

**OPTIMIZATION OF  
CALCINEURIN  
INHIBITOR  
TREATMENT  
AFTER SOLID ORGAN  
TRANSPLANTATION**

**DENNIS A. HESSELINK**

Optimization of Calcineurin Inhibitor Treatment  
after Solid Organ Transplantation

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# OPTIMIZATION OF CALCINEURIN INHIBITOR TREATMENT AFTER SOLID ORGAN TRANSPLANTATION

OPTIMALISATIE VAN DE BEHANDELING MET  
CALCINEURINEREMMERS NA SOLIDE  
ORGAANTRANSPLANTATIE

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Perdè dari mikuined ber kysr Caisar  
ankebut Bumi neubèt mizènedber  
kiumbeti Efrasiyab.

*The Spider has wove her Web in the  
Imperial Palace, the Owl has sung  
her watch Song upon the Towers, of  
Efrasiyab.*

Anonymous

[Quoted by Demetrius Cantemir in  
*The history of the growth and decay of  
the Othman Empire* (trans. N. Tindal,  
p.102, London, 1734)].

Nu weet ik: nergens vind ik vree,  
Op aarde niet en niet op zee,  
Pas aan die laatste smalle ree  
Van hout in zand.

J. Slauerhoff

*Voor Sophie en Juliette*



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## Chapter 1

# **GENERAL INTRODUCTION**

Based on *Transplant Proc* 2004;36(Suppl 2):S99-106

## GENERAL INTRODUCTION CONTENTS

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## 1.1

### HISTORICAL OVERVIEW

On December 23, 1954, Ronald Herrick donated a kidney to his identical twin brother Richard who was dying of renal failure. The kidney transplant produced urine immediately and in February 1955, Richard Herrick was discharged from hospital. He survived for another nine years (at which time his kidney allograft failed from recurrent glomerulonephritis), married the nurse who attended him, became father of two children and returned to work as a radio and television engineer. His brother Ronald lived on for more than 50 years after donating his kidney.<sup>1-3</sup>

In the years following this first successful human kidney transplantation, several more kidney transplants were performed between identical twins. Most of these patients had a return of normal kidney function and survived for a considerable period of time.<sup>4</sup> However, it was clear from previous experience that if kidney transplantation was to be extended successfully to genetically non-identical individuals, suppressing the recipient's immune system was necessary in order to prevent acute rejection. Early after World War II, a small number of human kidney transplantations had been performed in Europe and the United States. The recipients had received no immunosuppression except for a few cases in which short courses of ACTH or cortisone had been given. Although some of these kidney allografts did function for a limited period of time -most likely as a result of profound uremia<sup>5</sup>- most of the transplanted kidneys were acutely rejected or destroyed as a result of thrombosis or infection, and none of the recipients survived for a long period of time.<sup>3,6-10</sup>

The first attempts at immunosuppression included sublethal total body irradiation. By use of this treatment, which achieved immunosuppression by producing profound bone marrow aplasia, several kidneys were transplanted successfully. However, many patients developed overwhelming infections and despite this heavy immunosuppressive treatment, acute rejection still occurred frequently. As a result, the large majority of these patients died from sepsis or renal insufficiency.<sup>3,11-13</sup> Subsequently, total body irradiation was largely abandoned and the search for less toxic methods of immunosuppression continued. A major breakthrough came with the discovery of the immunosuppressive properties of 6-mercaptopurine in the late 1950s. Already in clinical use as an anticancer agent, 6-mercaptopurine was shown by Schwartz and Dameshek to suppress the immune response against human serum albumin in rabbits.<sup>14,15</sup> In addition, the drug prolonged the survival of kidney allografts in dogs although the actual survival of the animals was rather poor.<sup>16,17</sup> Shortly thereafter, 6-mercaptopurine and its pro-drug azathioprine (Imuran<sup>®</sup>), which was considered less toxic, were used in humans for the suppression of the immune response following kidney transplantation.<sup>13,18,19</sup> The initial results obtained with chemical immunosuppression were promising and in the words of Francis D. Moore, "gentle, feasible and practical" in comparison with the "tough, sledge-hammer medicine of whole body irradiation".<sup>13</sup>

During the next fifteen years, azathioprine became the base immunosuppressant for the prevention of acute rejection after kidney transplantation. As such, it was mostly used in combination with glucocorticoids with or without the addition of cytotoxic agents (such as

cyclophosphamide), actinomycin C or the newly-developed antilymphocyte serum. Besides the development of pharmacological immunosuppression, the 1960s and 1970s were further characterized by improvements in dialysis technique and refinements in the management of patients with renal allografts, a better understanding of transplantation biology, advances in preservation and storage of organs, the clinical introduction of tissue typing and cytotoxicity assays, as well as the first attempts at transplanting liver, lung and heart.<sup>3,20-24</sup> However, as the one-year kidney allograft survival rates increased to between 50% and 60% during this period, the downside of immunosuppression also became increasingly evident.<sup>21,22,25</sup> Serious and often fatal and atypical infections developed frequently among transplant recipients.<sup>3,21</sup> The incidence of cancer among transplanted patients was much higher as compared with the general population. Finally, all immunosuppressive drugs had their specific toxicities which resulted in considerable morbidity and an impaired quality of life.<sup>3</sup>

In the early 1970s, new strains of fungi imperfecti were isolated as part of an antifungal screening program in the microbiology department of Sandoz Ltd., Switzerland, from soil samples from Wisconsin in the United States (*Cylindrocarpon lucidum* Booth) and from the Hardanger Vidda in Norway (Sandoz employees away on business trips or on holiday used to take along plastic bags for collecting such samples). The Norwegian strains were originally classified as *Trichoderma polysporum* (Link ex. Pers.) Rifai, but were later termed after their correct taxonomic name *Tolypocladium inflatum* Gams.<sup>26</sup> Both strains turned out to produce unique metabolites that were called cyclosporins.<sup>26,27</sup> After perfecting and scaling up the fermentation process of *Tolypocladium inflatum* Gams, sufficient quantities of the mixture of cyclosporins (designated as compound 24-556) were produced for the initial screening of its pharmacologic activity. Compound 24-556 possessed only limited anti-fungal and no anti-tumor activity but it was remarkably immunosuppressive and non-toxic in rodents. Over the next few years, the major active metabolite of compound 24-556, cyclosporine A, was isolated and chemically characterized and studied further for its immunosuppressive effects.<sup>26,28-30</sup>

The immunosuppressive actions of cyclosporine A [now named cyclosporin(e) or ciclosporin] were first described by Jean-François Borel and colleagues.<sup>31</sup> They demonstrated that cyclosporine suppressed alloantibody formation, delayed skin allograft rejection in mice and graft-*versus*-host disease in mice and rats. In addition, the drug inhibited experimental allergic encephalitis and Freund's adjuvant-induced arthritis in rats (animal models for multiple sclerosis and rheumatoid arthritis, respectively). Importantly, and in contrast to cyclophosphamide and azathioprine, cyclosporine was not myelotoxic in immunosuppressive doses.<sup>31</sup> This classic study demonstrated that cyclosporine suppresses antibody- and cell-mediated immunity, is highly selective for (T-) lymphocytes, is not lymphocytotoxic but affects the induction phase of lymphocyte proliferation.

After the effectiveness of cyclosporine in preventing acute allograft rejection had been demonstrated further in several other animal experiments,<sup>32-34</sup> Roy Y. Calne and colleagues in Cambridge were the first to use the drug in human renal transplant patients.<sup>35</sup> In 1978, they reported on seven recipients of a kidney transplant from a mismatched cadaveric donor who had been treated with fixed-dose cyclosporine, initially as the sole immunosuppressive agent. Five of these patients were discharged from hospital with functioning grafts. Two of

these patients never required glucocorticoids although a cyclophosphamide analogue was added to the cyclosporine treatment in all but one. One patient died because of disseminated Aspergillosis and Candidiasis; Another patient required a nephrectomy because of pyelonephritis in the graft. Importantly, rejection episodes were of mild to moderate degree and “none of the patients had typical rejection crises with swollen allografts, pyrexia, and severe histological changes”.<sup>35</sup> In the same issue of *The Lancet*, Powles and co-workers<sup>36</sup> showed that cyclosporine was effective in treating acute cutaneous graft-versus-host disease after bone marrow transplantation. However, four of the five patients died because of liver failure.<sup>36</sup>

One year later, Calne *et al.*<sup>37</sup> reported on their continuing experience with cyclosporine in thirty-four organ transplant recipients who received a total of thirty-two kidneys, two pancreases and two livers. At the time of publication, twenty-six kidney allografts were still supporting life, among which three after more than one year. Fifteen patients had not required the use of any other immunosuppressive drug and the authors concluded that “... in man cyclosporine A is the most powerful immunosuppressive agent so far used in the management of patients with cadaveric renal allografts”.<sup>37</sup>

Despite these unprecedented results, there was, however, reason for concern. In the first patients receiving cyclosporine, the incidence of infectious complications and lymphomas was high. Second, cyclosporine proved to be nephrotoxic, a side effect which had not been observed in animal studies. As experience with the use of cyclosporine grew, it became clear that the initial dose of 25 mg/kg bodyweight per day -which was based on Calne's experience in dogs and pigs- was too high, resulting in overimmunosuppression and inhibition of kidney function. Since the nephrotoxic effects of cyclosporine were unknown at the time, impaired renal function was interpreted initially as acute rejection and additional immunosuppressive treatment was started, aggravating the already impaired renal function and further increasing the risk of infectious complications and lymphoma. By reducing the dose of cyclosporine (with or without the addition of low-dose prednisone), implementing several supportive measures (such as perioperative hydration and inducing forced diuresis), and early conversion to azathioprine-glucocorticoid combination therapy in case of cyclosporine-resistant rejection, much better results were obtained.<sup>38-40</sup>

Encouraged by these results, two multicentre trials were launched, one in Europe and one in Canada.<sup>41,42</sup> Both studies compared the efficacy of cyclosporine (with starting doses of 17 and 10 mg/kg per day, respectively), either as monotherapy or in combination with prednisone, with conventional treatment, consisting of azathioprine plus glucocorticoids with or without the addition of antilymphocyte globulin or cyclophosphamide according to local protocol. These trials demonstrated that treatment with cyclosporine resulted in a marked improvement of transplantation outcomes. In cyclosporine-treated patients, the severity of acute rejection episodes was markedly decreased and graft survival at one year after transplantation ranged between 70% and 80% as compared with a graft survival of between 50% and 60% in the control group.<sup>41,42</sup> The superiority of cyclosporine over conventional treatment was maintained with longer follow-up, with graft survival at three and five years after transplantation being roughly 15% higher in patients originally allocated to receive cyclosporine therapy.<sup>43-46</sup> Importantly, the incidence of infections and

malignancies was not higher in cyclosporine-treated patients.<sup>43-46</sup> Several other randomized trials that differed somewhat in their design compared with the European and Canadian studies, also demonstrated that cyclosporine-based immunosuppression had advantages over conventional immunosuppression as it resulted in a lower incidence of acute rejection and infections, and a reduced need to use glucocorticoids.<sup>47-49</sup>

In the fields of heart,<sup>50</sup> liver,<sup>51</sup> lung,<sup>52</sup> and heart-lung transplantation,<sup>53</sup> cyclosporine led to even more dramatic improvements in allograft- and patient survival and it was cyclosporine that first made these types of transplantation truly therapeutic interventions. After bone marrow transplantation, the use of cyclosporine resulted in faster engraftment and a lower incidence of severe graft-*versus*-host disease in comparison with methotrexate prophylaxis.<sup>54</sup> In addition, cyclosporine has been effective in the treatment of a number of autoimmune diseases including psoriasis,<sup>55,56</sup> rheumatoid arthritis,<sup>57</sup> colitis ulcerosa,<sup>58-60</sup> membranous glomerulonephritis,<sup>61</sup> asthma,<sup>62,63</sup> and uveitis.<sup>64</sup>

Although cyclosporine revolutionized transplantation medicine and offered new possibilities for treating autoimmune disease, there were several disadvantages to its use. First, in addition to being nephrotoxic, the drug turned out to have several other side effects including the induction of glucose intolerance, hypertension, and hyperlipidemia. Second, the pharmacokinetics of cyclosporine proved to be highly variable and its therapeutic window narrow, complicating its clinical use. As a result, other pharmaceutical companies developed an interest in transplantation medicine and sought to develop new immunosuppressive agents. In 1984, in a manner very much similar to the discovery of cyclosporine, a new strain of the bacterium *Streptomyces* was isolated from a soil sample obtained from Mount Tsukuba, Japan by workers of the Fujisawa Pharmaceutical Company. This strain, designated *Streptomyces tsukubaensis*, produced a metabolite, FK-506 or tacrolimus, that possessed powerful immunosuppressive activity both *in vitro* and *in vivo*.<sup>65-68</sup>

Thomas E. Starzl and his group in Pittsburgh were the first to test tacrolimus clinically. The new agent was remarkably effective as salvage therapy for hepatic allografts that rejected under conventional, cyclosporine-based immunosuppression. Encouraged by these results, tacrolimus was then used as the primary immunosuppressant in a pilot study in patients undergoing liver- or kidney transplantation. Compared with historical, cyclosporine-treated controls, tacrolimus reduced the incidence of acute rejection and lowered the requirement for glucocorticoids.<sup>69,70</sup> These findings caused a sensation and increasing numbers of patients in need of tacrolimus “rescue therapy” were referred to Pittsburgh from other transplant centres. Articles in the press, hailing tacrolimus as a wonder drug, contributed further to the hype.<sup>3</sup>

Yet, reports indicating toxicity of tacrolimus were reason for concern.<sup>71</sup> In addition, Starzl’s group was the only one to have access to the drug leading to scepticism among other transplant physicians and even allegations of observer bias.<sup>3,72</sup> The US Food and Drug Administration placed tacrolimus on the fast-track for evaluation but also mandated randomized controlled trials comparing the new agent with conventional immunosuppression as a prerequisite for approval. This decision, as well as the (design of the) two subsequent trials themselves, led to heated debates.<sup>72,73</sup> At the start of the multicentre trials in 1990, the Pittsburgh randomized

trial comparing tacrolimus plus prednisone to cyclosporine plus prednisone therapy, was half-finished (Starzl and co-workers were forced to perform this trial by the institutional review board of the Pittsburgh University).<sup>74,75</sup> The preliminary results demonstrated that tacrolimus halved the incidence of acute rejection compared to cyclosporine-treated liver transplant recipients. Considering the superiority of tacrolimus over cyclosporine to be settled, the Pittsburgh group condemned the two phase III multicentre trials as “cruel” and “unethical”.<sup>73-75</sup>

The European and American multicentre, randomized, controlled trials demonstrated that compared to a cyclosporine-based immunosuppressive regimen, tacrolimus was associated with significantly less acute and refractory acute rejection episodes after liver transplantation. Patient and graft survival were comparable in both groups but the incidence of neurological complications and disturbances of glucose metabolism were more common in the tacrolimus group.<sup>76,77</sup> These studies were followed by several trials comparing the efficacy and safety of tacrolimus to that of the oil-based cyclosporine formulation (Sandimmune®) in kidney transplant recipients.<sup>78-80</sup> Taken together, these trials showed that immunosuppression with tacrolimus resulted in comparable patient and graft survival at one year after renal transplantation compared with cyclosporine. However, tacrolimus significantly reduced the incidence of acute rejection and the need for antilymphocyte antibodies to treat rejection. The risk to develop post-transplant diabetes mellitus (PTDM) was about five times higher in patients receiving tacrolimus.<sup>81</sup> More recently, tacrolimus has been compared head-to-head with the microemulsified cyclosporin formulation (Neoral®), either in the context of conventional immunosuppressive regimens or together with newly-developed immunosuppressive agents such as mycophenolate mofetil (MMF). A meta-analysis comparing the positive and negative effects of tacrolimus and cyclosporin, and incorporating 30 trials, concluded that tacrolimus is superior to cyclosporine in preventing acute rejection after kidney transplantation. Importantly, tacrolimus treated patients had an improved short-term graft survival (censored for death) but this is at the expense of a higher incidence of PTDM.<sup>82</sup>

Over the past decade, cyclosporine and tacrolimus have remained the cornerstone of immunosuppressive therapy after solid organ transplantation. In the United States there has, however, been a shift towards a more frequent use of tacrolimus after kidney and liver transplantation.<sup>83</sup> At present, it is unclear if the reduction in the number of acute rejection episodes associated with the use of tacrolimus translates into improved long-term graft survival or whether its beneficial immunologic effects are outweighed by its potential to cause an excess of PTDM. From recent data it appears that in the current dosing strategies, the efficacy of both drugs is similar.<sup>84-86</sup> The choice for either cyclosporine or tacrolimus should therefore depend on the risks of an individual patient.

## 1.2

### PHARMACODYNAMICS OF CALCINEURIN INHIBITORS

Cyclosporine and tacrolimus exert their immunosuppressive effect mainly by inhibiting the activation and clonal expansion of T lymphocytes, although these agents also have inhibitory effects on B lymphocytes, natural killer cells and dendritic cells.<sup>87-89</sup> At the molecular level, these effects are mediated through the inhibition of the enzyme calcineurin (CN) and hence cyclosporine and tacrolimus are collectively referred to as calcineurin inhibitors (CNIs).

#### 1.2.1 T LYMPHOCYTE ACTIVATION

Binding of a T cell receptor (TCR) complex to its major histocompatibility complex (MHC)-peptide ligand is followed by the aggregation of several other pairs of TCR-peptide-MHC complexes and clustering of TCRs with their co-receptors CD4 or CD8. In addition, adhesion and co-stimulatory molecules on the T lymphocyte and antigen-presenting cell bind to their respective ligands to form an immunological synapse.<sup>88,90</sup> The co-aggregation of TCRs leads to the activation of a series of tyrosine kinases (first the Src-family kinases Lck and Fyn and then  $\zeta$  chain-associated protein 70) which in turn activate several major signal transduction pathways, including the phospholipase C (PLC)- $\gamma$  pathway. PLC- $\gamma$  activation results in the release of inositol triphosphate from the cell membrane which increases the intracytosolic calcium concentration by releasing calcium from the endoplasmic reticulum and by triggering the opening of calcium channels in the plasma membrane.<sup>87,88</sup>

A sustained rise in the intracellular calcium concentration leads to the activation of calmodulin and the serine/threonine phosphatase CN. Calcineurin is a heterodimer consisting of a catalytic subunit, calcineurin A (CNA), and a “regulatory” subunit, calcineurin B (CNB). The latter contains four calcium binding motifs, whereas CNA contains a calmodulin-binding domain and the so-called autoinhibitory domain. Upon binding of calmodulin and calcium to the A and B subunits of CN, respectively, a conformational change takes place which exposes the active site on CNA by releasing the autoinhibitory domain from the active site cleft.<sup>91,92</sup> Activation of CN allows the enzyme to dephosphorylate its substrate, the nuclear factor of activated T cell (NFAT) family. These proteins are located in the cytosol and inactive when phosphorylated at serine/threonine residues. Upon CN-mediated dephosphorylation, they localize to the nucleus where they complex with other transcriptional regulatory proteins such as activator protein-1 and induce the transcription of several genes required for T lymphocyte activation and proliferation, including interleukin (IL)-2, IL-4, IL-2 receptor, CD40 ligand, and interferon gamma (IFN- $\gamma$ ).<sup>93,94</sup> Recent data suggest that CN co-localizes with NFAT to the nucleus where it protects NFAT from kinases which would otherwise re-phosphorylate NFAT and cause it to be exported from the nucleus.<sup>87,88,91,92</sup>



### 1.2.2 MECHANISMS OF CALCINEURIN INHIBITOR-MEDIATED IMMUNOSUPPRESSION

Cyclosporine and tacrolimus inhibit CN after binding to their cytoplasmic receptors named immunophilins.<sup>96-98</sup> Each agent binds a different group of immunophilins: cyclosporin to the cyclophilins, tacrolimus to the FK-binding proteins (FKBP).<sup>99-101</sup> Cyclophilin and FKBP are peptidyl-prolyl *cis-trans* isomerases which are thought to be involved in protein folding.<sup>100-103</sup> Inhibition of immunophilin rotamase activity is, however, not responsible for the immunosuppressive effect of CNIs.<sup>104,105</sup> Rather, the cyclosporin-cyclophilin and tacrolimus-FKBP complexes bind to CNA, thereby blocking its phosphatase activity.<sup>96-98</sup> As a result, CN is unable to dephosphorylate NFAT, which remains in the cytoplasm and is unable to activate genes required for T lymphocyte activation.<sup>93,96-98</sup>

In addition to inhibiting the CN-NFAT pathway, cyclosporine and tacrolimus appear to exert their immunosuppressive effects by several other mechanisms, as well. Recently, it was demonstrated that these agents interfere with the activation of the JNK and p38 signaling pathways.<sup>106</sup> Moreover, one of the tacrolimus-binding immunophilins, FKBP-52, is associated with the cytoplasmic glucocorticoid receptor complex. By binding of tacrolimus to FKBP-52, glucocorticoid receptors may be released from the complex and migrate to the nucleus where they bind to glucocorticoid response elements in the regulatory regions of certain genes (resulting in a steroid-sparing or -mimetic effect).<sup>107</sup> Ultimately, the result of interfering by CNIs with these “alternative” pathways is, again, suppression of the transcription of genes involved in T lymphocyte activation and proliferation.

### 1.2.3 TOXICITY

In addition to T lymphocytes (which have a rather low CN content), CN is widely distributed throughout the human body. The protein is expressed, among others, in the brain, B lymphocytes, spleen, thymus, platelets, heart, liver, testes, pancreas, and the kidney.<sup>91,92</sup> Apart from T lymphocyte activation, numerous functions have been identified for CN, including among others, a role in the programmed cell death of neuronal cells, cardiac morphogenesis and the induction of cardiac hypertrophy, neutrophil migration, and regulation of the Na<sup>+</sup>, K<sup>+</sup>-ATPase in kidney and brain.<sup>91,92</sup> Not surprisingly, cyclosporine and tacrolimus display considerable toxicity (Table 1.1). The clinically most important side effects will now be shortly discussed.

The nephrotoxic effects of CNIs were already recognized in the first clinical trials exploring their immunosuppressive potential.<sup>38,70</sup> Almost three decades later, the intrinsic nephrotoxicity of cyclosporine and tacrolimus remains the Achilles heel of CNI-based immunosuppression. Clinically, an acute and a chronic form of CNI-induced nephrotoxicity can be distinguished.<sup>108</sup> Acute nephrotoxicity usually occurs within several days after starting CNI treatment. In patients who have received early malfunctioning grafts (for example as a result of prolonged ischemia time or when a kidney from an older donor is transplanted) it may present as an acute oligoanuric syndrome (delayed graft function). Acute CNI-induced nephrotoxicity can also present as a rise in serum creatinine that may resemble other causes of early graft dysfunction. In general, acute CNI-induced nephrotoxicity is rapidly and

completely reversible upon dose reduction or CNI withdrawal.<sup>108</sup> Pathophysiologically, it is characterized by constriction of the afferent glomerular arteriole leading to a decreased renal plasma flow and a reduction of the glomerular filtration rate.<sup>109</sup> This change in vascular tone appears to result from an imbalance in the secretion and metabolism of the vasodilatory prostaglandins and nitric oxide, and the vasoconstrictive thromboxane and endothelin, as well as an increased activity of the sympathetic nerve system. Second, CNIs have been reported to cause mesangial cell contraction thereby altering glomerular permeability. Finally, CNIs may interfere with normal tubular function causing sodium retention and edema, reduced excretion of potassium and uric acid (sometimes leading to hyperkalemia or gout), increased urinary magnesium excretion, and hyperchloremic metabolic acidosis.<sup>108,110,111</sup>

**Table 1.1** Side of effects of the calcineurin inhibitors cyclosporine and tacrolimus

---

Alopecia §
Gastrointestinal disturbances
Gingival hyperplasia †
Hemolytic uremic syndrome
Hepatotoxicity
Hyperkalemia
Hyperlipidemia
Hypertension
Hypertrichosis / hirsutism †
Hyperuricemia
Hypomagnesemia
Impaired glucose tolerance ‡
Nephrotoxicity
Neurotoxicity
Pancreatitis

---

§ Alopecia has been reported to occur more often during the use of tacrolimus

† These side effects occur more often during cyclosporine treatment

‡ The risk of developing diabetes mellitus is higher with tacrolimus than with cyclosporine

Chronic CNI-induced nephrotoxicity is associated with prolonged use of these agents and has been observed after all types of transplantation, as well as during treatment of autoimmune disease.<sup>108,112-114</sup> The clinical course is characterized by a slow decline in renal function that may become stable at a certain level of renal insufficiency or progress to end-stage renal failure. In addition, most patients have proteinuria and hypertension.<sup>108,112-115</sup> In contrast to the acute form, chronic CNI-induced renal insufficiency improves little, if at all, after dose reduction or cessation of CNIs. Moreover, although the risk of developing chronic renal insufficiency has been associated with longer use and higher doses of CNIs, this has not been a universal finding.<sup>116</sup> Some authors have speculated that the risk of CNI-induced nephrotoxicity results from individual susceptibility rather than pharmacokinetic determinants.<sup>116-118</sup> Histologically, chronic CNI-induced nephrotoxicity is characterized by extensive alterations in renal architecture that may include arteriolar hyalinosis, glomerular

sclerosis and thickening of Bowman's capsule, tubular atrophy, and interstitial (striped) fibrosis. Its pathogenesis is far from being understood but an increased expression of the pro-fibrotic transforming growth factor (TGF)- $\beta$  has been considered an important etiologic factor.<sup>107,108,112-115,117</sup>

A second CNI-related side effect which has raised concern over the long-term safety of these agents is their ability to induce arterial hypertension. Between 40% and 70% of renal transplant recipients were reported to have hypertension as a result of CNI use.<sup>111</sup> The pathophysiology of CNI-induced hypertension is unclear but appears to be closely linked to their nephrotoxic effects. Postulated mechanisms include sodium retention and expansion of the extracellular volume, direct vasoconstrictive effects (mediated through altered calcium responses, upregulation of angiotensin II receptors, changes in nitric oxide metabolism, *etc.*), as well as renal magnesium wasting.<sup>111,119</sup> The treatment of CNI-induced hypertension should include general measures such as body weight reduction, salt restriction and (if possible) dose reduction of CNIs or glucocorticoids, and treatment with antihypertensive drugs. Although calcium channel blockers have gained considerable popularity in the treatment of CNI-induced hypertension,<sup>120</sup> angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers appear to be equally effective. The choice for a particular agent should therefore depend on the specific conditions in an individual patient.

Cyclosporine and tacrolimus can both induce glucose intolerance, although the risk of developing PTDM appears to be higher in patients using tacrolimus.<sup>82</sup> An incidence of PTDM as high as 30% has been reported and has even further increased in recent years.<sup>121,122</sup> PTDM has been associated with a decreased quality of life and substantial morbidity resulting from an increased susceptibility to infections and most importantly, cardiovascular complications. CNIs may exert their diabetogenic effects by impairing the expression and secretion of insulin, whereas peripheral insulin resistance likely results from the concomitant use of glucocorticoids.<sup>123</sup> Hypercholesterolemia and to a lesser extent hypertriglyceridemia, are other metabolic complications resulting from the use of CNIs. The mechanism may be related to decreased bile acid synthesis and down-regulation of low density lipoprotein receptor levels leading to decreased cholesterol clearance from the peripheral circulation.<sup>124</sup> In view of the high incidence of cardiovascular morbidity and mortality after transplantation (among renal transplant recipients, cardiovascular disease accounts for one third of all deaths), aggressive management of hyperlipidemia and PTDM appears to be justified.

Neurotoxicity is another side effect of CNIs. The clinical features are diverse and CNI-related neurotoxicity may present as tremor of the hands, cortical blindness, a confusional state, speech abnormalities, and generalized tonic-clonic seizures. Neurotoxicity results from vasogenic edema which may progress to cytotoxic edema if exposure to CNIs is prolonged. Risk factors include hypocholesterolemia and hypomagnesemia.<sup>125</sup>

## 1.3

**PHARMACOKINETICS OF CALCINEURIN INHIBITORS****1.3.1 MOLECULAR STRUCTURE, PHYSICO-CHEMICAL PROPERTIES AND DOSAGE FORMS**

Cyclosporine A (cyclo[[*(E)*-(2*S*,3*R*,4*R*)-3-hydroxy-4-methyl-2-(methylamino)-6-octenoyl]-L-2-aminobutyryl-N-methylglycyl-N-methyl-L-leucyl-L-valyl-N-methyl-L-leucyl-L-alanyl-D-alanyl-N-methyl-L-leucyl-N-methyl-L-leucyl-N-methyl-L-valyl]; C<sub>62</sub>H<sub>111</sub>N<sub>11</sub>O<sub>12</sub>) is a neutral, cyclic peptide consisting of 11 amino acids and has a molecular weight of 1203 g/mol.<sup>28,29</sup> Tacrolimus ((3*S*,4*R*,5*S*,8*R*,9*E*,12*S*,14*S*,15*R*,16*S*,18*R*,19*R*,26*aS*)-8-allyl-5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26*a*-hexadecahydro-5,19-dihydroxy-3-[(*E*)-2-[(1*R*,3*R*,4*R*)-4-hydroxy-3-methylcyclohexyl]-1-methylvinyl]-14,16-dimethoxy-4,10,12,18-tetramethyl-15,19-epoxy-3*H*-pyrido[2,1-*c*][1,4]oxaazacyclotricosine-1,7,20,21(4*H*,23*H*)-tetron; C<sub>44</sub>H<sub>69</sub>NO<sub>12</sub> · H<sub>2</sub>O) is a macrolide lactone with a molecular weight of 804 g/mol.<sup>65</sup> Both compounds are poorly soluble in water but dissolve rapidly in organic solvents such as ethanol and acetone.

Cyclosporine was originally formulated as an oily solution, available in a liquid form or in soft gelatin capsules (Sandimmune<sup>®</sup>, Novartis, Basle, Switzerland). On contact with gastrointestinal fluids, cyclosporine Sandimmune is formed into a crude oil-in-water droplet mixture which needs emulsification by bile salts before cyclosporine can be absorbed. As this emulsification step is dependent on food intake, bile flow and gastrointestinal motility, the oral bioavailability of cyclosporin Sandimmune is poor and highly variable among individuals. The new cyclosporine formulation (Neoral<sup>®</sup>, Novartis, Basle, Switzerland) which was designed to overcome these problems, consists of cyclosporine in a lipophilic solvent (corn oil mono-, di- and triglycerides) and a hydrophilic solvent [propylene glycol (E490)], together with a surfactant [polyoxyl-40 hydrogenated castor oil (Cremophor)], an antioxidant [DL- $\alpha$ -tocopherol (E307)] and alcohol.<sup>126</sup> On contact with gastrointestinal fluids this formulation readily forms a homogeneous, monophasic microemulsion simulating a mixed micellar phase. This process is not dependent on the presence of bile and as a result, the absorption of microemulsified cyclosporin is more extensive and less variable compared with the oil-based solution.<sup>127-129</sup> Recently, several generic forms of cyclosporine were introduced.<sup>130</sup> In addition, inhaled cyclosporine was shown to be beneficial after lung transplantation.<sup>131</sup>

Tacrolimus [Prograf(t)<sup>®</sup>, Astellas Pharmaceuticals, München, Germany] is currently available as capsules for oral administration containing a solid dispersion of tacrolimus in hydroxypropylmethylcellulose.<sup>132</sup> Recently, a slow-release tacrolimus formulation was developed. In addition, tacrolimus is available as a solution for intravenous administration which contains tacrolimus, alcohol and a surfactant (HCO-16).<sup>132</sup> A tacrolimus ointment (Protopic<sup>®</sup>, Astellas Pharmaceuticals, München, Germany) has been approved for the treatment of atopic eczema.<sup>133</sup>

### 1.3.2 ABSORPTION

In general, the absorption of cyclosporine and tacrolimus is poor and variable between individuals. Oral bioavailability (F) averages around 30% but may be as high as 90% or as low as 5%.<sup>127-129,134-136</sup> The use of microemulsified cyclosporine is associated with a faster and higher absorption leading to an exposure that is around 40% higher when compared to the older oil-based formulation.<sup>137-139</sup> Peak concentrations ( $C_{\max}$ ) of (microemulsified) cyclosporine and tacrolimus are usually reached within 2 h although time-to-peak concentration ( $t_{\max}$ ) varies considerably between patients and may be as long as 6 h.

The absorption of CNIs occurs predominantly in the small intestine and is affected by several factors.<sup>140</sup> Concomitant ingestion of food may delay the absorption of CNIs and reduce exposure.<sup>141,142</sup> Biliary diversion (as may occur after liver transplantation) does not appear to influence the absorption of tacrolimus. In contrast, an open T-tube leads to a lower cyclosporine absorption although this effect is smaller for the microemulsion as compared with the oil-based formulation.<sup>127-129,134-136</sup> An important limiting factor for the absorption of CNIs is the expression on the intestinal surface of the multidrug-efflux pump Permeability-glycoprotein (P-glycoprotein). P-glycoprotein belongs to the family of adenosine triphosphate (ATP)-binding cassette (ABC) proteins which are able to pump many xenobiotics, including CNIs, from the cytoplasm or cell membrane to the extracellular space.<sup>143,144</sup> In the intestine, P-glycoprotein acts as a barrier to CNI absorption by actively extruding these drugs back into the gut lumen.<sup>145</sup>

### 1.3.3 DISTRIBUTION

Cyclosporine and tacrolimus distribute extensively into tissues and the cellular fraction of blood. The highest accumulation of cyclosporine has been observed in liver, pancreas, adrenal glands and fat tissue with lower concentrations in kidney, brain and heart.<sup>108,127-129</sup> Tacrolimus distributes in lung, spleen, heart, kidney, pancreas, brain, muscle and liver.<sup>134-136</sup> In human blood, cyclosporine and tacrolimus are mainly distributed into erythrocytes and leukocytes. Of the cyclosporine amount present in whole blood, 50% to 60% is located in erythrocytes, 10% to 20% is in leukocytes, and between 30% to 40% is located in the plasma. Compared with cyclosporine, tacrolimus appears to be more extensively located in the erythrocyte compartment with tacrolimus whole blood concentrations being about 15 to 35 times those measured in plasma. Erythrocytes have a high content of FK-binding proteins and since tacrolimus does not bind to hemoglobin, this appears to be the driving force behind its distribution in blood. More than 90% of cyclosporine and tacrolimus present in plasma is bound to plasma proteins. Cyclosporine binds to lipoproteins, whereas tacrolimus associates mainly with albumin and  $\alpha_1$ -acid glycoprotein. Both agents cross the placenta and reach the fetal circulation and are detectable in breast milk. The volume of distribution (Vd) at steady state (based on whole blood measurements) is around 3 to 5 L/kg bodyweight and between 0.5 and 1.4 L/kg for cyclosporine and tacrolimus, respectively.<sup>108,127-129,134-136,146,147</sup>

### 1.3.4 METABOLISM

Prior to elimination, cyclosporine and tacrolimus are almost completely metabolized (>99%). Biotransformation occurs mainly through the cytochrome P450 (CYP) isozymes CYP3A4 and CYP3A5 with hydroxylation and demethylation being the most important metabolic pathways.<sup>148-154</sup> For cyclosporine, around 30 metabolites have been identified, the most important being AM1, AM4N and AM9, which have an immunosuppressive activity which is generally less than that of the parent compound. Tacrolimus is metabolized to at least 15 metabolites, the predominant being 13-*O*-demethyl-tacrolimus. 13-*O*-demethyl-tacrolimus has an immunosuppressive activity that is one-tenth of that of the parent compound. The minor metabolite 31-*O*-demethyl-tacrolimus may have immunosuppressive activity comparable to that of tacrolimus.<sup>108,127-129,134-136,148,155,156</sup>

Biotransformation of cyclosporine and tacrolimus occurs primarily in the liver but also in the wall of the intestine and both agents are subject to a considerable first-pass effect.<sup>157-161</sup> The (whole blood) clearance of both agents varies considerably between patients but averages around 0.35 L/h/kg and 0.06 (range 0.03 - 0.09) L/h/kg for cyclosporine and tacrolimus, respectively. The corresponding terminal elimination half-lives are between 8 and 27 (range 4 - 50) h for cyclosporine and 12 (range 4 - 41) h for tacrolimus.<sup>108,127-129,134-136,146,147</sup>

### 1.3.5 ELIMINATION

Cyclosporine and tacrolimus metabolites are mainly (>90%) biliary eliminated with around 5% being excreted in the urine. Less than 1% of the absorbed amount of cyclosporine or tacrolimus is eliminated as unchanged drug in the urine.<sup>108,127-129,134-136,162</sup> Hepatic impairment leads to a reduced clearance of CNIs. This is consistent with the fact that both agents are extensively metabolized before elimination. However, increased concentrations of tacrolimus metabolites have also been reported in patients with liver failure, indicating an impaired biliary excretion. In contrast, renal failure does not have an important effect on the clearance of both compounds. Given their large volumes of distribution, dialysis is not effective in clearing cyclosporine or tacrolimus.<sup>108,127-129,134-136</sup>

## 1.4

### THE NEED FOR CALCINEURIN INHIBITOR DOSE INDIVIDUALIZATION

When Roy Y. Calne and co-workers first treated patients with cyclosporine, the drug was dosed according to bodyweight and administered once daily (intramuscularly for the first few days after transplantation and then orally). This cyclosporine dosing strategy was extrapolated from the results of their experiments with the drug in several animal species. Formal dose-finding studies in humans were not performed. Although this bodyweight-based cyclosporine dosing regimen was effective in preventing acute rejection after kidney transplantation, many patients developed severe infections or suffered from nephro- or

hepatotoxicity. Other patients, using the same (bodyweight-based) dose, did not experience cyclosporine-related side effects. A lowering of the cyclosporine dose proved to be effective in reducing the incidence of toxicity while efficacy was largely maintained.<sup>37</sup>

These observations suggested the existence of a cyclosporine concentration-effect relationship, as well as interindividual differences in the response to cyclosporine treatment. As experience with the use of cyclosporine increased, the practice of prescribing a fixed, bodyweight-based dose of cyclosporine was abandoned in favor of the more individualized strategy of therapeutic drug monitoring (TDM). TDM is the practice to adjust the dose of a drug according to blood concentration measurements, in order to reach a certain pre-defined (blood) concentration of that particular agent, the so-called target range. The rationale for performing TDM of CNIs will now be discussed, namely (1) the existence of a concentration-effect relationship (which is closer than the correlation between CNI *dose* and clinical events); (2) a narrow therapeutic window; and (3) large inter- and intra-individual variability in pharmacokinetics and pharmacodynamics.

#### 1.4.1 CONCENTRATION-EFFECT RELATIONSHIP (PHARMACOKINETICS-PHARMACODYNAMICS INTERACTION)

In 1981, Keown and co-workers<sup>163</sup> described 6 kidney transplant recipients in whom immunological reactivity and serum cyclosporine concentrations were measured in the early postoperative phase. They observed that the cyclosporine concentration in serum samples correlated positively with the ability of those particular samples to suppress the response to third-party cells in a mixed lymphocyte culture. Maintenance of patients at a cyclosporine serum predose concentration between 100 and 400 ng/mL resulted in almost complete suppression of immunological activity (as measured by various cytotoxicity assays) against their donors. One of the 6 patients experienced acute graft rejection. Serum cyclosporine concentrations during acute rejection were consistently below 100 ng/mL. When therapeutic cyclosporine concentrations were achieved (and methylprednisolone therapy was given), clinical and immunological resolution rapidly ensued.<sup>163</sup>

Since the publication of this landmark paper, other investigators have reported on the cyclosporine concentration-effect relationship and the poor correlation between cyclosporine dose and clinical outcomes. Halloran and others found that CN activity in whole blood or in peripheral blood leukocytes correlated inversely with the rise and fall of cyclosporine concentrations.<sup>164-166</sup> In addition, the *ex vivo* expression of the IL-2 gene was inhibited by cyclosporine in a concentration-dependent manner.<sup>167</sup> These experimental findings have been corroborated by clinical observations. Yee *et al.*,<sup>168</sup> studying 179 recipients of bone marrow grafts, demonstrated that low cyclosporine predose concentrations were significantly associated with an increased risk of developing acute graft-*versus*-host disease. In an analysis of 1868 whole-blood cyclosporine pharmacokinetic profiles obtained from 160 renal transplant recipients, Lindholm and Kahan<sup>169</sup> found that a cyclosporine clearance >325 mL/min and the resulting lower cyclosporine concentrations, were associated with an increased incidence of acute rejection and poorer graft survival. Cyclosporine doses were not different between rejecting and non-rejecting patients.<sup>169</sup>

A cyclosporine concentration-effect relationship has also been identified with regard to its side effects. Several investigators have reported that a high cyclosporine exposure increases the risk of developing acute nephrotoxicity.<sup>163,170-173</sup> Other cyclosporine-related adverse events have been less intensely studied. An association between high plasma cyclosporine trough levels and hepatotoxicity has been reported.<sup>170,174</sup> In pediatric renal transplant recipients, a high cyclosporine exposure was found to correlate positively with the incidence of hypertrichosis and tremor but not gingival hyperplasia.<sup>175</sup>

For tacrolimus, comparable correlations between tacrolimus exposure and the incidence of side effects and acute rejection have been described. In a dose-ranging trial performed among 120 *de novo* kidney transplant recipients, patients were randomized to a cyclosporine-based regimen or to one of three tacrolimus-based regimens designed to achieve low (5-14 ng/mL), medium (15-25 ng/mL) or high (26-40 ng/mL) predose whole blood concentrations. Corresponding starting doses were 0.2, 0.3, and 0.4 mg/kg per day, respectively. In the group that was maintained at the highest predose concentration range, a 62% incidence of toxicity was observed whereas only 10% of the patients experienced an acute rejection episode. In the lowest target range group, the reverse was observed with 33% of patients experiencing tacrolimus toxicity and an acute rejection incidence of 21%. Based on these results, a tacrolimus concentration range between 5 and 15 ng/mL was considered to provide optimal efficacy with minimal toxicity.<sup>176</sup> Other investigators have reported that higher tacrolimus exposure is associated with an increased risk of nephrotoxicity, severe neurotoxicity or the development of infections.<sup>177,178</sup>

#### 1.4.2 THERAPEUTIC WINDOW

The therapeutic index, or the ratio between toxic and effective concentrations, of CNIs is small, equaling 1 or less. This notion is based on the fact that the CNI concentration-effect relationship as discussed under 1.4.1, has not been a universal finding and appears not to be simply linear. For example, in the study by Lindholm and Kahan,<sup>169</sup> which is one of the largest and most detailed studies investigating the concentration-effect relationship of cyclosporine performed to date, no significant relationship between either nephrotoxicity nor hepatotoxicity and cyclosporine pharmacokinetics could be identified. Some of these “inconsistencies” between studies may be related to, for example, differences in the analytical methods or sampling strategies used, patient characteristics or immunosuppressive regimens. However, even investigators who did find a relationship between CNI concentrations and the occurrence of acute rejection or a particular side effect, noticed that some patients in the “toxic” concentration range did not suffer from any side effects and had excellent renal function. *Vice versa*, low CNI exposure does not necessarily result in rejection in all patients. In other words, the optimal CNI concentration range is individually determined and toxic and effective concentrations show considerable overlap between patients. Nevertheless, target ranges for both cyclosporine and tacrolimus (for the “average” patient) have been determined. For tacrolimus, in the early postoperative phase, many centers aim for tacrolimus predose concentrations between 10 and 15 ng/mL, a 50% difference between minimally effective and toxic concentrations.<sup>179</sup> For cyclosporine, Mahalati *et al.*,<sup>172,173</sup> recommended a target area-under the concentration *versus* time-curve (AUC) between 0 and 4 h of 4400 to 5500 ng/h per mL for kidney transplant patients in the first months after kidney transplantation, corresponding to a 25% difference.



### 1.4.3 VARIABILITY IN CALCINEURIN INHIBITOR PHARMACOKINETICS AND PHARMACODYNAMICS

A third reason for performing TDM for CNIs is their considerable interpatient variability in pharmacokinetics and pharmacodynamics. Studies investigating the pharmacokinetics of the oil-based cyclosporine formulation found bioavailability to average around 30% with a range between 10% and 90%, thus displaying a more than 9-fold variability. Time-to-peak concentration was generally between 3 h and 4 h but was sometimes as long as 22 h.<sup>137-139,146,169,180</sup> In addition, Lindholm<sup>169</sup> reported a 7-fold interindividual variability in cyclosporine clearance in renal transplant recipients, whereas Ptachcinski<sup>146</sup> reported a mean clearance of 0.34 L/h/kg with a range between 0.036 and 1.43 L/h/kg, representing a 40-fold difference. The findings of the latter study, however, may be somewhat limited by the fact that two renal transplant recipients used concomitant medication known to induce the CYP3A system.<sup>146,169</sup>

There also exists considerable within-patient variability for cyclosporine pharmacokinetics. Several investigators have observed a decrease in cyclosporine bioavailability and clearance, as well as a reduced cyclosporine dose-requirement with time after renal transplantation. These time-dependent changes occur mostly during the first 3 months after transplantation and have been attributed to changes in co-medication, alterations of gastrointestinal motility and changes in biochemical parameters such as hematocrit and serum albumin, which may alter cyclosporine distribution.<sup>138,169,171,181-184</sup> However, even in stable patients there is marked intraindividual variability in cyclosporine Sandimmune pharmacokinetics. Kovarik *et al.*,<sup>137</sup> studying 55 renal transplant patients who had used cyclosporine for a minimum of 6 months, found intraindividual coefficients of variation (CV) in  $t_{\max}$ , (dose-adjusted)  $C_0$ ,  $C_{\max}$ , and  $AUC_{0-12}$  of 74%, 20%, 33%, and 18%, respectively.

