

# Pharmacological treatments and monitoring strategies to improve outcome in solid organ transplants

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Oslo, June 2014

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## LIST OF PAPERS

- I. **Robertson I**, Åsberg A, Granseth T, Vetthe NT, Akhlaghi F, Ghareeb M, Molden E, Reier-Nilsen M, Holdaas H and Midtvedt K. More potent lipid lowering effect by rosuvastatin in everolimus treated renal transplant recipients. *Transplantation*. 2014. 27;97(12): 1266-1271
- II. **Robertson I**, Falck P, Andreassen AK, Næss NK, Lunder N, Christensen H, Gullestad L and Åsberg A. Whole blood, intralymphocyte and endomyocardial concentrations of CsA in heart transplant recipients. *Transplant Res*. 2013. 2(1): p.5.
- III. **Robertson I**, Vetthe NT, Midtvedt K, Falck P, Christensen H and Åsberg A. Closer to the site of action; everolimus concentrations in peripheral blood mononuclear cells correlate well with whole blood concentrations. (Submitted to *Therapeutic Drug Monitoring*)
- IV. **Robertson I**, Åsberg A, Ingerø AO, Vetthe NT, Bremer S, Bergan S and Midtvedt K. Use of generic tacrolimus in elderly renal transplant recipients – precaution is needed. (Accepted for publication in *Transplantation*, June 2014)

**ABBREVIATIONS**

ABC	ATP-binding cassette
ALERT	Assesment of Lescol in renal transplantation
AUC	Area under the concentration versus time curve
BCRP	Breast cancer resistance protein
BPAR	Biopsy proven acute rejection
C <sub>0</sub>	Concentration before drug intake
C <sub>2</sub>	Concentration 2 hours after drug intake
C <sub>3</sub>	Concentration 3 hours after drug intake
C <sub>4</sub>	Concentration 4 hours after drug intake
CI	Confidence interval
C <sub>max</sub>	Maximum plasma/whole blood concentration
CNI	Calcineurin inhibitors
CsA	Cyclosporine A
CV	Coefficient of variation
CYP	Cytochrome P450
eGFR	Estimated glomerular filtration rate
EMA	European Medicine Agency
EVE	Everolimus
FDA	US Food and Drug Administration
FKBP12	FK506 binding protein
GFR	Glomerular filtration rate
HDL	High-density lipoprotein
HT	Hypertension
IC <sub>50</sub>	50% inhibitory concentration
IL-2	Interleukin-2
LDL	Low-density lipoprotein
LVH	Left ventricular hypertrophy
MHC	Major histocompatibility complex
MMF	Mycophenolate mofetil
MRP2	Multidrug resistance protein 2
mTOR	Mammalian target of rapamycin
ND	Not determined

## ABBREVIATIONS

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NFAT	Nuclear factor of activated T cells
NTI	Narrow therapeutic index
OAT	Organic anion transporters
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporters
OKT3	Mouse monoclonal anti-CD3 T cell antibody
PBMC	Peripheral blood mononuclear cells
P-gp	P-glycoprotein
POR	Cytochrome P450 oxidoreductase
PPARA	Peroxisome proliferator-activated receptor alpha
PSI	Proliferation signal inhibitor
PTDM	Post transplant diabetes mellitus
SLC	Solute carrier
SNP	Single nucleotide polymorphism
TAC	Tacrolimus
TCR	T-cell receptor
TDM	Therapeutic drug monitoring

**ABSTRACT**

Following solid organ transplantation individualizing the immunosuppressive therapy to obtain an optimal balance between therapeutic efficacy and the occurrence of adverse events is the ultimately goal. This is complicated by the high intra- and interindividual pharmacokinetic variability and the narrow therapeutic index of the immunosuppressive drugs. Small variations in drug exposure may result in suboptimal immunosuppression or drug toxicity, with potentially adverse effects on patient outcomes. Therapeutic drug monitoring (TDM) is therefore mandatory in order to individualize the therapy. More knowledge and further improvements of drug treatment strategies and monitoring techniques are still desirable to further improve TDM and hence potentially both short- and long term outcomes after transplantation.

The primary objective of this thesis was to investigate some different pharmacological treatments and monitoring strategies to improve outcome in solid organ transplants. In this thesis results from three prospective clinical trials in solid organ transplants are presented. The lipid-lowering effect of rosuvastatin in comparison with fluvastatin, and the potential bilateral drug-drug interaction between rosuvastatin and everolimus (EVE) were assessed in renal transplant recipients at a stable phase following transplantation. Further, the relationship between both cyclosporine A (CsA) and EVE concentrations in different body compartments were evaluated as potential TDM tools in heart- and renal transplant recipients, respectively. Finally, the bioequivalence of an approved generic tacrolimus (TAC) was investigated with the original drug as reference in elderly stable renal transplant recipients.

In renal transplant recipients receiving EVE based immunosuppression and treated with fluvastatin, a switch to rosuvastatin induced a significant additional lipid lowering effect. The combination of EVE and rosuvastatin appears to be safe as EVE pharmacokinetics were unaffected following the switch to rosuvastatin. The systemic exposure of rosuvastatin was less than 3-fold higher compared to non-transplants reported in the literature when combined with EVE, and this is comparable to what is previously shown for fluvastatin in combination with CsA, a combination considered to be safe in renal transplant recipients. Safely achieving reduction in lipids could be of great importance in reducing cardiovascular risk in this high risk population.



No correlation between CsA concentrations in whole blood, T-lymphocytes or endomyocardial tissue was established in heart transplant recipients, potentially challenging traditional TDM based on whole blood CsA concentrations in these patients. In contrast, EVE concentrations in whole blood and PBMC correlated well and supports that TDM of EVE in whole blood is an appropriate choice.

The generic TAC formulation was not found to be bioequivalent to the original drug in elderly renal transplant recipients. Use of generic TAC resulted in a significantly higher systemic drug exposure. In the long run this may put the patients at higher risk of calcineurin inhibitor-related toxicity and impaired long-term outcomes. Importantly, the lack of bioequivalence would not have been detected by the standard monitoring parameter, TAC trough concentrations, as these concentrations were similar for both formulations. Generic TAC should be used with caution in elderly renal transplant recipients and it should be recognized that bioequivalence studies performed in healthy volunteers do not necessarily reflect the average transplant recipient.



# 1 INTRODUCTION

In 1956, just two years after the first successful renal transplantation had been performed between two monozygotic twins in Boston, USA, a renal transplantation from an unrelated donor to a patient with end stage renal disease was performed at Rikshospitalet, Oslo. The patient lived for 30 days with his new kidney, which is quite impressive given the insufficient immunosuppressive therapy available at that time.<sup>1</sup> During the last 10 years, between 250 and 300 renal transplantations have been performed annually in Norway, with 50% of the grafts functioning after about 11 years from deceased donors and 17-18 years from living donors.<sup>2</sup> The first heart transplantation in the Nordic countries was performed at Rikshospitalet, Oslo in 1983.<sup>3</sup> Due to donor organ shortage, only 30 to 35 heart transplantations are performed in Norway annually and mean survival is  $12.3 \pm 5.3$  years.<sup>4,5</sup>

## 1.1 Immunosuppressive therapy

The first attempts of inducing satisfactory immunosuppression in humans in order to make organ transplantation possible were the use of total body irradiation in combination with corticosteroids. This effort to control the immune system was proved either ineffectual or lethal, and it became evident that without chronic pharmacological immunosuppression, most grafts would be lost to acute/chronic rejection or recurrent kidney disease.<sup>6</sup> In the early 1960s the first successful pharmacological immunosuppressant, azathioprine was introduced for human use. In combination with corticosteroids, azathioprine quickly replaced alternative non-pharmacological approaches and renal transplantation became a viable treatment of end-stage renal disease, with a one-year graft survival of about 50%.<sup>6,7</sup> However, it was the introduction of the calcineurin inhibitor (CNI), cyclosporine A (CsA), in the beginning of the 1980s, which revolutionized transplant medicine, dramatically improved short-term graft survival for renal transplant recipients and made heart transplantation possible.<sup>8,9</sup> In the same period, the first reports on the use of mouse monoclonal anti-CD3 T cell antibody (OKT3) was also published.<sup>10</sup> The next advance came in the 1990s with the introduction of mycophenolate mofetil (MMF), tacrolimus (TAC) and sirolimus.<sup>11-13</sup> Additionally, anti T-cell agents were introduced for initial induction immunosuppression (to prevent early acute rejections) and as rescue therapy for steroid resistant rejections. These agents included anti-thymocytic globulins derived from horse or rabbit serum (e.g ATG<sup>®</sup> and Thymoglobulin<sup>®</sup>), and the anti-interleukin-2 (IL-2) receptor antibodies, daclizumab (withdrawn from the market

in 2009) and basiliximab.<sup>14,15</sup> An attempt was also made to improve the pharmacokinetic characteristics of both CsA and the proliferation signal inhibitor (PSI), sirolimus. CsA was formulated as a microemulsion pre-concentrate (Neoral<sup>®</sup>) and another PSI, everolimus (EVE) was introduced to the market in the early 2000s.<sup>16,17</sup> In 2011, the co-stimulation blocker belatacept was approved as the first biological agent for use in maintenance immunotherapy.<sup>18</sup>

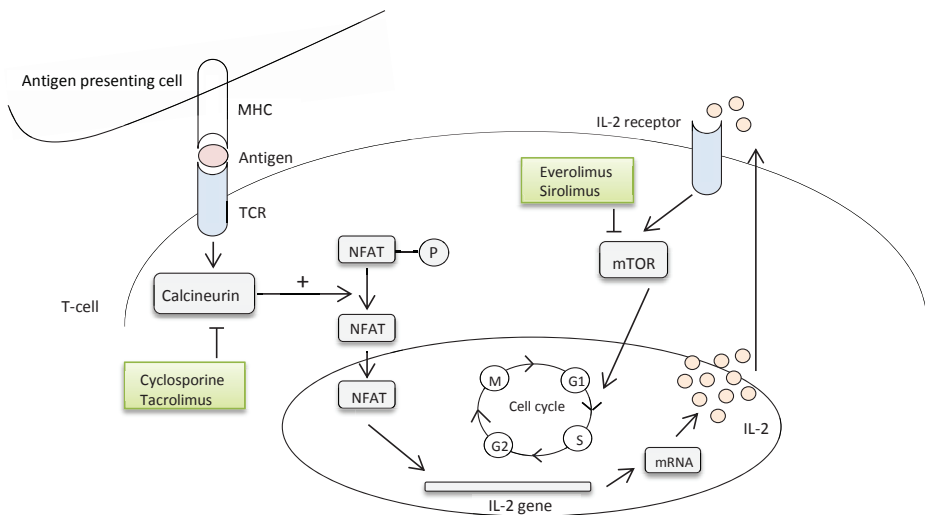
Immunosuppression is normally given as a combination of agents with different mechanism of action. By using combination regimens of the immunosuppressive drugs, the dosing and toxicity of each agent can be minimized without compromising the total immunosuppressive effect. The CNIs are still the backbone in most immunosuppressive regimens. TAC has since its introduction gradually replaced CsA and is now the dominant CNI in clinical transplantation. PSIs is used either in a combination with low dose CNI or as a substitute after CNI withdrawal or avoidance. In Norway, the current immunosuppressive protocol after renal transplantation is a quadruple regimen consisting of induction therapy with two doses of 20 mg basiliximab and a maintenance therapy of TAC (0.04 mg/kg) in combination with MMF (1.5 mg/day) and corticosteroids. The use of CsA is currently limited and is only administered to patients already treated with CsA. In renal transplant recipients with previous malignant disease (transplanted at the earliest one year after remission of malignancy) conversion from CNI to a PSI is considered seven weeks after transplantation. For heart transplant recipients, the immunosuppressive strategy is based on a triple drug regimen consisting of CNI, MMF and corticosteroids. In patients with deteriorating renal function, conversion from CNI to EVE is strongly considered.

Using modern powerful immunosuppressive drug combination therapy, the incidence of acute rejection has become low (in general <20%) and most centers have 1-year graft survival rates >90%. However, long term outcomes are still challenged by the adverse events of immunosuppressive drugs, contributing to late graft failure, cardiovascular morbidity, opportunistic infections and malignancies. Hence, optimization of immunosuppressive regimens is needed.

### **1.1.1 Pharmacodynamics**

Lymphocytes play a central role in cell-mediated immune response and are the site of action of immunosuppressive drugs. CsA and TAC depend on different intracellular mediators

(immunophilins) to achieve their action, but the target for both is the protein phosphatase calcineurin. CsA acts by binding to cyclophilin while TAC binds to another immunophilin, FKBP12 (FK506 binding protein 12). Both the CsA-cyclophilin complex and the TAC-FKBP12 complex inhibit the activity of calcineurin and thereby reduce its phosphatase activity in a dose proportional manner.<sup>19</sup> By inhibiting calcineurin, CsA and TAC suppress the dephosphorylation of nuclear factor of activated T cells (NFAT) and hence prevent the translocation of NFAT into the nucleus where it acts as a transcription factor. This results in an impaired synthesis of IL-2 and other important cytokines.<sup>20,21</sup> IL-2 serves as a cell cycle progression signal for T-cells, stimulating both their proliferation and differentiation.<sup>22</sup> EVE also complex with FKBP12, but unlike TAC, does not inhibit calcineurin activity. Instead, the EVE-FKBP12 complex is a highly specific inhibitor of mammalian target of rapamycin (mTOR), which is a cell-cycle specific kinase. Inhibition of the mTOR pathway blocks progression of the cell cycle from G1 into the S phase, which suppresses interleukin-driven T-cell proliferation. In contrast to the CNIs, EVE acts at a later stage in the cell cycle, not blocking the production of growth factors, but rather the proliferation signal that is provided by these factors.<sup>17,23</sup> A schematic figure of the mechanism of action for the CNIs and the PSIs is shown in Figure 1.



