburger, and others also performed a number of kidney transplantations.[3-6]

In 1954 the first successful kidney transplantation with a living kidney donor was performed between identical twins in Boston at the Peter Bent Brigham Hospital.[7,8] A kidney was transplanted from Ronald Herrick to his brother Richard Herrick. The transplantation kept Richard alive for eight more years. Joseph Murray who performed the procedure and is famous for his quote: *"If you're going to worry about what people say, you're never going to make any progress",* received the Nobel Prize for Medicine in 1990 for "his discoveries concerning organ transplantation in the treatment of human disease". Because Ronald and Richard were identical twins no immunologic problems occurred (the failure of the graft was caused by a recurrence of his primary kidney disease).

However, it was not until the development of immunosuppressive (drug) therapy, that kidney transplantation between non-genetically identical individuals became possible.[9] In the absence of such treatment, the recipient's immune system will treat the transplanted organ as 'strange' and reject it. Pharmacologic suppression of the immune system is for this reason crucial.

Currently, immunosuppressive therapy for the prevention of acute rejection after kidney transplantation mostly consists of the combination of a calcineurin inhibitor (CNI), either ciclosporine or tacrolimus, plus mycophenolic acid (MPA), and glucocorticoids with or without induction therapy with an interleukin (IL)-2 receptor blocker or a T-lymphocyte depleting agent. With immunosuppressive drug combination therapy, patient and kidney allograft survival have greatly improved.[10,11] However, prolonged use of immunosuppressive drugs leads to considerable toxicity, including an increased rate of infections and malignancies, as well as drug-specific toxicity.

Calcineurin inhibitors:

Pharmacokinetics, pharmacodynamics and pharmacogenetics

In the last three decades the incidence of acute rejection among kidney transplant recipients has been reduced dramatically as a result of the introduction of a number of

immunosuppressive drugs. Nowadays, renal allografts are infrequently lost as a result of uncontrollable acute rejection. Perhaps more than any other class of immunosuppressive drugs, the CNIs cyclosporine and tacrolimus have contributed to the decreased incidence of acute rejection. Cyclosporine revolutionized the transplant field following its introduction in the late 1970's. Fifteen years later tacrolimus entered the transplantation arena and is nowadays the preferred CNI. With tacrolimus-based immunosuppressive therapy, acute rejection rates during the first post-transplant year have now fallen below 20% and one-year graft survival has risen to above 90%. However, despite the remarkable improvement of the short-term transplantation results, the introduction of modern immunosuppressive drug therapy has not resulted in a comparable improvement of the long-term transplantation outcomes.[12]

There are numerous reasons for the disappointing long-term kidney transplant survival. The majority of kidney transplants is lost by death of the recipient with a functioning kidney allograft. In addition, many kidney transplants are lost because of recurrence of the primary kidney disease. A third important cause of chronic kidney transplant loss, is the poorly understood entity named "chronic allograft nephropathy". For many years this was considered to be caused by the chronic use of CNIs, that are notorious for their acute nephrotoxic effects.[13-15] However, the presumed chronic nephrotoxicity of CNIs is currently subject of much debate and several investigators believe that it is not an important cause for chronic kidney transplant loss and is in fact a misdiagnosis of immunologically-mediated damage to the transplant.[16, 17]

The search for new immunosuppressive compounds that are less toxic but equally effective as the CNIs has been disappointing. Over the last few years, the development of a number of promising immunosuppressive drugs was aborted because of unexpected toxicity, lack of efficacy or both [*e.g.* fingolimod (FTY720), sotrastaurin (AEB071), and tofacitinib (CP69,550)]. It is therefore to be expected that the current gold standard immunosuppressive drug regimen of a CNI plus MPA will be here to stay for the next few years.

Pharmacokinetics of tacrolimus

Tacrolimus is a macrolide antibiotic with a molecular weight of 806. Tacrolimus is produced by Streptomyces Tsukubaenis and is highly lipophilic and therefore poorly soluble in water.[18] The main mechanism of action of tacrolimus is the inhibition of T-lymphocyte signal transduction and interleukin (IL)-2 transcription by inhibiting the enzyme calcineurin. After entering the T-cell, tacrolimus binds to FK-binding protein (FKBP)-12, after which the tacrolimus-FKBP-12 complex binds to and inhibits calcineurin. By inhibiting calcineurin, tacrolimus prevents the dephosphorylation and activation of the nuclear factor of activated T-cells (NFAT). This eventually leads to the inhibition of the transcription of several genes important for T-cell activation and proliferation such as IL-2, IL-4 and gamma interferon.

The pharmacokinetics of tacrolimus are characterized by a highly variable, unpredictable, and in general, poor oral bioavailability. The oral bioavailability averages around 30%, but can be as low as 5% or as high as 90%. Tacrolimus distributes extensively into tissues and the cellular fraction of blood, where it is mainly distributed in erythrocytes and leucocytes. More than 90% of tacrolimus present in plasma is protein-bound. Besides a low oral bioavailability, the pharmacokinetics of tacrolimus are characterized by marked interindividual differences in first-pass metabolism and systemic clearance. The elimination half-life after oral intake of tacrolimus is between 8 and 12 hours. Following metabolism, tacrolimus is mainly excreted into bile (>90%).[19] The intestinal and hepatic metabolism of tacrolimus is mediated by the cytochrome P450 (CYP) isoenzymes 3A4 and 3A5. In addition, tacrolimus is a substrate of the drug transporter ATP-binding cassette (ABC) protein B1 (ABCB1, formerly known as permeability glycoprotein or P-gp).[20]

Pharmacokinetics of ciclosporine

Cyclosporine is an 11-amino acid cyclic peptide produced by the fungus Tolypocladium inflatum Gams.[21] Like tacrolimus, ciclosporine is a calcineurin inhibitor. Tacrolimus is however 10 to 100 time more potent at the molecular level.[22] Ciclosporine also has a different receptor than tacrolimus and binds to the intracellular protein cyclophilin-A with subsequent inhibition of the activation of calcineurin, again preventing IL-2 production by T-lymphocytes.[23] Ciclosporine has a high variability in its pharmacokinetics. The oral bioavailability of ciclosporine is 25-30%.[24] After oral administration the peak concentration in blood (Cmax) occurs at around two hours with a highly variable half-life of between 5 to 10 hours.[25] Ciclosporine in blood is distributed between erythrocytes and leukocytes with only about 4% in the plasma of which 70% is protein-bound.[26] Ciclosporine is metabolized by CYP3A4 and CYP3A5 expressed in the intestine and liver. It is also a substrate for ABCB1 expressed on the lymphocytes.

Therapeutic Drug Monitoring

Besides marked interindividual differences in its pharmacokinetics, the clinical use of calcineurin inhibitors is further complicated by their narrow therapeutic window. Calcineurin inhibitors are considered to be critical dose drugs and in most transplant centers the dose of these drugs is adjusted according to whole blood concentrations, a practice known as therapeutic drug monitoring (TDM). The target whole blood concentrations aimed for depend on various factors including the perceived immunological risk, type of organ transplanted, and co-medication used. Different analytical methods can be used to determine tacrolimus or ciclosporine whole blood concentrations. The most commonly used quantitative procedure for TDM for calcineurin inhibitors is that of the micro particle enzyme-linked immunoassay (MEIA). However, liquid chromatography with mass spectrometric detection (LC-MS) has been implemented as an alternative technology for monitoring.[27-29]

Pharmacogenetics of Calcineurin inhibitors

The clinical use of calcineurin inhibitors is complicated further by a high between-patient variability in their pharmacokinetics. In recent years it has become clear that much of the interindividual differences in the pharmacokinetics of CNIs results from variability in the activity of the metabolizing enzymes cytochrome P450 (CYP) 3A4 and CYP3A5.[30] In addition, several studies have established the importance of the drug transporter ABCB1 in the disposition of CNIs. The encoding genes of *CYP3A4*, *CYP3A5*, and *ABCB1* contain numerous single-nucleotide polymorphisms (SNPs) and these polymorphisms have been the subject of a considerable number of studies as they may explain the differences in calcineurin pharmacokinetics between patients.

CYP3A

The CYP enzyme family consists of more than 50 isoenzymes that are responsible for the oxidative metabolism of many endogenous and exogenous compounds.[31] The CYP3A subfamily (chromosome 7q21-q22.1), which represents the majority of CYP proteins in human liver, metabolizes more than 50% of all drugs currently in use (including tacrolimus and ciclosporine). The *CYP3A* locus consists of four genes and two pseudogenes: *CYP3A4, CYP3A5, CYP3A7, CYP3A43, CYP3AP1*, and *CYP3AP2*. A large number of SNPs has been identified in most of these genes (see http://www.imm.ki.se/CYPalleles).CYP3A4 and CYP3A5 have largely overlapping substrate specificities and, based on the amount of protein, are considered the most important CYP3A family members. CYP3A4 is constitutively expressed in liver, jejunum, colon, kidney and pancreas, but marked interindividual differences in its activity exist, which may vary by up to 40-fold.[32] For stable kidney transplant recipients, a ten-fold variation in enterocyte CYP3A4 content was reported.[33] CYP3A5 is also present in the liver, kidney and small intestine, although its expression is even more variable, and in general much lower, compared with CYP3A4. In Caucasian livers, the CYP3A5 protein was only detectable in 10-40% of all samples.[34] However, CYP3A5 may account for up to 50% of total hepatic CYP3A content in some individuals. The functional significance of the other two CYP3A family members, CYP3A7 and CYP3A43 is thought to be limited.[35]

In the last decade SNPs have been identified in the *CYP3A4* and *CYP3A5* genes. The *CYP3A4*1B* polymorphism located in the promoter region of the *CYP3A4* gene, has been associated with an increased expression of CYP3A4 protein.[36] However, the clinical relevance of this polymorphism is not yet clarified.[37,38] Besides the *CYP3A4*1B* polymorphism, the newly discovered *CYP3A4*22* in intron 6 of *CYP3A4* SNP may explain part of the variability in CNI pharmacokinetics.[39] The CYP3A4*22T-variant (C>T) allele was associated with decreased hepatic *CYP3A4* mRNA expression and with decreased CYP3A4 enzymatic activity. The *CYP3A4*22* allele is found with a relatively high allelic frequency of 3-4% in the Caucasian population.

At present however, the best-studied SNP is the *CYP3A5*3* SNP in intron 3 of the *CYP3A5* gene, genomic 6986A>G, which shows 100% linkage with the absence of CYP3A5 protein. The *CYP3A5*3* polymorphism has been shown to cause alternative splicing and

protein truncation, resulting in the absence of functional CYP3A5 protein.[30] At least one *CYP3A5*1* (or wild-type) allele is required for expression of CYP3A5 [30,34,40] and therefore mostly two groups are defined: *CYP3A5* expressers (*CYP3A5*1/*1* plus **1/*3*) and *CYP3A5* non-expressers (*CYP3A5*3/*3*). The frequencies of *CYP3A5*1* wild-type allele are: 5-16% in Caucasians, 15% in Japanese, 35% in Chinese, 51% in Asians and 60-85% in African-Americans.[30] In recent years, a variety of studies have clearly demonstrated that renal transplant recipients carrying the *CYP3A5*3* allele and who are therefore expected to lack functional CYP3A5 protein, require a significantly lower tacrolimus dose to reach target concentrations compared with patients homozygous for the wild-type allele (*CYP3A5* expressers).[37,41-43] A higher tacrolimus dose requirement has also been observed for *CYP3A5* expressers receiving a heart-, or lung transplant.[44] Because patients expressing *CYP3A5* need higher tacrolimus doses to reach target concentrations, they may be at an increased risk of under-immunosuppression, especially in the early phase after transplantation, and the subsequent development of acute rejection. Several authors have therefore suggested that the tacrolimus starting dose in these patients should be twice as high as that of *CYP3A5* non-expressers.[45]

ABCB1

ABCB1 is encoded by the *ABCB1* gene located on chromosome 7q21.1 and belongs to the family of the ABC membrane transporters (subfamily B). ABCB1 is an ATP-dependent transporter capable of pumping many endogenous substances, as well as a wide variety of drugs (including tacrolimus and ciclosporine), from the cytoplasm or cell membrane to the extracellular space.[31,46,47] Physiologically, ABCB1 is expressed in the kidney (brush border of proximal tubular cells), the liver (at the canalicular surface of hepatocytes), pancreas, and at the apical surface of mature enterocytes in the small intestine and colon.[48] The specific tissue expression of ABCB1 suggests that the protein functions as a protective barrier, by actively extruding xenobiotics and metabolites from the cell interior into bile, urine or gut lumen. ABCB1 is also expressed on various leukocytes, including T and B-lymphocytes and dendritic cells.[49,50] More than 25 SNPs have been discovered in *ABCB1*. The best-studied SNP is the *3435C>T* transition located in exon 26. This is a silent SNP, meaning that it does not lead to an amino acid change. However, it was recently demonstrated that this variant allele leads to an ABCB1 protein with an altered conformation and function that may have arisen as a result of an altered timing of translational folding.[48] Furthermore, Wang and colleagues demonstrated that the *3435C>T* variant affects *ABCB1* mRNA stability, although this finding has been questioned by others.[48,51] The *ABCB1 3435C>T* SNP is in strong linkage disequilibrium with the *1236C>T* (in exon 12) and the *2677G>T/A* (in exon 21; Ala893Ser/Thr) SNPs.[52] The effect of SNPs in *ABCB1* on tacrolimus dose-requirement, appears to be smaller when compared with the effects of genetic variation in *CYP3A5*.[44,53-56]

Perhaps even more important than its relation to pharmacokinetics is the effect of genetic variation on transplantation outcome. Identification of functional SNPs in genes encoding for drug metabolizing enzymes has great potential to influence the drug efficacy

and safety profile. *CYP3A5* expressers might have underexposure of tacrolimus, which can result in more early acute rejection.

In chapter 2 we report our studies on the relationship of single-nucleotide polymorphisms in CYP3A and ABCB1 and the pharmacokinetics and pharmacodynamics of calcineurin inhibitors.

Next to the interpatient variability, also the intrapatient variability (the variability in one patient) is a problem with the use of calcineurin inhibitors. A high intrapatient variability might put patients at risk for periods of over- or underimmunosuppression and might therefore lead to graft loss after transplantation. Only few studies have investigated this hypothesis. A study of Kahan *et al.*[21] studied this for ciclosporine and found that the incidence of chronic rejection was higher (40%) in patients with a high intrapatient variability compared to patient with a low intrapatient variability (rejection rate 24%). Borra *et al.*[57] studied this for tacrolimus and mycophenolic acid. They have found that a high intrapatient variability in the clearance of tacrolimus was a significant risk factor for treatment failure, a composite end point of graft loss, biopsy-proven chronic allograft nephropathy or a doubling of the plasma creatinine concentration. The impact of intra-patient variability in tacrolimus clearance in heart transplant adult recipients has not been studied.

In chapter 3 paragraph 3.2 we report our studies on the correlation between progression of graft vascular disease and intrapatient variability of tacrolimus in heart transplant patients.

As the pharmacogenetics plays a role in the pharmacokinetics of tacrolimus, the intrapatient variability might also be influenced by pharmacogenetics. Yong Chung have concluded that the *CYP3A5* genotype in tacrolimus treated patients is correlated to the intrapatient variability.[58] This observation might be explained by the fact that in patients without functional CYP3A5 enzyme the metabolism of tacrolimus depends exclusively on CYP3A4 and is therefore more sensitive to induction and inhibition.

In chapter 3 paragraph 3.1 we report our studies on the impact of CYP3A5 genotype and the intrapatient variability of tacrolimus in kidney transplant recipients.

A maximum efficacy with a minimum toxicity is the ultimate goal of immunosuppressive therapy in kidney transplantation. Although pharmacogenetics might influence the blood concentrations of calcineurin inhibitors the first questions that should be asked, is what blood concentrations should we achieve? It is surprising that the concentration-effect relationship for tacrolimus has been poorly defined. As mentioned previously TDM for tacrolimus is universally applied. To justify the use of TDM a concentration-effect relationship between tacrolimus and its effect (the prevention of acute rejection) should be present. However, a reliable association between tacrolimus exposure and pharmacodynamic effects is still missing.

In chapter 4 we report our studies on whether the currently used and empirically defined tacrolimus target concentrations are related to the incidence of acute rejection.

Mycophenolic Acid: PHARMACOKINETICS, PHARMACODYNAMICS AND PHARMACOGENETICS

Mycophenolic acid (MPA) was subsequently isolated from Penicillium stoloniferum. MPA is a selective, noncompetitive and reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH).[59] This enzyme catalyse the rate-limiting step in the *de novo* guanosine synthesis. In contrast to other cells, lymphocytes cannot rely on the salvage pathway as an alternative supply of guanosine nucleotides. MPA therefore selectively leads to inhibition of T- and B-lymphocyte proliferation.[60]

Mycophenolic acid (MPA) has complex pharmacokinetics.[61] After administration mycophenolate mofetil, which is a pro-drug, is extensively hydrolyzed to active MPA.[62] MPA binds for more than 95% to albumin.[59] Uridine diphosphat glucuronosyltransferases (UGTs) metabolise MPA via glucuronidation.[63] 7-O-mycophenolic acid glucuronide (MPAG) is the main metabolite and is not immunologically active.[64] Two other metabolites of MPA are formed, 7-O-glucoside and the pharmacologically active acyl glucuronide (AcMPAG). By enterohepatic circulation of MPAG a secondary plasma peak of MPA is formed.[65] MPAG is excreted into the bile via the multidrug resistance-associated protein (MRP2, encoded by *ABCC2*), which is expressed in the canalicular membrane of hepatocytes.[66] Other data suggest that organic anion transporting proteins (OATPs), encoded by solute carrier organic anion (SLCO1) genes, localized in the sinusoidal membrane of hepatocytes are involved in MPA and/or MPAG uptake in hepatocytes and therefore might affect MPA pharmacokinetics.[67] Over 95% of orally administered MPA is removed from the body by the kidneys as MPAG.

Mycophenolic acid pharmacogenetics and side effects

The interindividual differences in MPA pharmacokinetics can be caused by differences in the genetic composition of drug-metabolizing enzymes and drug transporters. Over the years numerous SNPs of the genes encoding for UGT isoenzymes, MRP2 and IMPDH have been reported. The use of MPA in clinical practice is complicated by the frequent occurrence of gastrointestinal adverse effects, such as nausea, abdominal cramps, and especially diarrhea. Diarrhea has been shown to reduce the quality of life of transplanted patients and is an important reason for noncompliance or discontinuation of MPA therapy.[68] The pathogenesis of MPA-related diarrhea in renal transplant recipients is unknown. A direct toxic effect of MPA or MPAG on enterocytes may be an explanation. Differences in the expression or function of drug-transporting enzymes present in the apical membrane of the intestine could lead to high local concentrations of MPA or MPAG at the level of the intestinal epithelium and thus predispose certain patients to develop gastrointestinal side effects. Sawamoto *et. al.* [69] demonstrated that MPA is a substrate for ABCB1. More

recently, these *in-vitro* findings were corroborated by an experimental *in-vivo* study in mice. Studying ABCB1 knockout mice, Wang *et al.* [70] observed that the MPA levels in plasma and tissue of these mice were markedly increased compared with wild-type mice, again suggesting that MPA is an ABCB1 substrate. Among the OATP transporters, OATP1B1 (SLCO1B1) and OATP1B3 (SLCO1B3) are the major OATPs expressed on hepatocytes. In addition, OATPs are also expressed in the kidney and intestine.[71] The OATPs are believed to be involved in the uptake of MPAG from the blood into hepatocytes .[72] Polymorphisms leading to altered OATP activity may therefore affect MPA pharmacokinetics.

In chapter 5 paragraph 5.1 we report our studies on whether genetic polymorphisms in ABCB1 and SLCO are related to MPA pharmacokinetics and the occurrence of MPA-related diarrhea after kidney transplantation. In chapter 5 paragraph 5.2 we report our studies on whether other SNPs were related to the side effects anemia and leucopenia of MPA.

The overall aim of this thesis was to explore ways of individualization of immunosuppressive therapy in transplant recipients.

The Aim of the thesis was to investigate:

1. Do the SNPs in *CYP3A* and *ABCB1* influence ciclosporine pharmacokinetics and pharmacodynamics? (Chapter 2.1)
2. Does the *CYP3A4*22 C>T* SNP influences tacrolimus pharmacokinetics? (Chapter 2.2)
3. Does the *CYP3A4*22 C>T* SNP affects the efficacy and toxicity of ciclosporine? (Chapter 2.3)
4. Do SNPs in *CYP3A* and *ABCB1* influence the cyclosporine-mediated nephrotoxicity? (Chapter 2.4)
5. Does the *ABCB1 3435C>T* variant allele cause increased intra-lymphocytic tacrolimus accumulation? (Chapter 2.5)
6. Do CYP3A5 expressers have a higher within-patient variability in the apparent oral clearance of Tac? (Chapter 3.1)
7. Is a high within-patient variability in the apparent oral clearance of Tac a risk factor for the subsequent development of transplant coronary artery disease? (Chapter 3.2)
8. What is the concentration-effect relationship between the currently accepted tacrolimus predose target concentrations and early acute rejection after kidney transplantation? (Chapter 4.1)
9. Do SNPs in *SLCO1B* and *ABCB1* influence the occurrence of MPA-related diarrhea? (Chapter 5.1)
10. Do SNPs in *IL12A, CYP2C8 and HUS1* affect the occurrence of MPA-related anemia and leucopenia? (Chapter 5.2)

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Chapter 2

Chapter 2.1

Polymorphisms in CYP3A5, CYP3A4 and ABCB1 are not associated with cyclosporine pharmacokinetics nor with cyclosporine clinical end points after renal transplantation

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ABSTRACT

The association of *CYP3A5, CYP3A4* and *ABCB1* SNPs with ciclosporin (CsA) pharmacokinetics is controversial. The authors studied the influence of these SNPs on CsA pharmacokinetics, as well as on the incidence of biopsy-proven acute rejection (BPAR), and renal function after kidney transplantation. One-hundred-seventy-one patients participating in an international, randomized-controlled trial were genotyped for *CYP3A5*3, CYP3A4*1B* and the *ABCB1* 1236 C>T, 2677 G>T/A, and 3435 C>T SNPs. The patients were treated with CsA, mycophenolate mofetil and glucocorticoids. CsA was dosed to reach pre-dose concentrations (C_0) or two hours post-dose concentrations (C_2). Pharmacokinetic parameters were measured on days 3 and 10, months 1, 3, 6 and 12 after transplantation. Renal function was assessed by measuring serum creatinine and calculating the creatinine clearance. The incidence of BPAR and delayed-graft function (DGF) was recorded. *CYP3A5*, *CYP3A4* and *ABCB1* genotype were not associated with dose-adjusted CsA C_0 or C_2. The incidence of BPAR in this cohort was 16% and was comparable between the different *ABCB1* genotype groups. No significant difference in the incidence of BPAR was found between *CYP3A5* expressers (10%) and non-expressers (18%), $p = 0.24$. Nor was there a difference in the incidence of BPAR between *CYP3A4*1* homozygotes (5%) *vs. CYP3A4*1B* carriers (18%), $p = 0.13$. There were no differences with regard to creatinine clearance between the different *CYP3A* and *ABCB1* genotype groups. According to the results, determination of *CYP3A* and *ABCB1* SNPs pre-transplantation is not helpful in determining the CsA starting dose and does not aid in predicting the risk of BPAR or worse renal function in an individual patient.

INTRODUCTION

The calcineurin inhibitor (CNI) ciclosporin (CsA) is an immunosuppressive agent used for the prevention of acute rejection after solid organ transplantation [1]. CsA blood concentrations have been shown to correlate with its efficacy (*i.e.* the absence of acute rejection) and toxicity [2]. However, a large interindividual pharmacokinetic variability and a narrow therapeutic window complicate the use of CsA. Therefore, therapeutic drug monitoring (TDM) is routinely performed for this agent after transplantation.

Ciclosporin is a substrate of the efflux transporter adenosine triphosphate (ATP) binding cassette (ABC) protein (ABCB1), which is encoded by the *ABCB1* gene. ABCB1 is a transmembrane transporter which is capable of pumping a wide variety of substances, including CsA, from the cytoplasm to the exterior of the cell [3]. Ciclosporin is metabolized by the cytochrome P450 (CYP) isoenzymes CYP3A4 and CYP3A5. Both ABCB1 and the CYP3A isoenzymes are expressed in the human intestine where they act synergistically as a barrier to limit the bioavailability of CsA. ABCB1 limits its absorption by active extrusion from the enterocyte interior back into the gut lumen, whereas exposure to CsA is reduced further by CYP3A-mediated metabolism in the enterocyte [3]. Hepatic CYP3A and biliary ABCB1 are responsible for the systemic clearance and elimination of CsA, respectively.

In the last decade several single-nucleotide polymorphisms (SNPs) have been identified in the *CYP3A5, CYP3A4*, and *ABCB1* genes. Several investigators have studied the association between these SNPs and the pharmacokinetics of CsA [reviewed in reference [9]]. Although several studies have demonstrated that SNPs in *ABCB1* influence the PK of drugs such as digoxin, simvasatin and atorvastatin, the effect of these SNPs on CsA disposition is less clearly defined [10-16]. For example, we reported a significant, albeit small, effect of the *CYP3A4*1B* SNP on CsA oral clearance [17], whereas other investigators did not observe an association. Similar conflicting results have been reported with regard to *CYP3A5*3* and various *ABCB1* SNPs [9, 14, 18-24]. Importantly, only limited data exist on the association of genetic variation in *CYP3A* and *ABCB1* and the efficacy and toxicity of CsA treatment in transplanted patients. Several studies did not observe an association between SNPs in *CYP3A* or *ABCB1* and the incidence of acute rejection after kidney transplantation [14, 19, 25]. Interestingly, in two recent studies an association between *ABCB1* genotype and the risk of developing clinically-defined CsA-associated nephrotoxicity, the most dreaded side-effect of CsA, was reported [1]

An important limitation of the majority of published genetic association studies in transplantation is that they were of a retrospective design, included limited numbers of patients, and that transplant recipients were not always uniformly treated. The aim of the present study was to investigate prospectively the association between *CYP3A4*, *CYP3A5* and *ABCB1* SNPs and CsA pharmacokinetics, as well as the relation of these genetic polymorphisms to the efficacy of CsA therapy and graft function after kidney transplantation. All patients reported in the current study participated in a randomized-controlled clinical trial (the so-called FDCC trial) [26]. The current pharmacogenetic sub-study

was performed as an integral part of this immunosuppressive drug trial allowing for an in-depth analysis of the association between genetic variability and CsA pharmacokinetics efficacy and adverse events.

PATIENTS AND METHODS

Patients and study design

The patients in this study were *de novo* kidney transplant recipients who participated in the Fixed-Dose vs. Concentration-Controlled trial. In the FDCC study, immunosuppressive therapy consisted of a CNI (either CsA or tacrolimus [Tac]) plus glucocorticoids and MMF. MMF treatment was either fixed-dose with patients randomised to the FD arm initially receiving 1000 mg MMF twice daily, or the MMF dose was adjusted on the basis of mycophenolic acid blood concentrations in patients randomised to the concentration-controlled arm. The choice of CsA or Tac and the target blood concentrations were in accordance with each center's protocol. Standard CNI and corticosteroid tapering regimens were left to the discretion of the investigators. Induction therapy with either anti-interleukin-2 receptor monoclonal antibody treatment or antithymocyte globulin was allowed. The study design and main results of the FDCC trial were published recently [26]. At the initiation of the study a pharmacogenetic sub-study was planned, and in the centers participating in this sub-study all patients provided two written informed consents, one for the FDCC study and one for the pharmacogenetic sub-study. The ethics committees of all participating centers and the relevant authorities in the participating countries approved the study protocol.

For this pharmacogenetic sub-study, data were available for 171 kidney transplant patients treated with CsA, MMF, and glucocorticoids. CsA dose was titrated to pre-dose concentrations (C_0) or to two hours post-dose concentrations (C_2) or both, according to local practice. Standard CNI and glucocorticoid tapering regimens were left to the discretion of the investigators. The use of drugs interfering with CsA PK, such as erythromycin, itraconazol etc. were not allowed. Induction therapy with either anti-interleukin-2 receptor monoclonal antibody treatment was allowed. The patients were instructed to take their evening dose of CsA exactly 12 hours before their morning dose.

C_0 and/or C_2 were measured on days 3 and 10, week 4, and months 3, 6 and 12 after transplantation, and whenever deemed necessary by the attending physician. Creatinine clearance was calculated by the Cockcroft-Gault formula [27]. Delayed graft function (DGF) was defined as the need for dialysis within the first week after transplantation. Biopsy-proven acute rejection (BPAR) was defined as any histologically confirmed episode for which a Banff score of 1 (mild, grades IA and IIA), 2 (moderate, grades IB and IIB), or 3 (severe, grade III) was recorded. The patients with panel reactive antibodies (PRA) above 50% within 6 months before study entry were excluded from the study. In the FDCC study, donor deoxyribonucleic acid (DNA) was not collected and no protocol kidney biopsies were performed.

Drug concentration measurements

Ciclosporin C_0 and C_2 were determined in whole blood in local laboratories in each of the participating centers by the use of immunoassays [CsA FPIA assay (AxSYM Abbott Laboratories) and Emit 2000 (Syva Company, Dade Behring Inc., Cupertino, CA)]. Proficiency testing was ensured by participation of all centers in the United Kingdom Quality Assessment Scheme (Prof. Holt, St George's, University of London, London United Kingdom). Dose-adjusted CsA concentrations were calculated by dividing the C_0 or the C_2 by the corresponding 24-h dose on a milligrams per kilogram basis. Mycophenolic acid (MPA) plasma concentrations were measured at three time points: before (pre-dose concentration), and 30 and 120 min after oral MMF administration. The MPA abbreviated AUC from 0 to 12 hr (AUC0–12) was calculated from these three MPA concentrations *(unpublished data).*

Genotype analysis

The patients were genotyped for *CYP3A4*1B, CYP3A5*3* and for *ABCB1 1236C>T* (exon 12), *2677G>T/A* (exon 21), and *3435C>T* (exon 26). Genomic DNA was isolated from 200 µL ethylenediaminetetraacetic acid-treated whole blood using a MagnaPure LC (Roche Diagnostics GmbH, Mannheim, Germany). Polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis for *CYP3A5*3* was performed as described previously [28-29]. Other rare *CYP3A5* alleles which result in the absence of functional CYP3A5 protein, such as *CYP3A5*6* [4], were not determined in the current study due to the low allele frequency of 0.1% in Caucasians [29]. Patients not carrying the *CYP3A5*3* allele were therefore assigned the *CYP3A5*1/*1* genotype by default. Genotyping for the *CYP3A4* –392A>G (*CYP3A4*1/*1B*) SNP and for *ABCB1 1236C>T, 2677G>T/A* and *3435C>T* was performed as described previously [21].

For the present analysis, patients carrying one *CYP3A5*1* allele were combined with *CYP3A5*1* homozygotes ("*CYP3A5* expressers") and were compared against *CYP3A5*3* homozygotes, the "*CYP3A5* nonexpressers". Likewise, *CYP3A4*1A* homozygotes were analyzed against the *CYP3A4*1B* carriers.

Statistical analysis

Student's *t*-test was used to compare means between groups at a single point in time. For the univariate analysis of the associations between categorical data (*e.g*. the incidence of acute rejection) we used Pearson's Chi Square test or Fisher's exact test, as appropriate. To identify risk factors for the development of BPAR a binary logistic regression was performed. To estimate the overall effect of *CYP3A5, CYP3A4* and *ABCB1* genotype on the outcome variables CsA daily dose and dose-adjusted CsA C_0 and C_2 we used mixed model analysis of variance (ANOVA). For all analyses, SPSS for Windows version 16.0 was used (SPSS, Chicago, IL, USA).

RESULTS

The characteristics of the 171 patients included in this study are summarized in Table 1. The allele frequencies of the various *CYP3A4, CYP3A5* and *ABCB1* SNPs were in Hardy-Weinberg equilibrium when analysed according to ethnic background (Caucasian, Black or Asian), except for *ABCB1 3435C>T* in Caucasians (Chi Square test, $p = 0.035$)

Table 1. Patient characteristics

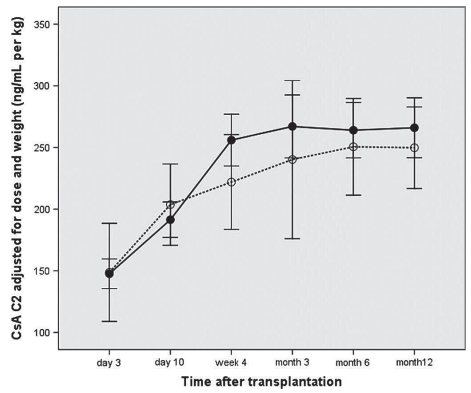
Sex (male / female)	105 / 66
Age (years)†	49.0 ± 11.9
First / Second Transplant	159 (93%) / 12 (7%)
Living / deceased donor	43 (25%) / 128 (75%)
CsA monitoring C_0/C_2/both	40 (23%)/ 25 (16%)/ 106 (62%)
FD / CC MMF therapy	94 (55%) / 77 (45%)
Induction therapy‡	96 (56%)
Primary kidney disease	
Diabetic nephropathy	9 (5 %)
Glomerulonephritis	46 (27%)
Hypertensive nephropathy	18 (11%)
Obstructive/reflux nephropathy	12 (7%)
Other	41 (24%)
Polycystic kidney disease	31 (18%)
Pyelonephritis / interstitial nephritis	5 (3%)
Unknown	9 (5%)
HLA mismatches§	2.9
Panel reactive antibodies	
less than 10% /10% or greater	155 (93%) / 12 (7%)
Ethnicity	
Asian	6 (3%)
Black	3 (2%)
Caucasian	150 (88%)
Other	12 (7%)

† Mean ± SD
‡ All of the 96 patients who received induction therapy were treated with antibodies against the IL-2 receptor and none was treated with anti-thymocyte globulin.
§ Mean
FD, Fixed-dose; CC, Concentration-controlled; MMF, Mycophenolate mofetil

CsA pharmacokinetics and CYP3A and ABCB1 genotype

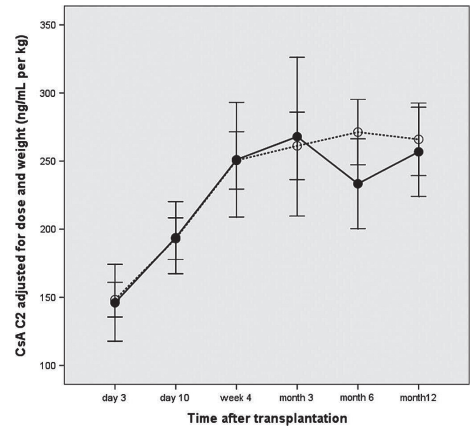
The daily dose of CsA and CsA exposure (measured as either C_0 or C_2) on days 3 and 10, month 1, 3, and 12 was comparable between patients carrying the *CYP3A5*1* allele and *CYP3A5* nonexpressers (data not shown). When we investigated CsA dose-adjusted concentrations, no statistically significant differences were observed between *CYP3A5* expressers and non-expressers throughout the first post-transplant year as shown in Figure 2 (C_2). No statistically significant differences between *CYP3A4*1A* homozygotes and *CYP3A4*1B* carriers were observed with regard to CsA daily dose, CsA exposure or dose-adjusted CsA concentrations (Figure 1). The C_0 revealed the same results, but the data are not shown.

Figure 1



Mean ciclosporine (CsA) dose-adjusted pre-dose concentrations C_2 according to *CYP3A4* genotype. The closed circles represent the *CYP3A4*1* homozygotes whereas the open circles represent the *CYP3A4*1B* carriers.

Figure 2



Mean CsA dose-adjusted C_2 according to *CYP3A5* genotype. The closed circles represent the *CYP3A5* expressers whereas the open circles represent the *CYP3A5* non-expressers.
Values are depicted as means. The error bars represent the corresponding 95% confidence intervals.

Also the *ABCB1* SNPs did not show a statistically significant association with CsA daily dose, CsA exposure or dose-adjusted CsA concentrations (data not shown). Next, we investigated the effects of unambiguous *ABCB1* haplotypes on CsA pharmacokinetics. For this analysis we compared patients that were homozygous for the *1236C, 2677G* and *3435C* alleles (*ABCB1 CGC* haplotype) with patients homozygous for *ABCB1 1236T, 2677T and 3435T* alleles (*ABCB1 TTT* haplotype). The *ABCB1 CGC* haplotype was present in 28 patients (16%) and the *ABCB1 TTT* haplotype was present in 20 patients (12%). There were no significant differences between the two haplotype groups with regard to CsA dose-adjusted C_0 or C_2 (data not shown) throughout the first post-transplant year. Finally, we performed an analysis combining the two *ABCB1* haplotypes with either the *CYP3A5* or the *CYP3A4* genotype to account for any interaction between *ABCB1* and *CYP3A*. No significant

differences were observed between these various genotype groups with regard to CsA dose-adjusted C_0 and C_2.

Clinical outcomes and CYP3A and ABCB1 genotype

Patient and graft survival

Of the 171 patients included in this study, 3 patients died during the first year after transplantation (overall one-year patient survival 98.2%). One patient died on day 21 after transplantation as a result of a myocardial infarction, one patient died of sepsis on day 41 after transplantation, and one patient died as a result of stroke on day 320 after transplantation. Throughout the first post-transplant year, 9 patients lost their graft, due to various causes (including one acute rejection), resulting in an overall graft survival of 94.7 %. Graft loss occurred at a median time of 55 days (range 8 to 338 days). Patient survival or graft loss was not associated with *CYP3A5*, *CYP3A4* or *ABCB1* genotype [15, 30]

Acute Rejection

The overall incidence of biopsy-proven acute rejection (BPAR) in this cohort of 171 patients was 16% (28 patients). Of the 28 patients with BPAR, 12 had grade 1 according to the Banff '97 classification and 16 patients had a grade 2 acute rejection. No difference in the incidence of BPAR was observed between *CYP3A5* expressers and non-expressers: 10% *vs.* 18%, respectively (p = 0.24). Likewise, patients carrying the *CYP3A4*1B* variant allele had an incidence of BPAR which was not statistically different compared to that of *CYP3A4*1* homozygotes: 5% *vs.* 18%, respectively (p = 0.13). In addition, no differences with regard to the severity of BPAR were observed between the different genotype groups. The incidence of BPAR was not related to *ABCB1* genotype when individual SNPs or haplotypes were analyzed (Table 2). Finally, *ABCB1* haplotypes stratified to *CYP3A4* or *CYP3A5* genotype were not related to the risk of acute rejection (data not shown).