Because of the large intra- and interindividual differences in the pharmacokinetics of cyclosporine Sandimmune, which are most pronounced during the absorption phase of the drug, the cyclosporine microemulsion formulation was developed. As outlined in paragraphs 1.3.1 and 1.3.2, this formulation has self-emulsifying properties making its absorption less dependent on gastrointestinal motility or on the presence of bile or food. Consistent with its physico-chemical properties, absorption of microemulsified cyclosporine is generally higher, faster and shows less intraindividual variability (reviewed in references 127-129). In renal transplant recipients who were converted from the oil-based to the microemulsion formulation on a milligram-to-milligram basis, the use of microemulsified cyclosporine resulted in a  $t_{\max}$  which was approximately 1 h shorter, and  $C_{\max}$  and  $AUC_{0-12}$  increased by 59% and 30%, respectively.<sup>137</sup> In studies in which patients were randomized to receive either the conventional or the microemulsion formulation, use of the latter resulted in a cyclosporine exposure that was on average between 40% and 60% higher and a shortening of  $t_{\max}$  to between 1 and 2 h.<sup>138,139</sup> Importantly, intraindividual variation in cyclosporine pharmacokinetics of patients using the microemulsion formulation was reduced compared with the oil-base formulation with a reduction of the intraindividual CV for  $t_{\max}$  (-45%),  $C_{\max}$  (-20%), and  $AUC_{0-12}$  (-10%).<sup>127-129,137-139</sup> However, the interindividual variability in cyclosporine pharmacokinetics appears to be comparable between the two formulations or at most only modestly decreased in favor of microemulsified cyclosporine.<sup>138,139</sup>

Tacrolimus also displays considerable interindividual variability in its pharmacokinetics and, comparable to cyclosporine, this variability is most marked during the absorption phase. Bioavailability is around 25% with a range of 4% to 93%. Interindividual differences in tacrolimus clearance appear to be less pronounced and have been reported to differ 3-fold, whereas terminal elimination half-life may vary up to 10-fold between individuals.<sup>134,147</sup> These interindividual differences in tacrolimus pharmacokinetics are reflected by the wide range of oral tacrolimus dosages (1 to 44 mg/day) that were needed to maintain tacrolimus whole blood trough concentrations between 5 and 20 ng/mL as reported by Venkataramanan *et al.*<sup>134</sup>

Intraindividual variability of tacrolimus pharmacokinetics has been less well studied. The apparent oral clearance of tacrolimus decreases after transplantation resulting in a reduction of the tacrolimus dose requirement.<sup>147,185</sup> These changes over time have been attributed to changes in hematocrit, serum albumin and tapering of glucocorticoids.<sup>186</sup> Not surprisingly, intraindividual variability in tacrolimus pharmacokinetics is largest during the first few months after transplantation and stabilizes thereafter. Nonetheless, changes in tacrolimus dose requirement may occur up to one year posttransplant. Over this period, mean intraindividual CV of tacrolimus AUC<sub>0-12</sub> is around 20%.<sup>178,185</sup>

Only limited data are available with regard to variability in CNI pharmacodynamics. No studies have been conducted at a population level to investigate the natural variability in CN activity or interindividual differences in the pharmacodynamic response to CNI administration. From several studies performed in a small number of individuals, it appears that the pharmacodynamic effects of CNIs display considerable variability between individuals. However, repeated administration of CNIs resulted in an inhibition of CN that was stable over time which argues against a significant adaptation to the pharmacodynamic effects of these agents.<sup>164-167</sup>

The consequence of the large variability in CNI pharmacokinetics is that, especially in the critical early phase after transplantation, large proportions of patients will have an exposure to CNIs that is well outside the therapeutic window.<sup>187</sup> Because of the poor correlation between CNI dose and exposure, most transplant centers have long since adopted the strategy to start patients on a certain dose of CNIs (based on their bodyweight) followed by rapid dose adjustments based on whole blood concentration measurements. Yet, even with intensive TDM in the early postoperative period, many patients (percentages of up to 50% have been reported) will have CNI exposure that is outside of the target range.<sup>182,184,185,188,189</sup> Obviously, this puts patients at risk for either acute rejection or toxicity. Pre-operative assessment of CNI pharmacokinetics has not solved these difficulties as the correlation between pre- and posttransplant values is poor.<sup>169</sup> In addition, for reasons outlined in section 1.5, some patients may experience changes in CNI exposure longer after transplantation.

## 1.5

# CAUSES OF VARIABILITY IN CALCINEURIN INHIBITOR PHARMACOKINETICS

### 1.5.1 P-GLYCOPROTEIN

P-glycoprotein, or ABCB1, belongs to subfamily B of the ABC membrane transporter family. The protein serves as an efflux pump capable of pumping a wide variety of endogenous and exogenous substances, including cyclosporine and tacrolimus, to the cell exterior.<sup>143</sup> P-glycoprotein is widely expressed throughout the human body and has, among others, been identified on the apical surface of mature enterocytes in the small intestine, at the canalicular surface of hepatocytes, the brush border of proximal tubular cells in the kidney, and on the luminal surface of endothelial cells in the brain. The specific tissue expression of P-glycoprotein suggests that its physiological role is to limit the exposure to potentially toxic substances and xenobiotics by reducing their absorption from the gut, facilitating their biliary or renal elimination, and by maintaining barriers between different compartments of the body (for example, the blood-brain barrier).<sup>144,145,190</sup>

The expression of P-glycoprotein is highly variable between individuals. In addition, the expression of P-glycoprotein has been shown to correlate with CNI pharmacokinetics. Several authors have reported case histories of small bowel transplant recipients in whom CNI dose requirement was positively correlated with intestinal P-glycoprotein expression.<sup>191-193</sup> Similarly, in liver transplant recipients with a high intestinal P-glycoprotein expression, an approximately 2-fold higher tacrolimus dose was needed to reach target concentrations compared with patients expressing P-glycoprotein at a low level. Importantly, high P-glycoprotein expression was associated with a significantly worse survival after liver transplantation (relative risk 1.63; 95% confidence interval 1.08-2.46).<sup>194</sup> In stable kidney transplant recipients, 17% of the variability in apparent oral cyclosporine clearance was accounted for by intestinal P-glycoprotein content. In addition, the P-glycoprotein concentration in the gut explained 30% of the variability in cyclosporine  $C_{max}$ .<sup>195</sup>

### 1.5.2 CYTOCHROME P450 3A

The activity of the isoenzymes CYP3A4 and CYP3A5, which are responsible for the metabolism of CNIs, displays considerable inter- and intraindividual variability.<sup>196,197</sup> Analogous to P-glycoprotein, variability in CYP3A activity explains part of the interindividual differences in CNI disposition. In kidney transplant recipients, more than half of the variability in apparent oral cyclosporine clearance was accounted for by variation in hepatic CYP3A activity as measured by the erythromycin breath test.<sup>195</sup> There is also evidence that heterogeneity in intestinal CYP3A activity translates into considerable differences in CNI pharmacokinetics between individuals. Combined administration of tacrolimus or cyclosporine with either ketoconazole (a potent CYP3A inhibitor) or rifampin (a CYP3A inducer) to volunteers or renal transplant recipients resulted in marked changes in CNI bioavailability (increased and decreased with ketoconazole and rifampin coadministration, respectively).

The observed changes could not be explained by alterations in hepatic bioavailability or hepatic metabolism alone and were thus attributed to changes in intestinal CNI metabolism. However, as ketoconazole and rifampin may also affect intestinal P-glycoprotein activity, the exact contributions of CYP3A and P-glycoprotein to the observed alterations in CNI pharmacokinetics could not be dissected.<sup>158-160,198-200</sup> Nonetheless, these data do indicate that variability in intestinal and hepatic P-glycoprotein and CYP3A activity explains much of the variation in the pharmacokinetics of CNIs.

### 1.5.3 DRUG INTERACTIONS

Drug interactions may also affect the outcome of CNI treatment. Interactions with other drugs may occur at any time during CNI therapy and can be divided into two types: pharmacodynamic and pharmacokinetic drug interactions.<sup>201,202</sup> A pharmacodynamic interaction occurs when coadministration of a particular drug enhances the immunosuppressive activity of CNIs or potentiates their toxicity. An example of the latter is the enhanced nephrotoxicity that may occur when CNIs are used in combination with other nephrotoxins such as aminoglycosides, amphotericin B or non-steroidal anti-inflammatory drugs.<sup>201,202</sup> Pharmacokinetic drug interactions may theoretically occur during the absorption, distribution, metabolism or elimination of a drug. For CNIs several drug interactions during the absorption phase have been described. The prokinetic agents cisapride and metoclopramide, for example, have been shown to increase CNI absorption.<sup>201,202</sup> However, inhibition or induction of both intestinal and hepatic CYP3A-mediated CNI metabolism is generally regarded as the most important drug interaction mechanism.<sup>201-203</sup> A large number of antimicrobial, anticonvulsant, and antiarrhythmic drugs has been shown to have clinically relevant interactions with CNIs via this mechanism (Table 1.2). Most of these agents also have inhibitory or inducing effects on P-glycoprotein which further contributes to changes in CNI bioavailability. In addition, it has been suggested that coadministration of P-glycoprotein inhibitors may increase the incidence of CNI-mediated neurotoxicity, not only by increasing blood concentrations but also by facilitating distribution into the central nervous system.<sup>202,204</sup>

### 1.5.4 DEMOGRAPHIC FACTORS AND OTHER PATIENT CHARACTERISTICS

Children require higher CNI doses to reach target concentrations compared with adults. This has been related to a decreasing clearance with advancing age -possibly due to changes in CYP3A isozyme expression- rather than changes in bioavailability.<sup>129,134,169,180,181,202</sup> The effect of gender on CNI pharmacokinetics appears to be less marked and dosage recommendations for male and female patients are the same.<sup>169,180,181,202,205</sup> Serum albumin and triglyceride concentration, as well as hematocrit have all been correlated with CNI disposition. Changes in these variables explain part of the alterations in CNI pharmacokinetics that occur shortly after transplantation.<sup>129,134,169,169,180,181,185,186,202</sup> As discussed in paragraph 1.3.5, decreased liver function leads to a reduced clearance of CNIs and to impaired biliary excretion of their metabolites, whereas impaired kidney function does not significantly affect CNI pharmacokinetics.<sup>129,134,169</sup>

**Table 1.2** Drugs having clinically relevant pharmacokinetic interactions with the calcineurin inhibitors cyclosporine and tacrolimus

<b>Drug</b>	<b>Clinical effect</b>
<i>Antibiotics</i>	
Clarithromycin	Increased exposure
Doxycyclin	Increased exposure
Erythromycin	Increased exposure
Rifampicin	Reduced exposure
<i>Antiepileptics</i>	
Carbamazepine	Reduced exposure
Phenobarbital	Reduced exposure
Phenytoin	Reduced exposure
<i>Antihypertensive and antiarrhythmic agents</i>	
Amiodarone	Increased exposure
Diltiazem	Increased exposure
Nicardipine	Increased exposure
Nifedipine	Increased exposure
Verapamil	Increased exposure
<i>Antimycotic drugs</i>	
Fluconazole	Increased exposure
Itraconazole	Increased exposure
Ketoconazole	Increased exposure
<i>Other</i>	
HIV protease inhibitors	Increased exposure
Theophyllin	Increased exposure

There has been much controversy with regard to the influence of ethnicity on CNI pharmacokinetics. The outcomes of transplantation in African Americans in general is worse compared to white transplant recipients.<sup>206</sup> Heightened immune reactivity and socioeconomic disparities have been suggested to account for these differences. However, several authors have reported that the oral bioavailability of both cyclosporine and tacrolimus is lower in black compared to white subjects.<sup>169,180,205-208</sup> It has been speculated that these inter-ethnic differences may have resulted from differences in CYP3A and P-glycoprotein expression or activity. An alternative explanation may be differences in meal habits. The latter hypothesis has been supported by the fact that cyclosporine exposure was comparable between black and white individuals under controlled dietary conditions.<sup>209,210</sup>

## 1.6

**STRATEGIES TO OPTIMIZE TREATMENT WITH  
CALCINEURIN INHIBITORS**

Over the past 50 years, solid organ transplantation has evolved from an experimental therapy for a limited number of selected patients to the treatment of choice for patients with end-stage organ failure.<sup>211</sup> This remarkable success is in part attributable to the development of immunosuppressive drug therapy. The introduction of CNIs and other immunosuppressive agents has led to a dramatic reduction in the incidence and severity of acute rejection and consequently, much improved short- and medium-term graft and patient survival. The long-term outcomes of solid organ transplantation have, however, not improved to a similar degree.<sup>86,212</sup> Today, the main obstacles to successful (long-term) engraftment are the high incidence of cancer, infection, and cardiovascular disease among transplant recipients, as well as late allograft loss due to chronic rejection.

Although there is some evidence that certain immunosuppressive agents are more carcinogenic than others, the high risk of transplant recipients to develop cancer is first and foremost related to the overall level of immunosuppression.<sup>213,214</sup> Likewise, the high frequency of infectious complications after transplantation is related to the net state of immunosuppression rather than to the use of a particular immunosuppressive agent.<sup>215,216</sup> In contrast, the use of CNIs probably contributes to the high incidence of cardiovascular disease among transplant recipients. These agents often cause hypertension, hyperlipidemia, and diabetes mellitus which are all established cardiovascular risk factors in the general population.<sup>217</sup>

Chronic rejection is characterized clinically by a gradual decrease in transplant function together with (transplanted) organ-specific histopathologic changes. It is now recognized that various nonimmunologic factors are involved in the pathogenesis of chronic rejection and therefore the more inclusive term “chronic allograft nephropathy” (CAN) has been introduced for kidney transplant recipients (in analogy, chronic rejection in heart, lung, and liver transplant recipients is also known as “transplant coronary artery disease”, “bronchiolitis obliterans” and “vanishing bile duct syndrome”, respectively). Among the factors that have been shown to contribute to the risk of chronic rejection, hyperlipidemia and hypertension can be induced by CNIs. In addition, cyclosporine appears to contribute directly to the development of chronic rejection by means of its pro-fibrotic properties.<sup>218,219</sup>

At present, the main challenge to transplant physicians is to improve the long-term outcomes of solid organ transplantation while maintaining the excellent short- and medium-term results that are currently being achieved. As an episode of (severe) acute rejection is one of the major risk factors for chronic rejection, any strategy to achieve this goal must not do so at the expense of an increased risk of acute rejection.<sup>218,220</sup>

### 1.6.1 CALCINEURIN INHIBITOR AVOIDANCE AND MINIMIZATION

Given the association between the use of CNIs and the risk of cardiovascular disease and CAN, complete avoidance of these agents is a logical strategy. In several randomized clinical trials in *de novo* primary kidney transplant recipients, the efficacy of a CNI-free, sirolimus-based immunosuppressive regimen has been explored. Treatment with sirolimus in combination with either MMF or azathioprine plus corticosteroids with or without basiliximab induction therapy, resulted in a patient and graft survival, as well as an incidence of biopsy-proven acute rejection that was comparable to cyclosporine-based therapy. In addition, these initial studies demonstrated a significantly better renal function in the absence of cyclosporine.<sup>221-223</sup>

However, data from more recent studies have not been able to confirm these benefits of CNI avoidance.<sup>224,225</sup> In SYMPHONY, a prospective, randomized study with four parallel arms, 1645 kidney transplant recipients were randomized to standard immunosuppression consisting of normal-dose cyclosporine, MMF and corticosteroids, or to one of three regimens consisting of daclizumab induction, MMF, and corticosteroids plus either low-dose cyclosporine, tacrolimus or sirolimus. At six and twelve months follow-up, the incidence of biopsy-proven acute rejection was highest in patients randomized to receive sirolimus-based immunosuppression (33% and 35%, respectively).<sup>224</sup> Another, smaller study has also demonstrated an unacceptably high risk of acute rejection in association with a sirolimus-based, CNI-free immunosuppressive protocol.<sup>225</sup> In addition, in the most recent studies, the main promise of CNI-free immunosuppression, namely an improvement of renal function and a lower incidence of CAN, was not achieved by completely avoiding the use of CNIs.<sup>224,226</sup>

An alternative approach to reduce the long-term toxicity of CNIs while capturing the benefit of low acute rejection rates associated with their use, is to treat patients for only a limited period of time with CNIs followed by CNI dose reduction or complete elimination. In the first studies that explored the safety of cyclosporine withdrawal, cyclosporine was either converted to azathioprine, or completely eliminated from azathioprine-containing immunosuppressive regimens. A meta-analysis of these trials demonstrated that cyclosporine withdrawal resulted in an overall 11% higher incidence of acute rejection compared with patients who continued cyclosporine treatment.<sup>227</sup> However, this increased risk of acute rejection was not associated with increased graft loss.<sup>227</sup> Similarly, the safety of cyclosporine withdrawal from MMF-based immunosuppressive regimens has been investigated. This strategy also resulted in a significantly higher incidence of acute rejection (ranging between 10% and 22%) but again not at the expense of increased graft loss at short-term follow-up. In addition, blood pressure and lipid profile improved after cyclosporine withdrawal.<sup>228-230</sup>

Finally, several investigators have studied the safety of early cyclosporine elimination from sirolimus-containing immunosuppressive regimens. In the Rapamune Maintenance Regimen Study, 430 kidney transplant recipients were randomized at month three posttransplantation to remain on sirolimus, cyclosporine and corticosteroid therapy or to have cyclosporine withdrawn and continue treatment with (increased dose) sirolimus and corticosteroids.<sup>231,232</sup> At one and two years follow-up, renal function was significantly better in the cyclosporine-withdrawal group but at the expense of a somewhat higher incidence of biopsy-proven acute rejection (9.8% *versus* 5.1% in the cyclosporine-withdrawal and

sirolimus-cyclosporine groups, respectively).<sup>231,232</sup> Following a somewhat similar approach, Gonwa *et al.*<sup>233</sup> also observed an improvement in renal function after early cyclosporine cessation but without an increased risk of acute rejection.

Although (early) cyclosporine withdrawal does not appear to result in increased graft loss at short-term follow-up, there has remained considerable concern that cessation of cyclosporine may not be the safest strategy to follow. Pascual and colleagues<sup>234</sup> therefore randomized stable kidney transplant recipients at one year after transplantation to either have their cyclosporine dose reduced by 50% or continue their maintenance dose. At six months follow-up, no acute rejection episodes were observed in either group. Importantly, cyclosporine dose reduction did result in a significantly improved renal function, whereas serum creatinine worsened in the control group.<sup>234</sup>

In summary, it appears that the lowest incidence of acute rejection is currently achieved by treating patients with immunosuppressive regimens that contain a CNI. At present it is unclear whether the benefits of early CNI withdrawal are outweighed by the increased risk of acute rejection associated with such an approach. Moreover, the side effects of newer immunosuppressive agents such as sirolimus (*e.g.* hyperlipidemia) are reason for concern as they may also negatively influence patient and graft survival. The question as to which is the optimal immunosuppressive strategy to follow can only be answered by longer follow-up of appropriately designed clinical trials.

### 1.6.2 DRUG FORMULATION

As discussed previously, the cyclosporine microemulsion formulation was developed to improve the unfavorable pharmacokinetic characteristics of the older oil-based formulation. This attempt has been partially successful. Compared with cyclosporine Sandimmune, microemulsified cyclosporine is more rapidly and completely absorbed and displays less intraindividual pharmacokinetic variability. However, the interindividual pharmacokinetic variability appears to be comparable between the two formulations and therefore the introduction of microemulsified cyclosporine has not abrogated the need to perform TDM.<sup>138,139</sup> Nonetheless, most transplant centers have abandoned cyclosporine Sandimmune in favor of the microemulsion formulation.<sup>83</sup>

### 1.6.3 THERAPEUTIC DRUG MONITORING

Given their narrow therapeutic window, variable pharmacokinetics, and the existence of a concentration-effect relationship, both cyclosporine and tacrolimus meet the requirements for a successful clinical application of TDM (see section 1.4). In addition, monitoring blood levels can be helpful to check for patient compliance. Over the years, the practice to adjust the CNI dose according to blood concentrations has gained widespread acceptance in the field of transplantation, although no trials comparing fixed-dose *versus* a TDM-based CNI dosing strategy (*i.e.* concentration-controlled) were ever conducted.



Which parameter to use for TDM is still a matter for debate. Traditionally, the CNI dose has been adjusted according to the predose concentration. However, for cyclosporine, the  $C_0$  correlates poorly with total drug exposure within a dosing interval and with clinical outcomes after transplantation.<sup>182-184,187</sup> As determination of full pharmacokinetic profiles is impractical for routine clinical use, there has been a search for simple alternatives to  $C_0$  for profiling cyclosporine pharmacokinetics. Because of the large variability in cyclosporine pharmacokinetics during its absorption phase, limited sampling strategies focusing on the first few hours after oral administration have become a popular method for monitoring cyclosporine therapy. In several studies it was demonstrated that the  $AUC_{0-4}$  correlated very well with  $AUC_{0-12}$  and accurately predicted clinical outcomes after kidney transplantation.<sup>172,173,182-184,187</sup> This strategy does, however, require the drawing of several blood samples and measurement of multiple cyclosporine concentrations (thereby increasing workload and costs), in addition to the application of a (simple) mathematical calculation step.

The cyclosporine whole blood concentration two hours after Neoral<sup>®</sup> administration ( $C_2$ ) has been shown to be the single-sampling point that correlates best with total drug exposure and the probability of developing acute rejection after transplantation.<sup>182-184,187</sup> Based on these studies, target levels for  $C_2$  were defined and patient management by  $C_2$  monitoring was subsequently shown to result in excellent short-term efficacy and safety among *de novo* renal transplant recipients.<sup>189,235,236</sup> However, to date only few studies have directly compared cyclosporine  $C_2$  (or other limited-sampling strategies) to traditional  $C_0$  monitoring. Levy *et al.*<sup>237</sup> studied 307 *de novo* liver transplant recipients who were randomized to receive cyclosporine either titrated to reach predefined  $C_2$  or  $C_0$  target level ranges. At 3 months follow-up, graft loss and the overall incidence of acute rejection were comparable between the two groups, although the incidence of moderate to severe histological grades of acute rejection was significantly higher among patients monitored by  $C_0$  (47% versus 73% for the  $C_2$  and  $C_0$  groups, respectively).<sup>237</sup> In the International Neoral Renal Transplant Study, there were no statistically significant differences between *de novo* renal transplant recipients ( $n = 204$ ) managed by either  $C_0$  or AUC monitoring with regard to rejection rate, rejection severity, graft survival or nephrotoxicity.<sup>184</sup> Although the absence of any statistically significant differences in major clinical endpoints in these studies may be explained by insufficient statistical power, these results do suggest that any benefits of limited sampling strategies are relatively small. Moreover, only few data exist on the benefits and safety of limited sampling strategies in stable patients longer after transplantation.<sup>238,239</sup>

With regard to tacrolimus, both poor and strong correlations between the  $C_0$  and total drug exposure have been reported. This correlation appears to be stronger than that reported for cyclosporine and most centers still rely on the tacrolimus  $C_0$ .<sup>179,240</sup> However, Kuypers and colleagues<sup>178</sup> demonstrated that tacrolimus exposure as determined by  $AUC_{0-12}$  is a more reliable pharmacokinetic parameter than  $C_0$  for predicting infectious complications in the early phase after renal transplantation. Recently, a more sophisticated method to guide tacrolimus dosing was reported.<sup>185</sup> However, although this limited sampling strategy adequately predicted tacrolimus exposure, it is unlikely to rapidly gain widespread clinical acceptance because of its rather complicated mathematics, the unfamiliarity of clinicians with Bayesian forecasting, the lasting need to sample at multiple time points and the lack of any data demonstrating improved clinical outcomes over  $C_0$  monitoring.<sup>185</sup>

#### 1.6.4 PHARMACOGENETICS AND PATIENT CHARACTERISTICS

Another strategy to attain CNI dose individualization is to consider patient characteristics that explain an important part of the interindividual variability in CNI pharmacokinetics and pharmacodynamics. As discussed in section 1.5, a large part of the variability in CNI disposition has been attributed to interindividual differences in the activity and expression of the metabolizing enzymes CYP3A4 and CYP3A5 and the drug transporter P-glycoprotein. In addition, an association between P-glycoprotein expression in the kidney allograft and the risk of CNI toxicity has been reported.<sup>241</sup> The possibility that differences in CYP3A and P-glycoprotein activity are in part explained by genetic variation was considered for a long time, but it was not until the recent identification of a number of single-nucleotide polymorphisms (SNPs) in the *CYP3A4*, *CYP3A5*, and *ABCB1* (or *MDR1*, the gene encoding P-glycoprotein) genes that further evidence for this hypothesis was provided.<sup>144,242</sup> At present, the impact of these SNPs on CNI pharmacokinetics or transplantation outcome is unknown. If the efficacy or toxicity of CNI treatment is (partially) genetically determined, then such information would theoretically be of great value in order to achieve a further individualization of CNI-based immunosuppressive therapy. A pharmacogenetics-based approach to CNI treatment could assist in determining the (starting) dose of these agents or identify patients at high risk for toxic effects. Importantly and unlike TDM, (pharmaco)genetic screening of a patient can be performed before transplantation. Moreover, genetic information is constant over an individual's lifetime, and steady-state conditions are not required for the interpretation of results. Pharmacogenetics may also provide a mechanistic explanation of drug behavior for single, as well as for multiple drugs.<sup>243-245</sup>

Finally, several other patient characteristics may assist in choosing a particular immunosuppressive regimen. For example, black patients may need higher CNI doses to reach target concentrations. Obese patients or other individuals at risk for developing post-transplant diabetes mellitus may benefit from treatment with cyclosporine rather than tacrolimus. In addition, an understanding of drug interactions may avoid under- or overexposure to CNIs when other drugs are added to or withdrawn from CNI-containing immunosuppressive regimens.

## 1.7

### AIMS OF THE THESIS

Despite the ongoing development of new immunosuppressive drugs, at present, the best transplantation results are arguably being achieved by using an immunosuppressive regimen that incorporates a CNI.<sup>246,247</sup> There is however, room for improvement. Therefore, the overall aim of this dissertation was to explore ways of optimizing CNI treatment. More specifically, we investigated the following:

1. The relationship between cyclosporine exposure and the occurrence of cyclosporine-related side effects in stable heart, kidney, and liver transplant recipients on maintenance therapy (*Chapter 2.1*).
2. The benefits and drawbacks of cyclosporine dosing based on  $C_2$  levels compared with conventional  $C_0$  level monitoring in stable heart, kidney, and liver transplant recipients (*Chapters 2.1 and 2.2*).
3. The effect of glucocorticoids on tacrolimus pharmacokinetics (*Chapter 3.1*).
4. The evidence for a pharmacokinetic interaction between cyclosporine and mycophenolic acid (*Chapter 3.2*).
5. The role of the multidrug resistance-associated protein 2 (MRP2) in the cyclosporine-mycophenolic acid interaction (*Chapter 3.3*).
6. The effect of single-nucleotide polymorphisms in the *MDR-1*, *CYP3A4* and *CYP3A5* genes on the pharmacokinetics of cyclosporine and tacrolimus (*Chapters 4.1, 4.2, and 4.3*).

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PART 2

# **THERAPEUTIC DRUG MONITORING**



## CHAPTER 2.1

# **THE RELATIVE IMPORTANCE OF CYCLOSPORINE EXPOSURE IN HEART, KIDNEY OR LIVER TRANSPLANT RECIPIENTS ON MAINTENANCE THERAPY**

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## ABSTRACT

We investigated the relationship between cyclosporine exposure and the presence of cyclosporine-related side effects and assessed the advantage of the cyclosporine concentration 2 h postdose ( $C_2$ ) over predose concentration ( $C_0$ ) monitoring. Cyclosporine area-under the concentration *versus* time-curves were measured during the absorption phase ( $AUC_{0-4h}$ ) in 49 liver, 28 heart and 26 kidney transplant recipients (time since transplantation >6 years) with or without cyclosporine-related side effects on maintenance therapy. The cyclosporine  $C_0$  correlated well with  $AUC_{0-4}$  ( $r = 0.77$ ), whereas  $C_2$  levels correlated strongly with  $AUC_{0-4}$  ( $r = 0.92$ ). Although we observed a trend towards higher cyclosporine concentrations in transplant recipients with side effects as compared with patients without cyclosporine toxicity, the large majority of those differences were not statistically significant. Thus, as cyclosporine exposure was not clearly related to the presence of side effects, and  $C_0$  correlated fairly with  $AUC_{0-4}$ , the advantage of monitoring cyclosporine treatment using  $C_2$  rather than  $C_0$ , may be limited for patients on cyclosporine maintenance therapy.

## INTRODUCTION

The introduction of cyclosporine in the early 1980s resulted in a significant improvement in the results of solid organ transplantation.<sup>1</sup> However, the clinical use of cyclosporine is complex, due to its narrow therapeutic index, many drug interactions and highly variable pharmacokinetics.<sup>2</sup> Moreover, cyclosporine has numerous side effects, such as nephrotoxicity, hypertension, hypercholesterolemia and the induction of glucose intolerance.<sup>1</sup> Most transplantation centers have adopted the strategy of monitoring cyclosporine using whole blood, predose or trough concentration ( $C_0$ ) measurements and adjusting the cyclosporine dose to reach a certain predefined  $C_0$  target range, the limits of which may differ, depending on the organ transplanted and the time since transplantation.<sup>3</sup>

The clinical utility of this approach suffers from the fact that, in *de novo* transplant recipients, the cyclosporine  $C_0$  does not predict total drug exposure over a 12 h or 24 h time period [as measured by the area-under the cyclosporine concentration *versus* time-curve (AUC)] at the individual level and does not correlate well with clinical outcome.<sup>4-8</sup> This is explained by the highly variable first-pass metabolism of cyclosporine that occurs mostly during the first 4 h following oral administration of the drug.<sup>9</sup> Therefore, a potential risk of the monitoring of cyclosporine using  $C_0$  is that low drug exposure may not be detected, possibly resulting in under-immunosuppression and the risk of acute rejection.

Likewise, high cyclosporine exposure may go unnoticed, resulting in (long-term) toxicity. Some have, therefore, advocated the use of an abbreviated AUC instead of the  $C_0$  to monitor cyclosporine therapy. The cyclosporine AUC in the first 4 h after oral administration ( $AUC_{0-4}$ ) has been shown to correlate well with the  $AUC_{0-12}$  and to predict clinical outcome after kidney transplantation.<sup>5,6,10</sup> Because the determination of an  $AUC_{0-4}$  is time consuming, expensive and not practicable for use in an outpatient clinic, there has been continuing interest in simpler parameters for therapeutic drug monitoring (TDM) of cyclosporine. The whole-blood cyclosporine concentration 2 h after administration of the drug ( $C_2$ ) was shown to be the single time point with the best correlation with total drug exposure.<sup>6,9,11</sup>

Subsequently,  $C_2$  monitoring has been used for TDM in several clinical trials and has, generally, resulted in a low or decreased incidence of acute rejection and excellent (renal) tolerability when compared with  $C_0$  monitoring.<sup>12-16</sup> Following the outcomes of these trials, target values for  $C_2$  have been identified. Currently, the recommended  $C_2$  values for liver and kidney transplant recipients more than 6 months after transplantation are 600 ng/mL  $\pm$  20% and 800 ng/mL  $\pm$  20%, respectively.<sup>9,11,16</sup>  $C_2$  target levels have not yet been established for heart transplant recipients. The measuring of  $C_2$  concentrations, however, does require a considerable effort to reliably draw blood at exactly the correct time point. Because of the practical limitations of this approach, many transplantation centers have not changed their policy of performing TDM on the basis of cyclosporine trough levels.

Although the correlation between acute rejection and nephrotoxicity and cyclosporine exposure as measured by an  $AUC_{0-4}$  or  $C_2$  has been established in *de novo* transplant recipients, the relation between drug exposure and other cyclosporine-related side effects is less clearly defined, especially in patients on long-term cyclosporine treatment. We feel that this is very

important because these other cyclosporine-related side effects negatively influence patient survival, quality of life and the long-term outcome after transplantation. Moreover, the occurrence of cyclosporine-related side effects may lead to patient non-compliance, with the risk of acute rejection. In our center we routinely measure cyclosporine  $C_0$  after kidney, liver and heart transplantation. We do acknowledge that individual patients may suffer from side effects that seem to be cyclosporine related, although predose concentrations are within, or even at the lower end of, the defined target range. Possibly, the use of another method for TDM, *i.e.* an  $AUC_{0-4}$  or  $C_2$ , would recognize the increased exposure to cyclosporine in these patients.

The aim of this study was twofold. First we investigated whether solid organ allograft recipients with cyclosporine-related side effects on maintenance therapy with cyclosporine and with cyclosporine  $C_0$  within the therapeutic range, had a higher exposure to cyclosporine than did a control group of transplant recipients, at similar cyclosporine  $C_0$  but without cyclosporine-related side effects. We therefore measured the cyclosporine  $AUC_{0-4}$  in 103 liver, kidney and heart allograft recipients more than 6 months after transplantation. Second we determined how many of those patients had  $C_2$  levels above the currently recommended target ranges.

## MATERIALS AND METHODS

### PATIENTS

During routine outpatient clinical visits, all patients who had received a heart, kidney or liver transplant at the Erasmus Medical Center in the Netherlands were asked to participate in the study. Patients had to have been on cyclosporine treatment, for at least 3 months without changes in cyclosporine dosage, during the 3 months before entry into the study. All patients used the cyclosporine microemulsion formulation (Neoral®, Novartis) twice daily in two equally divided doses. Patients taking medication known to interact with cyclosporine, such as the calcium-channel blockers diltiazem, nifedipine or verapamil, anti-epileptics (phenytoin and carbamazepine), antimycotics (fluconazole and ketoconazole) and macrolide antibiotics (erythromycin and clarithromycin), were not included in the study.

On the day of the pharmacokinetic study, patients were (physically) examined for the presence of renal insufficiency (serum creatinine  $\geq 125$   $\mu\text{mol/L}$  in liver and heart transplant recipients; not determined in kidney transplant recipients), hypertension (blood pressure  $\geq 150/100$  mmHg or the use of antihypertensive medication), hypercholesterolemia (total serum cholesterol  $>7.5$  mmol/L or the need for lipid-lowering drugs that was not present prior to transplantation), gum hyperplasia, hirsutism and hypertrichosis, polyneuropathy or tremor of the hands (not caused by diabetes mellitus or otherwise explained by co-medication such as theophyllin or sympathicomimetics), diabetes mellitus (defined by the need for glucose-lowering drugs that was not present before transplantation) and (post-transplantation) gout. If any of these symptoms was present, on the day of the study as well as during the 3-month period before entry into the study (determined by history taking



and patient chart review), patients were classified as having cyclosporine-related side effects. As a control group we selected solid organ allograft recipients who exhibited none of the above-mentioned cyclosporine-related side effects. The study was carried out in accordance with the declaration of Helsinki and was approved by the ethics committee of the Erasmus Medical Center. All patients gave written informed consent.

### **CYCLOSPORINE AUC<sub>0-4</sub> MEASUREMENT**

On the day of the AUC<sub>0-4</sub> measurement an intravenous cannula was inserted and maintained with 0.9% NaCl solution. After the C<sub>0</sub> whole-blood sample had been drawn, patients were asked to take their cyclosporine. Following cyclosporine administration, blood was drawn at 1, 2, 3 and 4 h. All patients had been instructed to take their regular cyclosporine dose 12 h before, on the previous day. The blood samples were frozen and stored at -30°C until the cyclosporine concentration was determined. Cyclosporine concentrations were determined by Emit 2000 assay (Syva, Dade Behring, Cupertino, CA, USA) on a Cobas Mira Plus analyzer (Roche). We used the trapezoidal rule to calculate the AUC<sub>0-4</sub>. The peak cyclosporine concentration (C<sub>max</sub>) and the time to peak cyclosporine concentration (t<sub>max</sub>) were obtained directly from the data.

### **STATISTICAL ANALYSIS**

We used Student's unpaired *t*-test, Fisher's exact test with Yates' continuity correction or one-way ANOVA followed by Tukey's post-hoc test, as appropriate, to compare pharmacokinetic parameters. For correlation analysis, we calculated Pearson's correlation coefficient, followed by linear regression and used Fisher's Z-transformation to compare correlation coefficients. Unless stated otherwise, data are presented as means ± SD. A *P* value of less than 0.05 was considered statistically significant.

## RESULTS

### GENERAL DATA

A total number of 103 patients was included, of whom 49 had received a liver transplant, 28 a heart transplant and 26 patients a kidney transplant. The mean age at the time of transplantation was  $45.7 \pm 11.4$  years for liver transplant recipients,  $45.7 \pm 14.3$  years for heart transplant recipients and  $46.2 \pm 14.4$  years for kidney transplant recipients and was not different between the three groups ( $P = 0.99$ , one-way ANOVA). Time after transplantation was comparable between the three groups, with a mean follow-up time of  $6.3 \pm 3.1$  years for liver transplant recipients,  $6.7 \pm 3.7$  years for heart transplant recipients and  $7.2 \pm 5.9$  years for kidney transplant recipients ( $P = 0.63$ ). The other patient characteristics are summarized in Table 2.1.1.

**Table 2.1.1** Characteristics of 103 solid organ transplant recipients.  
All values are expressed as means  $\pm$  SD

Characteristic	Liver	Heart	Kidney
Number of patients	49	28	26
Male / female sex (n)	20/29	22/6	17/9
Age at time of transplantation (years)	$45.7 \pm 11.4$	$45.7 \pm 14.3$	$46.2 \pm 14.4$
Range (years)	19 - 64	14 - 66	13 - 71
Time since transplantation (years)	$6.3 \pm 3.1$	$6.7 \pm 3.7$	$7.2 \pm 5.9$
Range (years)	1.4 - 12.6	0.9 - 15.3	1.6 - 26
Underlying disease			
Primary sclerosing cholangitis	8		
Primary biliary cirrhosis	10		
Hepatitis B	8		
Hepatitis C	2		
Alcohol-induced liver cirrhosis	5		
Acute or toxic liver failure	7		
Unknown cause	5		
Other	4		
Ischemic heart disease		13	
Cardiomyopathy		14	
Congenital heart disease		1	
Hypertensive nephropathy			6
Polycystic kidney disease			4
Diabetic nephropathy			1
Glomerulonephritis			2
Unknown cause			8
Other			5

## CYCLOSPORINE PHARMACOKINETICS

Daily cyclosporine dose was comparable between all three groups:  $216 \pm 80$  versus  $229 \pm 76$  versus  $238 \pm 61$  mg/day for liver, heart and kidney allograft recipients, respectively ( $P = 0.44$ ; Table 2.1.2). Cyclosporine dose, calculated on a mg per kg bodyweight basis, was equal in all three groups as well:  $2.9 \pm 1.2$  versus  $2.9 \pm 1.0$  versus  $3.1 \pm 0.9$  mg/kg/day for liver, heart and kidney allograft recipients, respectively ( $P = 0.79$ ; Table 2.1.2). However, liver transplant recipients were maintained at significantly lower cyclosporine predose concentrations than were kidney transplant recipients:  $115 \pm 46$  versus  $144 \pm 50$  ng/mL ( $P < 0.05$ ). The mean cyclosporine  $C_0$  of heart transplant recipients was not significantly different from those observed in either liver or kidney allograft recipients. In addition, cyclosporine  $C_1$ ,  $C_2$ ,  $C_3$ ,  $AUC_{0-4}$  and  $C_{max}$  were all significantly lower in liver transplant recipients compared with kidney allograft recipients, but not heart transplant recipients (Table 2.1.2). As illustrated in Figure 2.1.1,  $C_0$  correlated fairly with  $AUC_{0-4}$  and numerically less well with  $C_2$ ; Pearson's  $r$  ( $r^2$ ) 0.77 and 0.72 (0.59 and 0.51), respectively ( $P < 0.0005$ ). However, this difference was not statistically significant ( $P = 0.42$ , comparison of Pearson's correlation coefficients using Fisher's Z-transformation). The correlation between  $C_2$  and  $AUC_{0-4}$  was strong, with an  $r$  ( $r^2$ ) of 0.92 (0.85;  $P < 0.0005$ ). The difference in correlation of  $C_0$  and  $C_2$  with  $AUC_{0-4}$  was statistically significant ( $P < 0.0005$ ).

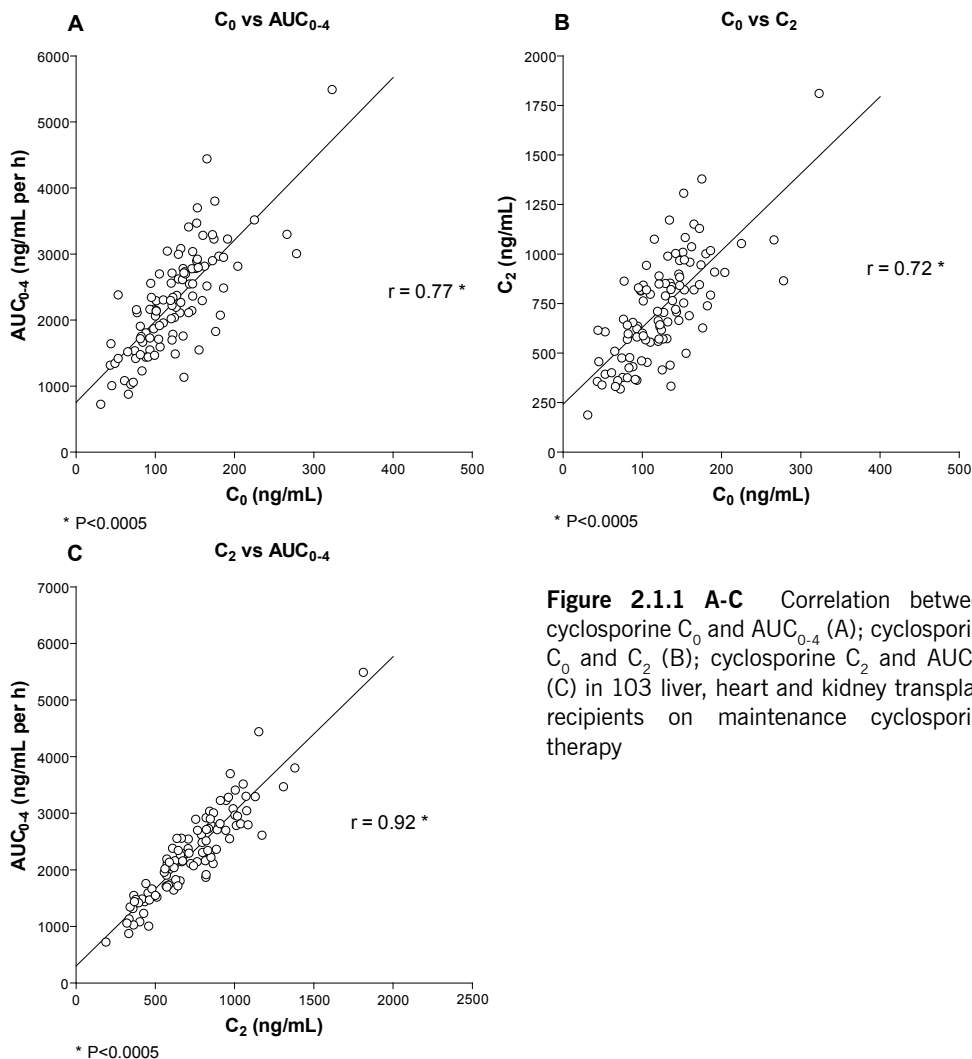
**Table 2.1.2** Cyclosporine (CsA) dosage and pharmacokinetics in three groups of solid organ transplant recipients. All values are expressed as means  $\pm$  SD. NS not significant

Transplantation type	Liver	Heart	Kidney	$P$ §
Number of patients	49	28	26	
CsA dose (mg/day)	$216 \pm 80$	$229 \pm 76$	$238 \pm 61$	NS
CsA dose (mg/kg per day)	$2.9 \pm 1.2$	$2.9 \pm 1.0$	$3.1 \pm 0.9$	NS
CsA $C_0$ (ng/mL)	$115 \pm 46$	$122 \pm 50$	$144 \pm 50$	$< 0.05$
CsA $C_1$ (ng/mL)	$754 \pm 350$	$926 \pm 352$	$1014 \pm 452$	$< 0.05$
CsA $C_2$ (ng/mL)	$641 \pm 248$	$729 \pm 290$	$884 \pm 191$	$< 0.001$
CsA $C_3$ (ng/mL)	$434 \pm 202$	$449 \pm 194$	$556 \pm 160$	$< 0.05$
CsA $C_4$ (ng/mL)	$305 \pm 143$	$312 \pm 140$	$382 \pm 112$	NS
CsA $AUC_{0-4}$ (ng/mL per h)	$2039 \pm 727$	$2321 \pm 827$	$2718 \pm 671$	$< 0.01$
$C_{max}$ (ng/mL)	$833 \pm 319$	$975 \pm 303$	$1116 \pm 365$	$< 0.01$
$t_{max}$ (h)	$1.4 \pm 0.6$	$1.3 \pm 0.4$	$1.4 \pm 0.5$	NS

§  $P$  values indicate differences in pharmacokinetic parameters between liver transplant recipients and kidney transplant recipients (one-way ANOVA with Tukey's post-hoc test).

## CYCLOSPORINE PHARMACOKINETICS AND CYCLOSPORINE-RELATED SIDE EFFECTS

Of the 49 liver transplant recipients included in the study, 30 patients (61.2%) had cyclosporine-related side effects, whereas 19 patients (38.8%) had none. Hypertension was present in 25 of the 30 patients with side effects (51.0%), renal insufficiency in 22 patients (44.9%) and hypertrichosis/hirsutism in 21 patients (42.9%). Gingival hyperplasia was found in 11 patients (22.4 %) and tremor of the hands and hypercholesterolemia were each found in seven patients (14.3%). No liver transplant recipients with gout were identified. Although there was an overall trend towards lower cyclosporine concentrations in patients without side effects as compared with the group of patients with cyclosporine toxicity, none of these differences reached statistical significance (Table 2.1.3).