**Figure 1.** Schematic and simplified figure of the mechanism of action for the calcineurin inhibitors (cyclosporine A and tacrolimus) and the proliferation signal inhibitors (everolimus and sirolimus). IL-2, interleukin-2; MHC, major histocompatibility complex; mTOR, mammalian target of rapamycin; NFAT, nuclear factor of activated T cells; TCR, T-cell receptor.

### 1.1.2 Adverse events

Despite their clinical efficacy, both CsA and TAC are limited by toxicity due to the wide tissue distribution of calcineurin. Calcineurin and NFAT isoform are not T-cell specific, and inhibition of this pathway by the CNIs give rise to toxicity beyond immunosuppression. Similar, mTOR is a ubiquitous kinase and consequently, the PSIs inhibit not only T cells, but also B lymphocytes and other immune cells. Hence, adverse effects of the PSIs reflect their lack of specificity for lymphocytes.<sup>24</sup>

#### *Calcineurin inhibitors*

Hypertension, dyslipidemia, neurotoxicity and post-transplant diabetes mellitus (PTDM) are well-known adverse effects of the CNIs.<sup>25-28</sup> CsA is more likely to cause dyslipidemia and hypertension, while TAC is more diabetogenic.<sup>29,30</sup> Treatment with the CNIs is however especially hampered by nephrotoxicity, which contributes to the late allograft loss in a substantial proportion of renal transplant recipients.<sup>31</sup> The acute nephrotoxicity may present as an acute oligoanuric syndrome (delayed graft function) or as a rise in serum creatinine. Acute nephrotoxicity usually occurs early after starting CNI treatment and in general, this acute CNI-induced nephrotoxicity is rapidly and completely reversible on dose reduction or CNI withdrawal.<sup>31,32</sup> It is characterized by constriction of the afferent arteriole, leading to a decreased renal plasma flow and a reduction of the glomerular filtration rate (GFR).<sup>33</sup> This reduction in GFR has been shown to be reduced following each given dose of CsA, primary via hemodynamic effects on the afferent arteriole.<sup>34,35</sup> Chronic CNI-induced nephrotoxicity is associated with prolonged use of these agents and has also been observed after transplantation of an organ other than the kidney. In fact, nearly 30% of heart transplant recipients develop renal dysfunction as early as one year post heart transplantation, an independent risk for both all-cause and cardiac mortality.<sup>36,37</sup> In contrast to the acute form, chronic CNI-induced renal insufficiency improves little after dose reduction or cessation of CNIs. It is associated with irreversible renal functional deterioration as a result of irreversible and progressive tubulo-interstitial injury and glomerulosclerosis.<sup>31,38</sup> Other adverse effects of the CNIs include increased susceptibility to infections and cancer due to the immunosuppressive effect *per se*.<sup>39-42</sup>

### *Everolimus*

The most frequent adverse effects of EVE are hypercholesterolemia and hypertriglyceridemia. In a review of 17 randomized controlled trials, EVE showed an increase in cholesterol and triglycerides levels in all but one study.<sup>43</sup> A large prospective trial found no difference in the occurrence of PTDM in the two EVE treatment groups compared to MMF.<sup>44</sup> Thrombocytopenia and anemia are frequent, though usually mild.<sup>44,45</sup> Rash, acne and mouth ulcers are the most frequent early complications reported by patients receiving treatment with PSIs.<sup>46</sup> Non-infectious pneumonitis is another complication associated with the PSIs.<sup>47,48</sup> Additionally, impaired wound healing has been described in renal transplant recipients receiving sirolimus.<sup>49</sup> However, wound healing did not differ between EVE and MMF treated patients in a large randomized trial.<sup>44</sup>

#### **1.1.3 Pharmacodynamic variability**

The correlation between drug exposure and pharmacodynamics is far from close. Drug concentrations within the therapeutic range do not guarantee absence of rejection or avoidance of toxicity in all patients. Thus, interindividual pharmacodynamic differences in response to the immunosuppressive drugs are also important in the determination of the overall clinical response. However, for the immunosuppressive drugs, there is no accurate “immunometer” to determine whether the level of immunosuppression is adequate, suboptimal or excessive. Previous work has shown different approaches of measuring the actual pharmacodynamic effect of each single immunosuppressive drug such as calcineurin activity, IL-2 production, expression of genes encoding cytokines and intralymphocyte ATP concentrations in CD4+ cells for the CNIs.<sup>50-53</sup> However, none of these approaches are currently in any broad clinical use. An even more valuable “immunometer” would be a method that covered the total immunosuppression in each patient, reflecting the combined effect of all immunosuppressive drugs used.

#### **1.1.4 Pharmacokinetics**

The intestinal absorption of both CsA and TAC is highly variable and the bioavailability of both drugs is low. The poor and unpredictable bioavailability of CsA is depending on the population studied (ranging from 10 to 89% for the Neoral® formulation).<sup>54</sup> For TAC, an average bioavailability of about 25% (ranging from 5 to 90 %) has been reported.<sup>55,56</sup> The

absolute oral bioavailability of EVE has not been assessed clinically, but based on animal investigations the bioavailability of EVE is considered to be low (16 %).<sup>57</sup> The low and variable bioavailability of both the CNIs and EVE is believed to be largely attributable to variability in expression and function of the metabolizing cytochrome P450 (CYP) 3A isoenzymes and of the multidrug efflux transporter P-glycoprotein (P-gp) both in the intestine and the liver, i.e. high first-pass effect.<sup>57-64</sup>

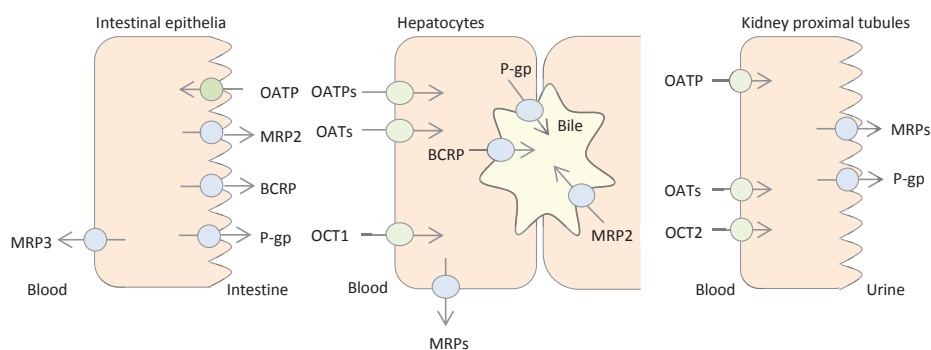
The human CYP3A isoform CYP3A4 is the most abundantly expressed CYP enzyme expressed in the liver and intestine for the majority of individuals and the main drug-metabolizing enzyme in humans.<sup>65-67</sup> Estimates suggest that the metabolism of approximately 40-50% of all drugs on the market involves CYP3A-mediated oxidation.<sup>65</sup> CYP3A4 expression is highly variable between individuals, with 10- to 100-fold differences in the liver and up to 30-fold differences in small intestine expression.<sup>68</sup> The CYP3A isoform CYP3A5 is closely related to CYP3A4 and shows significant overlap in substrate specificity, although the substrate affinity may differ.<sup>69</sup> The efflux pump P-gp is expressed in the liver, in pancreas, on enterocytes in the small intestine and colon, in the blood-brain barrier and in the human kidney. P-gp is also found in the membrane of lymphocytes.<sup>70</sup> The tissue distribution and the broad substrate specificity indicate that P-gp play a major role in protecting the body against xenobiotics.<sup>71</sup> CYP3A and P-gp have overlap in their substrate specificities, which allow CYP3A to have repeated contact with the substrate and its metabolites after extrusion by P-gp and subsequent reabsorption.<sup>68</sup>

Both CsA and TAC are extensively distributed in erythrocytes and more than 90% of CsA and TAC in plasma are bound to plasma proteins (lipoproteins and albumin/alpha 1-acid glycoprotein).<sup>72,73</sup> Similar to the CNIs, 75% of EVE is distributed into erythrocytes and approximately 75% of the plasma fraction is protein bound.<sup>74</sup> Metabolism of CsA and TAC occurs mainly in the liver and in the gastrointestinal epithelial cells predominantly by CYP3A4 and CYP3A5.<sup>75-78</sup> EVE is also metabolized by CYP3A4 and CYP3A5 and to a lesser extent by CYP2C8.<sup>78</sup> Metabolism of these drugs is virtually complete, with less than 1% of the parent drugs appearing in urine or feces. After metabolization, metabolites of CsA, TAC and EVE are eliminated in the bile and less than 5% is excreted in the urine.<sup>74,79-81</sup>

In addition to P-gp, several other drug transporters have been reported to play an important role in the absorption, distribution and elimination of CsA, TAC and EVE. These include transporters belonging to the ATP-binding cassette (ABC) transporter family such as the



multidrug resistance protein 2 (MRP2, also known as ABCC2) and breast cancer resistance protein (BCRP, also known as ABCG2) as well as transporters in the solute carrier family (SLC), including the organic anion-transporting polypeptides (OATPs).<sup>82-86</sup> An illustration of selected drug transporters in the intestinal epithelia, hepatocytes and kidney proximal tubules represented in Figure 2.



**Figure 2:** Illustration of selected human drug transporters in intestinal epithelia, hepatocytes and kidney proximal tubules. The uptake transporters, OATPs, OATs and OCTs are colored in green and the efflux transporters, P-gp, BCRP and MRPs are colored blue. Modified from Giacomini et al.<sup>87</sup> BCRP, breast cancer resistance protein; MRPs, multidrug resistance proteins; OATPs, organic anion transporting polypeptides; OAT, organic anion transporters; OCT, organic cation transporters; P-gp, P-glycoprotein.

### 1.1.5 Pharmacokinetic variability

Both the CNIs and EVE are characterized by a high inter- and intraindividual pharmacokinetic variability. Interindividual variability in the expression and activity of drug metabolizing enzymes and drug transporters are thought to be the major factors contributing to this highly variable pharmacokinetics of the CNIs and EVE. Variability in protein expression and activity in metabolizing enzymes and drug transporters could be determined by genetic and/or environmental factors. Environmental factors include foods, intoxicants, pollutions and drugs whereas genetic variability is usually the product of single nucleotide polymorphism (SNP). Other factors associated with pharmacokinetic variability are for example age, weight, organ function, disease state and protein binding.

***Genetic polymorphisms in cytochrome P450 enzymes and drug transporters***

The expression of CYP3A5 has been found to be largely determined by genetic polymorphism. A SNP in the third intron of *CYP3A5* (6986G>A, rs 776746) results in an alternatively spliced mRNA variant, which translates to a truncated non-functional protein.<sup>88</sup> This variant, designated as *CYP3A5\*3*, is the major allele among Caucasians and only individuals with at least one *CYP3A5\*1* allele are therefore classified as CYP3A5 expressers.<sup>88</sup> The association between *CYP3A5* genotype and TAC pharmacokinetics is well established, with patients expressing CYP3A5 (*CYP3A5\*1* carriers) requiring 2-fold higher doses of TAC compared with CYP3A5 non-expressers (*CYP3A5\*3/\*3*) to reach similar blood concentrations.<sup>89-91</sup> The impact of *CYP3A5* genotype status on the pharmacokinetics of CsA is less clear. The *in vitro* intrinsic metabolic clearance of CsA calculated from total metabolite formation is approximately 2.3 fold higher for CYP3A4 than for CYP3A5. Thus, CYP3A4 appears to play a more dominant role than CYP3A5 in the metabolism of CsA and the influence of the CYP3A5 polymorphism on the pharmacokinetics of CsA is limited.<sup>92-94</sup> No significant effect of CYP3A5 genotype on the pharmacokinetics of EVE has been observed and similar to CsA, CYP3A4 is most likely the predominant enzyme involved in the metabolic clearance of EVE.<sup>91,95-97</sup> In contrast to CYP3A5, the genetic basis for variable expression and activity of CYP3A4 remains poorly understood. However, a recently discovered SNP in intron 6 of the *CYP3A4* gene (c.522-191C>T; rs35599367; *CYP3A4\*22*) has been associated with reduced CYP3A4 activity.<sup>98,99</sup> Although the allele frequency is relatively low (5-7% in Caucasian population) studies show that carriers of the *CYP3A4\*22* requires lower CNI doses compared to patients expressing the wild type.<sup>94,99-101</sup> Contrary, *CYP3A4\*22* does not seem to substantially influence the pharmacokinetics of EVE.<sup>95</sup> Sequence variants located in the peroxisome proliferator-activated receptor-alpha (PPARA) and in the electron donor, cytochrome P450 oxidoreductase (POR) are other variants newly identified and potential contributors to the variability in CYP3A4 expression and activity.<sup>102,103</sup>

P-gp is encoded by the *ABCB1* gene which is polymorphically expressed with at least 50 SNPs identified to date. The most common and extensively studied SNPs include 3435C>T in exon 26, 1236C>T in exon 12 and 2677G>T/A in exon 21. The functional significance of these SNPs is controversial.<sup>104,105</sup> The majority of studies have focused on the *ABCB1* 3435C>T SNP and several studies have associated the homozygous 3435 TT variant genotype with lower intestinal P-gp expression and/or activity *in vivo*.<sup>106-108</sup> However, the results are

conflicting.<sup>109-112</sup> Variant alleles of the *ABCB1* 3435C>T, 1236C>T and 2677G>T/A usually occur together, suggesting that they are genetically linked. The *ABCB1* 1236T-2677T-3435T (T-T-T) haplotype is present in approximately 32% of Caucasians, and has been reported to significantly minimize the activity of P-gp.<sup>106</sup> The influence of this haplotype and the *ABCB1* 3435C>T, 1236C>T and 2677G>T/A SNPs on the pharmacokinetics of CsA and TAC remains uncertain, with inconsistent results and no relevant clinical effect has been presented so far.<sup>93</sup> For EVE, limited data regarding the impact of *ABCB1* polymorphism exists, and to date, no influence on the pharmacokinetics of EVE has been demonstrated.<sup>96,113,114</sup>