The median time to BPAR was 50 days post-transplantation (range: 3 to 181 days), with five BPARs occurring within the first ten days after transplantation. The mean CsA C_0 on day 3 after transplantation of these 5 patients was not different from that of patients who did not experience BPAR before day 10 after transplantation: 354 *vs.* 314 ng/mL (p = 0.63). The mean C_2 was also not different between these patients (993 *vs.* 1137 ng/mL, p = 0.54). In addition, on day 3 after transplantation, the MPA area-under the concentration time-curve (MPA $AUC_{0\text{-}12\ h}$) was not statistically significantly lower in patients experiencing an early episode of BPAR compared with those who did not have BPAR within the first 10 days after transplantation: 25.5 *vs.* 32.3 mg/L per hour (p = 0.19). In order to identify risk factors for BPAR we performed a binary logistic regression analysis (incorporating variables such as induction therapy, total number of HLA mismatches, exposure to CsA, PRA, *CYP3A5, CYP3A4* and *ABCB1* genotype). Only PRA was identified as a risk factor for the development of BPAR. Patients with PRA >10% had a significantly higher risk of developing BPAR compared to patients with PRA <10% (OR: 8.8, 95% CI: 2.0-39.5; p < 0.005).

Delayed Graft Function

DGF occurred in 44 of the 171 patients (26%). On day 3 after transplantation the mean CsA C_0 was not different between the patients with DGF and the patients who did not experience DGF: 291 *vs.* 324 ng/mL (p = 0.31). However, the mean CsA C_2 on day 3 between these patients did differ: 778 *vs.* 1233 ng/mL for patients with and without DGF, respectively (p < 0.0001). DGF occurred in 13/39 (33%) patients expressing *CYP3A5 vs.* 31/132 (23%) in *CYP3A5* nonexpressers (p = 0.216). The incidence of DGF was higher (p = 0.014) in patients carrying the *CYP3A4*1B* gene (10/21; 48%) compared to the *CYP3A4*1* homozygotes (34/150; 23%).

TABLE 2. Incidence of Biopsy-Proven Acute Rejection (BPAR) and Delayed Graft Function (DGF)

Genotype / Haplotype	No BPAR	BPAR	*P*	No DGF	DGF	*P*
CYP3A5 expressers	35 (90%)	4 (10%)	0.240	26 (67%)	13 (33%)	0.216
CYP3A5 nonexpressers	108 (82%)	24 (18%)		101 (77%)	31 (23%)	
CYP3A4*1B carriers	20 (95%)	1 (5%)	0.125	11 (52%)	10 (48%)	0.014*
CYP3A4*1 homozygotes	123 (82%)	27 (18%)		116 (77%)	34 (23%)	
ABCB1 3435 CT/TT	111 (83%)	23 (17%)	0.378	106 (79%)	28 (21%)	0.004*
CC	32 (89%)	4 (11%)		20 (56%)	16 (44%)	
ABCB1 1236 CT/TT	47 (86%)	8 (14%)	0.742	89 (77%)	26 (23%)	0.159
CC	96 (84%)	19 (16%)		37 (67%)	18 (33%)	
ABCB1 2677 GT/TT	95 (85%)	17 (15%)	0.727	88 (79%)	24 (21%)	0.065
GG/GA	48 (83%)	10 (17%)		38 (65%)	20 (35%)	
ABCB1 CGC	25 (90%)	3 (10%)	0.658	16 (57%)	12 (43%)	0.014*
ABCB1 TTT	17 (85%)	3 (15%)		18 (90%)	2 (10%)	

*$P < 0.05$.

We compared the association of the different *ABCB1* genotypes to incidence of DGF, as shown in Table 2. When we performed binary logistic regression analysis [incorporating the covariates gender, (donor) age, *CYP3A4, CYP3A5,* and *ABCB1* genotype, primary kidney disease and cold ischemia time], only longer cold ischemia time (OR: 1,11 95% CI: 1.04 – 1.18; p = 0.001) and higher donor age (OR: 1.05 95% CI: 1.01-1.08; P = 0.013) were significantly related to the risk of DGF.

Creatinine clearance

Throughout the first post-transplant year, the mean creatinine clearance increased from 31.6 mL/min on day 3 to 66.7 mL/min on year 1, respectively. When comparing all the patients the mean creatinine clearance was not associated with *CYP3A5* or *CYP3A4* genotype (data not shown). However, statistically significant differences in creatinine clearance on day 10 were observed for the different *ABCB1* genotypes (Table 3). A significant difference was also found for *CYP3A4* combined with the different *ABCB1* haplotypes. On day 10 after transplantation, *CYP3A4*1* homozygotes with the *ABCB1 TTT* haplotype had a creatinine clearance that was significantly higher compared with *CYP3A4*1* homozygotes with the *ABCB1 CGC* haplotype: 58.4 mL/min *vs.* 21.4 mL/min, respectively (p = 0.004). At week 4 *CYP3A4*1B carriers* with the *ABCB1 CGC* haplotype showed a significantly higher creatinine clearance compared to *CYP3A4*1B carriers* with *ABCB1 TTT* haplotype: 65,2 mL/min *vs.* 41,2 mL/min respectively (p = 0.044).

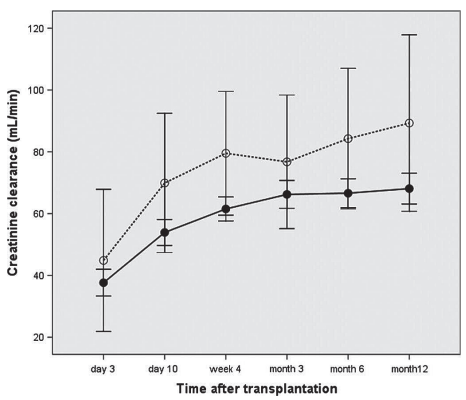
TABLE 3. Creatinine Clearance

Genotype	*ABCB1 3435*		*ABCB1 1236*		*ABCB1 2677*		*ABCB1* Haplotype	
Time > Treatment	*CT/TT* (N = 134)	*CC* (N = 36)	*CT/TT* (N = 115)	*CC* (N = 55)	*GT/TT* (N = 112)	*GG/GA* (N = 58)	*CGC* (N = 28)	*TTT* (N = 20)
Day 3	33 (25)	27 (22)	34 (26)	27 (21)	34 (26)*	26 (21)*	26 (21)*	42 (30)*
Day 10	49 (27)*	38 (23)*	49 (28)*	40 (22)*	50 (28)*	39 (24)*	37 (22)*	58 (21)*
Month 1	58 (24)	53 (18)	59 (24)	53 (19)	59 (23)	54 (22)	51 (16)*	63 (18)*
Month 3	64 (23)	63 (17)	65 (24)	62 (18)	65 (23)	62 (20)	62 (17)	68 (22)
Month 6	65 (23)	66 (16)	66 (24)	64 (15)	65 (23)	66 (20)	63 (13)	67 (19)
Month 12	67 (23)	67 (18)	67 (23)	68 (20)	66 (22)	69 (22)	65 (15)	70 (23)
Creatinine clearance (only patients with immediate graft function)								
Genotype	***ABCB1 3435***		***ABCB1 1236***		***ABCB1 2677***		***ABCB1* Haplotype**	
Time > Treatment	***CT/TT* (N = 106)**	***CC* (N = 20)**	***CT/TT* (N = 89)**	***CC* (N = 37)**	**GT/TT (N = 88)**	***GG/GA* (N = 38)**	***CGC* (N = 16)**	***TTT* (N = 18)**
Day 3	38 (25)	40 (22)	41 (26)	33 (21)	41 (26)	33 (21)	37 (22)	45 (29)
Day 10	56 (25)	51 (19)	58 (26)	50 (19)	58 (25)	50 (22)	49 (20)	61 (18)
Month 1	64 (22)	60 (17)	66 (22)	58 (17)	65 (21)	60 (21)	56 (15)	66 (17)
Month 3	67 (23)	66 (17)	69 (24)	64 (18)	68 (23)	65 (21)	65 (18)	70 (21)
Month 6	68 (23)	71 (12)	69 (24)	67 (15)	68 (23)	70 (21)	66 (9)	70 (18)
Month 12	69 (24)	76 (16)	69 (24)	72 (21)	68 (23)	74 (24)	70 (12)	73 (21)

Mean creatinine clearance in mL/min ± (standard deviation).
*$P < 0.05$.

However, when we exclude the patients with DGF different results were observed. Table 3 shows the analysis of *ABCB1* genotype and creatinine clearance for patients with immediate graft function. The creatinine clearance did not show significant differences between *ABCB1* genotypes anymore. However, according to the *CYP3A4* genotype the creatinine clearance was statistically higher in *CYP3A4*1B* carriers (n = 11) compared to *CYP3A4*1* homozygotes (n = 114) on day 10 (69.90 *vs.* 53.91 mL/min; *P* = 0.035), month 1 (79.53 *vs.* 61.51 mL/min (*P* = 0.013)) and month 6 (84.26 *vs.* 66.60 mL/min (*P* = 0.041)), as depicted in figure 3. The creatinine clearance in the different *CYP3A5* genotypes did not statistically differ when adjusted for DGF. However, when we performed a logistic regression analysis on month 12 to identify the factors that were associated with a lower creatinine clearance at month 12 after transplantation [including the co-variates: gender, (donor) age, *CYP3A4, CYP3A5* genotype, primary kidney disease, cold ischemia time, delayed graft function and BPAR], only a higher donor age (*p* = 0.024) was significantly related to a lower creatinine clearance, and the previously observed influence of *CYP3A4* genotype was no longer statistically significant (*p* = 0.094).

Figure 3



Mean creatinine clearance according to *CYP3A4* genotype. The closed circles represent the *CYP3A4*1* homozygotes (patients without DGF) whereas the open circles represent the *CYP3A4*1B* carriers without DGF.
Values are depicted as means. The error bars represent the corresponding 95% confidence intervals.

DISCUSSION

To our knowledge this is the second prospective trial to investigate the association between *CYP3A* and *ABCB1* genotype and CsA exposure [19]. Based on our present findings we conclude that polymorphic expression of *CYP3A4*, *CYP3A5* and *ABCB1* cannot explain the high interindividual pharmacokinetic variability of CsA. In a previous study [1] among kidney and heart transplant recipients, we observed a slightly higher CsA clearance in patients carrying the *CYP3A4*1B* allele compared to *CYP3A4*1* homozygotes. We believe that the apparent discrepancies between the current study and our previous study can be explained by the fact that in the study published in 2004 more elaborate CsA blood sampling was performed (i.e. AUC during the absorption phase) and a more precise pharmacokinetic analysis was used (nonlinear mixed-effects modelling). However, we feel that the effect of the *CYP3A4*1B* polymorphism, if any, is unlikely to be clinically relevant. Other studies have also failed to identify clinically significant effects of *CYP3A4* SNPs on CsA exposure [14, 21].

Possibly, polymorphisms in other genes encoding for proteins involved in the transport or metabolism of CsA are responsible for this as yet unexplained variability. For example, the Pregnane X receptor (PXR), which regulates ABCB1 expression appears to influence the bioavailability of CsA [31 32], which has also been shown for Tac [33]. However, results from a recently published study [34] have demonstrated that PXR does not appear to be very important in this respect. The large variation in dose-adjusted CsA concentrations suggests that factors other than genotype, must be considered to explain the variability in CsA pharmacokinetics [35].

In the current prospective study we have shown that the different genotypes do not lead to an increased incidence of acute rejection in kidney transplant patients who are treated with an immunosuppressive regimen consisting of CsA, MMF and glucocorticoids with or without anti-interleukin-2 receptor antibody treatment. This result was to be expected as the CsA concentrations did not differ significantly between these different genotypes.

With regard to the risk of developing DGF, logistic regression analysis showed that only cold ischemia time and donor age were related to the risk of DGF, which has also been reported by other authors [19, 36-38]. In contrast to our results, Cattaneo *et al* [19] reported that carriers of T allelic variants in *ABCB1 2677G>T/A* and *3435C>T* SNPs had a higher risk for DGF. They suggested that DGF in T allelic variant carriers might be the consequence of severe reperfusion injury, sustained by increased intra-lymphocytic CsA concentrations. However, it is questionable if intra-lymphocytic CsA accumulation leads to more DGF. It would be more logical to assume an effect of accumulation of CsA in renal tubular cells, in order to explain differences in DGF incidence. Hauser *et al* [23] observed that for renal transplant recipients the *ABCB1* genotype of the donor but not of the recipient, was a risk factor the occurrence of clinically-defined CsA-related nephrotoxicity. Obviously, it is the genotype of the donor that determines intragraft *ABCB1* expression and possibly intrarenal accumulation of CsA. Unfortunately, donor DNA was not collected in the present study.

With regard to renal transplant function, we found no association with *CYP3A* or *ABCB1* genotype. Only a higher donor age was identified as a risk factor for a lower creatinine clearance. This finding is in line with the recent findings of Naesens *et al.* who showed that that the age of the kidney transplant donor is a major determinant of its susceptibility to Tac-mediated nephrotoxicity, with kidneys from older donors have the most rapid deterioration of function [39-40] Future studies should take into account the role of genetic factors influencing metabolism and transportation of CNIs in both acceptor and donor.

CONCLUSION

The data from this prospective study provide evidence that the pharmacokinetics of CsA are not influenced by genetic variation in the metabolizing enzymes CYP3A4 and CYP3A5 or the drug transporter ABCB1. In addition, the incidence of BPAR and renal function were not associated with polymorphisms in these genes. Determination of *CYP3A* and *ABCB1* SNPs pre-transplantation is therefore not of help in determining the CsA starting dose and does not appear to identify patients at high risk of experiencing acute rejection or nephrotoxicity. Further research should be performed to determine other risk factors for developing CsA-induced nephrotoxicity, including the role of the genotype of the kidney donor.

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Chapter 2.2

A new functional CYP3A4 intron 6 polymorphism significantly affects tacrolimus pharmacokinetics in kidney transplant recipients

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ABSTRACT

Background Tacrolimus (Tac) is a potent immunosuppressant with considerable toxicity. Tac pharmacokinetics varies considerably between individuals, which complicates its use to prevent rejection after kidney transplantation. This variability might be caused by genetic polymorphisms in key metabolizing enzymes. METHODS: We evaluated the impact of a newly discovered single nucleotide polymorphism (SNP) in *CYP3A4* (rs35599367C>T) on Tac pharmacokinetics in 185 renal transplant recipients that participated in an international randomized-controlled clinical trial (FDCC-study). RESULTS: We showed that the overall Tac daily dose requirement was on average 33% lower for T-variant allele carriers compared to CC patients to reach the same predose blood concentrations ($CI_{95\%}$ -46% to -20%; p=0.018). When combined with *CYP3A5*3* genotype, the *CYP3A4* intron 6 SNP was also associated with the risk of supratherapeutic Tac levels (>15 ng/mL) during the first days following surgery, with OR=8.7 for *CYP3A4* T-variant allele carriers plus *CYP3A5*3/*3* (p=0.027) and OR=4.2 for *CYP3A4* CC-homozygotes plus *CYP3A5*3/*3* (p=0.002), compared to *CYP3A4* CC homozygotes having at least one *CYP3A5*1* allele. Overall increase in Tac dose-adjusted trough blood concentration was +179% for *CYP3A4* T-variant allele carriers with *CYP3A5*3/*3* (p<0.001), +101% for *CYP3A4* CC-homozygotes with *CYP3A5*3/*3* (p<0.001) and +64% for *CYP3A4* T-variant allele carriers with *CYP3A5*1* (p=0.020), as compared to *CYP3A4* CC-homozygotes with *CYP3A5*1*. CONCLUSION: The *CYP3A4* rs35599367C>T polymorphism is associated with a significantly altered Tac metabolism and therefore increases the risk of supratherapeutic Tac concentrations early after transplantation. Analysis of this *CYP3A4* allelic variant may help identifying patients at risk of Tac overexposure.

INTRODUCTION

CYP3A4 is the most abundant CYP enzyme in human liver and intestine and is responsible for the oxidative metabolism of approximately 45-60% of all prescribed drugs [1]. CYP3A4 activity varies widely in the population with a 10-100 fold inter-individual variability [2-5]. A recent study identified a functional single nucleotide polymorphism (SNP) in *CYP3A4* intron 6, which was associated with decreased CYP3A4 expression and activity. This SNP was correlated with statin dose requirement for lipid level control [6].

The immunosuppressive drug tacrolimus (Tac) is extensively metabolized by CYP3A4 and CYP3A5 [7-9]. The *CYP3A5*3* allele, encoding absence of CYP3A5 [10], was earlier associated with Tac predose concentrations (C_0) and Tac dose requirements. The *CYP3A5* genotype explains a major part of the Tac pharmacokinetic interindividual variability: carriers of two *CYP3A5*3* non-functional alleles require a significantly lower Tac dosage (about 50%) than patients carrying a *CYP3A5*1* active allele among kidney, liver, lung, and heart transplant recipients to reach identical C_0 concentrations [11-19]. However, the clinical benefit of *CYP3A5*-based Tac dosing remains debatable [20-21]. The polymorphic drug transporter *ABCB1* is also involved in Tac disposition. Variants have been associated with Tac drug disposition, although contradictory results have been published [22].

No studies performed to date have been able to identify SNPs in *CYP3A4* that could account for a substantial part of inter-individual variability in CYP3A4 activity. A newly discovered CYP3A4 SNP in intron 6 (rs35599367 C>T), may explain this. [6]. Main goal of our study was therefore to test specifically the hypothesis that this new *CYP3A4* SNP is correlated with an increased Tac exposure on a standard dosages, and might predict lower dose requirement in kidney transplant patients.

MATERIALS AND METHODS

Patients and study design

Patients were *de novo* kidney transplant recipients participating in a phase IV, open, prospective, randomized controlled, international multi-center trial comparing fixed-dose (FD) mycophenolate mofetil (MMF) treatment with concentration-controlled (CC) treatment (the 'FDCC-study') [23]. The randomization to FD or CC regimen was done in blocks of eight patients per center. Patients were randomized centrally, through an automated telephone system, in a one-to-one ratio. Parallel to the main study, also a pharmacogenetic substudy was started. The role of genetic polymorphisms in *UGT1A9* for MMF metabolism [24], in *UGT2B7* for Acyl-MPAG [25], and in *CYP3A5* and *ABCB1* for Tac exposure and acute rejection [26] in the FDCC-study have been published earlier. Patients provided separate written informed consents for both the FDCC-study and the substudy. The study protocol was approved by the ethics committees of all participating centers and relevant authorities in the participating countries.

Immunosuppressive therapy consisted of calcineurin inhibitor (CNI) and corticosteroids.

The choice of Tac or cyclosporine (CsA), and the target blood concentrations for each individual drug, were in accordance with each center's protocol. Tac treatment started within 48 hours prior to transplantation and was administrated orally. Therapeutic drug monitoring was routinely performed, and centers were free to aim for the target levels they considered appropriate. Retrospective analysis showed that all centers started Tac aiming for target levels 7-15 ng/mL, tapering to 5-12 ng/mL at month 3, and 4-10 ng/mL at month 12. Corticosteroid tapering was recommended but not mandatory and tapering regimens were left to the discretion of the investigators. In general, centers used higher dosages in the first 2 weeks (20-25 mg prednisolone equivalent daily), lower doses thereafter (15 mg on week 4, 5 mg at month 3) and low dose or no prednisolone between months 6 and 12. More details are reported in the original publication of the FDCC-study [23]. Genetic data were accessible for 185 kidney transplant recipients treated with Tac. However, pharmacokinetic data were not available for all patients at all time points. Tac predose concentrations (C_0) were measured on days 3, 10 and months 1, 3, 6 and 12 after transplantation, and whenever deemed necessary by the attending physician. Donor DNA was not collected and no protocol kidney biopsies were performed. Delayed graft function (DGF) was defined as the need for dialysis within the first week after transplantation. Biopsy-proven acute rejection (BPAR) was defined as any histologically confirmed episode with a Banff score of 1 or more. All biopsy samples were assessed locally by a pathologist.

Drug concentration measurement

Tac C_0 was determined in whole blood in local laboratories in each participating center by use of immunoassays, like the Tac II microparticulate enzyme immunoassay (MEIA; Abbott Laboratories, Abbott Park, IL, USA) and enzyme multiplied immunoassay technique (Emit 2000; Syva Company, Dade Behring Inc., Cupertino, CA, USA). The specificities of both assays were comparable and high correlations existed between immunoassays and high-performance liquid chromatography [27-28]. Although immunoassays slightly overestimate Tac concentrations due to concurrent measurement of metabolites, this methodology was feasible to assess differences in Tac concentrations regarding *CYP3A5* genotype [16]. A limited number of centers used LC-MS/MS to measure Tac concentrations. Proficiency testing was ensured by participation of all centers in the UK Quality Assessment Scheme (Dr Holt, St George's Hospital Medical School, London, UK). Dose-adjusted predose concentrations were calculated by dividing the C_0 by the corresponding 24-h dose on a mg/kg basis.

Genotype analysis

Genomic DNA was isolated from 200 µl EDTA whole blood using MagnaPure LC (Roche Diagnostics GmbH, Mannheim, Germany). Allelic discrimination reaction was realized using TaqMan® (Applied Biosystems, CA, USA) genotyping assays (C__59013445_10) for *CYP3A4* intron 6 C>T genotype on a ABI PRISM 7500® Fast real-time PCR Systems (Applied Biosystems, CA, USA) using 50 ng genomic DNA. *CYP3A5*3* and *ABCB1* 1236C>T, 2677G>T/A and 3435C>T analysis were performed as described earlier [26-29].

Statistical analysis

Statistical analyses were performed using Predictive Analytics Software (PASW) statistics, version 17.0 for Windows (IL, USA). Tac C_0 and dose-adjusted C_0 were normalized by logarithmic transformation. Log distributions were confirmed to be normally distributed by Kolmogorov–Smirnov tests. When only 2 genotype groups were compared, Student's independent t-tests were performed to compare means between groups at single time points. With more groups, analyses of variance (ANOVA) were performed under the null hypothesis that means of compared groups were equal. When differences between means were significant, post-hoc analysis consisted of a priori polynomial linear contrast test to assess any potential linear trend according to genotype classification. The corresponding linear contrast does test probability of a positive linear trend of the dependent variable across the ordered level of genotype classifications. Differences between groups were assumed for p-values <0.05. For univariate analysis of associations between categorical data (e.g. incidence of acute rejection), Fisher's exact test or Pearson's χ^2 test were used. Tac daily dose and dose-adjusted C_0 were compared between genotypes by mixed-model analysis based on the maximum likelihood ratio with patient genotype status as fixed factor and time after transplantation as repeated measurement. Sex, ethnicity and age of patients were introduced as random effects to adjust for these co-variables. No structure was imposed on variances and covariances between and within times of follow up of the repeated Tac measurements. We assumed levels of covariables (sex, ethnicity and age) uncorrelated and have a constant variance across time of follow up. Coefficients estimated from the mixed-model analysis of variance were back-transformed by taking their antilog, to become interpretable as percent differences in geometric mean values of untransformed outcomes. Multiple logistic regression analysis was carried out according to criteria defined by McMaster [30], with fixed Tac supratherapeutic threshold at 15 ng/mL. Genotype-specific odds ratios (OR) and 95% confidence intervals were computed using backward stepwise analysis based on maximum likelihood ratios to assess the impact of genotype on risk Tac plasma concentrations >15 ng/mL with p-values <0.05 being considered statistically significant for entry and p-values <0.10 for staying in the model. For these analyses, each genotype was coded as a 'dummy variable'.

RESULTS

CYP3A4 intron 6 genotype and tacrolimus exposure

Patient characteristics are reported in table 1. In total, 173 had the CC genotype, 11 the CT genotype and 1 patient was homozygous TT variant, resulting in a minor allele frequency of 3.5% in our study. The observed genotype distribution is in accordance with Hardy-Weinberg equilibrium (χ^2 test; p=0.25). Heterozygous CT and homozygous TT variants were grouped and analyzed together as T-variant allele carriers, against CC homozygous patients. We did not observe linkage disequilibrium between CYP3A4 intron 6 SNP and

either CYP3A5*3 or CYP3A4*1B alleles (χ^2 =0.24 and 1.36, p=1.0 and 0.46, respectively).

Table 1: Patient demographics

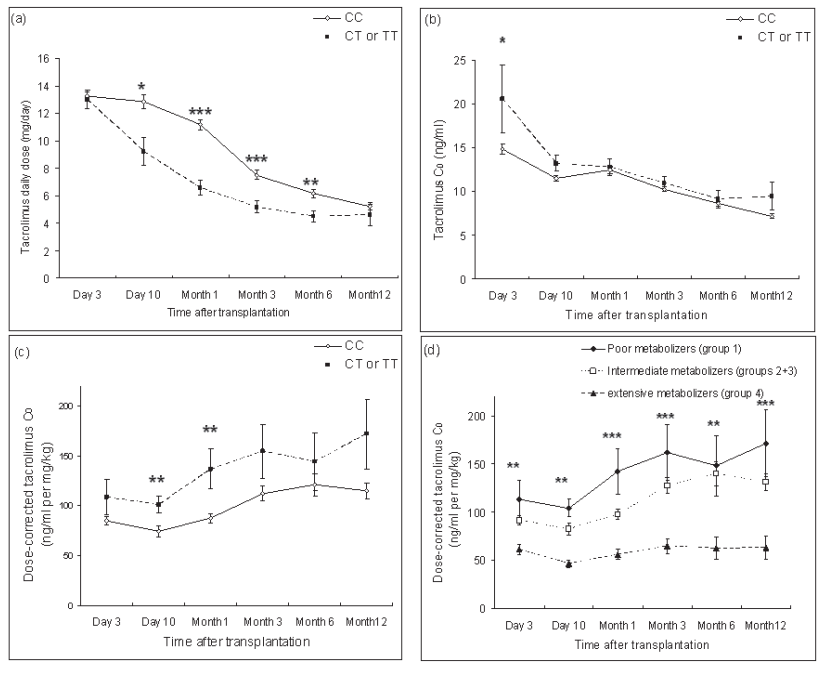
	All patients	*CYP3A4* intron 6 homozygous CC	*CYP3A4* intron 6 allele T carriers (CT plus TT)	p-value
N	**185**	**173**	**12**	**-**
Sex (male/female)	112 (60.5%) 73(39.5%)	108 (62.4%) 65(37.6%)	4 (33.3%) 8 (66.7%)	0.07
Age (years)[a]	47.9 ± 13.8	47.7 ± 14.0	51.8 ± 11.3	0.32
Weight (Kg)[a]	72.6 ± 14.0	72.8 ± 14.3	71.1 ± 10.4	0.69
Transplantation number				0.80
First	156 (84.3%)	145 (83.8%)	11 (91.7%)	-
Second	19 (10.3%)	18 (10.4%)	1 (8.3%)	-
Third or more	5 (2.7%)	5 (2.9%)	0 (0.0%)	-
Missing information	5 (2.7%)	5 (2.9%)	0 (0.0%)	-
Living/deceased donor	71 (38.4%) 114 (61.6%)	66 (38.2%) 107 (61.8%)	5 (41.7%) 7 (58.3%)	0.81
FD/CC MMF therapy	98 (53.0%) 87 (47.0%)	92 (53.2%) 81 (46.8%)	6 (50.0%) 6 (50.0%)	0.83
Induction therapy [b]	67 (36.2%)	62 (35.8%)	5 (41.5%)	0.69
Primary kidney disease				0.29
Diabetic nephropathy	16 (8.6%)	15 (8.7%)	1 (8.3%)	-
Glomerulonephritis	51 (27.6%)	48 (27.7%)	3 (25.0%)	-
Hypertensive nephropathy	18 (9.7%)	18 (10.4%)	0 (0.0%)	-
Obstructive/reflux nephropathy	9 (4.9%)	8 (4.6%)	1 (8.3%)	-
Other	42 (22.7%)	42 (24.3%)	0 (0.0%)	-
Polycystic kidney disease	29 (15.7%)	24 (13.9%)	5 (41.7%)	-
Pyelonephritis/interstitial nephritis	9 (4.9%)	8 (4.6%)	1 (8.3%)	-
Unknown	9 (4.9%)	8 (4.6%)	1 (8.3%)	-
HLA mismatches [c]	2.9 (3.0)	2.9 (3.0)	3.2 (3.0)	0.55
Panel reactive antibodies				
<10% / ≥10 %	167 (90.3%) 18(9.7%)	155 (89.6%) 18(10.4%)	12 (100.0%) 0(0.0%)	0.24
Ethnicity				0.65
Asian	9 (4.9%)	9 (5.2%)	0 (0.0%)	-
Black	8 (4.3%)	8 (4.6%)	0 (0.0%)	-
Caucasian	164 (88.6%)	152 (87.9%)	12 (100.0%)	-
Other	4 (2.2%)	4 (2.3%)	0 (0.0%)	-

CC, concentration-controlled; FD, fixed-dose; HLA, Human Leucocyte Antigen; MMF, mycophenolate mofetyl.
[a] Mean ± SD
[b] All patients who receive induction therapy were treated with antibody against the IL-2 receptor and none was treated with anti-thymocyte globulin.
[c]Mean (median)

Tac daily dose on day 3 after transplantation (without adjustments made based on TDM) was comparable between the two *CYP3A4* intron 6 genotype groups: 13.3 versus 13.0 mg/day for homozygous CC wild-type patients and carriers of at least one T-variant allele, respectively (p=0.84, figure 1a, table 2). However, with these comparable dosages, Tac C_0 was higher for T-variant carriers compared to CC patients: 20.5 versus 14.9 ng/mL, respectively (p=0.05, figure 1b, table 2). Later time points revealed that differences in C_0 between both groups were no longer observed (p>0.05, figure 1b, table 2), but that carriers of at least one T-variant allele required significantly lower Tac doses than CC patients from

day 10 to month 6 to reach this C_0 (figure 1a, table 2). The same trends were observed when dose was adjusted for weight (table 2). Repeated measurement in mixed-model analysis demonstrated overall daily Tac dose requirement (adjusted for covariates age, gender and ethnicity) to be on average 33% lower for T-variant allele carriers ($CI_{95\%}$ -46% to -20%; p=0.018) compared to CC patients. As a result, calculated Tac dose-adjusted C_0 was lower for CC patients compared to CT/TT patients. These differences were statistically different at day 10 and month 1 after transplantation (figure 1c, table 2), but not at later time points, which might be explained by a decrease in the number of subjects, resulting in a larger $CI_{95\%}$. The mixed-model for repeated measurements revealed that overall Tac dose-adjusted C_0 (adjusted for covariates [age, gender and ethnicity]) was on average 47% higher T-variant allele carriers ($CI_{95\%}$ 8-100%; p=0.001) compared to homozygous wild-type patients. For *ABCB1*, neither 2677G>T/A nor 1236C>T was associated with differences in Tac pharmacokinetic parameters during the whole study. By contrast, as reported (26), the 3435C>T SNP was significantly associated with Tac dose and dose-corrected Tac exposure in a linear mixed-model when *CYP3A4* genotype was not taken into account. When *ABCB1* haplotypes were generated, no differences were observed in Tac dose and dose-corrected Tac exposure between the *ABCB1* CGC (n=26) and TTT haplotype groups (n=20) (data not shown).

Figure 1.



A) Tacrolimus daily dose, B) tacrolimus predose concentration (C0), C) dose-corrected tacrolimus C0 according to the genetic status of patients for the CYP3A4 intron 6 C>T SNP at different time points and D) Dose-corrected tacrolimus C0 according to the genetic status of patients for the CYP3A4 intron 6 C>T and CYP3A5*3 SNPs at different time points. Data are represented as means (±SEM). *=p<0.05; **=p<0.01, ***=p<0.001.

Table 2: Tacrolimus dose, predose concentration (C_0) and dose-corrected predose concentration (C_0/Dose) according to the *CYP3A4* intron 6 C>T SNP genotype.

Tacrolimus dose (mg/day)	***CYP3A4* intron 6 homozygous CC**	**n**	***CYP3A4* intron 6 allele T carriers (CT plus TT)**	**n**	**p-value**
Day 3	13.3 [12.6-13.9]	136	13.0 [11.7-14.3]	9	0.84
Day 10	12.9 [11.9-13.9]	134	9.2 [7.2-11.7]	10	**0.05**
Month 1	11.2 [10.4-11.9]	137	6.6 [5.5-7.7]	10	**<0.001**
Month 3	7.5 [6.8-8.2]	131	5.2 [4.3-6.1]	11	**<0.001**
Month 6	6.2 [5.5-6.8]	120	4.5 [3.7-5.3]	10	**0.004**
Month 12	5.2 [4.7-5.8]	112	4.6 [3.1-6.1]	9	0.55
Weight-adjusted Tacrolimus dose (mg/day per kg b.w,)					
Day 3	0.181 [0.174-0.189]	135	0.193 [0.178-0.207]	9	0.45
Day 10	0.176 [0.162-0.190]	133	0.134 [0.101-0.168]	10	0.10
Month 1	0.156 [0.15-0.166]	136	0.097 [0.078-0.116]	10	**0.004**
Month 3	0.105 [0.095-0.115]	130	0.076 [0.061-0.091]	11	0.10
Month 6	0.087 [0.078-0.097]	119	0.066 [0.054-0.079]	10	0.25
Month 12	0.072 [0.063-0.081]	111	0.069 [0.041-0.097]	9	0.84
Tacrolimus C_0 (ng/mL)*					
Day 3	14.9 [13.8-16.0]	144	20.5 [15.2-27.7]	8	**0.05**
Day 10	11.5 [10.9-12.1]	133	13.2 [11.3-15.5]	9	0.21
Month 1	12.5 [11.8-13.2]	145	12.8 [10.9-15.0]	11	0.81
Month 3	10.2 [9.8-10.8]	145	11.0 [9.7-12.4]	11	0.47
Month 6	8.6 [8.1-9.2]	125	9.1 [7.6-11.0]	10	0.63
Month 12	7.2 [6.5-7.9]	110	9.5 [6.9-13.1]	8	0.12
Tacrolimus C_0/Dose (ng/mL per mg/kg)*					
Day 3	84.7 [78.2-91.8]	125	108.7 [82.9-142.6]	8	0.14
Day 10	74.3 [67.3-82.0]	120	101.3 [86.5-118.6]	8	0.006
Month 1	87.5 [80.3-95.4]	128	136.6 [107.9-173.1]	10	**0.006**
Month 3	112.2 [101.0-124.6]	124	154.2 [117.6-202.3]	10	0.10
Month 6	121.3 [106.4-138.3]	105	144.3 [111.7-186.3]	9	0.26
Month 12	114.7 [100.7-130.5]	98	171.8 [120.2-245.5]	8	0.09
Creatinine clearance (mL/min)					
Day 3	35.8 [31.7-39.9]	151	42.0 [20.1-63.8]	10	0.47
Day 10	45.1 [41.2-49.0]	148	47.4 [29.0-65.7]	11	0.77
Month 1	55.6 [52.0-59.3]	148	57.3 [42.1-72.5]	11	0.82
Month 3	60.1 [56.3-63.9]	144	66.2 [50.9-81.5]	11	0.40
Month 6	63.7 [59.8-67.7]	130	66.1 [50.0-82.3]	10	0.74
Month 12	65.6 [61.5-69.6]	114	59.1 [41.3-76.9]	9	0.40

* Values are expressed as geometric means with 95% confidence interval range in brackets

CYP3A4 intron 6 genotype, CYP3A5*3 and ABCB1 3435C>T combined effects

Subsequently, we investigated combined effects of *CYP3A4* intron 6 genotype, *ABCB1* 3435C>T and *CYP3A5*3* allelic status. Patients carrying at least one *CYP3A5*1* allele (CYP3A5 expressers) were compared with *CYP3A5*3/*3* nonexpressers. In a mixed-model analysis, adjusted for covariates age, gender and ethnicity, including *ABCB1* 3435C>T, *CYP3A5**3 and *CYP3A4* intron 6 genotype status as fixed effects, all investigated SNPs correlated significantly with dose-corrected Tac exposure. The overall Tac dose-adjusted C_0 was 43% higher among *CYP3A4* intron 6 CT/TT patients ($CI_{95\%}$ 13-88%; p<0.001) compared CC patients, and 43.3% lower among CYP3A5 expressers compared to nonexpressers ($CI_{95\%}$ -52.7 to -32.1%; p=0.001). Regarding *ABCB1* 3435TT individuals, patients with the *ABCB1* 3435CT and 3435CC genotypes had an overall Tac dose-adjusted C_0 that was 14.3% ($CI_{95\%}$ -26.2 to -0.5%; p=0.042) and 20.9% ($CI_{95\%}$ -32.7 to -7.1%; p=0.003) lower, respectively. However, only *CYP3A4* intron 6 and *CYP3A5* genotypes correlated significantly with Tac dose requirement as *ABCB1* 3435C>T genotype status did not appear as a significant fixed effect anymore in the mixed-model. In this final model, Tac dose requirement was 25% lower for T-variant allele carriers ($CI_{95\%}$ -43 to -7%; p=0.04) compared to CC patients, and 63.7% higher in patients who expressed CYP3A5 compared with nonexpressers ($CI_{95\%}$ 39.1 to 88.2%; p<0.001).