**Figure 2.1.1 A-C** Correlation between cyclosporine  $C_0$  and  $AUC_{0-4}$  (A); cyclosporine  $C_0$  and  $C_2$  (B); cyclosporine  $C_2$  and  $AUC_{0-4}$  (C) in 103 liver, heart and kidney transplant recipients on maintenance cyclosporine therapy

	Liver			Heart			Kidney		
	Side effects (n = 30)	No side effects (n = 19)	P	Side effects (n = 20)	No side effects (n = 8)	P	Side effects (n = 14)	No side effects (n = 12)	P
CsA dose (mg/day)	225 ± 92	201 ± 56	0.27	230 ± 83	225 ± 60	0.88	236 ± 60	242 ± 63	0.81
CsA dose (mg/kg per day)	3.0 ± 1.4	2.7 ± 0.8	0.32	2.9 ± 1.1	3.0 ± 1.0	0.87	3.0 ± 1.0	3.1 ± 0.7	0.85
CsA C <sub>0</sub> (ng/mL)	121 ± 48	107 ± 43	0.33	122 ± 57	124 ± 25	0.90	147 ± 50	141 ± 51	0.74
CsA C <sub>1</sub> (ng/mL)	814 ± 338	659 ± 357	0.13	943 ± 384	883 ± 273	0.69	1072 ± 488	947 ± 416	0.49
CsA C <sub>2</sub> (ng/mL)	678 ± 246	582 ± 244	0.19	722 ± 318	747 ± 225	0.85	899 ± 208	868 ± 178	0.69
CsA C <sub>3</sub> (ng/mL)	454 ± 215	403 ± 182	0.40	448 ± 217	451 ± 129	0.98	554 ± 170	559 ± 155	0.94
CsA C <sub>4</sub> (ng/mL)	322 ± 160	277 ± 108	0.29	311 ± 159	316 ± 83	0.94	376 ± 106	388 ± 122	0.79
CsA AUC <sub>0-4</sub> (ng/mL per h)	2167 ± 710	1837 ± 725	0.12	2329 ± 943	2300 ± 471	0.94	2786 ± 751	2638 ± 587	0.59
C <sub>max</sub> (ng/mL)	895 ± 303	735 ± 328	0.09	977 ± 331	969 ± 236	0.95	1147 ± 428	1080 ± 288	0.65
t <sub>max</sub> (h)	1.4 ± 0.6	1.5 ± 0.6	0.46	1.3 ± 0.4	1.3 ± 0.5	1.00	1.4 ± 0.5	1.4 ± 0.5	0.81

**Table 2.1.3** Cyclosporine (CsA)-related side effects and cyclosporine pharmacokinetics. No statistically significant differences in any of the cyclosporine pharmacokinetic parameters were observed between patients with and patients without side effects in the three groups. All values are expressed as means ± SD

Transplantation type	PK parameter	Gingival hyperplasia		Hypertrichosis/hirsutism		Tremor/polynuropathy		Hypercholesterolemia	
		Yes	No	Yes	No	Yes	No	Yes	No
Liver	n	11	38	21	22	7	22	7	42
	CsA dose (mg/kg per day)	3.6 ± 1.5	2.7 ± 1.0 <sup>§</sup>	3.3 ± 1.5	2.6 ± 0.8 <sup>§</sup>	3.4 ± 1.6	2.8 ± 1.1	3.2 ± 1.8	2.8 ± 1.1
	CsA C <sub>0</sub> (ng/mL)	128 ± 46	112 ± 46	124 ± 50	109 ± 42	125 ± 49	114 ± 46	122 ± 57	114 ± 45
	CsA C <sub>2</sub> (ng/mL)	775 ± 259	602 ± 233 <sup>§</sup>	705 ± 252	592 ± 237	746 ± 190	623 ± 253	691 ± 229	632 ± 252
	CsA AUC <sub>0-4</sub> (ng/mL per h)	2514 ± 727	1901 ± 676 <sup>§</sup>	2251 ± 734	1879 ± 692	2443 ± 614	1971 ± 729	2299 ± 753	1996 ± 723
Heart	C <sub>max</sub> (ng/mL)	1054 ± 340	769 ± 287 <sup>†</sup>	936 ± 325	755 ± 298 <sup>§</sup>	1042 ± 315	798 ± 310	1018 ± 336	802 ± 310
	n	9	19	18	10	10	18	12	16
	CsA dose (mg/kg per day)	3.3 ± 1.2	2.8 ± 0.9	3.0 ± 1.0	2.8 ± 1.0	3.3 ± 1.2	2.7 ± 0.9	3.0 ± 1.1	2.9 ± 1.0
	CsA C <sub>0</sub> (ng/mL)	145 ± 75	112 ± 29	126 ± 59	117 ± 29	128 ± 71	119 ± 34	133 ± 69	115 ± 28
	CsA C <sub>2</sub> (ng/mL)	858 ± 407	668 ± 201	749 ± 324	694 ± 228	771 ± 394	706 ± 224	798 ± 387	678 ± 188
Kidney	CsA AUC <sub>0-4</sub> (ng/mL per h)	2744 ± 1178	2121 ± 526	2415 ± 958	2152 ± 520	2478 ± 1143	2234 ± 610	2600 ± 1095	2112 ± 494
	C <sub>max</sub> (ng/mL)	1098 ± 374	916 ± 253	1014 ± 328	904 ± 251	1014 ± 357	953 ± 277	1082 ± 345	894 ± 248
	n	11	15	11	15	7	19	4	22
	CsA dose (mg/kg per day)	3.1 ± 1.1	3.1 ± 0.7	3.0 ± 0.8	3.1 ± 1.0	3.6 ± 1.0	2.8 ± 0.7 <sup>§</sup>	3.1 ± 0.8	3.1 ± 0.9
	CsA C <sub>0</sub> (ng/mL)	142 ± 52	146 ± 49	152 ± 55	139 ± 47	157 ± 61	140 ± 46	155 ± 24	142 ± 53
	CsA C <sub>2</sub> (ng/mL)	884 ± 148	885 ± 223	907 ± 204	868 ± 187	1017 ± 201	835 ± 167 <sup>§</sup>	1086 ± 233	848 ± 163 <sup>§</sup>
	CsA AUC <sub>0-4</sub> (ng/mL per h)	2789 ± 715	2665 ± 657	2816 ± 764	2646 ± 612	3061 ± 810	2591 ± 587	3440 ± 938	2587 ± 542 <sup>§</sup>
	C <sub>max</sub> (ng/mL)	1187 ± 452	1064 ± 290	1163 ± 450	1082 ± 300	1233 ± 495	1073 ± 309	1500 ± 582	1046 ± 277 <sup>§</sup>

§ P<0.05; † P<0.01

**Table 2.1.4**  
Cyclosporine (CsA)-related side effects and CsA pharmacokinetics in liver, heart and kidney transplant recipients. All values are expressed as means ± SD

Next, we compared cyclosporine exposure in liver transplant patients suffering from an individual side effect with that in patients who did not have that particular side effect (Table 2.1.4). Patients with gingival hyperplasia used significantly more cyclosporine than did with patients who did not have gingival hyperplasia:  $3.6 \pm 1.5$  versus  $2.7 \pm 1.0$  mg/kg per day, respectively ( $P = 0.025$ ). As a result, cyclosporine exposure ( $C_2$ ,  $C_{\max}$  and  $AUC_{0-4}$ ) was also significantly higher in patients with gingival hyperplasia (Table 2.1.4). Likewise, patients with hypertrichosis or hirsutism used significantly more cyclosporine than those patients with no excessive hair growth:  $3.3 \pm 1.5$  versus  $2.6 \pm 0.8$  mg/kg per day, respectively ( $P = 0.045$ ). This difference was reflected by a higher cyclosporine  $C_{\max}$  in the former patient group:  $936 \pm 325$  versus  $755 \pm 298$  ng/mL ( $P = 0.049$ ). For all other side effects studied (including nephrotoxicity and hypertension, data not shown), cyclosporine exposure in liver transplant recipients with a specific side effect was not statistically, significantly different from patients who did not have that side effect (Table 2.1.4).

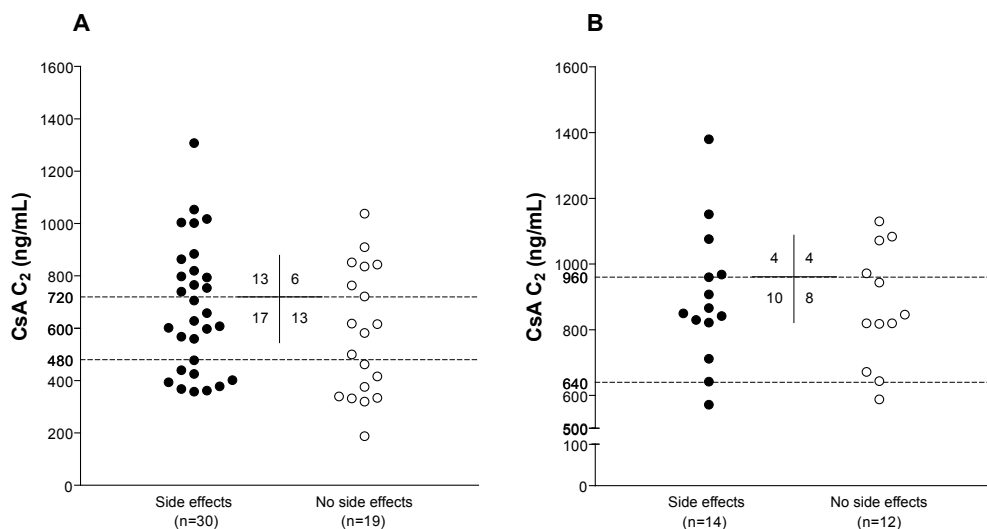
Of the 28 heart transplant recipients included in the study, 20 were identified as having side effects. Hypertension and hypertrichosis/hirsutism were the most common, each present in 18 (64.3%) patients, followed by hypercholesterolemia (12 patients; 42.9%), renal insufficiency (11 patients; 39.3%), tremor/polyneuropathy (10 patients; 35.7%) and gingival hyperplasia (nine patients; 32.1%). When cyclosporine pharmacokinetics were compared between heart transplant recipients with, and those without, any side effects, no significant differences were observed in any pharmacokinetic parameter (Table 2.1.3). The pharmacokinetics of the patients suffering from individual side effects (including nephrotoxicity, hypertension and gout) were not different from those of patients without those individual side effects (Table 2.1.4).

Finally, cyclosporine-related side effects were identified in 14 of the 26 renal transplant recipients included in the study. Hypertrichosis/hirsutism and gingival hyperplasia were the most frequently observed side effects and were each present in 11 patients (42.3%). Tremor or polyneuropathy was identified in seven patients (26.9%) and hypertension and hypercholesterolemia were present in four patients (15.4%); one patient (3.8%) suffered from gout. Because renal insufficiency in kidney transplant recipients is often determined by many factors and difficult to distinguish from cyclosporine nephrotoxicity (especially in the absence of a kidney biopsy), this side effect was not studied in this patient group. However, all patients that were classified as having no side effects did not show any clinical evidence for the presence of cyclosporine nephrotoxicity. As in liver and heart transplant recipients, no significant differences in any of the cyclosporine pharmacokinetic parameters were observed between the groups of patients with and without cyclosporine side effects (Table 2.1.3). When individual side effects were analyzed, we observed a higher cyclosporine dose and cyclosporine  $C_{\max}$  in patients with neurotoxicity than in patients who did not have tremor or polyneuropathy:  $3.6 \pm 1.0$  versus  $2.8 \pm 0.7$  mg/kg per day ( $P = 0.036$ ), and  $1017 \pm 201$  versus  $835 \pm 167$  ng/mL ( $P = 0.028$ ), respectively. In addition, the four patients with hypercholesterolemia had a higher cyclosporine exposure than those patients with normal serum cholesterol levels (Table 2.1.4). Cyclosporine exposure in patients with gingival hyperplasia, hypertrichosis/hirsutism, hypertension or gout was comparable to that in patients without those specific side effects (Table 2.1.4).

### CYCLOSPORINE-RELATED SIDE EFFECTS AND C<sub>2</sub>

Of the 49 liver transplant recipients, 19 patients (38.8%), had a C<sub>2</sub> value above the recommended target range of 600 ng/mL ± 20%. C<sub>2</sub> was below this target range in 16 (32.7%) liver transplant recipients (Figure 2.1.2A). The percentage patients with C<sub>2</sub> levels above target was not different between the group of liver transplant recipients with or without cyclosporine-related side effects ( $P = 0.55$ , Fisher's exact test with Yates' continuity correction). Of the 26 kidney transplant recipients, eight patients (30.8%) had a C<sub>2</sub> value above the recommended target value of 800 ng/mL ± 20%. The C<sub>2</sub> value was below this target range in two (7.7%) kidney transplant recipients (Figure 2.1.2B). Again, the number of patients with C<sub>2</sub> levels above target was not different between the group of kidney transplant recipients with or without cyclosporine-related side effects ( $P = 1.00$ ). Of the 28 heart transplant recipients, ten (35.7%) had a C<sub>2</sub> value above 600 ng/mL ± 20%. In four of those patients (14.3%) the C<sub>2</sub> value exceeded 800 ng/mL ± 20%.

**Figure 2.1.2 A-B** Cyclosporine (CsA) C<sub>2</sub> levels and CsA-related side effects in 49 liver (A) and 26 kidney (B) transplant recipients. The target level ± 20% range is indicated by *dotted lines*.





## DISCUSSION

The introduction of microemulsified cyclosporine and the publication of several clinical trials that compared the effectiveness of cyclosporine to tacrolimus, have led to a renewed interest in TDM and the pharmacokinetics of cyclosporine.<sup>17,18</sup> In recent years, both  $C_2$  and  $AUC_{0-4}$  have been demonstrated to be useful cyclosporine-monitoring tools that correlate well with the incidence of acute rejection and nephrotoxicity.<sup>5,6,10,12-15</sup> However, most of those studies were performed in *de novo* transplant recipients and did not relate cyclosporine pharmacokinetics to cyclosporine-related side effects other than renal insufficiency or hypertension.

The kidney transplantation program of the Erasmus Medical Center started in 1971 and was followed by the heart transplant program in 1984 and the liver transplant program in 1986. Since then, more than 1500 kidney, 400 heart and 350 liver transplantations have been carried out. For many years, cyclosporine was the calcineurin inhibitor of choice, but, in recent years we have switched to tacrolimus-based immunosuppressive regimens for our kidney and liver transplant recipients. However, many of our patients still use cyclosporine and often suffer from cyclosporine-related side effects. In the present study we therefore investigated the relationship between cyclosporine pharmacokinetics and cyclosporine-related side effects.

In our cohort of patients on cyclosporine maintenance therapy, cyclosporine pharmacokinetics did not correlate well with the presence of cyclosporine-related side effects. Although we did find an overall trend towards higher cyclosporine exposure in patients with (a specific) side effect(s), the majority of those differences did not reach statistical significance. In addition, the same cyclosporine exposure that was associated with the presence of a particular side effect in one type of transplant recipient was not related to the occurrence of that same side effect in patients who had had a different organ transplanted. In our opinion, this argues against a clear relationship between cyclosporine whole-blood concentrations and the presence of cyclosporine toxicity.

David-Neto *et al.* studied a pediatric kidney transplant cohort and found statistically significant correlations between cyclosporine pharmacokinetics and the occurrence of side effects.<sup>19</sup> An AUC greater than or equal to 4158 ng/mL predicted the presence of hypertrichosis, whereas a  $C_{max}$  greater than or equal to 878 ng/mL was the best predictor for the appearance of tremors. Gingival hyperplasia was not associated with any of the pharmacokinetic parameters studied.<sup>19</sup> Those results are not necessarily contradictory to our findings. The mean cyclosporine dose and  $AUC_{0-4}$  of our adult patients were much lower than those reported in the Brazilian pediatric study cohort. This could be explained by the fact that most of our patients were on long-term cyclosporine therapy and many of them had already undergone several cyclosporine dose reductions before the start of the study.

In many cases, the presence of side effects had been an important reason for cyclosporine dose reduction. Those previous dose reductions could have reduced a difference in cyclosporine exposure that might have existed between patients with or without side effects. As this was a cross-sectional study we do not have data on cyclosporine exposure at the time of emergence

of cyclosporine-related side-effects. Furthermore, a positive selection of the investigated patients might have occurred, as patients with severe side effects might have been switched to cyclosporine-free immunosuppressive regimens prior to the start of our study.

Our observations raise the question as to whether a further cyclosporine dose reduction in our population will result in a decrease in the incidence and severity of side effects. Cyclosporine exerts its immunosuppressive effect through inhibition of calcineurin (CN), an enzyme that is important for the activation of T cells. Several studies investigating the relationship between cyclosporine pharmacokinetics and CN inhibition demonstrated that, *in vivo*, CN is only partially inhibited. At  $C_0$  (ranging between 148 and 180 ng/mL), CN was inhibited by 50%, while cyclosporine peak concentrations (about 400 to 1800 ng/mL) resulted in around 70-80% CN inhibition.<sup>20-22</sup> Moreover, CN inhibition rarely reached 100% and was greater in some tissues due to drug accumulation.<sup>22</sup>

From these data it can be concluded that, at commonly used cyclosporine target levels, the maximal pharmacodynamic effect of the drug is obtained and that further increasing drug blood levels will probably result only in a high incidence of side effects and considerable drug toxicity. Therefore, many transplant patients probably receive too much cyclosporine and can undergo dose reduction, while the desired immunosuppressive effect of the drug is still maintained. More than one-third of our patients had  $C_2$  levels above currently recommended target ranges, and adaptation of  $C_2$  monitoring could result in (early) identification of cyclosporine “overexposure” and, subsequently, in (further) dose reductions. Levy *et al.* recently reported the results of conversion of liver transplant patients in the maintenance phase from  $C_0$  to  $C_2$  monitoring.<sup>23</sup> Of the 351 patients that were converted, 36% had  $C_2$  levels above the recommended target range. In those patients, a mean cyclosporine dose reduction of 16% was required to achieve target range, resulting in a significant improvement of renal function, blood pressure and serum cholesterol.<sup>23</sup>

Similar results have recently been reported for renal transplant recipients.<sup>24</sup> To study whether this approach will also lead to fewer (or less severe) side effects in our patient cohort, we are currently converting all liver transplant patients reported here to  $C_2$  level monitoring followed by dose reduction, if indicated. However,  $C_0$  correlated much better with  $AUC_{0-4}$  than has been reported previously.<sup>5,24</sup> Possibly, the difference in time after transplantation explains the difference between the results of Mahalati *et al.* and our own. Nonetheless, our results may indicate that the reported beneficial effects of  $C_2$  level monitoring might be limited for patients on cyclosporine maintenance therapy. Lowering currently used  $C_0$  target levels could result in a substantial cyclosporine dose reduction as well, without the logistic problems associated with (the implementation of)  $C_2$  level monitoring.

Alternatively, conversion of patients to tacrolimus is another possibility to decrease the incidence and severity of cyclosporine-related side effects. In 55 heart transplant recipients that were converted from cyclosporine to tacrolimus-based immunosuppressive therapy at our center, a significant improvement in blood pressure, serum cholesterol and gum hyperplasia, without signs of acute rejection, was observed, even in patients who were as long as 14 years after transplantation.<sup>25</sup>

In kidney transplant recipients, conversion to tacrolimus has been shown to be safe and to result in lower serum cholesterol levels with an improvement in gingival hyperplasia and hypertrichosis<sup>26,27</sup> and with an improved creatinine clearance in one study.<sup>27</sup> In a retrospective analysis of 94 liver transplant recipients, converted to tacrolimus for a variety of reasons, conversion resulted in a reduction of serum creatinine from  $167 \pm 36$  to  $119 \pm 28$  mmol/L (1 year after conversion).<sup>28</sup> Besides conversion to tacrolimus, complete cessation of cyclosporine is another possibility that has been studied. The results of the meta-analysis by Kasiske *et al.* demonstrate that discontinuation of cyclosporine results in an 11% higher risk for the development of acute rejection than in controls, in kidney transplantation.<sup>29</sup> However, the relative risk of graft failure was not significantly different from that of the control group.

In conclusion, we demonstrate no clear differences in cyclosporine exposure in solid organ transplant recipients with or without cyclosporine-related side effects. Cyclosporine  $C_2$  levels were above currently recommended target ranges in 38.8% of liver and 30.8% of kidney transplant recipients, but  $C_2$  levels above target were not more frequent in patients with side effects than in those with none. Cyclosporine dose reduction could be effective and safe in those patients. However, as the correlation between  $C_0$  and  $AUC_{0-4}$  was better than that previously reported, the advantage of  $C_2$  over conventional  $C_0$  level monitoring might be limited in patients on low or moderate dose cyclosporine maintenance therapy.

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## CHAPTER 2.2

# **CLINICAL OUTCOME AFTER CYCLOSPORINE DOSE REDUCTION BASED ON C<sub>2</sub> LEVELS IN LONG-TERM LIVER TRANSPLANT PATIENTS**

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## ABSTRACT

*Background* Recent studies suggest that cyclosporine dose adjustment based on  $C_2$  levels results in improvement of renal function. This study investigated the effect on renal function of dose reduction based on  $C_2$  levels in long-term liver transplant patients. *Methods* In 60 patients (>1 year after transplantation),  $C_2$  levels were assessed (target 600 ng/mL  $\pm$  20%). Dose reduction was performed if  $C_2$  exceeded 720 ng/mL. Serum creatinine concentrations were measured and creatinine clearance was calculated. *Results* Twenty-three patients (38%) had  $C_2$  values >720 ng/mL. After dose reduction, the mean cyclosporine dose decreased by 25% ( $P < 0.01$ ). The mean  $C_2$  value decreased by 42% ( $P < 0.01$ ). Serum creatinine concentrations remained stable. After dose reduction two patients experienced recurrence of primary biliary cirrhosis, in one patient autoimmune hepatitis recurred and rejection was diagnosed in one patient. *Conclusion* Cyclosporine  $C_2$  concentrations above 720 ng/mL are common in long-term liver transplant patients. Dose reduction of 25% did not improve kidney function and was accompanied by immune activation.



## INTRODUCTION

Renal dysfunction after transplantation is considered a problem for transplant recipients as it may progress towards end-stage renal failure requiring dialysis or renal transplantation.<sup>1,2</sup> The incidence of chronic renal failure is reported to be as high as 7% to 21% five years after transplantation of a non-renal organ. Forty-six percent of the patients in whom end-stage renal failure developed were placed on the waiting list for kidney transplantation.<sup>3</sup>

The incidence of chronic renal failure in liver transplant patients is 18% and seems to be higher compared with heart, lung and heart-lung transplant patients. Diabetes mellitus, hypertension and hepatitis C infection were independent risk factors for renal failure.<sup>3</sup>

Monitoring cyclosporine blood levels to avoid underdosing or toxicity is one of the essential issues in the follow-up of long-term liver transplant patients. Traditionally, cyclosporine dose is adjusted based on predose levels (C<sub>0</sub>). However, there is accumulating evidence that the cyclosporine concentration 2 h after administration (C<sub>2</sub>) is a more sensitive tool for optimizing cyclosporine dosing.<sup>4,5</sup> The C<sub>2</sub> level may be the most accurate predictor of the area-under the concentration *versus* time-curve (AUC) as a measure of total cyclosporine exposure.<sup>4</sup> Recent studies report a decreased incidence of acute rejection, as well as a decrease in cyclosporine-related side effects, in organ transplant patients by using C<sub>2</sub> monitoring instead of the conventional C<sub>0</sub> monitoring.<sup>6,7</sup> Dose reduction in overexposed patients according to C<sub>2</sub> levels is reported to result in improvements in renal function and blood pressure.<sup>8-10</sup>

So far, one study has examined the effects of dose adjustment based on C<sub>2</sub> levels in liver transplant patients more than 1 year post-transplantation.<sup>9</sup> At our center, the incidence of renal dysfunction shows an increase after onset of treatment with calcineurin inhibitors. In 177 patients we observed that 50.1% of the patients had a glomerular filtration rate below 60 mL/min 2 years after liver transplantation (unpublished results). This is then followed by a slow but continuing loss of renal function.

Therefore, we analyzed the effects of dose individualization by C<sub>2</sub> monitoring in liver transplant patients more than 1 year after transplantation. The aim of this study was twofold. First, we wanted to investigate the effects on renal function after dose reduction based on the C<sub>2</sub> levels in long-term liver transplant patients and secondly, to evaluate the possible risks of this dose reduction.

## PATIENTS AND METHODS

### PATIENTS

In February 2002, 60 stable liver transplant patients who received their first liver transplant between April 1988 and January 2001, were included in the study. The characteristics of the patients are presented in Table 2.2.1.

**Table 2.2.1** Patient characteristics

	All patients	Group 1 ( $C_2 \geq 720$ ng/mL)	Group 2 ( $C_2 < 720$ ng/mL)	<i>P</i>
Gender (male/female)	25/35	9/14	16/21	NS
Mean age at transplantation (year)	47.1 ± 11.6	46.7 ± 7.9	47.4 ± 13.6	NS
Mean time post-transplant (year)	6.0 ± 3.2	5.1 ± 2.8	6.5 ± 3.3	NS
Liver disease (HBV/HCV/ALD/PBC/other) (n)	10/3/5/25/17	3/0/2/11/7	7/3/3/14/10	NS
Conversion from Sandimmune to Neoral (n)	20	8	12	NS

HBV, hepatitis B; HCV, hepatitis C; ALD, alcohol-induced liver disease; PBC, primary biliary cirrhosis.

### INCLUSION CRITERIA

Patients with stable allograft function, who had received their transplant at least 1 year earlier and who used microemulsified cyclosporine as maintenance immunosuppressive drug, were eligible to participate in this study. Written informed consent was given by all participants.

### IMMUNOSUPPRESSION

Standardized immunosuppression protocols were used. All patients had previously been monitored by  $C_0$ . Cyclosporine was initiated within 24 h post-reperfusion and adopted to reach a  $C_0$  of between 200 and 400 ng/mL during the first 3 months after transplantation and between 100 and 200 ng/mL thereafter. The cyclosporine dose was adjusted in case of rejection or cyclosporine-related toxicity.

In September 1995, conversion from the oil-based cyclosporine formulation (Sandimmune; Novartis, Basle, Switzerland) to the microemulsion cyclosporine formulation (Neoral; Novartis) took place at our center. Before replacement of Sandimmune by Neoral, 20 patients were initially treated with Sandimmune. At the time of inclusion in this study, 38 patients (63.3%) were on Neoral monotherapy, 13 patients (21.7%) were treated with Neoral and prednisone, five patients (8.3%) were treated with Neoral, prednisone and azathioprine, two patients were treated with Neoral and azathioprine, one patient was treated with Neoral and mycophenolate mofetil (MMF) and one patient was treated with Neoral, prednisone and MMF.

## STUDY DESIGN

At the beginning of the study all 60 patients had their C<sub>0</sub> and C<sub>2</sub> levels assessed. For the purpose of this study, the target C<sub>0</sub> level was defined as 125 ng/mL ( $\pm$  20%). The target C<sub>2</sub> level was defined as 600 ng/mL ( $\pm$  20%).<sup>11</sup> Cyclosporine dose reduction was performed when the C<sub>2</sub> value exceeded 720 ng/mL using the formula: New dose = (old dose x 600) / actual C<sub>2</sub> level.<sup>12</sup> Cyclosporine dosage was left unaltered in patients whose C<sub>2</sub> levels were more than 20% below target (<480 ng/mL) in order to avoid cyclosporine overexposure in stable patients. Patients were divided into two subgroups based on whether or not they had cyclosporine dose reduction. Patients in group 1 had C<sub>2</sub> values above 720 ng/mL and the dose of cyclosporine was reduced. Patients in group 2 had C<sub>2</sub> values below 720 ng/mL and their dose of cyclosporine was not changed.

In order to assess changes in renal function, serum creatinine concentrations were collected 6 months before inclusion and measured at 2 weeks, 4 weeks, 8 weeks, 3 months and 6 months after cyclosporine dose reduction. Additionally, the creatinine clearance was calculated using the Modification of Diet in Renal Disease Study (MDRD) formula.<sup>13</sup> Clinical outcome was monitored by routine biochemical measurements at similar visits. Graft rejection and recurrence of liver disease were diagnosed by increased levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and bilirubin and were confirmed by histological examination of a liver biopsy.

## CYCLOSPORINE CONCENTRATION MEASUREMENT

After drawing the predose whole-blood sample, patients were asked to take their cyclosporine. Then, blood was drawn 2 h  $\pm$  15 minutes after cyclosporine intake. After collection of the blood samples, cyclosporine concentrations were determined using the Emit 2000 assay (Syva company, Dade Behring inc., Cupertino, CA) on a Cobas Mira Plus analyzer (Roche diagnostic systems).

## STATISTICAL ANALYSIS

Statistical analysis was conducted using the SPSS statistics software SPSS/PC 11.1. The paired *t*-test was used to test the difference between 6 months follow-up and start (*t* = 0) of the cyclosporine dose, C<sub>2</sub>, serum creatinine concentration and creatinine clearance separately for group 1 and in group 2. The Pearson's correlation coefficient between C<sub>0</sub> and C<sub>2</sub> was calculated. Differences in the distribution of gender, age at transplantation, time since transplantation, liver disease and conversion from Sandimmune to Neoral between group 1 and group 2 were assessed with the chi-square test for categorical variables and Student's *t*-test for continuous variables. *P* values smaller than 0.05 were considered statistically significant. All data are represented as means  $\pm$  standard deviation (SD).

## RESULTS

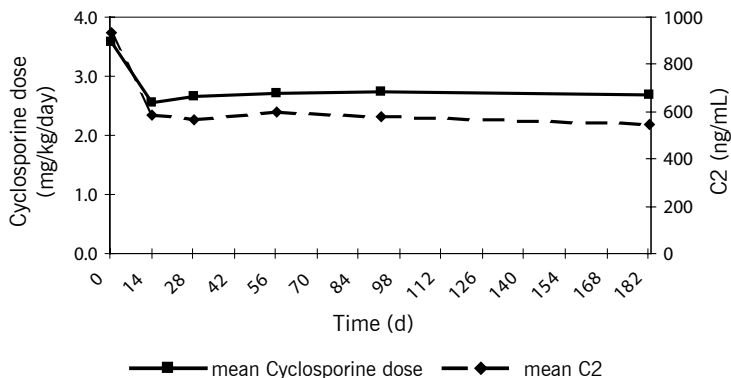
The mean cyclosporine  $C_0$  was  $122.1 \pm 54.9$  ng/mL and the mean  $C_2$  level was  $652.1 \pm 274.3$  ng/mL. There was a wide range of  $C_2$  values (188.0 - 1510.0 ng/mL). The correlation between  $C_0$  and  $C_2$  was weak ( $r = 0.59$ ). Twenty-three patients had  $C_2$  values above 720 ng/mL, 16 patients had  $C_2$  values within the target range of 480-720 ng/mL and 21 patients had  $C_2$  values below 480 ng/mL. Eleven of the 23 patients (48%) with high  $C_2$  had  $C_0$  values below or within the 100-150 ng/mL range (Table 2.2.2).

**Table 2.2.2**  $C_0$  and  $C_2$

$C_0 / C_2$	Low $C_2$ ( $\leq 480$ ng/mL)	Normal $C_2$ (480-720 ng/mL)	High $C_2$ ( $\geq 720$ ng/mL)	Total
Low $C_0$	15	5	1	21
Normal $C_0$	5	8	10	23
High $C_0$	1	3	12	16
Total	21	16	23	60

$C_0$ , cyclosporine predose concentration;  $C_2$ , cyclosporine concentration 2 h after administration.

**Figure 2.2.1** Cyclosporine dose and  $C_2$  level (group 1)



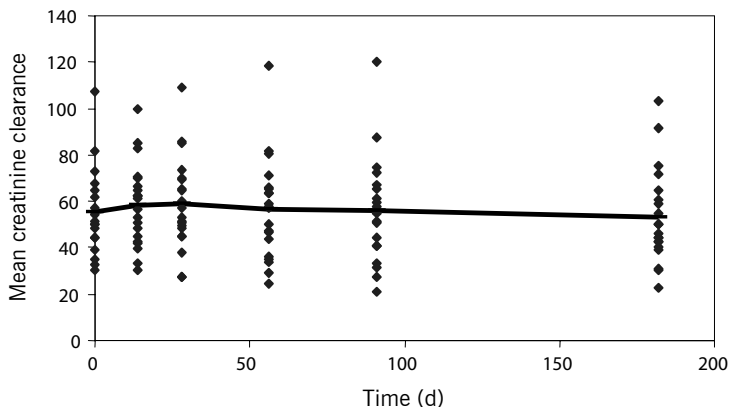
Accordingly, 23 patients with C<sub>2</sub> levels >720 ng/mL were selected for cyclosporine dose reduction. However, three of 23 patients did not agree with dose reduction and were not included in prospective part of the study. Dose reduction was performed in twenty patients (group 1). In 37 patients with the C<sub>2</sub> level within or below the desired target range, cyclosporine dosage was not reduced (group 2).

At 6 months follow-up, the mean cyclosporine dose was decreased from  $3.58 \pm 0.95$  mg/kg per day to  $2.69 \pm 0.91$  mg/k per day in group 1, representing a 25% reduction in cyclosporine dosage ( $P < 0.01$ ). The corresponding mean C<sub>2</sub> decreased from  $933.9 \pm 209.0$  ng/ml to  $545.3 \pm 228.3$  ng/mL ( $P < 0.01$ ), representing a 42% decrease, 6 months after dose reduction (Figure 2.2.1). Seventy-five percent of the patients were on target after 2 weeks.

**Table 2.2.3** Renal function, blood pressure and lipid levels before and after dose reduction

	t = 0	t = 6 months	P
Group 1 (C <sub>2</sub> ≥ 720 ng/mL; n = 20)			
Serum creatinine (mg/dL)	1.3 ± 0.4	1.4 ± 0.4	NS
Creatinine clearance (ml/min per 1.73m <sup>2</sup> )	55.2 ± 18.1	53.0 ± 19.1	NS
Systolic blood pressure (mmHg)	148 ± 20	144 ± 21	NS
Diastolic blood pressure (mmHg)	92 ± 8	83 ± 11	0.01
Total cholesterol (mmol/L)	5.3 ± 0.7	5.6 ± 1.6	NS
Triglycerides(mmol/L)	2.0 ± 1.1	1.9 ± 1.4	NS
Group 2 (C <sub>2</sub> < 720 ng/mL; n = 37)			
Serum creatinine (mg/dL)	1.4 ± 0.4	1.4 ± 0.4	NS
Creatinine clearance (ml/min per 1.73m <sup>2</sup> )	53.2 ± 20.2	53.1 ± 21.1	NS
Systolic blood pressure (mmHg)	143 ± 19	139 ± 16	NS
Diastolic blood pressure (mmHg)	85 ± 14	85 ± 9	NS
Total cholesterol (mmol/L)	5.4 ± 1.1	5.5 ± 1.5	NS
Triglycerides(mmol/L)	1.9 ± 0.6	1.9 ± 1.4	NS

**Figure 2.2.2** Mean creatinine clearance (group 1)



The mean creatinine concentration, creatinine clearance, systolic blood pressure and lipid level of group 1 remained stable during follow-up. None of the patients developed end-stage renal failure. Mean creatinine concentration 6 months before inclusion was measured and did not differ from the mean creatinine concentration at time of inclusion [6 months before inclusion:  $1.4 \pm 0.4$  mg/dL *versus* at time of inclusion:  $1.3 \pm 0.4$  mg/dL (NS)]. Mean diastolic blood pressure decreased significantly after cyclosporine dose reduction ( $P = 0.01$ ). In group 2, the renal function, blood pressure and lipid level did not change. Results of group 1 and 2 before and after follow-up are presented in Table 2.2.3. Mean creatinine clearance of group 1 during the 6 months follow-up is depicted in Figure 2.2.2.

In group 1, 1 patient (patient 1) experienced cellular rejection after 3 months follow-up, which was histologically classified as RAI 6. Corticosteroid pulse therapy was given as treatment and the cyclosporine dose was left unchanged. The  $C_2$  level at the time of diagnosis was 800 ng/mL. In 2 patients (patients 2 and 3) recurrence of primary biliary cirrhosis was diagnosed clinically at the week 8 and month 6 visit, respectively. The diagnosis was confirmed histologically. Both patients were treated with ursodeoxycholic acid and the cyclosporine dosage of patient 3 was increased. The  $C_2$  levels at the time of diagnosis were 555 ng/mL and 680 ng/mL, respectively. In a fourth patient (patient 4) autoimmune hepatitis recurred in week 8. Subsequently, the cyclosporine dosage was increased and allopurinol was given as additional treatment. In this patient, the  $C_2$  level at the time of diagnosis was 640 ng/mL.

Patients 1, 2 and 3 were on cyclosporine monotherapy and patient 4 was treated with cyclosporine and prednisolone at the time of diagnosis. Patients 1, 2, and 3 had a  $C_2$  value below the  $C_2$  target range at 1 visit. These events occurred before the diagnosis of rejection or recurrence. Patient 1 had a  $C_2$  level of 180 ng/mL at week 4, patient 2 had a  $C_2$  level of 470 ng/mL at week 4, and patient 3 had a  $C_2$  level of 395 ng/mL 8 weeks after dose reduction. In group 2, no rejection or recurrence of autoimmune liver disease was observed.

## DISCUSSION

This study of  $C_2$  monitoring in 60 stable liver transplant patients more than 1 year after transplantation shows that cyclosporine  $C_2$  concentrations above 720 ng/mL in this group of patients is common. Overexposure was observed in 23 of the 60 patients (38%). Eleven of the 23 patients (48%) had  $C_0$  values below or within target range, indicating the limitation of  $C_0$  monitoring. These findings confirm the role of  $C_2$  monitoring in detecting cyclosporine overexposure in long-term liver transplant patients.

In an earlier publication, Cole *et al.* reported a cyclosporine overexposure of 49% in renal transplant patients more than 3 months after transplantation.<sup>8</sup> In liver transplant patients, an overexposure of 68% was documented in patients more than 6 months after transplantation.<sup>14</sup> In 2 studies in stable renal transplant patients more than 1 year after transplantation, one described a  $C_2$  level exceeding 800 ng/mL in 29% of the patients, and the other observed a  $C_2$  above 850 ng/mL in 18% of the patients.<sup>15,16</sup> Our study showing an overexposure of 38% is in line with these findings.

Despite the linear relation in the used formula, a discrepancy was observed in our study between the cyclosporine dose and the measured C<sub>2</sub> level. In renal transplant patients, after a dose reduction of 27.5%, Cole *et al.* observed a 37.3% decrease in the mean C<sub>2</sub> value.<sup>8</sup> In liver transplant patients, Langers *et al.* reported that a 26.9 % dose reduction resulted in a 25.8% decrease in the mean C<sub>2</sub> value measured on day 2 after dose reduction.<sup>14</sup> We noticed that the discrepancy between the cyclosporine dose and the measured C<sub>2</sub> level in our study is considerable compared with the studies mentioned above. This may be because of high within-patient variability. Also differences in studied patient populations with the time post-transplantation and the use of co-medication differing between groups should be taken into consideration.

After dose reduction we observed a decline in mean diastolic blood pressure in group 1. This was in line with findings of previous studies in long-term renal, heart and liver transplant patients.<sup>8-10</sup> The mean serum creatinine concentration and mean creatinine clearance remained unchanged in our study. As the mean creatinine concentration 6 months before enrolment was comparable to that at the time of inclusion, we consider that there was no decline in slope of renal function in group 1 post-transplantation. Thus, the stabilized renal function during follow-up was not because of cyclosporine dose reduction. In contrast to other studies in non-renal transplant patients, we did not observe improvement in kidney function 6 months after cyclosporine dose reduction based on C<sub>2</sub> levels. In liver transplant patients, Langers *et al.* reported a significant improvement of 11.6% ( $P = 0.016$ ) in creatinine clearance at more than 6 months after transplantation (target C<sub>2</sub> was  $600 \pm 15\%$ ).<sup>14</sup> In a study by Cantarovich *et al.*, a 5.1% ( $P = 0.006$ ) decrease of the mean serum creatinine level was observed after cyclosporine dose reduction based on C<sub>2</sub> levels in liver transplant patients more than 1 year after transplantation. The target C<sub>2</sub> was between 300 and 600 ng/mL.<sup>9</sup> In a second study by Cantarovich *et al.* in heart transplant patients more than 1 year after transplantation, a 2.3% decrease of serum creatinine was reported after dose reduction based on C<sub>2</sub> levels, aiming at a range between 300 and 600 ng/mL.<sup>10</sup> The lack of improvement in renal function in our study can be related to the C<sub>2</sub> target range we used, which was higher than in the above-mentioned studies. Consistent with this idea, Cantarovich *et al.* reported an increase of the mean serum creatinine by 16% when a C<sub>2</sub> target level between 700 and 1000 ng/mL was used.<sup>9</sup> Therefore, the selected C<sub>2</sub> range is crucial in order to observe effects of dose adjustment based on C<sub>2</sub> levels performed in long-term liver transplant patients. Furthermore, our population had received a liver transplant at least 1 year before the enrolment. Consequently, irreversible damage to the kidney and its management by the reduction of cyclosporine dose already took place at the discretion of the attending hepatologist. The reversibility of cyclosporine nephrotoxicity is most likely to be more prominent early after transplantation. Cole *et al.* reported a significant decrease of serum creatinine levels in 54% of renal transplant patients at more than 3 months after transplantation by performing cyclosporine dose reduction based on C<sub>2</sub> levels.<sup>8</sup> Therefore, early introduction of dose adjustment based on C<sub>2</sub> is probably more effective.

However, during follow-up we observed a brief period of increase in mean creatinine clearance in the first 4 weeks after cyclosporine dose reduction. These improvements in renal function were temporary. We hypothesize that this event is an acute reaction of the kidney

to a lower cyclosporine dose, which cannot be maintained because of irreversible damage to the kidney. Therefore, we doubt the necessity of a longer follow-up.

It is noteworthy that cyclosporine dose reduction in long-term liver transplant patients may lead to allograft rejection and recurrence of autoimmune hepatitis or primary biliary cirrhosis. This might be attributable to a period of cyclosporine underexposure, which could not be confirmed, as the  $C_2$  levels at the time of diagnosis were within the target range. However, a short period of low immune suppression cannot be ruled out. In addition, patients using no or a small amount of co-medication are probably susceptible to experience complications of low immune suppression. Fluctuation of  $C_2$  levels during follow-up may easily cause immune activation as the patients are using only cyclosporine or cyclosporine plus prednisone as immunosuppressive drugs. Therefore, the use of co-medication should be taken into consideration when cyclosporine dose adjustment is performed. When a patient is on cyclosporine monotherapy, dose reduction should be done with caution and it is probable that the target  $C_2$  level for this group of patients is higher than 600 ng/mL ( $\pm 20\%$ ).

So far, none of the related studies in stable renal transplant patients reported such side effects.<sup>8,16</sup> After late conversion from  $C_0$  to  $C_2$  monitoring of cyclosporine in pediatric living-donor liver transplant recipients, rejection was reported in one patient (1,7%).<sup>17</sup> In adult stable liver transplant patients, rejection was reported in two patients after dose reduction. The AUC in these patients was below target.<sup>14</sup> The authors performed cyclosporine dose reduction in liver transplant patients at more than 6 months after transplantation with 19.4% of the patients on cyclosporine monotherapy.<sup>14</sup> In our study 63.3% (38 of 60) of the patients (more than 1 year after transplantation) were on cyclosporine monotherapy and 6.7% (4 of 60) of the patients had complications of immune activation. The problem of under-immunosuppression after cyclosporine dose reduction based on  $C_2$  levels is expected to be more prominent in long-term transplant patients because the number of patients who are using no immunosuppressive co-medication increases with time after transplantation.

In conclusion, cyclosporine dose reduction based on  $C_2$  levels in liver transplant patients more than 1 year post-transplantation is less profitable than in earlier stages and renal function may not be influenced. We realize that the risks of rejection and recurrence of autoimmune liver disease are not to be underestimated, especially in patients on cyclosporine monotherapy. By aiming for a  $C_2$  target range of 600 ng/mL ( $\pm 20\%$ ) no benefit in renal function was observed after cyclosporine dose reduction in long-term liver transplant patients.



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PART 3

# **PHARMACOKINETIC STUDIES**



## CHAPTER 3.1

# **TACROLIMUS DOSE REQUIREMENT IN RENAL TRANSPLANT RECIPIENTS IS SIGNIFICANTLY HIGHER WHEN USED IN COMBINATION WITH CORTICOSTEROIDS.**

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## ABSTRACT

*Aims* To evaluate the effect of corticosteroids on tacrolimus pharmacokinetics. *Methods* In a randomized trial, kidney transplant recipients were treated with tacrolimus and mycophenolate mofetil with either daclizumab (n = 31) or 3 months of prednisone (n = 34). Tacrolimus dose-adjusted predose concentrations ( $C_0$ ) at month 1-6 were compared between both groups and within the corticosteroid group before and after prednisone withdrawal. *Results* At month 1 the tacrolimus dose-adjusted  $C_0$  in the corticosteroid group was  $83 \pm 8$  versus  $119 \pm 17$  ng/mL per mg/kg in the daclizumab group. The tacrolimus dose-adjusted  $C_0$  within the corticosteroid group at month 1 and 2 was 42% and 29% lower compared with month 4 ( $P < 0.001$ ). *Conclusion* A higher tacrolimus dose is required to reach target concentrations when used in combination with corticosteroids.

## INTRODUCTION

Tacrolimus is a standard immunosuppressive drug in many transplant centers.<sup>1</sup> However, tacrolimus has a narrow therapeutic index and its pharmacokinetics show considerable interindividual variation. Therapeutic drug monitoring of tacrolimus whole-blood, predose concentrations ( $C_0$ ) is recommended and target ranges have been defined.<sup>2</sup> Nevertheless, in everyday clinical practice new and sometimes life-threatening interactions with tacrolimus are encountered.<sup>3</sup> These interactions may result in increased or decreased drug concentrations or in altered pharmacodynamic effects of tacrolimus, and deserve further and continued attention.<sup>4,5</sup>

Tacrolimus is metabolized by cytochrome P450 (CYP) 3A in liver and intestinal mucosa.<sup>6</sup> Intestinal phase I metabolism by CYP3A and active efflux of absorbed drug by P-glycoprotein are major determinants of oral bioavailability, which can be influenced by concomitant administration of inhibitors/inducers of these enzymes.<sup>7</sup> Corticosteroids induce the CYP system, but it is unclear whether commonly used doses of these drugs have a clinically relevant effect on tacrolimus concentrations. Therefore we investigated whether corticosteroid use causes important changes in the pharmacokinetics of tacrolimus.

## METHODS

### PATIENTS

Sixty-five kidney transplant recipients, participating in a multicenter randomized clinical trial, were treated with tacrolimus and mycophenolate mofetil in combination with either daclizumab or corticosteroids. Tacrolimus was dosed twice daily to achieve  $C_0$  concentrations of 15-20 ng/mL during the first 14 days, 10-15 ng/mL between weeks 3 and 6, and 5-10 ng/mL thereafter. Mycophenolate mofetil (1000 mg twice daily) was started 2 days after transplantation. All patients received 100 mg prednisolone i.v. on the first 3 postoperative days. Thereafter only patients in the corticosteroid group were given prednisone orally (dosed to bodyweight), which was tapered and stopped at month 3 post-transplantation. Patients in the daclizumab group received 1 mg/kg of the drug on day 0 and day 10.