OATP pharmacogenetics may also play a role in determining interindividual variability in drug exposure. Several of the OATPs show polymorphism and a large number of SNPs have been identified in the gene encoding OATP1B1, *SLCO1B1*.<sup>115,116</sup> A few relatively common polymorphisms in *SLCO1B1* have been associated with altered transport activity of OATP1B1. Individuals carrying the c.521T>C allele have shown impaired hepatic uptake and markedly increased plasma concentrations of OATP1B1 substrates, such as the statins.<sup>116,117</sup>

### *Age*

Progressive changes in body compositions and physiological processes affecting drug pharmacokinetics occur during aging. Declines in hepatic and renal blood flow, hepatic mass, and renal function are main contributors to decreased clearance of drugs in the elderly.<sup>118</sup> Despite extensive studies, the age-related changes in CYP3A expression and/or activity remain debated,<sup>119-122</sup> and most *in vitro* studies have reported CYP3A liver content to remain stable with age.<sup>123,124</sup> In elderly patients drug exposure is usually increased and lower doses are often sufficient to achieve therapeutic response compared with younger adults. In addition to pharmacokinetic differences, donor organ viability, multiple co-morbidities, polypharmacy, and immunological changes need to be considered when using immunosuppressive drugs in elderly transplant recipients.<sup>125-129</sup> As a result of an aging population, the number of elderly patients listed in transplant waiting programs and receiving kidney, liver, heart and lung transplants has been increasing the recent years. This trend has been most dramatic among renal transplant recipients. In 2012, about one third of the Norwegian renal transplant recipients were 65 years or older.<sup>2</sup>

**Drug-drug interactions**

Potential sites of pharmacokinetic drug-drug interactions include the gastrointestinal tract, protein- and tissue binding sites, drug metabolizing enzymes, drug transporters as well as biliary excretion. In general, however, inhibition and induction of the CYP3A mediated metabolism of the CNIs and EVE are regarded as the most common mechanism of drug-drug interactions. Clinically potent inhibitors, including the azole antifungals and calcium channel antagonists, have been demonstrated to increase the exposure of CNI and EVE significantly (Table 1). These drugs are also inhibitors or substrates of P-gp and the specific contribution of transporter and/or enzyme in the drug-drug interaction is difficult to determine.

**Table 1.** Examples of relevant interactions with the calcineurin inhibitors and everolimus

Type of concomitant drug	Concomitant drug	Effect on CNI exposure	Effect on EVE exposure	Ref.
Antifungals	Ketoconazole	↑	↑	130-132
	Fluconazole	↑	↑	133,134
Calcium channel antagonists	Diltiazem	↑	↑	114,135,136
	Verapamil	↑	↑	137,138
Antibacterials	Erythromycin	↑	↑	139-141
	Rifampicin	↓	↓	142-144
Food constituent	Grapefruit juice	↑	ND	145,146
Herbal preparation	St. John's wort	↓	ND	147,148

ND, not determined; CNI, calcineurin inhibitors; EVE, everolimus; Ref, references

Not all pharmacokinetic drug-drug interactions of CNIs and EVE can be attributed to CYP3A and P-gp. In the recent years, more focus has been given to other transporter-mediated drug-drug interactions as well.<sup>87</sup> For example, CsA interacts with mycophenolic acid by inhibiting MRP2 and thus the elimination of 7-O-mycophenolic acid glucuronide from the hepatocytes and into the bile.<sup>83,149</sup> Furthermore, CsA markedly raises the plasma concentrations of most statins.<sup>150</sup> Studies show that CsA raise the AUC of atorvastatin 7- to 15-fold, fluvastatin 2-fold and that of rosuvastatin 7-fold.<sup>151-155</sup> Since CsA is not a potent CYP3A4 inhibitor, the mechanism for this interaction is somewhat unclear. Although inhibition of CYP3A4 by CsA may partly explain the effects seen on atorvastatin, rosuvastatin and fluvastatin are not significantly metabolized by CYP3A4, indicating that inhibition of the uptake transporter OATP1B1 by CsA may be a major source of these drug-drug interactions.<sup>156,157</sup> TAC on the other hand was not found to inhibit OATP1B1 and does not seem to cause this interaction.<sup>155,158</sup> So far, limited data regarding the drug-drug interaction potential between EVE and statins exist.

### *Generic immunosuppressants*

In addition to biological variation, differences in drug formulations might also be a source of variability in drug exposure. The patents for several immunosuppressants, including CsA, TAC and MMF, have expired the last years and generic formulations are entering the market. Cost savings associated with generic substitution are often substantial and thus appear to be an attractive option to reduce the increasing costs of health care. Regulatory approval of generic drugs requires demonstration of bioequivalence to establish that the generic can be interchanged with the original drug without safety or efficacy concerns. Studies to determine bioequivalence are generally performed in small populations of healthy young adult volunteers using a single-dose crossover design. To establish bioequivalence the rate, determined by the maximum plasma concentration ( $C_{\max}$ ) and extent of absorption, defined by area under the concentration versus time curve (AUC) of the generic drug, cannot be significantly different from the original drug. Specifically, the 90% confidence interval (CI) of the ratio of the geometric means for the generic compared with the original formulation should be contained within the acceptance interval of 80 to 125%.<sup>159,160</sup> The European Medicine Agency (EMA) has adopted even stricter bioequivalence criteria for narrow therapeutic index (NTI) drugs, such as the immunosuppressive drugs, narrowing the acceptance limit to 90 to 111%.<sup>159</sup> Since no worldwide list of NTI drug exists, EMA is deciding this on a case-by-case basis. For generic TAC formulations, EMA requires that the acceptance interval should be 90 to 111% for AUC, but still allow a single dose  $C_{\max}$  interval of 80 to 125 % due to its high inpatient variability.<sup>161</sup> Recently, the transplant community has expressed concern whether single dose bioequivalence studies in healthy volunteers predict the actual therapeutic equivalence in patients receiving maintenance TAC.<sup>162,163</sup> So far, properly performed bioequivalence studies of generic TAC formulations in transplanted patients are limited.<sup>164,165</sup>

### **1.2 Therapeutic drug monitoring**

In a clinical setting the dosing of CsA, TAC and EVE is complicated by their intra- and interindividual variability, as well as their narrow therapeutic index. Therapeutic drug monitoring (TDM) of these drugs is therefore mandatory and is routinely performed.<sup>166-169</sup> Target concentrations have been empirically defined and whole blood concentrations of CsA, TAC and EVE are measured frequently, especially in the early phase after transplantation.

During the clinical development of CsA, the drug was dosed using mg/kg of body weight without performing drug concentration measurements. The drug was first administered as monotherapy (25 mg/kg), resulting in effective inhibition of rejections, but there was clear evidence of serious adverse effects such as nephrotoxicity and hepatotoxicity.<sup>170</sup> Thus, in the following trials, the CsA dose was reduced to 10 mg/kg, resulting in underexposure and an unacceptable rejection risk.<sup>171</sup> The following dose recommendation of CsA was 17.5 mg/kg, still as the sole immunosuppressive agent. After these initial experiences, it was realized that fixed doses of CsA were not optimal, as no relationship was found between administered doses and clinical effects. Consequently, it was concluded that to avoid adverse events, monitoring of CsA blood levels was required to individualize the doses. Initially, TDM using trough whole blood concentrations obtained before the morning dose ( $C_0$ ) seemed to be the solution to minimize CsA toxicity. However, the clinical outcome was still variable. Further investigations identified a link between the pharmacokinetics of CsA and clinical outcomes in individual transplant recipients. Estimates of drug exposure using  $AUC_{0-12}$  provided the most robust pharmacokinetic measure of CsA.<sup>172</sup> The correlation between  $C_0$  and  $AUC_{0-12}$  was, however, found to be poor,<sup>173</sup> but the concentration two hours after drug intake ( $C_2$ ) was identified to be a consistent predictor of  $AUC_{0-12}$ .<sup>174,175</sup>  $C_2$ -monitoring of CsA has therefore become the standard monitoring procedure in many centers. However, the clinical benefit from  $C_2$  over  $C_0$  monitoring has still not been fully elucidated.<sup>176-178</sup>

Only a few prospective concentration-controlled trials have been investigating the establishment of target TAC concentrations in relation to clinical outcome.<sup>179-183</sup> The lack of prospective randomized trials has made it difficult to reach any firm conclusions regarding the advantage of one particular TDM strategy over another. Most centers are using  $C_0$  to adjust the TAC dosage regimen, even though there is some debate regarding the correlation between  $C_0$  and  $AUC_{0-12}$ . Hence, other single time points have been studied. In contrast to CsA, TAC  $C_2$  does not seem to correlate significantly better with AUC than  $C_0$ , but some studies have however reported a stronger correlation between TAC  $C_3$  or  $C_4$  and  $AUC_{0-12}$  that could be relevant for TDM.<sup>184-186</sup> In the early years, TAC target ranges were relatively broad, ranging between 5 and 40 ng/mL,<sup>179,181</sup> subsequently lower trough concentrations were adopted varying between 10 and 20 ng/mL.<sup>180</sup> In the recently conducted Symphony trial the predefined "low dose" TAC trough concentrations targeted between 3 and 7 ng/mL were associated with the lowest acute rejection rate and the best allograft function after 1 year.<sup>187</sup> In Norway, TAC dose is adjusted to  $C_0$  targeting concentrations in the range 3 to 7 ng/mL in

standard risk patients. In high-risk patients, defined by panel reactive antibody of >20% and/or presence of donor specific antibodies, the TAC C<sub>0</sub> target range is 8 to 12 ng/mL during the first month post transplantation and subsequently 5 to 10 ng/mL.

The pharmacokinetic data collected from the phase 3 trials of EVE in kidney transplantation yielded a clear exposure-response relationship between EVE trough concentrations and various efficacy and safety responses.<sup>188-190</sup> Studies also demonstrated a good correlation between EVE trough concentrations and AUC in renal- and heart transplant recipients during the first year post transplantation.<sup>190,191</sup> The recommended therapeutic range for EVE trough concentrations is 3 to 8 ng/mL in adult renal- and heart transplant recipients and this has been validated in numerous studies in both patient populations.<sup>45,189,191-193</sup> The incidence of acute rejection was higher for patients with EVE trough concentrations < 3 ng/mL, and an association between higher trough concentrations and increasing incidence of adverse events such as thrombocytopenia, has been shown, making TDM of EVE worthwhile.

### **1.2.1 Drug concentrations at the sites of action**

Although intensive TDM in this patient population optimizes the immunosuppressive therapy quite significantly, patients still experience acute rejection episodes or nephrotoxicity despite C<sub>0</sub>/C<sub>2</sub> whole blood concentrations within target ranges.<sup>177</sup> Against this background, alternative ways, both pharmacodynamic and pharmacokinetic, to monitor the effect and toxicity of immunosuppressive drugs have been proposed. Since the lymphocytes are the site of action for the immunosuppressive drugs, it has been hypothesized that the fraction of the drug present within lymphocytes could be more directly related to the immunosuppressive efficacy than whole blood concentrations.<sup>194</sup> Additionally, several studies have demonstrated only a weak relationship between whole blood and intralymphocyte concentrations of both CsA and TAC.<sup>195-201</sup> A better strategy for drug optimization in transplanted patients could therefore include direct drug measurement at the target sites, i.e. in lymphocytes and allograft tissue. Indeed, previous studies have demonstrated that low immunosuppressant tissue exposure was significantly associated with a higher incidence of graft rejection, but not trough whole blood concentrations.<sup>199,202,203</sup> Furthermore, a previous study revealed a generally lower intracellular exposure of CsA in renal transplant recipients experiencing an acute rejection episode and demonstrated that a novel TDM method of measuring intracellular CsA concentration has the potential to predict acute rejection episodes.<sup>198</sup>

### ***P-gp expression and activity***

Since P-gp is expressed in lymphocytes and removes CsA, TAC and EVE from the intracellular compartment, the expression/activity of P-gp in these cells may be an important factor influencing the intracellular concentration of both CNIs and EVE.<sup>204</sup> Hence, the interindividual variability in the activity of P-gp may explain some of the variable immunosuppressive effect observed for these drugs. Interestingly, during acute rejection an up-regulation of P-gp mRNA expression has been shown in lymphocytes isolated from renal transplant recipients.<sup>205</sup> This up-regulation in P-gp could potentially lead to a lower concentration of the drug within the lymphocytes. Moreover, previous studies have demonstrated that P-gp polymorphism might influence the concentration of CsA and TAC both within the lymphocytes- and in allograft tissue compartments and thus modulate the immunosuppressive effect.<sup>196,200,206</sup>