As effects of *CYP3A4* intron 6 and *CYP3A5*3* SNPs appear independent, we generated groups of combined genotypes, with Group 1 containing CYP3A5 nonexpressers and *CYP3A4* intron 6 T-variant carriers (poor metabolizers); Group 2 contains CYP3A5 nonexpressers and *CYP3A4* intron 6 C-allele homozygotes (intermediate-1 metabolizers); Group 3 clustered CYP3A5 expressers carrying the *CYP3A4* intron 6 T-allele (intermediate-2 metabolizers) and Group 4 merged CYP3A5 expressers with *CYP3A4* intron 6 CC wild-types (extensive metabolizers) (table 3). Tac C_0 was significantly different among groups of genotypes at first visits (table 4). Tac daily dose requirement, based on reaching target Tac C_0 by TDM, was significantly different from day 10, and this remained significant (table 4). The same significant differences were observed when dose was adjusted for weight (table 4). Tac dose-adjusted C_0 was significantly different among groups at all time points (figure 1d, table 4). This was a linear trend in function of genotype category classification either for Tac predose concentrations at day 3 (Group 1 > Group 2 > Group 3 > Group 4, p<0.004; table 4) or for Tac dose requirement from day 10 to month 12 (Group 1 < Group 2 < Group 3 < Group 4, p=0.001; table 4). This trend was also observed for dose-adjusted Tac C_0 and showed high statistical significance at all investigated time points (Group 1 > Group 2 > Group 3 > Group 4, p=0.006; table 4). The mixed-model analysis revealed an overall increase in Tac dose-adjusted trough blood concentrations of +179.3% for poor (p<0.001), +101.4% for intermediate-1 (p<0.001) and +64.4% for intermediate-2 (p=0.020) clusters as compared to extensive metabolizers.

Table 3: *CYP3A4/CYP3A5* haplotype classification.

	CYP3A4 intron 6 CT or TT	*CYP3A4* intron 6 CC
CYP3A5*1 non carriers	**Group 1** Poor metabolizers n= 10 (5.4%)	**Group 2** Intermediate-1 metabolizers n= 142 (76.8%)
CYP3A5*1 carriers	**Group 3** Intermediate-2 metabolizers n= 2 (1.1%)	**Group 4** Extensive metabolizers n= 31 (16.8%)

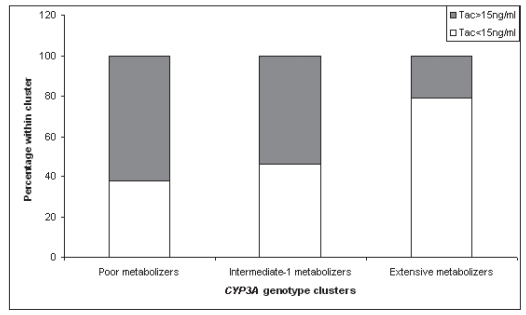
Table 4: Tacrolimus dose, predose concentration and dose-corrected predose concentration according to the combined *CYP3A4* intron 6 C>T SNP and *CYP3A5* genotype.

Tacrolimus dose (mg/day)	Group 1	n	Group 2	n	Group 3	n	Group 4	n	p-value ANOVA	p-value Polynominal#
Day 3	13.0 [10.9-15.1]	7	13.1 [12.4-13.8]	111	13.0 [0.3-25.7]	3	14.0 [12.0-15.9]	25	0.78	-
Day 10	8.3 [5.9-10.6]	8	11.8 [10.8-12.8]	109	13.0 [0.3-25.7]	2	17.6 [15.0-20.2]	25	**<0.001**	**<0.001**
Month 1	6.5 [5.0-8.0]	8	10.3 [9.5-11.0]	112	7.0 [-5.7-19.7]	2	15.2 [13.1-17.2]	25	<0.001	<0.001
Month 3	5.0 [3.8-6.2]	9	6.7 [6.2-7.3]	105	6.0 [6.0-6.0]	2	10.5 [8.5-12.6]	26	**<0.001**	**0.001**
Month 6	4.3 [3.2-5.3]	8	5.3 [4.8-5.9]	97	5.5 [-0.9-11.9]	2	9.7 [7.9-11.6]	23	**<0.001**	**<0.001**
Month 12	3.9 [2.9-4.9]	8	4.6 [4.1-5.1]	91	10.0	1	7.9 [6.2-9.6]	21	**<0.001**	**<0.001**
Weight-adjusted Tacrolimus dose (mg/day per kg b.w,)										
Day 3	0.190 [0.173-0.207]	7	0.178 [0.171-0.185]	110	0.201 [0.012-0.222]	2	0.197 [0.172-0.222]	25	0.18	-
Day 10	0.118 [0.089-0.146]	8	0.160 [0.147-0.174]	108	0.201 [0.012-0.390]	2	0.245 [0.212-0.277]	25	**<0.001**	**<0.001**
Month 1	0.094 [0.070-0.118]	8	0.143 [0.132-0.154]	111	0.108 [-0.084-0.300]	2	0.212 [0.189-0.235]	25	**<0.001**	**<0.001**
Month 3	0.072 [0.054-0.089]	9	0.093 [0.084-0.102]	104	0.093 [0.089-0.096]	2	0.152 [0.122-0.183]	26	**<0.001**	0.001
Month 6	0.062 [0.048-0.075]	8	0.074 [0.065-0.082]	96	0.085 [-0.010-0.180]	2	0.142 [0.112-0.173]	23	**<0.001**	**<0.001**
Month 12	0.058 [0.042-0.074]	8	0.062 [0.054-0.070]	90	0.155	1	0.114 [0.088-0.140]	21	**<0.001**	**<0.001**
Tacrolimus C0 (ng/mL)*										
Day 3	21.5 [14.2-32.5]	7	15.8 [14.7-17.1]	118	14.9	1	11.2 [9.1-13.7]	26	**<0.001**	**0.004**
Day 10	13.0 [10.5-16.0]	8	11.7 [11.0-12.4]	110	15.3	1	10.8 [9.3-12.5]	23	0.40	-
Month 1	12.8 [10.2-16.2]	9	12.7 [12.0-13.4]	120	12.4 [7.9-19.7]	2	11.5 [9.7-13.6]	25	0.62	-
Month 3	11.1 [8.9-13.0]	10	10.4 [9.9-11.0]	120	9.3	1	9.4 [8.4-10.5]	25	0.36	-
Month 6	9.1 [7.1-11.7]	9	8.7 [8.1-9.3]	105	9.0	1	8.2 [6.9-9.9]	20	0.90	-
Month 12	9.5 [6.4-14.0]	8	7.2 [6.6-8.0]	91	-	0	6.8 [5.1-9.0]	19	0.27	-
Tacrolimus C0/Dose (ng/mL per mg/kg)*										
Day 3	113.6 [78.4-164.7]	7	91.7 [83.9-100.3]	100	80.1	1	61.6 [53.3-71.1]	25	**<0.001**	**0.006**
Day 10	104.3 [84.5-128.9]	7	83.0 [74.6-92.3]	97	82.2	1	46.6 [39.8-54.4]	23	**<0.001**	**0.003**
Month 1	142.3 [100.9-200.6]	8	97.5 [89.3-106.3]	103	116.2 [12.3-1100.0]	2	56.2 [46.8-67.4]	25	**<0.001**	**<0.001**
Month 3	123.3 [115.9-226.2]	9	127.9 [114.9-142.3]	100	100.0	1	65.0 [52.3-80.7]	24	**<0.001**	**<0.001**
Month 6	148.3 [105.3-208.7]	8	140.2 [123.0-159.9]	86	116.1	1	62.8 [46.4-85.0]	19	**<0.001**	**0.001**
Month 12	171.8 [111.7-276.8]	8	131.2 [116.4-147.8]	80	-	0	63.0 [42.3-93.9]	18	**<0.001**	**<0.001**
Creatinine clearance										
Day 3	43.9 [16.4-71.3]	8	36.1 [31.6-40.6]	125	34.3 [-202.9-271.4]	2	34.6 [23.6-45.2]	26	0.85	-
Day 10	49.0 [25.9-72.0]	9	44.1 [39.8-48.3]	123	40.2 [-64.8-145.2]	2	50.0 [39.4-60.7]	25	0.67	-
Month 1	59.1 [40.0-78.2]	9	55.2 [51.1-59.2]	123	49.2 [19.5-78.9]	2	58.0 [48.8-67.2]	25	0.88	-
Month 3	67.6 [50.8-84.4]	10	60.0 [56.0-63.9]	118	52.5	1	60.9 [49.1-72.7]	26	0.77	-
Month 6	68.1 [50.5-85.8]	9	63.5 [59.2-67.7]	107	48.5	1	64.9 [53.6-76.1]	23	0.84	-
Month 12	60.9 [40.8-81.0]	8	65.4 [61.1-69.8]	94	45.0	1	66.1 [55.1-77.1]	20	0.75	-

* Values are expressed as geometric means with 95% confidence interval range in bracket
# The corresponding linear contrast tested the probability of a positive linear trend of the dependant variable across the ordered level of the genotype classification (a priori polynomial linear contrast test)

Patients from Group 1 and 2 had a geometric mean of Tac C_0 at day 3 above the consensus supratherapeutic threshold (15 ng/mL): 21.5 ng/mL for group 1 and 15.8 ng/mL for group 2. Logistic regression models showed that the risk of presenting supratherapeutic Tac C_0 at day 3 was significantly higher for Group 1 (OR=8.3 $CI_{95\%}$ [1.3–57.0], p=0.027) and Group 2 (OR=4.7; $CI_{95\%}$ [1.9–13.4], p=0.002), respectively, compared to Group 4 (Figure 2). Group 3 was excluded from analysis as data on Tac C_0 were available for only one patient (Tac C_0 = 14.9 ng/mL). No significant differences were observed regarding risk of Tac C_0 <10 ng/mL across the different genotype clusters (data not shown).

Figure 2. Percentage of patients within each *CYP3A* metabolizer cluster stratified by Tac C_0 levels at day 3 below or above the 15 ng/mL supratherapeutic threshold.



CYP3A4 genotype, delayed graft function, creatinine clearance and acute rejection

DGF was observed in 38/185 patients, of which two carried the CYP3A4 intron 6 T-variant allele. No statistically significant difference in incidence of DGF between variant carriers and CC patients was observed (χ^2=0.12, p= 0.72). Also no significant differences in creatinine clearance were observed between variant carriers and CC patients (table 2). No differences were observed regarding creatinine clearance or incidence of DGF between groups of combined genotypes for CYP3A4/5 SNPs (table 4 and 5, respectively). Biopsy proven acute rejection (BPAR) occurred in 37/185 patients, of which 4 carried the CYP3A4 intron 6 variant, but no statistically significant differences in BPAR incidence between variant carriers and CC patients was observed (χ^2=1.42, p=0.23). Similarly, we did not find any statistically significant differences in the incidence of either DGF or BPAR among different clusters of generated genotypes according to CYP3A4 intron 6 and CYP3A5*3 allelic status (χ^2=0.51 and 4.52, p=0.96 and 0.18, respectively).

DISCUSSION

In this study, we showed for the first time that the new *CYP3A4* intron 6 C>T SNP (rs35599367) is associated with a lower Tac dose requirement, in agreement with the reduced function of this CYP3A4 variant and thus expected reduced clearance of Tac [6]. We demonstrate that *de novo* kidney transplant recipients who carry at least one T-variant allele required significantly lower Tac doses to reach target Tac C_0 when compared to homozygous wild-type patients. During the first year after transplantation, T variant allele carriers required on average 33% lower Tac dose than homozygous CC patients.

Our findings are in agreement with the recent report of Wang *et al.* addressing the functional defect caused by this SNP [6]. These authors showed that the intron 6 C>T SNP rs35599367 is significantly linked to reduced *CYP3A4* mRNA expression and enzyme activity in human livers, being thus far the only CYP3A4 SNP with relative high allele frequency in Caucasians showing such a large effect. A recent paper by Jacobson *et. al* described three other *CYP3A4* polymorphisms with respect to Tac pharmacokinetics [31], but these were only observed in Africans. In our study, only eight patients were of African origin, which is why we did not include the Jacobson SNPs.

Using *CYP3A4/5* combined genotypes, the significance of the observed effects on Tac pharmacokinetics is increased compared to the *CYP3A4* or *CYP3A5* genotype alone. This effect was allele-dose dependent and the magnitude was quantitatively influenced by genotype classification: Tac dose requirement was lowest for poor metabolizers, followed by intermediate-1, intermediate-2 and then extensive metabolizers at all time points (p=0.001), except day 3 (p=0.78). This latter observation reflects that at this time point, no dose adjustments were made based on TDM and dosing thus was independent from genotype or metabolizer status. Similarly, Tac dose-adjusted C_0 was significantly affected

by CYP3A4/5 combined genotype and followed the order extensive < intermediate-2 < intermediate-1 < poor metabolizers, demonstrating that poor metabolizers require lower doses to achieve target Tac C_0 when compared to other groups at all time points, also at day 3 after transplantation. This suggests that this classification might lead to a better prediction of optimal Tac starting dose for immunosuppressive therapy.

The risk of supratherapeutic Tac C_0 (>15 ng/mL) on day 3 was significantly higher for poor and intermediate-1 metabolizers when compared to extensive metabolizers. This was even more pronounced among poor metabolizers compared to intermediate-1 metabolizers. We observed that both poor and intermediate-1 metabolizers had mean Tac C_0 at day 3 >15 ng/mL (21.5 and 15.8 ng/mL, respectively). We have reported earlier that a significantly larger proportion of patients carrying the *CYP3A5*1* allele had Tac C_0 <10 ng/mL [26]. When genetic status for *CYP3A4* intron 6 SNP was taken into account, no significant differences were observed regarding the risk of presenting Tac concentration below this threshold (data not shown). However, as recently suggested by several authors, it is likely that clinicians are able to target C_0 above this threshold rapidly after transplantation by performing simple concentration-controlled Tac dose adjustments without consideration of *CYP3A5* status [20]. In the present study, 15% of patients presented Tac C_0 <10 ng/mL at day 3, whereas 2 patients had Tac C_0 <5 ng/mL. Approximately 50% of patients presented Tac C_0 above 15 ng/mL. Neither subtherapeutic Tac C_0 at day 3 nor *CYP3A5*1* allele were associated with BPAR within 1 month after surgery (data not shown), in accordance with previous studies [16,26, 32-34]. We found that relatively many patients (50%) overshooted the Tac exposure upper limit and relatively few patients (15%) had Tac exposures <10 ng/mL, indicating that overexposure is a more frequently encountered problem than underexposure. This may be especially relevant in patients suffering from DGF. Regarding *ABCB1*, we found the 3435C>T SNP independently associated with Tac dose-adjusted C_0. The influence of this SNP (14.3% and 20.9% lower for heterozygotes and homozygotes, respectively) was modest when compared to the effects of *CYP3A4* and *CYP3A5* polymorphisms, and disappeared in a mixed-model analysis in which *CYP3A4* and *CYP3A5* genotype were included. The relatively minor contribution of *ABCB1* polymorphism on Tac pharmacokinetics is in line with previous investigations [35-37].

The present study has limitations. Although most participating centers used immunoassays to measure Tac concentrations, some centers applied LC-MS/MS. In an additional mixed-model analysis in which we adjusted for Tac assay, the effect of *CYP3A4* intron 6 genotype was still significant, both for Tac daily dose (-20% for T-variant allele carriers, p=0.007) as for Tac dose-adjusted C_0 (+37% for T-variant allele carriers, p<0.001). Secondly, corticosteroids are known to influence Tac exposure [38-39]. As corticosteroid tapering was recommended but not mandatory, there may have been differences in corticosteroid regimens used in the different centers. This may have influenced the analysis. If all patients had been treated with the same dose, the influence of genotype may have been stronger through reduction of uncontrolled variability generated by different tapering regimens. The corticosteroid dose could unfortunately not be included in the mixed-model analysis because different

formulations with different immunosuppressive potencies were used. Third, diabetic gastro-intestinal motility disorders can affect Tac pharmacokinetics. Although diabetic gastropathy may alter the curve of the Tac AUC during a dosing interval, Tac predose concentrations are in general much less affected [40] and we therefore believe that the influence of diabetes on the outcomes of the present study is limited. Fourth, there were occasionally data missing, which increased with follow-up. To overcome this, we have performed mixed-model analysis, which compensates for missing records. Nevertheless, a more complete data set may have provided stronger associations. Finally, we realize that our findings are significant with a 95% confidence level. Therefore, our results need to be confirmed with independent cohorts.

In conclusion, we have shown that the new genetic CYP3A4 intron 6 polymorphism 6 was associated with reduced Tac clearance in our patient cohort. Therefore, pretransplantation genotyping of the *CYP3A4* intron 6 C>T SNP, along with *CYP3A5*3*, could potentially benefit patients by reducing initial Tac doses among CYP3A poor metabolizers and thereby reduce the risk of reaching supratherapeutic Tac concentrations.

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Chapter 2.3

The new CYP3A4 intron 6 C>T polymorphism (CYP3A4*22) is associated with an increased risk of delayed graft function and worse renal function in cyclosporine-treated kidney transplant patients

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ABSTRACT

Objective Cyclosporine A (CsA) is a substrate of cytochrome P450 3A4 (CYP3A4). Recently, a newly discovered SNP in *CYP3A4* (rs35599367 C>T), defining the *CYP3A4*22* allele, has been linked to reduced hepatic expression and activity of CYP3A4. In the present study, the clinical impact of this SNP was investigated in a cohort of patients receiving a CsA-based immunosuppressive regimen.

Materials and methods 172 *de novo* kidney transplant recipients, receiving CsA/MMF as immunosuppressive therapy and participating in the FDCC study, were genotyped for the new *CYP3A4*22* allele. CsA C_0 and/or C_2 levels were measured on days 3 and 10, and on months 1, 3, 6 and 12 after transplantation. Plasma creatinine concentrations, delayed graft function (DGF) and biopsy-proven acute rejection (BPAR) were recorded.

Results The *CYP3A4*22* allele was significantly associated with a higher risk of DGF compared to *CYP3A4*1/*1* patients after adjustment for known risk factors (odds ratio (OR)=6.34, confidence interval ($CI_{95\%}$) (1.38-29.3, p=0.015). Mixed-model analysis demonstrated that the overall creatinine clearance was 20% lower in *CYP3A4*22* allele carriers compared to *CYP3A4*1/*1* patients [$CI_{95\%}$ (-33.1 to -7.2%, p=0.002]. For *ABCB1* 3435C>T, T-variant carriers had a decreased risk of developing DGF compared to CC patients (CT: OR=0.30, $CI_{95\%}$ [0.11-0.77], p=0.011; TT: OR=0.18, $CI_{95\%}$ [0.05-0.67], p=0.011).

Conclusion The *CYP3A4*22* constitutes a risk factor for DGF and worse creatinine clearance in patients receiving CsA-based immunosuppressive therapy. Therefore, pre-transplant genotyping for this *CYP3A4*22* allele might help clinicians to identify patients at risk of DGF and poor renal function when treated with CsA.

INTRODUCTION

The clinical use of Cyclosporine A (CsA) in suppressing the immune response is hampered by its narrow therapeutic index and its highly variable and relatively unpredictable pharmacokinetics [1]. Therapeutic drug monitoring has therefore been adopted by most transplant physicians as a means to improve the efficacy of CsA treatment and to reduce toxicity. CsA is extensively metabolized by CYP3A [2], but drug transporters are also involved in the disposition of CsA. One of them is ABCB1 (or P-glycoprotein), encoded by the *ABCB1* gene [3].

CYP3A4 is the most abundant CYP enzyme in human liver and intestine and is responsible for the oxidative metabolism of approximately 45% to 60% of all currently prescribed drugs [4]. CYP3A4 activity and protein content vary widely in the population, with a 10 to 100-fold inter-individual variability [5-8]. Studies aiming to unravel the genetic basis of variable CYP3A4 expression and/or activity failed thus far to identify nonsynonymous single-nucleotide polymorphisms (SNP) that could explain these inter-individual differences in pharmacokinetics. Genetic variants of *CYP3A4* that change amino acids are rare [9] and therefore can only account for a small portion of the observed variability. The most common *CYP3A4* variant allele is a promoter variant: *CYP3A4*1B*. This first described *CYP3A4* variant allele contains a nucleotide variation in the putative nifedipine response element [10]. The *CYP3A4*1B* allele was in initial studies associated with an increased CYP3A4 activity, but subsequent studies reported inconsistent results [5, 10-13].

A recent study identified a new functional SNP located in *CYP3A4* intron 6 (rs35599367 C>T), defining the *CYP3A4*22* allele (http://www.cypalleles.ki.se/), for which the T-variant allele was associated with both decreased hepatic *CYP3A4* mRNA expression and with decreased CYP3A4 enzymatic activity [14]. This SNP also proved to have clinical implications, since it correlated with statin dose requirement for optimal lipid level control [14]. The *CYP3A4*22* allele is found with a relatively high allelic frequency of 3-4% in the Caucasian population, strengthening its potential contribution to differential CYP3A4 activity. We have previously demonstrated in two independent studies that the *CYP3A4*22* allele might be clinically relevant when considering calcineurin inhibitor (CNI)-based therapy. We observed that kidney transplant recipients who carried the variant allele showed higher dose-adjusted CNI predose concentrations and were at a higher risk of reaching supratherapeutic whole-blood concentrations [15-16]. The goal of the present study was to validate the effect of the novel *CYP3A4*22* allele on CsA exposure, on dose requirement, and on efficacy/toxicity in a large cohort of 172 *de novo* kidney transplant patients participating in a prospective clinical trial.

MATERIALS AND METHODS

Patients and study design

Patients were *de novo* kidney transplant recipients who participated in a phase IV, open, prospective, randomized, controlled, international, multi-center trial comparing fixed-dose mycophenolate mofetil (MMF) treatment with concentration-controlled treatment (the FDCC trial) [17]. At the initiation of the study, a pharmacogenetic substudy was started. Pharmacogenetic results regarding the role of *UGT1A9* polymorphisms on MMF metabolism [18], the role of *UGT2B7* polymorphisms on acyl-glucuronide metabolite of mycophenolic acid (AcylMPAG) plasma concentrations [19], and the role of *CYP3A5* and *ABCB1* polymorphisms on tacrolimus (Tac) and CsA exposure and the risk of acute rejection [20-21] have been published earlier. All patients provided 2 written informed consents, one for the original FDCC study and one for the pharmacogenetic substudy. The FDCC and the pharmacogenetic (sub)study were approved by the ethics committees of all participating centers and relevant authorities in the participating countries. In the FDCC study, immunosuppressive therapy consisted of a CNI, MMF and corticosteroids. The choice of CsA or Tac and the target blood concentrations were in accordance with each center's protocol. Standard CNI and corticosteroid tapering regimens were left to the discretion of the investigators. For this pharmacogenetic study, genetic data were available for 172 kidney transplant recipients treated with CsA. However, pharmacokinetic data concerning CsA measurements and/or dosage were not available for all patients at all time points of follow up. CsA predose concentrations (C_0) and/or 2 hours after drug administration (C_2) were measured on days 3 and 10 and on months 1, 3, 6, and 12 after transplantation, and whenever deemed necessary by the attending physician. In the FDCC study, donor DNA was not collected and no protocol kidney biopsies were performed. Creatinine clearance was calculated by the Cockcroft-Gault formula [22]. Delayed graft function (DGF) was defined as the need for dialysis within the first week after transplantation. Biopsy-proven acute rejection (BPAR) was defined as any histologically confirmed episode for which a Banff score of 1 (mild, grades IA and IIA), 2 (moderate, grades IB and IIB), or 3 (severe, grade III) was recorded. The patients with panel reactive antibodies (PRA) above 50% within 6 months before study entry were excluded from the study.

Drug concentration measurements

CsA C_0 and C_2 were determined in whole blood in local laboratories in each of the participating centers by the use of immunoassays [CsA FPIA assay (AxSYM Abbott Laboratories) and Emit 2000 (Syva Company, Dade Behring Inc., Cupertino, CA)]. Proficiency testing was ensured by participation of all centers in the United Kingdom Quality Assessment Scheme (Prof. Holt, St George's, University of London, London, United Kingdom). Dose-adjusted CsA concentrations were calculated by dividing the C_0 or the C_2 by the corresponding 24-h dose on a milligrams per kilogram basis.

Genotype analysis

Genomic DNA was isolated from 200 µl ethylenediaminetetraacetic acid-treated whole blood using a MagnaPure LC (Roche Diagnostics GmbH, Mannheim, Germany). Allelic discrimination analysis was performed for the determination of *CYP3A4*22* allelic status (rs35599367). Allelic discrimination reaction was realized using TaqMan® (Applied Biosystems, CA, USA) genotyping assays (C__59013445_10) on the ABI PRISM 7500® Fast real-time PCR Systems (Applied Biosystems, CA, USA). The PCR cycle consisted of an initial step of 1 min at 60 °C, followed by a denaturation step at 95 °C for 30 sec and 40 cycles with 95°C for 3 sec and 60°C for 30 sec. The final post PCR read was made in 1 min at 60°C. The volume for each reaction was 11 µl, consisting of 5 µl TaqMan® GTXpress™ Master Mix, 0.125 µl of TaqMan® SNP genotyping assay (80x), containing the primers (64 µM) and the probes (16 µM), and 10ng genomic DNA. *CYP3A5*3, CYP3A4*1B* and *ABCB1* 3435C>T analysis were performed as described earlier [20].

Statistical analysis

Statistical analyses were performed using the Predictive Analytics Software (PASW) statistics, version 17.0 for Windows (IL, USA). CsA C_0 and dose-adjusted C_0 were normalized by a logarithmic transformation. The log and other pharmacokinetic parameters distributions were then confirmed to be normally distributed by the Kolmogorov–Smirnov test. As only 2 groups of genotype were compared, Student's independent *t*-tests were performed to compare means between groups at a single time point under the null hypothesis that the means of the compared groups were equal. For the univariate analysis of the association between categorical data (*e.g.* the incidence of acute rejection) we used Pearson's Chi Square test or Fisher's exact test, as appropriate. To estimate the overall effect of genotype on the outcome variables, mixed-model analysis were performed. Creatinine clearance was compared between genotypes by mixed-model based on the maximum likelihood ratio with the patient genotype status as fixed factors and the time after transplantation as repeated measurement. Sex, ethnicity and age of the donor and the recipient were introduced as random effects in the model in order to adjust the model for these co-variables. No structure was imposed on the variances and on the covariances between and within times of follow up of the repeated CsA measurements. Also, it was assumed that levels of covariables are uncorrelated and have a constant variance across time of follow up. Multiple logistic regression analysis was carried out to assess the influence of genotypes and other co-variables on the risk of DGF. Genotype-specific odds ratio (OR) and 95% confidence intervals were computed using backward stepwise analysis based on the maximum likelihood ratio with a p-value of less than 0.05 being considered statistically significant for entry and a p-value of less than 0.10 for remaining in the model. For these analyses, each genotype was coded as a distinct 'dummy variable'. Potential confounders considered for these analyses included sex, age of the donor and the recipient, primary kidney disease, PRA, and cold ischemia time. Subsequently, we also ran the analysis by taking into account the potential influence of other functional SNPs including *CYP3A4*1B*, *CYP3A5*3* and *ABCB1* 3435C>T.

RESULTS

CYP3A4 intron 6 genotype, CsA exposure, and clinical outcomes

The characteristics of the 172 patients included in the study are reported in Table 1. Of these patients, 161 had the *CYP3A4*1/*1* genotype, 11 were heterozygous for the *CYP3A4*22* variant allele, whereas no variant homozygotes were found. In total, this gives an allelic frequency of 3.2%. The observed genotype distribution was in accordance with Hardy-Weinberg equilibrium ($\chi^2 = 0.19$; $p = 0.66$).

Table 1: Demographics of the study population

	All patients	*CYP3A4*1/*1*	*CYP3A4*22* carriers	p-value
N	**172**	**161**	**11**	**-**
Sex (male/female)	106 (61.6%)/66 (38.4%)	96 (59.6%)/65 (40.4%)	10 (90.9%)/1 (9.1%)	**0.053**
Age (year)[a]	49 ± 12	49 ± 12	52 ± 13	0.47
Weight (kg)[a]	73 ± 15	73 ± 15	76 ± 10	0.43
Transplantation number				0.04
First	159 (92.4%)	151 (93.8%)	8 (72.7%)	
Second	13 (7.6%)	10 (6.2%)	3 (27.3%)	
Living/deceased donor	43 (25.0%)/129 (75.0%)	40 (24.8%)/121 (75.2%)	3 (27.3%)/8 (72.7%)	1.0
FD/CC MMF therapy	93 (54.1%)/79 (45.9%)	87 (54.0%)/74 (46.0%)	6 (54.5%)/5 (45.5%)	1.0
Induction therapy[b]	97 (56.4%)	93 (57.8%)	4 (36.4%)	0.21
Primary kidney disease				0.47
Diabetic nephropathy	9 (5.2%)	9 (5.6%)	0 (0.0%)	-
Glomerulonephritis	48 (27.9%)	46 (28.6%)	2 (18.2%)	-
Obstructive/reflux nephropathy	12 (7.0%)	12 (7.0%)	0 (0.0%)	-
Polycystic kidney disease	31 (18.0 %)	27 (16.8%)	4 (36.4%)	-
Pyelonephritis/interstitial nephritis	10 (2.9%)	8 (2.5%)	2 (9.1%)	-
Hypertensive nephropathy	17 (9.9%)	16 (9.9%)	1 (9.1%)	-
Other	36 (20.9%)	34 (21.1%)	2 (18.2 %)	-
Unknown	9 (5.2%)	9 (5.6%)	0 (0.0%)	-
HLA mismatches[c]	2.8 (3.0)	2.9 (3.0)	2.5 (3.0)	0.49
Panel reactive antibodies				
<10% / ≥10 %	160 (93.0%)/12 (7.0%)	150 (93.2%)/11 (6.8%)	10 (90.9%)/1 (9.1%)	0.57
Ethnicity				1.0
Asian	6 (3.5%)	6 (3.7%)	0 (0.0%)	-
Black	3 (1.7%)	3 (1.9%)	0 (0.0%)	-
Caucasian	151 (87.8%)	140 (87.0%)	11 (100.0%)	-
Other	12 (7.0%)	12 (7.4%)	0 (0.0%)	-
Cold ischemia time (hr)	13.1 ± 8.6	13.2 ± 8.8	9.8 ± 6.0	0.22
Donor age (year)	47.7 ± 14.9	47.8 ± 14.8	45.3 ± 17.6	0.58

We did not observe any linkage disequilibrium between the *CYP3A4*22* and either the *CYP3A4*1B* or the *CYP3A5*3* allele (χ^2=0.46 and 2.6; p=0.73 and 0.25, D=2.4x10^{-3} and 4.1x10^{-3}; r^2=2.6x10^{-3} and 4.8x10^{-3}, respectively). (Because of low minor allelic frequencies for the three SNPs, only 3 gametic types are found in the population. As a consequence, Lewontin D' is equal to 1 for both LD assessments and there is thus no need to invoke a low recombination rate and a complete LD to explain that value [23]).

Table 2: Cyclosporine dose, predose (C_0) and 2 hours after drug administration (C_2) concentrations, dose-corrected C_0 (C_0/Dose) and C_2 (C_2/Dose) according to the *CYP3A4*22* allelic status.

Cyclosporine dose (mg/day)	CYP3A4*1/*1	n	CYP3A4*22 carriers	n	p-value
Day 3	537 [499-574]	156	545 [389-670]	11	0.91
Day 10	495 [463-526]	151	516 [377-655]	11	0.73
Month 1	374 [354-394]	131	344 [248-441]	9	0.47
Month 3	255 [240-270]	108	250 [176-324]	6	0.87
Month 6	230 [217-244]	98	230 [196-264]	5	0.99
Month 12	213 [202-225]	93	210 [193-227]	5	0.90
Cyclosporine C_0 (ng/ml)*					
Day 3	276 [251-303]	118	283 [211-380]	11	0.87
Day 10	278 [253-305]	122	282.9 [198-405]	10	0.92
Month 1	244 [223-267]	103	269 [189-382]	8	0.56
Month 3	158 [145-173]	82	158 [72-346]	6	1.00
Month 6	136 [124-148]	75	123 [107-142]	5	0.57
Month 12	128 [117-140]	69	145 [120-175]	5	0.47
Cyclosporine C_2 (ng/ml)					
Day 3	1122 [1031-1212]	99	1331 [816-1846]	7	0.25
Day 10	1334 [1240-1428]	113	1680 [1040-2320]	6	0.11
Month 1	1299 [1200-1399]	101	1188 [486-1890]	5	0.63
Month 3	938 [856-1021]	86	919 [405-1434]	5	0.92
Month 6	847 [782-913]	80	796 [593-998]	5	0.70
Month 12	767 [708-827]	71	858 [621-1095]	5	0.43
Cyclosporine C_0/Dose (ng/ml per mg/kg)*					
Day 3	37 [34-40]	116	43 [33-55]	11	0.27
Day 10	42 [39-45]	120	45 [39-51]	10	0.65
Month 1	50 [45-55]	103	59 [44-80]	8	0.36
Month 3	47 [43-51]	82	48 [26-89]	6	0.90
Month 6	44 [40-49]	75	41 [28-59]	5	0.61
Month 12	46 [41-51]	69	52 [38-71]	5	0.54
Cyclosporine C_2/Dose (ng/ml per mg/kg)					
Day 3	148 [136-160]	99	154 [101-206]	7	0.82
Day 10	194 [180-207]	112	203 [138-268]	6	0.76
Month 1	254 [234-273]	101	203 [86-320]	5	0.26
Month 3	264 [240-289]	86	251 [137-366]	5	0.80
Month 6	264 [243-285]	80	268 [166-370]	5	0.92
Month 12	261 [239-283]	71	309 [210-408]	5	0.27
Creatinine clearance (ml/min)					
Day 3	32 [28-36]	160	28 [11-46]	11	0.67
Day 10	46 [42-51]	154	45 [24-65]	11	0.89
Month 1	85 [55-62]	131	53 [37-69]	9	0.47
Month 3	66 [62-70]	106	43 [35-51]	6	**0.007**
Month 6	67 [63-71]	98	53 [37-70]	5	0.15
Month 12	68 [64-73]	93	49 [37-61]	5	0.06

* Values are expressed as geometric means with 95% confidence interval range in brackets

All CsA pharmacokinetic parameters were comparable between the two *CYP3A4*22* genotype groups at all time points (Table 2). Regarding dose-adjusted parameters, similar results were observed without weight normalization (data not shown).

Biopsy-proven acute rejection occurred in 28 of the 172 patients (16%). Of these 28 patients, three carried a *CYP3A4*22* allele, whereas the remaining 25 were homozygous wild-type. No statistically significant difference in the incidence of BPAR between variant

allele carriers and homozygotes for the reference allele was observed (χ^2=1.04, p=0.39, Table 3).

Table 3: Influence of *CYP3A4*22* allele on delayed graft function (DGF) and biopsy-proven acute rejection (BPAR).

		*CYP3A4*1/*1*	*CYP3A4*22* carriers	χ^2	p-value
Delayed Graft Function (DGF)	**Yes**	39	5	2.44	0.15
	No	122	6		
Biopsy Proven Acute Rejection (BPAR)	**Yes**	25	3	1.04	0.39
	No	136	8		

Delayed graft function was observed in 44 of the 172 patients (26%), of which five carried a *CYP3A4* intron 6 variant allele. The difference observed in the proportion of patients experiencing DGF between T-allele variant carriers (46%) and homozygotes for the reference allele (24%) groups was in itself not significant (χ^2=2.44, p=0.15, OR=2.6, Table 3). However, when multiple binary logistic regression analysis was performed to assess the influence of patient genotype along with different co-variables [gender, age of the donor and the recipient, primary kidney disease, PRA, and cold ischemia time], the risk of experiencing DGF was significantly higher in patients that carried the *CYP3A4*22* variant allele compared to patients homozygous for CC (OR=6.34, $CI_{95\%}$ [1.38-29.3], p=0.015, Table 4). In addition, we observed that the carriers of a T-allele for the *ABCB1* 3435C>T SNP in *ABCB1* were less at risk of developing DGF than homozygous patients for the C-allele (OR=0.30 for CT, $CI_{95\%}$ [0.11-0.77], p=0.011, and OR=0.18 for TT, $CI_{95\%}$ [0.05-0.67], p=0.011, Table 4). By contrast, neither *CYP3A4*1B* nor *CYP3A5*3* allelic status was associated with the risk of DGF. In the logistic regression model, patients with PRA >10% had a significantly higher risk of developing DGF compared to patients with PRA <10% (OR=9.63, $CI_{95\%}$ [2.02-45.9], p=0.005, Table 4). Finally, longer cold ischemia time (OR=1.14, $CI_{95\%}$ [1.06-1.21], p<0.001) and higher donor age (OR=1.04, $CI_{95\%}$ [1.01-1.08], p=0.006, Table 4) were significantly related to the risk of DGF. The risk of DGF was not influenced by CsA C_0 at day 3, recipient age, gender or primary kidney disease. By contrast, it was significantly correlated with the CsA C_2 levels, with higher concentrations being protective (OR=0.997 $CI_{95\%}$=[0.996-0.998], p<0.001). When all non-significant potential confounders (age of the recipient, sex and primary kidney disease) along with CsA C_2 levels were forced into the logistic regression model, it did not modify the association between *CYP3A4*22* allele and the risk of DGF, which was still higher in carriers of the *CYP3A4*22* allele with an OR of 7.5 CI95%=[0.65-87.5] but no more significant (p=0.11), maybe because C_2 levels were only available for 7 of the 11 *CYP3A4*22* allele carriers. There is evidence that a prior transplant may predispose to DGF [24] and because among *CYP3A4*22* variant allele carriers significantly more patients received a second renal transplant as compared with the homozygous wild-type group (p=0.04, Table 1), this may have confounded our results.

However, no association was observed between the number of prior transplants and the risk of DGF, neither in the univariate nor in the multiple logistic regression models (data not shown). Consistently, when we excluded recipients of a second transplant from our analysis, the increased risk of DGF among patients carrying the *CYP3A4*22* variant allele was still observed (p=0.03) with an OR of 7.66 ($CI_{95\%}$=[1.24-47.4]).