In all patients tacrolimus dose, tacrolimus  $C_0$ , bodyweight and serum creatinine were recorded at months 1, 2, 3, 4, 5 and 6. Dose-adjusted tacrolimus concentrations were calculated by dividing  $C_0$  by the corresponding tacrolimus dose (mg/kg). Patients did not take drugs known to interact with CYP3A or P-gp. Patients using the calcium-channel blockers nifedipine or amlodipine were included in the study, but patients using verapamil, nifedipine or diltiazem were not.

### ETHICS

The study was performed in accordance with the Declaration of Helsinki and its amendments. The protocol was approved by the Ethics Committee of the Erasmus Medical Center. Written informed consent was obtained from all subjects.

## DRUG CONCENTRATION MEASUREMENTS

Tacrolimus  $C_0$  were determined using the Emit 2000 assay (Syva Company, Dade Behring Inc., Cupertino, CA) on a Cobas Mira Plus analyzer (Roche Diagnostic Systems Inc.). In our laboratory, the coefficients of variation of Bio-Rad control samples with mean concentrations of 4.5, 11.8 and 24.1 ng/mL, were 10.7%, 5.2% and 5.4%, respectively. More details on the sensitivity, reproducibility and specificity of the tacrolimus assay in our laboratory have been published previously.<sup>8</sup> Proficiency samples were obtained from the United Kingdom Quality Assessment Scheme (Dr Holt, St George's Hospital Medical School, London, UK). The performance of the tacrolimus assay in our laboratory meets proficiency standards. Corticosteroid concentrations were not determined.

## STATISTICAL ANALYSIS

We used linear mixed effect (LME) models to compare tacrolimus dose and concentration between the two groups over 6 months, with patients as the random effect. Residuals were checked for normality. A Student's unpaired *t*-test was used for comparisons at each individual time point when overall comparisons based on the LME were significant. We used paired *t*-tests to test within group differences. Results are presented as means  $\pm$  SEM (95% CI). *P* values  $\leq 0.05$  were considered statistically significant.

## RESULTS

Of the 65 patients included, 31 were treated with daclizumab and 34 patients received prednisone for 3 months. All patients received mycophenolate mofetil and tacrolimus. Renal function, bodyweight and tacrolimus  $C_0$  were not significantly different between the two groups at any time point between months 1 and 6. The incidence of acute rejection was comparable: five *versus* three patients in the corticosteroid and daclizumab group, respectively ( $P = 0.71$ , Fisher's exact test). These patients received 1000 mg methylprednisolone on 3 consecutive days.

Tacrolimus dose-adjusted  $C_0$  was lower at month 1 in the corticosteroid group compared with the daclizumab group:  $83 \pm 8$  *versus*  $119 \pm 17$  ng/mL per mg/kg, respectively. Although this difference was also observed at months 2 and 3 (Figure 3.1.1A), the overall differences were not statistically significant (LME  $P = 0.06$ ). Within the corticosteroid group, significant differences in tacrolimus dose-adjusted  $C_0$  were observed before and after prednisone withdrawal. Thus, tacrolimus dose-adjusted  $C_0$  at months 1 and 2 was lower compared with months 4-6 ( $P = 0.001$  and  $P < 0.05$ , respectively; Figure 3.1.1A, Table 3.1.1).

When patients using nifedipine or amlodipine (nine patients in each treatment group) were excluded from the analysis, tacrolimus dose-adjusted  $C_0$  was again lower in the corticosteroid group compared with the daclizumab group [LME, 95% CI 15.6, 58.5,  $P < 0.001$ ; at month 1:  $83 \pm 9$  *versus*  $138 \pm 22$  ng/mL per mg/kg (95% CI -102, -7,  $P = 0.03$ ; Figure 3.1.1B)]. This difference in tacrolimus dose-adjusted  $C_0$  resulted from a significantly higher overall mean tacrolimus dose required for patients treated with corticosteroids compared



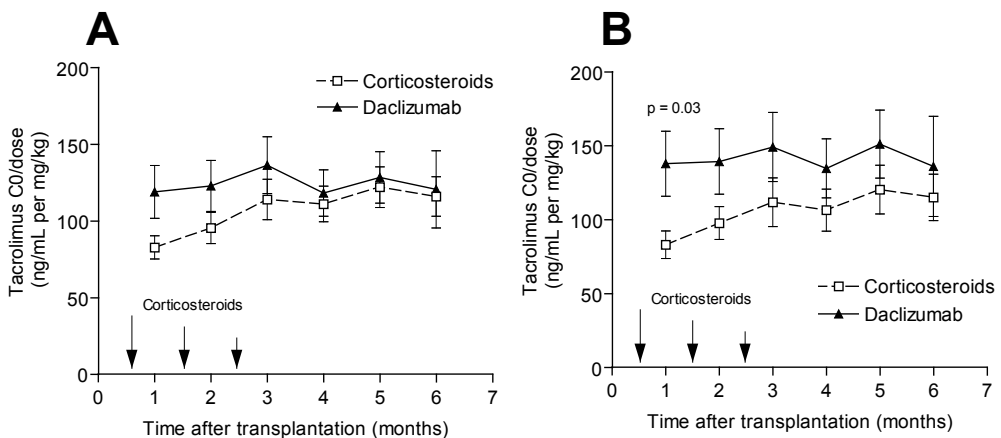
with patients treated with daclizumab [LME, 95% CI -3.4, -1.0,  $P < 0.001$ ]. At month 1 the mean tacrolimus dose was:  $13.4 \pm 1.2$  versus  $9.5 \pm 1.0$  mg/day (95% CI 0.8, 7.1,  $P = 0.015$ ). This difference was still present at month 2 ( $11.3 \pm 1.1$  versus  $7.6 \pm 1.0$  mg (95% CI 0.6, 6.6,  $P = 0.021$ ), and diminished thereafter. The same between-group difference existed for tacrolimus dose corrected for bodyweight (LME, 95% CI -0.01, -0.04,  $P < 0.001$ ). At month 1 the mean tacrolimus dose per kg bodyweight was:  $0.18 \pm 0.013$  versus  $0.13 \pm 0.014$  mg/kg (95% CI 0.01, 0.09,  $P = 0.013$ ) and at month 2 it was  $0.15 \pm 0.012$  versus  $0.10 \pm 0.013$  mg/kg (95% CI 0.01, 0.08,  $P = 0.021$ ). However, at month 2 the tacrolimus  $C_0$  was also higher in the corticosteroid group:  $11.6 \pm 0.9$  versus  $9.2 \pm 0.7$  ng/mL (95% CI 0.06, 4.6,  $P = 0.04$ ).

**Table 3.1.1** Mean % differences in tacrolimus dose-adjusted predose concentrations (ng/mL per mg/kg) for patients in the corticosteroid group ( $n = 34$ ), before (month 1-3) and after (month 4-6) prednisone withdrawal. Differences are expressed as absolute values and as percentage of the corresponding values before corticosteroid withdrawal.

Month	1	2	3
4	33.5 <sup>§</sup> (42%)	25.4 <sup>§</sup> (29%)	-2.6 (-2%)
5	44.3 <sup>§</sup> (54%)	33.4 <sup>†</sup> (37%)	7.3 (6%)
6	44.0 <sup>§</sup> (58%)	22.5 (25%)	1.8 (3%)

<sup>§</sup>  $P \leq 0.001$ , <sup>†</sup>  $P \leq 0.01$

**Figure 3.1.1** Mean tacrolimus dose-adjusted predose concentrations [ $C_0$ ; ng/mL per mg/kg ( $\pm$  SEM)] in all patients (A). At months 1-3 the tacrolimus dose-adjusted  $C_0$  is higher for patients treated with daclizumab [ $n = 31$  (closed triangles)] compared with patients treated with corticosteroids [ $n = 34$  (open squares)], but the overall difference between the two groups was not significantly different (LME  $P = 0.06$ ). Within the corticosteroid group, the tacrolimus dose-adjusted  $C_0$  is significantly lower before prednisone withdrawal (months 1 and 2) compared with after prednisone withdrawal (months 4-6). When patients using calcium-channel blockers were excluded (B), a lower tacrolimus dose-adjusted  $C_0$  was observed in patients treated with corticosteroids [ $n = 25$  (open squares)] compared with patients treated with daclizumab [ $n = 22$  (closed triangles)].



## DISCUSSION

We have demonstrated that for the same tacrolimus  $C_0$  value, a higher tacrolimus dose was required for renal transplant recipients who were concomitantly treated with corticosteroids, compared with patients treated with daclizumab. The difference in tacrolimus dose-adjusted  $C_0$  between the corticosteroid and daclizumab groups was maximal at month 1 (around 30%). It is questionable whether this statistical difference is clinically relevant. Interindividual variability in tacrolimus dose-adjusted  $C_0$  is large and could exceed the effect of corticosteroids. Moreover, renal function was the same in both groups and did not deteriorate after corticosteroid discontinuation. However, in individual patients the latter may occur.<sup>9</sup>

Another finding was the influence of nifedipine and amlodipine on tacrolimus concentrations. Verapamil, diltiazem and nicardipine are known to interact with cyclosporine, but nifedipine and amlodipine have no or only minor effects on cyclosporine pharmacokinetics.<sup>10-12</sup> Inhibition of tacrolimus metabolism has been demonstrated *in vitro* and in a retrospective analysis for nifedipine, but not amlodipine.<sup>13,14</sup> Interactions between calcineurin inhibitors and calcium-channel blockers are believed to result from competitive inhibition of CYP3A by the latter.<sup>6</sup> Because prednisone induces CYP3A, its effects on calcineurin inhibitor metabolism are opposite to those of calcium-channel blockers.<sup>7</sup> Our observation that the differences in tacrolimus dose and dose-adjusted  $C_0$  between the corticosteroid and daclizumab groups were larger when patients using calcium-channel blockers were excluded, could be explained by this phenomenon.

In conclusion, tacrolimus dose requirement was higher when used in combination with corticosteroids. The effects of corticosteroids on tacrolimus pharmacokinetics appeared smaller when patients were treated with calcium-channel blockers. Changes in prednisone dosage can result in altered tacrolimus  $C_0$ . Therefore monitoring of tacrolimus concentrations is necessary in corticosteroid weaning protocols.

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CHAPTER 3.2

**THE INFLUENCE OF  
CYCLOSPORINE ON  
MYCOPHENOLIC  
ACID PLASMA  
CONCENTRATIONS:  
A REVIEW**

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## ABSTRACT

If mycophenolate mofetil (MMF) treatment is combined with cyclosporine, mycophenolic acid (MPA) plasma concentrations decrease, mycophenolic acid glucuronide (MPAG) increases, and the second peak in the MPA pharmacokinetic profile disappears. This is presumed to be caused by a cyclosporine-induced inhibition of the biliary excretion of MPAG, probably at the level of one of the drug transporters in the apical (canalicular) membrane of the hepatocyte. The most likely candidate for this inhibitory effect is canalicular multiple organic anion transporter. In patients switched from cyclosporine therapy to tacrolimus, as a result of this switch with unchanged MMF dose, the MPA concentrations will increase. It is not impossible that, after patients are switched from cyclosporine to tacrolimus, suddenly patients will have MMF-related side effects, although they may have been fine with that same dose before discontinuation of cyclosporine. Clinically, the difference in MPA concentrations between cyclosporine- and non-cyclosporine-containing regimens is also important in view of the accumulating evidence relating drug concentrations to efficacy. A potential strategy of increasing the MMF dose early after transplantation to reach the target concentration and tapering the dose at later points could reduce the incidence of acute rejection and avoid toxicity. In March 2003, the so-called Fixed Dose *versus* Concentration Controlled trial was started. In the FDCC trial, 900 patients will be randomized for either standard-dose therapy or concentration-controlled MMF therapy. The final results of this trial are expected in early 2006.

## INTRODUCTION

The prodrug mycophenolate mofetil (MMF) is now a standard immunosuppressive drug in patients after organ transplantation and is mostly used in combination with the calcineurin inhibitors cyclosporine or tacrolimus.<sup>1</sup> After oral administration, MMF is rapidly absorbed from the gut and then converted to mycophenolic acid (MPA), the active immunosuppressant.<sup>1</sup> After an initial peak ( $C_{max}$ ) at 1 h, a second increase in MPA plasma concentration occurs 8 to 12 h after administration, which is caused by enterohepatic recirculation of MPA.<sup>2</sup> Thus the total area-under the MPA plasma concentration *versus* time-curve (AUC) results from the following 2 processes: (1) intestinal absorption and de-esterification of MMF, and (2) enterohepatic recirculation of MPA.<sup>3</sup>

Most pharmacokinetic drug interactions with cyclosporine and tacrolimus are caused by these immunosuppressants being substrates for both the adenosine triphosphate-binding cassette (ABC) transporter P-glycoprotein and for cytochrome P450 3A enzymes. In contrast, MPA is not a P-glycoprotein or P450 substrate and is mainly eliminated by conjugation reactions by the uridine diphosphate glucuronosyltransferase (UGT) enzyme family.<sup>4</sup> The main MPA metabolite, 7-hydroxy-glucuronide MPA (MPAG), is excreted in bile and then contributes to the enterohepatic recirculation after deglucuronidation in the gastrointestinal tract.<sup>5</sup> Finally, MPAG is eliminated by the kidneys. Zucker *et al.* reported significantly higher MPA predose plasma concentrations and MPA AUCs in renal transplant recipients treated with MMF and tacrolimus than in patients treated with MMF and cyclosporine.<sup>6</sup> They also found a difference in MPAG concentrations between cyclosporine- and tacrolimus-treated patients. After all patients with impaired renal function were eliminated, MPAG AUC was significantly higher in the cyclosporine-treated group.<sup>7</sup> Hübner *et al.* also found significantly higher MPA predose concentrations in renal transplant patients treated with tacrolimus than in a control group of cyclosporine-treated patients.<sup>8</sup> Both Zucker *et al.* and Hübner *et al.* concluded that the difference in MPA concentrations was the result of a tacrolimus-induced augmentation of the amount of MPA and that in the tacrolimus-treated patients the MPA concentrations were elevated compared with those in the cyclosporine group.<sup>7,8</sup>

These findings led to the hypothesis that the major cause for increased MPA concentrations during coadministration of tacrolimus is inhibition of the glucuronidation of MPA by tacrolimus.<sup>6</sup> This hypothesis is supported by an *in vitro* study which showed that tacrolimus, but not cyclosporine, inhibits UGT-mediated formation of the major MPA metabolite, MPAG.<sup>9</sup> For this study UGT was extracted from human liver and kidney tissue, and both the conversion of MPA to MPAG and the dose-dependent inhibition of this conversion by adding cyclosporine and tacrolimus were studied.

The pharmacokinetic data for both groups are, however, also consistent with the hypothesis that cyclosporine decreases MPA concentrations. The data from both studies are, in fact, insufficient to choose either explanation for the observed difference. What is missing is a control group of patients treated with MMF in the absence of cyclosporine or tacrolimus to more objectively indicate which group is “abnormal”.

Reference	Patient type	Treatment	MMF dose (mg daily)	Number of patients	Time after trans-plantation	MPA predose concentration (mg/L)	MPA AUC <sub>0-12</sub> (mg/L · h)	Assay
Zucker <i>et al.</i> <sup>6</sup>	Adult	MMF + CsA	2000	12	NA	1.2 ± 0.4	32.1 ± 6.3	HPLC
		MMF + Tac	2000	18	NA	2.8 ± 0.3	50.2 ± 3.9	
Hübner <i>et al.</i> <sup>8</sup>	Adult	MMF + CsA	1500 ± 500	10	1-6 mo	1.9 ± 1.1	ND	EMIT
		MMF + Tac	1700 ± 300	5	1-6 mo	3.4 ± 1.3	ND	
Smak Gregoor <i>et al.</i> <sup>10</sup>	Adult	MMF + CsA	2000	18	3-10 mo	1.98 ± 0.12	ND	EMIT
		MMF + CsA	2000	11	1 yr	4.38 ± 0.40	ND	
Smak Gregoor <i>et al.</i> <sup>11</sup>	Adult	MMF + CsA	2000	19	9 mo	1.53	ND	EMIT
		MMF	2000	19	9 mo	3.16	ND	
Filler <i>et al.</i> <sup>13</sup>	Pediatric	MMF + CsA	1158 ± 301	15	6.6 ± 3.5 yr	3.6 ± 2.2	62.7 ± 29.1 (0.084) <sup>§</sup>	EMIT
		MMF + Tac	555 ± 289	14	1.2 ± 2.2 yr	4.8 ± 2.9	62.8 ± 23.3 (0.122) <sup>§</sup>	
		MMF	866 ± 401	13	4.4 ± 4.2 yr	4.4 ± 1.5	64.5 ± 19.3 (0.067) <sup>§</sup>	
Pou <i>et al.</i> <sup>14†</sup>	Adult	MMF + CsA	2000	107	0-3 mo	2.14 ± 1.2	ND	EMIT
		MMF + Tac	2000	33	0-3 mo	3.63 ± 2.6	ND	
		MMF	2000	22	0-3 mo	3.82 ± 2.2	ND	
Brown <i>et al.</i> <sup>15</sup>	Pediatric	MMF + CsA	500	10	>1 yr	1.68	29.2	HPLC
		MMF + Tac	250	11	>1 yr	1.41	26.0	

EMIT, enzyme multiplied immunoassay technique; HPLC, high-performance liquid chromatography; NA, not applicable; ND, not determined

<sup>§</sup> Dose-normalized AUC ( $\mu\text{g} \times \text{h} \times \text{m}^2/\text{mL} \times \text{mg}$ )

<sup>†</sup> Kidney and lung transplant recipients

**Table 3.2.1** Studies comparing MPA concentrations between cyclosporine (CsA)- and tacrolimus (Tac)-based regimens in kidney transplant patients



In contrast to the design of the clinical studies by Zucker *et al.* and Hübner *et al.*, a clinical study by Smak Gregoor *et al.* included a group of kidney transplant patients who were treated with MMF and steroids and no calcineurin inhibitors (Table 3.2.1).<sup>6,8,10</sup> The pharmacokinetic data from these patients were very similar to those of patients treated with the combination of tacrolimus, MMF and prednisone.<sup>6</sup> Mean MPA predose concentrations of the patients on a regimen of MMF and prednisone were  $4.38 \pm 0.4$  mg/L versus  $1.98 \pm 0.12$  mg/L in a control group of cyclosporine-, MMF- and prednisone-treated patients (all patients received 2 g of MMF daily). Hübner *et al.* reported MPA predose concentrations of  $3.4 \pm 1.3$  mg/L in tacrolimus-treated patients and  $1.87 \pm 1.1$  mg/L in cyclosporine-treated patients, with slightly lower MMF doses (1.5 and 1.7 g daily, respectively).<sup>8</sup> On the basis of our results, we concluded that cyclosporine reduces MPA predose concentrations. This conclusion contrasts with those of Zucker *et al.* and Hübner *et al.*, who proposed that treatment with tacrolimus increases MPA exposure.<sup>6,8</sup>

The data from the cross-sectional study reported by Smak Gregoor *et al.* were confirmed in a subsequent prospective longitudinal study.<sup>10,11</sup> A cohort of patients treated with cyclosporine, MMF and prednisone during the first 6 months after kidney transplantation were randomized to either continuation of triple-drug treatment or discontinuation of either cyclosporine or prednisone at 6 months posttransplantation. MPA predose concentrations were measured at the time of randomization and at 9 months after transplantation. In the patients continuing triple-drug treatment and in the patients discontinuing prednisone, MPA predose concentrations did not change, whereas after discontinuation of cyclosporine a highly significant rise in MPA predose concentrations was observed. A similar rise in MPA concentrations was observed by Shipkova *et al.* in 5 patients with deteriorating renal function in whom cyclosporine was discontinued.<sup>12</sup> The clinical studies showed that mean MPA predose concentrations in kidney transplant recipients treated with MMF and prednisone were significantly higher (3.16 mg/L) than in patients treated with MMF, cyclosporine, and prednisone (1.87 mg/L).<sup>10,11</sup> This strongly indicates that cyclosporine does reduce MPA exposure, thus explaining the relative increase in MPA exposure after conversion from cyclosporine to tacrolimus as previously noted and ascribed solely to effects of tacrolimus on MPA metabolism.<sup>6,8</sup> As indicated by our data, a comparison of MPA pharmacokinetics in patients treated with MMF and cyclosporine versus patients treated with MMF and tacrolimus is misleading without a control group of patients treated with MMF monotherapy. In fact, the valuable observations from Hübner *et al.* and Zucker *et al.* are fully consistent with the hypothesis that cyclosporine decreases MPA concentrations.<sup>6,8</sup>

Filler *et al.* published their results on pharmacokinetics of MMF in pediatric renal transplant patients.<sup>13</sup> They compared pharmacokinetic profiles from 13 patients who received MMF without calcineurin inhibitors with profiles from 14 patients treated with MMF and tacrolimus and 15 patients treated with MMF and cyclosporine.<sup>13</sup> Also, in their study, dose-normalized MPA AUC was higher in tacrolimus-treated patients than in cyclosporine-treated patients. Surprisingly, dose-normalized MPA AUC in patients with no calcineurin inhibitor was significantly lower than dose-normalized MPA AUC in patients treated with tacrolimus and not significantly different from that in patients treated with cyclosporine.

More recently, Pou *et al.* studied MPA concentrations in kidney and lung transplant patients.<sup>14</sup> They observed significantly higher MPA trough levels in both tacrolimus-treated patients

(3.63 mg/L) and patients who were not treated with a calcineurin inhibitor (3.82 mg/L) compared with the cyclosporine-treated group (2.14 mg/L).<sup>14</sup> Brown *et al.* studied pediatric liver transplant patients and found significantly higher MPA pre-dose concentrations in tacrolimus-treated patients than in cyclosporine-treated patients.<sup>15</sup>

## MECHANISM OF INHIBITORY EFFECT OF CYCLOSPORINE ON MPA PLASMA CONCENTRATIONS

In comparisons of clinical studies in transplant patients, different patient groups represent heterogeneous populations, leading to the influence of several variables on the interpretation of causes for pharmacokinetic drug-drug interactions. These variables include time since transplantation, concomitant medication, and coexisting diseases.<sup>10,11</sup>

To understand the influence of tacrolimus and cyclosporine on MMF pharmacokinetics more completely, van Gelder *et al.* eliminated the influence of confounding variables in clinical studies by performing drug interaction studies in inbred Lewis rats under controlled conditions.<sup>17</sup> In this study, 3 groups of rats were treated once daily with either MMF plus cyclosporine, MMF plus tacrolimus, or MMF plus vehicle. Rats in the MMF plus tacrolimus group and in the MMF plus vehicle group showed a second peak in the MPA AUC, which is consistent with enterohepatic recirculation of MPA. The MPA AUC for the animals treated MMF plus cyclosporine did not show this second MPA peak, resulting in a mean plasma MPA AUC at 24 h ( $AUC_{0-24}$ ) for the cyclosporine-treated animals that was significantly lower than that in rats treated with MMF plus tacrolimus and MMF plus vehicle. Furthermore, coadministration of cyclosporine and MMF significantly increased MPAG  $AUC_{0-24}$ , suggesting that cyclosporine inhibits MPAG excretion into bile. This explains the well-known increased MPA exposure in organ transplant patients caused by conversion from cyclosporine- to tacrolimus-based immunosuppression. Because cyclosporine increases the MPAG plasma concentrations and decreases the MPA/MPAG ratio, the most likely mechanism for the pharmacokinetic interaction between MMF and cyclosporine is the inhibition of MPAG excretion from the hepatocytes into bile by cyclosporine. Although potential species differences may restrict the extrapolation of the data from our rat study to patients, there is good evidence that enterohepatic recirculation in humans has a major role in the pharmacokinetics of MMF as well. Studies with oral carbon 14-labeled MMF in bile-cannulated rats showed that 24 h after study drug administration 77% and 21% of the dose was recovered from bile and urine, respectively.<sup>18</sup> In humans, the mean contribution of the enterohepatic recirculation to the overall pharmacokinetic profile for MPA was found to be 37% (range 10%-61%).<sup>1</sup> In healthy volunteers, disruption of enterohepatic recirculation by pretreatment with cholestyramine for 4 days before MMF treatment decreased the MPA  $AUC_{0-24}$  of a single dose of MMF by 40%.<sup>19</sup>

Hepatic uptake and biliary excretion are of importance for the disposition of a wide array of drugs. Transporters such as ABC proteins play important roles in the biliary elimination of xenobiotics, including many glucuronides.<sup>20</sup> Cyclosporine is an inhibitor of P-glycoprotein and may also be responsible for the inhibition of a variety of other biliary ABC transporters,

including canalicular multiple organic anion transporter [cMOAT or multidrug resistance-associated protein (MRP)-2].<sup>21-24</sup> We speculate that cyclosporine inhibits active transport mechanisms in the biliary membrane responsible for the excretion of MPAG into bile. The hepatobiliary transport of MPAG, however, has not yet been studied. A further understanding of the hepatobiliary transport of MPAG can be obtained by studying animal models, isolated hepatocytes, or cell lines transfected with genes of the drug transporters under investigation.<sup>20</sup> Animal models, consisting of specific strains of animals with a mutation (either spontaneous or transgenic) that gives rise to disturbances in transport, have the advantage that transport can be studied in an integrated model that is defined by the presence or absence of a single gene.<sup>20</sup> The transport-deficient rat (TR) is an example of an animal strain with a spontaneous mutation, resulting in a defect in cMOAT. Identification of the TR rat has greatly contributed to the functional characterization of cMOAT. The most prominent substrate of this transporter is conjugated bilirubin, and TR rats have often been used as a model for the human Dubin-Johnson syndrome.<sup>25</sup> Dutch investigators have discovered a single-nucleotide deletion in the cMOAT gene responsible for the TR phenotype.<sup>26</sup> In cMOAT-transfected cell lines, cyclosporine was shown to inhibit apical transport.<sup>27</sup> The hyperbilirubinemia associated with high-dose cyclosporine treatment is probably also caused by inhibition of cMOAT.<sup>25</sup> It is interesting that cyclosporine is a more potent inhibitor than is its analogue PSC 833 and produces higher bilirubin levels, whereas PSC 833 is a more potent inhibitor of P-glycoprotein.<sup>28</sup> It is our hypothesis that cyclosporine inhibits active transport of MPAG by cMOAT in the biliary membrane and thus inhibits the elimination of MPAG into bile. We are currently investigating the role of cMOAT in the pharmacokinetics of MPA by comparing normal Wistar rats and a cMOAT-deficient mutant strain during *in vivo* treatment with MMF alone or in combination with cyclosporine.

## CLINICAL RELEVANCE OF THE DRUG INTERACTION

In the 3 so-called pivotal trials that have led to the registration of MMF for the prevention of acute rejection after kidney transplantation, it was found that treatment with a standard dose of 2 g daily had similar efficacy and better tolerability compared with 3 g daily.<sup>29-31</sup> In all 3 studies, patients were receiving cyclosporine. These studies have also led to the recommendation to use a standard dose of 2 g daily in all patients. Although our data clearly show that MPA exposure is significantly higher in tacrolimus-treated patients, the dose recommendation for MMF in the context of tacrolimus treatment is the same.

In patients in whom MMF is combined with sirolimus, preliminary data show that MPA exposure is comparable to that of combined MMF and tacrolimus treatment.<sup>32</sup> From these studies, it is clear that the MPA concentrations will increase in patients switched from cyclosporine- to tacrolimus-based therapy, even with unchanged MMF dose. It is not impossible that suddenly patients will have MMF-related side effects after they switch from cyclosporine to tacrolimus, although they may have been fine with that same dose before discontinuation of cyclosporine. We have seen such patients at our center. Clinicians should be aware of this effect of changing calcineurin inhibitor therapy. It is likely that the optimal maintenance dose for MMF therapy in a tacrolimus-based regimen is less than the recommended 1000 mg twice daily in cyclosporine-treated patients. Another clinically

relevant consequence of the described interactions occurs in patients on a regimen of MMF therapy in whom broad-spectrum antibiotics are started. Because antibiotics may change the bowel flora, their use can result in decreased deglucuronidation activity and interruption of enterohepatic recirculation. For kidney transplant patients, treatment with broad-spectrum antibiotics for prolonged time periods is unusual, but in bone marrow transplantation or lung transplantation this is a likely cause of reduced MPA exposure.

Clinically the difference in drug levels between regimens that do and do not contain cyclosporine is also important in view of the accumulating evidence relating drug concentrations to efficacy of MMF. Most published pharmacokinetic data come from patients within the first 6 months posttransplantation. The largest series of patients in whom repetitive MPA AUCs were determined is the randomized, concentration-controlled trial (RCCT) of MMF in the prevention of acute rejection after kidney transplantation, performed in 7 centers in The Netherlands and Belgium in 1994-1995.<sup>33</sup> The aim of the study was to maintain the MPA AUC at 12 h ( $AUC_{0-12}$ ) at a predefined target value. Patients ( $n = 154$ ) were randomized for low-, intermediate-, or high-target AUC groups. All patients were treated with cyclosporine, prednisone, and MMF. Blood samples were taken at regular intervals, MPA  $AUC_{0-12}$  was measured, and then, if necessary, the MMF dose was adjusted accordingly. This study showed a statistically significant correlation between drug concentrations (for MPA  $AUC_{0-12}$  and, to a lesser extent, also for MPA predose concentrations) and likelihood of developing an acute rejection episode.<sup>34</sup>

Other groups have also shown a relationship between pharmacokinetic markers and outcome in MMF treatment.<sup>35-40</sup> Recently, a proposal for MPA target concentrations was published by Shaw *et al.*<sup>41</sup> A target MPA  $AUC_{0-12}$  of at least 30 mg/L x h up to 60 mg/L x h was considered sufficient, both for the immediate posttransplant period and for maintenance therapy. Corresponding MPA predose concentrations for this range are 1.0 to 3.5 mg/L. Independently from Shaw *et al.*, in their recommendations, Oellerich *et al.* also recommend the interval between 30 and 60 mg/L x h as the optimal target values.<sup>37,41</sup> Potentially, a strategy of adjusting MMF dose on the basis of MPA target concentrations could reduce the incidence of acute rejection by increasing the dose early after transplantation to reach target as soon as possible and by tapering the dose at later time points not to exceed target and to avoid toxicity.

Although several investigators have shown that drug exposure (either MPA  $AUC_{0-12}$  or MPA predose concentrations) predicts outcome, a clinical trial comparing a group of patients receiving standard-dose therapy with a group of patients treated with concentration-controlled MMF therapy has not yet been performed. A trial with this design would be able to determine the added value of performing therapeutic drug monitoring in MMF therapy. In March 2003, the so-called Fixed Dose *versus* Concentration Controlled trial was started. In the Fixed Dose *versus* Concentration Controlled trial, 900 patients will be randomized to either standard-dose therapy or concentration-controlled MMF therapy. The final results of this trial are expected in early 2006. Because both cyclosporine- and tacrolimus-treated patients will be entered into this trial, this study will also allow a further evaluation of the different effects of these drugs on MPA exposure. Subgroup analyses will include studies on the influence of renal function, co-medication, gene polymorphisms for enzymes involved in pharmacokinetics, and genes involved in the generation of an immune response.

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CHAPTER 3.3

**CYCLOSPORINE  
INTERACTS WITH  
MYCOPHENOLIC ACID  
BY INHIBITING THE  
MULTIDRUG  
RESISTANCE-ASSOCIATED  
PROTEIN 2**

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## ABSTRACT

In mycophenolate mofetil (MMF)-treated organ transplant recipients, lower mycophenolic acid (MPA) plasma concentrations have been found in cyclosporine- compared with tacrolimus-based immunosuppressive regimens. We previously demonstrated that cyclosporine decreases exposure to MPA and increases exposure to its metabolite MPA-glucuronide (MPAG), possibly by interfering with the biliary excretion of MPAG. To elucidate the role of the multidrug resistance-associated protein (Mrp)-2 in the interaction between MMF and cyclosporine, we treated three groups of 10 Mrp2-deficient rats (TR-rat) for 6 days with either vehicle, cyclosporine (8 mg/kg) or tacrolimus (4 mg/kg) by oral gavage. Hereafter, co-administration with MMF (20 mg/kg) was started in all groups and continued through day 14. The 24-h MPA/MPAG area-under the concentration *versus* time-curve (AUC) was determined after single (day 7) and multiple MMF doses (day 14). On both study days, there were no significant differences in the mean MPA and MPAG AUC between cyclosporine- and tacrolimus-treated animals. We conclude that the pharmacokinetics of MMF are comparable in Mrp2-deficient rats receiving either cyclosporine or tacrolimus as co-medication. This finding suggests that cyclosporine-mediated inhibition of the biliary excretion of MPAG by the Mrp2 transporter is the mechanism responsible for the interaction between cyclosporine and MMF.

## INTRODUCTION

Mycophenolate mofetil (MMF) is a pro-drug that is rapidly and almost completely absorbed from the gut where it is de-esterified to form mycophenolic acid (MPA), the active immunosuppressant. MPA is converted by the uridine diphosphate glucuronosyl transferase (UGT) enzyme family into 7-hydroxy-glucuronide mycophenolic acid (MPAG) which is excreted into bile and is not pharmacologically active. In the gut, bacterial deconjugation transforms MPAG back into MPA, which is absorbed from the colon. Because of this enterohepatic circulation, the initial MPA plasma concentration peak at 1 h is followed by a second increase in the MPA plasma concentration, occurring 6-12 h after oral administration. In human subjects, interference with the enterohepatic circulation reduces the MPA area-under the concentration *versus* time-curve (AUC) by 35% to 40%.<sup>1,2</sup> Finally, the majority of the absorbed MMF is eliminated by the kidneys as MPAG.<sup>1,2</sup>

We and others previously demonstrated that co-administration of MMF with cyclosporine to solid organ transplant recipients leads to a reduction of MPA plasma concentrations and an increase in the plasma levels of MPAG as compared with patients treated with MMF plus tacrolimus or corticosteroids.<sup>3-6</sup> These clinical findings were confirmed in an animal study that compared MPA and MPAG exposure between Lewis rats that were treated with MMF plus cyclosporine, MMF plus tacrolimus or MMF plus placebo.<sup>7</sup> Rats in the MMF plus tacrolimus and MMF plus placebo groups showed a second peak in the plasma MPA AUC, consistent with enterohepatic recirculation. In contrast, animals treated with MMF plus cyclosporine showed a marked reduction of the second MPA peak, resulting in a significantly lower mean MPA AUC. Furthermore, co-administration of cyclosporine significantly increased the AUC of MPAG, suggesting a cyclosporine-induced inhibition of MPAG excretion into bile.<sup>7</sup>

At present, the exact mechanism responsible for the pharmacokinetic interaction between MMF and cyclosporine is unknown. We hypothesized that cyclosporine impairs biliary MPAG elimination through inhibition of the multidrug resistance-associated protein (MRP) 2 (or ABCC2, previously known as canalicular multispecific organic anion transporter). MRP2 is expressed at the apical (canalicular) surface of hepatocytes, where it functions to excrete endogenous conjugates as well as conjugation products of drug metabolism into bile.<sup>8,9</sup> Evidence for the implication of MRP2 in the MMF-cyclosporine interaction comes from the observation that cyclosporine can cause a conjugated hyperbilirubinemia (a MRP2 substrate) *in vivo* and is an inhibitor of MRP2 function *in vitro*.<sup>10-12</sup> Furthermore, it was recently demonstrated that Eisai hyperbilirubinemic rats (EHBRs), lacking *Mrp2* due to a genetic mutation, can only excrete MPAG to a limited degree in bile after intravenous administration of MPA, resulting in high MPAG plasma concentrations.<sup>13</sup>

In this study, we aimed to elucidate the role of MRP2 in the interaction between MMF and cyclosporine by repeating our previous pharmacokinetic study that was performed in wildtype rats, in transport-deficient (TR-) Wistar rats.<sup>7</sup> TR- rats have a mutation in the *mrp2* gene which results in the absence of functional *Mrp2* protein. Phenotypically, TR- rats are characterized by a defective hepatobiliary excretion of bilirubin glucuronides and other amphiphilic anions.<sup>12,14,15</sup> We show that in the absence of *Mrp2*, the previously described

effects of cyclosporine on MMF pharmacokinetics are no longer present, giving equal MPA and MPAG plasma concentrations as compared with rats treated with MMF and tacrolimus. For the first time, these *in vivo* data provide evidence for the hypothesis that inhibition of MRP2 by cyclosporine is the main mechanism responsible for the interaction between cyclosporine and MPA.

## MATERIALS AND METHODS

### ANIMALS

Adult male TR- Wistar rats (HRD-AMC Abcc2) were purchased from Harlan (Horst, the Netherlands). The animals were housed in microisolation cages (three per cage) and had free access to food and water. Rats were acclimated under a 12-h light/dark cycle for 2 weeks before the start of the study. All rats were 12 weeks of age and weighed 250 g at the start of the experiment.

### DRUG FORMULATIONS

*Vehicle*: As placebo (hereafter called vehicle) we used Basis pro Suspension (Fagron Pharmaceuticals B.V., Nieuwerkerk a/d IJssel, the Netherlands) which consisted of 0.75 mg methylhydroxybenzoate, 0.20 mg propylhydroxybenzoate, 10.0 mg aluminiummagnesium silicilate, 10.0 mg carmellose sodium 500 mPas.s, 0.75 mg citric acid 1 *aq*, 263.0 mg sirupus simplex, and 783.30 mg purified water per mL.

*Mycophenolate mofetil*: MMF powder (Cellcept<sup>®</sup>, Roche Bioscience, Palo Alto, CA, USA) was suspended in vehicle for oral gavage every three days to produce a 2% solution which was stored at 4°C.

*Cyclosporine*: cyclosporine oral microemulsion formulation (100 mg/mL; Neoral<sup>®</sup>, Novartis Pharma AG, Basel, Switzerland) was freshly diluted in vehicle once daily to produce a 0.8% solution for oral gavage.

*Tacrolimus*: tacrolimus solution for intravenous injection (10 mg/mL; Prograf<sup>®</sup>, Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan) was diluted in vehicle once daily to produce a 0.4% solution which was administered by oral gavage.

All drugs were kindly supplied by the manufacturers.

## STUDY DESIGN

The study was a three-arm, two-period pharmacokinetic drug interaction study. Thirty adult, male TR- rats were allocated to three study groups (n = 10 each). The possible drug interactions between MPA and cyclosporine and between MPA and tacrolimus were studied after cyclosporine and tacrolimus reached steady-state, and after single and multiple MMF doses. The drug dosages used were chosen on the basis of previous experience demonstrating their ability to prevent the occurrence of acute rejection after kidney transplantation in rats.<sup>7</sup>

*Vehicle group:* After daily oral gavage with vehicle on days 0-6, MMF treatment (20 mg/kg bodyweight) was started on day 7 and was administered daily through day 14. Blood samples for MPA and MPAG pharmacokinetic analysis were collected during the 24 h subsequent to dosing on day 7 (single dose pharmacokinetic profile). Additional MPA and MPAG 24-h pharmacokinetic profiles (multiple dose) were determined subsequent to dosing on study day 14.

*Cyclosporine group:* From study day 0 through day 14, one group of rats was treated with 8 mg/kg bodyweight cyclosporine daily. On day 7, co-administration of MMF (20mg/kg bodyweight) was started and continued through day 14. Blood samples for MPA and MPAG pharmacokinetic profiles were collected during 24 h subsequent to dosing on study days 7 (single dose) and 14 (multiple dose). In addition, blood was collected at these time points for predose cyclosporine concentration measurements.

*Tacrolimus:* The dosing schedule and the schedule for the collection of samples for pharmacokinetic profiles was identical to those described for cyclosporine. The rats received a daily tacrolimus dose of 4 mg/kg bodyweight per day instead of cyclosporine.

All drugs were given once daily by oral gavage at 9:00 am. When drugs were co-administered, they were given within 5 min. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes by tail bleeding under isoflurane (Rhodia Organique Fine Ltd., Bristol, United Kingdom) anesthesia before and 0.5, 1, 2, 6, 12 and 24 h after dosing. After collection, EDTA blood samples were immediately centrifuged at 11750 g, after which the plasma was frozen at -80 °C.

## ETHICS

The experimental protocol was approved by the Animal Experiments Committee of the Erasmus Medical Center under the national Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of “Guidelines on the protection of experimental animals” by the Council of Europe (1986), Directive 86/609/EC.

## QUANTIFICATION OF PLASMA LEVELS OF THE STUDY DRUGS

Cyclosporine and tacrolimus whole-blood concentrations were determined with the Emit 2000 assay (Syva company, Dade Behring Inc., Cupertino, CA, USA) on a Cobas Mira Plus analyzer (Roche Diagnostic Systems, Basel, Switzerland). Details on the sensitivity and

reproducibility of the Emit assay in our laboratory were published previously.<sup>16</sup> Proficiency samples were obtained from the United Kingdom Quality Assessment Scheme (Dr. Holt, St George's Hospital Medical School, London, United Kingdom).

MPA and MPAG in rat plasma were simultaneously measured with high-performance liquid chromatography (HPLC) according to the method described by Shipkova with several modifications.<sup>17</sup> The assay was validated for determination of MPA and MPAG in rat plasma according to FDA guidelines.<sup>18</sup> The limit of quantification was arbitrarily set at 0.25 mg/L for MPA and 2.5 mg/L for MPAG. The assay was found to be linear in a concentration range from 0.25 mg/L to 30 mg/L for MPA and from 2.5 mg/L to 100 mg/L for MPAG (correlation coefficient >0.99). In the same concentration range, the within-day CV ranged from 2.0% to 3.1% for MPA and from 1.5% to 3.9% for MPAG. The between-day CV ranged from 1.9% to 8.5% for MPA and from 3.0 to 6.9% for MPAG. The accuracy of the assay, defined as the percentage of recovery of MPA and MPAG from the control samples was for MPA in the range of 108-110% and for MPAG in the range of 98-113%.

### PHARMACOKINETIC ANALYSIS

The concentration-time data were analyzed using WinNonlin version 4.1 (Pharsight Corporation, Mountain View, CA, USA). A non-compartmental model with extravascular input for plasma data was used to obtain estimates for MPA and MPAG maximum concentration ( $C_{max}$ ), time to maximum concentration ( $t_{max}$ ) and AUC. Since the AUC on study day 7 was measured after the first MMF dose,  $AUC_{0-\infty}$  was estimated. The decline of MPA or MPAG concentration between two subsequent time points that was most representative for elimination was used for extrapolation beyond 24 h. On day 14 steady-state was assumed and  $AUC_{0-24}$  was calculated. AUCs were calculated by using the logarithmic trapezoidal rule.

### BIOCHEMISTRY

Before the start of the study and on study days 7 and 14, blood urea nitrogen, serum albumin and serum total bilirubin were determined on an ELAN analyzer (Eppendorf-Merck, Darmstadt, Germany) using the UV test/GIDH method, BCG method, and DPD method, respectively (Merck Diagnostica, Darmstadt, Germany).

### STATISTICAL ANALYSIS

All data are expressed as means  $\pm$  standard deviation unless otherwise stated. For comparisons between groups we used one-way ANOVA or Kruskal-Wallis test, as appropriate. Post-hoc analysis was performed using Bonferroni's test for multiple comparisons or using the Mann Whitney U test. For comparisons within groups, the paired *t*-test was used. *P* values at  $\alpha \leq 0.05$  were considered statistically significant. Statistical analysis was performed using SPSS for Windows version 11.5.0 (SPSS, Chicago, IL, USA).

## RESULTS

### MYCOPHENOLIC ACID AND GLUCURONIDATED MYCOPHENOLIC ACID PHARMACOKINETICS

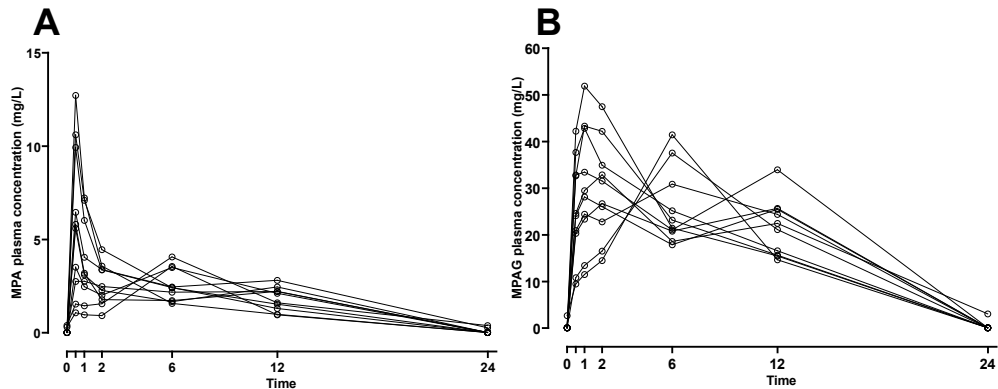
A total of 30 MPA and MPAG pharmacokinetic profiles was obtained on study day 7 (single MMF dose). In all three treatment groups, the MPA concentration *versus* time profiles were characterized by a first peak within the first hour followed by a second peak at 6-12 h after drug administration, consistent with enterohepatic recirculation of MPA (Figures 3.3.1A and 3.3.2A).

On day 7, after the first MMF dose, the mean  $AUC_{0-\infty}$  of MPA was significantly different between the three treatment groups:  $32.0 \pm 8.0$  *versus*  $24.5 \pm 6.1$  *versus*  $21.8 \pm 6.4$  mg x h/L for the vehicle, cyclosporine and tacrolimus groups, respectively ( $P = 0.007$ ; Figure 3.3.2A and Table 3.3.1). This overall difference resulted from a significantly higher  $AUC_{0-\infty}$  of MPA in the vehicle group as compared with the tacrolimus group ( $P = 0.008$ ). When the MPA exposure was compared between the vehicle and the cyclosporine groups a similar trend was observed, although this difference was not statistically significantly different ( $P = 0.065$ ). The  $AUC_{0-\infty}$  of MPA in the cyclosporine and tacrolimus groups were not different ( $P = 1.00$ ).