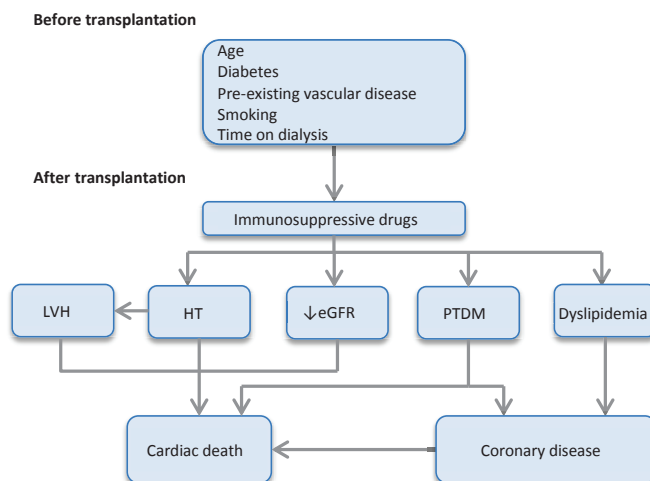
The activity of P-gp and following change in local drug exposure could in addition to influence the efficacy of the immunosuppressive drugs, also affect the toxicity associated with these drugs. It has been suggested that interindividual variability in renal P-gp expression might contribute to the local susceptibility to CNI nephrotoxicity.<sup>31</sup> The most plausible hypothesis to explain an association between P-gp expression and CNI nephrotoxicity is local accumulation of the CNIs when apical P-gp expression (and hence activity) is low. Naesens et al. did in fact observe that a lower P-gp expression in kidney transplant biopsies was a risk factor for chronic histologic damage in patients receiving TAC, but the literature is inconsistent.<sup>207-209</sup>

### **1.3 Cardiovascular disease in renal transplant recipients**

Despite a significant improvement in rejection rates and short-term graft survival in renal transplant recipients the last decades, long-term survival has remained essentially the same. Cardiovascular disease continue to be a major cause of graft loss and the leading cause of death in this patient population.<sup>210</sup> The incidence of cardiovascular disease in renal transplant recipients is 3 to 5 times that of age-matched patients in the general population. Risk factors for the development of cardiovascular disease following renal transplantation include PTDM, obesity, hypertension, dyslipidemia, smoking, treatment with immunosuppressive drugs, reduced GFR and proteinuria.<sup>211-218</sup> Strategies that reduce the prevalence and impact of cardiovascular disease would be expected to prolong graft and patient survival. A schematic



figure of selected risk factors contributing to cardiovascular disease both before and after transplantation are shown in Figure 3.



**Figure 3.** A schematic figure of selected risk factors for cardiovascular disease after transplantation. Patients accumulate risk during the time before transplantation and after transplantation the immunosuppressive drugs contribute to the cardiovascular risk. eGFR, estimated glomerular filtration rate; HT, hypertension; LVH, left ventricular hypertrophy; PTDM, post transplantation diabetes mellitus. Modified from Jardine et al.<sup>210</sup>

### 1.3.1 Dyslipidemia in renal transplant recipients

Dyslipidemia is common in renal transplant recipients. Dyslipidemia is defined by elevated plasma total cholesterol, elevated low-density lipoprotein (LDL), elevated triglycerides and/or low high-density lipoprotein (HDL), all factors that may contribute to the development of atherosclerosis.<sup>219</sup> Alterations in the lipid levels of renal transplant recipients typically occur early post-transplant, and although many factors contribute to post transplant dyslipidemia, the immunosuppressive drugs play a major role. Total cholesterol is typically increased by 30%, in addition to similar increases in LDL and triglycerides as well as high levels of atherogenic proteins such as apolipoprotein B and lipoprotein A.<sup>220</sup> Among the immunosuppressive agents, corticosteroids and CsA are especially associated with elevations in lipid levels and more recently, the PSIs have also been recognized as a major cause of dyslipidemia.<sup>221</sup> Treatment with the PSIs significantly increases both cholesterol and

triglycerides in a dose-dependent pattern. The pathogenesis of PSI induced dyslipidemia is unclear, but could possibly be due to a decrease in the catabolism of apolipoprotein B100, inhibition of insulin-like growth factor signals, and/or alterations in hepatocytes synthesis of lipid moieties.<sup>43,221</sup> The consequences of long-term PSI treatment is however uncertain, because of the potential benefits on atherosclerotic plaques.<sup>222</sup>

### **1.3.2 Treatment of dyslipidemia in renal transplant recipients**

There has only been one large prospective randomized study in transplant recipients comparing statin treatment (fluvastatin) with placebo. In the Assessment of LEscol in Renal Transplantation (ALERT) study it was shown a 35% reduction in the incidence of nonfatal myocardial infarctions or cardiac deaths in patients treated with fluvastatin.<sup>223</sup> Given the increased risk of cardiovascular disease in renal transplant recipients, treatment with lipid lowering agents, normally HMG-CoA reductase inhibitors (statins), is generally recommended.<sup>224</sup>

#### ***Statins***

Statins are competitive inhibitors of HMG-CoA reductase, the rate-limiting step in cholesterol biosynthesis. By blocking HMG-CoA reductase, statins reduce intracellular cholesterol in the liver and stimulate the expression of LDL receptors, thereby lowering total cholesterol and LDL by uptake into the liver.<sup>225</sup> Due to its low interaction potential with the immunosuppressive drugs and as a consequence of the ALERT study, fluvastatin is commonly the lipid-lowering drug of choice in renal transplant recipients. In contrast to several other statins, fluvastatin is primarily metabolized by CYP2C9 and to a lesser extent by CYP3A4 and CYP2C8.<sup>226</sup> However, fluvastatin is a low potency statin and may not be adequate in patients with significant hyperlipidemia. In these patients a high potency statin such as atorvastatin or rosuvastatin may be necessary. Rosuvastatin, the latest member in the statin family, has been shown to be a more potent lipid-lowering drug compared to the other statins in a non-transplant population.<sup>227,228</sup> As opposed to atorvastatin, rosuvastatin is minimally metabolized and similar to fluvastatin, has a low risk of metabolic pharmacokinetic interactions.<sup>229</sup> Rosuvastatin has however a high affinity for several drug transporters, including OATP1B1 and BCRP.<sup>85,230,231</sup> Limited data on the use of rosuvastatin in transplant recipients with concomitant immunosuppressive therapy exists.

## 2 AIMS OF PRESENT STUDIES

Overall aim was to investigate pharmacological treatments and monitoring strategies to improve outcome in solid organ transplants.

Specific aims were as follows:

- assess the lipid-lowering effect of rosuvastatin compared to fluvastatin (**paper I**)
- study how whole blood concentrations of CsA and EVE is associated with concentrations in other body compartments (**paper II and III**)
- investigate the drug-drug interaction potential of the EVE and rosuvastatin combination in renal transplant recipients receiving EVE (**paper I**)
- investigate bioequivalence of an approved generic TAC formulation with the original drug as reference (**paper IV**)

### 3 SUMMARY OF PAPERS

#### Paper I

*More potent lipid lowering effect by rosuvastatin compared to fluvastatin in everolimus treated renal transplant recipients*

In this study we aimed to assess the lipid-lowering effect of rosuvastatin compared to fluvastatin in renal transplant recipients receiving EVE. Safety was assessed as the pharmacokinetic (PK) interaction potential of a rosuvastatin/everolimus combination in RTR. A 12-hour everolimus PK-investigation was performed in twelve stable RTR receiving everolimus and fluvastatin (80 mg/day). Patients were then switched to rosuvastatin (20 mg/day) and a follow-up 12/24-hour PK-investigation of everolimus/rosuvastatin was performed after one month. In renal transplant recipients already receiving fluvastatin, a switch to rosuvastatin further decreased LDL-cholesterol and total cholesterol by  $30.2 \pm 12.2\%$  ( $P < 0.01$ ) and  $18.2 \pm 9.6\%$  ( $P < 0.01$ ), respectively. Everolimus  $AUC_{0-12}$  was not affected by concomitant rosuvastatin treatment,  $80.3 \pm 21.3$  before and  $78.5 \pm 21.9 \mu\text{g} \cdot \text{h/mL}$  after, respectively ( $P = 0.61$ ). Mean rosuvastatin  $AUC_{0-24}$  was  $157 \pm 61.7 \text{ ng} \cdot \text{h/mL}$ , about 3-fold higher than reported in the literature for non-transplants. Rosuvastatin showed a superior lipid-lowering effect compared to fluvastatin in stable renal transplant recipients receiving everolimus. The combination of everolimus/rosuvastatin appears to be as safe as the everolimus/fluvastatin combination.

#### Paper II

*Endomyocardial, intralymphocyte and whole blood concentrations of ciclosporin A in heart transplant recipients*

The aims of the present study were to evaluate the relationships between CsA concentrations at different target sites as potential TDM tools in heart transplant recipients. Ten heart transplant recipients (8 men, 2 women) on CsA-based immunosuppression were enrolled in this prospective single center pilot study. Blood samples were obtained once to twice weekly up to 12 weeks posttransplant. One of the routine biopsies was allocated to this study at each sampling time. Three patients experienced mild rejections. In the study period, the mean (range) intralymphocyte CsA trough concentrations were 10.1 (1.5 to 39) and 8.1 (1.3 to 25)  $\text{ng}/10^6$  cells in the rejection and non-rejection group, respectively ( $P = 0.21$ ). Corresponding

whole blood CsA concentrations were 316 (153 to 564) and 301 (152 to 513) ng/mL ( $P=0.33$ ). There were no correlations between whole blood, intralymphocyte or endomyocardial concentrations of CsA ( $P>0.11$ ). The study did not support an association between decreasing intralymphocyte CsA concentrations and acute rejections. Further, there were no association between blood concentrations and concentrations at sites of action, potentially challenging TDM in these patients.

### **Paper III**

*Closer to the site of action; everolimus concentrations in peripheral blood mononuclear cells correlate well with whole blood concentrations*

In this study we aimed to investigate whether there was a correlation between EVE concentrations in whole blood and in peripheral blood mononuclear cells (PBMC) with the special emphasis to investigate the potential influence of P-gp activity on this association. Twelve renal transplant recipients (5 men, 7 female) treated with everolimus (EVE) underwent a pharmacokinetic investigation where both whole blood EVE concentrations and EVE concentrations in PBMC were determined. In addition, the activity of P-gp in PBMC was determined using the Rhodamine123 efflux assay and the patients' genotypes of *ABCB1* were determined. There was a significant correlation between EVE dose adjusted  $AUC_{0-6}$  in whole blood and in PBMC ( $r = 0.88$ ,  $P<0.01$ ) and no association was demonstrated between the P-gp activity and EVE trough concentrations in PBMC ( $r = -0.46$ ,  $P=0.18$ ). Furthermore, *ABCB1* 1236C>T, 3435C>T, 2677G>T/A polymorphism did not influence PBMC concentrations of EVE. A high degree of association between EVE whole blood and PBMC concentrations was demonstrated. The results may therefore indicate that P-gp efflux from PBMC is of minor importance for the distribution of EVE.

### **Paper IV**

*Use of generic tacrolimus in elderly renal transplant recipients – precaution is needed*

In this open label, single-center, prospective, randomized, crossover study we aimed to compare steady state pharmacokinetics of a generic tacrolimus formulation (Tacni®) with the original (Prograf®) in renal transplants above 60 years. Twenty-five patients, median age 69 years, were randomized at time of transplantation to receive original or generic tacrolimus and provided two full 12-hr pharmacokinetic profiles. The investigations were performed in a

stable phase, early after transplantation; approximately 6 and 8 weeks posttransplant. Following the first investigation, tacrolimus formulations were switched in a 1:1 dose ratio. Generic tacrolimus did not meet the bioequivalence criteria;  $AUC_{0-12}$  was 17% ( $P < 0.01$ ) and  $C_{max}$  was 49% ( $P < 0.01$ ) higher compared to the original. The generic formulation also showed a shorter time to reach  $C_{max}$  ( $T_{max}$ ) ( $P = 0.03$ ). Importantly, the lack of bioequivalence was not reflected in the standard monitoring parameter, trough concentrations ( $P = 0.80$ ). The tested generic tacrolimus did not show bioequivalence in elderly renal transplant recipients. The significantly higher systemic exposure of tacrolimus, despite similar trough concentrations, may in the long-run increase the risk of adverse effects.

## 4 DISCUSSION

Individualizing a patient's drug therapy to obtain the optimal balance between therapeutic efficacy and avoidance of adverse events is the ultimately goal in immunosuppressive therapy. Due to the large intra- and interindividual variations and the narrow therapeutic index for immunosuppressive drugs, correct dosing is challenging. More knowledge and further improvements of dosing strategies and monitoring techniques are thus desirable to potentially improve both short- and long term outcomes after transplantation.

### 4.1 Cardiovascular risk in renal transplant recipients

Efforts to reduce cardiovascular risk factors and hence improve long term outcome have become a priority in post transplant care. In **paper I** the lipid lowering effect of rosuvastatin in comparison with fluvastatin, the current gold standard treatment, was assessed. The results from the study demonstrated that in renal transplant recipients receiving EVE based immunosuppression and treated with full dose fluvastatin (80 mg/day), a switch to rosuvastatin (20 mg/day) induced a significant additional lipid-lowering effect. Total cholesterol, LDL-cholesterol and triglycerides were significantly reduced from the fluvastatin treatment values by another 20 to 30% after the switch to rosuvastatin. These results (**paper I**) are in agreement with previous findings in the non-transplant population, where rosuvastatin has been consistently found to be the most potent statin.<sup>227,228,232</sup>

The patients in **paper I** were already treated with the highest available dose of fluvastatin, and had probably already obtained a LDL-cholesterol reduction of about 38.6 mg/dL (1 mmol/L) from the early post-transplant phase before entering the study.<sup>223</sup> Treatment with rosuvastatin reduced LDL-cholesterol further by a mean of 1.1±0.5 mmol/L. Results from the ALERT study showed that lowering LDL cholesterol by 1 mmol/L reduced cardiac death or myocardial infarction by approximately 30%.<sup>218,223</sup> Implicit this suggests that renal transplant recipients at high risk for cardiovascular events might benefit from more intensive lipid-lowering therapy. Safely achieving a larger LDL-cholesterol reduction could be of great importance in reducing the cardiovascular risk in these patients. Hence, the additional lipid-lowering effect of rosuvastatin observed in **paper I** may have a potential to further improve long-term outcomes in renal transplant recipients.