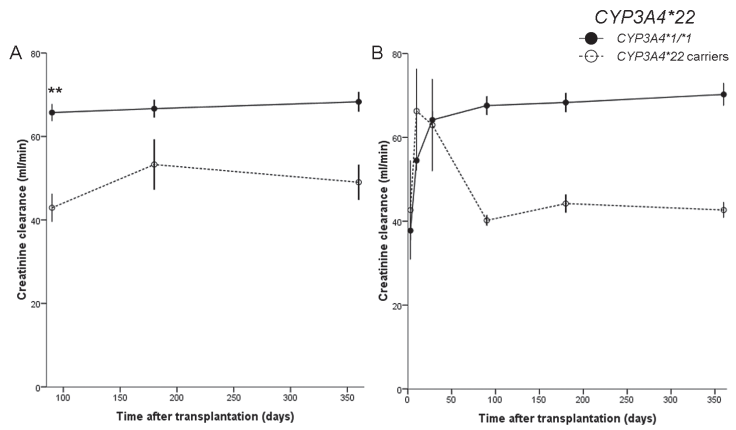
Table 4: Multiple logistic regression modeling for the risk of presenting DGF

Risk factor	OR	$CI_{95\%}$	p-value
Reference class*	1	-	-
CYP3A4 intron 6 CT	6.34	[1.38-29.3]	0.015
ABCB1 3435CT	0.30	[0.11-0.77]	0.013
ABCB1 3435TT	0.18	[0.05-0.67]	0.011
PRA >10%	9.63	[2.02-45.9]	0.005
Donor age	1.04	[1.01-1.08]	0.006
Cold ischemia time	1.14	[1.06-1.21]	<0.001

* *CYP3A4* intron 6 CC, *ABCB1* 3435CC, Panel Reactive Antibodies <10%, Donor age 47.6, Cold ischemia time 13.1 hours.

Renal function, assessed by calculation of creatinine clearance, was statistically significantly lower among *CYP3A4*22* allele carriers at 3 months of follow-up compared with wildtype patients (p=0.006, Table 2). This difference did not remain significant at later time points (p=0.15 and 0.06 at months 6 and 12, respectively). In the FDCC trial the duration of DGF was not systematically recorded. Therefore, we chose to study renal function of the whole cohort from month 3 onwards as by that time, most patients will likely have recovered from DGF or their grafts will have been considered as “never functioning”. Creatinine clearance values at earlier time points (*i.e.* <3 months) may reflect the influence of dialysis in cases of DGF and were therefore not considered for the analysis. Repeated measurement in mixed-model analysis demonstrated that the overall creatinine clearance was 26% lower in *CYP3A4*22* variant allele carriers when considering data from the third month onwards ($CI_{95\%}$ [-41.3 to -11.3%], p=0.001. Figure 1A). We also performed the analyses considering the entire 12-month study period after excluding all patients that experienced DGF. Results showed that the creatinine clearance did not differ between the two *CYP3A4* genotype groups at the individual time points anymore (p=0.06 at month 3 and p=0.10 at month 6 and 12). The overall creatinine clearance throughout the first year after transplantation remained 13% lower in DGF-free *CYP3A4*22* variant allele carriers although this difference was not statistically significantly different (p=0.13, Figure 1B).

Figure 1: Creatinine clearance (mL/min) according to the genetic status of patients for *CYP3A4*22* allelic status when considering (A) late follow-up time points in all patients and (B) the entire follow-up time in DGF-free patients only. Data are represented as means (±SD). **=p<0.01. Open circles: *CYP3A4*22* allele carriers, filled circles: *CYP3A4*1/*1* homozygotesDISCUSSION



Our study is the first retrospective analysis investigating the association between the newly discovered *CYP3A4* intron 6 rs35599367 C>T SNP (*CYP3A4*22*), CsA exposure and response to CsA therapy in a prospective trial. We show here that the *CYP3A4*22* variant allele is a risk factor for DGF and poorer renal function during CsA treatment. As the *CYP3A4*22* allele is not in substantial LD with any Hapmap SNPs and is not referred to in Hapmap, association studies using tag SNPs failed to correlate this SNP with differential CYP3A4 clinical phenotypes. Given the fact that genome-wide human SNP arrays only contain SNPs that have a minor allele frequency of more than 5%, the impact of the *CYP3A4*22* allele will not be detected in GWAS using these genotyping tools.

We hypothesize that the higher risk of DGF in carriers of the *CYP3A4*22* allelic variant is real and the consequence of a reduced CYP3A4 activity. The *CYP3A4*22* variant allele has been functionally linked to reduced *CYP3A4* mRNA expression and 6β-testosterone hydroxylation in human liver microsomes [14]. The exact mechanism underlying the increased DGF risk of *CYP3A4*22* carriers is at present unclear. Although the nephrotoxicity of CsA is well-known, its contribution to the occurrence of DGF after kidney transplantation is controversial. Indeed, in the BENEFIT trial, no differences in the incidence of DGF were observed between patients treated with CsA and patients who were treated with a CsA-free protocol [25]. In agreement with those results, we showed here an inverse relationship between CsA C_2 levels and the risk of DGF, which might be explained by the fact that the physician would have decided to initially lower the CsA dose for patients considered as at higher risk of DGF but, hence, argues against an association between CsA exposure and risk of DGF (*i.e.* lower is the CsA $C_{2,}$ higher is the risk). Therefore, the higher risk of DGF observed in *CYP3A4*22* carriers may not have been caused by an altered metabolism of CsA. CYP3A4 plays a role in oxidative stress and its activity is related to the production of reactive oxygen species (ROS). Therefore, one explanation might be that the reduced

CYP3A4 expression of *CYP3A4*22* carriers leads to differential levels of ROS. This could eventually lead to a worse outcome of ischemia-reperfusion injury and a higher risk of DGF, although this remains highly speculative at this moment. On the other hand, a recent meta-analysis that aimed to identify the risks and benefits of reduced CNI exposure by pooling data from 56 studies (including the above-mentioned BENEFIT trial) demonstrated that CNI-sparing strategies were associated with a reduction in DGF rate [26]. Therefore, despite the fact that the meta-analysis was conducted irrespective of the CNI used (*i.e.* Tac or CsA), we cannot exclude that the higher risk of DGF in *CYP3A4*22* allele carriers was the consequence of an altered CYP3A4-mediated oxidative metabolism of CsA.

In addition to a higher risk of DGF, *CYP3A4*22* variant allele carriers had a poorer renal function. In contrast to DGF, there is considerable evidence that CsA exposure has an impact on long term kidney allograft function [27]. Indeed, in the BENEFIT trials, it was shown that renal function was better in patients receiving belatacept and no CsA as compared to patients who did receive CsA [25, 28-29]. These observations may seem contradictory to our results as, in the present study, the reduced renal function (and higher risk of DGF) of *CYP3A4*22* allele carriers was not explained by differences in CsA exposure between the different genotype groups. Nonetheless, there remains a possibility that differences may have existed between the *CYP3A4*22* genotype groups in terms of CsA systemic exposure. We showed previously for the *CYP3A4*1B* allele, that a patient's genotype does affect CsA oral clearance (as determined from an area-under the concentration *versus* time curve), which was not apparent when C_0 or C_2 were studied [30]. If overexposure to CsA did indeed exist, one would expect an increased incidence of other CsA-related adverse events to have occurred in association with the *CYP3A4*22* allele, as well. Blood pressure and lipid levels were not systematically recorded in the FDCC trial. However, when studying the risk of opportunistic infections in binary logistic regression, we observed a trend towards an increased risk of experiencing an opportunistic infection in *CYP3A4*22* carriers (OR=2.9, p=0.09, $CI_{95\%}$ [0.85-10.0]). This argues in favour of our hypothesis that patients carrying the *CYP3A4*22* SNP allele may have been exposed to higher CsA levels and may have suffered from over-immunosuppression.

In addition, CsA levels measured by immunoassays should be interpreted with caution, as these assays are known to cross-react with CsA metabolites [31]. It is possible that the *CYP3A4*22* allele influenced the ratio of CsA to its metabolites which was not detected because of these analytical issues. A reduced CsA metabolite production in *CYP3A4*22* carriers would result in higher levels of the parent drug, which is considered to exert the nephrotoxic effects unlike its metabolites [32-36].

The poorer long-term renal function of *CYP3A4*22* carriers may have resulted from their increased occurrence of DGF. However, we feel that these two outcome variables were independently related to *CYP3A4* genotype. First, we showed that, when only later time points were considered (*i.e.* when kidney function for patients that experienced DGF likely had recovered), the creatinine clearance was significantly lower for *CYP3A4*22* carriers. Secondly, when patients experiencing DGF were excluded from the analysis, the overall

creatinine clearance remained poorer in *CYP3A4*22* carriers although the difference between the two genotype groups was numerically smaller and no longer statistically significantly different. This may have resulted from a loss of statistical power as 5 out of 11 *CYP3A4*22* carriers suffered from DGF and the number of data available for DGF-free *CYP3A4*22* variant allele carriers decreased when time of follow-up increased (day 3: n=6; day 10: n=6; month 1: n=4, month 3, 6 and 12: n=2). Still, we believe that the association between *CYP3A4* genotype and renal function should be interpreted with caution, given the relatively low number of *CYP3A4*22* carriers and hence the possibility of a type II statistical error or spurious associations. The observed differences in renal function between the different *CYP3A4*22* genotype groups are not likely due to differences in intra-renal CYP3A4 activity, as CYP3A4 is not expressed in the kidney [37-39].

We have previously reported that *CYP3A4*22* variant allele carriers require lower Tac doses to achieve target blood concentrations and are at risk of Tac overexposure as compared with homozygous wildtype patients [15, 16]. These results are in agreement with the idea of a reduced CYP3A4-mediated metabolism in *CYP3A4*22* variant allele carriers. In contrast to the observation made in the present study, the *CYP3A4*22* allele was associated with higher CsA dose-adjusted predose concentrations in a cross-sectional study of stable renal transplant [16]. These discrepancies might be ascribed to differences in study design. In our previous study, only 50 patients were included and no more than 5 *CYP3A4*22* carriers were identified. In addition to the restricted cross-sectional design, the small size of studied groups could have increased the probability of a spurious association. However, and in line with the current study, we observed in our previous cross-sectional study that the creatinine clearance was 15.5% lower in patients harboring a *CYP3A4*22* allele [16].

For *ABCB1* 3435C>T, multivariate analysis showed that the T-variant allele is protective for DGF. As the T-allele has been previously associated with decreased expression of ABCB1 in different organs [40-43], our hypothesis is that patients carrying the T-allele accumulate more CsA in body compartments (other than the kidney) where ABCB1 is expressed. The transplanted kidney, which expresses the donor genotype, would then be protected from drug overexposure. Indeed, it has been shown by Hauser *et al.* that the *ABCB1* genotype of the kidney donor was a risk factor for CsA-related nephrotoxicity [44]. Unfortunately, donor DNA was not collected in the present study. In contrast to our results, Cattaneo *et al.* [45] showed that CsA-treated kidney transplant recipients carrying the 3435T-allelic variant had a three-fold increased risk for DGF and a trend to a slower recovery of renal function than patients homozygous for the C-allele. Their hypothesis was that oxidative stress in grafts transplanted in carriers of the 3435T allele could have been amplified by ROS generated in increased amount by infiltrating leukocytes. The underlying assumption is that intra-leukocyte CsA concentrations are increased in 3435T allele carriers. However, Ansermot *et al.* did not detect any significant difference in wildtype and variant haplotype carriers for 2677G>T/A and 3435C>T SNPs when considering CsA t_{max}, $t_{1/2}$, C_{max} and AUC_{0-24} in peripheral blood mononuclear cells (PBMCs) [46]. The observation arising from our study is corroborated by the data reported earlier by Fellay *et al*. [47] on the influence of the *ABCB1* 3435C>T genotype on anti-HIV drugs distribution. We have previously demonstrated that when *CYP3A4*22* allelic status was not taken into account, *ABCB1* 3435C>T SNP was no longer a significant covariate for the risk of DGF [20]. This indicates that the *ABCB1* 3435C>T recipient genotype has only a weak impact on CsA pharmacokinetics. To fully address this issue, further studies which take into account *ABCB1* genotype of the donor, along with the *CYP3A4*22* genotype of the recipient, should be performed. Finally, we confirmed the results of previous reports showing that donor age and longer cold ischemia time were both related to the risk of developing DGF [45, 48-50].

In conclusion, we demonstrated in a large cohort of patients that the *CYP3A4* intron 6 SNP, representing the *CYP3A4*22* allele, predisposes kidney transplant recipients receiving CsA to worse renal function and is associated with an increased risk of DGF. Therefore, pre-transplant genotyping of the *CYP3A4*22* allele could potentially help the clinician to identify patients at risk of impaired renal function associated with CsA-based therapy. Prospective studies are needed to confirm this contribution of *CYP3A4*22* pharmacogenetic screening for CsA therapy.

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Chapter 2.4

Genetic polymorphisms in ABCB1 influence the pharmacodynamics of tacrolimus

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ABSTRACT

Introduction Tacrolimus has a large inter-individual pharmacokinetic variability and quantification of its effect is difficult. It is a substrate of ABCB1, an efflux-pump expressed more on CD8+ T cells than on CD4+ T cells. The *ABCB1* 3435C>T single-nucleotide polymorphism (SNP) has been associated with inter-individual differences in ABCB1 activity and may influence drug efficacy. Here the influence of this SNP on the biological effect of tacrolimus was studied.

Methods Rhodamine (Rh123) efflux was used to study ABCB1 activity, with or without the addition of the ABCB1-inhibitor verapamil. Intracellular IL-2 production in T cells was used to measure the pharmacodynamic effect of tacrolimus after PMA/ionomycin stimulation of whole blood. In addition, the *ABCB1* genotype of 36 tacrolimus-treated renal transplant patients was related to ABCB1 activity and tacrolimus-efficacy.

Results The mean Rh123 efflux was higher in CD8+ T cells compared to CD4+ T cells: 40% vs. 19% of cells, resp. ($p<0.001$). Verapamil almost completely blocked Rh123 efflux (to 1.8% of CD4+ T cells and 0.5% of CD8+ T cells), while tacrolimus did not change Rh123 efflux. Tacrolimus 10 ng/mL reduced the production of IL-2 in CD4+ and CD8+ T cells by 28.9% and 45.4% ($p < 0.05$). Tacrolimus-mediated inhibition of IL-2 was enhanced by verapamil ($p<0.05$). This effect on tacrolimus pharmacodynamics was associated with *ABCB1* 3435C>T SNP in renal transplant patients: verapamil reduced the percentage of IL-2 producing CD4+ and CD8+ T cells by 14% and 22% in patients with the CC genotype ($p < 0.05$) but not in patients with the TT genotype. Moreover, the ratio tacrolimus C_0 over % IL-2 producing CD8+ T cells in CC genotype patients was significantly higher compared to TT genotype patients ($p<0.05$), showing a smaller pharmacodynamic effect in CC genotype patients.

Conclusion The *ABCB1* 3435C>T SNP influences ABCB1 activity of T cells and the pharmacodynamic effect of tacrolimus in kidney transplant patients.

INTRODUCTION

Combination therapy of the calcineurin inhibitor (CNI) tacrolimus with mycophenolate mofetil, with or without glucocorticoids forms the most frequently used immunosuppressive regimen after solid organ transplantation [1]. Tacrolimus forms a complex with the intracellular FKBP12 (FK506 binding protein), which binds and inhibits the phosphatase activity of calcineurin [2]. This complex subsequently blocks the activation of the nuclear factor of activated T cells (NFAT), leading to inhibition of the expression of various cytokines, such as interleukin (IL)-2, which decreases inflammatory responses of alloreactive T cells [2]. Tacrolimus has a narrow therapeutic range, a large inter-individual variability in its pharmacokinetics, and quantification of its effect is difficult [3].

CNIs are substrates of ABCB1 (ATP-binding cassette sub-family B member 1, previously known as P-glycoprotein (P-gp) or multidrug resistance protein-1 (MDR1) [4], a protein that acts as an efflux pump and is encoded by the *ABCB1* gene. Physiologically, ABCB1 is expressed in the liver, pancreas, on enterocytes in the small intestine and colon, in the blood-brain barrier, and in the human kidney. ABCB1 is also found on various leukocytes, including T and B lymphocytes [5]. The specific tissue expression of ABCB1 suggests that it functions as a protective barrier. Its expression in the intestine is thought to limit the absorption of xenobiotics, whereas its expression in the biliary tract and kidney may facilitate the elimination of metabolic waste products and toxins [5]. In addition, ABCB1 removes CNIs from the intracellular compartment of lymphocytes, their main therapeutic target [4, 6-7]. For this reason, the activity of ABCB1 may affect intracellular tacrolimus concentrations and inter-individual variability in ABCB1 activity may influence the immunosuppressive effect of tacrolimus. The efflux-pump has a higher expression on CD8+ T cells than on CD4+ T cells [8-10], and hence will influence the intralymphocytic tacrolimus concentrations especially in CD8+ T cells. Several single-nucleotide polymorphisms (SNPs) have been identified in *ABCB1*. The SNP that has been studied most is the C to T transition at position 3435 within exon 26 (rs1045642), a synonymous SNP. The *ABCB1* 3435C>T SNP has been associated with reduced mRNA expression [11] and stability [12], and more recently, with changes in substrate specificity [13]. The *ABCB1* 3435CC genotype is associated with a higher ABCB1 function compared to the 3435CT and 3435TT genotypes [13].

Up until now, however, only few reports have been published on the functional effects of genetic variation in *ABCB1* on immunosuppressant pharmacokinetics and pharmacodynamics. Crettol *et al* [7] have shown in cyclosporine-treated patients that 3435TT carriers had 1.7 fold increased intracellular drug concentrations and 1.2 fold increased whole blood concentration. To the best of our knowledge no such data exist on the effects of this SNP with regard to tacrolimus. Therefore, in the present study, we investigated the influence of the *ABCB1* 3435C>T SNP on the pharmacodynamic effects of tacrolimus *in vitro* and *ex vivo* in blood from tacrolimus-treated renal transplant patients.

MATERIAL AND METHODS

Renal transplant patients

For the *ex vivo* analysis we studied blood samples from 36 renal transplant patients, who were treated with a maintenance immunosuppressive regimen of oral tacrolimus (Prograft®, Astellas Pharma Inc., Tokyo, Japan) and mycophenolate mofetil (Cellcept®, Roche Laboratories, New Jersey, NJ). The number of patients was chosen to have approximately 10 individuals in each genome group at an expected 3435CT frequency of 50% and a 3435TT frequency of 25% [14-15]. Tacrolimus doses ranged from 1 to 3 mg twice daily, aiming for predose concentrations (C_0) between 4 and 8 ng/mL, and oral mycophenolate mofetil was dosed between 500 and 1000 mg twice daily. Blood samples were collected before drug-intake in heparin and EDTA-containing vacutainer tubes (BD Vacutainer, Becton Dickinson, Plymouth, UK). Tacrolimus concentrations were determined in EDTA whole blood using the ACMIA-Flex immunoassay on a Dimension XPand analyzer (Siemens HealthCare Diagnostics, Inc, Newark, DE) in accordance with the manufacturers' instructions. The study protocol was approved by the local ethics committee and informed consent was obtained from each individual.

Rhodamine 123 assay

For the Rh123 assay peripheral blood mononuclear cells (PBMC) were used. PBMC were isolated from heparinized normal human whole blood by density-gradient centrifugation over Ficoll-paque (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's protocol. PBMC were rested overnight in RPMI 1640-DM medium (Gibco BRL, Paisley, Scotland, UK) supplemented with 2 mmol/L L-glutamine (Gibco BRL), 100 IU/mL penicillin (Gibco BRL), 100 µg/mL streptomycin (Gibco BRL) and 10% heat inactivated fetal bovine serum (FBS) (BioWhittaker, Verviers, Belgium). Next PBMC were loaded for 45 minutes with Rh123 (0.5 ug/mL; Sigma Aldrich, Steinheim, Germany) at 37 ^{0}C and washed twice with Rh123-free medium, Rh123-free medium containing 40 µmol/L verapamil (Sigma Aldrich) or Rh123-free medium containing 10 ng/mL tacrolimus (Astellas Pharma Inc.). Rh123 was allowed to efflux from the cells for two hours at 37 ^{0}C before cells were washed twice with phosphate buffered saline + 0.5% BSA (bovine serum albumin) at 4 ^{0}C. PBMC were surface-labeled with antibodies for 20 minutes on ice with the following lineage-specific monoclonal antibodies (mAb): CD3-peridinin chlorophyll protein complex (PERCP), CD4-pacific blue (PB) and CD8-PE-cyanin7 (PE-CY7; BD Biosciences, San Jose, CA). Samples were analyzed on a FACS Canto II flow cytometer (BD Biosciences). At least ten thousand gated cell events were acquired from each tube and Rh123 fluorescence was measured from 505 to 550 nm using an excitation wave length of 488 nm. Negative FMO (fluorescence minus one) control tubes were included. FMO controls were set up by leaving out the Rh123 in the staining panel.

Intracellular IL-2 analysis

Two hundred µL heparinized whole blood was spiked for one hour at 37 °C with vehicle or verapamil (Sigma-Aldrich) followed by one hour incubation with vehicle or tacrolimus in concentrations of 5, 10 or 50 ng/mL (Astellas Pharma Inc., Tokyo, Japan). Samples were activated with phorbol-12-myristate-13-acetate 0.8 µM and ionomycin 10 µg/mL (PMA/ionomycin) (Sigma-Aldrich) for four hours at 37 °C. To retain cytokines within the cells, Golgistop (BD Biosciences) was added during stimulation with PMA/ionomycin. After 4 hours the stimulation was stopped with EDTA, final concentration 2 µmol/L. Cells were fixed and lysed for 10 minutes with 4mL FACS lysing solution (BD Biosciences). After washing PBMC were permeabilized for 10 minutes with FACS perm solution II (BD Biosciences). Thirty minutes surface and intracellular staining was performed simultaneously using CD3-PERCP, CD4-PB, CD8-PE-CY7, and IL-2-allophycocyanin (APC; BD Biosciences). Samples were analyzed on a FACS Canto II flow cytometer and ten thousand gated cell events were acquired from each tube. Isotype control IgG1-APC (BD Biosciences) and FMO control tubes were included.

Genotype for ABCB1 3435C>T

Genotyping for the ABCB1 3435C>T was performed using Taqman® allelic discrimination assays on the ABI Prism 7000 Sequence detection system (Applied Biosystems, California, USA). The assay consisted of two allele-specific minor groove binder (MGB) probes, labelled with the fluorescent dyes VIC® and FAM™. PCRs were performed in a reaction volume of 12 µL, containing assay-specific primers, allele-specific Taqman® MGB probes, Abgene Absolute™ QPCR Rox Mix and genomic DNA (5 ng). The thermal profile consists of an initial denaturation step at 95°C for 15 minutes, followed by 40 cycles of denaturation at 92°C for 15 seconds, annealing and extension at 60°C for 1 minute. Genotypes were scored by measuring allele-specific fluorescence using the SDS 1.2.3 software (Applied Biosystems).

Analysis of data

Percentage of IL-2 and Rh123 positive cells were generated by data-analysis with Diva version 6.0 software (BD Biosciences) and statistical analysis was done using Graph Pad Prism 5.0 (Graph Pad Software Inc., La Jolla, CA, USA).

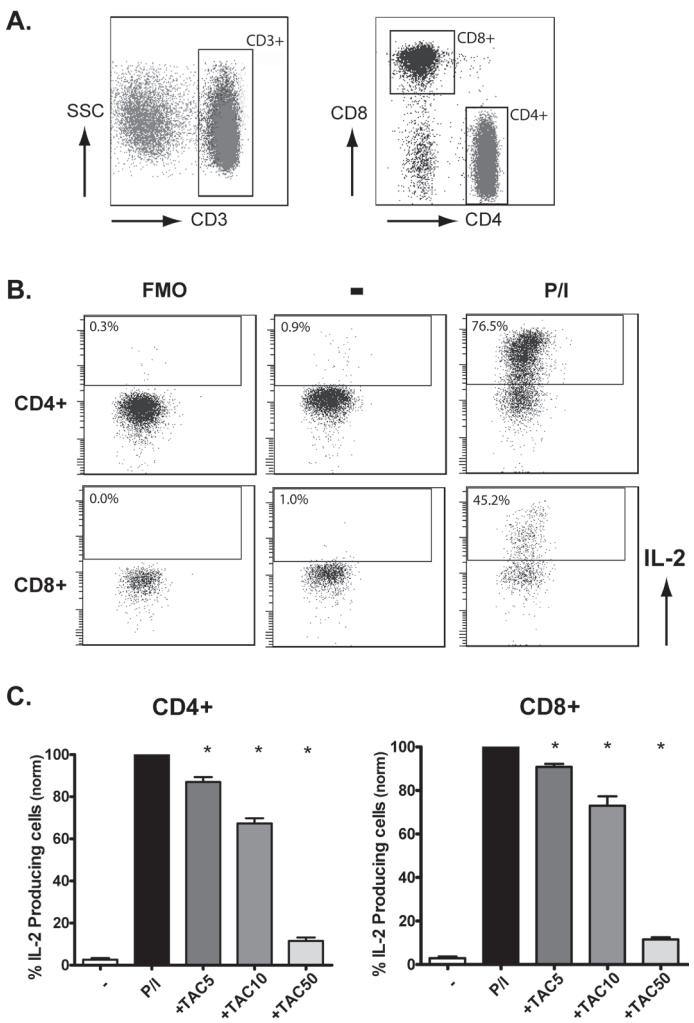
RESULTS

Tacrolimus inhibits intracellular IL-2 production in T cells

First, the pharmacodynamic effect of tacrolimus was studied by measuring intra-lymphocytic IL-2 production after *ex vivo* stimulation of whole blood from healthy volunteers by PMA/ionomycin. A typical example of the flow cytometric measurement is given in figure 1A and B. Stimulation induced the expression of intracellular IL-2 in 76.9 % of CD4+ and 41.8 % of CD8+ T cells (mean, n = 5, figure 1B). Tacrolimus inhibited the percentage of IL-2 producing

CD4+ and CD8+ T cells in a dose dependent manner (figure 1C). At 10 ng/mL tacrolimus, the inhibition was 34.5% ($p<0.05$) in CD4+ T cells and 27.3% ($p<0.05$) in CD8+ T cells, compared to no drug. For our next experiments this 10 ng/mL tacrolimus concentration was used to study the effect of blocking ABCB1 pump activity by verapamil.

Figure 1. Inhibition of intracellular IL-2 production by tacrolimus

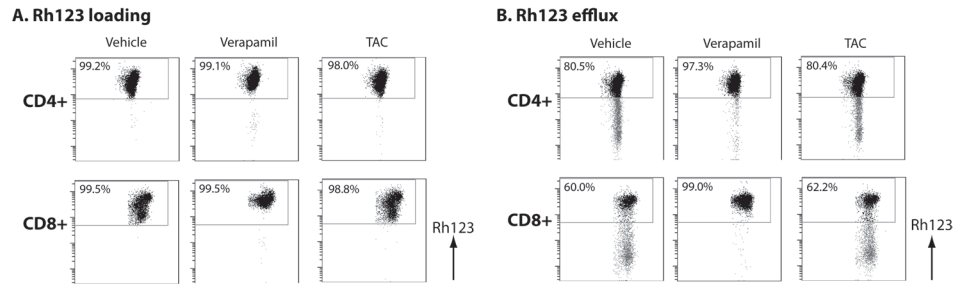


A) Example dot plots of gating strategy used to measure the interleukin (IL)-2 production in CD4+ T cells and CD8+ T cells, after *ex vivo* stimulation of whole blood from healthy volunteers by phorbol-12-myristate-13-acetate (PMA)/ionomycin. On the left plot the CD3+ T cells are separated from the PBMC fraction by CD3+ monoclonal antibody surface staining. On the right plot the CD3+ T cells are shown and separated into CD4+ and CD8+ T cells. **B)** These plots show the IL-2 expression in CD4+ (above) and CD8+ T cells (below) of a fluorescence minus one control (FMO, left), an unstimulated sample (middle) and a PMA/ionomycin stimulated sample (right). **C)** Average percentages of IL-2 producing CD4+ T cells (left) and CD8+ T cells (right) for unstimulated samples, PMA/ionomycin stimulated samples and samples incubated with 5, 10 and 50 ng/mL tacrolimus before stimulation (means + SEM of 5 independent experiments are depicted, * $p < 0.05$ compared to stimulated sample).

Effect of verapamil on ABCB1 efflux pump activity

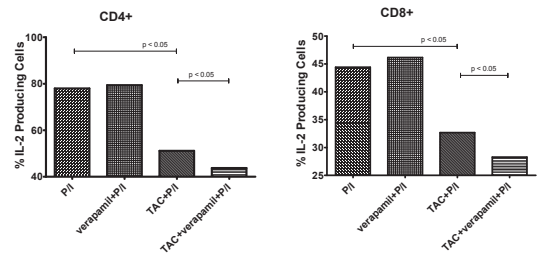
ABCB1 pump activity can be studied by loading PBMC with Rh123 and measuring the efflux after incubation of the cells in Rh123 free medium [16-17]. Here Rh123 efflux was measured by flow cytometry in CD4+ and CD8+ T cells with or without the addition of tacrolimus and the ABCB1 inhibitor verapamil. After treatment of cells for 45 minutes with Rh123 more than 98% of T cells were loaded with dye (figure 2A). Cells were incubated in Rh123-free medium for two hours in the presence of vehicle, 40 µmol/L verapamil or 10 ng/mL tacrolimus. A typical example of Rh123 loading and efflux are shown in figure 2.

Figure 2. Rh123 efflux inhibited by verapamil



A) PBMC from healthy volunteers were loaded with rhodamine (Rh) 123 for 45 min. Rh123 uptake by CD4+ and CD8+ T cells is shown in the presence of medium, verapamil 40 µmol/L or tacrolimus 10 ng/mL. **B)** Left plots show the efflux of Rh123 after two hrs incubation of the samples in vehicle without Rh123. CD4+ and CD8+ T cells effluxed the dye in the presence of vehicle and tacrolimus while verapamil strongly inhibited dye efflux. Data are representative of 3 independent experiments.

Figure 3. Inhibition of ABCB1 increases the effect of tacrolimus on T cells



Whole blood from healthy volunteers was stimulated by phorbol-12-myristate-13-acetate/ionomycin for 4 hrs and analyzed as shown in figure 1A and 1B. The ABCB1 inhibitor verapamil (40 µmol/L) enhanced the inhibitory effect of 10 ng/mL tacrolimus on the interleukin (IL)-2 producing cells by 28.9% ($p<0.05$) in CD4+ T cells and by 45.4% ($p<0.05$, n=5) in CD8+ T cells. Verapamil alone did not influence the number of IL-2 producing T cells.

In the presence of vehicle, Rh123 was effluxed by 19% of CD4+ T cells (median, range 18-19%; three independent experiments) and 40% of CD8+ T cells (median, range 37-40%). In line with previous studies [9-10], CD8+ T cells had a higher ABCB1 efflux activity compared to CD4+ T cells ($p<0.001$, n=3). Tacrolimus did not influence Rh123 efflux compared to vehicle, 18% in CD4+ T cells (median, range 16-19%, n=3) and 38% in CD8+ T cells (range 37-39%),

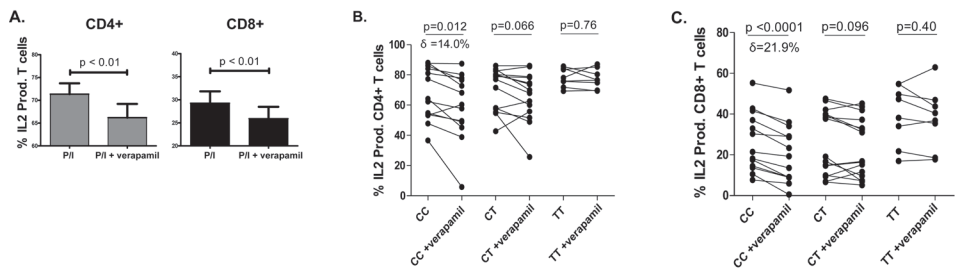
while addition of the ABCB1-inhibitor verapamil almost completely blocked Rh123 efflux from CD4+ and CD8+ T cells (efflux in only 1.8% (median, range 1.3-3.7%) and 0.5 % (range 0.1-1.0%, p<0.05 compared to vehicle).

Next, we investigated the effect of verapamil on the pharmacodynamic effect of tacrolimus. After *ex vivo* stimulation of whole blood from healthy volunteers the induced intracellular IL-2 expression in T cells was measured. In the absence of tacrolimus, the addition of verapamil did not influence the percentage of IL-2 producing CD4+ or CD8+ T cells (n = 5, p>0.05; figure 3). However, in the presence of 10 ng/mL tacrolimus, verapamil enhanced the inhibitory effect of tacrolimus on the IL-2 producing cells by 28.9% (p<0.05) in CD4+ T cells and 45.4% (p<0.05, n=5) in CD8+ T cells (figure 3), presumably mediated through intra-lymphocytic tacrolimus accumulation.

ABCB1 pump activity in T cells of renal transplant patients

A total of 36 renal allograft patients treated with tacrolimus were enrolled in our study. Their mean age was 53.9 years (median 55 years), mean time after transplantation was 48 months (median 34 months), the ratio of men to women was 18:19 and 35 out of 36 patients received concomitant treatment with mycophenolate mofetil and/or corticosteroids while one patient received tacrolimus monotherapy. The tacrolimus C_0 concentrations ranged from 2.7 to 13.8 ng/mL (median 6.7 ng/mL). These demographic variables were not significantly different across the three genotype groups containing 13 patients with the *ABCB1 3435CC* genotype, 15 with the CT and 8 with the TT genotype.

Figure 4. In CC genotype renal transplant patients blocking ABCB1-activity enhances the immunosuppressive effect of tacrolimus



A) Whole blood from tacrolimus-treated renal transplant patients (n = 36) was stimulated by phorbol-12-myristate-13-acetate/ionomycin in the presence of verapamil 40 µmol/L. Verapamil decreased the percentage of interleukin (IL)-2 producing CD4+ and CD8+ T cells by inhibiting efflux of tacrolimus (means + SEM are depicted). **B)** If the patients from figure 4A are categorized according to their ABCB1 3435C>T genotype verapamil reduced the percentage of IL-2 producing cells by 22% in CD8+ T cells and 14% in CD4+ T cells of patients with a CC genotype (n=13), whereas no significant effect of verapamil was found in the group of patients with a CT (n=15) or TT (n=8) genotype.

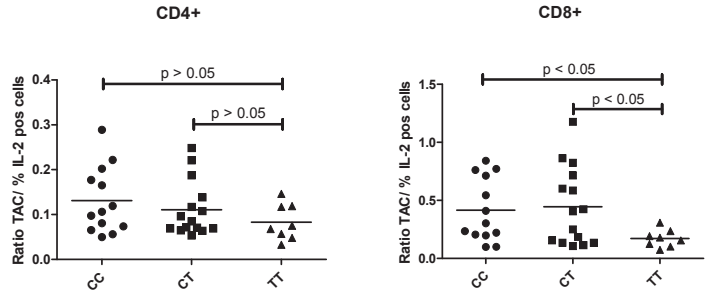
Whole blood from these patients was stimulated with PMA/ionomycin in the presence of verapamil or vehicle, and intracellular IL-2 expression was measured. Overall verapamil decreased the percentage of IL-2 expressing CD4+ and CD8+ T cells (p<0.01, figure 4A). In

patients with the CC genotype, verapamil reduced the percentage of IL-2 producing CD4+ T cells by 14% (p<0.05) and of IL-2 producing CD8+ T cells by 22% (p<0.0001), whereas no significant effect of verapamil was found in patients with the CT or TT genotype (p>0.05; figures 4B and 4C). Of note, due to individual variation in some patients IL-2 production increased after the addition of verapamil. These exceptions were however not seen in the CD8+ T cells of CC genotype patients where the effect of ABCB1-inhibition was the strongest.

Ratio tacrolimus C_0 over IL-2 producing T cells

Another way to show the influence of the *ABCB1* 3435C>T genotype on the pharmacodynamic effect of tacrolimus is to correlate tacrolimus whole blood concentrations with inhibition of IL-2 production by T cells, in the three genotype groups. This is illustrated in figure 5 where the ratio tacrolimus C_0 over percentage of IL-2 producing T cells was studied. In patients with the CC genotype this ratio was significantly higher (p<0.05) compared to the TT genotype indicating that in CC-genotype patients, tacrolimus has a smaller pharmacodynamic effect (Figure 5).

Figure 5. CC genotype patients need more tacrolimus for inhibition of IL-2 production in T cells



The ratio between tacrolimus C_0 and percentage of IL-2 producing T cells after whole blood stimulation with phorbol-12-myristate-13-acetate/ionomycin is plotted on the Y-axis for the three different *ABCB1* 3435C>T genotype groups.

DISCUSSION

Tacrolimus is a substrate for the ABCB1 efflux pump [5]. Here we hypothesized that the 3435C>T SNP in the *ABCB1* gene can modulate the immunosuppressive activity of tacrolimus by altering the activity of the pump. The data from the Rh123 efflux assay demonstrated that the functionality of the ABCB1 pump can be inhibited by the ABCB1 inhibitor verapamil, especially in CD8+ T cells (figure 2). Other studies have also shown higher functionality of ABCB1 in CD8+ T cells compared to CD4+ T cells [18-20]. The T cells with the highest ABCB1 efflux activity are memory CD4+ and CD8+ T cells [9, 21], a population with

low susceptibility to tacrolimus [22] and important in the transplantation setting since they are pivotal for allo-reactivity [23-26].

Moreover, *in vitro* in whole blood from healthy volunteers we saw that verapamil augmented the biological effect of tacrolimus, i.e. inhibition of IL-2 production in T cells (Figure 3). Although this study is the first to relate ABCB1 activity to tacrolimus efflux and its pharmacodynamic effect, the inhibition of ABCB1 by verapamil has been extensively reported elsewhere [27-28], especially in anti-cancer drug treatment. Verapamil is extensively used as an ABCB1 inhibitor in clinical trials [27-28], it increases the AUC of different anti-cancer drugs [29-32] and recently Neerati *et al.* showed that the ABCB1 inhibition by verapamil results in increased AUC and peak plasma concentrations of phenytoin, another ABCB1 substrate [33].