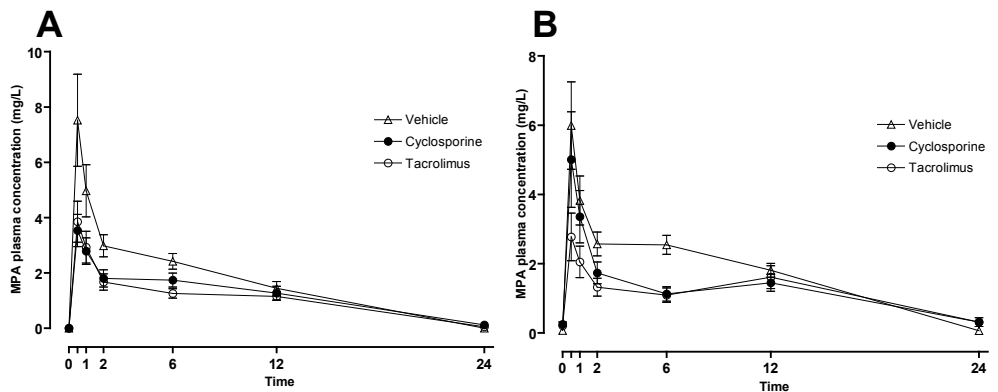
After the first week, two rats in the tacrolimus group died because of aspiration and therefore only 28 MPA and MPAG pharmacokinetic profiles were available for study day 14 (after multiple MMF doses). Again, we found an overall difference in MPA exposure between the different study groups ( $P = 0.018$ ) but there was no difference in the  $AUC_{0-24}$  of MPA between rats receiving tacrolimus or cyclosporine:  $28.1 \pm 10.3$  *versus*  $30.0 \pm 13.3$  mg x h/L, respectively ( $P = 1.00$ ; Figure 3.3.2B and Table 3.3.1). When the MPA exposure in the tacrolimus and cyclosporine groups was compared with the vehicle group, only the difference between the tacrolimus and vehicle group was significant, although a similar trend was observed between the cyclosporine and vehicle groups ( $P = 0.033$  and  $P = 0.056$ , respectively; Figure 3.3.2B and Table 3.3.1).

Table 3.3.1 summarizes the  $AUC_{0-\infty}$  and  $AUC_{0-24}$  of MPAG values in the three different treatment groups at the two time points. Individual MPAG concentration *versus* time profiles are depicted in Figure 3.3.1B. In line with our observations for MPA, the MPAG exposure was never significantly different between the tacrolimus and cyclosporine groups. However, on study day 7, there was an overall difference in MPAG exposure between the three groups which was caused by a significantly lower  $AUC_{0-\infty}$  of MPAG in the vehicle group as compared with the tacrolimus group but not the cyclosporine group (overall  $P = 0.022$ ). On study day 14, this difference was no longer present, although the  $AUC_{0-24}$  of MPAG remained numerically highest in the rats treated with a calcineurin inhibitor ( $P = 0.28$ ; Table 3.3.1, Figure 3.3.3).

**Figure 3.3.1** Individual mycophenolic acid (A) and mycophenolic acid-glucuronide (B) pharmacokinetic profiles after once daily administration of 20 mg MMF/kg bodyweight to male Mrp2 transport-deficient (TR-) Wistar rats. Depicted are the concentration versus time profiles of the rats in the vehicle group that were obtained on study day 14 (after multiple MMF dosing).



**Figure 3.3.2** Mean ( $\pm$  SEM) mycophenolic acid (MPA) plasma concentrations in Mrp2 transport-deficient (TR-) rats after once daily administration of 20 mg MMF/kg bodyweight in combination with either vehicle (triangles), cyclosporine (solid circles) or tacrolimus (open circles)(n = 10 in each group). Depicted are the MPA pharmacokinetic profiles obtained on study day 7 (after a single MMF dose; panel A) and on day 14 (after multiple MMF doses; panel B).





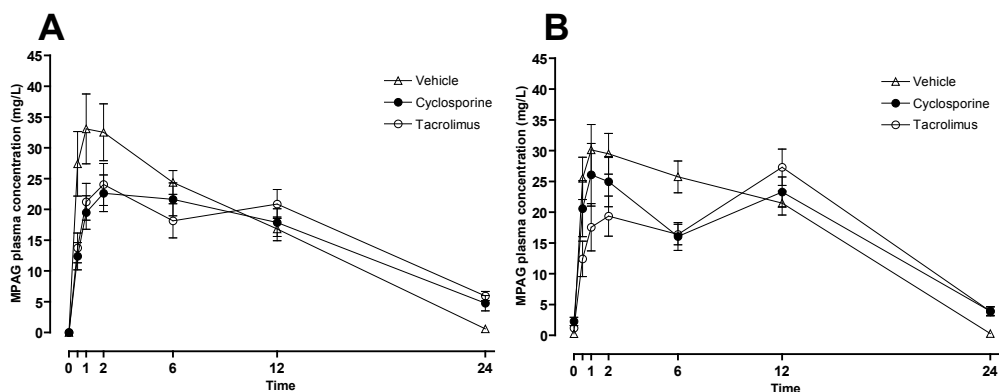
**Table 3.3.1** Pharmacokinetic data of MPA and MPAG in Mrp2 transport-deficient (TR-) rats treated with MMF in combination with either vehicle, cyclosporine (CsA) or tacrolimus (Tac)(n = 10 in each group). Data represent the mean  $\pm$  SD.

Study Day	Analyte	Parameter	Treatment Group		
			MMF + Vehicle	MMF + CsA	MMF + Tac
Day 7 (single dose)	MPA	AUC <sub>0-∞</sub> (mg x h/L)	32.0 $\pm$ 8.0	24.5 $\pm$ 6.1	21.8 $\pm$ 6.4 ‡
		C <sub>max</sub> (mg/L)	7.9 $\pm$ 4.9	4.0 $\pm$ 1.3 †	4.0 $\pm$ 2.2 †
		t <sub>max</sub> <sup>§</sup> (h)	0.5 (0.5 - 6.0)	0.5 (0.5 - 12.0)	0.8 (0.5 - 24.0)
	MPAG	AUC <sub>0-∞</sub> (mg x h/L)	324.4 $\pm$ 76.2	376.8 $\pm$ 87.2	422.3 $\pm$ 53.9 †
		C <sub>max</sub> (mg/L)	38.3 $\pm$ 13.3	27.8 $\pm$ 7.0	30.8 $\pm$ 7.4
		t <sub>max</sub> (h)	2.0 (0.5 - 12.0)	4.0 (1.0 - 24.0)	4.0 (1.0 - 12.0)
Day 14 (multiple dose)	MPA	AUC <sub>0-24</sub> (mg x h/L)	41.7 $\pm$ 6.4	30.0 $\pm$ 13.3	28.1 $\pm$ 10.3 †
		C <sub>max</sub> (mg/L)	6.5 $\pm$ 3.4	5.1 $\pm$ 4.3	3.2 $\pm$ 1.8 †
		t <sub>max</sub> (h)	0.5 (0.5 - 6.0)	0.5 (0.5 - 2.0)	0.5 (0.5 - 2.0)
	MPAG	AUC <sub>0-24</sub> (mg x h/L)	328.4 $\pm$ 30.1	365.0 $\pm$ 75.2	366.2 $\pm$ 58.3
		C <sub>max</sub> (mg/L)	36.9 $\pm$ 7.9	33.0 $\pm$ 11.3	30.7 $\pm$ 7.9
		t <sub>max</sub> (h)	2.0 (1.0 - 12.0)	7.0 (1.0 - 12.0)	12.0 (1.0 - 12.0)

§ For t<sub>max</sub> data represent the median (range)

† P < 0.05, significantly different from the vehicle group

‡ P < 0.01, significantly different from the vehicle group

**Figure 3.3.3** Mean ( $\pm$  SEM) mycophenolic acid glucuronide (MPAG) plasma concentrations in Mrp2 transport-deficient (TR-) rats after once daily administration of 20 mg MMF/kg bodyweight in combination with either vehicle (triangles), cyclosporine (solid circles) or tacrolimus (open circles)(n = 10 in each group). Depicted are the MPAG pharmacokinetic profiles obtained on study day 7 (after a single MMF dose; panel A) and on day 14 (after multiple MMF doses; panel B).

**Table 3.3.2** Serum biochemistries of transport-deficient rats treated with MMF in combination with either vehicle, cyclosporine or tacrolimus (n = 10 in each group). Data represent the mean  $\pm$  SD.

		Vehicle	Cyclosporine	Tacrolimus
Albumin (g/L)	day 0	31.2 $\pm$ 4.0	31.4 $\pm$ 0.7	30.8 $\pm$ 2.4
	day 7	29.8 $\pm$ 1.8 <sup>§</sup>	27.6 $\pm$ 1.2	30.4 $\pm$ 1.2 <sup>§</sup>
	day 14	29.7 $\pm$ 2.1 <sup>†</sup>	27.4 $\pm$ 1.8	30.0 $\pm$ 1.4 <sup>†</sup>
Total bilirubin ( $\mu$ mol/L)	day 0	18.1 $\pm$ 10.2	29.1 $\pm$ 10.6	33.5 $\pm$ 9.6
	day 7	18.9 $\pm$ 4.6	65.5 $\pm$ 24.3 <sup>‡</sup>	43.4 $\pm$ 18.7 <sup>§,‡</sup>
	day 14	24.5 $\pm$ 10.0	68.3 $\pm$ 15.2 <sup>‡</sup>	61.6 $\pm$ 23.3 <sup>#</sup>
Blood Urea Nitrogen (mmol/L)	day 0	8.8 $\pm$ 0.3	8.8 $\pm$ 0.3	9.3 $\pm$ 0.7
	day 7	8.4 $\pm$ 0.8	9.0 $\pm$ 0.7	8.9 $\pm$ 0.9
	day 14	7.0 $\pm$ 0.7	7.2 $\pm$ 0.9	7.3 $\pm$ 0.3

<sup>§</sup>  $P < 0.001$ , significantly different from the cyclosporine group

<sup>†</sup>  $P < 0.05$ , significantly different from the cyclosporine group

<sup>‡</sup>  $P < 0.001$ , significantly different from the vehicle group

<sup>#</sup>  $P < 0.01$ , significantly different from the vehicle group

The MPA to MPAG-AUC<sub>0-∞</sub> ratio was significantly different between the three treatment groups on study day 7: 0.10  $\pm$  0.01 *versus* 0.07  $\pm$  0.02 *versus* 0.05  $\pm$  0.02 for the vehicle, cyclosporine and tacrolimus groups, respectively ( $P < 0.001$ ). The MPA to MPAG-AUC<sub>0-24</sub> ratio on study day 14 was also significantly different between the three groups: 0.13  $\pm$  0.01 *versus* 0.08  $\pm$  0.03 *versus* 0.08  $\pm$  0.03 for the vehicle, cyclosporine and tacrolimus groups, respectively ( $P = 0.001$ ). On study days 7 and 14, this difference was caused by a significantly higher MPA:MPAG ratio in the vehicle group as compared to either the cyclosporine or the tacrolimus group ( $P < 0.001$  and  $P = 0.002$ , for study days 7 and 14, respectively), whereas the cyclosporine and tacrolimus groups did not differ significantly on study days 7 or 14 ( $P = 0.17$  and  $P = 1.00$ , respectively).

### CYCLOSPORINE AND TACROLIMUS WHOLE-BLOOD CONCENTRATIONS

The mean cyclosporine predose concentrations at study days 7 and 14 were 456  $\pm$  234 and 367  $\pm$  137 ng/mL, respectively and were not significantly different ( $P = 0.44$ ). The tacrolimus predose concentrations were also comparable between the two time points: 3.1  $\pm$  1.7 *versus* 2.3  $\pm$  0.8 ng/mL for day 7 and 14, respectively ( $P = 0.55$ ).

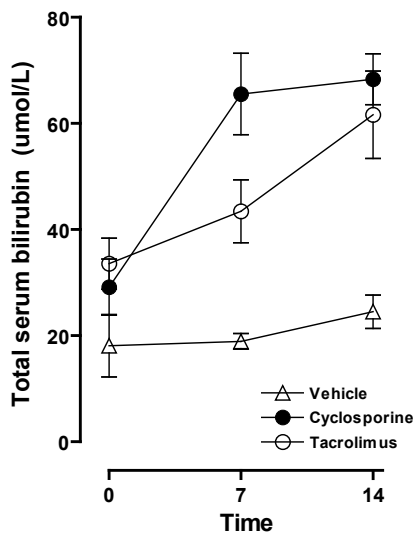
### SERUM CHEMISTRIES

To exclude significant nephrotoxicity caused by cyclosporine or tacrolimus as a cause of possible differences in MPA and MPAG pharmacokinetics, we measured blood urea nitrogen concentrations at baseline and on study days 7 and 14. Throughout follow-up, the mean blood urea nitrogen was comparable between the three groups (Table 3.3.2). For serum albumin, there existed a significant overall difference between the three groups at study days 7 and 14 (overall  $P < 0.001$  and  $P < 0.01$ , respectively; Table 3.3.2), which was caused

by a lower serum albumin in the cyclosporine group as compared with both the vehicle and tacrolimus groups.

The serum total bilirubin concentrations at baseline were markedly elevated but not different between the three groups ( $P = 0.22$ ; Table 3.3.2). However, on study days 7 and 14, the serum total bilirubin concentration was significantly higher in the cyclosporine and tacrolimus groups as compared with the vehicle group (Table 3.3.2 and Figure 3.3.4). On study day 7, there also existed a difference in serum total bilirubin between the cyclosporine and tacrolimus group, but at the end of the study period, the bilirubin concentrations in the rats receiving a calcineurin inhibitor were comparable (Table 3.3.2 and Figure 3.3.4).

**Figure 3.3.4** Mean serum total bilirubin concentrations ( $\pm$  SEM) in Mrp2 transport-deficient (TR-) rats after once daily administration of 20 mg MMF/kg bodyweight in combination with either vehicle (triangles), cyclosporine (solid circles) or tacrolimus (open circles).



## DISCUSSION

Several authors have shown that MPA exposure is significantly lower in cyclosporine-compared with tacrolimus-based immunosuppressive regimens.<sup>3-6</sup> The reported difference is as high as 30% to 40% and is clinically relevant. As a consequence of this difference in MPA exposure, the optimal MMF maintenance dose in cyclosporine-treated patients could be different from that in tacrolimus-treated patients. Also, discontinuation of cyclosporine treatment will lead to increased MPA exposure without a change in the MMF dose and can lead to the occurrence of new MMF-related side effects.<sup>4,19</sup> With the increasing interest in therapeutic drug monitoring of MMF therapy, the calcineurin inhibitor of choice is of high relevance.

In this study, we show that in the absence of the drug-transporting protein Mrp2, the pharmacokinetics of MPA and MPAG are comparable between rats receiving either cyclosporine or tacrolimus as co-medication. In addition, the ratio of the MPA to MPAG AUC of TR- rats treated with MMF and vehicle (which averaged around 1:9) was comparable to the MPA:MPAG ratio that we observed previously in wildtype rats treated with MMF and cyclosporine.<sup>7</sup> In the current study, identical cyclosporine and tacrolimus doses on a bodyweight basis were used, resulting in similar predose concentrations. For the first time, these results demonstrate *in vivo*, that Mrp2 is the transporter mainly responsible for the excretion of MPAG into bile and that inhibition of Mrp2 by cyclosporine is the mechanism underlying the interaction between cyclosporine and MPA. Because the difference in MPA exposure between cyclosporine and tacrolimus co-administration appears to be present in both rats and transplanted patients, the results of the present experimental study are relevant for the human situation. There only appears to be a quantitative difference between both species. In humans, the contribution of enterohepatic recirculation to total MPA exposure averages around 40%, whereas in rats it can account for as much as 70%.

Further evidence for the role of MRP2 in the excretion of MPAG comes from the results of Sallustio *et al.* who perfused isolated rat livers obtained from normal and TR- rats, with MPA and measured MPA and MPAG concentrations in both perfusate and bile.<sup>20</sup> In normal rats, more than 90% of the administered MPA dose was recovered as MPAG in bile and no MPAG was present in the perfusate. In marked contrast, less than 1% of the MPA dose was recovered as MPAG from the bile of TR- rats, and around 80% was recovered as MPAG in the perfusate. Importantly, glucuronidation of MPA to MPAG appeared to be comparable between normal and Mrp2-deficient rats.<sup>20</sup> A markedly reduced excretion of MPAG into bile was also demonstrated in EHBRs (that lack Mrp2) after intravenous MPA administration.<sup>13</sup> However, in this report, the interaction between MMF and calcineurin inhibitors was only directly studied in normal, but not in Mrp2-deficient animals. Moreover, all drugs were administered intravenously and were not in steady-state.<sup>13</sup> For the current study, we compared the results of the Mrp2-deficient Wistar rat with those from our previous experiment in wildtype Lewis rats. Wildtype Wistar rats have been shown to metabolize several drugs by glucuronidation in the liver, with subsequent biliary excretion and enterohepatic recirculation. For diclofenac and valproic acid it was recently demonstrated that biliary excretion of their respective metabolites is mediated by Mrp2.<sup>21,22</sup> This shows

that, similar to normal Lewis rats, wildtype Wistar rats possess a functional Mrp2-mediated enterohepatic recirculation of glucuronidated substances and obviates the need for a control group of wildtype Wistar rats.

Interestingly, in this study, we observed marked differences in the MPA and MPAG plasma concentrations between the vehicle group and the groups receiving a calcineurin inhibitor: the mean MPA exposure in the vehicle group was higher compared with rats receiving a calcineurin inhibitor, while the opposite was true for MPAG exposure. There are several possible explanations for this finding. First, in TR- rats, the absence of Mrp2 may be partly compensated for by (the induction of) other organic anion transporters. Alternative drug-transporting enzymes that could theoretically provide an escape mechanism for MPAG excretion into bile may include the breast cancer resistance protein (or ABCG2), the bile salt export pump (or ABCB11) and others.<sup>23-25</sup> Calcineurin inhibitors are known to inhibit a variety of these drug-transporters.<sup>26-29</sup> P-glycoprotein (or ABCB1) is a less likely candidate as this transporter is not known to transport glucuronidated substances. If alternative MPAG-elimination pathways are operational in the liver of the TR- rat, then their blockade by cyclosporine or tacrolimus would theoretically lead to an increased MPAG exposure and, as less MPAG is available for enterohepatic recirculation, to a decreased MPA exposure compared with rats receiving vehicle. The observation that bilirubin concentrations were highest in rats who were treated with calcineurin inhibitors suggests that inhibition of alternative MPAG-excretory mechanisms may indeed have occurred in our experiment.

Another, not mutually exclusive, escape mechanism would be that of an increased secretion of MPAG into sinusoidal blood in the calcineurin inhibitor groups. It may be speculated that (induction of) MRP3/Mrp3 is involved. Mrp3 has a high affinity for glucuronide substrates and is known to be overexpressed in the EHBR, as well as in humans with the Dubin-Johnson syndrome who are deficient for MRP2.<sup>30-32</sup> MRP3/Mrp3 is located on the basolateral membrane of the hepatocyte and mediates the transport of organic anions from the hepatocyte into sinusoidal blood.<sup>32-34</sup> Xiong *et al.* reported an increased basolateral egress of acetaminophen-glucuronide in the TR- rat, suggesting up-regulation of an organic anion transporter on the basolateral membrane of Mrp2-deficient rat livers.<sup>35</sup>

A second mechanism that may have contributed to the differences in MPA/MPAG exposure between the vehicle and cyclosporine/tacrolimus-treated rats, could be an increased MPA clearance in the latter group. The observation that the MPA peak concentrations were lower in the rats treated with a calcineurin inhibitor is suggestive in this respect. High bilirubin or elevated MPAG plasma concentrations may result in a decreased binding of MPA to plasma albumin, thus increasing the free fraction of MPA, resulting in a more rapid conjugation of MPA to MPAG.<sup>2,36,37</sup> Alternatively, a lowering of serum albumin, as we observed in the cyclosporine-treated rats, could also have increased the free fraction of MPA. However, the reduction of serum albumin was limited and we feel that this mechanism is unlikely to have influenced the disposition of MPA or MPAG in our experiment to a significant degree.<sup>37</sup>

Finally, the observation that co-administration of MMF with cyclosporine or tacrolimus, lowered MPA peak concentrations compared with rats in the vehicle group, may also suggest that treatment with calcineurin inhibitors decreased the absorption of MPA. It has previously

been demonstrated that treatment with cyclosporine is associated with overexpression of P-glycoprotein in parenchymal cells of human kidney allografts.<sup>38</sup> This indicates that cyclosporine may induce its own detoxification by upregulation of P-glycoprotein expression in the kidney. Although Koziolk *et al.* did not investigate the expression levels of P-glycoprotein in the small intestine, increased P-glycoprotein levels would in theory result in a lowering of the oral bioavailability of P-glycoprotein substrates.<sup>38</sup>

In conclusion, our data provide evidence that the pharmacokinetic interaction between cyclosporine and MPA is caused by inhibition of Mrp2 by cyclosporine.

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PART 4

# **PHARMACOGENETIC STUDIES**



CHAPTER 4.1

**GENETIC  
POLYMORPHISMS OF  
THE *CYP3A4*, *CYP3A5*,  
AND *MDR-1* GENES AND  
PHARMACOKINETICS  
OF THE CALCINEURIN  
INHIBITORS  
CYCLOSPORINE AND  
TACROLIMUS**

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## ABSTRACT

**Background** The calcineurin inhibitors cyclosporine (INN, ciclosporin) and tacrolimus have a narrow therapeutic index and show considerable interindividual variability in their pharmacokinetics. The low oral bioavailability of calcineurin inhibitors is thought to result from the actions of the metabolizing enzymes cytochrome P450 (CYP) 3A4 and CYP3A5 and the multidrug efflux pump P-glycoprotein, encoded by *MDR-1*. **Objective** Our objective was to determine the role of genetic polymorphisms in *CYP3A4*, *CYP3A5* and *MDR-1* with respect to interindividual variability in cyclosporine and tacrolimus pharmacokinetics. **Methods** Kidney transplant recipients receiving cyclosporine (n = 110) or tacrolimus (n = 64) were genotyped for *CYP3A4*\*1B and \*3, *CYP3A5*\*3 and \*6, and *MDR-1* C3435T. Dose-adjusted trough levels were determined and correlated with the corresponding genotype. **Results** Tacrolimus dose-adjusted trough levels were higher in *CYP3A5*\*3/\*3 patients (n = 45) than in \*1/\*3 plus \*1/\*1 patients (n = 17), as follows: median and range, 94 (34-398) ng/mL per mg/kg versus 61 (37-163) ng/mL per mg/kg ( $P < 0.0001$ , Mann-Whitney test). *CYP3A4*\*1B allele carriers (n = 10) had lower tacrolimus dose-adjusted trough levels compared with those in patients with the wildtype (\*1/\*1) genotype (n = 54): median and range, 57 (40-163) ng/mL per mg/kg versus 89 (34-398) ng/mL per mg/kg ( $P = 0.003$ , Mann-Whitney test). No evidence was found supporting a role for the *MDR-1* C3435T polymorphism in tacrolimus dose-requirement. None of the polymorphisms studied correlated with cyclosporine dose-adjusted predose concentrations. **Conclusion** As a group, patients with the *CYP3A5*\*3/\*3 genotype require less tacrolimus to reach target predose concentrations compared with *CYP3A5*\*1 allele carriers, whereas *CYP3A4*\*1B carriers need more tacrolimus to reach target trough concentrations compared with *CYP3A4*\*1 homozygotes.

## INTRODUCTION

The calcineurin inhibitors cyclosporine (INN, ciclosporin) and tacrolimus are highly effective in preventing acute rejection after solid organ transplantation. However, both drugs have a narrow therapeutic index and show highly variable pharmacokinetics. In addition, calcineurin inhibitors have interactions with many other widely prescribed drugs that can lead to altered blood concentrations of cyclosporine or tacrolimus.<sup>1-4</sup> Cyclosporine and tacrolimus are dosed according to blood concentrations rather than bodyweight to avoid overimmunosuppression or underimmunosuppression. Currently, the parameter most widely used for therapeutic drug monitoring of calcineurin inhibitors is the predose, or trough, concentration ( $C_0$ ).<sup>5-7</sup>

The variability in the pharmacokinetics of calcineurin inhibitors is largely determined by differences in oral bioavailability. The oral bioavailability of cyclosporine and tacrolimus is poor and varies between patients or within a single patient over time.<sup>1-4</sup> In recent years much research has focused on the possible causes of these interindividual and intraindividual differences in the pharmacokinetics of calcineurin inhibitors. It has become clear that the biologic activity of the permeability-glycoprotein and the cytochrome P450 (CYP) enzyme system play an important role in this respect.<sup>8,9</sup> P-glycoprotein is the product of the multidrug-resistance 1 (*MDR-1*) gene. The protein serves as a transporter and is capable of pumping a wide variety of endogenous substances, as well as drugs (including cyclosporine and tacrolimus), from the cytoplasm to the exterior of the cell.<sup>8-10</sup> Physiologically, P-glycoprotein is present in the liver, kidney, adrenal gland, pancreas and the small intestine. In the small intestine P-glycoprotein is expressed at the apical surface of mature enterocytes, where it prevents the absorption of (possible toxic) xenobiotics from the intestinal lumen by active extrusion from the cell interior.<sup>8,9</sup> The CYP system is an enzyme family that is responsible for the oxidative metabolism of many molecules and consists of more than 50 isozymes. CYP3A is the subfamily that accounts for the metabolism of many drugs, including cyclosporine and tacrolimus.<sup>11,12</sup> The CYP3A subfamily consists of the isozymes CYP3A4,<sup>11</sup> CYP3A5,<sup>12,13</sup> and CYP3A7,<sup>14,15</sup> as well as the recently discovered CYP3A43.<sup>16</sup> CYP3A4 and CYP3A5 have largely overlapping substrate specificities. CYP3A4 is, like P-glycoprotein, abundantly and constitutively expressed in hepatic and intestinal epithelium, whereas CYP3A5 appears to be more variably expressed.<sup>8,9,11-13,17,18</sup> CYP3A7 is expressed mainly during fetal life.<sup>14,15,19</sup> CYP3A43 appears to have a low expression in the adult liver, but its relative contribution to drug metabolism is as yet unknown.<sup>16</sup>

Many drugs already undergo substantial metabolism in the intestine, after absorption from the gut lumen, in addition to metabolism in the liver.<sup>8,9,20</sup> CYP3A and P-glycoprotein are jointly present in the gut, and it is believed that the low oral bioavailability of calcineurin inhibitors results largely from the actions of these 2 enzymes.<sup>8,9,17,20-25</sup> The interindividual differences in the pharmacokinetics of calcineurin inhibitors have been attributed to interindividual heterogeneity in enzymatic activity of P-glycoprotein and CYP3A. However, the cause of this heterogeneity in enzymatic activity remains to be elucidated. Recently, a number of single-nucleotide polymorphisms (SNPs) were described for the *MDR-1*,<sup>26</sup> *CYP3A4*,<sup>27,28</sup> and *CYP3A5*<sup>18,29,30</sup> genes. The *C3435T* mutation in the *MDR-1* gene has been associated with

a decreased protein expression, whereas the *CYP3A5\*3* and the *CYP3A5\*6* alleles were found to cause alternative splicing and protein truncation, resulting in the absence of functional *CYP3A5* from liver tissue.<sup>29,30</sup> For the *CYP3A4\*1B* allele, an increased transcription was demonstrated *in vitro*, which would theoretically result in higher enzymatic activity *in vivo*.<sup>31</sup> Therefore these genetic polymorphisms may provide an explanation for the observed variability in calcineurin inhibitor pharmacokinetics. To determine the effects of *MDR-1* and *CYP3A* SNPs on the pharmacokinetics of calcineurin inhibitors, we analyzed 174 patients who underwent renal transplantation and were receiving maintenance treatment with either cyclosporine or tacrolimus. Cyclosporine and tacrolimus dose-adjusted  $C_0$  values were determined and correlated with the corresponding *MDR-1*, *CYP3A4*, and *CYP3A5* genotypes. Patients were analyzed for the *MDR-1 C3435T*,<sup>26</sup> *CYP3A4\*1B*,<sup>27</sup> *CYP3A4\*3*,<sup>28</sup> *CYP3A5\*3*,<sup>29</sup> and *CYP3A5\*6*<sup>29</sup> variant alleles.

## MATERIALS AND METHODS

### PATIENTS

All renal transplant recipients visiting the outpatient clinic of the Erasmus Medical Center, Rotterdam, The Netherlands, who had received a renal graft at least 1 year before the start of the study were eligible for entry into this study. During routine visits, blood samples were drawn for genotyping. Cyclosporine and tacrolimus dose and predose concentrations, as well as demographic and clinical data were obtained at 3 and 12 months ( $\pm 1$  month) after transplantation. Both tacrolimus and cyclosporine were given in 2 equally divided doses. All patients treated with cyclosporine used the microemulsion formulation (Neoral; Novartis Pharmaceuticals Corporation, Hanover, NJ). Patients taking medication known to interact with calcineurin inhibitors, such as calcium channel blockers (diltiazem, nifedipine, and verapamil), antiepileptics (phenytoin and carbamazepine), antimycotics (fluconazole and ketoconazole), and macrolide antibiotics (erythromycin and clarithromycin), were not eligible for entry into the study.

### ETHICS

The study was performed in accordance with the declaration of Helsinki and its amendments. The protocol was approved by the Ethics Committee of the Erasmus Medical Center, and written informed consent was obtained from all subjects.

### DRUG CONCENTRATION MEASUREMENTS

Cyclosporine and tacrolimus  $C_0$  values were determined in whole blood with the Emit 2000 assay (Syva company, Dade Behring Inc, Cupertino, CA) on a Cobas Mira Plus analyzer (Roche Diagnostic Systems, Basel, Switzerland). Details on the sensitivity and reproducibility of the Emit assay in our laboratory were published previously.<sup>32</sup> Proficiency samples were obtained from the United Kingdom Quality Assessment Scheme (Dr Holt, St George's



Hospital Medical School, London United Kingdom). Dose-adjusted predose concentrations were calculated by dividing the  $C_0$  by the corresponding 24-h dose on a milligrams per kilogram basis.

## **DEOXYRIBONUCLEIC ACID ISOLATION AND GENOTYPE DETERMINATION**

Genomic deoxyribonucleic acid was isolated from 200  $\mu$ L ethylenediaminetetraacetic acid-treated whole blood using a MagnaPure LC (Roche Diagnostics GmbH, Mannheim, Germany).

## **POLYMERASE CHAIN REACTION-RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS FOR CYP3A4\*1B AND \*3, CYP3A5\*3 AND \*6, AND MDR-1 (C3435T) VARIANT ALLELES**

Polymerase chain reaction (PCR)-restriction fragment length polymorphism analyses for *CYP3A4\*1B* and \*3 and *CYP3A5\*3* and \*6 were performed as described previously.<sup>33-35</sup> In brief, 50 ng of genomic deoxyribonucleic acid was used in a PCR volume of 50  $\mu$ L containing 1x buffer [10-mmol/L Tris-hydrochloric acid, pH 8.3; 1.5-mmol/L magnesium chloride; 50-mmol/L potassium chloride; and 0.001% (wt/vol) gelatin (Perkin-Elmer Inc, Wellesley, MA)], 0.2-mmol/L each deoxyribonucleoside triphosphate (Roche), 1.25 U of *AmpliTaq* Gold (Perkin-Elmer), and 40 pmol of each of forward and reverse primer. For *MDR-1*, forward primer 5'-CATGCTCCCAGGCTGTTTAT-3' and reverse primer 5'-GTAACCTGGCAGTTTCAGTG-3' were used. PCR conditions were as follows: 7 minutes at 94 °C; 35 cycles of 1 minute at 94 °C, 1 minute at 55 °C, and 1 minute at 72 °C; and finally 7 minutes at 72 °C. The PCR product (10  $\mu$ L) was digested with *Pst*I (*CYP3A4\*1B*), *Ssp*I (*CYP3A5\*3*), or *Dpn*II (*MDR-1*) in a total volume of 15  $\mu$ L for 2 h at 37 °C, and subsequently analyzed on agarose/Tris-borate-ethylenediaminetetraacetic acid gel with ethidium bromide staining.

## **STATISTICAL ANALYSIS**

Between-group differences were calculated by use of the Mann-Whitney *U* test and one-way ANOVA (Kruskal-Wallis test), followed by the Dunn post hoc test for multiple comparisons. A *P* value of  $\leq 0.05$  was considered statistically significant. All values are expressed as median and range unless stated otherwise.

## RESULTS

A total of 174 patients were included, of whom 110 were treated with cyclosporine and 64 were treated with tacrolimus. The characteristics of these patients are summarized in Table 4.1.1.

**Table 4.1.1** Characteristics of 174 renal transplant recipients treated with cyclosporine or tacrolimus

	Cyclosporine	Tacrolimus	All patients
Patients (male : female)	110 (69:41)	64 (34:30)	174 (103:71)
Transplantation number			
First	89	47	136
Second	15	14	29
Third or more	6	3	9
Primary kidney disease			
Glomerulonephritis	23	11	34
Chronic pyelonephritis	8	3	11
Immunoglobulin A nephropathy	6	4	10
Diabetic nephropathy	9	6	15
Hypertensive nephropathy	8	5	13
Polycystic kidney disease	15	13	28
Unknown	14	12	26
Other	27	10	37
Race			
Asian	12	6	18
Black	11	9	20
White	87	49	136

### **MDR-1 GENOTYPE**

Of the 110 patients treated with cyclosporine, the *MDR-1* genotype was determined in 109. The wildtype genotype (3435CC) was observed in 22 patients (20.2%), whereas 56 (51.4%) were heterozygous (3435CT) and 31 (28.4%) homozygous (3435TT) for the variant allele. There were no significant differences among the 3 groups in cyclosporine dose (mg/kg), cyclosporine  $C_0$  (ng/mL), or cyclosporine dose-adjusted  $C_0$  (ng/mL per mg/kg) at month 3 or 12 (Table 4.1.2). Of the 64 patients treated with tacrolimus, the wildtype genotype was present in 15 patients (23.4%), whereas 34 (53.2%) were heterozygous and 15 (23.4%) were homozygous for the variant allele. Again, no significant differences were observed among the 3 groups in tacrolimus dose (mg/kg), tacrolimus  $C_0$  (ng/mL) or tacrolimus dose-adjusted  $C_0$  (ng/mL per mg/kg) at month 3 or 12 (Table 4.1.2).

**Table 4.1.2** *MDR-1* genotype and dose requirement for cyclosporine (n = 109) or tacrolimus (n = 64) in 173 renal transplant recipients

	3435 CC	<i>MDR-1</i> genotype	
		3435 CT	3435 TT
Patients taking cyclosporine (No.)	22	56	31
Cyclosporine C <sub>0</sub> (ng/mL)			
3 months	217.5 (80-400)	217.5 (40-370)	210 (90-380)
12 months	145 (80-430)	140 (20-320)	125 (75-290)
Cyclosporine C <sub>0</sub> /dose (ng/mL per mg/kg)			
3 months	45.3 (16.0-82.6)	41.5 (10.4-78.0)	40.2 (19.1-87.6)
12 months	37.3 (14.9-84.3)	38.3 (9.2-142.9)	38.8 (22.0-79.5)
Patients taking tacrolimus (No.)	15	34	15
Tacrolimus C <sub>0</sub> (ng/mL)			
3 months	9.2 (4.7-19.7)	9.1 (5.1-17.5)	10.0 (7.2-14.5)
12 months	7.1 (4.3-9.4)	6.8 (4.0-19.2)	7.1 (3.0-17.1)
Tacrolimus C <sub>0</sub> /dose (ng/mL per mg/kg)			
3 months	66.8 (46.2-369.4)	86.8 (33.8-397.8)	91.2 (53.4-270.1)
12 months	80.4 (40.9-202.5)	96.8 (27.5-432.0)	100.9 (26.0-192.1)

No significant differences in dose requirement of either drug were observed between patients with the 3 *MDR-1* genotypes. All values are expressed as median with range in parentheses. C<sub>0</sub>, Predose or trough concentration.

### CYP3A4 GENOTYPE

For patients treated with cyclosporine, the *CYP3A4* wildtype genotype (*\*1/\*1*) was observed in 94 patients (87.1%), whereas 9 (8.3%) were heterozygous and 5 (4.6%) were homozygous for the *CYP3A4\*1B* allele. The *CYP3A4\*3* allele was not found among patients taking cyclosporine. For the *CYP3A4\*1/\*1B* polymorphism, no significant differences in cyclosporine dose (mg/kg), cyclosporine C<sub>0</sub> (ng/mL), or cyclosporine dose-adjusted C<sub>0</sub> (ng/mL per mg/kg) were observed among individuals with the *CYP3A4\*1/\*1*, *CYP3A4\*1/\*1B* and *CYP3A4\*1B/\*1B* genotypes at 3 and 12 months after transplantation (Table 4.1.3).

Of the 64 patients treated with tacrolimus, 7 (10.9%) were heterozygous and 3 (4.7%) were homozygous for the variant *CYP3A4\*1B* allele. A trend was observed toward a lower dose-adjusted C<sub>0</sub> in patients heterozygous or homozygous for the *CYP3A4\*1B* allele, as compared with the *CYP3A4\*1/\*1* genotype ( $P = 0.01$ , one-way ANOVA; data not shown). When patients carrying the *CYP3A4\*1B* allele were compared to patients with the *CYP3A4\*1/\*1* genotype, a significant difference in tacrolimus dose-adjusted C<sub>0</sub> was found, as follows: 57.0 ng/mL per mg/kg versus 89.3 ng/mL per mg/kg, respectively;  $P = 0.003$  (Table 4.1.3 and Figure 4.1.1A). This difference remained statistically significant at month 12 (Table 4.1.3). These findings are in line with the assumption that carriers of a *CYP3A4\*1B* allele will display higher *CYP3A4* activity as a result of increased expression of this allele. Because tacrolimus C<sub>0</sub> was similar in both groups at month 3 and month 12 ( $P = 0.84$  and  $P = 0.57$ , respectively) (Table 4.1.3), the observed differences in tacrolimus dose-adjusted C<sub>0</sub> are explained by a significantly higher tacrolimus dose requirement in patients with the *CYP3A4\*1B* allele compared with patients

with the *CYP3A4*\*1/\*1 genotype at month 3 [0.18 (0.04-0.27) mg/kg versus 0.09 (0.03-0.23) mg/kg,  $P = 0.01$ ] and at month 12 [0.10 (0.04-0.23) mg/kg versus 0.06 (0.03-0.21) mg/kg,  $P = 0.03$ ]. Two patients taking tacrolimus carried the *CYP3A4*\*3 allele. The tacrolimus dose-adjusted  $C_0$  in these 2 patients was higher than that in patients with the wildtype genotype ( $n = 62$ ), as follows: 134.9 (106.6-163.2) ng/mL per mg/kg versus 83.83 (33.83-397.8) ng/mL per mg/kg, respectively. This difference was also observed at 12 months after transplantation, as follows: 147.3 (117.0-177.5) ng/mL per mg/kg versus 88.8 (26.0-432.0) ng/mL per mg/kg. Because of the small number of *CYP3A4*\*3 variant alleles, the relationship between tacrolimus dose-adjusted  $C_0$  and the *CYP3A4*\*3 allele could not be analyzed statistically.

**Table 4.1.3** *CYP3A4* genotype and dose requirement for cyclosporine ( $n = 108$ ) or tacrolimus ( $n = 64$ ) in 172 renal transplant recipients

	*1/*1	<i>CYP3A4</i> genotype *1/*1B plus *1B/*1B	$P$ value <sup>§</sup>
Patients taking cyclosporine (No.)	94	14	
Cyclosporine $C_0$ (ng/mL)			
3 months	210 (40-400)	217 (65-370)	NS
12 months	137.5 (20-430)	135 (85-190)	NS
Cyclosporine $C_0$ /dose (ng/mL per mg/kg)			
3 months	41.5 (10.4-87.6)	41.7 (17.9-68.2)	NS
12 months	38.3 (9.2-142.9)	35.4 (21.1-69.0)	NS
Patients taking tacrolimus (No.)	54	10	
Tacrolimus $C_0$ (ng/mL)			
3 months	9.2 (5.1-19.7)	10.3 (4.7-13.9)	NS
12 months	7.0 (3.0-19.2)	7.5 (4.0-9.4)	NS
Tacrolimus $C_0$ /dose (ng/mL per mg/kg)			
3 months	89.3 (33.8-397.8)	57.0 (39.5-163.2)	0.003
12 months	107.0 (26.0-432.0)	65.5 (28.0-177.5)	0.027

A significant difference in tacrolimus dose requirement was observed between patients carrying the variant *CYP3A4*\*1B allele and patients with the *CYP3A4*\*1/\*1 genotype. For cyclosporine no such difference was observed. All values are expressed as median with range in parentheses.

NS, Not significant.

<sup>§</sup>Two-tailed, Mann-Whitney  $U$  test.

### **CYP3A5 GENOTYPE**

The majority of the patients who were treated with cyclosporine were homozygous for the *CYP3A5*\*3 variant allele [ $n = 78$  (71.6%)] and are thus expected to lack *CYP3A5* activity. It was determined that 26 patients (23.8%) carried 1 *CYP3A5*\*1 allele, and the *CYP3A5*\*1/\*1 genotype was observed in 5 patients (4.6%). Of the 31 *CYP3A5*\*1 allele carriers, 5 were also heterozygous for the *CYP3A5*\*6 variant allele. It is difficult to assess whether these patients are compound heterozygotes (with no active *CYP3A5*) or whether they carry both polymorphisms on the same allele (leaving 1 active *CYP3A5* allele). Therefore, we excluded these 5 patients from further analysis. There were no significant differences between the 3

CYP3A5 genotypes in cyclosporine dose (mg/kg), cyclosporine  $C_0$  (ng/mL) or cyclosporine dose-adjusted  $C_0$  (ng/mL per mg/kg) at month 3 or 12 (Table 4.1.4). Moreover, no significant differences were found when we compared carriers of the CYP3A5\*1 allele to CYP3A5\*3 homozygotes.

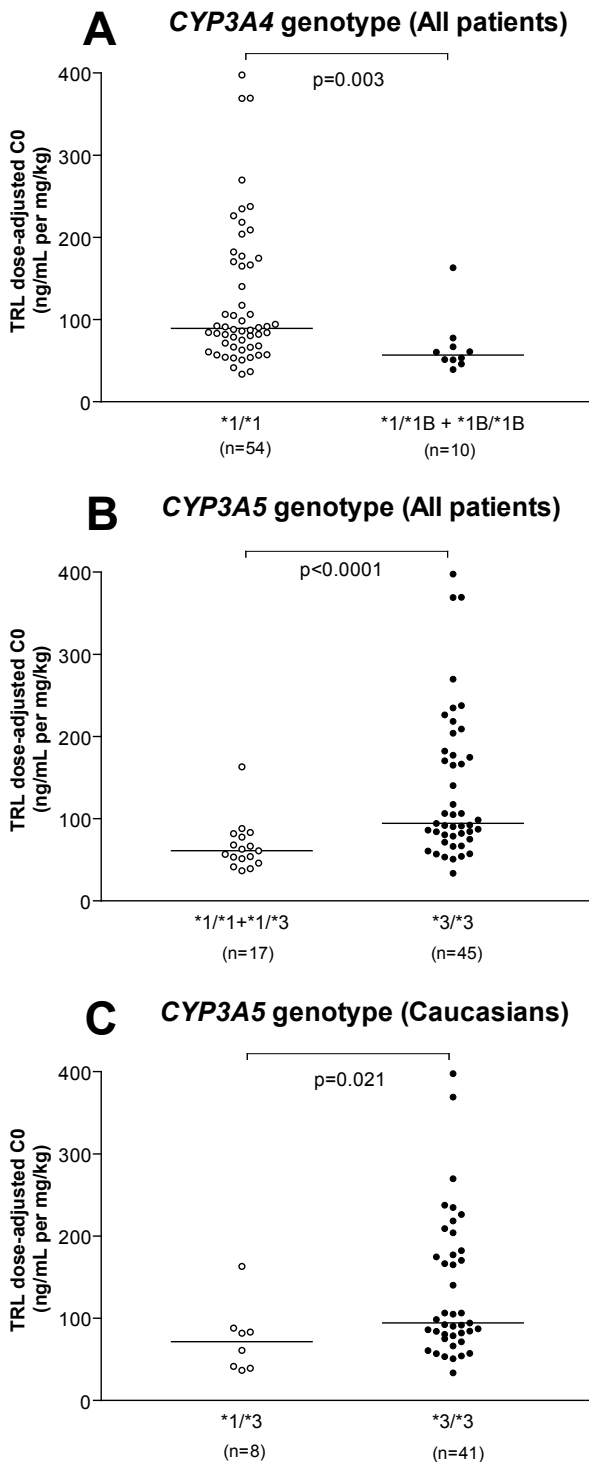
The CYP3A5\*1/\*1 genotype was observed in 3 (4.7%) of the 64 tacrolimus-treated patients, whereas 16 patients (25%) were heterozygous and 45 homozygous (70.3%) for the CYP3A5\*3 variant allele. Of the 19 patients carrying a wildtype allele, 2 were heterozygous for the CYP3A5\*6 allele. These patients were not analyzed further. A significant difference was found in tacrolimus dose (mg/kg) and tacrolimus dose-adjusted  $C_0$  (ng/mL per mg/kg) among the 3 groups, as shown in Table 4.1.4 and Figure 4.1.1B. A significantly lower tacrolimus dose-adjusted  $C_0$  was found in patients carrying a CYP3A5\*1 allele compared to patients with the CYP3A5\*3/\*3 genotype, both at month 3 and month 12 after transplantation. Again, because the tacrolimus  $C_0$  was not significantly different among the 3 groups, the observed differences in tacrolimus dose-adjusted  $C_0$  can only be explained by a higher dose requirement for patients carrying a wildtype allele. According to the expectation that CYP3A5\*1 allele carriers have CYP3A5 enzyme activity, the CYP3A5\*1/\*1 and \*1/\*3 groups were analyzed against the CYP3A5\*3/\*3 group. The earlier observed difference in tacrolimus dose-adjusted  $C_0$  remained highly significant: 61.0 ng/mL per mg/kg versus 94.4 ng/mL per mg/kg,  $P < 0.0001$  (Table 4.1.4 and Figure 4.1.1B).