## 4.2 Monitoring immunosuppressive drugs at their sites of action

The pharmacokinetics of CsA, TAC and EVE are complex and unpredictable. Our increasing knowledge and understanding of both the pharmacokinetics and pharmacodynamics of these drugs emphasize the need for continuous revision of TDM strategies.

### 4.2.1 Correlations at different target sites

In **paper II**, no correlations between CsA concentrations in whole blood, T-lymphocytes or endomyocardial tissue were demonstrated in heart transplants. This pilot study was, to our knowledge, the first to report CsA concentrations in endomyocardial tissue and to show the absence of correlation with both whole blood and intralymphocyte CsA concentrations. A similar weak correlation between CsA whole blood and intralymphocyte concentrations was also evident in the CsA data presented in **paper III**. This weak correlation between whole blood and intralymphocyte CsA concentrations are in agreement with results also from other studies,<sup>195-197</sup> suggesting that whole blood concentrations measured for TDM is not an optimal predictor of the target site concentration of CsA. A poor relationship between whole blood and PBMC concentrations has been demonstrated for TAC as well, both studies in liver- and heart transplant recipients report of weak correlations.<sup>201,233</sup> Against this background and since limited data exist on monitoring of EVE within the target compartment, the correlation between EVE concentrations in whole blood and in PBMC was investigated (**paper III**). Surprisingly, the results showed that whole blood and PBMC EVE AUC<sub>0-6</sub> correlated well. This was in contrast to a study conducted in heart transplant recipients where a weak correlation between trough concentrations of EVE in whole blood and PBMC were observed.<sup>234</sup> However, the patients in that study were also treated with CsA and only trough concentrations were measured, both factors may contribute to the observed discrepancy between the two studies. Interestingly and in contrast to CsA (**paper II**), the results (**paper III**) indicate that TDM of EVE in whole blood gives valuable information of the concentration at the site of action, i.e. within the lymphocytes.

### 4.2.2 Influence of P-glycoprotein

It has been suggested that the variability in expression and activity of P-gp in lymphocytes is a plausible explanatory factor for the weak relationship between whole blood and intralymphocyte concentration of immunosuppressive drugs. In **paper III** the potential influence of P-gp activity on EVE concentrations in PBMC was investigated. Even though the



P-gp activity showed considerable interpatient variability, no significant correlation between EVE dose adjusted trough concentrations in PBMC and the P-gp activity, measured by Rhodamine123 (Rh123) efflux method, was demonstrated. The Rh123 efflux method is a commonly used method to investigate the functional activity of P-gp in human lymphocytes. Rh123 is a cationic, fluorescent dye that is readily taken up by cells and actively pumped out of the cells by P-gp and other efflux transporters. The efflux of Rh123 in the presence of a selective P-gp inhibitor is decreased. The ratio of intracellular accumulation of Rh123 in the presence and absence of this inhibitor is hence a measure of P-gp activity in lymphocytes.<sup>235</sup> The Rh123 efflux method used in **paper III** has shown satisfactory intra- and interday variability with coefficient of variations (CV) below 20% (data not shown). In addition, *ABCB1* polymorphism did not have any impact on EVE concentrations in PBMC (**paper III**). These findings as well as the high association between EVE AUC<sub>0-6</sub> in whole blood and in PBMC might suggest that P-gp efflux is of minor importance in the distribution of EVE.

A few previous studies have investigated the potential influence of P-gp on CsA concentrations in PBMC.<sup>195,196</sup> Ansermot et al. demonstrated that CsA PBMC pharmacokinetics was influenced by P-gp activity in healthy volunteers, showing a significant negative correlation between P-gp activity and CsA concentrations in PBMC.<sup>195</sup> However, *ABCB1* polymorphism did not influence the pharmacokinetics of CsA in PBMC.<sup>195</sup> The influence of *ABCB1* polymorphism (*ABCB1* 1199G>A and 3435C>T) was however demonstrated in another study in renal, liver and lung transplant recipients.<sup>196</sup> In addition to P-gp, PBMC express several others efflux transporters, including MRP2 and BCRP, both of which CsA has been shown to be an inhibitor of.<sup>83,236</sup> Hence, variation in expression and activity of these efflux transporters might also contribute to the poorly correlated concentrations of CsA in whole blood and lymphocytes. To our knowledge, limited data regarding the effect of EVE on these specific drug transporters exists.

#### 4.2.3 Clinical interest and limitations

The potential clinical interest of monitoring concentrations of the immunosuppressive drugs at their target sites (lymphocytes and graft tissue) would significantly increase if a link to a clinical endpoint, such as rejection or drug toxicity, were established. The study in **paper II** failed to show correlation between intralymphocyte concentrations of CsA and acute rejection episodes in heart transplants, and does not support the previous findings of decreased CsA concentrations within lymphocytes prior to rejection episodes in renal transplant recipients.<sup>198</sup>

However, only three patients experienced acute rejection episodes during the study period. Thus, the small sample size clearly limits the conclusions that could be drawn. Additionally, CsA concentrations were measured at trough, not  $C_2$ , which have shown to correlate better with acute rejections compared to trough concentrations.<sup>237</sup> Further investigations in properly powered trials are needed to elucidate this relation between acute rejection episodes and drug concentrations at target sites in transplant recipients.

Up until now, the main clinical evidence for the interest of monitoring immunosuppressive drugs in PBMC was provided in a study by Capron et al. in liver transplant recipients.<sup>233</sup> They showed that although no differences in whole blood concentrations were observed, TAC concentrations in PBMC were lower in patients with histological rejection compared to patients without rejection in an early phase following transplantation. Importantly, the study also demonstrated that intrahepatic TAC concentrations significantly correlated with TAC PBMC concentrations, suggesting that TAC concentrations in PBMC might be reliable markers of the immunosuppressive efficacy of TAC.<sup>233</sup> Although, this study shows encouraging results, monitoring of the immunosuppressive drugs in PBMC is still in its early stages. Further studies are warranted, especially studies relating drug concentrations of immunosuppressive drugs in PBMC and graft tissue with clinical endpoints, such as acute rejection and maybe more importantly, toxicity. Currently, there is however many analytical constraints that make it difficult to implement this monitoring approach in a clinical setting. Monitoring of immunosuppressive drug in PBMC requires isolating and purifying of PBMC from whole blood, a relatively time-consuming procedure. In addition, access to an analytical method of sufficient sensitivity, as well as a reliable cell counting system to relate the concentrations obtained to the number of cells, are essential. Future work should focus on further optimization of the complex isolation procedure. Furthermore, in addition to direct drug concentration measurements at target sites, i.e. graft tissue and lymphocytes, identification and validation of pharmacodynamic biomarkers may be other potential strategies for drug optimization in transplant recipients. Finally, results from the present studies (**paper II and paper III**) should be interpreted with caution; the sample size is small, so additional conformational large studies are required.

### 4.3 Factors contributing to interindividual pharmacokinetic variability

An increased understanding of the processes underlying pharmacokinetic variability is of great interest to further optimize TDM of immunosuppressive drugs to ensure a safe and effective management of transplant recipients.

#### 4.3.1 Drug-drug interactions

Pharmacokinetic drug-drug interactions make immunosuppressive therapy in renal transplant recipients a challenge. When introducing a new drug to the existing multidrug therapy of transplant recipients, it is always a concern whether a clinically relevant interaction may occur. The potential pharmacokinetic interaction between EVE and rosuvastatin was investigated in **paper I** and was, to our knowledge, the first investigation of the EVE and rosuvastatin combination in renal transplant recipients. The EVE pharmacokinetics was not influenced by concomitant rosuvastatin treatment (**paper I**). Previous single dose studies in healthy volunteers investigating the interaction between EVE and simvastatin, atorvastatin or pravastatin have not shown any evidence of clinically relevant interactions.<sup>238,239</sup> Our results thus support the previous findings, indicating that rosuvastatin does not influence EVE pharmacokinetics to any relevant degree in renal transplant recipients.

Everolimus is extensively metabolized via CYP3A and is a substrate for P-gp.<sup>78,240</sup> Rosuvastatin, on the other hand, is subjected to a minimal degree of metabolism, and appears to not be a P-gp substrate,<sup>231,241-243</sup> although the literature is somewhat contradictory on the latter.<sup>244,245</sup> Based on this, it does not seem to be a potential pharmacokinetic risk in combining EVE with rosuvastatin. However, the important role of hepatic transport of rosuvastatin is well recognized, and OATP1B1 transport is an essential mechanism mediating its hepatic uptake. OATP1B1 has previously been shown to be a transporter that is subjected to high degree of interactions between other immunosuppressive drugs and statins.<sup>150,158</sup> In fact, Simonson et al. reported a 7-fold increase in the steady state AUC and an 11-fold increase in  $C_{max}$  of rosuvastatin in heart transplant recipients on CsA based immunosuppression, and suggest that CsA inhibition of OATP1B1-mediated rosuvastatin hepatic uptake may be the mechanism of the drug-drug interaction.<sup>152</sup> EVE has also been shown, *in vitro*, to inhibit OATP1B1. In this study, the 50% inhibitory concentration ( $IC_{50}$ ) of OATP1B1 was found to be 4.1  $\mu$ M for EVE. This value is above EVE blood levels usually observed in post transplantation settings (1-10 nM), and the inhibition of OATP1B1 by EVE

is thus not expected to cause any clinical relevant drug-drug interactions.<sup>85</sup> To our knowledge, no *in vivo* data regarding the influence of EVE treatment on the disposition of statins exist.

Against this background, the influence of EVE on the pharmacokinetics of rosuvastatin was also investigated (**paper I**). In the present study, mean rosuvastatin steady state  $AUC_{0-24}$  and  $C_{max}$  values were 2.8-fold and 2.5-fold higher, respectively, compared to literature data in non-transplant patients (**paper I**).<sup>246</sup> This less than 3-fold higher systemic exposure of rosuvastatin when combined with EVE is comparable to what is shown for fluvastatin in combination with CsA, a combination that is considered safe in renal transplant recipients.<sup>150</sup> The use of a control group is necessary in pharmacokinetic studies where the patients cannot serve as their own controls. This was the case in **paper I** where historical control group (literature data) had to be used for rosuvastatin baseline pharmacokinetics, as EVE treatment could not be withdrawn in these patients. Hence, the 2.8-fold higher systemic exposure of rosuvastatin compared to non-transplant patients observed in **paper I** may not necessarily be due to a pharmacokinetic interaction, but could be a result of different features between the patients and the historical control group. The historical control group was considered to be the most optimal comparator found in the published literature, and consisted of eighteen healthy men participating in a trial designed to assess the dose proportionality of rosuvastatin.<sup>246</sup> In contrast to the present study where the steady state  $AUC_{0-24}$  was estimated, the participants in the historical control group was given a single dose of rosuvastatin and the AUC was calculated from time 0 to time of the last measureable concentration. Ideally, the steady state  $AUC_{0-24}$  obtained from the present study (**paper I**) should be compared to AUC from time 0 to infinity, but this parameter was not estimated in the historical control group due to secondary peaks present within individual plasma concentration-time profiles. Consequently, the observed difference in AUC between our patients and the historical control group may be overestimated. Furthermore, for six of the patients in our study the  $AUC_{0-24}$  was estimated using the  $C_0$  sample as the 24-hr sample and this probably overestimate AUC. By using a developed pharmacokinetic population model for rosuvastatin, the AUC for these six patients were estimated to be lower, making the difference in AUC between our patients and the historical control group 20% smaller (2.2 fold increase in rosuvastatin AUC) (data not shown). This use of literature data for the comparison of systemic exposure of rosuvastatin is obviously not an optimal study design. We do, however, believe that it is an informative comparison considering the ethical and practical difficulties to obtain data from transplanted patients with and without their immunosuppressive drugs. Although a slight increase in the

risk of statin induced side effects cannot be ruled out, these data indicate that rosuvastatin treatment most probably is safe in combination with EVE in renal transplant recipients.

#### 4.3.2 Genetic polymorphism in drug metabolizing enzymes and drug transporters

Interindividual differences in drug response can result from sequence variants in genes encoding drug-metabolizing enzymes and drug transporters. Due to the small sample size and the study design of the present studies the genotyping results should be carefully interpreted. In general however, pharmacogenetic information may identify patients with a greater chance of effective response or a higher susceptibility of adverse events and could therefore give additional value to the traditional TDM.

Genetic polymorphism in *CYP3A5* is well known to influence the pharmacokinetics of TAC and this was also shown in the present study (**paper IV**), however no effect on EVE disposition was observed (**paper I**). Results from **paper IV** demonstrated that patients without functional *CYP3A5* (*CYP3A5* \*3/\*3) had a 2-fold higher systemic exposure compared to *CYP3A5* expressers (*CYP3A5* \*1/\*3), confirming that *CYP3A5* expressers need approximately double TAC doses to reach target concentration.<sup>90,247</sup> The lack of effect of the presence of functional *CYP3A5* enzymes on EVE disposition (**paper I**) was also in consistency with previous findings.<sup>97,113</sup> *ABCB1* TTT-haplotype has been associated with reduced function of P-gp.<sup>106</sup> There was however no differences in the pharmacokinetics of CsA, EVE and TAC in patients with this haplotype in the present studies (**paper I, II and IV** (data not shown)). Similar, no influence of the *ABCB1* 3435C>T variant on either CsA, TAC or EVE pharmacokinetics were observed (data not shown) (**paper I, II and IV**), supporting findings from previous studies.<sup>114,248,249</sup> Furthermore, recent clinical data has identified polymorphisms in *PPARA* (rs4253728 and rs4823613) as potential sources of variability in *CYP3A4* activity.<sup>102</sup> Interestingly, one patient was homozygote carrier for both *PPARA* variant alleles (rs4253728 and rs4823613) and showed higher systemic exposure of EVE compared to heterozygote and/or homozygote wild type genotypes (**paper I**).