Tacrolimus intracellular concentrations have been reported to be a more reliable marker of its immunosuppressive efficacy compared to whole blood concentrations [34]. Therefore we hypothesized that intracellular tacrolimus concentrations and its effect on T cells are increased by verapamil by blocking the efflux pump. Accordingly, intracellular concentrations and the immunosuppressive effect can be affected by the inter-individual variability in the ABCB1 pump. Kimchi-Sarfaty *et. al.* [13] have found that the 3435C>T transition affects the timing of co-translational folding and insertion of ABCB1 into the plasma membrane, thereby altering the structure of the substrate interaction sites and differentiates verapamil specificities to the ABCB1 pump. The genetically determined differences in ABCB1 correlate with the activity of the pump; different studies have shown that 3435CC carriers had a higher ABCB1 pump activity than those with the 3435TT genotype. [7, 35-36]. The current study showed in tacrolimus-treated renal transplant patients with the 3435CC genotype, the more active ABCB1 variant, that verapamil decreased the IL-2 production by T cells i.e. enhanced the biological effect of tacrolimus. In contrast, verapamil did not influence IL-2 production in patients with the TT genotype (figure 4).

To relate the immunosuppressive effect of tacrolimus to the three genotypes, the ratio of tacrolimus trough concentration and percentage of IL-2 producing T cells, as a measure for the requirement of tacrolimus in renal transplant patients was compared. This ratio was significantly higher in CD8+ T cells of patients that have the 3435CC genotype compared to the ratio in TT genotype patients, showing that tacrolimus had a smaller effect in renal transplant patients with the CC genotype. This could be explained by the fact that in CC-genotype patients tacrolimus is more effectively pumped out of the cells, which leads to lower tacrolimus concentrations at its target. CC genotype patients needed higher tacrolimus concentrations to achieve the same IL-2 inhibition compared to the TT genotype, in line with the study of Hoffmeyer *et. al*. [11] that shows healthy volunteers with the 3435 *ABCB1* TT genotype, after oral administration, have higher plasma concentrations of digoxin compared to subjects that have a CC genotype. In addition, Crettol *et. al.* [7] have shown that carriers of the variant 3435T allele had higher intracellular cyclosporine concentrations, in line with our results. The importance of IL-2 production by CD8+ T cells for allo-reactivity was shown in liver transplant patients where it was undoubtedly associated with acute cellular rejection [37]. Additional studies are necessary to confirm our preliminary findings and to relate haplotype polymorphisms of the ABCB1 genotype [13] to the pharmacodynamic effect of CNI, which needs higher patient numbers than used in the current study.

The current study shows the *ABCB1* 3435C>T SNP affects the pharmacodynamic effect of tacrolimus in renal transplant patients. For clinical use these effects shed new perspectives for measurements of intracellular tacrolimus concentrations and are a move towards optimization of the immunosuppressive regimen after kidney transplantation.

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Chapter 2.5

The pharmacogenetics of calcineurin inhibitor related nephrotoxicity

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ABSTRACT

Chronic calcineurin inhibitor (CNI)-induced nephrotoxicity is associated with prolonged use of cyclosporine and tacrolimus and has been observed after all types of transplantation, as well as during treatment of autoimmune disease. Extensive alterations in the renal architecture including glomerular sclerosis, tubular atrophy and interstitial fibrosis may lead to end-stage renal failure. Increasing evidence shows that pharmacogenetic factors explain part of the between-patient differences in susceptibility to developing CNI-induced nephrotoxicity. In this paper this evidence is reviewed, with special emphasis on the role of genetic factors influencing metabolism and transportation of CNIs in both acceptor and donor.

INTRODUCTION

The nephrotoxic side effects of cyclosporin A (CsA) were already recognized by Calne and co-workers when they prescribed the drug for the first time to human kidney transplant recipients in the late 1970s.[1] The nephrotoxicity of CsA had not been apparent in their experiments in animals that were performed prior to the clinical introduction of CsA. However, it was soon discovered that the acute CsA-mediated nephrotoxic effects were reversible on dose reduction or cessation of the drug. It was not until CsA was used for prolonged periods in patients with autoimmune disease and in nonrenal transplant recipients that the chronic nephrotoxic effects of CsA became clear. After the clinical introduction of tacrolimus (Tac) in the early 1990s, this drug also turned out to have nephrotoxic side effects comparable to those associated with the use of CsA. At present, after more than 20 years of clinical use, the chronic nephrotoxicity of the calcineurin inhibitors (CNIs) CsA and Tac is one of the biggest challenges facing transplant physicians. Whether or not ISA247, a novel CsA analog, will be associated with less nephrotoxicity than CsA and Tac is unclear at present. A recent phase 3 trial with this drug in patients with psoriasis showed mild to moderate reductions in renal function in only 2% of the patients but only indirect comparisons with CsA can be made as this was a placebo-controlled study.[2] In this review, we will describe recent advances in our understanding of CNI-mediated nephrotoxicity with a focus on the drug transporter adenosine triphosphate-binding cassette protein B1 (ABCB1) and cytochrome P450 3A (CYP3A) and the variability in their encoding genes.

Pathophysiology

Clinically, an acute and a chronic form of CNI-induced nephrotoxicity can be distinguished.[3] Acute nephrotoxicity usually occurs within several days after starting CNI treatment. It may present as an acute oligoanuric syndrome [delayed graft function (DGF)] or as a rise in serum creatinine that may resemble other causes of early graft dysfunction such as, for example, acute rejection. In general, acute CNI-induced nephrotoxicity is rapidly and completely reversible on dose reduction or CNI withdrawal.[3] Pathophysiologically, it is characterized by constriction of the afferent glomerular arteriole, leading to a decreased renal plasma flow and a reduction of the glomerular filtration rate (GFR).[4] This change in vascular tone seems to result from an imbalance in the secretion and metabolism of the vasodilatory nitric oxide and prostaglandins, and the vasoconstrictive thromboxane and endothelin and an increased activity of the sympathetic nervous system. Second, CNIs have been reported to cause mesangial cell contraction, thereby altering glomerular permeability. Finally, CNIs may interfere with normal tubular function causing sodium retention and edema, reduced excretion of potassium and uric acid, increased urinary magnesium excretion, and hyperchloremic metabolic acidosis.[3,5,6]

Chronic CNI-induced nephrotoxicity is associated with prolonged use of these agents and has been observed after all types of transplantation and during treatment of autoimmune disease.[3,7-9] The clinical course is characterized by a slow decline in renal function that

may progress to end-stage renal disease. In addition, most patients have hypertension. Although some patients with chronic CNI-induced nephrotoxicity may have proteinuria, most patients do not as a result of the vasoconstrictive effects of CNIs (thereby lowering glomerular filtration pressure). In fact, the absence of proteinuria in a patient with a failing transplant can be of help in distinguishing CNI-induced nephrotoxicity from other causes of chronic renal transplant failure such as chronic (humoral) rejection or recurrence of the primary kidney disease in the transplant. In contrast to the acute form, chronic CNI-induced renal insufficiency improves little, if at all, after dose reduction or cessation of CNIs. Histologically, chronic CNI-induced nephrotoxicity is characterized by extensive alterations in the renal architecture that may include arteriolar hyalinosis, glomerular sclerosis and thickening of Bowman's capsule, tubular atrophy (TA), and interstitial (striped) fibrosis and diagnostic pathologic criteria have been described.[10-12] Nonetheless, it may be difficult to distinguish histologic changes associated with prolonged use of CNIs from those induced by for example, hypertension, diabetes, aging, or chronic humoral rejection, conditions that may coexist with CNI nephrotoxicity.[13]

The pathogenesis of chronic CNI-induced nephrotoxicity is far from being understood, but hemodynamic changes leading to ischemia, direct toxic effects of CNIs on the tubules, and an increased expression of the profibrotic transforming growth factor-β are considered important etiologic factors. For an in-depth review of the pathogenesis of chronic CNI-mediated nephrotoxicity, the reader is referred to the literature.[12]

The clinical problem

Although the use of CNIs has resulted in a dramatic decrease in the incidence of acute rejection and, consequently, a large improvement in short-term transplantation results, the long-term results of kidney transplantation have not improved to a similar degree over the last few decades.[14] In fact, the long-term results of kidney transplantation may even have worsened in recent years.[15] Although late kidney allograft loss has many causes, there is compelling evidence that the prolonged use of CNIs is an important contributing factor.

Nankivell *et al*. prospectively followed a cohort of kidney-pancreas transplant recipients treated with CsA-based immunosuppression for up to 10 years after transplantation.[16,17] All patients received kidneys of pristine quality [defined as the absence of vascular changes, glomerulosclerosis and TA and interstitial fibrosis (IF) at time of implantation], and protocol kidney biopsies were obtained regularly after transplantation. Ten years after transplantation, the cumulative incidence of histologic changes in the kidney, indicative of chronic CNI-related nephrotoxicity, was nearly 100%.[16,17] More recently, Naesens *et al.* described a cohort of 252 renal transplant recipients who all received Tac-based immunosuppression.[18] In this study, protocol biopsies were obtained regularly from the time of implantation for up to 3 years post transplantation. In line with the observations made by Nankivell *et al*. in CsA-treated patients, in this cohort, the cumulative incidence of vascular intimal thickening, glomerulosclerosis, TA, and IF all increased with time after transplantation.[16-18]

In addition, chronic renal dysfunction is common after nonrenal transplantation. Ojo *et al.* observed that chronic renal failure (defined as a GFR of 29 mL per minute per 1.73 m^2 of body surface area or less or the development of end-stage renal disease) developed in 11426 (16.5%) of 69321 patients 5 years after nonrenal transplantation.[19] End-stage renal disease (defined as the initiation of dialysis or preemptive kidney transplantation) developed in 3297 (28.9%) of the patients with newly diagnosed chronic renal failure. The 5-year risk of chronic renal failure varied according to the type of organ transplanted and ranged from 6.9% among recipients of heart-lung transplants to 21.3% among recipients of intestine transplants.[19] In this registry study, the large majority of patients were treated with a CNI-containing immunosuppressive regimen and no (protocol) kidney biopsies were performed. Therefore, the cause of chronic renal failure was not established. Nonetheless, these observations do indicate that renal dysfunction in CNI-treated transplant recipients is a huge clinical problem.

Finally, studies in patients treated with CNIs for autoimmune disease such as uveitis and psoriasis have demonstrated that chronic renal dysfunction is a complication of prolonged CNI treatment.[8,9,20] For example, among 41 patients treated with CsA for idiopathic uveitis, the GFR decreased from 102 mL/min per 1.73m^2 at the start of treatment to 88 mL/min per 1.73m^2 after 2 years of CsA therapy. Importantly, this decrease in renal function was accompanied by histologic changes over time with significant increases in glomerular sclerosis, thickening of Bowman's capsule and TA and IF.[9]

Although the risk of developing chronic renal insufficiency has been associated with longer use and higher doses of CNIs, this has not been a universal finding (reviewed in Naesens *et al.*[12]). In fact, some authors have speculated that the risk of developing CNI-induced nephrotoxicity depends on individual susceptibility and is not directly related to systemic exposure to CNIs if drug levels are kept within the current target ranges by use of therapeutic drug monitoring.[12,21] In recent years, there has been an increasing interest in the role of drug transporters and drug-metabolizing enzymes and their possible roles in the pathophysiology of chronic CNI-induced nephrotoxicity. We will now discuss the evidence pointing towards a role of ABCB1 and CYP3A in the development of CNI-related nephrotoxicity.

ABCB1

ABCB1 (formerly known as permeability glycoprotein or P-gp) belongs to the family of the -binding membrane transporters (subfamily B).[22] It is an 170 kDa adenosine triphosphate-dependent transporter capable of pumping many endogenous substances and a wide variety of drugs (including CsA and Tac) from the cytoplasm or cell membrane to the extracellular space.[23,24] Based on its specific expression in among others, the intestine, liver, and kidney, it has been postulated that ABCB1 functions as a protective barrier by actively extruding xenobiotics and metabolites from the cell interior into the gut lumen, bile, or urine. ABCB1 is also expressed on various leukocytes, including T and B lymphocytes and dendritic cells.[25, 26]

In the normal human kidney, ABCB1 is expressed in the brush border of proximal tubular epithelial cells.[18,27-29] In addition, ABCB1 is expressed in epithelial cells of Bowman's capsule, glomerular mesangial cells, on the apical membrane of the thick ascending limb of Henle's loop, intracellularly in distal tubules, and on the apical membrane of the collecting duct.[30,31] ABCB1 expression in endothelial cells of renal arteries, arterioles, and glomerular and peritubular capillaries was found to be absent or low.[18,29,31] Intrarenal ABCB1 is thought to play an important role in the renal elimination of metabolic waste products and toxins. Interestingly, renal ischemia-reperfusion injury in mouse kidney resulted in an upregulation of Abcb1 expression, which may represent an adaptive response in the renal regeneration process after injury.[32]

Evidence for a role of ABCB1 in the pathogenesis of CNI-related nephrotoxicity was first provided by García del Moral *et al.* who demonstrated that *in vitro* exposure of Madin-Darby canine kidney tubule cells to CsA leads to an increased expression of ABCB1.[33] In human arterial endothelial cells and rat proximal tubule cells, ABCB1 expression and activity was also induced by incubation with CsA.[34] Treatment of these cells with Tac had the same effect, although this required supratherapeutic concentrations of the drug.[34] *In vivo*, it was demonstrated that chronic treatment of rats with CsA induced the expression of ABCB1 in renal tubular cells, which increased with a longer duration of CsA treatment.[35,36] This induction was shown to be reversible because after cessation of CsA treatment, ABCB1 expression levels decreased toward normal.[35] Importantly, in these rat studies, ABCB1 expression was inversely related to the presence of hyaline arteriopathy and periglomerular -and peritubular atrophy.[36]

In human kidney transplant recipients, chronic CsA treatment has also been associated with upregulated ABCB1 expression.[31,33] In a histological case-control study, Koziolek *et al.* observed that the intrarenal expression of ABCB1 was higher in CsA-treated kidney transplant recipients who suffered from acute tubular necrosis, acute rejection or chronic allograft nephropathy as compared with normal kidneys or kidney transplants from recipients treated with a CsA-free immunosuppressive regimen.[31] By contrast, in kidney transplant biopsies from patients suffering from CsA-related nephrotoxicity, ABCB1 expression was not upregulated when compared with controls.[31] Similar observations were reported by Joy *et al.* in a small retrospective study.[37] More recently, Naesens *et al*. observed that a lower ABCB1 expression (as determined by immunohistochemistry) in protocol kidney transplant biopsies was a risk factor for the development of chronic histologic damage in Tac-treated renal transplant recipients.[18]

Taken together, these observations suggest that treatment with CNIs induces ABCB1 expression both *in vitro* and *in vivo*. This upregulation of ABCB1 may serve to protect the kidney from the injurious effects of CNIs by facilitating their extrusion. Failure to adequately upregulate ABCB1 expression or a constitutively low ABCB1 expression in renal parenchymal cells could lead to intrarenal accumulation of CNIs and predispose patients to the occurrence of CNI-related nephrotoxicity.

Although this hypothesis remains to be formally tested, experimental studies have provided evidence that ABCB1 inhibition does indeed cause increased intrarenal CNI concentrations, leading to renal dysfunction. In rats, it was demonstrated that sirolimus potentiated the nephrotoxic effects of CsA through a pharmacokinetic interaction, which caused increased intrarenal CsA concentrations.[38] Anglicheau *et al.* demonstrated *in vitro* that this pharmacokinetic interaction likely results from sirolimus-mediated ABCB1 inhibition.[39]

CYP3A

The CYP enzyme family consists of more than 50 isoenzymes that are responsible for the oxidative metabolism of many endogenous and exogenous compounds.[40] The CYP3A subfamily, which represents the majority of CYP proteins in human liver, metabolizes more than 50% of all drugs currently in use.[40] The CYP3A isoenzymes CYP3A4 and CYP3A5 are responsible for the metabolism of CsA and Tac in adults.[41-43] Both CYP3A4 and CYP3A5 are expressed in the small intestine and liver with CYP3A4 being the most important CYP3A isoenzyme in terms of expression and activity. By contrast, in the kidney, the expression and activity of CYP3A5 by far exceed that of CYP3A4.[44-47] In normal kidney, the expression of CYP3A5 was highest in proximal tubular epithelial cells and collecting duct epithelium with a lower expression in distal tubular epithelial cells.[45]

There exist large differences in the activity of intestinal and hepatic CYP3A between individuals, and this variability in enzyme activity is in part responsible for the marked interindividual variability in the pharmacokinetics of CNIs.[48-52] Given the predominance of CYP3A5 in CYP3A-mediated metabolism in the kidney, it has been hypothesized that intrarenal CYP3A5 expression may be a risk factor for CNI-induced renal dysfunction. In a case-control study, Joy *et al.* compared CYP3A5 expression in kidney biopsies from renal transplant recipients with histological evidence of CNI-induced nephrotoxicity (n = 29) with biopsy specimens from patients with various renal diagnoses including acute interstitial nephritis, diabetes mellitus and acute cellular rejection (n = 30).[53] Compared with controls, the expression of CYP3A5 in the apical membrane and cytoplasm of both proximal and distal tubules was found to be lower in nephrotoxic biopsies.[53] These findings suggest that reduced CYP3A5 expression may be a risk factor for the development of structural nephrotoxicity secondary to CNI treatment, possibly by reduced intrarenal detoxification. However, this was a relatively small and retrospective study in which the control group appeared to consist of both renal transplant recipients and nontransplanted patients suffering from various kidney diseases. In addition, the type of CNI treatment (CsA *versus* Tac) was not specified, the use of drugs interacting with CNIs was allowed, and no genotyping for *CYP3A5* was performed (see below).

Genetic variability in *ABCB1* and *CYP3A* and CNI-related nephrotoxicity

Both the *ABCB1* and *CYP3A4* and *CYP3A5* genes are polymorphically expressed. More than 25 single nucleotide polymorphisms (SNPs) have been discovered in *ABCB1*. The best-

studied SNP is the 3435C>T transition located in exon 26, which leads to the formation of an ABCB1 protein with an altered conformation and function.[54] The *ABCB1* 3435C>T SNP is in strong linkage disequilibrium with the 1236C>T (in exon 12) and the 2677G>T/A (in exon 21; Ala893Ser/Thr) SNPs.[55] With regard to CYP3A4 and CYP3A5, a large number of SNPs have been identified in their encoding genes (see http://www.imm.ki.se/CYPalleles).[56] At present, the best-studied SNP is that in intron 3 of the *CYP3A5* gene, genomic 6986A>G, which shows 100% linkage with the absence of CYP3A5 protein. This variant, referred to as the *CYP3A5*3* allele, occurs homozygously in 80% of the white and 30% of the African American population.[57-59]

Over the past few years, there have been numerous studies investigating the association between the various SNPs in *ABCB1* and *CYP3A* and the pharmacokinetics of CNIs. With regard to CsA, these SNPs appear to have only a limited, if any, effect on its pharmacokinetics (reviewed in de Jonge and Kuypers[60]). However, renal transplant recipients carrying the *CYP3A5*3* allele require a significantly lower Tac dose to reach target concentrations compared with patients homozygous for the wild-type allele (6986A, designated as *CYP3A5*1*).[60] A higher Tac dose requirement has also been observed for *CYP3A5* expressers (individuals with the *CYP3A5*1/*1* or *CYP3A5*1/*3* genotype) receiving a heart, liver, or lung transplant. In contrast, the effect of SNPs in *ABCB1* and *CYP3A4* on Tac dose requirement seems to be much smaller when compared with the *CYP3A5*3* SNP.[60]

The focus of genetic association studies in transplantation has recently shifted from CNI pharmacokinetics to transplantation outcomes, including renal allograft dysfunction. Hauser *et al.* were the first to report on the influence of *ABCB1* genotype on the occurrence of CsA-related nephrotoxicity after renal transplantation.[61] In this case-control study, which included 97 patients, the *ABCB1* genotype of both the acceptor and the donor was determined and the latter proved to be a major risk factor for the occurrence of clinically defined CsA-related nephrotoxicity.[61] Among patients with CsA-related nephrotoxicity (n = 18), kidney transplants with the *ABCB1* 3435TT genotype were overrepresented compared with patients without toxicity [odds ratio (OR) = 3.2; 95% confidence interval 1.4-7.6]. The donor's but not the recipient's *ABCB1* genotype was highly predictive as 2½ years after transplantation, approximately 40% of all patients who received a kidney transplant from a donor homozygous for the 3435T allele developed CsA-related nephrotoxicity compared with only 10% of patients who received a kidney with the 3435CT or 3435CC genotype. To determine whether the *ABCB1* genotype was an independent risk factor for CsA-related nephrotoxicity, a multivariate logistic regression analysis was performed. In the final model, which included several nongenetic factors, only the donor's *ABCB1* 3435TT genotype was strongly associated with clinically defined CsA-related nephrotoxicity (OR = 13.4; 95% confidence interval 1.2-148).[61] Because both the CsA dose and exposure to CsA were not significantly different between patients with and those without CsA nephrotoxicity, these observations were explained by a reduced ABCB1-mediated CsA elimination from renal tubular epithelium in patients with the *ABCB1* 3435TT genotype. Further evidence for a decreased renal elimination of CNIs in *ABCB1* 3435TT homozygotes comes from a

recent Belgian study.[18] A cohort of 252 adult renal transplant recipients, who received a kidney transplant from a deceased donor in 93% of cases and who were treated with Tac, mycophenolate mofetil (MMF) and glucocorticoids, was followed prospectively for 3 years after transplantation. During this period, a total of 744 kidney transplant biopsies (both protocol and indication biopsies) was obtained. A gradual increase in chronic histologic damage of renal allografts was observed during the follow-up period. Both donor and recipient homozygosity for the *ABCB1* T allele at position 3435 were significantly associated with a higher grade of IF and TA. This association was most prominent when both donor and recipient had the *ABCB1* 3435TT genotype with and OR of 3.9 (donor plus recipient homozygous for *ABCB1* T *versus* no homozygosity) of having a higher IF/TA score. Systemic exposure to Tac was not associated with the chronic histologic appearance of kidney transplants. Importantly, graft function at 3 years post transplant was also worse in individuals with the combined donor-recipient *ABCB1* TT genotype, although graft survival was not associated with *ABCB1* genotype.[18] In a retrospective study including 832 renal transplant recipients treated with CNIs, kidney allograft survival at 1 year post transplant was also found not to be associated with *ABCB1* genotype.[62]The incidence of DGF after renal transplantation was recently reported to be associated with recipient *ABCB1* genotype.[63] Among 147 renal transplant patients who were followed prospectively and treated with CsA, glucocorticoids and mycophenolate mofetil or azathioprine as part of a randomized controlled clinical trial, carriers of *ABCB1* T allelic variants at positions 2677 and 3435 (in exons 21 and 26, respectively) had a more than 3-fold higher risk of developing DGF compared with noncarriers. After a median follow-up of 65.5 months, GFR was about 5 mL/min per 1.73 m^2 lower in carriers of the T allelic variants in exons 21 and 26 compared with noncarriers.[63] This difference could not be explained by differences in systemic exposure to CsA. The authors speculated that the higher incidence of DGF in T allele carriers could have resulted from higher intracellular CsA concentrations in infiltrating leucocytes (which express ABCB1) causing a higher production of reactive oxygen species and more severe oxidative stress.[63] In patients treated with Tac, however, *ABCB1* genotype was not found to be a risk factor for DGF.[18,64] In addition, in an animal study, mice deficient for Abcb1 (*mdr1a/1b*$^{(-/-)}$) were in fact protected against ischemia-reperfusion injury. It was suggested that the smaller degree of renal damage in *mdr1a/1b*$^{(-/-)}$ mice kidneys after ischemia was caused by a decreased number of apoptotic cells causing tubular obstruction.[65] Obviously, more research is needed to clarify the exact role of ABCB1 in ischemia-reperfusion injury.

Fewer data are available regarding the role of genetic variation in *CYP3A* in CNI-induced renal dysfunction. Kuypers *et al.* reported a higher incidence of (indication)biopsy-proven Tac nephrotoxicity in 95 renal transplant recipients (not the donors) with the *CYP3A4*1/CYP3A5*1* and *CYP3A4*1B/CYP3A5*1* genotypes than among patients with the *CYP3A4*1/CYP3A5*3* genotype.[66] In contrast, Quteineh reported a nonsignificant trend between *CYP3A5* genotype of the transplant recipient and the incidence biopsy-proven Tac nephrotoxicity (being highest in *CYP3A5*3* homozygotes).[67] Other studies did not find significant associations between *CYP3A* genotype and either renal function or allograft

survival after renal transplantation.[62,64,68] Interestingly, in one study it was observed that kidney transplant recipients carrying a *CYP3A5*1* allele had a better survival compared with noncarriers.[69]

CNI-related nephrotoxicity after nonrenal transplantation

The association between CNI-induced nephrotoxicity and genetic variation in ABCB1 and CYP3A has not been extensively studied in recipients of a nonrenal organ transplant. In a case-control study including 120 white liver transplant recipients treated with CNIs (74% receiving CsA-based and 26% Tac-based immunosuppression), patients who were homozygous for the ABCB1 2677 T allele (which is in linkage disequilibrium with the 3435 T allele) were less than 50% as likely to experience symptoms of chronic renal dysfunction (defined as a serum creatinine ≥ 141 µmol/L) compared with 2677GT or 2677GG individuals 3 years after transplantation.[70] In this study, a nonsignificant trend toward a lower incidence of chronic renal dysfunction was observed among 3435 T allele homozygotes.[70] In contrast, in a recent study conducted among 51 pediatric liver transplant recipients, patients with an ABCB1 variant allele at positions 2677 or 3435 were found to be at an increased risk of developing renal dysfunction.[71] In a case-control study including 106 heart transplant recipients (treated with CsA or Tac), the ABCB1 2677G>T/A SNP was not found to be associated with renal dysfunction.[72] A recent retrospective single-center analysis including 294 heart transplant recipients treated with CsA also found no association between the ABCB1 1236C>T, 2677G>T/A, and 3435C>T SNPs or ABCB1 haplotype and renal function 1 year after transplantation.[73]

With regard to the effect of *CYP3A5* genotype on the incidence of renal dysfunction after nonrenal transplantation even fewer data are available. Recently, Fukudo *et al.* studied 60 adult liver transplant recipients who received Tac-based immunosuppression.[74] They observed that renal dysfunction was not associated with the *CYP3A5* genotype of the transplanted liver. In contrast, recipients (and thus the native kidneys) homozygous for *CYP3A5*3* had a significantly higher risk of developing nephrotoxicity compared with *CYP3A5*1* allele carriers: 46% *versus* 17%; hazard ratio 3.16 (1.01 - 6.16).[74] *CYP3A5* genotype was not associated with renal dysfunction after cardiac transplantation.[72]

When interpreting the results of genetic association studies investigating the pharmacogenetics of CNI-related nephrotoxicity after nonrenal transplantation, several things should be kept in mind. First of all, and unlike transplanted kidneys, native kidneys are innervated. One of the possible mechanisms of CNI-induced nephrotoxicity is the ability of these drugs to stimulate the sympathetic nervous system which in turn may lead to an increased renal vascular resistance and reduced glomerular filtration.[12] Second, patients receiving a heart or liver transplant already may have (undiagnosed) renal disease (*e.g.* immune-complex glomerulonephritis) at the time of transplantation or may develop renal insufficiency because of causes other than CNI-induced nephrotoxicity.[75] In many of the above-mentioned studies investigating renal insufficiency after nonrenal transplantation, kidney biopsies were not routinely taken to firmly establish the cause of renal failure.

As such, cases of renal insufficiency attributed to the use of CNIs may in fact have been misdiagnosed. Finally, the pharmacokinetics of CNIs in liver transplant recipients is complex and influenced by both donor and recipient genotype. Fukudo *et al.* demonstrated that in the early phase after liver transplantation, intestinal *ABCB1* expression and intestinal *CYP3A5* genotype contributed to the interindividual variation in the oral clearance of Tac.[74] After the first month, however, it was the *CYP3A5* genotype of the grafted liver that significantly affected Tac dose requirement. These findings suggest that the major organ influencing Tac disposition changes from the native intestine in the early phase after transplantation to the transplanted liver in the stable phase when the metabolic function of the graft has recovered.[74]

Conclusions and future directions

There is substantial evidence that intrarenally expressed ABCB1, and to a lesser extent CYP3A5, is implicated in the pathogenesis of CNI-induced renal dysfunction. In addition, genetic variation in the *ABCB1* and *CYP3A5* genes explains some of the interindividual differences in the susceptibility to the nephrotoxic effects of CNIs.

However, at present many questions are left unanswered and problems unsolved. First of all, we should improve our ways to diagnose true CNI-induced nephrotoxicity and distinguish it from other diseases and conditions that may also cause histologic damage of the kidney transplant and lead to renal insufficiency (such as, e.g., chronic antibody-mediated rejection and polyomavirus infections). Studies using gene expression profiling or newly-identified biomarkers for renal damage have shown promising results.[76-78] Second, there is no direct evidence in humans that the association between ABCB1 expression/genotype and CNI-induced nephrotoxicity is indeed caused by higher intrarenal concentrations of CNIs. It is therefore to be expected that future studies will pursue this hypothesis by measuring intrarenal CNI concentrations in animal models of CNI-related nephrotoxicity and possibly also in human studies. Such investigations would also allow a further study of the role of various CsA and Tac metabolites in CNI-induced nephrotoxicity.[79] In addition to this "pharmacokinetic hypothesis", alternative explanations for the association between ABCB1 and chronic CNI-related nephrotoxicity will likely be studied further. Such alternative explanations include the role of ABCB1 in proximal tubular epithelial cell apoptosis.[80] Moreover, the role of other polymorphically expressed genes such as, for example, transforming growth factor-β, vascular endothelial growth factor, and caveolin-1 requires further study.[81-83] Finally, there is at present only limited evidence that ABCB1 and CYP3A genotype ultimately influence graft (or patient) survival.

Nonetheless, we feel that in the near future interventional trials will be initiated to investigate whether patients who are predicted to be at increased risk of CNI-induced nephrotoxicity will indeed benefit from CNI-free immunosuppressive protocols or CNI-sparing regimens.

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Chapter 3

Chapter 3.1

CYP3A5 genotype is not related to the intrapatient variability of tacrolimus clearance

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ABSTRACT

Background The risk of long-term chronic allograft nephropathy and graft loss after kidney transplantation is increased in patients with a high intra-patient variability of tacrolimus (Tac) clearance.

Methods To test whether this intra-patient variability is associated with an individual's *CYP3A5* genotype, we measured the intra-patient variability in Tac clearance in a cohort of 208 kidney transplant recipients treated with Tac and mycophenolate mofetil.

Results Tac dose requirement was significantly higher in patients expressing *CYP3A5*. However, intra-individual variability of Tac clearance was not related to *CYP3A5* genotype.

Conclusion Intraindividual variability in Tac clearance is not related to CYP3A5 genotype. Other factors, including patient adherence, may explain the variability in Tac clearance within an individual patient over time.

INTRODUCTION

In a previous study, we observed that the risk of long-term chronic allograft nephropathy and graft loss after kidney transplantation is increased in patients with a high intra-patient variability of tacrolimus (Tac) clearance.[1] The causes of this variability in Tac clearance within individual patients are incompletely understood. An explanation could be an individual's cytochrome P450 3A (CYP3A) genotype. Tac is metabolised by the CYP isoenzymes CYP3A4 and CYP3A5. Both enzymes are polymorphically expressed and several studies have demonstrated that patients homozygous for the *CYP3A5*3* single-nucleotide polymorphism (SNP), which leads to the absence of functional CYP3A5 protein, require a lower Tac dose compared with patients who express CYP3A5 protein due to the presence of the *CYP3A5*1* allele.[2-6]

Korean investigators have recently suggested that not only inter-patient variability but also intra-patient variability is correlated with *CYP3A5* genotype.[7] In healthy individuals taking Tac as part of a bioequivalence study, *CYP3A5* expressers had less intra-patient variability as compared to *CYP3A5* non-expressers. This observation was explained by the fact that in patients without functional CYP3A5 enzyme, the metabolism of Tac depends exclusively on the activity of CYP3A4.[7,8] Because the CYP3A4 enzyme is more sensitive to induction and inhibition, *CYP3A5* non-expressers could be more prone to variable Tac clearance over time.[8]

To investigate this hypothesis, we correlated the intra-patient variability in Tac clearance to the *CYP3A5* genotype in a large cohort of renal transplant patients.

MATERIALS AND METHODS

Study population

All patients who participated in our previous study and of whom the *CYP3A5* genotype was known, were included.[1] *CYP3A5* genotyping and Tac whole-blood concentration measurement in these patients was previously performed as part of and as described in several studies investigating the genetic basis of inter-patient variability in Tac pharmacokinetics.[9-11] Patients not carrying the *CYP3A5*3* allele were assigned the *CYP3A5*1/*1* genotype by default.

Study parameters

The primary outcome variable of the present study was the intra-patient variability in apparent oral Tac clearance (in the rest of this manuscript referred to as "clearance"). The Tac clearance of an individual was calculated by taking the reciprocal of the ratio of the whole-blood Tac concentrations (in nanogram per millilitre) obtained between 6 and 12 months after transplantation and the corresponding Tac doses (in milligram per day).

The within-patient variability in Tac clearance was then calculated as described previously[1] using the following formula:
{[(Xmean –X1) + (Xmean –X2) …… + (Xmean -Xn)] / n} / Xmean * 100= intra-patient variability (%)
Where Xmean is the mean Tac clearance of the available samples, X1 is the first available Tac clearance, X2 is the second and so on. The patients were divided into low and high intra-individual variability groups using the median variability of Tac clearance as the cut-off value.

Statistical analysis

To test the null hypothesis of no difference in CYP3A5 genotype frequency between patients with a high and low intra-patient variability in Tac clearance, we compared CYP3A5 expressers (patients with the CYP3A5*1/*1 or CYP3A5*1/*3 genotype) with non-expressers (patients with the CYP3A5*3/*3 genotype). Statistical analysis was performed by use of the Chi square test, Fischer's exact test or Student's t test, as appropriate with an α of 0.05. All values are depicted as means (± SD) unless stated otherwise.

RESULTS

Of the 297 patients included in our original study cohort, DNA for *CYP3A5* genotyping was available for 208 patients. For all patients there were at least 2 samples available for calculation of the intra-patient variability of Tac clearance with a mean number of 4.6 ± 1.8 samples per patient. The baseline characteristics of these 208 patients are depicted in Table 1. We observed no differences in these characteristics between *CYP3A5* expressers and non-expressers, except for mean body weight and ethnicity. In line with previous observations, the *CYP3A5*1* allele was more prevalent among black transplant recipients compared with Caucasian patients.[12] In addition, Tac dose requirement was 1.8-fold higher in patients expressing *CYP3A5* compared with *CYP3A5* non-expressers, confirming earlier observations.[11]

Of the 208 patients included, 104 had a low intra-patient variability of Tac clearance, whereas 104 patients displayed a high within-patient variability in Tac clearance. Ethnicity was not related to intra-patient variability of Tac clearance. When comparing *CYP3A5* expressers with *CYP3A5* non-expressers, no differences in the intra-patient variability of Tac clearance were observed (P = 0.28; Table 2). In addition, we observed no differences in the intra-individual variability of Tac clearance between patients with the *CYP3A5*1/*1 vs. CYP3A5*1/*3 vs. CYP3A5*3/*3* genotypes (P = 0.43; Table 2)

Depicted are the numbers of patients with a low or a high intrapatient variability in dose-corrected Tac predose concentration according to *CYP3A5* genotype. The apparent oral clearance was calculated by taking the reciprocal of this ratio (for example, a dose-corrected Tac predose concentration of 0.52 ng/ml per mg per day gives an apparent oral

Table 1. Baseline characteristics.

	CYP3A5 expressers (n = 59)	*CYP3A5* non-expressers (n = 149)	P-value
Male / female	38 / 21	88 / 61	0.48
Mean age recipient at transplantation (years)	45.7 ± 13.1	44.3 ± 14.4	0.50
Mean transplant number	1.3 ± 0.7	1.2 ± 0.5	0.25
Mean bodyweight (kg)	72.0 ± 14.0	77.9 ± 14.2	0.0075
Ethnicity			
Asian	9 (15.3%)	8 (5.4%)	
Black	16 (27.1%)	2 (1.3%)	
Caucasian	19 (32.2%)	114 (76.5%)	
Other	4 (6.8%)	5 (3.4%)	
Unknown	11 (18.6%)	20 (13.4%)	
Mean number of HLA mismatches	2.6	2.5	0.60
Living donor	40.7%	58.4%	0.22
Primary kidney disease			
Chronic pyelonephritis	2 (3.4%)	7 (4.7%)	
Diabetes mellitus	8 (13.6%)	8 (5.4%)	
Glomerulonephritis	4 (6.8%)	7 (4.7%)	
Hypertensive nephropathy	15 (25.4%)	21 (14.1%)	
IgA nephropathy	5 (8.5%)	11 (7.4%)	
Polycystic kidney disease	3 (5.1%)	22 (14.8%)	
Reflux/obstructive nephropathy	3 (5.1%)	11 (7.4%)	
Unknown	5 (8.5%)	23 (15.4%)	
Other	14 (23.7%)	39 (26.2%)	
Tac C_0 (ng/ml)			
t = 6 months	7.5 ± 2.5	8.0 ± 2.8	0.15
t = 12 months	7.0 ± 2.4	6.8 ± 2.4	0.50
Tac C_0/dose (ng/ml per mg per day)			
t = 6 months	1.08 ± 1.0	1.92 ± 0.9	<0.0001
t = 12 months	1.09 ± 0.6	1.90 ± 1.0	<0.0001

Table 2. Intra-patient variability in Tac clearance and *CYP3A5* genotype.