**Table 4.1.4** CYP3A5 genotype and dose requirement for cyclosporine (n = 104) or tacrolimus (n = 62) in 166 renal transplant recipients (patients carrying a CYP3A5\*6 allele were excluded)

	CYP3A5		
	*1/*1	*1/*3	*3/*3
Patients taking cyclosporine (No.)	4	22	78
Cyclosporine $C_0$ (ng/mL)			
3 months	205 (160-340)	217.5 (40-370)	207.5 (45-400)
12 months	170 (145-225)	132.5 (75-270)	135 (20-430)
Cyclosporine $C_0$ /dose (ng/mL per mg/kg)			
3 months	42.6 (33.2-57.0)	41.3 (10.4-78.0)	40.7 (14.4-87.6)
12 months	46.3 (33.7-59.4)	33.1 (14.9-70.2)	38.3 (9.2-143.0)
Patients taking tacrolimus (No.)	2	15	45
Tacrolimus $C_0$ (ng/mL)			
3 months	9.7 (7.7-11.7)	9.1 (5.7-15.7)	9.4 (4.7-19.7)
12 months	7.5 (6.9-8.0)	7.2 (4.0-10.6)	7.0 (3.0-19.2)
Tacrolimus $C_0$ /dose (ng/mL per mg/kg)			
3 months	67.3 (56.8-77.8)	61.0 (36.8-163.2)	94.4 (33.8-397.8) §
12 months	78.8 (73.6-84.0)	57.6 (27.5-177.5)	124.2 (26-432.0) §

Patients carrying a CYP3A5\*1 allele require a significantly higher tacrolimus dose to reach similar tacrolimus predose concentrations as compared with patients homozygous for the CYP3A5\*3 allele. For cyclosporine no such difference was observed. All values are expressed as median with range in parentheses.

§  $P < 0.001$ , CYP3A5\*1/\*3 versus CYP3A5\*3/\*3, Kruskal-Wallis test with Dunn's multiple comparison test.



**Figure 4.1.1** Tacrolimus (TRL) dose-adjusted predose concentration ( $C_0$ ) and *CYP3A* genotype at month 3 after transplantation in 64 renal transplant recipients. A significant difference in tacrolimus dose requirement was observed between patients carrying the *CYP3A4*\*1*B* allele and patients with the *CYP3A4*\*1/\*1 genotype ( $P = 0.003$ , Mann-Whitney  $U$  test; panel A). A significant difference in tacrolimus dose-adjusted  $C_0$  was also observed between patients carrying the *CYP3A5*\*1 allele and patients homozygous for *CYP3A5*\*3 ( $P < 0.0001$ , Mann-Whitney  $U$  test; panel B) (patients carrying a *CYP3A5*\*6 allele were excluded from the analysis). When white patients were analyzed separately, this difference in tacrolimus dose requirement was still present ( $P = 0.021$ , Mann-Whitney  $U$  test; panel C).

## INFLUENCE OF ETHNICITY

The Dutch transplant patient population consists mainly of patients of Caucasian descent. This is reflected by the large cohort of white patients in our study population [136/174 patients (78.2%)]. Only 20 patients (11 in the cyclosporine and 9 in the tacrolimus group) were black, with the remaining 18 patients being of Asian descent. The low number of patients in the last 2 groups indicates that conclusions regarding the influence of ethnicity should be interpreted with caution. Nevertheless, we performed all previously mentioned analyses for each ethnic group separately. With ethnic diversity taken into account, no influence of the *MDR-1* genotype was found, for either cyclosporine or tacrolimus. The previously discovered correlation of the *CYP3A4\*1B* allele with tacrolimus dose requirement was lost, probably because of the limited number of *CYP3A4\*1B* allele carriers (3 white and 7 black patients, respectively; data not shown). However, the reported correlation of the *CYP3A5* genotype with tacrolimus dose requirement remained significant in the white population, both at month 3 and at month 12 after transplantation ( $P = 0.021$  and  $P = 0.01$ , respectively) (Figure 4.1.1C). It is interesting that, for white patients taking cyclosporine, a significant influence of *CYP3A5* genotype on cyclosporine dose requirement was now found but only at month 12 after transplantation, whereas this difference was not present for the whole study population [*CYP3A5\*1/\*3* ( $n = 14$ ) versus *CYP3A5\*3/\*3* ( $n = 71$ ); median and range, 27.85 (14.93-69.0) ng/mL per mg/kg versus 38.03 (9.2-142.9) ng/ml per mg/kg;  $P = 0.03$ ].

## DISCUSSION

The clinical use of calcineurin inhibitors is complicated by their narrow therapeutic index and highly variable and unpredictable pharmacokinetics in individual patients. Although therapeutic drug monitoring is routinely performed for this class of drugs, both acute and chronic calcineurin inhibitor toxicity occur in everyday clinical practice. Moreover, some patients do not reach target concentrations with recommended starting doses of calcineurin inhibitors and therefore have an increased risk of underimmunosuppression and acute rejection.<sup>1-4</sup> The role of P-glycoprotein and the CYP3A enzymes in calcineurin inhibitor pharmacokinetics has been recognized for some time, but until the recent identification of a number of SNPs in the *MDR-1* and *CYP3A* genes, the (genetic) basis for the observed interindividual differences in pharmacokinetics was unclear.<sup>8,9,26,27,29</sup>

We observed a highly significant association between tacrolimus dose requirement and the *CYP3A5\*1/\*3* polymorphism. Our results show that a lower tacrolimus dose is required to reach target concentrations in patients homozygous for *CYP3A5\*3* compared with *CYP3A5\*1* allele carriers. This is in agreement with the fact that the *CYP3A5\*3* allele results in the loss of hepatic CYP3A5 activity.<sup>29,30</sup> Moreover, it may explain the recently published findings of MacPhee *et al.*<sup>36</sup> who suggested that a SNP in the *CYP3API* pseudogene (A/G<sub>-44</sub>) is strongly correlated with tacrolimus dose requirement. Although the *CYP3API* pseudogene is strongly associated with hepatic CYP3A5 activity, being a pseudogene, it cannot be responsible for polymorphic expression. In fact, the results of MacPhee *et al.* may have been caused by linkage of the *CYP3API* pseudogene to the *CYP3A5\*1/\*3* SNP we studied.<sup>29,36</sup> Our findings could be of clinical importance because patients carrying the *CYP3A5\*1* allele may theoretically run

the risk of underimmunosuppression and, subsequently, of acute rejection. In our patient cohort the incidence of biopsy-proven acute rejection was not significantly different between *CYP3A5\*1* allele carriers and patients with the *CYP3A5\*3/\*3* genotype (23.5% versus 20.0%, respectively;  $P = 0.74$  Fisher's exact test). However, this was a cross-sectional study in a selected group of patients with a graft survival of at least 1 year and no severe drug toxicity necessitating discontinuation of tacrolimus. A prospective study is needed to further address these possible clinical consequences. Eventually, determination of the *CYP3A5* genotype before transplantation may identify patients at risk for underimmunosuppression or toxicity and alert clinicians to a high likelihood of development of drug levels outside target ranges. It is tempting to speculate that our results provide a genetic basis for the poorer outcome after transplantation in black patients. One of the factors that possibly explains this observation is the fact that the oral bioavailability of tacrolimus (and cyclosporine) is lower in black kidney transplant recipients as compared with white patients.<sup>37,38</sup> Indeed, in our study group the allelic frequency of *CYP3A5\*1* was much higher in patients of African origin compared with white patients, which is fully consistent with the literature.<sup>29,30</sup>

A relationship between *CYP3A4* genotype and cyclosporine dose requirement could not be demonstrated in our study, confirming the results of 2 previous studies.<sup>39,40</sup> These findings argue against a major influence of the *CYP3A4\*1B* promoter variant allele on cyclosporine pharmacokinetics. In contrast, our results are the first to show that patients carrying the *CYP3A4\*1B* allele require a higher tacrolimus dose to reach target concentrations. Until now, the effect of the *CYP3A4\*1B* allele on *CYP3A4* activity remained controversial. From clinical studies, it was postulated that the *CYP3A4\*1B* allele results in a decreased enzymatic activity.<sup>27,41</sup> However, subsequent microsomal studies could not confirm an effect of the *CYP3A4\*1B* allele on enzymatic function,<sup>42,43</sup> whereas a higher *CYP3A4* expression of the *CYP3A4\*1B* allele was reported *in vitro*.<sup>31</sup> The fact that we observed a correlation between the *CYP3A4* genotype and tacrolimus, but not cyclosporine, dose requirement, could be explained by the fact that the molecular structure of these 2 drugs is entirely different.<sup>4</sup> We believe that this is an unlikely mechanism, because the *CYP3A4\*1/\*1B* SNP is located in the 5' transcriptional regulatory element of the *CYP3A4* coding region, more likely causing changes in protein expression rather than activity. Alternatively, we may not have detected an effect of the *CYP3A4\*1B* allele on cyclosporine pharmacokinetics. The variability in the pharmacokinetics of orally administered calcineurin inhibitors is largely determined by interindividual differences in first-pass elimination. However, the (dose-adjusted)  $C_0$  value reflects clearance from the systemic circulation rather than first-pass extraction. An effect of the *CYP3A4\*1B* allele on tacrolimus, but not cyclosporine, dose requirement, could have been found because the tacrolimus  $C_0$  correlates better with total drug exposure (as measured by the tacrolimus 12-h area-under the concentration versus time-curve) than does the cyclosporine  $C_0$ .<sup>6,7</sup>

Another issue that needs to be addressed is the combined effect of the genetic polymorphisms in *CYP3A4* and *CYP3A5* on tacrolimus pharmacokinetics. Because tacrolimus is metabolized by both *CYP3A4* and *CYP3A5*, this dual pathway partially obscures the (clinical) effects of genetic polymorphisms of either enzyme. In our population, 80% of the patients homozygous for *CYP3A5\*3* were homozygous for *CYP3A4\*1* (associated with a lower tacrolimus dose



requirement in comparison with that of *CYP3A4\*1B* allele carriers). It is interesting that we also found linkage between the *CYP3A5\*3* and *CYP3A4\*1* alleles in 500 white patients who were described previously (references 33 and 35; van Schaik *et al.*, unpublished observations). Recently, linkage between these two SNPs was described by a second group.<sup>44</sup>

Finally, our results do not demonstrate an association between cyclosporine or tacrolimus dose requirement and the *C3435T* polymorphism in exon 26 of the *MDR-1* gene. Although it was originally associated with lower duodenal P-glycoprotein expression in patients homozygous for the variant allele,<sup>26</sup> a later study found no significant correlation between the *C3435T* polymorphism and cyclosporine  $C_0$ .<sup>39</sup> Nevertheless, these results should be interpreted with some caution. Intestinal P-glycoprotein content accounts for 30% of the interindividual variability in peak blood concentrations of orally administered cyclosporine but has markedly less effect on the variation in oral cyclosporine clearance.<sup>45</sup> By analyzing the full pharmacokinetic profiles of 14 healthy volunteers, Min and Ellingrod<sup>46</sup> found that individuals carrying the variant *MDR-1 C3435T* allele had a 15% higher peak blood concentration and a 22% higher area-under the concentration *versus* time-curve from 0 to 24 h.<sup>46</sup> However, these differences failed to reach statistical significance, possibly because of the limited number of *MDR-1 C3435TT* homozygotes.

In conclusion, our study demonstrates for the first time that a strong correlation exists between the *CYP3A* genotype and tacrolimus dose requirement. Patients carrying a *CYP3A5\*1* allele require significantly more tacrolimus to reach target concentrations compared to *CYP3A5\*3* homozygotes and may thus have a higher likelihood of underimmunosuppression and acute rejection. Carriers of the *CYP3A4\*1B* allele also required more tacrolimus to reach adequate whole blood levels compared with patients with the *CYP3A4\*1/\*1* genotype, although its exact contribution needs to be elucidated because of linkage of the *CYP3A4\*1* allele to the *CYP3A5\*3* allele. No evidence was found supporting a role for the *MDR-1 C3435T* SNP in the pharmacokinetics of calcineurin inhibitors.

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## CHAPTER 4.2

# **POPULATION PHARMACOKINETICS OF CYCLOSPORINE IN KIDNEY AND HEART TRANSPLANT RECIPIENTS AND THE INFLUENCE OF ETHNICITY AND GENETIC POLYMORPHISMS IN THE *MDR-1, CYP3A4, AND CYP3A5* GENES**

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## ABSTRACT

*Objective* Our objective was to determine the relationship between single-nucleotide polymorphisms (SNPs) in the multidrug resistance 1 (*MDR-1*) gene and the cytochrome P450 (CYP) genes *CYP3A4* and *CYP3A5* and the pharmacokinetics of cyclosporine (INN, ciclosporin). *Methods* Cyclosporine pharmacokinetics of 151 kidney and heart transplant recipients undergoing maintenance therapy was described by use of nonlinear mixed-effects modeling (NONMEM) according to a 2-compartment pharmacokinetic model with first-order absorption and elimination. All patients were genotyped for the *CYP3A4\*1B* and \*3, *CYP3A5\*3* and \*6, and *MDR-1* 3435C→T SNPs. *Results* For a typical 70-kg white patient, the following parameters were estimated: absorption rate constant, 1.27 h<sup>-1</sup>; absorption time lag, 0.47 h; oral volume of distribution of the central and peripheral compartment, 56.3 and 185.0 L, respectively; oral clearance (Cl/F), 30.7 L/h; and oral intercompartmental clearance, 31.7 L/h. Estimated interpatient variability of Cl/F was 28%. Cl/F was significantly correlated with weight and ethnicity; Cl/F was 13% higher (95% confidence interval, 8%-18%;  $P < 0.005$ ) in white patients than in black and Asian patients. In carriers of a *CYP3A4\*1B* variant allele, Cl/F was 9% (95% confidence interval, 1%-17%;  $P < 0.05$ ) higher compared with *CYP3A4\*1* homozygotes, and this effect was independent of ethnicity or weight. Incorporation of these covariates into the NONMEM model did not markedly reduce interpatient variability of Cl/F. None of the other SNPs studied significantly influenced any of the pharmacokinetic parameters. *Conclusion* Patients carrying a *CYP3A4\*1B* variant allele have a significantly higher oral cyclosporine clearance compared with patients homozygous for *CYP3A4\*1*. However, this genetic effect on cyclosporine disposition was small, and genotyping of transplant recipients for *CYP3A4* is thus unlikely to assist in planning initial cyclosporine dosing.



## INTRODUCTION

The clinical use of the immunosuppressive agent cyclosporine (INN, ciclosporin) is hampered by its many side effects, narrow therapeutic index, and highly variable and unpredictable pharmacokinetics, as well as the frequent occurrence of drug interactions.<sup>1</sup> Therapeutic drug monitoring has, therefore, been adopted by most transplant physicians as a means to improve the efficacy and reduce the toxicity of cyclosporine treatment. Pharmacogenetics has the potential to assist in determining the starting dose of immunosuppressive drugs.<sup>2</sup> For renal transplant recipients, it has been demonstrated that early adequate exposure to such agents is critical and that failure to reach target concentrations as early as the third postoperative day may result in acute rejection.<sup>3</sup>

The multidrug resistance 1 (*MDR-1*) gene encodes P-glycoprotein, an adenosine triphosphate-dependent transmembrane transporter that is capable of pumping a wide variety of endogenous substances, xenobiotics, and drugs from the cytoplasm to the exterior of the cell. Cytochrome P450 (CYP) 3A4 and CYP3A5 are enzymes with overlapping substrate specificities that are largely responsible for the phase I metabolism of the majority of drugs currently in use.<sup>2</sup> Physiologically, P-glycoprotein and the CYP3A enzymes are expressed in human liver and gut, where they act synergistically to limit systemic exposure to a large number of xenobiotics and drugs. In the small intestine, P-glycoprotein limits the absorption of these substances by active extrusion from the enterocyte interior back into the gut lumen. The oral bioavailability of many drugs is reduced further by intestinal metabolism by CYP3A. In addition, *CYP3A* and *MDR-1* expression in liver and biliary canaliculi is largely responsible for systemic drug clearance.<sup>4</sup>

There exist marked interindividual differences in *MDR-1* and *CYP3A* expression that may be attributed to a number of recently discovered single-nucleotide polymorphisms (SNPs) in the *MDR-1* and *CYP3A* genes. The *MDR-1* 3435C→T SNP was originally associated with a decreased protein expression in individuals carrying a T allele, resulting in higher probe drug blood concentrations.<sup>5-7</sup> A large number of drugs, including anticancer agents, antiarrhythmics, and antiviral drugs, are P-glycoprotein substrates; therefore polymorphic expression of this drug transporter potentially has an important clinical impact. However, the exact functional significance of this SNP is still a matter for debate (for a review, see reference 8). For *CYP3A5*, the *CYP3A5*\*3 and the *CYP3A5*\*6 variant alleles have been described, which cause alternative splicing and protein truncation, resulting in the absence of functional CYP3A5 from liver tissue.<sup>9,10</sup> For the *CYP3A4*\*1B variant allele, an increased transcription has been demonstrated *in vitro*.<sup>11</sup>

Cyclosporine and its counterpart tacrolimus are both P-glycoprotein and CYP3A substrates,<sup>12-14</sup> and their well-recognized pharmacokinetic variability is explained in part by marked interindividual heterogeneity in intestinal and hepatic *MDR-1* and *CYP3A* expression.<sup>15-19</sup> Several groups have studied the association between the above-mentioned SNPs and tacrolimus dose requirement in renal,<sup>20-24</sup> heart,<sup>25</sup> liver,<sup>26</sup> and lung transplant recipients.<sup>27</sup> In summary, the tacrolimus dose requirement was consistently found to be higher in *CYP3A5* expressors, whereas the *MDR-1* genotype appears to have a minor or no effect on tacrolimus pharmacokinetics. Interestingly, several large cohort studies in renal

transplant recipients could not detect a correlation between these SNPs and the cyclosporine dose requirement.<sup>22,28-30</sup> A possible explanation for these conflicting results could be that in most studies the effect of genotype on cyclosporine dose requirement was investigated by use of the cyclosporine predose concentration ( $C_0$ ), which has a notoriously poor correlation with total drug exposure.<sup>3</sup> For tacrolimus, the  $C_0$  appears to reflect total tacrolimus exposure much better.<sup>31</sup> A possible effect of the *MDR-1* or *CYP3A* genotype on cyclosporine dose requirement could thus have remained undetected.

In this study we investigated the relationship between cyclosporine pharmacokinetics and *MDR-1* and *CYP3A* genotypes in more detail. The presence of the *MDR-1* 3435C→T,<sup>5</sup> *CYP3A4*\*1B,<sup>32</sup> *CYP3A4*\*3,<sup>33</sup> *CYP3A5*\*3, and *CYP3A5*\*6,<sup>9,10</sup> variant alleles was determined in 151 kidney and heart transplant recipients who were all treated with cyclosporine. Pharmacokinetic analysis was carried out by use of nonlinear mixed-effects modeling (NONMEM). In contrast to more classic pharmacokinetic analysis methods (*i.e.*, noncompartmental method), this technique has the advantage that individual pharmacokinetic parameters can be assessed when only a limited number of blood concentrations are available for each patient.

## METHODS

### PATIENTS

All patients described herein participated in 1 of 2 studies. For the current study, the demographic, clinical, pharmacokinetic, and pharmacogenetic data of these patients were pooled and analyzed together. The design and aims of these 2 studies were as follows:

*Study 1.* The aim of study 1 was to investigate the relationship between cyclosporine exposure and the presence of cyclosporine-related side effects in solid organ transplant recipients receiving maintenance therapy (Hesselink DA, unpublished data, 2004). All heart, kidney, and liver transplant recipients visiting our outpatient clinic were asked to participate in the study. Inclusion criteria were (1) unchanged cyclosporine dose within the last 3 months before entry into the study, (2) stable graft function, (3) use of the cyclosporine microemulsion formulation (Neoral; Novartis Pharmaceuticals Corporation, Hanover, NJ), and (4) no treatment with drugs known to interact with cyclosporine at the *CYP3A* or *MDR-1* level. The use of azathioprine, mycophenolate mofetil, or prednisone as part of the immunosuppressive regimen was allowed. In all patients the cyclosporine exposure was determined by drawing blood at 0, 1, 2, 3 and 4 h after oral administration of the normal morning dose. Cyclosporine pharmacokinetics was then compared between patients with and patients without cyclosporine-related side effects. In addition, genotyping for *MDR-1*, *CYP3A4*, and *CYP3A5* was performed in all patients as an integral part of the study. The *CYP3A* and *MDR-1* genotype of the transplanted organs was not determined. Because cyclosporine is metabolized by hepatic and intestinal *CYP3A* enzymes and its metabolites are almost exclusively excreted by the biliary route (around 95%), we only included the data from the heart ( $n = 28$ ) and renal allograft recipients ( $n = 26$ ) in this study.

*Study 2.* The objective of study 2 was to determine the role of SNPs in *MDR-1*, *CYP3A4*, and *CYP3A5* with respect to interindividual variability in cyclosporine and tacrolimus dose requirement. The results of this study were published recently.<sup>22</sup> In brief, all renal transplant recipients who were examined in the outpatient clinic of our hospital and had received a renal allograft at least 1 year before the start of the study were asked to participate. During routine visits, blood samples were drawn for genotyping. Cyclosporine and tacrolimus dose and predose concentrations, as well as demographic and clinical data, were recorded at 3 and 12 months ( $\pm 1$  month) after transplantation. Again, all patients were treated with microemulsified cyclosporine and did not take any drugs known to interact with cyclosporine. Patients were included if their cyclosporine dose remained unchanged for at least 1 week before predose concentration sampling at the 3- and 12-month time points.

## ETHICS

Both studies were performed in accordance with the declaration of Helsinki and its amendments. Protocols were approved by the Ethics Committee of the Erasmus Medical Center, Rotterdam, The Netherlands, and written informed consent was obtained from all subjects.

## DEOXYRIBONUCLEIC ACID ISOLATION AND POLYMERASE CHAIN REACTION-RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS

Polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis for *CYP3A4\*1B* and \*3, *CYP3A5\*3* and \*6, and *MDR-1* 3435C>T variant alleles was performed as described previously.<sup>22,34-36</sup> In brief, genomic deoxyribonucleic acid was isolated from 200  $\mu$ L ethylenediaminetetraacetic acid-treated-whole blood by use of a total nucleic acid extraction kit on a MagnaPure LC (Roche Diagnostics GmbH, Mannheim, Germany). Next, 50 ng of genomic deoxyribonucleic acid was used in a PCR volume of 50  $\mu$ L containing 1x buffer [10-mmol/L Tris-hydrochloric acid, pH 8.3; 1.5-mmol/L magnesium chloride; 50-mmol/L potassium chloride; and 0.001% (wt/vol) gelatin (Perkin-Elmer Inc, Wellesley, MA)], 0.2-mmol/L each deoxyribonucleoside triphosphate (Roche Diagnostics GmbH), 1.25 U of *AmpliTaq* Gold (Perkin-Elmer), and 40 pmol of each of forward and reverse primer (described in references 22 and 34-36). PCR conditions were as follows: 7 minutes at 94°C; 35 cycles of 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C; and finally, 7 minutes at 72°C. The PCR product (10  $\mu$ L) was digested with *Pst*I (*CYP3A4\*1B*), *Ssp*I (*CYP3A5\*3*), or *Dpn*II (*MDR-1*) in a total volume of 15  $\mu$ L for 2 h at 37°C and subsequently analyzed on agarose/Tris-borate-ethylenediaminetetraacetic acid gel with ethidium bromide staining. Because the *CYP3A5\*3* and *CYP3A5\*6* variant alleles both result in the absence of functional CYP3A5 protein from liver,<sup>9,10</sup> the *CYP3A5\*6* allele was only determined when patients carried at least 1 *CYP3A5\*1* (wildtype) allele.

## CYCLOSPORINE CONCENTRATION MEASUREMENTS

Cyclosporine whole-blood concentrations were determined with the Emit 2000 assay (Syva company, Dade Behring Inc, Cupertino, CA) on a Cobas Mira Plus analyzer (Roche Diagnostic Systems, Basel, Switzerland). Proficiency samples were obtained from the United Kingdom Quality Assessment Scheme (Dr Holt, St George's Hospital Medical School, London, United Kingdom).

## PHARMACOKINETIC ANALYSIS

Pharmacokinetic models were fitted to data from all individuals simultaneously by use of NONMEM. The NONMEM model accounts for interpatient and residual pharmacokinetic variability (random effects), as well as pharmacokinetic differences predicted by patient factors (fixed effects). The typical population parameters, interpatient and residual variances, were estimated by use of the NONMEM software program (double precision; version V, level 1.1). The first-order method was used throughout the analysis. The pharmacokinetics of cyclosporine was described according to compartmental models, which were parameterized in terms of clearance and volume with rate constants used only to describe the absorption process. For instance, for a 2-compartment pharmacokinetic model with first-order absorption and elimination, the following parameters were estimated: absorption rate constant ( $K_a$ ), time lag between intake and start absorption ( $T_{lag}$ ), volume of distribution of the central compartment ( $V1$ ), clearance from the central compartment ( $Cl$ ), volume of distribution of the peripheral compartment ( $V2$ ), and intercompartmental clearance ( $Q$ ). Because cyclosporine was administered orally, the terms *volume of distribution* and *clearance* represent the ratios of these parameters to the unknown bioavailability [oral volume of distribution of central compartment ( $V1/F$ ), oral volume of distribution of peripheral compartment ( $V2/F$ ), oral clearance from central compartment ( $Cl/F$ ), and oral intercompartmental clearance ( $Q/F$ )]. Simultaneous analysis of the data from all patients requires statistical models for interpatient and residual variances. Interpatient variability of the pharmacokinetic parameters was estimated by use of a proportional error model. For instance, interindividual variability in  $Cl/F$  was estimated as follows:

$$Cl/F_i = Cl/F_{pop} (1 + \eta_i)$$

in which  $i$  represents the number of the individual,  $Cl/F_i$  is the clearance of the  $i^{th}$  individual,  $Cl/F_{pop}$  is the  $Cl/F$  value of a typical individual, and  $\eta$  is the interindividual random effect with mean 0 and variance  $\omega^2$ . In addition to interpatient variability of the pharmacokinetic parameters, the covariance between those parameters was also estimated.

For a NONMEM model, the residual variance corresponds to the difference between the observed concentration ( $C_{obs}$ ) and the predicted concentration ( $C_{pred}$ ). The latter is predicted on the basis of individual parameters ( $Cl/F_i$ ,  $V1/F_i$ , and so on). Residual variance was modeled with an additive error model as follows:

$$\ln(C_{obs} \ i) = \ln(C_{pred} \ i) + \epsilon$$

where  $\epsilon$  is an independent random variable with mean 0 and a variance of  $\sigma^2$ .

On the basis of the derived population model and the observed individual concentrations, individual pharmacokinetic parameter estimates were obtained by Bayesian (POSTHOC) analysis. The individual estimates were plotted against demographic factors (sex, ethnicity, weight, daily dose, time after transplantation) and the tested genotypes (*MDR-1*, *CYP3A4*, *CYP3A5*) for visual inspection according to the method described by Maitre *et al.*<sup>37</sup> Covariates that showed a correlation with a pharmacokinetic parameter were entered into the population pharmacokinetic model. In the NONMEM model the relationship between categoric covariates (genotype, sex, ethnicity) and pharmacokinetics was statistically tested by use of the log-likelihood test.<sup>38</sup> For instance, a change in the cyclosporine Cl/F in *CYP3A4\*1B* carriers was evaluated by use of the following equation:

$$Cl / F = \theta_1 \times \theta_2^{FLAG}$$

where the indicator variable FLAG has the value 0 [wildtype (*\*1/\*1*)] or 1 (*CYP3A4\*1B* allele carrier),  $\theta_1$  is the typical cyclosporine Cl/F with *CYP3A4\*1/\*1* (FLAG = 0), and  $\theta_2$  is the fractional change in  $\theta_1$  with *CYP3A4\*1B* carriers. The minimum value of the objective function (MVOF) generated by NONMEM was used to evaluate the increase in goodness of fit of the model. The MVOF is approximately proportional to -2 times the logarithm of the likelihood of the data. The model was first evaluated with FLAG fixed to 0 for all genotypes (wildtype and *\*1B* carriers) and subsequently with FLAG equal to 0 or 1 as explained. A decrease in the MVOF of at least 3.8 identified a covariate as being significant ( $P < 0.05$ ). This criterion is based on the objective function having an approximate chi square distribution with 1 *df*.

Relationships between continuous covariates and pharmacokinetic parameters were evaluated in a similar manner. For instance, the relationship between Cl/F and weight was described as follows:

$$Cl/F_{pop} = \theta_3 \cdot \left( \frac{Weight}{70} \right)^{\theta_4}$$

where  $Cl/F_{pop}$  is a typical population value of Cl/F,  $\theta_3$  is  $Cl/F_{pop}$  of a patient with weight of 70 kg, and  $\theta_4$  is an exponent.

## STATISTICAL ANALYSIS

For comparisons between groups, 1-way ANOVA was performed with SPSS for Windows, version 11.5.0 (SPSS, Chicago, IL).

## RESULTS

The demographic, clinical, pharmacokinetic and pharmacogenetic data of the 151 transplant recipients are depicted in Tables 4.2.1 and 4.2.2. For 2 typical patients, cyclosporine plasma concentration *versus* time profiles are given in Figure 4.2.1. Because the cyclosporine dose was not changed during a period of at least 1 week immediately before blood sampling, steady-state pharmacokinetics was assumed. Consequently, the predose concentration reflects the concentration at the end of the dosing interval. By use of a 1-compartment model, adequate population estimates were obtained for  $T_{lag}$ ,  $K_a$ ,  $V1/F$  and  $Cl/F$  and the interpatient variability of the latter 3 parameters. However, the diagnostic plot of weighted residuals (WRES) *versus* time showed a U-shaped trend, which is indicative of model misspecification (plot not shown). The addition of a peripheral compartment improved the goodness of fit. The residual error decreased, the objective function was approximately 180 units lower, and WRES *versus* time plots showed no trend. The objective function for a 3-compartment model did not further improve the fit. Estimation of interpatient variability of  $T_{lag}$  further improved the fit of the 2-compartment model. Typical population values for the pharmacokinetic parameters are summarized in Table 4.2.3 (model 1). Interpatient variability of  $K_a$  and  $V1/F$  was high (with corresponding values of 152% and 128%, respectively), whereas interpatient variability of  $Cl/F$  was moderate (28%). Correlations between  $K_a$  and  $V1/F$ ,  $K_a$  and  $Cl/F$ , and  $V1/F$  and  $Cl/F$  were high, with corresponding values of 0.98, 0.74 and 0.81, respectively. No interpatient variability could be estimated for  $V2/F$  and  $Q/F$ . This should not be interpreted to indicate an absence of variability but simply that the data did not contain enough information to quantify the variance of these parameters. Several plots were produced to judge the goodness of fit of the derived model (plots not shown). In plots of model-predicted and Bayesian individually predicted concentrations *versus* observed concentrations, the data points were symmetrically distributed around the line of unity (predicted concentration equal to observed concentration), indicating the adequacy of the derived NONMEM model. The model slightly underestimated the observed maximal concentrations. Several models accounting for a more complex absorption process (*e.g.*, time-dependent absorption, Weibull absorption and a dual-sequential first-order absorption process) were tried but did not improve the fit significantly.

Plots of the individual pharmacokinetic parameters  $K_a$ ,  $V1/F$ , and  $Cl/F$  *versus* the covariates sex, ethnicity, weight, daily dose, and time after transplantation indicated the following relationships: ethnicity, sex, and weight with  $Cl/F$  and weight with  $V1/F$ . After the black and Asian patients were combined,  $Cl/F$  in this group was 13% (95% confidence interval, 8%-18%) lower than in white patients (Figure 4.2.2). The MVOF was reduced by 8.9 points ( $P < 0.005$ ). Subsequently, a possible difference in  $Cl/F$  between black and Asian patients was evaluated by inclusion of an extra parameter in the population model. Oral clearance in Asian patients was slightly lower than in black patients (reduction of 15% and 12%, respectively, compared with white patients). This difference was not significant, however, because the MVOF of the population model was reduced with only 1.2 points. Therefore the extra parameter was not included in the model. To continue the population analysis, the MVOF was further reduced with 7.8 points when the relationship between weight and  $Cl/F$  was introduced in the NONMEM model ( $P < 0.005$ ). Mean bodyweight was not different among the 3 different ethnic groups ( $P = 0.35$ , 1-way ANOVA). Introduction of relationships between sex and  $Cl/F$  and between weight and  $V1/F$  did not further improve the model.

**Table 4.2.1** Demographic, clinical, and pharmacokinetic characteristics of 151 renal and heart transplant recipients

Characteristic	Kidney		Heart	
	Study 1	Study 2	Study 1	
No. of patients	26	109 <sup>§</sup>	28	
Male/female	17/9	67/42	22/6	
Ethnicity (white/black/Asian)	20/6/0	87/11/11	27/0/1	
Age at time of transplantation (y)	46.2 ± 14.4	44.9 ± 12.6	45.7 ± 14.3	
Time after transplantation (mo)	86.9 ± 71.2	3.0 ± 1.0	12.0 ± 1.0	80.6 ± 44.4
CsA dose (mg/d)	239 ± 61	372 ± 127	286 ± 84	229 ± 76
Body weight (kg)	81.4 ± 21.9	73.5 ± 14.0	76.9 ± 14.7	80.3 ± 17.9
CsA dose per bodyweight (mg · kg <sup>-1</sup> · d <sup>-1</sup> )	3.06 ± 0.88	5.13 ± 1.72	3.80 ± 1.22	2.94 ± 1.03
CsA C <sub>0</sub> (ng/mL)	144 ± 50	210 ± 76	150 ± 60	122 ± 50
CsA C <sub>2</sub> (ng/mL)	884 ± 191	ND	ND	729 ± 290
CsA AUC <sub>0-4</sub> (ng/mL · h)	2718 ± 671	ND	ND	2321 ± 827
C <sub>max</sub> (ng/mL)	1116 ± 365	ND	ND	975 ± 303
t <sub>max</sub> (h)	1.39 ± 0.50	ND	ND	1.25 ± 0.44
Primary kidney disease				
Glomerulonephritis	2	24		
Polycystic kidney disease	4	15		
Unknown	8	14		
Diabetic nephropathy	1	9		
Chronic pyelonephritis	0	8		
Hypertensive nephropathy	6	7		
Immunoglobulin A nephropathy	0	6		
Focal segmental glomerulosclerosis	0	5		
Other	5	21		
Primary heart disease				
Ischemic heart disease			13	
Cardiomyopathy			14	
Congenital abnormality			1	

All values are depicted as mean ± SD or number of patients.

CsA, Cyclosporine; C<sub>0</sub>, trough concentration; C<sub>2</sub>, concentration at 2 hours, AUC<sub>0-4</sub>, area under the concentration versus time-curve between 0 and 4 hours; C<sub>max</sub>, peak concentration; ND, not determined; t<sub>max</sub>, time to peak concentration.

<sup>§</sup> Twelve patients participated in studies 1 and 2.

**Table 4.2.2** Allele frequencies of *MDR-1* and *CYP3A* single-nucleotide polymorphisms in 151 renal and heart transplant recipients receiving cyclosporine maintenance therapy

Single-nucleotide polymorphism	Allele	Ethnicity and frequency		
		White (n = 126)	Asian (n = 13)	Black (n = 12)
<i>MDR-1</i> 3435C>T	C	0.42	0.50	0.65
	T	0.58	0.50	0.35
<i>CYP3A4</i> *1/*1B	*1	0.96	1.00	0.54
	*1B	0.04	0.00	0.46
<i>CYP3A4</i> *1/*3	*1	0.99	1.00	1.00
	*3	0.01	0.00	0.00
<i>CYP3A5</i> *1/*3	*1	0.08	0.42	0.42
	*3	0.92	0.58	0.58

**Table 4.2.3** Population pharmacokinetic parameter estimates for cyclosporine

	Model 1: Estimate and CV%	Model 2: Mean and CV%
Population parameters		
K <sub>a</sub> (1/h)	1.39 (11%)	1.27 (9%)
V1/F (L)	55.7 (6%)	56.3 (5%)
Cl/F (L/h)	30.5 (3%)	30.7 <sup>§</sup> (3%)
V2/F (L)	166 (11%)	185 (16%)
Q/F (L/h)	35.4 (8%)	31.7 (11%)
T <sub>lag</sub> (h)	0.551 (9%)	0.470 (16%)
Fractional change in oral clearance (Cl/F) <sup>†</sup>		
Ethnicity = black or Asian	-	0.870 (3%)
<i>CYP3A4</i> genotype = *1/*1B or *1B/*1B	-	1.09 (4%)
Exponent clearance - weight	-	0.172 (35%)
Interpatient variability		
K <sub>a</sub> (%)	152 (22%)	155 (32%)
V1/F (%)	128 (22%)	125 (29%)
Cl/F (%)	28 (23%)	29 (29%)
T <sub>lag</sub> (%)	75 (53%)	110 (89%)
Residual error		
ε	0.225 (8%)	0.222 (8%)
Minimal value objective function		
Value	-633.7	-654.4

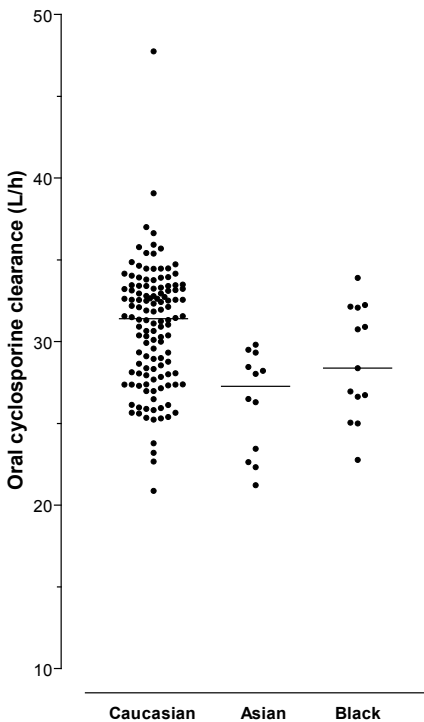
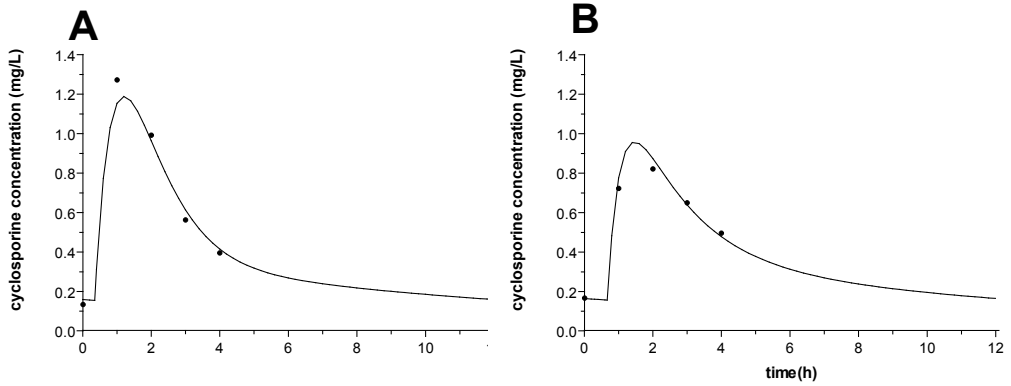
CV, Coefficient of variation; K<sub>a</sub>, absorption rate constant; V1/F, oral volume of distribution of central compartment; Cl/F, oral clearance from central compartment; V2/F oral volume of distribution of peripheral compartment; Q/F, oral intercompartmental clearance; T<sub>lag</sub>, time lag between intake and start absorption; ε, residual error.

<sup>§</sup>Typical Cl/F in a 70-kg white patient with *CYP3A4*\*1/\*1 genotype.

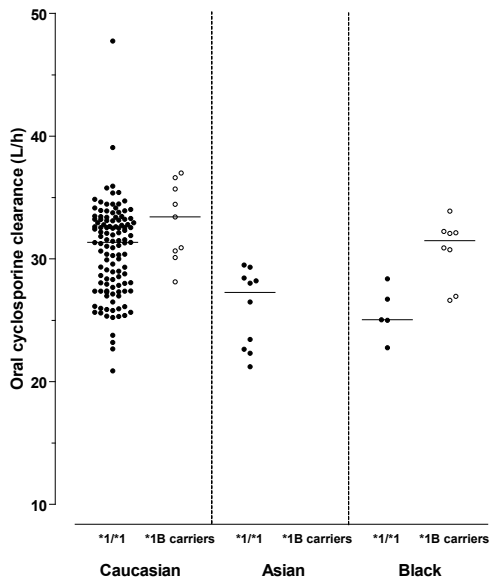
<sup>†</sup> The typical Cl/F can be calculated as follows:  $Cl/F_{pop} = 30.7 \times (\text{Weight}/70)^{0.172}$ . For black and Asian patients and carriers of a *CYP3A4*\*1B variant allele, Cl/F<sub>pop</sub> can be obtained by multiplication with the factors 0.870 and 1.09, respectively.



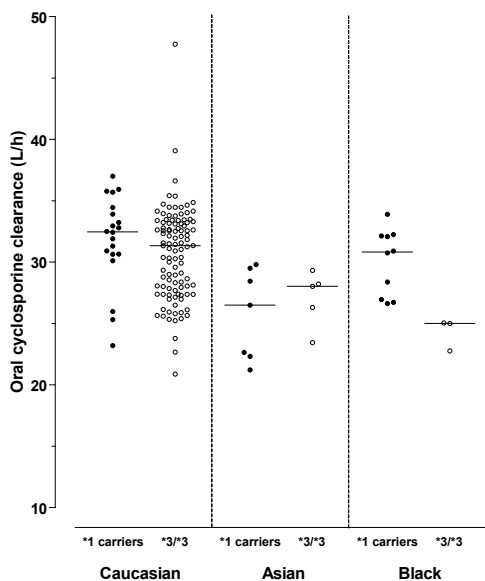
**Figure 4.2.1** Cyclosporine plasma concentration *versus* time profiles for 2 typical patients. The *solid circles* represent observed concentrations. The *solid line* represents the Bayesian fit obtained on the basis of the individual concentrations and population model 2.



**Figure 4.2.2** Bayesian oral cyclosporine clearance versus ethnicity. Individual estimates were obtained on basis of the final NONMEM model and individual concentrations. The *solid circles* represent the individual patients. The *solid line* represents the median.



**Figure 4.2.3** Bayesian oral cyclosporine clearance versus *CYP3A4* genotype. The *solid circles* represent the  $*1/*1$  patients, and the *open circles* represent the  $*1/*1B$  and  $*1B/*1B$  patients. The *solid line* represents the median.



**Figure 4.2.4** Bayesian oral cyclosporine clearance versus *CYP3A5* genotype. The *solid circles* represent the  $*1/*1$  and  $*1/*3$  patients, and the *open circles* represent the  $*3/*3$  patients. The *solid line* represents the median.

Individual Bayesian estimates of the pharmacokinetic parameters were generated on the basis of the intermediate NONMEM model and plotted *versus* the different genotypes. The plot of Cl/F *versus* CYP3A4\*1/\*1B genotype indicated an increased Cl/F in patients carrying a CYP3A4\*1B variant allele compared with CYP3A4\*1 homozygotes. Evaluation of this hypothesis in the NONMEM model demonstrated a small (9%; 95% confidence interval, 1%-17%) but significant ( $P < 0.05$ ) increase in Cl/F; the MVOF was reduced with 4.0 points on introduction of CYP3A4\*1/\*1B genotype into the model (Figure 4.2.3). The effect of the CYP3A4\*1/\*1B genotype appeared to be different for white and black patients (Figure 4.2.3). However, no statistical significance was obtained when this difference was evaluated in the population model. On further inspection of the plots, Cl/F appeared to be slightly lower in patients who were homozygous for CYP3A5\*3. However, this small difference (6%) was not statistically significant ( $P = 0.06$ ) when CYP3A5\*1/\*3 genotype was implemented in the population model (Figure 4.2.4). One white patient carried a CYP3A4\*3 variant allele. The cyclosporine Cl/F of this individual was 33.8 L/h, which was not markedly different from the median cyclosporine Cl/F of white patients (Table 4.2.3). No other significant changes in any of the pharmacokinetic parameters were detected in patients with MDR-1 3435C→T, CYP3A4\*3, or CYP3A5\*6 variant alleles.

The final population pharmacokinetic parameters are summarized in Table 4.2.3 (model 2). The diagnostic plots of predicted *versus* observed concentrations indicated adequacy of the derived NONMEM model (plot not shown). Inclusion of the covariates (sex, ethnicity, weight, daily dose, and time after transplantation) did not improve the slight underprediction of maximal concentrations. Figure 4.2.1 represents Bayesian individually predicted concentrations for 2 typical patients. No trend was observed in the plot of the WRES *versus* time (not shown).