The large interindividual pharmacokinetic variability observed with statin therapy has at least in part been associated with altered expression and/or function of OATP1B1 (*SLCO1B1*).<sup>116</sup> Two patients in **paper I** with *SLCO1B1* c.521CC genotype had a substantially higher systemic exposure of rosuvastatin compared to the patients expressing the wild-type genotype

(*SLCO1B1* c.521TT). These results mirror previous studies and support that genotyping of *SLCO1B1* could be relevant to identify patients at risk of statin-induced side effects.<sup>250,251</sup>

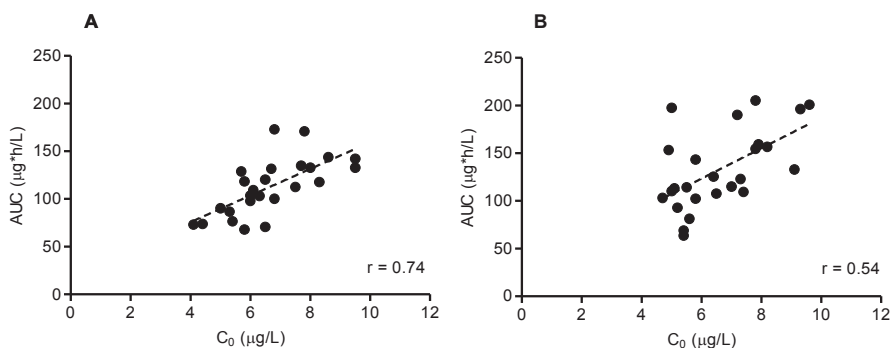
In **paper II**, the relation between a reduced renal function and an increased concentration of the secondary metabolites of CsA was evaluated. No significant relation was observed between a reduced renal function and an increased concentration of the secondary metabolites and functional CYP3A5 genotypes in the present study (**paper II**). This is in contrast to previous studies indicating that elevated blood and urine concentrations of the secondary metabolites AM19, AM1c and AM1c9 may be associated with renal dysfunction in CsA treated patients, and that CYP3A5 expressers have higher formation of the secondary metabolites AM19 and AM1c9.<sup>92,252-254</sup>

### 4.3.3 Drug formulation

In **paper IV** we aimed to investigate the bioequivalence of an approved generic TAC formulation (Tacni<sup>®</sup>) in elderly renal transplant recipients at steady state, using the original drug (Prograf<sup>®</sup>) as reference. This was the first prospective randomized study in elderly stable renal transplant recipients investigating bioequivalence of a generic TAC formulation. Despite being an approved generic TAC formulation available in most European countries this generic formulation did not fulfill bioequivalence criteria when investigated in a relevant clinical setting of the intended patient population. Importantly, the lack of bioequivalence would not have been detected by the standard monitoring parameter, TAC trough concentrations, as these concentrations were similar for both formulations.

The fact that the systemic drug exposure associated with the generic formulation was significantly higher than the original formulation, together with no differences in generic and original trough concentrations of TAC is especially worrisome (**paper IV**). This effect would not have been detected without a full pharmacokinetic investigation. Similar findings were demonstrated in a study by Min et al. where no differences in trough concentrations were observed despite a significantly higher exposure of the generic TAC formulation, Tacrobell<sup>®</sup> (Chung Kun Dang Pharmaceutical Corp., Seoul, Korea).<sup>165</sup> Results from both this and our study thus emphasize that studies of generic TAC formulations drawing conclusions based solely on TAC trough concentrations should be interpreted with great caution. For generic formulations, there is no requirement to demonstrate that the relationship between trough concentrations and AUC is identical with the original drug, and that the same trough

concentration can be used as target. In our study there was as well a weaker correlation between  $C_0$  and  $AUC_{0-12}$  for the generic TAC formulation compared to the  $C_0$  and  $AUC_{0-12}$  correlation for the original drug (Figure 5). Thus, in the absence of information regarding  $C_0$  and AUC correlation it cannot be presumed that the same  $C_0$  will achieve the same AUC. The results of the present study strongly suggest that such data need to be provided for generic formulations, to allow routine TDM to be performed under valid assumptions.



**Figure 5:** Scatter plots of trough concentrations ( $C_0$ ) versus  $AUC_{0-12}$  for original (A) and generic (B) tacrolimus. The correlations were estimated using a Spearman's rank order correlation test. Dotted linear trend lines are added for visualization purposes.

The reasons for the observed lack of bioequivalence between the two TAC formulations in **paper IV** are not obvious and remain to be investigated. In the before mentioned study by Min et al, the generic TAC formulation (Tacrobell<sup>®</sup>) failed to meet the bioequivalence criteria both ten days and six months after renal transplantation in patients aged between 18 and 65 years.<sup>165</sup> Min et al. published these results when our study was in the final stage and interestingly their results are similar to the findings in the present study despite being conducted in a considerable younger group of patients. This may indicate that age might not be the main parameter causing the non-bioequivalence observed in our study. Bioequivalence in different age groups was unfortunately not investigated in the subpopulation analysis of the study by Alloway et al.<sup>255</sup>

Authorities require that generic drug manufactures meet the same batch-to-batch requirements for strength, purity, and quality as the original manufacturer. However, since a number of drugs are manufactured in foreign countries or use foreign-made ingredients, it has been raised question regarding the pharmaceutical quality of generic drugs. Interestingly, it was

recently reported that five approved generic TAC formulations did not meet the pharmaceutical quality criteria.<sup>256</sup> It is assumed that drugs with marketing authorization meet the authorities' strict quality standards and we can thus only speculate whether there could be a quality issue with the generic TAC formulation studied in **paper IV**.

As mentioned earlier, EMA has adopted stricter bioequivalence criteria for NTI drugs to ensure that true bioequivalence is established for these drugs. In contrast, the US Food and Drug Administration (FDA) have not changed their policy and the 80 to 125% criteria are also applied to NTI drugs, including all immunosuppressants. Regardless of whether one applies EMA or FDA requirement in **paper IV**, the investigated generic TAC formulation did not show bioequivalence. There is an ongoing debate whether the current FDA standards regulating bioequivalence are restrictive enough to ensure that generic formulations of NTI drugs are therapeutic equivalent to the original drug. The FDA has been discussing the application of scaled average bioequivalence approach for TAC, in which the 90% confidence interval is tightened based on the CV of the original drug, with bioequivalence limits of 0.80-1.25 for CV higher than 21.42%.<sup>257,258</sup> Incorporation of these more restrictive limits for NTI drugs are steps in the right direction. However, the study in **paper IV** also raises an important discussion on a need to perform proper bioequivalence studies on the intended patient population of all ages in a realistic setting prior to approval.

High within-subject variability in immunosuppressant drug exposure is known to have serious consequences in organ transplant recipients and could lead to increased rates of rejection and graft loss. With multiple generic products the potential variability in drug exposure may be further increased. In the largest retrospective analysis of *de novo* renal transplant recipients, patients receiving generic CsA had a higher rate of biopsy-proven acute rejection (BPAR) despite achieving comparable blood levels with those on the original drug (Neoral®).<sup>259</sup> Although mean 12-hr trough concentrations of CsA were similar with the two formulations, patients treated with the generic CsA formulation had significantly higher within subject variability for CsA trough concentrations than those treated with the original drug.<sup>259</sup> The lack of interchangeability is a concern as multiple substitutions between various formulations could lead to considerable variability in exposure that may result in impaired long-term outcome.

The approval of generic drug using bioequivalence studies is based on the fundamental assumption that if two formulations are shown to be bioequivalent, they will provide the same



therapeutic effect. In **paper IV** bioequivalence between the two TAC formulations were not established, and use of the generic was associated with a significantly higher systemic exposure. Long-term studies of the original TAC formulation show that increased drug exposure is associated with a higher risk of nephrotoxicity, neurotoxicity, hypertension, dyslipidemia, and diabetes.<sup>26,27,31,221,260</sup> This emphasizes the need of properly designed bioequivalence studies, rather than extrapolating data from simpler designs, to assure that potential issues with long-term outcome are not overlooked. Our study addresses only the conversion between the generic TAC formulation, Tacni<sup>®</sup> and the original drug and we can only speculate whether our findings could be similar in other generic TAC formulations as well as in different age groups.

The main purpose of generic drug development is to reduce the price of marketed drugs, ultimately to lower public health costs. However, cost savings may be outweighed by the cost of adverse consequences such as a more intense TDM of generic immunosuppressive drugs. There is invested considerable amount of time, effort and resources in tailoring immunosuppressive treatment to meet the individual patient's characteristics and avoid graft loss. The so far unquantified risk of using generic immunosuppressive drugs and the lack of comprehensive information regarding their efficacy and safety seems incompatible with the current focus on an individualized immunosuppressive therapy and a patient centered medicine.

## 5 CONCLUSION

Treatment with rosuvastatin showed a clinically relevant superior lipid-lowering effect compared to fluvastatin in EVE treated renal transplant recipients. The combination of EVE and rosuvastatin seems to be safe, but a slightly increased risk of statin-induced side effects cannot be ruled out. Safely achieving a larger LDL-cholesterol reduction will most probably be of great importance in reducing the cardiovascular risk in these patients.

No correlation between CsA concentrations in whole blood, T-lymphocytes or endomyocardial tissue was established in heart transplant recipients. This might potentially challenge traditional TDM based on whole blood CsA concentrations in these patients. In contrast, EVE concentrations in whole blood and PBMC correlated well in renal transplant recipients and supports TDM of EVE in whole blood to be an appropriate choice.

The generic TAC formulation was not found to be bioequivalent to the original drug in elderly renal transplant recipients. Importantly, the lack of bioequivalence was not detected by the standard monitoring parameter, TAC trough concentrations, as these concentrations were similar for both formulations. Generic TAC should therefore be used with caution in elderly renal transplant recipients and it should be recognized that bioequivalence studies performed in healthy volunteers do not necessarily reflect the average transplant recipient.

## 6 CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

Findings in **paper I** assured us that rosuvastatin is a safe statin to use in renal transplant recipients treated with EVE. Furthermore, the results showed that in EVE treated renal transplants where the lipid lowering effect of fluvastatin often is not sufficient, treatment with rosuvastatin could now be an appropriate choice to achieve an the desired lipid-lowering effect and thus a potentially reduce cardiovascular risk in this high risk population.

After the completion of **paper IV** the generic TAC formulation (Tacni<sup>®</sup>) is no longer in use at Oslo University Hospital, Rikshospitalet. Additionally, the Norwegian Medicines Agency has showed great interest in the results, and is currently looking into this particular generic formulation based on the findings from our study. In the light of our results, we hope the discussion regarding the limitations in extrapolating results from healthy volunteers receiving single drug doses to a patient population on maintenance therapy continues and question whether the current bioequivalence criteria for immunosuppressive drugs are appropriate.

The findings from **paper II and III** could potentially be used in the development of pharmacokinetic population models. Population approaches to pharmacokinetic modeling are increasingly used and could offer a more accurate TDM tool than the current strategies. In a population model, the pharmacokinetic parameters are calculated from both population data and individual information. Amongst the advantages of this methodology, is the possibility to include covariates that influence the pharmacokinetic parameters. Such covariates could include drug concentrations at different target sites, various genotypes of metabolizing enzymes and drug transporters as well as the activity of P-gp. This approach could allow for more individualized dosing recommendations based on targeting the concentration at the site of interest and hence a possible favorable effect on short- and long term outcome in transplant recipients.



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RESEARCH

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# Endomyocardial, intralymphocyte, and whole blood concentrations of ciclosporin A in heart transplant recipients

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## Abstract

**Background:** In the early phases following heart transplantation a main challenge is to reduce the impact of acute rejections. Previous studies indicate that intracellular ciclosporin A (CsA) concentration may be a sensitive acute rejection marker in renal transplant recipients. The aims of this study were to evaluate the relationships between CsA concentrations at different target sites as potential therapeutic drug monitoring (TDM) tools in heart transplant recipients.

**Methods:** Ten heart transplant recipients (8 men, 2 women) on CsA-based immunosuppression were enrolled in this prospective single-center pilot study. Blood samples were obtained once to twice weekly up to 12 weeks post-transplant. One of the routine biopsies was allocated to this study at each sampling time. Whole blood, intralymphocyte, and endomyocardial CsA concentrations were determined with validated HPLC-MS/MS-methods. Mann–Whitney *U* test was used when evaluating parameters between the two groups of patients. To correlate whole blood, intralymphocyte, and endomyocardial CsA concentrations linear regression analysis was used.

**Results:** Three patients experienced mild rejections. In the study period, the mean (range) intralymphocyte CsA trough concentrations were 10.1 (1.5 to 39) and 8.1 (1.3 to 25) ng/10<sup>6</sup> cells in the rejection and no-rejection group, respectively ( $P=0.21$ ). Corresponding whole blood CsA concentrations were 316 (153 to 564) and 301 (152 to 513) ng/mL ( $P=0.33$ ). There were no correlations between whole blood, intralymphocyte, or endomyocardial concentrations of CsA ( $P > 0.11$ ).