	Low variability group		High variability group	
	n (%)	Dose-corrected Tac predose concentration (ng/ml per mg per day)	n (%)	Dose-corrected Tac predose concentration (ng/ml per mg per day)
***CYP3A5* non-expressers (*3/*3)**	78 (75.0%)	0.52 ± 0.27	71 (68.3%)	0.45 ± 0.30
***CYP3A5* expressers (*1/*1 plus *1/*3)**	26 (25.0%)	0.33 ± 0.30	33 (31.7%)	0.24 ± 0.15
***CYP3A5* *1/*1**	5 (4.8%)	0.53 ± 0.45	9 (8.7%)	0.20 ± 0.11
***CYP3A5* *1/*3**	21 (20.2%)	0.28 ± 0.24	24 (23.1%)	0.25 ± 0.16

clearance of 1.92 L/day). These numbers are given as absolute values and as percentage of the total population (in parentheses). The median variability in Tac clearance of the whole population was 15.6% and this value was used to define patients as having either a high (above the cut-off of 15.6%) variability or a low (below the cut-off) variability. The mean Tac clearance of each genotype group is also shown. No differences were observed in the distribution of patients with the various *CYP3A5* genotypes among the high and low variability groups (p = 0.28 for expressers *vs.* non-expressers and p = 0.43 for non-expressers *vs. CYP3A5 *1/*3 vs. CYP3A5 *1/*1*, respectively).

DISCUSSION

In the present study we found no significant association between intra-patient variability of Tac clearance and *CYP3A5* genotype. Our findings are in contrast with the observations of Chung *et al.*[7] who observed a higher proportion of *CYP3A5* non-expressers among patients with a high intra-patient variability of Tac clearance.

There could be several reasons for these seemingly conflicting data. First of all, the pharmacokinetic data of Chung *et al.* were more detailed than ours. For our study, only Tac pre-dose concentrations obtained during routine patient care at the outpatient clinic were available for the analysis, whereas Chung and colleagues performed detailed pharmacokinetic measurements over a twelve hour Tac dosing interval. The participants in the latter study were healthy volunteers (n = 29) who participated in a pharmacokinetic bioequivalence study. As these volunteers received two single doses of two different Tac formulations, the intra-patient variability in the study of Chung *et al.* may in part be due to differences between the two Tac formulations.

Our study population consisted of transplanted patients using a number of drugs other than Tac. This co-medication was not systematically recorded but may have had an effect on intra-individual variability in Tac clearance by inducing or inhibiting *CYP3A4*-mediated Tac metabolism. Two recent studies[13-14] have shown that *CYP3A5* non-expressers are more susceptible to the inhibitory effects of fluconazole and ketoconazole on Tac metabolism. Obviously, this factor does not confound the results when studying a selected group of healthy volunteers participating in a cross-over pharmacokinetic study.[15] Another possible explanation for the differences between our results and those of Chung *et al.*, could be the different ethnic make-up of the study populations. Our study population was of mixed ethnic background, whereas the population of Chung *et al*. consisted exclusively of Korean subjects. We can not rule out the possibility that the CYP3A4 enzyme of Koreans is more susceptible to induction or inhibition than the CYP3A4 enzyme of Caucasians. Of the 208 patients in our study less than 10% were of Asian descent and our study therefore lacks power to perform a meaningful analysis of Asian patients separately.

Non-adherence to the immunosuppressive medication may be another cause for high within-patient variability in Tac clearance, especially in observational cohort studies. In our previous analysis[1] we observed that the intra-patient variability for Tac clearance was poorly correlated with the intra-patient variability for mycophenolic acid clearance. We assumed that non-adherence would have resulted in a similar variability in clearance of both drugs. However, we cannot rule out the possibility that non-compliance did influence our results. Future studies using sensitive methods for medication adherence such as the Q-methodology are needed to resolve this matter.[16]

CONCLUSION

The intra-patient variability of Tac clearance is not associated with *CYP3A5* genotype in stable kidney transplant patients. More research is needed to provide insights about what causes intra-patient variability of Tac clearance in patients using this immunosuppressive drug.

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Chapter 3.2

High intra-patient variability in tacrolimus clearance does not predict progression of cardiac allograft vasculopathy after heart transplantation

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Chapter 4

Tacrolimus predose concentrations do not predict the risk of acute rejection after renal transplantation: a pooled analysis from three randomized controlled clinical trials

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[¥] This article is dedicated to Henrik Ekberg, who sadly passed away on December 29th, 2012.

ABSTRACT

TDM for tacrolimus (Tac) is universally applied. However, the concentration-effect relationship for Tac is poorly defined. This study investigated whether Tac concentrations are associated with acute rejection in kidney transplant recipients. Data from three large trials were pooled. We used univariate and multivariate analysis to investigate the relationship between BPAR and Tac predose concentration at 5 time points (day 3, 10, and 14, and month 1 and 6 after transplantation). A total of 136/1304 patients experienced BPAR, giving an overall incidence of 10.4%. We did not find any significant correlations between Tac predose concentrations and the incidence of BPAR at the different time points. In the multivariate analysis, only Delayed Graft Function (DGF) and the use of induction therapy were independently correlated with BPAR, with an odds ratio of 2.7 [95% CI: 1.8 - 4.0; $p < 0.001$] for DGF and 0.66 [95% CI: 0.44 - 0.99; $p = 0.049$] for induction therapy. The other variables, including the Tac predose concentrations, were not statistically significantly associated with BPAR. We did not find an association between the Tac predose concentrations measured at 5 time points after kidney transplantation and the incidence of acute rejection occurring thereafter. Based on this study it is not possible to define the optimal target concentrations for Tac.

INTRODUCTION

Tacrolimus (Tac) has almost replaced cyclosporine A (CsA) as the drug of first choice for the prevention of graft rejection after kidney transplantation [1]. Therapeutic Drug Monitoring (TDM) for Tac is universally applied. Requirements for a drug to implement TDM in clinical practice include a high between-patient variability in pharmacokinetics, a relatively low within-patient variability, and a concentration-effect relationship. In order to do TDM, assays to measure drug concentrations also need to be available and ideally, randomized trials should show an improvement in clinical outcome when a drug is dosed based on measured drug concentrations compared to a fixed-dose approach. For Tac several assays are available, but randomized trials showing a benefit of TDM are not available. However, it is not realistic to expect that for Tac such a trial will ever be performed.

Contrary to the belief of many physicians and surgeons, the concentration-effect relationship for Tac is poorly defined. As the most important reason to prescribe Tac to a transplant recipient is the prevention of acute rejection, it is surprising that there are so few data on the concentration-effect relationship of Tac. Based on the current literature there is little support to promote the use of a specific therapeutic window and aim for certain target concentrations.

Several investigators have attempted to identify the optimal Tac concentration range, *i.e.* the one which is associated with the lowest incidence of rejection and with acceptable toxicity, as shown in Table 1.

Table 1. Literature

Author, Year	Number of patients	Conclusion
Borobia et al, 2009 [15]	57 kidney	The Tac predose concentrations within the first post-operative week are an important predictor of acute rejection
Staatz t al, 2001 [16]	29 kidney	Significant relationship between acute rejection and median Tac predose concentrations in the first month.
Bottiger et al, 1999 [17]	14 kidney	Concentrations below 10 ng/mL seem to be beneficial with respect to side effects
Kershner et al, 1996 [18]	92 kidney	Significant relationship between the Tac concentrations and toxicity
Undre et al, 1999 [19]	56 kidney	Mean 12-hour Tac area-under the concentration *vs.* time-curve (AUC_{0-12}) on day 2 after transplantation was significantly lower in 17 patients who experienced acute rejection than in the 39 patients who remained rejection-free
Kershner et al, 1996 [18]	721 liver	No relationship between the Tac concentrations and toxicity
Laskow et al, 1996 [20]	92 kidney	No significant difference among three different Tac-ranges (5-14 ng/mL, 15-25 ng/mL, and 26-40 ng/mL) with respect to the incidence of rejection
Nashan et al, 2009 [21]	60 liver	Tac predose concentrations of 5-8 ng/mL in the first month of transplantation resulted in the same rejection rates as Tac concentrations of 10-15 ng/mL.

The findings of many of these reports are conflicting and limited by the fact that they were of a retrospective design, included limited numbers of patients, and that the co-immunosuppressive medication used was different from that which is currently considered the gold standard. For the interpretation of the studies that are available an important additional problem is the fact that not all investigators studied Tac concentrations at the same time point after transplantation.

Rodriguez *et al* [2] recently performed a meta-analysis of 64 studies investigating the correlation between the Tac predose concentration and the incidence of rejection in liver transplant recipients. They concluded that the mean Tac predose concentration during the first month was not correlated with acute rejection. Nevertheless, they suggested that lower Tac predose levels would be more appropriate after liver transplantation to prevent Tac toxicity.

Despite limited evidence for performing TDM for Tac and the exact predose concentrations to aim for, in most transplant centers considerable time and effort is spent on the precise dosing of Tac in order to reach the predefined Tac target concentrations rapidly. Once on target, maintaining patients within the target concentration range also requires careful monitoring.

The aim of the present study therefore was to investigate whether the currently used and empirically-defined Tac target predose concentrations are indeed associated with the risk of developing acute rejection in kidney transplant recipients. We pooled the data of three large randomized-controlled trials (RCTs) and studied the relation between Tac exposure and the incidence of biopsy-proven acute rejection (BPAR).

PATIENTS AND METHODS

Patients and clinical trials

For the present analysis we combined the data of three large, randomized-controlled clinical trials in kidney transplant recipients, the FDCC (*3*), Symphony [4] and OptiCept [5] trials. In brief, the main common elements of the three studies were the randomized, open-label, parallel-arm, multicenter design, and the fact that they included a broad spectrum of patients. In general, these patients had a low-to-medium immunological risk and were treated under the respective protocols for at least one year after kidney transplantation. In addition to adults, the FDCC and OptiCept studies enrolled paediatric patients, who were, however, not included in our analysis.

Tac target concentrations

For the present analysis we included only the patients from these three RCTs who received Tac as part of their immunosuppressive regimen from the day of transplantation and had a minimum of 1 known Tac level. The Tac levels were targeted differently between the studies. For the FDCC study, Tac dosing was according to each center's protocol, and on average was between 10 and 14 ng/mL in the first month, with gradual tapering thereafter. In the Symphony study, Tac levels were targeted at 3-7 ng/mL for the study period. In the OptiCept trial, the Tac predose concentrations were 8-12 ng/mL within the first month, 4-6 or 8-10 ng/mL in the second and third months (depending on the randomization group), and 3-5 or 6-8 ng/mL from the fourth month onwards. Data on Tac dose and predose concentrations, as well as other demographic and clinical characteristics were collected from the databases of the three RCTs and pooled. Tac predose concentrations were studied at day 3 (± 2 days), day 10 (± 2 days), day 14 (± 3 days), month 1 (± 7 days), month 6 (± 4 weeks). We changed the Tac levels that were higher than 30 ng/mL (24 measuring points in total) into missing values, to prevent that non-predose Tac concentrations would be included in the analysis. However, we also performed the analysis with all the Tac levels (including the ones that were higher than 30 ng/mL).

Acute rejection

BPAR was defined as any histologically-confirmed episode for which a Banff score of 1 (mild, grades IA and IIA), 2 (moderate, grades IB and IIB), or 3 (severe, grade III) was recorded. In all three trials, all biopsy samples were assessed by a local pathologist, and rejection was classified according to the revised Banff grading system [6]. For the present analysis, only the first episode of BPAR was investigated. Ongoing or recurrent rejections were not studied.

Statistical analyses

The correlations between Tac concentrations and BPAR were done for BPARs occurring after the time of the Tac concentration measurement, within the remainder of the first post-transplant year tested with the non-parametric Mann Whitney U test at the five different time points. We also did the same analysis for BPARs occurring within the month following the Tac concentration measurement, again for all five time points. We also performed a similar analysis categorizing the patients as high-risk if they had one or more of the following characteristics: delayed graft function (DGF), second or third transplantation, panel reactive antibodies (PRA) of more than 15%, four or more human leukocyte antigen (HLA) mismatches, or were of African descent (black). All other patients were considered as low-risk. We have previously used the same definition for high and low risk [7]. The significance level was stated at 5%. Induction therapy (yes/no)(either ATG of anti ILR monoclonal antibody induction), HLA mismatches (<4 / ≥4), DGF (yes/no), PRA (<15 / ≥15) and number of transplant (first / ≥second transplant) were correlated with the occurrence of BPAR within one month and one year after transplantation by using the Chi Square test. To identify independent risk factors for the development of BPAR, a binary logistic regression was performed, including all the above mentioned variables, plus median levels of Tac predose concentrations. Statistical analysis was carried out using SPSS version 19 (SPSS / IBM Inc., Chicago, Illinois, USA).

RESULTS

Patient characteristics

In the three clinical trials a total of 1363 renal transplant patients were treated with Tac after transplantation. Of these patients, 1304 met the inclusion criteria and were used for further analysis (Fig. 1). Of these 1304 patients, 358 (27%) participated in the FDCC study, 385 (30%) in the Symphony study, and 561 (43%) in the Opticept study.

Figure 1. Included patients from the three clinical trials and reasons for exclusion from the study.

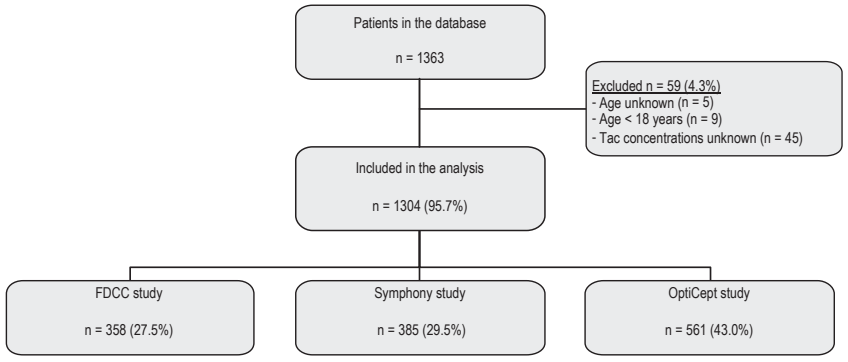
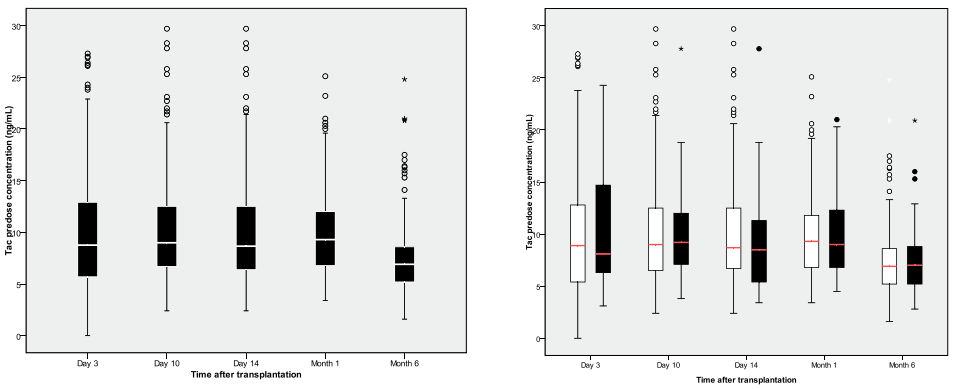


Figure 2.



A Boxplots depicting the Tac predose concentrations of all patients at the five different time points after transplantation.

B Boxplots depicting the Tac predose concentrations of patients experiencing BPAR (black boxes) and patients without BPAR (white boxes) at the five different time points after transplantation.

Bottom, middle, and top lines of each box correspond to the 25th percentile, the 50th percentile (median), and the 75th percentile, respectively. The caps show the 5th and 95th percentiles. The points represent the outliers and the asterisks represent the extreme outliers (more than three times the height of the boxes).

The patient characteristics are listed in Table 2. A total of 4953 Tac predose concentrations of 1304 patients were available for the analysis (Total predose concentrations of 818 on day 3; 1127 on day 10; 804 on day 14; 1167 on month 1 and 1019 on month 6).

The Tac predose concentrations show a substantial range and are depicted in Figure 2A. Twenty-four Tac concentrations were >30 ng/mL (n =13 on day 3; n =4 on day 14, n = 4 on month 1 and n = 3 on month 6). As we were unable to check whether these concentrations were truly predose concentrations or in fact post-dose concentrations, these values were classified as “missing values” and excluded from the primary analysis.

Relationship between Tac and BPAR

In this cohort the overall incidence of BPAR was 10.4% (n = 136) within one year after transplantation. The vast majority of BPARs occurred within the first month after transplantation (91/136 = 7%). We univariately tested the relationship between median Tac predose concentrations and the occurrence of BPAR within the first post-transplant year at 5 different time points, as shown in Table 3A and Figure 2B. We did not find any significant relationship between the Tac concentration and the incidence of BPAR. The results for BPAR within the first month after the Tac measurements did show similar results: again patients that developed a BPAR had Tac predose concentrations that were not different compared to patients without a BPAR, as shown in Table 3B. As for only 61% of the patients a Tac predose concentration was available for day 3 (Table 3A), we have studied the mean Tac predose concentration for each patient, based on samples drawn between day 3 and day 14 and correlated this to BPAR. Again, these Tac concentrations were not significantly different between patients with BPAR and patients without BPAR (10.02 *vs.* 9.97; p = 0.90).

The data were further analyzed by stratification into two groups: patients with a predose concentration < 5 ng/mL *vs.* patients with a predose concentration > 5 ng/mL, and patients with a predose concentration < 10 ng/mL *vs.* patients with a predose concentration > 10 ng/mL. The results are shown in Table 4. There were no statistically significant associations between the Tac predose concentrations and the occurrence of BPAR within one month after the measurement or throughout the rest of the first year after transplantation.

To analyse the risk of BPAR further, we divided the group into high and low immunological risk patients according to the definition described above. The total number of patients defined as being low-risk was 499 (39%) whereas 786 (61%) patients were considered to be high-risk. Nineteen patients were not included in this analysis, because one or more of the variables needed to define their immunological risk were not known. The incidence of BPAR was higher in patients in the high-risk group (100/786 = 12.7%) compared to the low-risk group (36/499 = 7.2%), with an odds ratio of 1.9 for patients in the high-risk group *vs.* the low-risk patients [95% CI: 1.3 - 2.8; p < 0.05]. First we analysed the Tac concentrations at the different time points for the high-risk group *vs.* the low-risk group. At all the time points the median Tac predose concentrations were not statistically significantly different between the high and low risk groups. We further analysed the Tac concentrations at the different time points within the high and low risk group separately, as shown in Table 5.

Again no significantly differences could be found between the patients that developed BPAR and patients without BPAR for the low (Table 5A) as well as for the high risk patients (5B).

We have changed the Tac levels that were higher than 30 ng/mL (24 measuring points in total) into missing values, to prevent that non predose Tac concentrations would be included in the analysis. However, we have also performed the analysis with all the Tac levels (including the ones that were higher than 30 ng/mL), but the results did not change (data not shown).

Explaining BPAR

Next to the Tac predose concentrations, in the univariate analysis, induction therapy, HLA mismatches, DGF, PRA and number of transplants were tested with the occurrence of BPAR within one year after transplantation. Of all 1304 patients 68% used induction therapy, and 9.6% of these patients suffered from a BPAR whereas this percentage was 12.3% in patients who did not use induction therapy after transplantation (p = 0.13). We also correlated the incidence of BPAR and the mean Tac concentration of day 3 to day 14 only within patients that did not use induction therapy. The Tac concentration in this group was not statistically different between patients with BPAR and patients without BPAR (p = 0.53). To test the influence of HLA mismatching we divided the group into patients that had 0-3 HLA mismatches *vs.* patients that had more than 3 HLA mismatches. There was a significant correlation between the number of HLA mismatches and the occurrence of BPAR. In patients with more than 3 HLA mismatches 12.3% had BPAR *vs.* 8.9% in patients that had 0-3 HLA mismatches (p = 0.046). Also for DGF we found a significant correlation with the occurrence of BPAR (19.7% in patients with DGF *vs.* 8.3 % in those without DGF), p < 0.001). The PRA status was not significantly related to the development of BPAR. PRA was separated into patients that had a PRA < 15% and patients with a PRA > 15%, in the first group 10.5% developed BPAR and in the last group 8.6% (p = 0.54). We have also studied the development of BPAR within patients that had a first kidney transplantation and compared this to patients that had one or more transplants before. Patients who had been transplanted before had a higher risk of developing BPAR (17.9%) compared with patients who received their first kidney allograft (9.9%; p = 0.021). The variables are listed in Table 6A. Because of the different designs of the studies we have also tested the incidence of BPAR within the different studies (Symphony, Opticept and FDCC). The patients in the Opticept trial suffered significantly less from a BPAR than in the other studies (7.5% vs. 12.2% (Symphony) and 13.1% (FDCC); p = 0.01.

In order to exclude the possibility that some of the other factors associated with the incidence of BPAR have confounded the relationship between Tac concentrations and BPAR we have adjusted for observed confounders and we performed a multivariate analysis which included these variables, as well as the Tac concentrations. Multivariate analysis demonstrated that only DGF and the use of induction therapy were independently correlated to BPAR, with an odds ratio of 2.7 [95%-CI: 1.8 - 4.0; p < 0.001] for DGF, and

0.66 [95%-CI: 0.44 - 0.99; p = 0.049] for the use of induction therapy. The other variables, including the Tac predose levels, were not significantly associated with the risk of developing BPAR as shown in Table 6B.

DISCUSSION

We did not find a correlation between the Tac predose concentration measured at 5 time points after transplantation and the occurrence of acute rejection in the period thereafter, within the first post-transplant year. The same was true for BPARs within the first month following the Tac measurement. We investigated a large and heterogeneous study population, and the Tac concentrations measured showed a substantial range, despite rather tight target concentrations defined in the protocols.

The situation for Tac seems to be quite different from mycophenolic acid (MPA). For MPA, a concentration-effect relationship has been shown repetitively [8-9] and for MPA it was also shown that in contrast to patients at low-risk for BPAR for high-risk patients there was a significant difference in the incidence of BPAR depending on the MPA concentrations reached [7]. In the present study, in neither the high-risk nor in the low-risk patients the incidence of acute rejection was dependent on the Tac concentrations. A bit to our surprise the mean Tac concentrations in high risk patients were not different from the Tac concentrations found in the low risk population. We had expected that physicians responsible for dosing Tac would aim for higher Tac concentrations in patients considered to be at presumed higher risk for BPAR, and that they would allow for lower concentrations in patients with a lower risk of rejection. Also in the multivariate analysis the Tac concentrations did not surface as predictor for BPAR.

TDM is generally considered to be required for managing Tac therapy. Often transplant centers have specified the target concentrations for Tac, depending on time post-transplant, on co-medication and presumed risk of rejection. One would think that for a drug so extensively used the evidence for the optimal Tac concentration would be compelling. We show that this is not the case. In the past 15 years we have seen a substantial change in the target Tac concentrations, with targets as high as 20 ng/mL in the early years, and with targets as low as 3-7 ng/mL in the Symphony study. This change in target concentrations was largely reached empirically, and there is only limited evidence for the different targets. This does not imply that TDM for Tac is useless. Without TDM the large between-patient variability in Tac pharmacokinetics would go unnoticed, and extremes in Tac exposure would occur, exposing some patients to toxic levels and others to very low levels. Based on our analysis however it is not possible to conclude that the Tac target concentrations should be above for example 5 or 10 ng/mL. Possibly the threshold for efficacy is at a concentration that is even lower than the currently applied targets, and it is possible that only when concentrations reach values as low as 1 or 2 ng/mL the incidence of BPAR starts to increase. The same was suggested by Rodriguez [2] who proposed to further lower the Tac

concentration in liver transplantation. They even recommended the regulatory authorities and pharmaceutical industry to change the regulatory drug information for lowering the target levels.

This study is a combined analysis of three large clinical trials, and a large number of kidney transplant recipients was included. In spite of the considerable number of patients studied, we could not show an association between the development of acute rejection in 1 month or 1 year after transplantation and the Tac whole blood concentrations. Also adjusting for confounders in a multivariate analysis the results stayed negative. Recently Capron *et al.* [10] also showed that there is no correlation between Tac whole blood concentrations and rejection after liver transplantation. However, they did find a strong correlation between Tac concentrations within peripheral blood mononuclear cells (PBMCs), the site of action of Tac, and the staging of rejection in liver transplant recipients. However, as indicated above, the currently clinically employed assays measure the Tac concentration in whole blood, which is determined to a large extent by the Tac concentration in the erythrocyte fraction. Tac concentrations in PBMCs are not 1:1 correlated with whole blood (or erythrocyte) concentrations, for example due to the presence of drug transporting enzymes in the cell membranes of PBMCs. Therefore Tac concentration within PBMCs might be a better marker of immunosuppressive efficacy than the whole blood predose concentration. Future studies should study the relationship between intracellular Tac concentrations and rejection risk in kidney transplant recipients in more detail.A limitation to this study is that donor specific anti-HLA antibodies were not routinely measured, and therefore we have no data on correlations between tacrolimus exposure and DSA. Next to this, we had only access to Tac concentrations drawn at predefined time points. These Tac concentrations might not be the last measured concentration prior to diagnosing BPAR and we cannot exclude the possibility that a similar analysis with the last levels drawn would show an association. However, intrapatient variability of Tac is limited and we do not think that we would have achieved another outcome by using the last levels drawn. Another limitation is that the pre-dose concentrations that were investigated in this study do not adequately reflect the exposure to Tac. Kuypers *et al.* in 2004 showed that in contrast to Tac predose concentrations the Tac area under the concentration curve from 0 to 12 hours [AUC(0-12)] was correlated with clinical efficacy, at different time points after transplantation [11]. However, a good correlation between Tac predose concentrations and AUC has been demonstrated. In clinical practice predose concentrations are the preferred method to monitor Tac treatment [12]. In a multivariate analysis also Australian investigators [13] did not find a correlation between Tac pre-dose concentrations or Tac AUC and incidence of acute rejection, whereas in their study MPA-AUC was correlated to BPAR.

Another explanation might be that other mechanisms, such as innate immunity, which are not calcineurin driven might play a role in the development of acute rejections. These rejections could not be prevent by the use of calcineurin inhibitors, such as Tac and for these type of rejections it is therefore not useful to aim for a specific Tac target. Although T-cells, inhibited by Tac, have a critical role in acute rejection it is known that there is an upregulation of proinflammatory mediators in the allograft before the T cell response, this is due to innate immunity and it is independent of the adaptive immune system [14].

In this study we have focused only on efficacy, as the incidence of nephrotoxicity was not prospectively collected. Therefore it is not possible form this study to define the upper threshold for Tac treatment.

In conclusion, we did not find an association between the Tac predose concentrations and the incidence of acute rejection after kidney transplantation. Even though it is generally accepted that TDM is essential to maintain the efficacy of Tac, the analysis in this study does not show that TDM, at the used whole blood target ranges, adds to lowering the risk of acute rejection. We do not want to suggest that TDM for Tac can be abolished, but a more critical perception on the relevance of the presumed optimal target concentrations is recommended.

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Table 2. Patient Characteristics

Gender (female / male)	450 (34%) / 854 (66%)
Age (yr; mean (SD)	48 (13.8)
Ethnicity (%): - Black - Non- Black	 161 (12%) 1143 (88%)
Transplantation (1st / ≥2)	1219 (94%) / 84 (6%)€
Delayed Graft Function: Yes / No	238 (18%) / 1066 (82%)
Panel reactive antibodies (< 15% / ≥ 15%)	1124 (91.5%) / 105 (8.5%)€
HLA-mismatches (< 4 / ≥4)	709 (54%) / 595 (46%)
Living related / living unrelated / deceased donor	338 (26%) / 183 (14%) / 783 (60%)
Induction therapy: Yes / No	890 (68%) / 414 (32%)

€: For transplantation and PRA there were missing values in 1 and 6 patients, respectively.

Table 3A. Median Tac predose concentrations and their association with BPAR occurring within the remainder of the first posttransplant year after the Tac concentration measurement.

Post-transplant Time point	Median predose Tac concentration (ng/mL) in patients with BPAR	Median predose Tac concentration (ng/mL) in patients without BPAR
Day 3	Tac: 10.3 [6.5; 17.1; 27.6]Ω n = 135 (61%)¥	Tac: 9.5 [6.0; 14.5; 29.5]Ω n = 1168 (63%)¥
Day 10	Tac: 9.0 [7.0; 11.8; 25.8]Ω n = 92 (85%)¥	Tac: 9.1 [6.6; 12.2; 28.2]Ω n = 1013 (87%)¥
Day 14	Tac: 7.8 [5.6; 10.4; 26.2]Ω n = 65 (72%)¥	Tac: 8.1 [6.2; 11.4; 29.7]Ω n = 722 (62%)¥
Month 1	Tac: 8.7 [5.8; 12.7; 20.2]Ω n = 45 (84%)¥	Tac: 9.7 [7.0; 12.5; 27.6]Ω n = 1050 (90%)¥
Month 6	Tac: 7.5 [6.3; 10.5; 11.0]Ω n = 15 (80%)¥	Tac: 6.8 [5.3; 8.6; 23.6]Ω n = 924 (79%)¥

¥ The percentage of patients of whom the Tac levels were available for analysis at this post-transplant time point. Tac concentrations were related to BPAR occurring after the date of the Tac concentration measurement.

Ω The numbers show the 25th percentile, 75th percentile and the range respectively. For all comparisons no statistically significant differences were found, all p-values were > 0.05.

Table 3B. Median Tac predose concentrations and their association with BPAR occurring within 1 month after the Tac concentration measurement.

Post-transplant Time point	Median predose Tac concentration (ng/mL) in patients with BPAR	Median predose Tac concentration (ng/mL) in patients without BPAR
Day 3	Tac: 11.1 [6.3; 10.5; 11.0]Ω N = 60 (66%)¥	Tac: 9.5 [6.3; 10.5; 11.0]Ω N = 1212 (62%)¥
Day 10	Tac: 9.0 [6.3; 10.5; 11.0]Ω N = 51 (86%)¥	Tac: 9.1 [6.3; 10.5; 11.0]Ω N = 1047 (87%)¥
Day 14	Tac: 8.5 [6.3; 10.5; 11.0]Ω N = 24 (71%)¥	Tac: 8.1 [6.3; 10.5; 11.0]Ω N = 1209 (62%)¥
Month 1	Tac: 8.0 [6.3; 10.5; 11.0]Ω N = 7 (71%)¥	Tac: 9.7 [6.3; 10.5; 11.0]Ω N = 1206 (90%)¥
Month 6	Tac: 7.4 [6.3; 10.5; 11.0]Ω N = 5 (100%)¥	Tac: 6.8 [6.3; 10.5; 11.0]Ω N = 1178 (79%)¥

¥ The percentage of patients of whom the Tac levels were available for analysis at this post-transplant time point. Tac concentrations were related to BPAR occurring after the date of the Tac concentration measurement.

Ω The numbers show the 25th percentile, 75th percentile and the range respectively. For all comparisons no statistically significant differences were found, all p-values were > 0.05.

Table 4. Numbers of patients with Tac concentrations below or above 5 ng/mL (Table 4A) and numbers of patients with Tac concentrations below or above 10 ng/mL (Table 4B) at 5 post-transplant time points, and incidence of BPAR in these patients following that time point.

4A. Tac predose concentrations < / > 5 ng/mL

Time point	Tac < 5ng/mL	BPAR	Tac > 5ng/mL	BPAR	P -value
Day 3	146	10 (6.8%)	671	73 (10.9%)	0.14
Day 10	129	7 (5.7%)	962	71 (7.4%)	0.42
Day 14	92	8 (8.7%)	677	39 (5.8%)	0.27
Month 1	86	2 (2.3%)	1002	36 (3.6%)	0.54
Month 6	185	2 (1.1%)	751	10 (1.3%)	0.79

4B. Tac predose concentrations < / > 10 ng/mL

Time point	Tac < 10ng/mL	BPAR	Tac > 10ng/mL	BPAR	P -value
Day 3	426	40 (9.4%)	391	43 (11%)	0.48
Day 10	619	49 (7.9%)	472	29 (6.7%)	0.26
Day 14	495	32 (6.5%)	274	15 (5.5%)	0.58
Month 1	573	22 (3.8%)	515	16 (3.1)	0.58
Month 6	797	9 (1.1%)	139	3 (2.2%)	0.32

Table 5. Median Tac predose concentrations at different time points after transplantation in patients with BPAR and in patients without BPAR divided into low (5A) and high risk patients (5B).

5A Low Risk patients

LOW-RISK PATIENTS (n = 499) (Total BPAR incidence: 36/499 (7,2%))			
Post-transplant Time point	Median predose Tac concentration (ng/mL) in patients with BPAR	Median predose Tac concentration (ng/mL) in patients without BPAR	p-value
Day 3	Tac: 10.3 [6.3; 10.5; 11.0]Ω n = 17 ¥	Tac: 10.1 [6.3; 10.5; 11.0]Ω n = 269 ¥	0.46
Day 10	Tac: 9.5 [6.3; 10.5; 11.0]Ω n = 20 ¥	Tac: 9.0 [6.3; 10.5; 11.0]Ω n = 394 ¥	0.68
Day 14	Tac: 8.9 [6.3; 10.5; 11.0]Ω n = 11 ¥	Tac: 7.9 [6.3; 10.5; 11.0]Ω n = 302 ¥	0.73
Day 3- day 14	Tac: 9.2 [6.3; 10.5; 11.0]Ω n = 36 ¥	Tac: 9.2 [6.3; 10.5; 11.0]Ω n = 454 ¥	0.63
Month 1	Tac: 10.1 [6.3; 10.5; 11.0]Ω n = 10 ¥	Tac: 9.3 [6.3; 10.5; 11.0]Ω n = 421 ¥	0.64

¥ The patients from whom the Tac levels were available for analysis at this post-transplant time point. For month 6 after transplantation the number of patients was too low to perform the analysis and this time point is therefore excluded from the analysis.
Ω The numbers show the 25th percentile, 75th percentile and the range respectively

5B. High Risk patients

HIGH-RISK PATIENTS (n = 786) (Total incidence of BPAR: 100/786 (12,7%))			
Post-transplant Time point	Median predose Tac concentration (ng/mL) in patients with BPAR	Median predose Tac concentration (ng/mL) in patients without BPAR	p-value
Day 3	Tac: 10.6 n = 66 ¥	Tac: 9.4 n = 454 ¥	0.26
Day 10	Tac: 8.7 n = 58 ¥	Tac: 9.1 n = 600 ¥	0.98
Day 14	Tac: 7.8 n = 36 ¥	Tac: 8.1 n = 402 ¥	0.28
Day 3- day 14	Tac: 9.1 n = 99 ¥	Tac: 9.3 n = 673 ¥	0.63
Month 1	Tac: 8.7 n = 28 ¥	Tac: 9.9 n = 614 ¥	0.24

¥ The patients from whom the Tac levels were available for analysis at this post-transplant time point. For month 6 after transplantation the number of patients was too low to perform the analysis and this time point is therefore excluded from the analysis.
Ω The numbers show the 25th percentile, 75th percentile and the range respectively.

Table 6A. Other variables related to BPAR (univariate analysis).

	Patients (%)	Patients with BPAR (%)	P-value
DGF	18.3	19.7	< 0.001
No DGF	81.7	8.3	
HLA mismatches > 4	46	12.3	
HLA mismatches < 4	54	8.9	0.046
Number transplantation > 1	6.4	17.9	0.021
Number transplantation = 1	93.6	9.9	
PRA > 15%	13.8	8.6	0.54
PRA < 15%	86.2	10.5	
Induction therapy	68	9.6	0.13
No Induction therapy	32	12.3	

Table 6B. Multivariate analysis

	OR (95% CI)	P-value
DGF	2.7 (1.8 – 4.0)	0.0001
Induction	0.66 (0.44 – 0.99)	0.049
Mean Tac concentration day 3 – day 14	0.98 (0.94 – 1.03)	0.48
HLA mismatches < 4	1.47 (1.02 – 2.13)	0.07
Number transplantation > 1	1.71 (0.91 – 3.23)	0.09
PRA > 15%	0.51 (0.17 – 1.53)	0.23

DGF = Delayed graft function; PRA = panel reactive antibody

Chapter 5

Chapter 5.1

Mycophenolic acid-related diarrhea is not associated with polymorphisms in SLCO1B nor with ABCB1 in renal transplant recipients

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ABSTRACT

Objective We investigated the association between genetic polymorphisms in *ABCB1* and *SLCO1B* and mycophenolic acid (MPA) pharmacokinetics, and MPA-related diarrhea and leucopenia in 338 kidney transplant recipients.

Methods 338 patients participating in an international, randomized-controlled clinical trial were genotyped for *ABCB1* and *SLCO1B.* The patients were all treated with mycophenolate mofetil plus either ciclosporine (CsA) or tacrolimus (Tac). MPA-AUCs, MPA-glucuronide (MPAG) AUCs and acylglucuronide (AcMPAG)-AUCs were measured on days 3 and 10, and months 1, 3, 6, and 12 after kidney transplantation.

Results The risk of developing diarrhea was 1.8-fold higher in patients co-treated with Tac as compared with patients co-treated with CsA (95% CI: 1.03-3.13; *P* = 0.038). *ABCB1* and *SLCO1B* SNPs were not associated with dose-adjusted exposure to MPA, MPAG or AcMPAG nor with the incidence of diarrhea or leucopenia.

Conclusion Genotyping for *ABCB1* or *SLCO1B* pre-transplantation is unlikely to be of clinical value for individualization of MPA therapy.

INTRODUCTION

Mycophenolate mofetil (MMF) is the most frequently used antiproliferative agent for the prevention of acute rejection after kidney transplantation [1]. MMF is a pro-drug and is itself not pharmacologically active. After oral administration, MMF is rapidly hydrolyzed to form mycophenolic acid (MPA). MPA is immunologically active and is inactivated by uridine diphosphate glucuronosyltransferase (UGT)-mediated glucuronidation to form 7-hydroxy-glucuronide mycophenolic acid (MPAG) [1-2]. In addition, two minor metabolites are formed: the inactive phenolic glucoside and the acylglucuronide (AcMPAG), which has immunosuppressive properties *in vitro.* MPAG is the main MPA metabolite and is excreted into bile through multidrug-resistance protein (MRP)-2 mediated transport [3-4]. In the gut, bacterial deconjugation transforms MPAG back into MPA, which is absorbed from the colon. Because of this enterohepatic circulation, the initial MPA plasma concentration peak at 1-2 h is followed by a second increase in the MPA plasma concentration, occurring 6-12 h after oral administration. Finally, the majority of the absorbed MMF is eliminated by the kidneys as MPAG [2, 4].