## DISCUSSION

There has been much controversy about the effects of genetic polymorphisms in MDR-1 and CYP3A on the pharmacokinetics of the calcineurin inhibitors cyclosporine and tacrolimus. Several association studies have reported effects of the MDR-1 3435C→T,<sup>20,25</sup> CYP3A4\*1B,<sup>22</sup> and CYP3A5\*3,<sup>21-25,27</sup> SNPs on the disposition of tacrolimus, whereas most studies reported to date have not found a correlation between MDR-1, CYP3A4, or CYP3A5 genotype and the pharmacokinetics of cyclosporine.<sup>22,28-30,39,40</sup> We hypothesized that these contrasting results may have arisen through underdetection of an effect of genotype on cyclosporine pharmacokinetics through the use of a too crude pharmacokinetic analysis (dose-adjusted  $C_0$ )<sup>22,28,29</sup> or underpowering in the studies that performed more detailed pharmacokinetic profiling.<sup>39,40</sup>

With the use of NONMEM, a sensitive and precise method to describe population pharmacokinetics, we did indeed find a significant, albeit numerically small, effect of the CYP3A4\*1B SNP on cyclosporine pharmacokinetics. Patients carrying the variant CYP3A4\*1B allele were found to have an increased oral cyclosporine clearance (+9%). This finding is in agreement with our previous observation that renal transplant recipients with

the *CYP3A4*\*1/\*1*B* or \*1*B*/\*1*B* genotype require a higher tacrolimus dose to reach target  $C_0$  compared with patients homozygous for *CYP3A4*\*1.<sup>22</sup> The current findings are supported by the results of the *in vitro* study by Amirimani *et al.*,<sup>11</sup> who described in eukaryotic luciferase reporter constructs that the *CYP3A4*\*1*B* promoter sequence caused a higher expression compared with the *CYP3A4*\*1 promoter. This expression was found to be 1.4-fold higher in HepG2 cells carrying the variant promoter, and was 1.9-fold higher in MCF7 cells.<sup>11</sup> Min and Ellingrod<sup>41</sup> reported an association between the *CYP3A4*\*1*B* polymorphism and cyclosporine pharmacokinetics in healthy volunteers. By use of noncompartmental analysis (WinNonLin program; Pharsight Corporation, Mountain View, CA), the mean area-under the concentration *versus* time-curve between 0 and 24 h divided by dose was found to be significantly higher in *CYP3A4* wild-type (\*1/\*1) subjects compared with individuals homozygous for the variant allele (\*1*B*/\*1*B*) (21.5 ng · h/mL · mg<sup>-1</sup> *versus* 11.7 ng · h/mL · mg<sup>-1</sup>, respectively). Oral clearance was lower in wildtype individuals compared with *CYP3A4*\*1*B* allele homozygotes (49.4 L/h *versus* 83.5 L/h, respectively), suggesting increased enzymatic activity or expression in individuals with the variant \*1*B* allele.<sup>41</sup>

Although the effect of *CYP3A4* genotype on cyclosporine pharmacokinetics was statistically significant, one could argue about its clinical relevance for the individual patient. Incorporation of *CYP3A4* genotype into the final NONMEM model did not markedly reduce interpatient variability of  $K_a$ , Cl/F, V1/F, or  $T_{lag}$ , indicating that the *CYP3A4* genotype made only a limited contribution to the observed marked interindividual differences in cyclosporine pharmacokinetics. The influence of environmental factors, such as smoking habits, diet, and comedication, or perhaps not-yet-identified genetic factors may, therefore, be more important factors determining the pharmacokinetics of this drug. Because the current study population consisted of stable transplant patients receiving maintenance therapy, the results may be different in patients in the early phase after transplantation when adequate immunosuppressive drug dosing is most critical. Furthermore, this study used a cross-sectional design, and the possibility of a selection bias must, therefore, be considered. We believe, however, that several points argue against a significant selection bias. First, all the allele frequencies in the different ethnic groups were in Hardy-Weinberg equilibrium and were in agreement with published allele frequencies.<sup>5,9,10,32-36</sup> Second, therapeutic drug monitoring for cyclosporine was performed in all patients. Patients with drug concentrations well outside the target range (possibly as a result of extremely high or low clearance) are, therefore, likely to have been identified, resulting in appropriate dose adjustments. Third, cyclosporine-related side effects were quite common among the “stabilized” patients of the study group. Moreover, our current practice in cases of cyclosporine toxicity is to reduce the dosage of this drug and not to withdraw cyclosporine until the first year after transplantation. Fourth, with modern triple immunosuppression, the percentage of patients who lose their graft within the first year after renal transplantation is below 10% and is largely a result of either technical failure (thrombosis of vessels) or grafts that never functioned.

An alternative explanation for these findings could be that the observed correlation between *CYP3A4* genotype and oral cyclosporine clearance may, in fact, have been caused by differences in *CYP3A5* expression. Several authors have suggested that it is *CYP3A5* rather than *CYP3A4* that determines an individual’s *CYP3A* enzymatic activity and there is

substantial evidence that the *CYP3A4\*1B* and the *CYP3A5\*1* alleles are linked.<sup>9,22,35,36,42</sup> In our study 67% of the white patients carrying the *CYP3A4\*1B* allele also carried the *CYP3A5\*1* allele. In the black patients, all of the *CYP3A4\*1B* carriers had a *CYP3A5\*1* allele, suggesting linkage disequilibrium between these 2 alleles. The importance of *CYP3A5* for tacrolimus disposition has been reported by several independent research groups. Renal and heart transplant recipients with a genetic inability to express *CYP3A5* (homozygous for *CYP3A5\*3* or \*6) required less of the drug to reach target concentrations.<sup>20,22-25</sup> Recently, a similar *CYP3A5* effect on tacrolimus dose requirement was found in lung transplant recipients.<sup>27</sup> In our study, patients homozygous for the *CYP3A5\*3* allele (nonexpressors) did have a reduced oral cyclosporine clearance (-6%) compared with carriers of the *CYP3A5\*1* allele, but this difference was not statistically significant ( $P=0.06$ ). In agreement with this finding, Haufroid *et al.*<sup>24</sup> reported a small effect of the *CYP3A5\*3* polymorphism on cyclosporine  $C_0$  adjusted for the last dose in 50 stable renal transplant recipients. However, this genotype effect was much smaller than that observed for the tacrolimus dose requirement. In a stepwise multiple regression analysis, Haufroid *et al.*<sup>24</sup> showed that *CYP3A5* genotype only explained 9% of the total variance in cyclosporine dose-adjusted  $C_0$  in their study population.

The *MDR-1* 3435C→T SNP was not related to cyclosporine pharmacokinetics, confirming earlier reports in kidney and heart transplant recipients.<sup>22,29,39,40</sup> This SNP is a silent polymorphism, which means that it does not cause an amino acid change.<sup>5</sup> However, there is substantial evidence that the 3435C→T polymorphism is linked to other, nonsynonymous SNPs in *MDR-1* (among others, the 2677G→T/A SNP in exon 21).<sup>8</sup> Haplotype analysis of *MDR-1* may, therefore, be a superior method to analyze the effects of genetic variability in this gene on drug pharmacokinetics.<sup>8</sup> An effect of *MDR-1* haplotype has been described for tacrolimus<sup>21</sup> and for cyclosporine<sup>43</sup> in a small number ( $n = 9$ ) of Asian heart transplant recipients. However, in a larger study ( $n = 98$ ) in renal transplant recipients,<sup>30</sup> the latter finding could not be confirmed, suggesting that genetic variability in *MDR-1* is unlikely to contribute much to interindividual differences in cyclosporine pharmacokinetics.<sup>30</sup>

Finally, a remarkable finding was the observed effect of ethnicity on oral cyclosporine clearance. Black and Asian transplant recipients were found to have a reduced oral cyclosporine clearance, independent of their *CYP3A* genotype. This finding may seem in contradistinction with the literature, because several authors have previously reported a higher cyclosporine clearance and a lower oral bioavailability in black transplant recipients.<sup>44-46</sup> However, these studies were conducted in *de novo* renal transplant recipients who used the older oil-based cyclosporine formulation<sup>44,45</sup> or were conducted in healthy volunteers without controlling for diet.<sup>46</sup> Administration of microemulsified cyclosporine to white and black volunteers under controlled (dietary) conditions did not result in statistically significant differences in cyclosporine pharmacokinetics between ethnic groups.<sup>47</sup> Comparable cyclosporine exposure between black and white patients after administration of either the oil-based or the microemulsified preparation has also been observed in *de novo* renal transplant recipients.<sup>48</sup> In this study patients fasted overnight and later ate a standardized meal.<sup>48</sup> On the basis of the findings of our study, the comparable cyclosporine pharmacokinetics between black and white patients may be explained by the fact that the reduced oral cyclosporine clearance (-13%) in black patients is counterbalanced by the greater presence of the *CYP3A4\*1B* variant

allele (resulting in a +9% higher Cl/F) in this patient group (allelic frequency of 0.46 *versus* 0.04 in black and white patients, respectively). The finding that Asian patients had a slightly lower oral cyclosporine clearance compared with white individuals may seem surprising, because the transplant survival in the former group has been reported to be comparable or even superior to that of white patients.<sup>49</sup> However, the influence of immunologic, physiologic, and socioeconomic factors on long-term allograft survival may well outweigh small interethnic differences in cyclosporine pharmacokinetics. Moreover, to the best of our knowledge, no studies have been published that were primarily designed to compare the pharmacokinetics of microemulsified cyclosporine between white and Asian subjects. In addition, the ancestry of “Asians” is often not specified in clinical trials. In our study all of the Asian patients were of Indian or Indonesian descent, and there exist important differences in genetic background, as well as in dietary habits, between these populations and Chinese and Japanese patients.

In summary, we found a statistically significant effect of the *CYP3A4\*1/\*1B* genotype on the pharmacokinetics of cyclosporine in stable renal and heart transplant recipients. Patients carrying a *CYP3A4\*1B* variant allele had a higher oral cyclosporine clearance, whereas no effect of the *MDR-1* 3435C→T SNP or the *CYP3A4\*3* or *CYP3A5\*3* and *\*6* variant alleles was observed. However, because the effect of the *CYP3A4\*1B* polymorphism on cyclosporine disposition was small, genotyping of transplant recipients for *CYP3A* is unlikely to assist in planning initial dosing of this immunosuppressant.

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CHAPTER 4.3

**THE PHARMACOGENETICS  
OF CALCINEURIN  
INHIBITORS: ONE STEP  
CLOSER TOWARD  
INDIVIDUALIZED  
IMMUNOSUPPRESSION?**

Dennis A Hesselink, Teun van Gelder, Ron HN van Schaik

## ABSTRACT

The immunosuppressive drugs cyclosporine and tacrolimus are widely used to prevent acute rejection following solid organ transplantation. However, the clinical use of these agents is complicated by their many side effects, a narrow therapeutic window, and highly variable pharmacokinetics. The variability in cyclosporine and tacrolimus disposition has been attributed to interindividual differences in the expression of the metabolizing enzymes cytochrome P450 (CYP) 3A4 and 3A5, and in the expression of the drug transporter P-glycoprotein (encoded by the *ABCB1* gene, formerly known as the multidrug resistance 1 gene). Variation in the expression of these genes could in turn be explained by several recently-identified single-nucleotide polymorphisms (SNPs). Determination of these SNPs in (future) transplant recipients has the potential to identify individuals who are at risk of underimmunosuppression or the development of adverse drug reactions. Ultimately, genotyping for *CYP3A* and *ABCB1* may lead to a further individualization of immunosuppressive drug therapy for the transplanted patient.

## INTRODUCTION

Since the first human kidney was successfully transplanted half a century ago, transplantation has grown from an experimental therapy to become the treatment of choice for patients with end-stage organ failure.<sup>1,2</sup> A large part of this success can be attributed to the development of powerful and more specific immunosuppressive drugs during the past three decades. With the availability of these agents, the incidence of acute rejection has been reduced dramatically, and presently this is a rare cause for allograft loss.<sup>1,2</sup>

However, the introduction of modern immunosuppressive drug therapy has not resulted in a comparable improvement in the long-term transplantation outcomes. Late allograft loss most often results from the death of a patient with a functioning graft, or from chronic allograft nephropathy (CAN), which is an incompletely understood process characterized clinically by a slowly progressive decline in transplant function.<sup>2</sup> Paradoxically, immunosuppressive drug treatment itself negatively influences long-term patient and allograft survival. First, by its very nature, immunosuppressive therapy promotes the development of malignancy and infectious disease. Second, transplant recipients have an increased risk of cardiovascular disease, which is the leading cause of death in this population. The use of immunosuppressive drugs, such as cyclosporine and tacrolimus, contributes to this risk, as their side effects include the induction of glucose intolerance, hypertension and hyperlipidemia.<sup>3</sup> Third, some immunosuppressants appear to be directly involved in the development of CAN.<sup>2</sup> Finally, most immunosuppressive drugs have specific side effects that compromise the quality of life of transplanted patients.

At the present time, the main challenge facing transplant physicians involves further improving the long-term transplantation outcomes, while maintaining the excellent short-term results that are currently achieved. As the benefits of modern immunosuppressive therapy are partially outweighed by its adverse effects, there has been an ongoing search for more specific and less toxic immunosuppressive agents.<sup>4</sup> However, a more immediate option that could reduce iatrogenic morbidity may be the further individualization of the currently-available immunosuppressive treatment strategies. Pharmacogenetics offers great promise in this respect, as it has the potential to assist in “identifying the right drug and the right dose for an individual patient”.<sup>5</sup> In this paper we discuss the current status of pharmacogenetics in solid organ transplantation. As pharmacogenetic research in this field has mainly focused on the immunosuppressants cyclosporine and tacrolimus, the discussion is limited to these two agents and the genes that play a central role in their disposition, the adenosine triphosphate (ATP)-binding cassette (*ABC*)*B1* gene [in the older nomenclature known as the multidrug resistance 1 (*MDR1*) gene], and the genes encoding the cytochrome P450 (CYP) isoenzymes 3A4 and 3A5.

## CALCINEURIN INHIBITORS

The immunosuppressive actions of the calcineurin inhibitors (CNIs) cyclosporine and tacrolimus were first described in 1976 and 1987, respectively.<sup>6,7</sup> Both drugs exert their immunosuppressive effect by inhibiting several signaling processes in T cells, most importantly by blocking the phosphatase calcineurin, which leads to a decreased transcription of the interleukin (IL)-2, IL-4, CD40 ligand and interferon- $\gamma$  genes, which finally results in the inhibition of T cell activation and proliferation.<sup>8,9</sup> The clinical introduction of cyclosporine in the early 1980s led to a marked reduction in the incidence and severity of acute rejection, and with the availability of cyclosporine, heart and lung transplantation first became realistic options.<sup>10</sup> In the 1990s, tacrolimus proved to be equally effective, and possibly even superior to cyclosporine.<sup>11</sup> At present, in most transplant centers, CNIs form the cornerstone of immunosuppressive therapy after organ transplantation. Generally, in such therapy, either cyclosporine or tacrolimus is used in combination with an anti-proliferative agent, such as azathioprine or mycophenolate mofetil (MMF), with or without the addition of corticosteroids.

Despite their effectiveness, the clinical use of CNIs is hampered by many side effects, which include nephrotoxicity, hypertension, the induction of diabetes mellitus and dyslipidemia, and cosmetic side effects. Moreover, both drugs have a narrow therapeutic window, highly variable and unpredictable pharmacokinetics, and interactions with many other drugs.<sup>8,9</sup> Attempts to improve CNI therapy have resulted in the widespread application of therapeutic drug monitoring (TDM). TDM is the practice of monitoring blood drug concentrations and adjusting the drug dose, in order to reach a predefined target concentration that has been associated with the optimal balance between efficacy and toxicity.<sup>12</sup>

Although TDM of CNIs has resulted in a more individualized therapy, there are several limitations to this practice. First, there is still uncertainty regarding the best parameter for pharmacokinetic monitoring of CNIs. Traditionally, these drugs are dosed according to the predose concentration ( $C_0$ ). However, it has become clear that the correlation between the cyclosporine  $C_0$  and total drug exposure is poor, and that the predictive value of this parameter for the occurrence of acute rejection or nephrotoxicity is limited.<sup>13</sup> This is explained by the fact that cyclosporine pharmacokinetics are most variable during the absorption phase, whereas the  $C_0$  mainly reflects systemic clearance. Therefore, the cyclosporine area-under the concentration *versus* time-curve during the first 4 h after oral administration ( $AUC_{0-4}$ ) and the cyclosporine 2-h postdose concentration ( $C_2$ ) have recently been advocated as more appropriate tools for TDM.<sup>13-15</sup> Second, TDM can only be performed after the start of drug treatment. Therefore, TDM has no predictive value and is of no aid when determining the starting dose of a drug for an individual patient. This is especially relevant for transplant recipients, as failure to reach target cyclosporine concentrations as soon as three days after renal transplantation, is critical in order to prevent acute rejection.<sup>15</sup> Third, TDM is performed invasively and requires considerable effort to reliably draw blood at the correct time point. Finally, TDM does not provide any mechanistic information regarding the factors that underlie a drug's pharmacokinetics.

## PHARMACOKINETICS OF CALCINEURIN INHIBITORS

The pharmacokinetics of CNIs are characterized by a highly variable, unpredictable, and, in general, poor oral bioavailability, which has an average value of 30%, ranging from 4%-89%.<sup>8,9</sup> Following absorption from the gut, both drugs are metabolized in the intestine and liver into 15-30 metabolites. Elimination of CNIs is mainly biliary, with renal clearance accounting for < 1 and 6% of total body clearance for tacrolimus and cyclosporine, respectively.<sup>8,9</sup> Besides a low oral bioavailability, the pharmacokinetics of CNIs are characterized by marked interindividual differences in first-pass metabolism and systemic clearance. Recently, it has become clear that much of the interindividual differences in CNI pharmacokinetics result from variability in the activity of the permeability glycoprotein (P-glycoprotein) and the CYP isoenzymes 3A4 and 3A5 (Figure 4.3.1).

### P-GLYCOPROTEIN

P-glycoprotein is encoded by the *ABCB1* gene located on chromosome 7q21.1, and belongs to the family of the ABC membrane transporters (subfamily B). P-glycoprotein is an ATP-dependent transporter capable of pumping many endogenous substances, as well as a wide variety of drugs (including CNIs, sirolimus, and glucocorticoids), from the cytoplasm or cell membrane to the extracellular space.<sup>5,16-20</sup> Physiologically, P-glycoprotein is expressed in the liver (at the canalicular surface of hepatocytes), kidney (brush border of proximal tubular cells), pancreas, and at the apical surface of mature enterocytes in the small intestine and colon.<sup>21</sup> The specific tissue expression of P-glycoprotein suggests that the protein functions as a protective barrier, by actively extruding xenobiotics and metabolites from the cell interior into bile, urine or gut lumen. P-glycoprotein is also expressed in testes, placenta (trophoblasts), on the luminal surface of capillaries in the brain, and at the choroid plexus where it serves to maintain the blood-testis, maternal-fetal, blood-brain and blood-cerebrospinal fluid barriers, respectively.<sup>21</sup> In addition, P-glycoprotein is expressed on various leukocytes, including T and B lymphocytes and dendritic cells (Figure 4.3.1).<sup>22,23</sup>

### CYP3A

The CYP enzyme family consists of more than 50 isozymes that are responsible for the oxidative metabolism of many endogenous and exogenous compounds (Figure 4.3.1).<sup>5</sup> The CYP3A subfamily, which represents the majority of CYP proteins in the human liver, metabolizes more than 50% of all drugs currently in use (including CNIs, sirolimus and glucocorticoids)<sup>5,24-26</sup> and consists of the isozymes CYP3A4, CYP3A5, CYP3A7, and CYP3A43.<sup>24,26,27</sup> CYP3A4 and CYP3A5 have largely overlapping substrate specificities and, based on the amount of protein, are considered the most important CYP3A family members. CYP3A4 is constitutively expressed in liver, jejunum, colon, kidney, and pancreas, but marked interindividual differences in its activity exist, which may vary by up to 40-fold.<sup>28</sup> For stable kidney transplant recipients, a ten-fold variation in enterocyte CYP3A4 content was reported.<sup>29,30</sup> CYP3A5 is also present in the liver, kidney, and small intestine, although its expression is even more variable, and in general much lower, compared with CYP3A4. In Caucasian livers, the CYP3A5 protein was only detectable in 10%-40% of all samples.<sup>31</sup>

However, CYP3A5 may account for up to 50% of total hepatic CYP3A content in some individuals.<sup>32</sup> The functional significance of the other two CYP3A family members, CYP3A7 and CYP3A43, is incompletely understood. CYP3A7 appears to be most important during fetal life. However, in approximately 3% of adults, CYP3A7 is expressed due to inheritance of the *CYP3A7\*1C* allele. In this allele, part of the *CYP3A4* promoter has replaced the *CYP3A7* promoter, thereby preventing downregulation after birth.<sup>33</sup> CYP3A43 accounts for less than 1% of CYP3A transcripts in adult human liver, and therefore, its contribution to CYP3A-mediated metabolism is thought to be minimal.<sup>27</sup>

### **P-GLYCOPROTEIN AND CYP3A AS BARRIERS TO CALCINEURIN INHIBITOR EXPOSURE**

Several studies have established the importance of P-glycoprotein and CYP3A in the disposition of CNIs (Figure 4.3.1). First, P-glycoprotein limits the absorption of these drugs by active extrusion from the enterocyte interior back into the gut lumen. Kaplan and colleagues<sup>34</sup> described a small bowel transplant recipient with an extremely low oral bioavailability of both tacrolimus and cyclosporine. This patient was found to have a high small bowel P-glycoprotein content. Administration of fluconazole, an inhibitor of P-glycoprotein, resulted in an increase in CNI concentrations in whole-blood. A similar relationship between CNI dose requirement and intestinal *ABCB1* expression has been described for small bowel,<sup>35,36</sup> liver,<sup>37,38</sup> and kidney<sup>30</sup> transplant recipients. Besides extrusion by P-glycoprotein, systemic exposure to CNIs is limited by substantial intestinal metabolism. Kolars and co-workers<sup>39</sup> introduced cyclosporine into the small bowel of patients during the anhepatic phase of liver transplant surgery. Cyclosporine metabolites were readily detectable in portal venous blood, indicating that the small intestine is a major site of cyclosporine breakdown. More recently, Tuteja and colleagues<sup>40</sup> provided evidence for intestinal tacrolimus metabolism. In stable renal transplant recipients, coadministration of tacrolimus and ketoconazole (an inhibitor of CYP3A and P-glycoprotein, like fluconazole ) resulted in a decrease in tacrolimus clearance and an increase in the tacrolimus bioavailability. This effect was greater following oral ketoconazole administration, compared with intravenous dosing, suggesting intestinal CYP3A-mediated tacrolimus metabolism.<sup>40</sup> The importance of intestinal CNI metabolism has been substantiated further in animal and human-volunteer studies.<sup>41-44</sup> Finally, the interindividual variability in CNI disposition correlates with variation in hepatic CYP3A activity.<sup>29</sup>

In summary, the pharmacokinetics of CNIs are influenced by the synergistic actions of intestinal and hepatic P-glycoprotein and CYP3A. In the gut, these enzymes act as a barrier to absorption by active extrusion into the gut lumen and through intestinal phase I metabolism, whereas hepatic CYP3A and P-glycoprotein activity are responsible for systemic drug clearance.<sup>18</sup>



**Table 4.3.1** Cyclosporine pharmacokinetics and single-nucleotide polymorphisms in the *ABCBI* gene.

Author [reference]	Subjects	n	SNP(s)	PK parameter	Effect
von Ahlsen [66]	Renal transplant recipients	124	3435C>T	C <sub>0</sub>	No effect on CsA dose requirement
Hesselink [67]	Renal transplant recipients	109	3435C>T	C <sub>0</sub>	No effect on CsA dose requirement
Mai [72]	Renal transplant recipients	98	2677G>T/A 3435C>T	C <sub>0</sub> <sup>p</sup> , C <sub>2</sub> , AUC <sub>0-4</sub>	No effect of haplotypes on CsA pharmacokinetics
Anglicheau [73]	Renal transplant recipients	106	1236C>T 2677G>T/A 3435C>T	Calculated AUC <sub>0-12</sub>	No effect of haplotypes on CsA pharmacokinetics
Haufroid [74]	Renal transplant recipients	50	1236C>T 2677G>T/A 3435C>T	C <sub>0</sub>	No effect of haplotypes on CsA pharmacokinetics
Kuzuya [68]	Renal transplant recipients	97	3435C>T -129T>C 1236C>T 2677G>T/A 3435C>T	C <sub>2</sub>	No effect of individual SNPs on CsA dose requirement
Yates [75]	Renal transplant recipients	10	3435C>T	AUC <sub>0-12</sub>	Higher CsA dose requirement in T allele carriers
Hesselink [69]	Renal and heart transplant recipients	151	3435C>T	AUC <sub>0-4</sub>	No effect on oral CsA clearance or dose requirement
Balram [71]	Heart transplant recipients	10	3435C>T	AUC <sub>0-4</sub>	No effect on CsA pharmacokinetics
Chowbay [54]	Heart transplant recipients	14	1236C>T 2677G>T/A 3435C>T	AUC <sub>0-12</sub>	Lower CsA exposure in C-G-C compared with T-T-T haplotype
Min [77]	Healthy volunteers	14	3435C>T	AUC <sub>0-24</sub>	No effect on CsA pharmacokinetics
Bonhomme-Faivre [76]	Liver transplant recipients	44	3435C>T	C <sub>2</sub>	Lower CsA dose requirement in TT homozygotes
Hebert [70]	Liver transplant recipients	89	2677G>T/A 3435C>T	C <sub>0</sub>	No effect of individual SNPs on CsA dose requirement

AUC, area-under the concentration *versus* time-curve; C<sub>0</sub><sup>p</sup>, predose concentration; C<sub>2</sub>, 2-h postdose concentration; CsA, cyclosporine; PK, pharmacokinetic; SNP, single-nucleotide polymorphism.

## GENETIC VARIABILITY IN *ABCB1* AND *CYP3A*

Interindividual variability in *CYP3A* and P-glycoprotein activity can be partly explained by differences in age, gender, environmental factors (concomitant medication, smoking, and diet), and co-morbidity. In addition, the possibility that this variability has an important genetic basis has been recognized for a long time, but it was not until the recent identification of several single-nucleotide polymorphisms (SNPs) in the *ABCB1*, *CYP3A4*, and *CYP3A5* genes that further evidence for this hypothesis was provided (for a review see references 16 and 27).

To date, more than 25 SNPs have been discovered in *ABCB1*. The best-studied SNP is the 3435C→T transition located in exon 26. This is a silent SNP, meaning that it does not lead to an amino acid change. In the original report by Hoffmeyer and co-workers<sup>45</sup>, individuals homozygous for the T allele were found to have a lower duodenal P-glycoprotein content and higher plasma digoxin (a P-glycoprotein substrate) concentrations under noninduced, as well as under rifampin-induced, conditions. These findings suggested reduced P-glycoprotein activity in T allele carriers<sup>45</sup>, and this was confirmed by several other studies.<sup>46-48</sup> Healthy Caucasian volunteers with the 3435TT genotype had higher  $AUC_{0-4}$  and  $C_{max}$  values for digoxin than those with the 3435CC genotype.<sup>46</sup> In a study of Japanese volunteers, P-glycoprotein expression was numerically lower in placentas of 3435TT individuals.<sup>47</sup> A reduced P-glycoprotein activity associated with the T allele was also demonstrated in an *in vitro* study. Hitzl and colleagues<sup>48</sup> showed that rhodamine (a P-glycoprotein substrate) efflux from CD56<sup>+</sup> natural killer cells and leukocyte *ABCB1* mRNA levels were significantly lower in 3435TT subjects as compared with CC and CT individuals. However, the functionality of the 3435C→T SNP is controversial, as some investigators have reported a higher P-glycoprotein activity in association with the T allele. For example, *ABCB1* mRNA levels in the proximal small bowel of healthy Japanese volunteers were significantly higher in 3435TT individuals compared with 3435CC individuals, whereas heterozygotes (CT) had intermediate mRNA expression levels.<sup>49</sup> In addition, the fexofenadine  $AUC_{0-4}$  after single-dose administration was significantly lower in 3435TT individuals compared with CC subjects, suggesting a higher P-glycoprotein expression *in vivo*.<sup>50</sup> Finally, others have not found a relation between the 3435C→T SNP and intestinal *ABCB1* expression/P-glycoprotein content<sup>38,51</sup> or the pharmacokinetics of various drugs, including digoxin, talinolol, and fexofenadine.<sup>51-53</sup>

There is substantial evidence that the exon 26 SNP is linked to other SNPs in *ABCB1*.<sup>16</sup> Linkage disequilibria have been established between *ABCB1* 3435C→T and 1236C→T in exon 12, and with 2677G→T/A in exon 21.<sup>46,51,54,55</sup> Therefore, effects ascribed to 3435C→T may have been caused by the fact that this SNP is inherited together with other causative SNP(s). The 2677G→T/A SNP is a good candidate for such a relationship. This SNP is a missense mutation resulting in an 893Ala to 893Ser or 893Thr amino acid change. However, the reported functional consequences of this 2677G→T/A transition are also far from clear, as the absence of 2677G has been associated with both increased and decreased expression.<sup>16</sup> A difference in the contribution of either the T or A allele may effectively obscure the effects of the 2677G→T/A SNP because the 2677T variant is correlated with decreased *ABCB1* expression, whereas the 2677A variant has been associated with an increased activity.<sup>56</sup> It

may be that a marked phenotypic effect only arises when an individual possesses a specific combination of SNPs (haplotype), meaning that assessment of a patient's haplotype could prove to be a superior method to be used in investigating genotype-phenotype relationships. With haplotype analysis, even the functional consequences of as yet unidentified SNPs can be detected once the key polymorphisms representative of a certain haplotype (which do not necessarily have to be the SNPs responsible for phenotypic differences) have been defined.<sup>55</sup> The number of studies using *ABCBI* haplotype analysis is still limited but it has become clear that important inter-racial differences in the frequencies of such haplotypes exist.<sup>16,55,57,58</sup>

The human *CYP3A* gene cluster on chromosome 7q21-q22.1 consists of four genes and two pseudogenes: *CYP3A4*, *CYP3A5*, *CYP3A7*, *CYP3A43*, *CYP3AP1*, and *CYP3AP2*.<sup>27</sup> At present, more than 30 SNPs have been identified in *CYP3A4* (see <http://www.imm.ki.se/CYPalleles>). The first *CYP3A4* SNP described in the literature was *CYP3A4-V*, later renamed *CYP3A4\*1B*, which is encoded by -290A→G in the nifedipine-specific element of the *CYP3A4* promoter. This variant allele was originally identified by linkage to a worse presentation of prostate cancer<sup>59</sup>, and *CYP3A4\*1B* was hypothesized to cause a decreased *CYP3A4* transcription. However, *in vitro*, the *CYP3A4\*1B* allele resulted in a 1.5-fold increase in transcription<sup>60</sup>, whereas other investigators did not observe a change in enzyme activity.<sup>61</sup>

*CYP3A5*, the second important *CYP3A* subfamily member, is highly homologous to *CYP3A4* (83%). Recently, a SNP in intron 3 of the *CYP3A5* gene, genomic 6986A→G, showed 100% linkage with the absence of *CYP3A5* protein. This variant, referred to as the *CYP3A5\*3* allele,<sup>31,32</sup> occurred homozygously in 80% of the Caucasian and 30% of the African-American population.<sup>31,32,62,63</sup> The *CYP3A5\*3* allele encodes a splice variant mRNA resulting in only a small amount of normally-spliced *CYP3A5* mRNA.<sup>32</sup> Interestingly, linkage was observed between the *CYP3A4\*1B* and *CYP3A5\*1* alleles,<sup>32,64</sup> which makes it difficult to assign increased *CYP3A* activity to either of these SNPs. In addition, another splice variant was identified, leading to deletion of exon 7: *CYP3A5\*6*.<sup>32</sup> This deletion causes a frameshift, resulting in a truncated protein at amino acid 184. This *CYP3A5\*6* allele was found in 3 out of 20 African-Americans,<sup>32</sup> and in 1 out of 500 Caucasians.<sup>62</sup>

The -44G→A SNP in *CYP3AP1* (originally identified as a -45A→G) was initially believed to represent a variant allele of *CYP3A5*, in which the -44A allele correlated with absence of *CYP3A5* activity.<sup>65</sup> However, this observation was later explained by genetic linkage of *CYP3AP1* -44G→A with *CYP3A5\*3*.<sup>32</sup> Although the *CYP3AP1* -44G→A SNP has been used as an indicator for *CYP3A5* activity, this does not seem to be a reliable predictor as the linkage is incomplete.<sup>64</sup>

## PHARMACOGENETICS OF CALCINEURIN INHIBITORS

### ABCB1

Von Ahnsen and colleagues<sup>66</sup> were the first to study the relationship between the *ABCB1* genotype and cyclosporine dose requirement. In a cohort of 124 stable renal transplant recipients, the *ABCB1* 3435C→T SNP was not associated with cyclosporine dose requirement (determined by dividing the cyclosporine  $C_0$  by the corresponding dose per bodyweight), a finding which was subsequently confirmed by other studies in renal,<sup>67-69</sup> liver,<sup>70</sup> and heart transplant recipients<sup>69,71</sup> (Table 4.3.1).<sup>54,66-77</sup> More recently, the effect of *ABCB1* genotype on cyclosporine disposition was studied in more detail with the use of *ABCB1* haplotype analysis. Nevertheless, in the largest cohort studies,<sup>72-74</sup> no significant correlation between *ABCB1* haplotype and cyclosporine pharmacokinetics was identified, although in several smaller study groups both a higher<sup>75</sup> and lower<sup>54,76</sup> cyclosporine dose requirement has been associated with possession of a T allele at position 3435 (Table 4.3.1).

The effect of genetic variation in *ABCB1* on tacrolimus pharmacokinetics has also been investigated extensively (Table 4.3.2).<sup>38,67,70,74,78-84</sup> As in the case of cyclosporine, a large number of studies have not reported an association between *ABCB1* genotype and tacrolimus pharmacokinetics.<sup>38,67,70,74,81-83</sup> These studies were conducted among kidney and liver transplant recipients, and a possible *ABCB1* genotype effect was studied using both individual SNP, as well as haplotype analysis. However, in the largest cohort of kidney transplant recipients studied to date (n = 180), MacPhee and co-workers<sup>78</sup> did find an association between tacrolimus dose requirement and the *ABCB1* 3435C→T SNP. Patients with the *ABCB1* 3435CC genotype were found to have the highest tacrolimus dose requirement but this genotype effect was relatively small. Anglicheau and co-workers<sup>80</sup> determined *ABCB1* haplotypes in 81 renal transplant recipients. The majority of patients in this cohort had the 1236C–2677G–3435C haplotype (haplotype 1) or the 1236T–2677T/A–3435T haplotype (haplotype 2). In line with the observations of MacPhee and colleagues<sup>78</sup>, haplotype 1 individuals were found to have a significantly higher tacrolimus dose requirement compared with haplotype 2 individuals: 0.18 mg/kg per day *versus* 0.15 mg/kg per day.<sup>80</sup> A higher tacrolimus dose requirement of patients homozygous for *ABCB1* 2677GG or 3435CC was also described for pediatric heart and adult lung transplant recipients.<sup>79,84</sup> These results may be explained by differences in the pharmacokinetic ( $C_0$ ,  $C_2$  or AUC) or genetic (single SNP *versus* haplotype) analyses that were performed in the various studies. Moreover, the sample size of the study cohorts and the time (after transplantation) points at which a possible *ABCB1* genotype effect was assessed, differed between studies. However, taken together, it appears that the variability in the pharmacokinetics of CNIs is, at most, only explained to a limited degree by the various *ABCB1* SNPs that have been studied to date.

**Table 4.3.2** Tacrolimus pharmacokinetics and single-nucleotide polymorphisms in the *ABCBI* gene.

Author [reference]	Subjects	n	SNP(s)	PK parameter	Effect
Hesselink [67]	Renal transplant recipients	64	3435C>T	C <sub>0</sub>	No effect on Tac dose requirement
Haufroid [74]	Renal transplant recipients	50	1236C>T	C <sub>0</sub>	No effect of haplotypes on Tac dose requirement
			2677G>T/A		
Mai [81]	Renal transplant recipients	73	3435C>T	C <sub>0</sub>	No effect of haplotypes on Tac dose requirement
			2677G>T/A		
Tsuchiya [82]	Renal transplant recipients	30	3435C>T	AUC <sub>0,12</sub>	No effect of individual SNPs on Tac dose requirement
			2677G>T/A		
Anglicheau [80]	Renal transplant recipients	81	1236C>T	C <sub>0</sub>	Higher Tac dose requirement in C-G-C compared with T-T-T haplotype
			2677G>T/A		
MacPhee [78]	Renal transplant recipients	180	3435C>T	C <sub>0</sub>	Higher Tac dose requirement in CC individuals
			3435C>T		
Asano [94]	Renal transplant recipients	29	3435C>T	C <sub>0</sub>	Higher Tac dose requirement in TT individuals
			1236C>T		
Goto [38]	Liver transplant recipients	69	1236C>T	C <sub>0</sub>	No effect of haplotypes on Tac dose requirement
			2677G>T/A		
Hebert [70]	Liver transplant recipients	31	3435C>T	C <sub>0</sub>	No effect of individual SNPs on Tac dose requirement
			2677G>T/A		
Goto [83]	Liver transplant recipients	143	3435C>T	C <sub>0</sub>	No effect of individual SNPs on Tac dose requirement
			2677G>T/A		
Zheng [79]	Pediatric heart transplant recipients	65	3435C>T	C <sub>0</sub>	Higher Tac dose requirement in 2677GG and 3435TT individuals
			2677G>T/A		
Zheng [84]	Lung transplant recipients	83	2677G>T/A	C <sub>0</sub>	Higher Tac dose requirement in 2677GG and 3435TT individuals
			3435C>T		

AUC, area-under the concentration *versus* time-curve; C<sub>0</sub>, predose concentration; PK, pharmacokinetic; SNP, single-nucleotide polymorphism; Tac, tacrolimus.

**Table 4.3.3** Calcineurin inhibitor pharmacokinetics and single-nucleotide polymorphisms in cytochrome P450 3A4.

Author [reference]	Subjects	n	SNP	Drug	PK parameter	Effect
Rivory [85]	Renal transplant recipients	117	*1B	CsA	C <sub>0</sub>	No effect on dose requirement
von Ahlsen [66]	Renal transplant recipients	124	*1B	CsA	C <sub>0</sub>	No effect on dose requirement
Hesselink [67]	Renal transplant recipients	108	*1B	CsA	C <sub>0</sub>	No effect on dose requirement
Min [86]	Healthy volunteers	14	*1B	CsA	AUC <sub>0-24</sub>	Higher clearance in *1B homozygotes
Hesselink [69]	Renal and heart transplant recipients	151	*1B	CsA	AUC <sub>0-4</sub>	Higher clearance in *1B homozygotes
Hesselink [67]	Renal transplant recipients	64	*1B	Tac	C <sub>0</sub>	Higher dose requirement in *1B carriers

AUC, area-under the concentration *versus* time-curve; C<sub>0</sub>, predose concentration; CsA, cyclosporine; PK, pharmacokinetic; SNP, single-nucleotide polymorphism; Tac, tacrolimus.

Author [reference]	Subjects	n	SNP(s)	Drug	PK parameter	Effect
Hesselink [67]	Renal transplant recipients	109	*3 / *6	CsA	C <sub>0</sub>	No effect on dose requirement
Yates [75]	Renal transplant recipients	10	*3	CsA	AUC <sub>0-12</sub>	Lower dose requirement in CYP3A5 expressors
Anglicheau [73]	Renal transplant recipients	106	*3	CsA	Calculated AUC <sub>0-12</sub>	No effect on pharmacokinetics
Haufroid [74]	Renal transplant recipients	50	*3 / *6	CsA	C <sub>0</sub>	No effect on pharmacokinetics
Hesselink [69]	Renal and heart transplant recipients	151	*3 / *6	CsA	AUC <sub>0-4</sub>	No effect on pharmacokinetics
Hesselink [67]	Renal transplant recipients	64	*3 / *6	Tac	C <sub>0</sub>	Dose requirement higher in CYP3A5 expressors
Haufroid [74]	Renal transplant recipients	50	*3 / *6	Tac	C <sub>0</sub>	Dose requirement higher in CYP3A5 expressors
Thervet [88]	Renal transplant recipients	80	*3	Tac	C <sub>0</sub>	Dose requirement higher in CYP3A5 expressors
MacPhee [78]	Renal transplant recipients	180	-44A>G <sup>§</sup>	Tac	C <sub>0</sub>	Dose requirement higher in CYP3A5 expressors
Tsuchiya [82]	Renal transplant recipients	30	*3	Tac	AUC <sub>0-12</sub>	Dose requirement higher in CYP3A5 expressors
Mai [81]	Renal transplant recipients	73	*3	Tac	C <sub>0</sub>	No effect on dose requirement
MacPhee [89]	Renal transplant recipients	180	*3	Tac	C <sub>0</sub>	Dose requirement higher in CYP3A5 expressors
Goto [83]	Liver transplant recipients	143	*3	Tac	C <sub>0</sub>	Dose requirement higher in CYP3A5 expressors
Zheng [79]	Pediatric heart transplant recipients	65	*3	Tac	C <sub>0</sub>	Dose requirement higher in CYP3A5 expressors
Zheng [84]	Lung transplant recipients	83	*3	Tac	C <sub>0</sub>	Dose requirement higher in CYP3A5 expressors

<sup>§</sup> The -44A>G SNP is located in CYP3A11

AUC, area-under the concentration *versus* time-curve; C<sub>0</sub>, predose concentration; CsA, cyclosporine; PK, pharmacokinetic; SNP, single-nucleotide polymorphism; Tac, tacrolimus.

**Table 4.3.4** Calcineurin inhibitor pharmacokinetics and single-nucleotide polymorphisms in cytochrome P450 3A5.

## CYP3A4

A limited number of studies has investigated the effect of the *CYP3A4\*1B* promoter variant allele on the pharmacokinetics of CNIs (Table 4.3.3).<sup>66,67,69,85,86</sup> In renal transplant recipients, the *CYP3A4\*1B* allele was not associated with cyclosporine dose requirement, nor with the incidence of biopsy-proven acute rejection or the occurrence of cyclosporine-related nephrotoxicity.<sup>66,67,85</sup> Interestingly, we found that renal transplant recipients carrying a *CYP3A4\*1B* allele had an approximately one-third higher tacrolimus dose requirement compared with *CYP3A4\*1* homozygotes.<sup>67</sup> It is possible that the *CYP3A4\*1B* allele also affected cyclosporine pharmacokinetics, but this effect may have been obscured as only the cyclosporine  $C_0$  was investigated in these studies. This parameter correlates poorly with total cyclosporine exposure, whereas the tacrolimus  $C_0$  better reflects total tacrolimus exposure.<sup>13-15,87</sup> To test this hypothesis, we described cyclosporine pharmacokinetics of 151 kidney and heart transplant recipients undergoing maintenance therapy by use of nonlinear mixed-effects modeling.<sup>69</sup> By use of this sensitive method, the oral clearance of cyclosporine (clearance divided by bioavailability or Cl/F) of *CYP3A4\*1B* allele carriers was indeed found to be 9% higher compared with *CYP3A4\*1* homozygotes, an effect independent of ethnicity. These observations suggest a higher CYP3A4 expression in patients carrying a *CYP3A4\*1B* allele, which is in accordance with the reported increased expression of the *CYP3A4\*1B* promoter sequence *in vitro*.<sup>60</sup> In a healthy volunteer study, and using a noncompartmental analysis, Min and Ellingrod<sup>86</sup> also found that the mean cyclosporine Cl/F was significantly higher in *CYP3A4\*1B* homozygotes compared with wildtype individuals.<sup>86</sup>

Although these results indicate that the *CYP3A4\*1B* allele may have functional effects *in vivo*, one could argue about its clinical relevance. The *CYP3A4* genotype only explains a small part of the interindividual variability in cyclosporine pharmacokinetics, and genotyping for *CYP3A4\*1B* is thus unlikely to assist in planning initial cyclosporine dosing.<sup>69</sup> Moreover, the observed correlation between *CYP3A4* genotype and cyclosporine clearance may have been caused by differences in *CYP3A5* expression. There is evidence that the *CYP3A4\*1B* and the *CYP3A5\*1* alleles are linked and, therefore, differences in *CYP3A5* rather than *CYP3A4* activity could explain the observed differences in cyclosporine pharmacokinetics.<sup>27,32,62,64,67,69</sup>

## CYP3A5

In contrast to the various *ABCB1* and *CYP3A4* SNPs, the correlation between tacrolimus dose requirement and *CYP3A5* genotype has been established more clearly (Table 4.3.4).<sup>67,69,73-75,78,79,81-84,88,89</sup> Studies in kidney, liver, lung, and heart transplant recipients have demonstrated that patients who do not express functional CYP3A5 (for instance, individuals homozygous for *CYP3A5\*3*, representing 80% of the Caucasian population<sup>62</sup>) require significantly less tacrolimus to reach target concentrations compared with patients who do express CYP3A5 (*CYP3A5\*1* allele carriers).<sup>67,74,79,82-84,88,89</sup> In addition, the -44G→A SNP in the *CYP3A* pseudogene *CYP3A1* was also associated with tacrolimus dosing in renal transplant recipients.<sup>78</sup> This SNP is (incompletely) linked to the *CYP3A5\*3* allele, which explains the observed correlation with tacrolimus dose requirement.<sup>64</sup> The patient cohort of MacPhee and colleagues was recently directly genotyped for *CYP3A5\*3*, confirming that individuals genetically predicted to express CYP3A5 required twofold higher tacrolimus doses to achieve target concentrations.<sup>89</sup>



Most studies published to date have not found significant associations between the *CYP3A5*\*3 SNP and cyclosporine pharmacokinetics (Table 4.3.4). In one study<sup>69</sup>, patients homozygous for the *CYP3A5*\*3 SNP (nonexpressors) did have a reduced cyclosporine Cl/F (-6%) compared with carriers of the *CYP3A5*\*1 allele. However, this small difference was not statistically significant ( $P = 0.06$ ). Yates and co-workers<sup>75</sup> reported a higher cyclosporine Cl/F in renal transplant recipients with a genetic inability to express *CYP3A5*. The pharmacokinetic analysis used in this study was sensitive, but its results are in marked contrast with the findings of many other studies. In particular, *CYP3A5* nonexpressors had the highest cyclosporine dose requirement, which is exactly the opposite of what might have been expected.

## DISEASE OUTCOME

In addition to the direct effect on the pharmacokinetics of CNIs, genetic variation in *ABCBI* and *CYP3A* may also influence transplantation outcomes. MacPhee and colleagues<sup>89,90</sup> assessed the time to achieve tacrolimus target concentrations among renal transplant recipients. Despite the use of TDM, patients who expressed *CYP3A5* had a significantly lower mean tacrolimus  $C_0$  during the first two weeks after transplantation and experienced a delay in achieving target concentrations. Acute rejection episodes occurred earlier in *CYP3A5* expressors than in nonexpressors (median of day 8 *versus* day 13), although there was no statistically significant difference in the overall rate of biopsy-confirmed acute rejection.<sup>89,90</sup> The absence of a relation between *ABCBI* or *CYP3A* genotype and the incidence of acute rejection has also been described by other authors.<sup>66,67</sup> However, these studies were all retrospective and were not designed nor powered to detect such an effect. Larger studies are needed to answer this question, as with modern immunosuppressive protocols the incidence of acute rejection has fallen below 20%.