**Conclusions:** The study did not support an association between decreasing intralymphocyte CsA concentrations and acute rejections. Further, there were no association between blood concentrations and concentrations at sites of action, potentially challenging TDM in these patients.

**Keywords:** Ciclosporin A, Endomyocardial biopsies, Heart transplantation, Acute rejection, T-lymphocytes

## Background

Heart transplantation is the final treatment option in end-stage heart failure and even though the procedure shows good results there is still room for improvement. In the early post-transplant phase a main challenge is to reduce the impact of acute rejections. The negative effects of the immunosuppressive therapy used to avoid acute rejection is however also a challenge in these patients. Hence, in the early phases following transplantation a combination of therapeutic drug monitoring (TDM) of immunosuppressive

drugs and weekly endomyocardial biopsies are used to optimize the treatment for heart transplant recipients. A method with high specificity and accuracy to prevent graft rejection is an unmet clinical need.

Ciclosporin A (CsA) has been a cornerstone in the immunosuppressive therapy since its introduction in the mid 1980s. CsA is metabolized by the cytochrome P-450 3A (CYP3A) subfamily to >30 more or less pharmacologically active metabolites [1]. In addition, CsA is both a substrate and an inhibitor of the efflux transporter P-glycoprotein (P-gp) [2]. P-gp, coded by the *ABCB1* gene, is expressed in T-lymphocytes and transports CsA out of the cell [2-4]. A

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previous study has shown that polymorphism in the *ABCB1* gene may influence the intralymphocyte CsA concentration [5]. These pharmacokinetic properties are the basis for the substantial intra- and interindividual variation in CsA concentration. CsA is associated with a numerous of severe side effects, resulting in a narrow therapeutic range which makes the TDM of the drug extra demanding. The current routine TDM of CsA is performed by measuring whole blood concentrations, either in trough samples or lately also in C2 samples. However, since CsA exerts its immunosuppressive effect within T-lymphocytes [6], measurement of CsA within these cells may provide more relevant information regarding the immunosuppressive effect of CsA than whole blood concentrations. Several groups have shown data that support this hypothesis in transplant recipients [5,7-10]. We have recently shown that intracellular CsA concentration in T-lymphocytes decreased several days before an acute rejection was possible to diagnose in renal transplants by current standard clinical methods [7]. Intracellular CsA concentration monitoring therefore seems to have a potential as a semi-invasive method for prediction of acute rejection episodes. The purpose of the study was to evaluate the relationships between CsA concentrations at different target sites, that is whole blood, lymphocytes, and endomyocardial tissue, and to investigate CsA concentrations in isolated T-lymphocytes from heart transplant recipients in order to further examine intracellular monitoring as a potential TDM tool. In addition, the patients' genotype of P-gp was determined to investigate if genetic polymorphism in the *ABCB1* gene could explain differences in the intralymphocyte concentration of CsA.

## Patients and methods

### Patients and study design

Ten heart transplant recipients (8 men and 2 women) with a mean age of  $52 \pm 12$  years were enrolled in this single-center prospective pilot study. The patients were included  $17 \pm 6$  days after transplantation and followed for a period of  $70 \pm 8$  days. They all applied to standard post-transplant procedures at Oslo University Hospital, Rikshospitalet. All the patients were treated with C0-monitored CsA, mycophenolate mofetil (MMF), and steroids according to the center immunosuppressive protocol at that time. The CsA treatment was initiated with 10 mg/kg orally on the day of transplantation followed by C0 monitoring with target concentrations of 250 to 350 ng/mL after 1 month and further tapered to 60 to 120 mg/mL after 1 year of treatment. All patients received 1.5 g MMF twice daily from the day of transplantation, the doses was further adjusted according to side effects. The patients received 0.2 mg/kg/day oral prednisolone from the second postoperative day and were further tapered to 0.1 mg/kg/day within the following months. None of the patients were given induction

therapy. Patients were not allowed to use concomitant drugs that could interact with CsA pharmacokinetics.

Study specific whole blood samples (EDTA vacutainer tubes) for CsA analyses and T-lymphocyte isolation were taken in association with routine blood samples for standard clinical follow-up; twice weekly during the first weeks and thereafter weekly samples for the rest of the investigation period. Whole blood samples and isolated T-lymphocytes were frozen and stored at  $-20^{\circ}\text{C}$  until analysis. Routine monitoring of these patients include series of six endomyocardial biopsies at post-transplant week 1, 2, 5, 7, 10, and 12. One of the six biopsies taken at each time-point was allocated for CsA analysis in this study. The biopsy was wrapped in a piece of aluminum foil and stored at  $-20^{\circ}\text{C}$  until analysis. In addition, EDTA whole blood was drawn once during the study for determination of the recipients *ABCB1* (1199G>A, 1236C>T, 2677G>A, 2677G>G, and 3435C>T) and *CYP3A5* (\*3 (6986A>G, splicing defect)) genotypes. All acute rejections were verified with a biopsy and classified according to the International Society for Heart and Lung Transplantation (ISHLT) standardized cardiac biopsy grading [11,12].

The study was performed in accordance with the Declaration of Helsinki, local laws, and other regulations, and all patients signed a written informed consent before study start. The study was evaluated by the Regional Committee for Medical Research Ethics and approved by the Norwegian Medicines Agency. The study is registered on ClinicalTrials.gov (NCT00139009).

### T-lymphocyte isolation

T-lymphocytes were isolated from freshly drawn heparin whole blood using Prepacyte<sup>®</sup> (BioE, St Paul, MN, USA) [13]. An aliquot of 100  $\mu\text{M}$  of verapamil was pre-added to the heparin vacutainers to inhibit P-gp from pumping CsA out of the cells [14]. Prepacyte<sup>®</sup> uses a negative selection process and facilitates the agglutination and precipitation of erythrocytes, B-lymphocytes, and mature myeloid cells like granulocytes, monocytes, and platelets, producing a supernatant of lymphocytes, highly enriched for T-cells. The excess of erythrocytes in the supernatant was removed by lysis using Vitalyse<sup>™</sup>. After centrifugation (400 g) and washing, the remaining supernatant contains >97% lymphocytes comprising 88% to 96% of the resultant cell population [15]. To relate the intracellular concentration to a physiological parameter, cell count using a *Bürker Chamber* was performed. The cells were isolated within 4 h post sampling. The isolating method starts with 7 mL of whole blood and produces a T-lymphocyte isolate pellet to which was finally added 1 mL methanol:ACN:water (1:1:3) for cell lysis and protein precipitation. The mixture was stored at  $-20^{\circ}\text{C}$  until solid phase extraction and subsequent analysis of CsA concentrations.

### CsA and metabolite concentrations

Concentrations of CsA and six of its main metabolites were determined in whole blood, intracellularly in isolated T-lymphocytes, and in endomyocardial biopsies. The whole blood and intracellular CsA and metabolite concentrations were determined with a validated high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method previously described [16]. In brief, the analytes were extracted and purified by protein precipitation with methanol and centrifugation before the supernatants were subjected to solid phase extraction using Oasis hydrophilic-lipophilic balance cartridges. CsA and metabolites were separated chromatographically on a C8-column before MS/MS detection. The intracellular concentration of CsA was related to the number of T-lymphocytes in the sample (ng/10<sup>6</sup> cells).

The concentration of CsA and two metabolites, AM1 and AM9, were determined in endomyocardial biopsies by using a modification of the method described above [16]. After moistening the biopsy with 20 µL water for 5 min, the biopsy was weighed before homogenized in 150 µL de-ionized water with an automated tissue homogenizer; Precellys<sup>®</sup> 24 (Bertin Technologies, France), programmed to 2×50 s cycles with a 20-s pause. Fifty µL of the internal standard (0.5 µg/mL ciclosporin C (CsC) in methanol: acetonitrile (ACN): water (1:1:3)) was added to 100 µL homogenate and this mixture was protein precipitated with 100 µL ACN. Particulate matter was removed by centrifugation (30 min, 12,000 g, 4°C) and the supernatant was evaporated to dryness under a stream of nitrogen gas. The eluate was reconstituted in 50 µL of 65% mobile phase A consisting of ACN/20 mM ammonium formate buffer (NH<sub>4</sub><sup>+</sup>COO<sup>-</sup>) pH 3.6 (20:80 v/v), and 35% mobile phase B, consisting of ACN/ NH<sub>4</sub><sup>+</sup>COO<sup>-</sup> (80:20 v/v), before injecting 20 µL on the LC-MS/MS system. The analytical system consisted of Aquity ultra performance liquid chromatography<sup>™</sup> (UPLC) connected to a Micromass Quattro micro<sup>™</sup> triple quadrupole mass spectrometry (MS) detector (Waters Corporation, USA) using electrospray ionization (ESI) interface. The detector was operated in a positive ion mode. Separation of the analytes was carried out on a reversed phase UPLC C18 column (100 × 2.1 mm, 1.7 µm) (Acquity UPLC BEH Shield C18, Waters, USA) and the column was heated to 70°C. The analytes were eluated using a stepwise gradient at the flow rate of 0.6 mL/min. The gradient program was as follows: 62% mobile phase A for 14 min followed by a gradually increase of mobile phase B to 100% for 7 min. One hundred per cent mobile phase B was held constant for 10 min and the system was finally re-equilibrated at start conditions for 5 min. Analysis run time per sample was 36 min. Calibration curves were produced from stock solutions of CsA, AM1, and AM9, which were mixed with the internal standard (CsC), evaporated to dryness under a stream of

nitrogen gas and reconstituted in 65% mobile phase A and 35% mobile phase B. All standard curves comprised of at least eight concentration levels, including a blank sample (0.0 to 80 ng/mL). The regression coefficients ( $r^2$ ) of the linear standard curves were >0.998 and for both CsA and the metabolites the validation parameters for precision and accuracy (intra- and inter-run) were <9%.

### Genotyping

Genotyping was performed as previously described, using a polymerase chain reaction (PCR) - restriction fragment length polymorphism assay [17]. Restriction enzyme digestion generated DNA fragments that were separated by electrophoresis on 3% agarose gels. All the patients were screened for relevant polymorphism in *CYP3A5* (\*3 (6986A>G, splicing defect)) and *ABCB1* (1199G>A, 1236C>T, 2677G>T, 2677G>A, 2677G>G, and 3435C>T). Dr D Katz (Abbott Laboratories, Abbot Park, IL (MDR1)) and Dr R van Schaik (Department of Clinical Chemistry Erasmus MC, The Netherlands (CYP3A5)) kindly supplied positive controls.

### Statistics and calculations

Mann-Whitney *U* test was used when evaluating parameters between the two groups of patients. To correlate whole blood, intralymphocyte, and endomyocardial CsA concentrations linear regression analysis was used. Statistical significant differences were considered for *P* values <0.05. All statistical analyses were performed using SPSS version 19. The renal function was estimated using the Modification of Diet in Renal Disease (MDRD) formula [18,19].

## Results

### Patients

All 10 heart transplant recipients completed this 3-month-long pilot study. Three patients experienced biopsy-proven acute rejection episodes during the study at an average of 58 ± 16 days after transplantation, and one of these patients experienced in total three rejection episodes during the study period. One of the patients in the no-rejection group developed renal failure during the study. Demographic data at inclusion are summarized in Table 1. No significant differences were observed between the rejecting and the no-rejection patients.

### Intracellular T-lymphocyte and whole blood concentrations of CsA

An average of 12.3 (range, 7 to 20) samples per patient was analyzed for both intracellular and whole blood concentration of CsA. In total, 139 whole blood samples and 121 intralymphocyte samples was analyzed during the study period. Both intracellular and whole blood concentrations of CsA showed large intra- and interindividual



**Table 1 Demographic data at time of inclusion**

	All	No-rejection group	Rejection group	P value
Gender (male/female)	8/2	2/5	3/0	-
Weight (kg)	76.7 ± 18.0	73.9 ± 19.5	83.3 ± 15.0	0.517
Age (years)	51.9 ± 11.9	51.0 ± 12.9	54.0 ± 11.5	0.833
CsA dose (mg/day)	330 ± 115	293 ± 116	417 ± 57.7	0.183
CsA C0 (ng/mL)	245 ± 59.3	239 ± 71.7	257 ± 10.4	0.383
Plasma creatinine (μmol/L)	131 ± 55	146 ± 59.8	96.5 ± 16.5	0.117
Creatinine clearance (mL/min)	58.0 ± 21.4	50.3 ± 18.9	77.6 ± 14.7	0.067
Serum urea (mmol/L)	10.5 ± 5.3	10.8 ± 6.0	9.8 ± 3.8	1.000
Hematocrit (%)	32.3 ± 4.2	32.3 ± 4.9	32.5 ± 0.7	0.500
Steroid dose (mg/day)	14.8 ± 3.8	13.6 ± 3.7	17.5 ± 2.5	0.137
Treated with MMF	10/10	7/7	3/3	-

Data are means ± SD. All variables were analyzed with a Mann-Whitney U test. CsA, cyclosporine A; MMF, mycophenolate mofetil.