The clinical use of MPA is complicated by its large inter-individual pharmacokinetic variability. Exposure to MPA may vary up to 10-fold in transplant recipients [5]. This variability in MPA pharmacokinetics is in part explained by both non-genetic and genetic factors [6]. With regard to the latter, it was recently demonstrated that single-nucleotide polymorphisms (SNPs) in the MPA-metabolizing enzymes UGT1A8 and UGT1A9, as well as in the drug transporter MRP2, explain part of the variability in MPA pharmacokinetics [7-9]).

The use of MPA is further complicated by the frequent occurrence of gastrointestinal adverse effects, such as nausea, abdominal cramps and especially diarrhea. Diarrhea has been shown to reduce the quality of life of transplanted patients and is an important reason for non-compliance or discontinuation of MPA therapy [10]. In daily clinical practice the MMF dose is reduced in patients suffering from diarrhea. Although this does lead to less gastrointestinal discomfort in most patients, MPA dose reduction is not without risk as it may result in acute rejection [11-13].

The pathogenesis of MPA-related diarrhea in renal transplant recipients is unknown. Some authors have speculated that AcMPAG may cause an erosive enterocolitis by covalently binding to villous proteins in the intestinal mucosa [14]. However, data from the Fixed-dose (FD) *versus* Concentration-controlled (CC) trial have shown that AcMPAG plasma-concentrations were not different between patients with and those without diarrhea [15]. Moreover, no association was found between the occurrence of diarrhea and genetic polymorphisms in *UGT2B7* which is responsible for the formation of AcMPAG [16-17]. A direct toxic effect of MPA or MPAG on enterocytes may be an alternative explanation. Differences in the expression or function of drug transporting enzymes present in the apical membrane of the intestine could lead to high local concentrations of MPA or MPAG at the level of the intestinal epithelium and thus predispose certain patients to develop gastrointestinal side effects. Membrane transporters play a critical role in the absorption

and disposition of many drugs. However, at present there are only limited data considering the role of membrane transporters, such as *ABCB1* and *SLCO1B1* in the pharmacokinetics of MPA and MPAG.

ABCB1 (P-glycoprotein; encoded by *ABCB1)* is an adenosine triphosphate (ATP) binding cassette (ABC) transmembrane transporter that is expressed in the intestinal epithelium where it limits the absorption of many drugs by actively extruding them back into the gut lumen [18-19]. More than 25 SNPs have been discovered in *ABCB1*, some of which have been associated with interindividual variability in drug disposition [20-21]. In an *in vitro* study, by use of a transfected cell line, Sawamoto *et al.* demonstrated that MPA is a substrate for ABCB1 [22]. More recently, these *in vitro* findings were corroborated by an experimental *in vivo* study in mice. Studying *ABCB1* knockout mice, Wang *et al.* observed that the MPA levels in plasma and tissue of these mice were markedly increased as compared to wildtype mice, again suggesting that MPA is an ABCB1 substrate [23]. Among the organic anion transporting polypeptide (OATP) transporters, encoded by SLCO (solute carrier organic anion) genes, OATP1B1 (*SLCO1B*) and OATPB3 (*SLCO1B3*) are the major OATPs expressed on hepatocytes. In addition, OATPs are also expressed in the kidney and intestine [24]. The OATPs are believed to be involved in the uptake of MPAG from the blood into hepatocytes [25]. Polymorphisms leading to altered OATP activity may therefore affect MPA pharmacokinetics. Miura *et al.* [26] demonstrated that the MPA AUC in carriers of the *SLCO1B3* 334G allele was higher compared with TT carriers in 87 Japanese patients who had undergone renal transplantation. Picard *et al.* also investigated this SNP in renal transplant recipients but reported the contrary, namely a higher MPA exposure in *SLCO1B3* 334T allele carriers compared with patients with the *SLCO1B3* 334 GG genotype.

The aim of this study was to investigate whether genetic polymorphisms in *ABCB1* and *SLCO* are related to MPA pharmacokinetics and the occurrence of MPA-related diarrhea after kidney transplantation. In addition, we studied the relation between these polymorphisms and the occurrence of leucopenia (another common side effect of MPA), an association which was recently observed in a paediatric kidney transplant population [27]. To this end, we performed a subgroup analysis of patients who participated in the FDCC clinical trial [28]. The current pharmacogenetic substudy was performed as an integral part of this immunosuppressive drug trial.

METHODS

Patients and study design

The patients in this study were *de novo* kidney transplant recipients who participated in a phase IV, open, prospective, randomized, controlled, international, multicenter trial comparing Fixed-Dose MMF (Cellcept™, Roche Pharmaceuticals, Basle, Switzerland) treatment with Concentration-Controlled MMF treatment (the so-called "FDCC trial"). The primary outcomes of the FDCC trial have been presented in a separate publication [28]. At the initiation of the study a pharmacogenetic substudy was planned, and in the centers participating in this substudy, all patients provided two written informed consents, one for the FDCC study and one for the pharmacogenetic substudy. The ethics committees of all participating centers and the relevant authorities in the participating countries approved the study protocol.

In the FDCC study, immunosuppressive therapy consisted of MMF, a calcineurin inhibitor and glucocorticoids. The choice for ciclosporine (CsA) or tacrolimus (Tac), was according to each centre's protocol. Standard calcineurin inhibitor and glucocorticoids tapering regimens were left to the discretion of the investigators. Induction therapy with either anti-interleukin-2 receptor monoclonal antibody treatment or antithymocyte globulin was allowed. From the patients participating in the pharmacogenetic substudy, a single 3 mL EDTA blood sample was collected during one of the pharmacokinetic assessments. Samples were shipped to the Erasmus MC in Rotterdam, the Netherlands, where DNA was isolated and stored.

Diarrhea was defined as a frequency of more than four loose stools per day, if this was a change from the patient's normal pattern. Diarrhea was defined as a secondary safety objective in the FDCC-trial. A possible infectious etiology of diarrhea was not systematically investigated. Leucopenia was defined as a total leukocyte count below 3.0 x 10^9/L.

Drug concentration measurements

In all patients, a mini area-under the concentration *versus* time-curve (AUC; three samples within the first two hours after administration) was measured to calculate the corresponding $AUC_{0\text{-}12h}$ on day 3, day 10, week 4 and months 3, 6 and 12 after transplantation and whenever deemed necessary by the attending physician [28]. Tac pre-dose concentrations and CsA pre-dose and two-hour post-dose concentrations were measured on day 3, day 10, week 4 and months 3, 6 and 12 after transplantation and whenever deemed necessary by the attending physician. The CsA-treated patients were analyzed separately from the Tac-treated patients, as CsA is a known inhibitor of ABCB1 and CsA is also known to interact with MPA pharmacokinetics through inhibition of MRP2-mediated excretion of MPAG in the gut lumen [29-30].

DNA isolation and genotyping

For the present analysis, patients were genotyped for 3 *ABCB1* SNPs [21]: 1236C>T (exon 12), 2677G>T/A (exon 21) and 3435C>T (exon 26), for 2 *SLCO1B1* SNPs (521T>C and 388A>G) and 2 *SLCO1B3* SNPs (344T>G and 699G>T).

Genotyping for the *ABCB1* 1236C>T, 2677G>T/A and 3435C>T was performed using Taqman allelic discrimination assays on the ABI Prism 7000 Sequence detection system. Each assay consisted of two allele-specific MGB probes, labelled with the fluorescent dyes VIC and FAM. The primer and probe sequences, designed by Applied Biosystems (Assay-by-Design service) are listed in Table 5. For the tri-allelic variant 2677G>T/A two separate assays were designed, one detecting G2677A and one detecting G2677T. PCRs

were performed in a reaction volume of 12 µL, containing assay-specific primers, allele-specific Taqman MGB probes, Abgene Absolute QPCR Rox Mix and genomic DNA (5 ng). The thermal profile consists of an initial denaturation step at 95°C for 15 minutes, followed by 40 cycles of denaturation at 92°C for 15 seconds and annealing and extension at 60°C for 1 minute. Genotypes were scored by measuring allele-specific fluorescence using the SDS 1.2.3 software for allelic discrimination (Applied Biosystems).

The *SLCO1B1* 388A>G, 521T>C and the *SLCO1B3* 334T>G, 699G>A genotyping was done using predesigned DME Taqman allelic discrimination assays on the ABI Prism 7000 HT Sequence detection system. The assay ID's are listed in Table 5. Each assay consisted of two allele-specific minor groove binding (MGB) probes, labeled with the fluorescent dyes VIC and FAM. PCR were performed in a reaction volume of 10 µL, containing assay-specific primers, allele-specific Taqman MGB probes, Abgene Absolute QPCR Rox Mix and genomic DNA (15 ng). The thermal profile consists of an initial denaturation step at 95°C for 15 minutes, followed by 40 cycles of denaturation at 92°C for 15 seconds and annealing and extension at 60°C for 1 minute. Genotypes were scored by measuring allele-specific fluorescence using the SDS 1.2.3 software for allelic discrimination (Applied Biosystems).

Statistical analysis

As the distribution of the data followed a normal distribution, MPA-AUCs are expressed as means ± SD. Statistical significance between the different genotype groups was assessed using ANOVA. The association between the occurrence of diarrhea/leucopenia and different SNPs was analyzed using the Chi-square test or Fisher's exact test, where appropriate. In all analyses the significance level was 5%. We used a binary logistic regression model to investigate predictive factors for MPA-related leukopenia and diarrhea. When a consistent (a minimum of five of six time points) pattern in MPA $AUC_{0\text{-}12hr}$ or MPAG/MPA ratio difference was observed for one genotype group, multivariate analyses were performed, using mixed-model analysis of variance. In these models, the overall difference in MPA $AUC_{0\text{-}12hr}$ or MPAG/MPA ratio was analyzed after logarithmic transformation, adjusted for FD/CC arm, gender, age, enzyme-multiplied immunoassay technique/high-performance liquid chromatography analysis, delayed graft function, and creatinine clearance. Explanatory variables in the linear model were: study visit as within-patient categorical variable with six levels and various between-patient variables that were considered constant in time, such as gender and age at transplantation. No structure was imposed on the variances per visit and covariances between visits of repeated measurements of the outcome variables considered. Coefficients estimated from the mixed-model analysis of variance were back-transformed (antilog) to become interpretable as percentage changes in geometric mean values of the untransformed outcome variable. All statistical analyses were performed by use of SPSS version 16.0 software (Chicago, IL, USA).

For the haplotype analysis we performed a PHASE analysis [31]. The program PHASE implements methods for estimating haplotypes from population genotype data. All the haplotypes that were estimated with > 98% and had a frequency of > 5% were included in the analysis [32].

RESULTS

The baseline characteristics of the 338 patients included in this study are summarized in Table 1.

Table 1. Patient characteristics

Gender (female / male)	130 / 208
Age mean yr (range)	49 (18-78)
Ethnicity (%):	
- Caucasian	297 (88)
- Black	10 (3)
- Asian	14 (4)
- unknown	17 (5)
Transplantation (1st, 2nd, 3rd, unknown)	298 (88%) /30 (9%) / 5 (2%) / 5 (2%)
Fixed-dose / Concentration-controlled MMF	180 / 158
Delayed Graft Function: Yes / No	77 / 260
Tacrolimus / Ciclosporine	157 / 172
Panel reactive antibodies (< 10% / ≥ 10%)	308 / 26
HLA-mismatches	
A	0.91
B	1.12
DR	0.86
Living / deceased donor	110 / 228
Induction therapy: Yes / No	154 / 184

MPA Pharmacokinetics

ABCB1

The genotype frequencies of the various *ABCB1* SNPs are shown in Table 2. The frequencies were all in Hardy-Weinberg equilibrium (Chi-Square test). The daily MMF dose was not significantly different between the different *ABCB1* genotype groups. The *ABCB1* 1236C>T, 2677G>T/A and 3435C>T SNPs were not associated with dose-adjusted MPA-AUC, as shown in Table 3 (co-medication Tac) and Table 4 (co-medication CsA). Next, we studied the effects of the *ABCB1* haplotypes on the MPA-AUCs. Only 5 of the 18 estimated *ABCB1* haplotypes were observed with high frequency (>5%). These haplotype frequencies were: *ABCB1* TT-TT-TT (13%), CC-GG-CC (17%), CT-GG-CC (12%), CT-GT-CT (31%), TT-GT-CT (8%). Each of the 13 remaining genotypes represented less than 3% of the patients' combined *ABCB1* genotypes. None of the MPA pharmacokinetic parameters was significantly associated with one of the *ABCB1* genotypes (data not shown).

SLCO1B

The allelic frequencies of the *SLCO1B1* and the *SLCO1B3* polymorphisms are shown in Table 2. The frequencies were all in Hardy-Weinberg equilibrium. *SLCO1B3* 699G>A and *SLCO1B3* 344T>G have been reported to be in linkage disequilibrium and carriers of *SLCO1B3* 699A are often also carriers of the *SLCO1B3* 344G allele [25]. The same was seen in our study population (data not shown). For this reason and also because in the literature the most data exist on the *SLCO1B3* 344T>G genotype only the results of this genotype are provided. The daily MMF dose was not significantly different between the different *SLCO1B3* genotypes. We analyzed the dose-adjusted MPA-AUC for patients co-treated with CsA and co-treated with Tac separately. In the CsA group no significant associations were found with regard to dose-adjusted MPA-AUC and the different polymorphisms in *SLCO1B1* and *SLCO1B3* (Table 4). For the patients co-treated with Tac no differences in dose-adjusted MPA-AUC were found in relation to *SLCO1B1*. On the other hand, when studying the *SLCO1B3* SNP, we observed a significantly lower dose-adjusted MPA-AUC in patients with the *SLCO1B3* 344 TT genotype at month 1 and 3 compared with patients with the *SLCO1B3* 344 GT genotype (Table 3). At month 6 there was a trend to significance (P = 0.072). When we studied the corresponding MPAG/MPA metabolic ratio, there was a significant difference. The patients with the 344 TT genotype had a 24% lower MPAG/MPA metabolic ratio (P = 0.038) compared to the 344 GG genotype and the patients with the 344 GT genotype had a 21% lower (P < 0.0001) MPAG/MPA metabolic ratio compared the 344 GG genotype. Next, we performed a mixed model analysis, adjusting for FD/CC arm, gender, age, enzyme-multiplied immunoassay technique/high-performance liquid chromatography analysis, delayed graft function, UGT and MRP2 SNPs and creatinine clearance. This did not result in identifying the *SLCO1B3* genotype as a risk factor for lower MPA-AUCs, nor for the MPAG/MPA metabolic ratio.

Incidence of diarrhea

Diarrhea was observed in 77 of the 338 patients, giving an overall incidence of 23%. The mean time to the first episode of diarrhea among these patients was 21 days after kidney transplantation (range 3 to 323 days). In 49 of the 77 patients (64%) the MMF dose was reduced, whereas no dose reduction was necessary in 28 episodes of diarrhea (36%). Mean daily MMF doses and MPA exposure were not different between the patients that experienced diarrhea compared to patients who did not suffer from diarrhea. This was observed when we analysed the whole follow-up period, or only the month 1 and month 3 post-transplantation time points.

However when we split up the group into Tac and CsA treated patients we observed a difference: in the Tac treated patients 45 patients (29%) suffered from diarrhea, while in the CsA group 33 patients (19%) suffered from diarrhea. As a result a 1.8 higher risk (OR: 1.79, 95% CI 1.03 – 3.13; P = 0.038) of diarrhea was observed for patients co-treated with Tac compared with patients co-treated with CsA.

In the Tac group the MPA AUCs were not different at any time point for patients suffering from diarrhea and patients that did not suffer from diarrhea. However, the MPAG/MPA metabolic ratio on day 3 in the Tac group was significantly lower for the patients that suffered from early diarrhea (diarrhea within the first 3 months after transplantation) compared to patients that did not suffer from diarrhea: 7 (diarrhea) *vs.* 5 (no diarrhea); P = 0.03. The MPAG/MPA ratio on days 3 or day 10 did not show any differences between patients with or without diarrhea that occurred within month 1 posttransplantation.

When we compared the incidence of diarrhea in the Tac group between the different *ABCB1* genotypes, the incidence of diarrhea was comparable between these groups (Table 2). In the CsA group 33 of the 172 patients (19%) developed diarrhea with no significant difference between the different *ABCB1* genotype groups (Table 2). Next, we investigated the effects of the *ABCB1* haplotypes on incidence of diarrhea. In the unambiguous haplotype *ABCB1* CC-GG-CC group, 11 (19%) patients developed diarrhea and in the *ABCB1* TT-TT-TT group, 11 (26%) of the patients had diarrhea (Table 2). For the other ABCB1 haplotypes the following incidences for diarrhea were observed: CT-GG-CC: 9 (23%), CT-GT-CT: 22 (21%), TT-GT-CT: 7 (26%). None of the haplotypes was associated with the occurrence of diarrhea (P = 1.0).

Table 2. Frequencies of the genotypes, incidence of diarrhea and leukopenia.

Genotype			No Diarrhea	Diarrhea	p-value	No Leucopenia	Leucopenia	p-value
ABCB1		(n)						
1236C>T	CC	117 (35%)	80%	20%	0.786	94 (80%)	23 (20%)	0.546
	CT	155 (47%)	77%	23%		131 (85%)	23 (15%)	
	TT	60 (18%)	75%	25%		51 (85%)	9 (15%)	
2677G>T/A	GG	110 (34%)	79%	21%	0.868	88 (80%)	22 (20%)	0.589
	GT/TT	200 (62%)	77%	24%		168 (84%)	31 (16%)	
	GA	14 (4%)	79%	21%		12 (86%)	2 (14%)	
3435C>T	CC	81 (24%)	79%	21%	0.683	70 (86%)	11 (14%)	0.172
	CT	173 (52%)	79%	21%		138 (80%)	35 (20%)	
	TT	78 (24%)	74%	26%		68 (88%)	9 (12%)	
Haplotype*	CGC	58 (17%)	81%	19%	0.999	48 (83%)	10 (17%)	0.433
Haplotype	TTT	43 (13%)	74%	26%		38 (88%)	5 (12%)	
SLCO1B1		(n)						
T521C	CC	11 (3%)	80%	20%	0.654	9 (90%)	1 (10%)	0.395
	TC	91 (27%)	75%	25%		67 (79%)	18 (21%)	
	TT	234 (70%)	80%	20%		183 (85%)	33 (15%)	
A388G	AA	102 (30%)	79%	21%	0.246	72 (81%)	17 (19%)	0.497
	AG	147 (44%)	75%	25%		116 (83%)	24 (17%)	
	GG	76 (23%)	85%	15%		64 (88%)	9 (12%)	
SLCO1B3		(n)						
T344G	GG	224 (66%)	78%	22%	0.007¥	176 (85%)	32 (15%)	0.336
	GT	100 (30%)	84%	16%		74 (82%)	16 (18%)	
	TT	13 (4%)	46%	54%		9 (69%)	4 (31%)	

¥: significant (p < 0.05), between 344GG and 344TT
* Also the other *ABCB1* haplotypes did not show any differences in incidence of diarrhea or leucopenia

To study the influence of the MMF treatment arm on the incidence of diarrhea we analyzed the patients that received MMF in a fixed-dose separately from patients that received MMF in a concentration-controlled form. Again no statistically significant differences were found between the different *ABCB1* genotypes (data not shown).

When studying the *SLCO1B* SNPs, no association between diarrhea and *SLCO1B1* genotype was observed. However, patients with the *SLCO1B3* 344 TT genotype had a significantly higher incidence of diarrhea (54%) compared to patients with the *SLCO1B3* 344 GG genotype (22%; *P* = 0.007; Table 2) and to patients with the GT genotype (16%; *P* = 0.006).

To identify the risk factors for the development of diarrhea within one month, a logistic regression was performed. The following variables were included: *ABCB1* genotype and haplotype, *SLCO1* genotype, gender, age, MMF treatment (FD *vs.* CC), living/deceased donor, type of calcineurin inhibitor, creatinine values at day 10 and finally AcMPAG-AUC, MPAG-AUC, MPA-AUC, MPAG/MPA ratio (all day 10 levels). We did not identify any risk factors for the development of diarrhea. When leaving the AcMPAG out of the analysis (as this metabolite was only measured in 66 patients), this did not alter the results and we still could not identify any risk factors for diarrhea.

Table 3. Dose adjusted MPA-AUC in patients using tacrolimus as co-medication according to *ABCB1* and *SLCO* genotype

Dose-adjusted MPA-AUC (mg.h/L) ± SD								
			Day 3	Day 10	Month 1	Month 3	Month 6	40 ± 80
ABCB1		(n)						
1236C>T	CC	61 (39%)	50 ± 23	50 ± 32	61 ± 33	74 ± 47	80 ± 49	40 ± 80
	CT	65 (41%)	46 ± 21	41 ± 21	55 ± 23	65 ± 22	69 ± 27	40 ± 73
	TT	31 (20%)	44 ± 14	40 ± 18	53 ± 19	79 ± 27	24 ± 74	21 ± 78
2677G>T/A	GG	55 (36%)	53 ± 23 ¥	50 ± 32	62 ± 32	71 ± 34	81 ± 45	84 ± 48
	GT/TT	89 (59%)	44 ± 19	40 ± 20	55 ± 23	72 ± 30	74 ± 34	76 ± 45
	GA	8 (5%)	36 ± 14	40 ± 14	41 ± 11	82 ± 83	53 ± 23	52 ± 16
3435C>T	CC	44 (28%)	49 ± 24	48 ± 33	58 ± 33	75 ± 48	76 ± 48	78 ± 52
	CT	76 (48%)	47 ± 20	43 ± 21	55 ± 23	66 ± 28	73 ± 34	71 ± 36
	TT	37 (24%)	45 ± 18	42 ± 22	58 ± 26	77 ± 27	76 ± 29	86 ± 51
SLCO1B1		**(n)**						
T521C	CC	3 (2%)	72 ± 0.7	44 ± 17	57 ± 7	63 ± 8	49 ± 7	43 ± 11
	TC	40 (25%)	49 ± 24	47 ± 31	55 ± 32	83 ± 52	82 ± 43	77 ± 45
	TT	117 (73%)	46 ± 19	43 ± 23	57 ± 25	68 ± 27	72 ± 35	77 ± 45
A388G	AA	53 (33%)	46 ± 22	46 ± 29	60 ± 30	74 ± 34	79 ± 41	79 ± 50
	AG	77 (48%)	46 ± 19	44 ± 24	57 ± 26	71 ± 30	74 ± 36	81 ± 46
	GG	26 (16%)	48 ± 20	43 ± 21	50 ± 24	69 ± 52	62 ± 34	55 ± 20
SLCO1B3		**(n)**						
T344G	GG	107 (66%)	49 ± 22	42 ± 25	54 ± 25	66 ± 31	70 ± 36	79 ± 50
	GT	46 (29%)	44 ± 18	52 ± 25	68 ± 31	88 ± 43	86 ± 40	73 ± 31
	TT	8 (5%)	44 ± 16	37 ± 17	44 ± 14 ¥	60 ± 20 ¥	62 ± 24	62 ± 16

¥: significant ($p < 0.05$), but only for GT vs. TT

Incidence of leucopenia

In this study, 56 patients (17%) developed leucopenia. The daily MMF dose on days 3 and 10, month 3, 6 and 12 was comparable between patients suffering from leucopenia and patients who did not experience leucopenia. Only at month 1 did we observe a significant difference in MMF dose: 2541 mg (95% CI: 2264-2817) *vs.* 2088 mg (95% CI: 1998-2178); *P* < 0.001 for patients suffering from leukopenia (n = 55) and those not suffering from leukopenia (n = 245), respectively. However, the corresponding MPA-AUC at day 3, month 1, 3 and 6 was not different between patients with and those without leucopenia. Only at month 12 did we observe a significant difference in MPA-AUC between patients with (n = 32) and those without leucopenia (n = 164): (77 mg*h/L [95% CI: 60-95] *vs.* 61 mg*h/L [95% CI: 55-66; *P* = 0.03]), respectively. No differences were found when we analysed the incidence of leucopenia separately in Tac-treated patients and CsA-treated patients. In order to identify the relationship of *ABCB1* SNPs and *SLCO* SNPs with leucopenia we investigated the different SNPs in relation to the incidence of leucopenia. No relationship between SNPs in these genes and the risk of developing leucopenia was identified as shown in Table 2.

To identify other risk factors for leucopenia we performed a logistic regression. The following variables were included: *ABCB1* genotype and haplotype, *SLCO1* genotype, gender, age, MMF treatment (FD *vs.* CC), living/deceased donor, type of calcineurin inhibitor, induction therapy, creatinine values at day 10 and finally AcMPAG-AUC, MPAG-AUC, MPA-AUC, MPAG/MPA ratio (all day 10 levels). We did not identify any risk factors for the development of leucopenia. Again when leaving the AcMPAG out of the analyses we still did not identify any risk factors for the development of leukopenia.

Table 4. Dose adjusted MPA-AUC in patients using ciclosporine as co-medication according to *ABCB1* and *SLCO* genotype

		Dose-adjusted MPA-AUC (mg.h/L) ± SD						
			Day 3	Day 10	Month 1	Month 3	Month 6	*Month 12*
ABCB1		**(n)**						
1236C>T	CC	55 (33%)	31 ± 10	29 ± 13	34 ± 12	41 ± 15	48 ± 14	46 ± 16
	CT	84 (51%)	31 ± 10	31 ± 12	34 ± 17	44 ± 23	49 ± 24	48 ± 21
	TT	27 (16%)	36 ± 16	30 ± 10	32 ± 11	41 ± 16	46 ± 14	45 ± 16
2677G>T/A	GG	53 (31%)	32 ± 10	30 ± 14	35 ± 13	43 ± 17	50 ± 15	45 ± 16
	GT/TT	111 (65%)	32 ± 12	31 ± 12	33 ± 16	43 ± 21	48 ± 21	48 ± 19
	GA	6 (4%)	30 ± 5	23 ± 3	27 ± 9	25 ± 10	35 ± 5	49 ± 10
3435C>T	CC	36 (22%)	32 ± 8	28 ± 9	32 ± 10	41 ± 15	48 ± 15	43 ± 16
	CT	90 (54%)	31 ± 11	30 ± 12	35 ± 17	43 ± 23	48 ± 22	47 ± 18
	TT	40 (24%)	33 ± 14	33 ± 14	33 ± 11	43 ± 16	48 ± 17	49 ± 20
SLCO1B1		**(n)**						
T521C	CC	8 (5%)	38 ± 15	29 ± 8	37 ± 24	47 ± 19	54 ± 10	45 ± 13
	TC	49 (29%)	31 ± 12	29 ± 12	33 ± 19	43 ± 29	50 ± 25	50 ± 26
	TT	110 (66%)	32 ± 10	31 ± 13	34 ± 12	42 ± 15	47 ± 17	47 ± 17
A388G	AA	48 (29%)	30 ± 9	27 ± 8	30 ± 12	36 ± 12	42 ± 15	45 ± 16
	AG	67 (40%)	33 ± 13	32 ± 14	35 ± 17	44 ± 17	49 ± 16	48 ± 17
	GG	46 (27%)	32 ± 9	32 ± 13	35 ± 15	45 ± 25	51 ± 24	45 ± 20
SLCO1B3		**(n)**						
T344G	GG	111 (66%)	31 ± 10	29 ± 12	33 ± 15	41 ± 17	45 ± 18	49 ± 21
	GT	51 (31%)	33 ± 13	33 ± 13	36 ± 14	48 ± 26	56 ± 22	46 ± 17
	TT	5 (3%)	37 ± 14	33 ± 8	26 ± 9	39 ± 17	52 ± 5	43 ± 14
G699A	AA	110 (66%)	31 ± 10	29 ± 12	33 ± 15	41 ± 17	45 ± 18 ¥	48 ± 19
	GA	50 (30%)	33 ± 14	33 ± 13	37 ± 14	49 ± 26	56 ± 22	46 ± 17
	GG	5 (3%)	37 ± 14	33 ± 8	26 ± 9	39 ± 17	52 ± 5	43 ± 14

¥: significant ($p < 0.05$)

Table 5. Primer and probe sequences of *ABCB1* and *SLCO1B* SNPs

SNP	Sequence	
***ABCB1* C1236T**	Forward primer	TCTCACTCGTCCTGGTAGATCTTG
	Reverse primer	CACCGTCTGCCCACTCT
	VIC probe	TCAGGTTCAGGCCCTT
	FAM probe	TCAGGTTCAGACCCTT
***ABCB1* G2677A**	Forward primer	AATACTTTACTCTACTTAATTAATCAATCATATTTAGTTTGACTCA
	Reverse primer	GTCTGGACAAGCACTGAAAGATAAGA
	VIC probe	TTCCCAGCACCTTC
	FAM probe	CTTCCCAGTACCTTC
***ABCB1* G2677T**	Forward primer	CTTAGAGCATAGTAAGCAGTAGGGAGT
	Reverse primer	GAAATGAAAATGTTGTCTGGACAAGCA
	VIC probe	TTCCCAGCACCTTC
	FAM probe	TTCCCAGAACCTTC
***ABCB1* C3435T**	Forward primer	ATGTATGTTGGCCTCCTTTGCT
	Reverse primer	GCCGGGTGGTGTCACA
	VIC probe	CCCTCACGATCTCTT
	FAM probe	CCCTCACAATCTCTT
	Rs-number	**Assay ID**
***SLCO1B1* 388A>G**	Rs 2306283	C__1901697_20
***SLCO1B1* 521T>C**	Rs 4149056	C__30633906_10
***SLCO1B3* 334T>G**	Rs 4149117	C__25639181_40
***SLCO1B3* 699G>A**	Rs 7311358	C__25765587_40

DISCUSSION

This study provides evidence that the pharmacokinetics of MPA is not influenced by the investigated *SLCO1B* or *ABCB1* SNPs. Although, in the univariate analysis we found that patients homozygous for the *SLCO1B3* 344T allele had significantly lower dose-adjusted MPA-AUC compared to the *SLCO1B3* 344GT genotype, the multivariate regression did not identify the *SLCO1B3* genotype as a risk factor for lower MPA-AUCs. The same was shown for the corresponding MPAG/MPA metabolic ratio.

In a study from Miura *et al.* the dose-adjusted MPA-$AUC_{6\text{-}12}$ was significantly higher in patients with the *SLCO1B3* 344GG genotype compared to the 334TT genotype [26]. Miura *et al.* however studied only 87 patients for a shorter time period. Conversely, Picard *et al.* reported a higher MPA exposure in *SLCO1B3* 344T allele carriers compared with patients with the *SLCO1B3* 344GG genotype, which was attributed to a probably reduced enterohepatic cycling in these patients. These conflicting results were not confirmed by our study as we observed no significant association between MPA exposure and *SLCO1B3* SNPs. Recently Jacobson *et al.* [33] also found no association with MPA toxicity and *SLCO1B3* SNPs.

We did not find associations between the different genotypes and the incidence of diarrhea. However, a 1.8 higher risk (OR: 1.79, 95% CI (1.03 – 3.13 $P = 0.038$)) of diarrhea was observed for patients co-treated with Tac compared with patients co-treated with CsA. This is in line with the literature. The lower incidence of diarrhea in patients receiving CsA, confirmed in this study, may be due to inhibition of biliary excretion of MPAG or the AcMPAG metabolite in the gut [14, 34-35]. Other pathophysiological mechanisms however may also play a role. For example, recently, Liu *et al.* [36] have shown that MPA dramatically increased the secretion of IL-1ß. This increased IL-1ß concentration could result in more diarrhea through an increased apoptosis of gastrointestinal cells. Alternatively, MPA is a selective inhibitor of IMPDH, which is involved in de production of purines for lymphocytes and is also involved in the replication of gastrointestinal epithelial cells. If IMPDH would be inhibited, the replication of gastrointestinal epithelial cells would decrease, which could result in disruption of fluid absorption and in diarrhea [37-38]. However, IMPDH activity was not measured routinely in the FDCC cohort.

In the literature there is not so much evidence for the relationship between polymorphisms in *ABCB1* and the development of leucopenia. Fukuda *et al.* reported a trend for patients that were homozygous for *ABCB1* 3435CC genotype and the development of leucopenia. However, only 8 pediatric kidney transplant recipients developed leucopenia in that cohort after receiving MMF, whereas there were only three patients with the *ABCB1* 3435CC genotype. We studied a larger cohort prospectively and could not replicate the findings of Fukuda et al. [27]. We also did not find a relationship for the *SLCO1B* genotype and leucopenia. Other genes, such as UGT might be associated with leucopenia. In a study in 38 pediatric renal transplant recipients an association between the *UGT1A9* polymorphism and leucopenia was identified. The patients that were homozygous for UGT1A9 -331T>C

developed leukopenia [39]. However, after studying 2724 SNPs by Jacobson *et al.* [33] none of these SNPs were associated with leucopenia.

In the present study, *SLCOB1* and *ABCB1* genotype was not found to be associated with changes in the MPA pharmacokinetics nor with the incidence of diarrhea or leucopenia in MMF-treated renal transplant recipients. Sawamoto *et al.* and also Wang *et al.* [22-23] previously showed *in vitro* and in an animal study, that MPA is a substrate for ABCB1. However, they both studied the expression and function of the ABCB1 protein and not the effect of polymorphisms in the *ABCB1* gene [22-23]. The latter may have only a limited effect on ABCB1 protein expression and function. Another explanation for the absence of any significant association between MPA pharmacokinetics and *ABCB1* SNPs in this study could be that the relative contribution of the ABCB1 transporter to MPA disposition is limited compared to that of other transporter proteins. Diarrhea is a frequent side effect of MMF, and as this frequently results in a MMF dose reduction, diarrhea may lead to graft loss because of under-immunosuppression. For that reason it is important to initiate studies that aim to find risk factors for this side effect. Based on the present findings, genotyping for *ABCB1* or *SLCO1B* pre-transplantation is unlikely to be of clinical value for individualisation of MPA therapy.

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Chapter 5.2

Mycophenolic acid related anemia and leucopenia in renal transplant recipients are related to genetic polymorphisms in CYP2C8

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With great interest we read the article by Jacobson *et al.* [1] who observed that single-nucleotide polymorphisms (SNPs) in *IL12A* (rs568408G>A)*, CYP2C8* (rs11572076*G>A*), and *HUS1* (rs1056663C>T) are associated with the occurrence of mycophenolic acid (MPA)-related anemia but not leucopenia. These SNPs were identified by studying 978 renal transplant recipients by use of customized, commercially available gene chips that included a total of 2724 polymorphisms. Although the exact pathophysiological mechanism of these genotype-phenotype associations are not known, IL-12 mRNA levels were previously shown to be elevated in patients with aplastic anemia, HUS1 appears to be involved in the cellular response to DNA damage, and CYP2C8 may produce the 6-*O*-desmethyl-MPA metabolite.

However, the data of Jacobson *et al.* [1] were not confirmed in an independent cohort. The aim of our study was to investigate whether these SNPs are indeed associated with the occurrence of MPA-related anemia and leucopenia after kidney transplantation.

The patients described herein were *de novo* kidney transplant recipients who participated in a trial comparing Fixed-Dose (FD) mycophenolate mofetil (MMF) treatment with Concentration-Controlled (CC) MMF treatment. The primary outcomes of this so-called FDCC trial were reported previously [2]. Immunosuppressive therapy consisted of MMF combined with ciclosporine or tacrolimus, and prednisolone according to local practice. Anemia was defined as a hemoglobin level <11.3 g/dL (7.0 mmol/L) occurring after day 28 post-transplantation. Leucopenia was defined as a total leukocyte count <3.0 x 10^9/L. Of the total of 901 patients participating in the FDCC trial, 338 agreed to participate in a pharmacogenetic substudy. Genomic DNA was isolated from 200 µL EDTA whole-blood using a MagnaPure LC (Roche Diagnostics GmbH, Mannheim, Germany). Allelic discrimination reactions were realized using TaqMan® (Applied Biosystems, CA, USA) genotyping assays (C___2423981_10, C___1825446_20 and C__31658115_10) for rs568408G>A in *IL12A,* rs1056663C>T in *HUS1*, and rs11572076G>A in *CYP2C8* on a ABI PRISM 7500® fast real-time PCR System (Applied Biosystems) using 20 ng genomic DNA according to the manufacturer's instructions. For this analysis, data on the incidence of anemia and leucopenia of 332 of the 338 patients were available. Patients who received fixed-dose MMF (n=178) were analyzed separately from those that received concentration-controlled MMF (n=154). Only in the concentration-controlled group was the MMF dose adjusted on the basis of repetitive pharmacokinetic sampling results [3].

The allele frequencies of the various SNPs were as follows: *CYP2C8* G 99.5%, *CYP2C8* A 0.5%, *IL12A* A 15.1% and *IL12A* G 84.9%, and *HUS1* C 51.1% and *HUS1* T 48.9%. They were all in Hardy-Weinberg equilibrium. When we studied the allele frequencies in the fixed-dose and the concentration-controlled group separately, comparable allele frequencies were observed, with the exception of the distribution of the *CYP2C8* SNP in the concentration-controlled group: In this group all patients had the GG genotype.