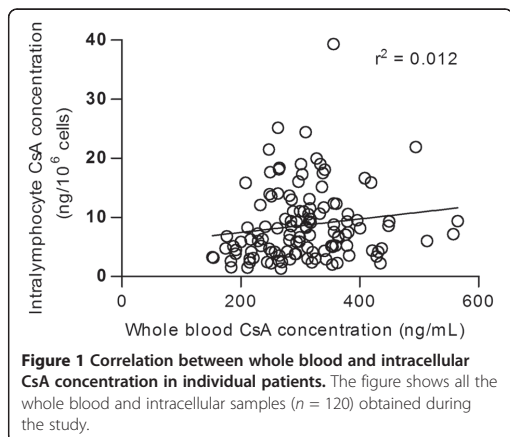
variations in both groups, and there were no correlation between whole blood and intracellular CsA concentration throughout the study ( $r^2=0.012$ ,  $P=0.11$ ; Figure 1). In the study period, the mean (range) intracellular CsA trough concentrations were 10.1 (1.5 to 39) and 8.1 (1.3 to 25) ng/ $10^6$  cells in the rejection and no-rejection groups, respectively ( $P=0.21$ ). The corresponding mean (range) whole blood CsA concentrations were 316 (153 to 564) and 301 (152 to 513) ng/mL, respectively ( $P=0.33$ ).

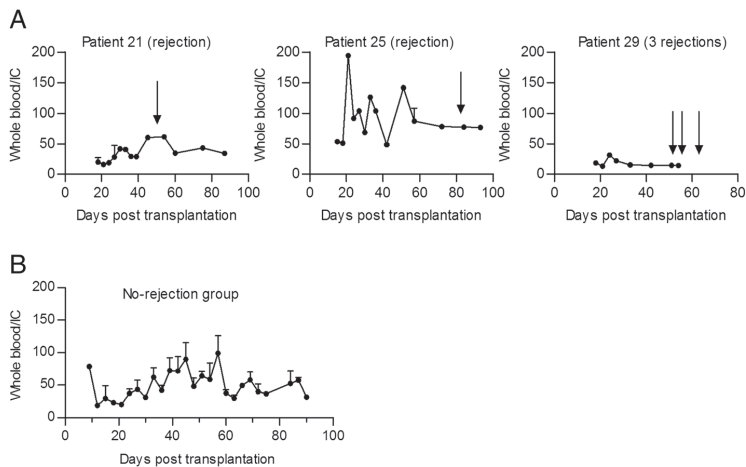
Figure 2 shows the individual ratio of whole blood to intralymphocytic CsA concentration for the three patients experiencing rejection and for the mean ratios for the no-rejection patients during the study period. One of the rejection patients (patient 21) showed an increase in the whole blood/intracellular ratio at time of rejection, due to a combination of declined intracellular concentration and a slight increase in the whole blood concentration. In the two other rejection patients (patients 25 and 29) no change

was observed in the whole blood/intracellular ratio in conjunction to the rejection episode, but interestingly patient 25 showed substantially increased ratio on several occasions prior to the rejection episode as compared to the mean ratio for the no-rejection group (Figure 2A). In the no-rejection group the mean individual whole blood/intracellular ratio ranged from 33.6 to 86.4 with a corresponding standard deviation range of 17.8 to 46.7. The absolute average intracellular CsA concentration to the time of rejection was 10.4 (1.5 to 39) ng/ $10^6$  cells in the rejection group and the corresponding average CsA concentration to the mean time of rejection (day 58) was 8.2 (1.3 to 25) ng/ $10^6$  the no-rejection group ( $P=0.38$ ). At the rejection day the absolute intracellular CsA concentration for the three rejecting patients were 9.4, 7.2, and 18.4 ng/ $10^6$  cells.

#### CsA metabolites, genotypes, and renal function

Genotyping results for both *ABCB1* (*1199G>A*, *1236C>T*, *2677G>T*, *2677G>A*, *2677G>G*, and *3435G>T*) and *CYP3A5* (\*3) are presented in Table 2. Two out of three patients in the rejection group were homozygote *ABCB1* TTT carriers, but all patients were potential carriers of this reduced P-gp function haplotype. Three of the 10 patients expressed functional *CYP3A5* enzymes (*CYP3A5\*1*), one in the rejection group. It was observed that the patients expressing functional *CYP3A5* enzymes tended to have higher concentrations of the metabolites AM19 ( $P=0.21$ ), AM1c9 ( $P=0.57$ ), AM1c ( $P=0.73$ ), AM4N ( $P=0.27$ ), similar concentration of AM9 ( $P=0.43$ ), and a decreased concentration of AM1 ( $P=0.57$ ) compared to the patients without functional *CYP3A5* (Figure 3). We did not observe a significant difference in renal function between patients expressing functional *CYP3A5* (eGFR of  $51 \pm 23$  mL/min) and patients not expressing functional *CYP3A5* (eGFR of  $66 \pm 19$  mL/min,  $P=0.38$ ). One of the three patients





**Figure 2** The ratio ( $\pm$  SEM) of whole blood to intralymphocytic CsA trough concentration days after transplantation. **(A)** The CsA whole blood/intracellular ratio ( $\pm$  SEM) for the three rejection patients. The arrows mark the point where the patients experienced an acute rejection episode. High levels of the ratio represent a drop in intracellular CsA concentration compared to whole blood concentration. **(B)** The mean whole blood/intracellular ratio ( $\pm$  SEM) for the patients with no rejection.

expressing functional CYP3A5 experienced renal failure during the study period.

#### Concentration of CsA and metabolites in endomyocardial biopsies

Nineteen biopsies, from seven out of the 10 patients, were obtained for the current study. Only one out of these seven patients was in the rejection group. In these patients an average of 2.7 (range, 1 to 6) biopsies per patient were analyzed for concentrations of CsA and two metabolites, AM1 and AM9. CsA concentration varied from 216 to 833 pg/mg heart tissue. No correlations were found between endomyocardial CsA concentrations and whole blood ( $r^2=0.029$ ,  $P=0.48$ ) or intralymphocyte concentrations

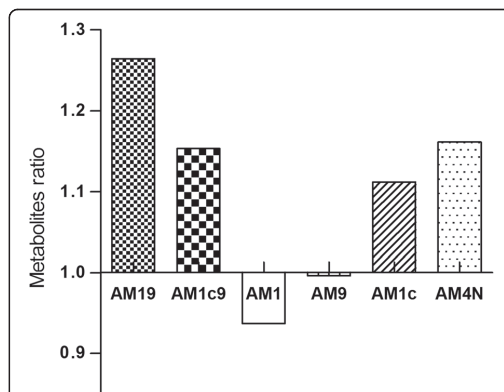
( $r^2=0.055$ ,  $P=0.35$ ). There was no obvious association between the endomyocardial concentration of CsA and rejection episodes.

#### Discussion

The present pilot study does not support the hypothesis of decreased intracellular T-lymphocyte concentration of CsA prior to rejection episodes. The main finding, however, was that there were no correlations between CsA

**Table 2** Patient's genotyping of ABCB1 and CYP3A5

Patient	ABCB1	1236C>T	3435C>T	CYP3A5
	2677G>A/T	1236C>T	3435C>T	*3
21	T/T	T/T	T/T	*3/*3
22	G/T	C/T	C/T	*1/*3
23	G/T	C/T	C/T	*3/*3
24	G/T	C/T	C/T	*3/*3
25	G/T	C/T	C/T	*3/*3
26	T/T	T/T	T/T	*3/*3
27	G/T	C/T	T/T	*1/*3
28	G/T	C/T	T/T	*3/*3
29	T/T	T/T	T/T	*1/*3
30	G/T	C/T	T/T	*3/*3



**Figure 3** Ratio between the mean concentration of the metabolites AM19, AM1c9, AM1, AM9, AM1c, and AM4N in patients with CYP3A5\*1/3 and in patients with CYP3A5\*3/\*3.

concentrations in whole blood, T-lymphocytes, or endomyocardial tissue.

Gustafsson and colleagues are, to our knowledge, the only group who previously has measured intralymphocyte CsA concentration in heart transplant recipients [10]. The study discovered a close association between whole blood CsA C2 concentrations and lymphocyte CsA AUC<sub>0-12</sub> in MMF treated patients. This is contradictory to our findings where no correlation between CsA in whole blood and T-lymphocytes was found. A possible explanation to this discrepancy could be the fact that Gustafsson *et al.* performed measurement of whole blood CsA concentration in C2 samples and determined lymphocyte CsA AUC<sub>0-12</sub>, while in the present study CsA concentration were measured in C0 samples. C2 monitoring leads to an improvement in the clinical outcomes in heart transplant recipients [20,21] and measuring whole blood C2 concentrations could perhaps more precisely predict the CsA concentration and, in turn AUC, within lymphocytes. Nevertheless, our results are in agreement with previous studies reporting of no correlation between CsA concentration in whole blood and lymphocytes [22,23]. Although these studies were performed in different patient populations (renal transplant recipients and healthy volunteers), the findings demonstrate that whole blood CsA concentrations may not be a good predictor of the target site concentration of CsA. To the best of our knowledge, the present pilot study is the first to report of CsA concentration in endomyocardial tissue and to show the absence of correlation with both whole blood and intralymphocyte CsA concentrations in heart transplant recipients. In a recent study, Capron *et al.* evaluated the correlation of intrahepatic, peripheral mononuclear cells (PBMC) and blood concentrations of tacrolimus (Tac), another calcineurin inhibitor, in liver transplant recipients. In this study, no correlation was found between mean Tac blood concentration and PBMC or intrahepatic concentration of Tac. However, it was discovered that intrahepatic Tac concentration significantly correlated with Tac PBMC concentrations [24]. Capron *et al.* have earlier showed that hepatic tissue concentrations of Tac correlated with early acute rejection after liver transplantation, this in contrast to blood concentrations [25]. These findings also suggest that direct drug measurement at the target sites (lymphocytes and graft tissue) could be a better approach than measuring whole blood concentration to predict the efficacy of immunosuppressive drugs.

The present pilot study failed to show correlation between intracellular CsA concentration in T-lymphocytes and acute rejection episodes. Several other groups have however shown a possible correlation between low intracellular CsA concentration and rejection episodes in renal transplant recipients. A study conducted by Barbari *et al.* demonstrated that rejecting patients exhibited a low CsA

lymphocyte content despite a higher or similar CsA blood concentration [8]. Similarly, we have shown that renal transplant recipients experiencing a rejection episode had a lower intracellular exposure of CsA several days before clinical diagnosis of acute rejection episodes [7]. The difference observed between renal and heart transplant recipients in this respect have no obvious explanation. However, as mentioned before C2 concentrations are known to correlate better with acute rejections compared to trough concentrations [20] and it was C2 concentrations that were used in our previous study [7]. Further, it cannot be ruled out that the renal transplant recipients experiencing an acute rejection episode had a stronger immune response compared to rejecting patients in the present study.

Since CsA is both a substrate and an inhibitor of P-gp, the patients' genotype for this efflux pump was determined as it is expressed in T-lymphocytes. The *ABCB1* haplotype TTT (1236T, 2677T and 3435T) has previously been associated with impaired functional transport activity [26]. In the present study only three patients experienced an acute rejection episode. Two of the three rejection patients were homozygote *ABCB1* TTT haplotypes, but all patients included in the study were potential TTT haplotypes. This makes the interpretation of the data difficult, but if the hypothesis that acute rejection episode are associated with lower intracellular CsA concentrations should hold true, it would be expected that rejection patients have high transport activity of P-gp, contradictory to our findings [7,27].

Renal failure is a frequent and recognized complication following heart transplantation and CsA has been implicated as a potential risk factor [28-31]. Previous studies indicate that elevated blood and urine concentrations of the secondary metabolites AM19, AM1c, and AM1c9 may be associated with renal dysfunction in CsA treated patients [31-35], and that CYP3A5 expressers have higher formation of the secondary metabolites AM19 and AM1c9 [36]. Contrary, in renal transplant recipients on Tac-based immunosuppression, a protective role of CYP3A5 expression in the kidney has been observed [37]. By contrast to previous findings [31-35,38], the present study did not show any tendencies of a reduced renal function by an increased concentration of the secondary metabolites or functional CYP3A5 genotypes. This should however be carefully interpreted as the power is relatively low as outlined below.

#### Study limitations

The main limitation of this pilot study is the low sample size and only three patients experienced acute rejection episodes. This clearly limits the conclusion that could be drawn. In addition, CsA concentrations were measured in trough samples and not in C2 samples. The

intralymphocyte CsA concentration displayed a high intra- and interindividual variation, and this could partly be explained by the complex isolation procedure and the low level of automatization of the T-lymphocyte isolation method.

## Conclusions

The main finding of the present pilot study was that no correlation between CsA concentrations in whole blood, T-lymphocytes or endomyocardial tissue was present in heart transplant recipients. In addition, results from the present study do not support previous findings that CsA concentrations within T-lymphocytes decrease days before acute rejection episodes are diagnosed. The small sample size clearly limits the extent to which any definitive conclusion could be drawn. However, both findings are relevant with regards to TDM of CsA in this population and should be further investigated in properly powered clinical trials.

## Abbreviations

ACN: Acetonitrile; AUC: Area under the concentration versus time curve; C<sub>0</sub>: Concentration before dose (trough); C<sub>2</sub>: Concentration 2 hours after dose; CsA: Cyclosporin A; C<sub>5c</sub>: Cyclosporin C; CYP: Cytochrome P-450; HPLC-MS/MS: High performance liquid chromatography-tandem mass spectrometry; MDRD: Modification of diet in renal disease; MMF: Mycophenolate mofetil; PBMC: Peripheral blood mononuclear cells; PCR: Polymerase chain reaction; P-gp: P-glycoprotein; Tac: Tacrolimus; TDM: Therapeutic drug monitoring; UPLC: Ultra performance liquid chromatography.

## Competing interests

The authors of this manuscript have no conflicts of interest to disclose.

## Authors' contributions

PF, AKJ, LG, and AA designed the study and collected samples. AKJ and LG recruited patients. IR, PF, NKN, and NL analyzed data. IR and AA wrote the paper, whereas all authors have been involved in discussion of results and have contributed to, read, and approved the final manuscript.

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