Anemia occurred in 65 patients (37%) in the fixed-dose group and in 61 patients (40%) in the concentration-controlled group, which was not significantly different (*P*=0.56). No

significant differences in the frequency of the investigated *IL12A* and *HUS1* SNPs were found between patients with and those without anemia in the fixed-dose and concentration-controlled groups (Table 1). For the *CYP2C8* SNP a significant association (*P*=0.021) was observed in the fixed-dose group with 100% of patients with the *CYP2C8* rs11572076GA genotype having anemia (Table 1). However, only three patients (2%) had the GA genotype, yielding low statistical power. In fact, based on the reported (ref Jacobson) increased relative risk (2.1) of developing anemia in association with the *CYP2C8 A* variant allele, a sample size of 2200 patients (22 patients with anemia and 2178 control subjects) would have been necessary to have an 80% power with an alpha of 0.05% to reject the null hypothesis. However, this being a substudy of the completed FDCC trial, we feel that one should be cautious about performing such posthoc sample size calculations

The incidence of leucopenia was comparable in the fixed-dose and concentration-controlled groups: 24 patients (14%) *versus* 32 patients (21%), respectively (*P*=0.10). No significant associations were observed between the different *IL12A* and *HUS1* SNPs and the incidence of leucopenia, both in the concentration-controlled and the fixed-dose groups (Table 1). However, a significant association between the *CYP2C8* rs11572076G>A SNP and leucopenia was observed in the fixed-dose group with patients carrying the variant allele having a higher risk of developing this adverse event: 67% (n=2) *versus* 13% (n=22), respectively (*P*=0.007; Table 1).

Our results support the observation made by Jacobson *et al*. [1] that *CYP2C8* rs11572076*A* allele carriers have a higher risk to develop anemia. However, the actual number of patients carrying this variant allele was rather small and therefore -despite the fact that this association was confirmatory- the possibility of a spurious finding remains. The association of the *CYP2C8* rs11572076*A* allele with anemia was observed in the fixed-dose MMF group. In contrast to patients who received a concentration-controlled MMF dose (where any effects of genetic variation may be obscured by therapeutic drug monitoring), no dose adjustment based on plasma MPA concentrations was made in the fixed-dose group, allowing for the full expression of the clinical consequences of this SNP. A similar difference on the impact of pharmacogenetics between fixed-dose and concentration-controlled treated patients was reported previously [3]. We could not confirm the association with anemia and the *IL12A* rs568408G>A and the *HUS1* rs1056663C>T SNPs. This may be explained by the fact that the size of the population we studied was smaller than that studied by Jacobson *et al.* [1] (n = 332 *versus* n = 978, respectively) and the resulting lower statistical power to detect such an association [4-6].

In addition, we found a significant association of the *CYP2C8* genotype with the incidence of leucopenia. However, the frequency of the *CYP2C8* rs11572076*A* variant allele was only 0.5% and is therefore alone insufficient to explain the much higher incidences of anemia and leucopenia that frequently complicate the use of MPA [7]. Other factors, such as exposure to MPA, are in our view much more important determinants of hematologic MPA toxicity [8]. Moreover, following transplantation, blood counts are frequently monitored and after a MMF dose reduction, anemia and leucopenia are often rapidly reversible. We

therefore conclude that although the *CYP2C8* genotype appears to be associated with the risk of developing anemia and leucopenia, a pharmacogenetic approach is at present not helpful to reduce the risk MPA-related hematologic toxicity.

Table 1. Incidence of anemia and leucopenia in relation to SNPs in the *CYP2C8, IL12A,* and *HUS1* genes in 332 patients (fixed-dose group = 178 and concentration-controlled group = 154) receiving MMF as part of the FDCC trial.

SNP and number of patients (frequencies)	Fixed-Dose group (n = 178)				Concentration-controlled group (n = 154)			
	Anemia	*P*-value	Leucopenia	*P*-value	Anemia	*P*-value	Leucopenia	*P*-value
CYP2C8 (rs11572076)								
GA	3 (100%)	*0.021#*	2 (67%)	*0.007#*	0 (0%)	Not	0 (0%)	Not
GG	62 (35%)		22 (13%)		61 (40%)	tested	32 (21%)	tested
IL12A (rs568408)								
AA	0 (0%)	0.31	0 (0%)	0.80	4 (50%)	0.27	2 (25%)	0.15
GA	20 (44%)		7 (15%)		17 (50%)		3 (9%)	
GG	45 (35%)		17 (13%)		40 (36%)		27 (24%)	
HUS1 (rs1056663)								
CC	16 (41%)	0.31	8 (21%)	0.32	11 (26%)	0.10	9 (21%)	0.99
CT	31 (32%)		12 (12%)		34 (43%)		16 (20%)	
TT	18 (44%)		4 (10%)		16 (49%)		7 (21%)	

= significant; $P < 0.05$

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Chapter 6

General Discussion

Kidney transplantation is the preferred therapy for patients with end-stage renal disease [1]. Unfortunately, there is a shortage in donor kidneys, resulting in long waiting times for kidney transplantation. Waiting time is on average more than 4 years in the Netherlands (Nederlandse Transplantatie Stichting). Transplantation is not a cure, it's an on-going treatment and the patients are required to take immunosuppressive drugs for the rest of their lives. Transplanted kidneys have a limited life span and many patients require a re-transplantation. These re-transplantations further contribute to the shortage in available kidneys. Optimizing longevity of the kidney transplant and using 'the right' immunosuppressive treatment after transplantation is essential. But what is 'the right' immunosuppressive treatment? 'The right' immunosuppressive treatment could be different for every patient. Individualization of immunosuppressive therapy after organ transplantation remains a challenge for every clinician. Individualization or personalization of drug treatment involves the implementation of clinical pharmacological technology to adjust for the differences between individuals. This includes the application of pharmacogenetics and therapeutic drug monitoring.

In chapter 2 we have studied the pharmacogenetics of calcineurin inhibitors. In chapter 2.1 we have studied whether the polymorphisms in *CYP3A5*, *CYP3A4* and *ABCB1* affect clinical endpoints in kidney transplant recipients treated with ciclosporine. This study has shown that determination of *CYP3A* and *ABCB1* SNPs before transplantation are not helpful in identifying patients at high risk of experiencing acute rejection or nephrotoxicity. A pharmacokinetic association with ciclosporine and the already mentioned genes could also not be found in this study. The genetic polymorphisms in *CYP3A5, CYP3A4* and *ABCB1* are therefore not useful in determining the appropriate dose of ciclosporine for individual patients. Possibly a study of the pharmacogenetics of the donor might be more promising, especially with respect to the risk of developing severe nephrotoxicity [2, 3]. One could imagine that the activity of the ABCB1 protein (P-glycoprotein) would determine the intracellular accumulation of ciclosporine, and thus be linked to nephrotoxicity. In chapter 2.2 we conclude that *CYP3A4*22* T-variant allele significantly affects the pharmacokinetics of tacrolimus. Although there is a statistically significant association between genotype and pharmacokinetics this does not necessarily mean that using the genotype for individualized dosing of the drug will improve the clinical outcome. How much of the variability between patients will the genotype explain and what is the clinical benefit from the use of this genotype? The ultimate goal is not a better pharmacokinetic profile of tacrolimus, but a better clinical outcome, *i.e.* less toxicity or more efficacy of the drug. However, *CYP3A4*22* might be a guide to reach this goal. So far, *CYP3A5*1* stays the strongest predictor for tailoring tacrolimus treatment, but also for CYP3A5, before implementation into the routine clinical care of transplant recipients, prospective clinical trials are needed to decide whether such a pharmacogenetic dose approach will improve the clinical outcomes. We are currently performing a randomized-controlled clinical trial (Dutch trial registry number NTR2226, www.trialregister.nl). In this trial patients are randomized to receive either a tacrolimus dose based on their *CYP3A5* genotype of a standard tacrolimus dose only based on their

body weight. However, not all the pharmacokinetic variability is explained by the *CYP3A5* genotype [4]. The *CYP3A4*22* T-variant allele might help to explain the additional variation. We have found that kidney transplant patients carrying the *CYP3A4*22* T allele had a lower tacrolimus dose requirement. This effect was independent of CYP3A5. So the *CYP3A4*22* allele might be useful to fine-tune the tacrolimus starting dose after transplantation. Recently Elens *et al.* [5] confirmed this with a more detailed pharmacokinetic analysis in a Belgium population. She provided evidence for refining the genotype-based tacrolimus dosage by adding the *CYP3A4*22* genotype to the *CYP3A5*3* genotype. Using the algorithm for tacrolimus dosing of Passey *et al.* (6 2011) and incorporating the *CYP3A4*22* allele improved the precision of this algorithm [5]. In chapter 2.3 we have shown that this recently identified *CYP3A4*22* SNP is also a risk factor for developing delayed graft function and for impaired renal function, although, these were renal transplant patients who were treated with ciclosporine. This adds more evidence to incorporate the *CYP3A4*22* genotype into a genotype-based dosage guideline.

In chapter 2.4 we have tested whether the *ABCB1* 3435C>T genotype influences the intracellular IL-2 production (as a measurement for the immunosuppressive effect of tacrolimus) in T-cells. We have found that the *ABCB1* 3435C>T SNP influences ABCB1 activity and therefore the pharmacodynamic effect of tacrolimus in kidney transplant patients. We have performed *in vitro* experiments and show that if we block the ABCB1 pump with a ABCB1 blocker verapamil the cytokine production was enhanced in patients with the *ABCB1 3435CC* genotype (the active form of the ABCB1 pump), but not in patients with the *ABCB1 3435TT* genotype. These results show that tacrolimus has a smaller effect in patients with an active ABCB1 pump, as the intracellular tacrolimus will be pumped out to the extracellular fluid by the ABCB1 pump. The evidence is supported by a study from Capron *et al.* [7] who described that renal transplant recipients carrying the *ABCB1 3435T* allele had higher tacrolimus concentration within the lymphocytes. While these results are promising, more studies are needed to conclude whether the *ABCB1 3435C>T* genotype will be a biomarker for the tacrolimus dose.

An already extensively used tool to individualize the immunosuppressive therapy is therapeutic drug monitoring (TDM) [8]. As part of clinical practice in the vast majority of transplant centers the tacrolimus concentrations are monitored. A drug should fulfill certain criteria to be suitable for therapeutic drug monitoring [9]. These criteria include availability of a cost-effective drug assay, established target concentration range, a narrow therapeutic window, significant inter-individual pharmacokinetic variability and a reasonable relationship between the plasma (or whole-blood) concentration and the clinical effect. For tacrolimus, which is often used, one would think that a 'reasonable relationship between the whole-blood concentration and the clinical effect' and therefore an 'established target concentration range' has been shown extensively. Surprisingly this is not the case. In chapter 4 we studied whether the tacrolimus predose concentrations are related to the risk of acute rejection after transplantation. A large cohort of 1304 transplant recipients was used. No clear concentration-effect relationship was found. For example,

patients with lower tacrolimus concentration did not have more rejections. One could argue that the lack of a clear concentration-effect relationship would be a strong argument to stop with TDM. Our study however does not imply that TDM for tacrolimus is useless. Without TDM the large between-patient variability in tacrolimus pharmacokinetics would go unnoticed, and extremes in tacrolimus exposure would occur, exposing some patients to toxic levels and others to very low levels. Based on our analysis however it is not possible to conclude that the tacrolimus target concentrations should be above, for example, 5 or 10 ng/mL. Possibly the threshold for efficacy is at a concentration that is even lower than the currently applied targets, and it is possible that only when concentrations reach values as low as 1 or 2 ng/mL the incidence of BPAR starts to increase. Probably the used tacrolimus doses nowadays are too high. Unfortunately, in the dataset used for this study we could not investigate the correlation between toxicity and tacrolimus exposure. With the current dosing regimen, toxicity of tacrolimus might be a larger problem than underexposure. Recently however, Israni *et al*. [10] did find an association with risk of rejection in the first six months and tacrolimus trough levels after month 3. The tacrolimus concentrations which are measured for TDM purposes in daily practice are in whole blood. However, the site of action of tacrolimus is within the lymphocyte. Tacrolimus concentrations in peripheral blood mononuclear cells are not 1:1 correlated with whole blood (or erythrocyte) concentrations, for example, due to the presence of drug transporting enzymes in the cell membranes of lymphocytes [11], (see also chapter 2.4). Therefore tacrolimus concentrations within lymphocytes might be a better predictor of immunosuppressive efficacy than the whole blood predose concentration. Assays to measure tacrolimus in lymphocytes have been described [12]. Capron *et al.* [13] have studied the correlation between acute rejection and the tacrolimus concentration within the lymphocytes. They have found that the tacrolimus concentration in the lymphocytes is inversely correlated to the risk of acute rejection in liver transplant recipients; lower intracellular tacrolimus concentration means a higher incidence of acute rejection. Interestingly, they also concluded that the whole-blood tacrolimus concentrations were not related to the rejection risk. Future studies should study the relationship between intracellular tacrolimus concentrations and rejection risk in kidney transplant recipients in more detail and the tissue concentration of tacrolimus in relation to its toxicity should also be included.

The general opinion regarding the need for TDM for tacrolimus is different for mycophenolic acid (MPA). For MPA a concentration–effect relationship has been shown repetitively and in a randomized-controlled clinical trial it was also shown that with TDM it is possible to reduce the incidence of acute rejection in kidney transplant patients at low to moderate immunological risk [14]. Despite compelling evidence for the added value of TDM for MPA, many transplant centers do not perform TDM for MPA. In chapter 5 we have performed studies on the pharmacogenetics of MPA, with a focus on its toxicity. In clinical practice the use of MPA is complicated by the frequent occurrence of gastrointestinal adverse effects, especially diarrhea. The gastrointestinal side effects are an important reason for noncompliance [15]. In chapter 5.1 we have studied whether polymorphisms in

SLCO1B and *ABCB1* are associated with MPA-related diarrhea. On the basis of our findings, genotyping for *ABCB1* or *SLCO1B* pre-transplantation is unlikely to be of clinical value for individualization of MPA therapy. Hematologic side effects, such as anemia and leucopenia are another common side effect of MPA. Jacobson *et al.* [16] found that SNPs in *IL12A*, *CYP2C8* and *HUS1* are associated with the occurrence of MPA-related anemia but not leucopenia. In chapter 5.2 we tried to confirm these findings. Our results support the observation made by Jacobson *et al*. that *CYP2C8A* (rs11572076*G>A*) allele carriers have a higher risk to develop anemia. However, the actual number of patients carrying this variant allele was small and therefore the possibility of a spurious finding remains. We could not confirm the association with anemia and the *IL12A* (rs568408G>A) and *HUS1* (rs1056663C>T). This may be explained by the fact that the size of the population we studied was smaller than that studied by Jacobson *et al*. (n = 332 *vs*. n = 978, respectively) and the resulting lower statistical power to detect such an association. Although the CYP2C8A allele carriers have a higher risk to develop anemia, blood counts are frequently monitored after transplantation and after a MMF dose reduction, anemia and leucopenia are often rapidly reversible. Therefore the conclusion form this study is that although the *CYP2C8* genotype seems to be associated with the risk of developing anemia and leucopenia, a pharmacogenetic approach is at present not helpful to reduce the risk of MPA-related hematologic toxicity. It is therefore not recommended to dose the MPA differently or to choose for example for azathioprine in transplant patients with the CYP2C8A allele.

The studies mentioned above investigated the inter-patient variability, *i.e.* the differences in pharmacokinetic *between* patients. However, tacrolimus pharmacokinetics is also characterized by *intra-patient* variability, the variability *within one* patient over time. In daily practice this is visualized by fluctuating tacrolimus concentrations in patients with a stable tacrolimus dose. Recently, Borra *et al.* [17] demonstrated that a high variability in tacrolimus predose concentrations was a risk factor for graft loss in renal transplant patients. A determinant for higher intra-patient variability could be an individual's cytochrome P450 3A (*CYP3A*) genotype. Korean investigators [18] have suggested that not only inter-patient variability but also intra-patient variability is correlated with *CYP3A5* genotype. This was explained by the fact that in patients without functional CYP3A5 enzyme, the metabolism of tacrolimus depends exclusively on the activity of CYP3A4. Because the CYP3A4 enzyme is more sensitive to induction and inhibition, CYP3A5 nonexpressers could be more prone to variable tacrolimus clearance over time. In chapter 3.1 this hypothesis was investigated, by correlating the intra-patient variability in tacrolimus clearance to the *CYP3A5* genotype in the renal transplant patient used by Borra *et al*. [17] A relationship between the CYP3A5 genotype and the tacrolimus clearance was not found. The study from Yong Chung *et al.* who did find a relationship performed a more detailed pharmacokinetics analysis and the study population was different. Our study population (N = 208) consisted of renal transplant patients, whereas Yong Chung studied healthy volunteers (N = 29) with no co-medication and who used two different formulations of tacrolimus. Moreover the study

population by Yong Chung *et al.* consisted exclusively of Korean subjects and we had mainly Caucasian subjects and only 10% Asian subjects. We cannot rule out the possibility that the CYP3A4 enzyme of Koreans is more susceptible to induction or inhibition than the CYP3A4 enzyme of whites.

Another factor that might explain a higher intra-patient variability in tacrolimus pharmacokinetics is poor adherence to medication in timing and/or frequency of ingestion. In chapter 3.2 we have also studied the influence of intra-patient variability in heart transplant patients. In the heart transplant recipients, in contrast to the renal transplant recipients, a relationship between the intra-patient variability in tacrolimus pharmacokinetics and the risk on cardiac allograft vasculopathy was not found. This discrepancy between renal and heart transplant patients might be explained by the use of more control visits in heart transplant recipients compared to renal transplant recipients. Although the main immunosuppressive regimen in both groups consists of MPA and tacrolimus and prednisolone, the heart transplant patients use the prednisolone for a longer period of time and they have more out-patient visits in the first year compared to renal transplant patients. A progression in the disease might be noticed at an earlier stage, but also non-adherence to the therapy might be noticed earlier. This difference may have contributed to the contrasting outcomes of the two studies. Moreover, the number of investigated patients was relatively low, and therefore our study may not have enough statistical power to identify the association between intra-patient variability and the progression of vascular disease. Studies focusing on the intra-patient variability should be performed to clarify the causes for this phenomenon.

The promise of individualizing immunosuppressive drugs has not yet been realized. Some investigators have designed a dosing algorithm that incorporated all the factors that influenced the pharmacokinetics of the immunosuppressive drugs, in order to reach 'the right immunosuppressive treatment' after solid organ transplantation (6, 19, 20, 21 novel polymoprhimsms). However, a validation of the recently developed DeKAF algorithm for dosing tacrolimus failed to accurately predict the tacrolimus clearance [22]. Using real time tacrolimus doses and blood concentrations from an independent cohort did not show a good performance of the algorithm. The DeKAF algorithm took into account several clinical parameters, and for pharmacogenetics it only incorporated the *CYP3A5* genotype. A polygenic algorithm incorporating also other genes known to influence the clearance of tacrolimus, including *ABCB1*, *CYP3A4*22*, *POR*28* and *PXR* may be more predictive. However to conclude that the additional genetic variants (next to the *CYP3A5* genotype) explain the residual variability in tacrolimus requirement, the reported associations should be confirmed in independent cohorts. It then may become possible to develop an accurate starting dose for tacrolimus and under- or overexposure to tacrolimus may be prevented. We should however also include the pharmacogenetics of the donor. After identification of the different polymorphisms that have an influence on the immunosuppressive pharmacokinetics and pharmacodynamics, we might have a better 'dosing algorithm' for the transplanted patients.

However even when we use this pharmacogenetic dosing approach, a large proportion of overall variability will remain unexplained. Therefore the pharmacogenetics must go hand in hand with TDM, which is used to correct for the remainder of variability in the pharmacokinetics. As already mentioned intracellular tacrolimus concentrations might be more valuable to measure, and assays applying LC-MS are being developed, also in our center. However also here randomized clinical trials should be performed to define the optimal intra-cellular tacrolimus therapeutic window. When we would genotype the transplanted patients before their transplantations and after the transplantation we follow them with the right TDM measurements, we might help physicians to adapt the daily dose of the immunosuppressant to avoid major episodes of under- or overdosing and aspire to the 'right immunosuppressive treatment' after transplantation.

CONCLUSION OF THIS THESIS

- The SNPs in CYP3A and ABCB1 do not influence the ciclosporine pharmacokinetics and pharmacodynamics.
- The tacrolimus dose-adjusted trough blood concentration is higher in CYP3A4*22 T variant allele carriers.
- The CYP3A4*22 T variant allele carriers have also more delayed graft function and a poorer renal function when patients are treated with ciclosporine.
- The SNPs in CYP3A and ABCB1 do not influence the ciclosporine mediated nephrotoxicity.
- The ABCB1 3435C>T variant allele causes increased intralymphocytic tacrolimus accumulation.
- CYP3A5 expressers do not have a higher within patient variability in the apparent oral clearance of tacrolimus.
- A high within-patient variability in the apparent oral clearance of tacrolimus is not a risk factor for the progression of cardiac allograft nephropathy.
- Tacrolimus predose concentrations do not predict the risk of acute rejection after renal transplantation.
- Polymorphisms in the SLCO1B and ABCB1 do not influence the occurrence of MPA related diarrhea.
- CYP2C8A allele carriers have more MPA related anemia and leucopenia.

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Appendices

Scientific summary

Currently, immunosuppressive therapy for the prevention of acute rejection after kidney transplantation mostly consists of the combination of a calcineurin inhibitor (CNI), either cyclosporine or tacrolimus, plus mycophenolic acid (MPA), and glucocorticoids with or without induction therapy with an interleukin (IL)-2 receptor blocker or a T-lymphocyte depleting agent. With immunosuppressive drug combination therapy, patient and kidney allograft survival have greatly improved.

Chapter 2 discusses the studies on the relationship between single-nucleotide polymorphisms (SNPs) in *CYP3A* and *ABCB1* and the pharmacokinetics and pharmacodynamics of CNIs. In the first chapter (2.1) we have shown that the studied SNPs in *CYP3A4*, *CYP3A5* and *ABCB1* cannot explain the high interindividual pharmacokinetic variability of cyclosporine. In addition, the incidence of biopsy-proven acute rejection and the renal function were not associated with SNPs in these genes. In the next two chapters (2.2 and 2.3) we studied the recently discovered *CYP3A4*22* SNP. In paragraph 2.2 we show that in kidney transplant recipients on cyclosporine therapy, the CYP3A4*22 T allele emerges as a risk factor for delayed graft function and impaired renal function. Chapter 2.3 describes the study of the *CYP3A4*22* SNP with respect to tacrolimus PK. It is shown for the first time that the *CYP3A4*22* SNP in kidney transplant recipients is associated with reduced tacrolimus clearance. We have demonstrated in this study that kidney transplant recipients who carry 1 or 2 T alleles require a 33% lower mean tacrolimus dose to reach their target predose concentration compared to wild-type patients. The fourth paragraph of this chapter studied the influence of the *ABCB1* 3435C>T SNP on the pharmacodynamics of tacrolimus. In an *in vitro* study it was demonstrated that the tacrolimus-mediated inhibition of cytokine production in T-cells was enhanced by the ABCB1 inhibitor verapamil. We show that in kidney transplant recipients who were on tacrolimus therapy and have the *ABCB1* 3435CC genotype, which encodes the active ABCB1 variant, verapamil decreased IL-2 production. On the other hand, in patients with the *ABCB1* 3435TT genotype, the inactive variant, verapamil did not influence cytokine production. We have also calculated the requirement of tacrolimus in renal transplantation, by using the ratio of tacrolimus trough levels and percentage of IL-2 producing T-cells. The ratio was significantly higher in CD8+ T-cells of patients who have an active ABCB1 pump (3435CC genotype) compared to 3435TT genotype. This implies that tacrolimus had a smaller effect in kidney transplant recipients with the 3435CC genotype. In the last paragraph (2.5) we have studied the literature to review whether pharmacogenetic factors explain part of the interpatient variability in susceptibility to develop calcineurin-induced nephrotoxicity. We have found evidence in the literature that ABCB1 and to lesser extent CYP3A5 is implicated in the pathogenesis of CNI-induced renal dysfunction. In addition, SNPs in *ABCB1* and *CYP3A5* explain part of the interindividual differences in the susceptibility to the nephrotoxic effect of CNIs, although conflicting results have been published.

Next to the interindividual differences in the pharmacokinetics of immunosuppressive therapy we have studied in chapter 3 the intrapatient variability of tacrolimus pharmacokinetics. In the first paragraph (3.1) we studied kidney transplant recipients and found no significant associations between the intrapatient variability in tacrolimus clearance and the *CYP3A5* genotype. In the second paragraph we have studied the intrapatient variability in tacrolimus clearance in heart transplant recipients. We have studied whether a high within-patient variability in tacrolimus clearance is associated with more rapid progression of cardiac allograft vasculopathy. However, the study shows that the intrapatient variability in tacrolimus pharmacokinetics is not correlated with graft vascular disease at 4 year after transplantation nor to acute rejection in heart transplant recipients.

Chapter 4 is dedicated to therapeutic drug monitoring. After treatment with tacrolimus TDM is universally applied. However, the concentration-effect relationship for tacrolimus is poorly defined. We have investigated whether the tacrolimus concentrations are associated with acute rejection in kidney transplant recipients. We have pooled data from three large clinical trials and studied the relationship between biopsy proven acute rejection and tacrolimus predose concentration in 1304 patients. We did not find a significant correlation between tacrolimus predose concentrations and the incidence of biopsy-proven acute rejection.

In chapter 5 we have reported the studies on mycophenolate mofetil. We have studied whether polymorphisms in the *SLCO1B* gene and in the *ABCB1* gene in renal transplant patients are related to MPA pharmacokinetics or to the occurrence of MPA-related diarrhea. We have found that the pharmacokinetics of MPA are not influenced by the investigated *SLCO1B* nor by the *ABCB1* SNPs. Moreover, the incidence of diarrhea in the MPA treated patients could not be explained by the differences in the SLCO1B or the ABCB1 genotypes. In chapter 5.2 we have studied if the polymorphisms in the *IL12A, CYP2C8* and *HUS1* genes are associated with the occurrence of MPA related anemia and leucopenia. We concluded that the *CYP2C8A* allele carriers have a higher risk to develop anemia and leucopenia, but the *IL12A* and *HUS1* gene polymorphisms were not associated with the incidence of anemia.

Samenvatting

Het huidige immunosuppressieve regime voor de preventie van acute afstoting na niertransplantatie bestaat voornamelijk uit de combinatie van een calcineurineremmer (CNI), hetzij ciclosporine of tacrolimus, met mycofenolaat mofetil (MMF) en glucocorticoïden met of zonder inductie therapie. Met de ontwikkeling van deze combinatietherapie is de overleving van zowel de getransplanteerde nier als van de patiënt sterk verbeterd.

Hoofdstuk 2 beschrijft de resultaten van de studies over de relatie tussen genetische polymorfismen ("single-nucleotide polymorphisms; SNPs) in het CYP3A en ABCB1 gen en de farmacokinetiek en farmacodynamiek van calcineurineremmers. In de eerste paragraaf (2.1) hebben we aangetoond dat de polymorfismen in het CYP3A4, CYP3A5 en ABCB1 gen de grote interindividuele farmacokinetische variabiliteit van cyclosporine niet kunnen verklaren. De nierfunctie en de incidentie van acute afstoting bleek ook niet geassocieerd met polymorfismen in deze genen. De volgende twee hoofdstukken (2.2 en 2.3) beschrijven we het onderzoek naar het CYP3A4*22 polymorfisme. In paragraaf 2.2 tonen we aan dat in niertransplantatie patiënten die behandeld worden met ciclosporine, het CYP3A4*22 T variant allel een risicofactor is voor een vertraagde transplantaat functie en een slechtere nierfunctie. In hoofdstuk paragraaf 2.3 hebben we het CYP3A4*22 polymorfisme onderzocht in relatie tot de farmacokinetiek tacrolimus. In deze hfdts hebben we voor de eerste keer laten zien dat het CYP3A4*22 polymorfisme in niertransplantatie patiënten is geassocieerd met een verminderde klaring van tacrolimus. We hebben in deze studie aangetoond dat niertransplantatie patiënten die 1 of 2 T allelen hebben van het CYP3A4*22 gen, een 33% lagere gemiddelde dosis van tacrolimus nodig hebben voor de beoogde tacrolimus concentratie (C_0) vergeleken met wild-type patiënten. De vierde paragraaf van hoofdstuk 2 bestudeerde de invloed van het ABCB1 3435C>T polymorfisme op de farmacodynamiek van tacrolimus. In een ***in vitro*** studie hebben wij aangetoond de cytokine productie in T cellen, die geremd wordt door tacrolimus, door de ABCB1 remmer verapamil verder wordt geremd, vermoedelijk doordat verapamil zorgt voor een hogere intra-cellulaire tacrolimus concentratie. We hebben laten zien dat in niertransplantatie patiënten die behandeld worden met tacrolimus en die het ABCB1 3435CC genotype hebben, de actieve ABCB1 variant, verapamil de IL-2 productie verlaagt. Daarentegen heeft verapamil bij patiënten met het inactieve ABCB1 genotype, ABCB1 3435TT, geen invloed op de IL-2 productie. Ook hier speelt de accumulatie van tacrolimus intra-cellulair een belangrijke rol. We hebben ook 'de behoefte aan tacrolimus' berekend, door de ratio te berekenen tussen tacrolimus dalspiegels en het percentage IL-2 producerende T-cellen. De ratio was significant hoger in patiënten met een actieve ABCB1 pomp (3435CC genotype) vergeleken met patiënten met het 3435TT genotype. Dit impliceert dat tacrolimus minder effect heeft in niertransplantatie patiënten met het 3435CC genotype. In de laatste paragraaf (2.5) hebben we de literatuur bestudeerd met betrekking tot farmacogenetische

factoren en calcineurin geïnduceerde nefrotoxiciteit. We hebben bewijs gevonden in de literatuur dat ABCB1 en in mindere mate CYP3A5 betrokken zijn bij de pathogenese van CNI geïnduceerde renale dysfunctie. Daarnaast zouden polymorfismen in het ABCB1 en CYP3A5 gen een deel van de interindividuele verschillen in de gevoeligheid voor de nefrotoxische werking van calcineurineremmers verklaren, maar er worden tegenstrijdige resultaten gevonden.

Naast de interindividuele verschillen in de kinetiek van immunosuppressieve therapie hebben we in hoofdstuk 3 de intra-patiënt variabiliteit van tacrolimus farmacokinetiek bestudeerd. In de eerste paragraaf (3.1) hebben we onderzocht of het CYP3A5 genotype de intra-patient variabiliteit in tacrolimus farmacokinetiek kan verklaren. We hebben gevonden dat het CYP3A5 genotype geen invloed heeft op de intra-patient variabiliteit van tacrolimus. In de tweede paragraaf hebben we de intra-patiënt variabiliteit in tacrolimus klaring onderzocht na harttransplantatie. We hebben onderzocht of een hoge intra-patiënt variabiliteit in tacrolimus klaring geassocieerd is met een snellere progressie van cardiale allograft vasculopathie. Uit deze studie blijkt dat de intra-patiënt variabiliteit in tacrolimus farmacokinetiek niet is gecorreleerd met cardiale allograft vasculopathie 4 jaar na transplantatie, noch met acute afstoting na harttransplantatie.

Hoofdstuk 4 is gewijd aan het meten van concentraties van geneesmiddelen in het bloed (TDM). Tijdens behandeling met tacrolimus wordt TDM universeel toegepast. De concentratie - effect relatie voor tacrolimus is echter slecht gedefinieerd. Wij hebben onderzocht of de tacrolimus concentraties een relatie hebben met acute afstoting na niertransplantatie. We hebben gegevens samengevoegd uit drie grote klinische studies en hebben de relatie tussen biopsie bewezen acute afstoting en tacrolimus predose-concentraties in 1304 patiënten onderzocht. Wij zagen geen significante correlatie tussen tacrolimus predose concentraties en de incidentie van acute afstoting.

In hoofdstuk 5 zijn de studies gerapporteerd over mycofenolaat mofetil. Er is onderzocht of polymorfismen in het SLCO1B gen en in het ABCB1 gen bij niertransplantatiepatiënten zijn gerelateerd aan de farmacokinetiek van MPA en het optreden van de MPA-gerelateerde diarree. Wij hebben gevonden dat de farmacokinetiek van MPA niet wordt beïnvloed door de onderzochte SLCO1B noch door de ABCB1 SNPs. Bovendien wordt de incidentie van diarree in de MPA behandelde patiënten niet verklaard door de verschillende SLCO1B of de ABCB1 genotypes. In hoofdstuk 5.2 hebben we onderzocht of polymorfismen in het IL12A, CYP2C8 en HUS1 gen geassocieerd zijn met het optreden van MPA gerelateerde anemie en leukopenie. De conclusie luidt dat patiënten met het CYP2C8A allel een hoger risico hebben om anemie en leukopenie te ontwikkelen, maar de IL12A en HUS1 polymorfismen niet geassocieerd zijn met de incidentie van anemie of leukopenie.

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2. *Tacrolimus: Pharmacokinetics and pharmacogenetics; Optimized use of immunosuppressive drugs in transplantation*; **R. Bouamar.** D.A. Hesselink, T. van Gelder; International Transplantation Updates 2009; ISBN: 978-84-9926-055-6
3. *Functionality test for drug safety alerting in computerized physician order entry systems.* van der Sijs H, **Bouamar R,** van Gelder T, Aarts J, Berg M, Vulto A. Int J Med Inform. 2010 Apr;79(4):243-51.
4. *The pharmacogenetics of calcineurin inhibitor-related nephrotoxicity.* Hesselink DA, **Bouamar R,** van Gelder T. Ther Drug Monit. 2010 Aug;32(4):387-93.
5. *Polymorphisms in CYP3A5, CYP3A4, and ABCB1 are not associated with cyclosporine pharmacokinetics nor with cyclosporine clinical end points after renal transplantation.* **Bouamar R,** Hesselink DA, van Schaik RH, Weimar W, Macphee IA, de Fijter JW, van Gelder T; Ther Drug Monit. 2011 Apr;33(2):178-84.
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8. *Mycophenolic acid-related anemia and leucopenia in renal transplant recipients are related to genetic polymorphisms in CYP2C8.* **Bouamar R,** Elens L, Shuker N, van Schaik RH, Weimar W, Hesselink DA, van Gelder T. Transplantation. 2012 May 27;93(10):e39-40
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11. *ATP-binding cassette transporters as pharmacogenetic biomarkers for kidney transplantation.* Shuker N, **Bouamar R,** Weimar W, van Schaik RH, van Gelder T, Hesselink DA. Clin Chim Acta. 2012 Sep 8;413(17-18):1326-37.
12. *Tacrolimus predose concentrations do not predict the risk of acute rejection after renal transplantation: a pooled analysis from three randomized-controlled clinical trials*. **Bouamar R,** Shuker N, Hesselink DA, Weimar W, Ekberg H, Kaplan B, Bernasconi C, van Gelder T. *Am J Transplant. 2013 May;13(5):1253-61.*
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15. *Clinical implementation of pharmacogenetics in kidney transplantation: CNIs in the starting blocks.* Elens L, **Bouamar R,** Shuker N, Hesselink DA, van Gelder T, van Schaik RH. Br J Clin Pharmacol. 2013 Oct 10.
16. *Genetic polymorphisms in IL-2, IL-10, TGF-B1 and IL2RB and acute rejection in renal transplant patients.* Z. Chen, **R. Bouamar,** R.H.N. van Schaik, J.W.de Fijter, A. Hartmann, M. Zeier, K. Budde, D.R.J. Kuypers, W. Weimar, D. A. Hesselink, T. van Gelder; Submitted
17. *The role of pharmacogenetics in the disposition of and response to tacrolimus in solid organ transplantation.* D A. Hesselink, **R. Bouamar,** L.Elens, R.H.N. van Schaik, T. van Gelder, Invited Review Clinical Pharmacokinetics; 2013 Nov 19.

PhD portfolio

Name PhD student: Drs. R. Bouamar	PhD period: 2009 - 2013
Erasmus MC Department: Hospital Pharmacy & Internal Medicine	Promotor(s): Prof. dr. T. van Gelder & Prof. dr. W. Weimar
	Supervisor: Dr. D.A. Hesselink

1. PhD training

	Year
General courses	
- Cursus Methodologie van Patiëntgebonden Onderzoek en Voorbereiding van Subsidie aanvragen	2009
- Presentation training skills Erasmus Medical Center	2009
- Biostatistics for Clinicians	2010
- Course Regression analysis	2010
- Introduction to data analysis	2010
- There's no excuse for writing	2010
- Het leren doen van onderzoek	2010
- Cursus Moleculaire Biologie, Molecular Medicine	2011
- Biomedical English Writing an Communication	2011
- Presentation training skills	2011
- NIH course Principles of Clinical Pharmacology	2012
- Course Endnote	2012
Seminars and workshops	
- Department Journal Club	2008 - 2013
Presentations (oral)	
- International Congress of Therapeutic Drug Monitoring and Clinical Toxicology (Germany)	2011
- Annual congress Dutch Transplantation Society, (the Netherlands)	
- Annual congress Dutch Transplantation Society, (the Netherlands)	2012
- Figon Dutch Medicine Days (The Netherlands)	2013
	2013
Presentations (poster)	
- Figon Dutch Medicines Days (the Netherlands)	2009
- American Transplant Congress (Boston, United States)	2009
- Molmed Day (The Netherlands)	2010
- Middle Eastern Society of Organ Transplantation (Tunisia)	2010
- International Congress of Therapeutic Drug Monitoring and Clinical Toxicology (Stuttgart, Germany)	2011
(Inter)national conferences	
- Annual congress Dutch Transplantation Society, The Netherlands (participated every year)	2008 - 2013
- Middle Eastern Society of Organ Transplantation (Tunisia)	2010
- International Congress of Therapeutic Drug Monitoring and Clinical Toxicology (Stuttgart, Germany)	2011
- Siemens European Symposium on Clinical and Translational Immunosuppression (Barcelona, Spain)	2011
- Klinisch Review Symposium, (Utrecht, The Netherlands)	2009 - 2013
- Molmed Day (Rotterdam, The Netherlands)	2010 – 2012

2. Teaching

	Year
- Education in pharmacy 5th/6th year medical students, Erasmus Medical Center	2008 - 2013
- Education in pharmacology HBO students, Hoge School Rotterdam	2012
Supervising	
- Nilufar Pashaee	2011
- Zhigang Chen	2012
Other	
- Speakers award Talent day	2010
- Speakers award Annual congress Dutch Transplantation Society	2012
- Speakers award Annual congress Dutch Transplantation Society	2013
- Novartis Personalized Immunosuppression Award Annual congress Dutch Transplantation Society	2013
- Editorial Board of Transplantation	2013

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