One of the major limitations of CNI treatment is the frequent occurrence of nephrotoxicity. Evidence for a role of P-glycoprotein in the pathogenesis of CNI-related nephrotoxicity was provided by Koziolok and co-workers<sup>91</sup> who demonstrated in a histological study that treatment with cyclosporine was associated with increased expression of P-glycoprotein in arterial endothelia, proximal tubules, and endothelial cells of the Bowman's capsule in kidney transplants with acute tubular necrosis, acute rejection or CAN. By contrast, in kidney biopsies from patients suffering from cyclosporine-related nephrotoxicity, P-glycoprotein expression was not increased when compared with controls. These findings suggest that cyclosporine treatment induces P-glycoprotein expression, in order to facilitate cyclosporine detoxification. Failure to adequately upregulate P-glycoprotein expression, or a constitutively low P-glycoprotein expression in renal parenchymal cells, may lead to intrarenal accumulation of cyclosporine, and predispose patients to the occurrence of cyclosporine-related nephrotoxicity (Figure 4.3.1).<sup>91</sup>

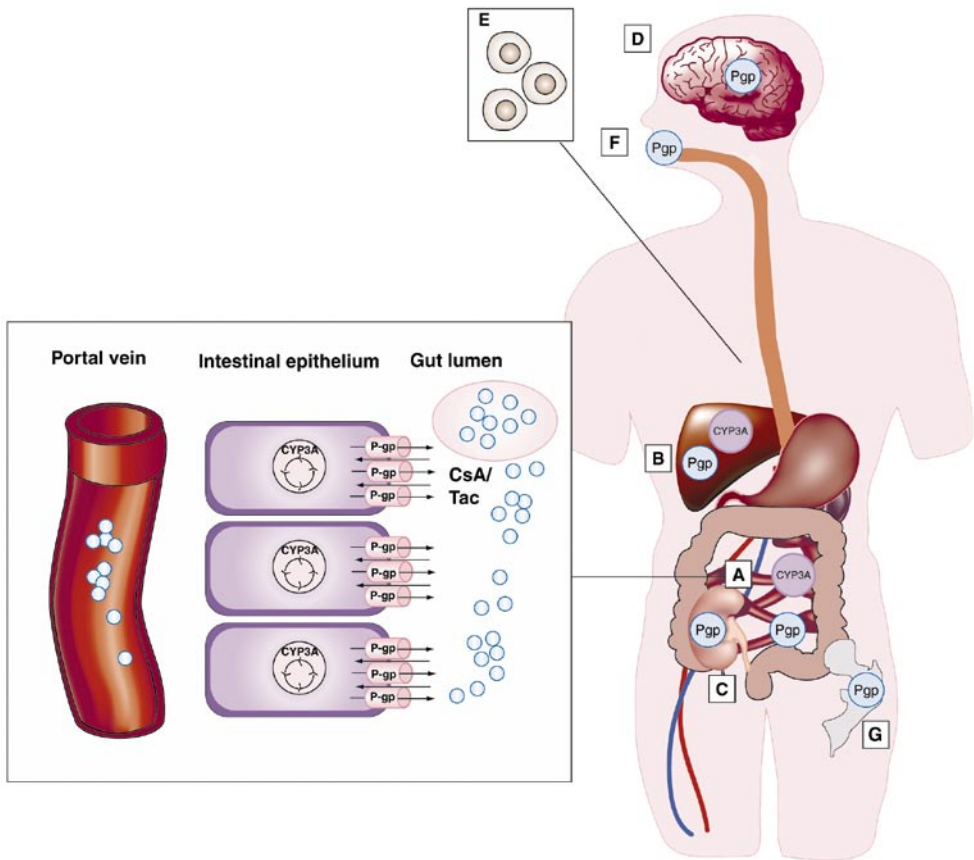
The influence of *ABCBI* genotype on the occurrence of CNI-related nephrotoxicity was studied in a case-control study among liver transplant recipients.<sup>70</sup> Patients treated with CNIs, who were homozygous for the *ABCBI* 2677T allele, were less than 50% as likely to experience symptoms of chronic renal dysfunction compared with 2677GT or 2677GG individuals.

The cause for this observation is at present unclear, but could possibly be explained by a lower renal P-glycoprotein efflux activity in G allele carriers, leading to a higher intrarenal CNI exposure.<sup>70</sup> However, the genotype of the liver transplant itself was not determined in this study, and an alternative explanation for these findings may be a difference in hepatic cyclosporine metabolism and exposure between patients with and without cyclosporine-related nephrotoxicity. In another case-control study, Hauser and colleagues<sup>92</sup> recently reported the influence of *ABCB1* genotype on the occurrence of cyclosporine-related nephrotoxicity after renal transplantation. In this study, the *ABCB1* genotype of the donor was also determined and proved to be a major risk factor for the occurrence of cyclosporine-related nephrotoxicity.<sup>92</sup> Among patients with cyclosporine-related nephrotoxicity, kidney transplants of the *ABCB1* 3435TT genotype were over-represented compared with patients without toxicity [odds ratio (OR) = 3.2; 95% confidence interval (CI) 1.4-7.6]. The donor's but not the recipient's, *ABCB1* genotype was highly predictive as 2½ years after transplantation, approximately 40% of all patients who received a kidney transplant from a donor homozygous for the 3435T allele developed cyclosporine-related nephrotoxicity, compared with only 10% of patients who received a kidney with the 3435 CT or CC genotype. To determine whether the *ABCB1* genotype was an independent risk factor for cyclosporine-related nephrotoxicity, a multivariate logistic regression analysis was performed. In the final model, which included several nongenetic factors, only the donor's *ABCB1* 3435TT genotype was strongly associated with cyclosporine-related nephrotoxicity (OR = 13.4; 95% CI 1.2-148).<sup>92</sup> Although the seemingly contradictory findings of the latter two studies require further investigation, they do indicate that it may become possible to perform a risk assessment for the occurrence of CNI-related nephrotoxicity by utilizing pharmacogenetics. Patients receiving a transplant from a donor with a genotype that renders them more susceptible to the development of CNI-related nephrotoxicity should then be (more) closely monitored, and might benefit from early CNI dose reduction or conversion to a CNI-free immunosuppressive regimen.

Finally, possession of a *ABCB1* 2677T/A allele has been associated with an increased risk of tacrolimus-induced neurotoxicity.<sup>93</sup> In addition, kidney transplant recipients with the *ABCB1* 3435TT genotype had a lower risk of developing corticosteroid-induced necrosis of the femoral head,<sup>94</sup> but this SNP was not related to the development of cyclosporine-induced gingival overgrowth (Figure 4.3.1).<sup>95</sup>

**Figure 4.3.1** The effects of P-glycoprotein and cytochrome P450 3A on the pharmacokinetics of calcineurin inhibitors and transplantation outcomes

**A.** In the gut, P-glycoprotein and CYP3A form a barrier to the absorption of the CNIs cyclosporine and tacrolimus. **B.** Following absorption, cyclosporine and tacrolimus are metabolized in the liver and excreted into bile. **C.** A small amount of absorbed cyclosporine and tacrolimus is eliminated by the kidneys. In addition, the *ABCB1* genotype of the kidney (allograft) may predict the development of CNI-related nephrotoxicity. **D.** P-glycoprotein also maintains the blood-brain barrier and the *ABCB1* genotype appears to be a risk factor for CNI-related neurotoxicity. **E.** Furthermore, P-glycoprotein limits entry of CNIs into various leukocytes. **F.** The *ABCB1* genotype is not related to the development of cyclosporine-induced gingival hyperplasia but does appear to predict the risk of corticosteroid-induced necrosis of the femoral head (**G**).



ABC, Adenosine triphosphate-binding cassette; CNI, Calcineurin inhibitors; CsA, cyclosporine; CYP, cytochrome P450; P-gp, P-glycoprotein; Tac, tacrolimus

## EXPERT COMMENTARY

In summary, most pharmacokinetic studies in transplantation have not identified a substantial effect of the various SNPs in *ABCB1*, *CYP3A4* or *CYP3A5* on the pharmacokinetics of cyclosporine. In contrast, tacrolimus dose requirement has been repeatedly found to be 30-50% higher in patients expressing *CYP3A5*, whereas SNPs in *ABCB1* appear to contribute little, if at all, to the interindividual variability in tacrolimus pharmacokinetics. The role of the *CYP3A4\*1B* allele is still controversial as the reported association with tacrolimus dose requirement may have arisen through linkage with *CYP3A5\*1*.

The most important promise of pharmacogenetics, namely to provide the genetic basis for the interindividual variability in the pharmacokinetics of CNIs, has not yet been fulfilled. It may have been too optimistic to expect that cyclosporine or tacrolimus pharmacokinetics would depend on a single SNP in only one gene. More likely, the causes of the variability in the pharmacokinetics of CNIs are multifactorial, to which multiple SNPs in different genes may contribute. In addition, the polymorphic expression of *ABCB1* and *CYP3A* not only depends on genetic polymorphisms, but also on variation in transcriptional regulation. Nevertheless, pharmacogenetics has made some important contributions to our understanding of the pharmacokinetics of tacrolimus. The association between tacrolimus dose requirement and *CYP3A5* genotype has been most significant in this respect, and indicates that pharmacogenetic analysis before transplantation may assist in guiding individual tacrolimus dosing. Although this remains to be formally tested, some transplant centers have already adopted the strategy to give a twofold higher tacrolimus dose to *CYP3A5* expressors (MacPhee IA, personal communication). Second, the high frequency of the *CYP3A5\*1* allele among black people<sup>31,32</sup> may explain the low oral bioavailability of tacrolimus in this ethnic group, and could help to reduce the incidence of acute rejection in black patients.<sup>96</sup> Finally, the *ABCB1* genotype of the (transplanted) kidney appears to predict the risk for the development of CNI-related nephrotoxicity, which is one of the major limitations to the successful long-term use of these immunosuppressants.

Future clinical studies are needed to further characterize the complex interplay between inherited factors and individual variation in CNI response and transplantation outcomes. Such studies should investigate the combined effect of different SNPs in *ABCB1* and *CYP3A* on the pharmacokinetics of CNIs, preferably by use of haplotype analysis and the most advanced pharmacokinetic assessment. In addition, as all studies conducted to date are of a cross-sectional or retrospective design and generally have a limited sample size, there is a need for larger numbers of well-characterized patients who have been uniformly treated and systematically evaluated, to make it possible to quantify drug response objectively. In order to limit spurious genotype-phenotype relationships, the possibility of co-medication interfering with CNI pharmacokinetics should also be carefully monitored in such studies.<sup>34,40,44,97-99</sup> A pharmacogenetic (sub)study as an integral part of a large prospective immunosuppressive drug trial would be most appropriate in this respect. Such trials may also answer questions regarding the efficacy, safety, and cost effectiveness of pharmacogenetics-guided immunosuppressive therapy. In the mean time, fundamental research will continue to provide deeper insights into the transcriptional regulation of *CYP3A* and *ABCB1*, and will

hopefully discover more specific activity probes for the individual CYP3A isozymes.<sup>27</sup> The recent elucidation of the crystal structure of human CYP3A4 is likely to be helpful in this respect.<sup>100</sup> Finally, there are other questions left unanswered. For example, it is not clear why CYP3A5 genotype affects tacrolimus, but not cyclosporine, pharmacokinetics. The relative contribution of CYP3A4 and CYP3A5 to the metabolism of each of these two drugs should therefore be better defined. Moreover, the recently reported effect of the CYP3A genotype on the novel immunosuppressant sirolimus requires confirmation.<sup>101</sup> It is also to be expected that the genetic basis of the variability in the pharmacokinetics of the immunosuppressive drug MMF will be investigated.

## OUTLOOK

Pharmacogenetics has generated considerable enthusiasm in transplantation medicine. Drug therapy in this field is characterized by the frequent occurrence of toxicity and drug interactions, and the need for close monitoring of drug concentrations in order to ensure adequate immunosuppression. Pharmacogenetic studies in transplant recipients have recently led to a further understanding of the genetic basis of the variable pharmacokinetics of immunosuppressants, although its full complexity is only beginning to be understood. In order to meet the ultimate goal of pharmacogenetics (*i.e.*, to realize a further individualization of immunosuppressive drug treatment) future study is needed. It is most likely that pharmacogenetics will not replace traditional TDM, but will have an additional value. Nonetheless, it is to be expected that pharmacogenetics will influence our everyday practice of medicine in the near future. It will become possible to perform genotype screens that identify patients with a smaller chance of an effective response, or an increased risk of adverse reactions. Ultimately, for transplanted patients the genetic profile may be added to well-known risk factors, such as the recipient's ethnicity, panel reactive antibody titers and previous transplant history, when selecting immunosuppressive drug protocols.

## HIGHLIGHTS

- Cyclosporine and tacrolimus are critical-dose immunosuppressive agents that display highly variable pharmacokinetics.
- Cyclosporine and tacrolimus are metabolized by the cytochrome P450 (CYP) isozymes 3A4 and 3A5 and are a substrate for P-glycoprotein, which is encoded by the adenosine triphosphate-binding cassette (*ABC*)*B1* gene.
- Interindividual differences in cyclosporine and tacrolimus pharmacokinetics have been attributed to variability in the expression and activity of P-glycoprotein and the CYP3A enzymes.
- Several recently-identified single-nucleotide polymorphisms (SNPs) may form the genetic basis for the polymorphic expression of CYP3A and *ABC**B1*.

- The *ABCB1* 3435C→T and 2677G→T SNPs do not appear to contribute much to the interindividual variability in cyclosporine- and tacrolimus pharmacokinetics, but may be associated with the occurrence of cyclosporine- or tacrolimus-related nephrotoxicity.
- The *CYP3A4\*1B* variant allele has been associated with a higher tacrolimus, and to a lesser extent cyclosporine, dose requirement but these observations may have been caused by linkage to the *CYP3A5\*1* allele
- The *CYP3A5\*3* allele, which results in the absence of functional CYP3A5 protein, has been strongly associated with a lower tacrolimus, but not cyclosporine, dose requirement.
- Patients who do express *CYP3A5* (\*1 allele carriers) not only need more tacrolimus, but are also slower to reach their target concentration when compared with non-expressors, despite the use of therapeutic drug monitoring.
- Carriers of a *CYP3A5\*1* allele may have an increased risk of the development of acute rejection.
- Screening transplant recipients for *CYP3A5* expression has the potential to optimize tacrolimus therapy, although this needs to be confirmed prospectively.

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The pharmacogenetics of calcineurin inhibitors: one step closer toward individualized immunosuppression?



## CHAPTER 5

# **SUMMARY AND CONCLUSIONS**

## SUMMARY

The calcineurin inhibitor (CNI) cyclosporine revolutionized transplantation medicine. Its potent immunosuppressive activity resulted in a dramatic decrease in the incidence of acute rejection after solid organ transplantation. As a result, kidney allograft survival improved markedly and other forms of solid organ transplantation first became realistic options with the availability of this drug. In the nineties, tacrolimus was approved for the prevention of acute rejection after solid organ transplantation and like cyclosporine, exerts its immunosuppressive effect by inhibiting the enzyme calcineurin. Today, more than 25 years after CNIs were first introduced into the clinic, these agents remain the cornerstone of immunosuppressive therapy after solid organ transplantation.<sup>1</sup>

However, cyclosporine and tacrolimus are not ideal drugs. Most important, they are toxic and can cause, among others, renal insufficiency, hypertension, hyperlipidemia, and diabetes mellitus. These side effects have a negative influence on graft and patient survival, and decrease the quality of life of transplant recipients. Furthermore, the pharmacokinetics of CNIs are highly variable both between and within individuals, which makes these agents difficult to use and close monitoring of CNI therapy necessary.<sup>1</sup>

The overall aim of this dissertation was to explore ways of optimizing treatment with CNIs. In this regard, several questions were addressed which are formulated in **Chapter 1**. In addition, the history of CNIs, their pharmacokinetics and pharmacodynamics, as well as current CNI treatment strategies are reviewed in the first chapter.

In the second part of the dissertation, (new strategies for) therapeutic drug monitoring (TDM) of cyclosporine was investigated. More specifically, we studied (1) the relationship between cyclosporine exposure and the occurrence of cyclosporine-related side effects in stable heart, kidney, and liver transplant recipients on maintenance cyclosporine therapy (**Chapter 2.1**), and (2) the benefits and drawbacks of cyclosporine dosing based on the cyclosporine whole-blood concentration 2 h after oral administration ( $C_2$ ) compared with conventional predose ( $C_0$ ) concentration monitoring (**Chapter 2.1** and **Chapter 2.2**). The rationale for these studies was the following:<sup>2-6</sup>

1. Cyclosporine exposure (within a dosing interval) correlates with the occurrence of its side effects and its efficacy.
2. The cyclosporine  $C_0$  poorly reflects total cyclosporine exposure and does not adequately predict its efficacy (*i.e.*, absence of acute rejection) nor the occurrence of adverse clinical events (toxicity).
3. The cyclosporine area-under the concentration *versus* time-curve within the first 4 h after oral administration ( $AUC_{0-4}$ ) and the cyclosporine  $C_2$  better reflect total cyclosporine exposure than  $C_0$ .
4. The cyclosporine  $AUC_{0-4}$  and  $C_2$  correlate with clinical events.



Because of the above, it has recently been propagated that TDM of cyclosporine should be based on cyclosporine  $AUC_{0-4}$  or  $C_2$  levels.<sup>7</sup> However, this recommendation is based on studies that were mostly performed in *de novo* transplant recipients. In addition, side effects other than nephrotoxicity have not been studied intensively.<sup>8</sup> Importantly, there have been no studies that have demonstrated improved graft or patient survival when cyclosporine was monitored (and dosed) according to  $AUC_{0-4}$  or  $C_2$  levels.<sup>5,6,9</sup> Given the fact that  $AUC_{0-4}$  and  $C_2$  level monitoring are more expensive and time-consuming than  $C_0$  level monitoring, and pose logistic problems, further study of these new strategies for TDM of cyclosporine was justified.<sup>10,11</sup>

In **Chapter 2.1**, we investigated the relationship between cyclosporine exposure and the occurrence of cyclosporine-related side effects [renal insufficiency, hypertension, hyperlipidemia, neurotoxicity (polyneuropathy and tremor of the hands), and cosmetic side effects (gingival hyperplasia, hirsutism and hypertrichosis)]. A total of 49 liver, 28 heart, and 26 kidney transplant recipients, all stable and on cyclosporine maintenance therapy (>6 months after transplantation) were included in the study. In all patients, the cyclosporine  $AUC_{0-4}$  was measured. Cyclosporine exposure was compared between patients with and those without a particular cyclosporine-related side effect. In addition, the correlation coefficients between  $C_0$ ,  $C_2$ , and  $AUC_{0-4}$  were calculated. The correlation between the cyclosporine  $C_0$  and  $AUC_{0-4}$  was weaker than the correlation between the latter and the cyclosporine  $C_2$  ( $r^2$  of 0.59 and 0.84, respectively). However, cyclosporine exposure (as measured by  $AUC_{0-4}$ ) was not significantly different between patients with and those without toxicity, and as such, we could not demonstrate a cyclosporine concentration-effect relationship in patients on maintenance therapy. Possibly, the negative findings in this study can be explained by previous cyclosporine dose reductions in patients experiencing side effects.

In **Chapter 2.2**, we investigated the effect of cyclosporine dose reduction based on  $C_2$  levels, on renal function in long-term liver transplant recipients. Cyclosporine  $C_2$  levels were measured in 60 stable liver transplant recipients (>1 year after transplantation) and dose reduction was performed if  $C_2$  levels exceeded the recommended target range of 600 ng/mL  $\pm$  20%.<sup>7</sup> In twenty-three patients (38%),  $C_2$  levels were above the upper limit of the  $C_2$  target range, whereas  $C_2$  levels were within the target range in 27% of the patients. Twenty of the 23 patients with cyclosporine “overexposure” agreed to have their cyclosporine dose reduced. Although cyclosporine  $C_2$  target levels were reached rapidly in these patients and cyclosporine dose was reduced by a mean of 25%, at 6 months follow-up, no improvement of renal function, lipid levels or systolic blood pressure was observed. However, after dose reduction, 1 patient experienced an episode of acute cellular rejection, in 2 patients primary biliary cirrhosis recurred, and 1 patient had a recurrence of autoimmune hepatitis. We concluded that according to current recommendations, cyclosporine overexposure is common among stable liver transplant recipients. However, cyclosporine dose reduction aimed at reaching recommended  $C_2$  levels did not result in an improvement of renal function and posed a risk of immune activation.

In part 3 of the dissertation, pharmacokinetic drug interactions with CNIs were studied. In addition to CNIs, most transplant recipients are also treated with several other immunosuppressive drugs. The rationale for using a “cocktail” of immunosuppressive agents

is to ensure adequate immunosuppression (through the synergistic actions of multiple, differently-acting agents), while limiting the toxicity of an individual drug.<sup>1</sup> Because pharmacokinetic interactions can result in increased or decreased blood concentrations of an individual drug, such interactions may be clinically relevant.

In **Chapter 3.1**, we studied the effect of corticosteroids on the pharmacokinetics of tacrolimus. In a randomized, controlled trial, *de novo* kidney transplant recipients were treated with tacrolimus plus mycophenolate mofetil (MMF) in combination with either daclizumab ( $n = 31$ ) or 3 months of prednisone ( $n = 34$ ). Tacrolimus dose-adjusted  $C_0$  at months 1-6 were compared between and within the 2 groups. The tacrolimus dose-adjusted  $C_0$  at months 1-3 was lower in the corticosteroid group compared with the daclizumab group, although the overall difference was not statistically significant ( $P = 0.06$ ). Within the corticosteroid group, a lower tacrolimus dose-adjusted  $C_0$  was observed at months 1 and 2 compared with months 4-6 (after corticosteroid withdrawal). These findings demonstrate that tacrolimus dose requirement is higher when tacrolimus is used in combination with corticosteroids. Changes in corticosteroid dosage may thus result in altered tacrolimus exposure causing toxicity or underimmunosuppression.

In **Chapter 3.2**, we reviewed the pharmacokinetic interaction between CNIs and mycophenolic acid (MPA), the active metabolite of MMF. There is controversy regarding the effects of cyclosporine and tacrolimus on MPA concentrations.<sup>12-15</sup> We conclude that co-administration of cyclosporine and MMF leads to a decreased MPA concentration, whereas tacrolimus does not have a clinically relevant effect on MPA concentrations. In addition, we propose that cyclosporine decreases exposure to MPA by inhibiting the biliary excretion of its metabolite MPA-glucuronide (MPAG).

The mechanism of the interaction between cyclosporine and MPA was investigated in **Chapter 3.3**. We hypothesized that cyclosporine impairs biliary elimination of MPAG through inhibition of the multidrug resistance-associated protein 2 (MRP2). Three groups of 10 rats deficient for Mrp2 were treated for 6 days with either placebo, cyclosporine or tacrolimus. Hereafter, co-administration with MMF was started in all groups and continued through day 14. The 24 h AUC ( $AUC_{0-24}$ ) of MPA and MPAG was determined after single (day 7) and multiple MMF doses (day 14). On both study days, there were no significant differences in the mean MPA and MPAG  $AUC_{0-24}$  between cyclosporine and tacrolimus-treated animals. In a previous study in normal rats (*i.e.*, rats expressing Mrp2), significant differences in MPA and MPAG exposure between cyclosporine and tacrolimus-treated rats had been observed (MPA being lower and MPAG higher in the cyclosporine-treated animals).<sup>16</sup> Therefore our findings suggest that cyclosporine-mediated inhibition of the biliary excretion of MPAG by MRP2 is the mechanism responsible for the interaction between cyclosporine and MPA. Nonetheless other mechanisms may also be important. MRP2 is expressed in proximal renal tubular cells and cyclosporine-mediated inhibition of renal MPAG clearance could explain the higher MPAG concentrations observed in cyclosporine-treated patients, although such a mechanism does not readily explain their reduced exposure to MPA.<sup>17</sup> In addition, recent data suggest that cyclosporine may also inhibit hepatic glucuronidation of MPA in Wistar rats.<sup>18</sup>

Part 4 of the dissertation focused on the pharmacogenetics of CNIs. The pharmacokinetics of these drugs vary widely between individuals and this has been attributed to interindividual differences in the expression of the CNI-metabolizing enzymes cytochrome P450 (CYP) 3A4 and CYP3A5, as well as the drug transporter P-glycoprotein.<sup>19</sup> Recently, several single-nucleotide polymorphisms (SNPs) in the genes encoding CYP3A4, CYP3A5, and P-glycoprotein have been described.<sup>20,21</sup> We hypothesized that these SNPs may lead to altered CYP3A or P-glycoprotein activity or expression, which in turn may result in differences in CNI pharmacokinetics.

The aim of **Chapter 4.1** was to determine the role of genetic polymorphisms in *CYP3A4*, *CYP3A5*, and *MDR-1* (encoding P-glycoprotein) with respect to interindividual variability in cyclosporine and tacrolimus pharmacokinetics. Kidney transplant recipients receiving cyclosporine (n = 110) or tacrolimus (n = 64) were genotyped for *CYP3A4\*1B* and \*3, *CYP3A5\*3* and \*6, and *MDR-1* 3435C→T. Dose-adjusted  $C_0$  were determined and correlated with the corresponding genotype. Tacrolimus dose-adjusted  $C_0$  were significantly higher in *CYP3A5\*3/\*3* patients than in \*1/\*3 plus \*1/\*1 patients, being roughly one-third higher in *CYP3A5* non-expressors (patients with the *CYP3A5* \*3/\*3 genotype). *CYP3A4\*1B* allele carriers had lower tacrolimus dose-adjusted  $C_0$  compared with those in patients with the wildtype (\*1/\*1) genotype. No evidence was found supporting a role for the *MDR-1* 3435C→T SNP in tacrolimus dose-requirement. None of the SNPs studied correlated with cyclosporine dose-adjusted  $C_0$ . We concluded that as a group, patients with the *CYP3A5\*3/\*3* genotype require less tacrolimus to reach target  $C_0$  compared with *CYP3A5\*1* allele carriers, whereas *CYP3A4\*1B* carriers need more tacrolimus to reach target  $C_0$  compared with *CYP3A4\*1* homozygotes.

In **Chapter 4.2**, we aimed to elucidate why tacrolimus dose requirement was found to correlate with SNPs in *CYP3A4* and *CYP3A5*, whereas cyclosporine dose requirement did not (the main finding of **Chapter 4.1**). We hypothesized that these contrasting results arose by using a (too) crude pharmacokinetic analysis (dose-adjusted  $C_0$ ) resulting in underdetection of an effect of genotype on cyclosporine pharmacokinetics. We therefore described cyclosporine pharmacokinetics of 151 kidney and heart transplant recipients undergoing maintenance therapy by use of nonlinear mixed-effects modeling (NONMEM) according to a 2-compartment pharmacokinetic model with first-order absorption and elimination. All patients were genotyped for the *CYP3A4\*1B* and \*3, *CYP3A5\*3* and \*6, and *MDR-1* 3435C→T SNPs. Oral cyclosporine clearance was found to be 9% higher in carriers of a *CYP3A4\*1B* variant allele compared with *CYP3A4\*1* homozygotes, and this effect was independent of ethnicity. Incorporation of *CYP3A4* genotype into the final NONMEM model did not markedly reduce interpatient variability of oral clearance, indicating that *CYP3A4* genotype made only a limited contribution to the observed interindividual differences in cyclosporine pharmacokinetics. None of the other SNPs studied significantly influenced any of the pharmacokinetic parameters. We concluded that patients carrying a *CYP3A4\*1B* variant allele have a significantly higher oral cyclosporine clearance compared with patients homozygous for *CYP3A4\*1*. However, this genetic effect on cyclosporine disposition was small, and genotyping of transplant recipients for *CYP3A4* is therefore unlikely to assist in planning initial cyclosporine dosing.

In **Chapter 4.3**, we discussed the current status of pharmacogenetics in transplantation medicine. Drug therapy in this field is characterized by the frequent occurrence of drug interactions and toxicity, and the need for close monitoring of drug concentrations in order to ensure adequate immunosuppression. Pharmacogenetics has generated considerable enthusiasm among transplant physicians as it has the potential to identify “the right drug and the right dose for an individual patient”. In this respect, polymorphisms in the *MDR-1* and *CYP3A5* genes appear at present to be the most promising. Although the *MDR-1* 3435C→T and 2677G→T SNPs do not appear to contribute much to the interindividual variability in CNI pharmacokinetics, they may predict the risk of developing CNI-related nephrotoxicity. The *CYP3A5*\*3 allele, which results in the absence of functional *CYP3A5* protein, is strongly correlated with tacrolimus, but not cyclosporine, dose requirement. Therefore, patients who express *CYP3A5* (\*1 allele carriers) need more tacrolimus to reach target concentrations and are at risk of developing acute rejection.

## CLINICAL IMPLICATIONS

What are the (clinical) implications of our findings for the individual transplant recipient? First of all, our findings demonstrate that after 20 years of clinical use, the optimal strategy for TDM of cyclosporine remains to be determined. Although it was recently recommended in an international consensus statement,<sup>7</sup> to dose cyclosporine according to  $C_2$  levels (or some other limited sampling strategy), the studies on which these recommendations were based have been criticized heavily.<sup>10,11,22</sup> The most important argument against  $C_2$  monitoring has been that to date, no controlled clinical trial has demonstrated unequivocally that it leads to improved clinical outcomes.<sup>10,22</sup> In our studies, no cyclosporine concentration-effect relationship was observed. Due to the cross-sectional study design the relationship between drug exposure and adverse events may have been obscured as patients not tolerating cyclosporine may already have been switched to tacrolimus-based therapy or have been subject to dose reductions. Adoption of  $C_2$  monitoring did not result in any clinically relevant benefit and in certain patients possibly even caused immune reactivation. Although our findings may be limited by the fact that a relatively small number of (selected) patients was studied, it appears that cyclosporine dosing based on  $C_2$  or  $AUC_{0-4}$  holds no advantage over conventional  $C_0$  level monitoring, at least in stable patients on maintenance therapy. As such, we agree with Campbell and Johnson<sup>10</sup> that “there is not enough science yet to justify the practice of  $C_2$  monitoring”.

Second, until recently, the only evidence for a pharmacokinetic interaction between corticosteroids and tacrolimus came from *in vitro* and animal studies.<sup>23,24</sup> We demonstrate that in humans, co-administration of these drugs is indeed associated with a higher dose requirement of tacrolimus. This finding is in line with the recently published observations of two other research groups.<sup>25,26</sup> In addition, evidence for the proposed mechanism of this pharmacokinetic interaction, namely induction of *CYP3A*-mediated tacrolimus metabolism by corticosteroids, comes from the work of Lemahieu and colleagues.<sup>27</sup> The interaction between tacrolimus and corticosteroids appears to be clinically relevant as tapering of the latter (which is nowadays common practice in many transplant centers) may

cause an increase in tacrolimus exposure and subsequently nephrotoxicity, which may be misinterpreted as acute rejection. *Vice versa*, treatment of an episode of acute rejection with high-dose corticosteroids may reduce tacrolimus exposure which may then lead to so-called “steroid-resistant” acute rejection. Therefore, monitoring of tacrolimus concentrations is necessary in corticosteroid-weaning protocols and during episodes of acute rejection.

Third, we show that co-administration of cyclosporine and MMF leads to a decreased exposure to MPA. No clinically important pharmacokinetic interaction appears to exist between MMF and tacrolimus. In addition, we elucidated the mechanism underlying this interaction, namely cyclosporine-mediated inhibition of the biliary excretion of MPAG by the MRP2 transporter. The corollary of these findings is that the MMF dose should be higher in patients treated with cyclosporine than in patients treated with tacrolimus, in order to ensure adequate MPA exposure. Cessation of cyclosporine treatment or a switch from cyclosporine to tacrolimus-based immunosuppression may cause increased exposure to MPA and MPA-related toxicity. Finally, inhibition of MRP2 by cyclosporine may have important clinical consequences for the (co-) administration of other drugs.<sup>28</sup>

Lastly, our pharmacogenetic studies demonstrated that this rapidly evolving field may help to individualize and optimize treatment with CNIs. Genetic screening of (future) transplant recipients may identify patients at risk for adverse drug reactions or under immunosuppression. However, this hypothesis remains to be tested prospectively in clinical trials. One possibility is to genotype patients on the waiting list for *CYP3A5* polymorphisms and adjust the starting tacrolimus dose accordingly. Based on our data, *CYP3A5* expressors should start with a tacrolimus dose that is between 30% and 50% higher compared with non-expressors. This approach may result in a more rapid achievement of tacrolimus target concentrations and thus in a reduction of the incidence of acute rejection. Given the low allelic frequency of these polymorphisms among white and Asian individuals, as well as the low rejection rates that are achieved with modern immunosuppressive regimens, such a trial would require a considerable number of patients in order to have adequate discriminative power. Hopefully, the transplant community will unite and perform such a study. Moreover, it is at present unclear why polymorphisms in *CYP3A* and *MDR-1* are associated with tacrolimus but not with cyclosporine pharmacokinetics. The relative contribution of the different *CYP3A* isozymes and *MDR-1* polymorphisms to the pharmacokinetics of CNIs, which has hitherto only been studied *in vitro* and by use of liver microsomes, should therefore also be investigated in human transplant recipients under the conditions of everyday clinical practice.<sup>29-32</sup>

## CONCLUSIONS

1. In solid organ transplant recipients on cyclosporine maintenance therapy, cyclosporine exposure as measured by  $AUC_{0-4}$ , is not correlated with the occurrence of cyclosporine-related side effects.
2. Cyclosporine dosing based on  $C_2$  levels in stable liver transplant recipients does not lead to an improvement of renal function and is associated with the risk of immune activation.
3. Co-administration of corticosteroids and tacrolimus increases tacrolimus dose requirement.
4. Co-administration of cyclosporine and mycophenolate mofetil leads to a decreased mycophenolic acid concentration, whereas tacrolimus does not have a clinically relevant effect on mycophenolic acid concentrations.
5. Cyclosporine interacts with mycophenolic acid by impairing the biliary excretion of mycophenolic acid-glucuronide through inhibition of the multidrug resistance-associated protein 2.
6. Patients expressing *CYP3A5* require more tacrolimus to reach target concentrations than *CYP3A5* non-expressors. Genetic screening of transplant recipients for *CYP3A5* expression may thus guide individual tacrolimus dosing.
7. Single-nucleotide polymorphisms in *CYP3A4*, *CYP3A5*, and *MDR-1* have no clinically relevant effects on cyclosporine pharmacokinetics, and genotyping of transplant recipients for *CYP3A* or *MDR-1* is unlikely to assist in planning initial cyclosporine dosing.

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## CHAPTER 6

# **SAMENVATTING EN CONCLUSIES**

## SAMENVATTING

De calcineurineremmer ciclosporine veroorzaakte een revolutie binnen de transplantatiegeneeskunde, omdat het gebruik van dit krachtige immunosuppressieve geneesmiddel leidde tot een sterke afname van het aantal acute afstotingen na orgaantransplantatie. Dientengevolge trad er een aanzienlijke verbetering op van de overleving van niertransplantaten en met het beschikbaar komen van dit geneesmiddel werd transplantatie van andere organen voor het eerst een realistische behandelingsmogelijkheid. Tacrolimus werd in de jaren negentig geregistreerd voor de preventie van acute afstoting na orgaantransplantatie en oefent, net als ciclosporine, zijn immunosuppressieve werking uit door remming van het enzym calcineurine. Vandaag de dag, meer dan 25 jaar na hun klinische introductie, vormen de calcineurineremmers nog steeds de hoeksteen van de immunosuppressieve behandeling na solide orgaantransplantatie.<sup>1</sup>

Ciclosporine en tacrolimus zijn echter verre van ideale geneesmiddelen. In de eerste plaats zijn zij toxisch en kan het gebruik ervan leiden tot, onder andere, nierinsufficiëntie, hypertensie, hyperlipidemie, en diabetes mellitus. Deze bijwerkingen hebben een ongunstige invloed op de overleving van het getransplanteerde orgaan en de patiënt, en kunnen de kwaliteit van leven van de ontvanger van een orgaan verminderen. Daarnaast is de farmacokinetiek van calcineurineremmers zeer variabel, zowel tussen individuen als binnen één persoon in de tijd. Dit maakt het klinische gebruik van deze geneesmiddelen moeilijk aangezien éénzelfde dosis bij de ene patiënt kan resulteren in het gewenste effect maar bij een ander kan leiden tot het optreden van bijwerkingen. Het nauwgezet vervolgen van de behandeling met calcineurineremmers is dan ook noodzakelijk.<sup>1</sup>

Het doel van het onderzoek zoals beschreven in dit proefschrift was om te onderzoeken óf en hoe de behandeling met calcineurineremmers zou kunnen worden verbeterd. Hiertoe werden enkele onderzoeksvragen bedacht welke zijn geformuleerd in **Hoofdstuk 1**. Daarnaast worden in het eerste hoofdstuk de geschiedenis van calcineurineremmers, hun farmacokinetiek en farmacodynamiek, alsmede de huidige behandelingsstrategieën met deze geneesmiddelen uiteen gezet.

In het tweede gedeelte van dit proefschrift, werd het doseren van ciclosporine op geleide van bloedspiegels, het zogenaamde “therapeutic drug monitoring” (TDM), onderzocht. Wij onderzochten hiertoe het volgende: (1) de relatie tussen ciclosporine expositie en het optreden van ciclosporine-gerelateerde bijwerkingen in stabiele hart-, lever-, en niertransplantaatontvangers die een onderhoudsbehandeling met ciclosporine kregen (**Hoofdstuk 2.1**); (2) de voor- en nadelen van het doseren van ciclosporine op geleide van de ciclosporine volbloed concentratie 2 uur na inname van het geneesmiddel ( $C_2$ ) in vergelijking met de traditioneel gebruikte dalspiegel ( $C_0$ ) (**Hoofdstuk 2.1** en **Hoofdstuk 2.2**). De uitgangspunten voor deze onderzoeken waren de volgende:<sup>2-6</sup>

1. De ciclosporine blootstelling (binnen een doseringsinterval) correleert met het risico op het krijgen van ciclosporine-gerelateerde bijwerkingen en het risico op het doormaken van een acute afstoting.
2. De ciclosporine  $C_0$  vormt een povere afspiegeling van de totale blootstelling aan het geneesmiddel en voorspelt in onvoldoende mate de effectiviteit (d.w.z. het vrijblijven van acute afstoting) en de toxiciteit.
3. De oppervlakte onder de curve van de ciclosporine concentratie uitgezet tegen de tijd ("area under the curve"; AUC) gedurende de eerste 4 uur na inname ( $AUC_{0-4}$ ) en de ciclosporine  $C_2$  zijn een betere afspiegeling van de blootstelling aan ciclosporine dan de  $C_0$ .
4. De ciclosporine  $AUC_{0-4}$  en  $C_2$  correleren met het risico op acute afstoting en toxiciteit.

Vanwege het bovenstaande is recentelijk voorgesteld om ciclosporine te doseren op geleide van de  $AUC_{0-4}$  of  $C_2$ .<sup>7</sup> Echter, deze aanbeveling is gebaseerd op onderzoeken welke grotendeels werden verricht onder *de novo* niertransplantaatontvangers. Daarnaast zijn de bijwerkingen van ciclosporine, anders dan nefrotoxiciteit, niet uitvoerig onderzocht.<sup>8</sup> Bovendien zijn er tot op heden geen onderzoeken gepubliceerd die een verbetering van transplantaat- of patiëntoverleving hebben aangetoond als ciclosporine werd gedoseerd op geleide van de  $AUC_{0-4}$  of  $C_2$ .<sup>5,6,9</sup> Aangezien het doseren van ciclosporine op geleide van de  $AUC_{0-4}$  of  $C_2$  duurder en tijdrovender is dan het doseren op geleide van de  $C_0$  en bovendien aanzienlijke logistieke problemen met zich meebrengt, lijkt verder onderzoek naar de (meer)waarde van deze nieuwe doseringsstrategieën gerechtvaardigd.<sup>10,11</sup>

In **Hoofdstuk 2.1** onderzochten wij de relatie tussen de blootstelling aan ciclosporine en het optreden van ciclosporine-gerelateerde bijwerkingen [nefrotoxiciteit, hypertensie, hyperlipidemie, neurotoxiciteit (polyneuropathie en tremor van de handen), en cosmetische bijwerkingen (gingiva hyperplasie, hirsutisme en hypertrichose)]. Een totaal van 49 lever-, 28 hart- en 26 niertransplantatiepatiënten werd geïncludeerd in het onderzoek. Alle patiënten waren klinisch stabiel, minstens 6 maanden eerder getransplanteerd en ontvingen een onderhoudsbehandeling met ciclosporine. In alle patiënten werd de ciclosporine  $AUC_{0-4}$  gemeten. De blootstelling aan ciclosporine werd vervolgens vergeleken tussen patiënten met en patiënten zonder een bepaalde ciclosporine-gerelateerde bijwerking. Daarnaast werden de correlatiecoëfficiënten tussen de  $C_0$ ,  $C_2$  en  $AUC_{0-4}$  berekend. De correlatie tussen de ciclosporine  $C_0$  en  $AUC_{0-4}$  was zwakker dan de correlatie tussen de  $C_2$  en  $AUC_{0-4}$  ( $r^2$  van respectievelijk 0.59 en 0.84). Echter, de blootstelling aan ciclosporine (gemeten aan de  $AUC_{0-4}$ ) was niet significant verschillend tussen patiënten met en patiënten zonder ciclosporine-gerelateerde bijwerkingen. Een ciclosporine concentratie-effect relatie kon dus niet worden aangetoond in patiënten die een onderhoudsbehandeling ciclosporine kregen. Mogelijk was dit het gevolg van het reeds eerder naar beneden bijstellen van de ciclosporine dosis bij patiënten die bijwerkingen hadden.

In **Hoofdstuk 2.2** onderzochten wij de gevolgen van ciclosporine dosisreductie, op geleide van  $C_2$  spiegels, op de nierfunctie van stabiele levertransplantatiepatiënten. Hiertoe werden  $C_2$  spiegels gemeten in 60 stabiele levertransplantaatontvangers (>1 jaar na levertransplantatie). De ciclosporine dosis werd vervolgens gereduceerd als de  $C_2$  spiegel

boven het aanbevolen niveau van  $600 \text{ ng/mL} \pm 20\%$  lag.<sup>7</sup> Bij 23 patiënten (38%) werd een  $C_2$  spiegel boven de bovengrens van het aanbevolen niveau gemeten. In 27% van de gevallen lag de  $C_2$  binnen het aanbevolen niveau. Twintig van de 23 patiënten met een “te hoge” blootstelling aan ciclosporine stemden in met het verlagen van de ciclosporine dosis. Het aanbevolen  $C_2$  niveau werd snel bereikt met een gemiddelde ciclosporine dosisreductie van 25%. Echter, deze dosisreductie leidde na 6 maanden follow-up niet tot een verbetering van de nierfunctie, het lipidenspectrum, of de systolische bloeddruk. Met betrekking tot de effectiviteit kreeg één patiënt een acute afstoting en bij 3 andere patiënten keerde de oorspronkelijke ziekte terug in het transplantaat (2 gevallen van primaire biliaire cirrose en 1 geval van autoimmuun hepatitis). Wij concludeerden derhalve dat, uitgaande van de huidige aanbevelingen, een te hoge blootstelling aan ciclosporine frequent voorkomt onder stabiele levertransplantaatontvangers. Echter, een dosisreductie met als doel het realiseren van de aanbevolen  $C_2$  spiegels, leidt niet tot een verbetering van de nierfunctie en geeft mogelijk zelfs een risico op immunosuppressie.

In deel 3 van dit proefschrift werden farmacokinetische interacties tussen calcineurineremmers en enkele andere geneesmiddelen onderzocht. De meeste transplantatiepatiënten krijgen naast een calcineurineremmer, diverse andere immunosuppressieve geneesmiddelen. Het idee achter het gebruik van een “cocktail” van immunosuppressiva is om voldoende immunosuppressie te bewerkstelligen (door de synergistische werking van diverse geneesmiddelen met een verschillend werkingsmechanisme) en tegelijkertijd de toxiciteit van de individuele geneesmiddelen te beperken.<sup>1</sup> Aangezien farmacokinetische interacties tot verhoogde of verlaagde bloedspiegels van een individueel geneesmiddel kunnen leiden (en daarmee mogelijk resulteren in acute afstoting of toxiciteit), zijn dergelijke interacties klinisch relevant.

In **Hoofdstuk 3.1** bestudeerden wij het effect van glucocorticoiden op de farmacokinetiek van tacrolimus. In een gerandomiseerde, gecontroleerde studie werden *de novo* niertransplantaatontvangers behandeld met tacrolimus en mycofenolaat mofetil (MMF) in combinatie met ofwel daclizumab ( $n = 31$ ) of prednison gedurende 3 maanden ( $n = 34$ ). De voor dosisgecorrigeerde tacrolimus dalspiegels tijdens maand 1 t/m 6 na niertransplantatie werden vergeleken tussen de 2 groepen. De voor dosisgecorrigeerde tacrolimus dalspiegels tijdens maand 1 t/m 3 waren lager in de met prednison behandelde groep maar het overall verschil tussen de 2 groepen was niet statistisch significant verschillend ( $P = 0.06$ ). Binnen de prednison groep was de voor dosisgecorrigeerde tacrolimus  $C_0$  tijdens maand 1 en 2 lager dan tijdens maand 4 t/m 6 (na het staken van prednison). Deze bevindingen tonen aan dat de tacrolimus dosisbehoefte hoger is wanneer tacrolimus wordt gecombineerd met glucocorticoiden. Veranderingen in de glucocorticoïd dosering kunnen derhalve aanleiding geven tot een veranderde blootstelling aan tacrolimus wat mogelijk resulteert in te weinig immunosuppressie of toxiciteit.

**Hoofdstuk 3.2** is een overzicht van de literatuur over de farmacokinetische interactie tussen calcineurineremmers en mycofenolzuur (mycophenolic acid; MPA), de actieve metabooliet van MMF. Er bestaat controverse over het effect van ciclosporine en tacrolimus op de MPA concentratie.<sup>12-15</sup> Wij concluderen dat gelijktijdig gebruik van ciclosporine en MMF leidt tot een verlaging van de MPA concentratie, terwijl tacrolimus geen klinisch belangrijk effect op

de MPA concentratie heeft. Daarnaast postuleren we dat ciclosporine de blootstelling aan MPA verlaagt door remming van de biliare excretie van haar metaboliet MPA-glucuronide (MPAG).

Het onderzoek naar het mogelijke pathofysiologische mechanisme dat ten grondslag ligt aan de interactie tussen ciclosporine en MPA wordt beschreven in **Hoofdstuk 3.3**. De hypothese was dat ciclosporine de biliare excretie van MPAG remt door inhibitie van het “multidrug resistance-associated protein 2” (MRP2). Drie groepen van elk 10 ratten deficiënt voor Mrp2 werden gedurende 6 dagen behandeld met placebo, ciclosporine, of tacrolimus. Op dag 7 werd tevens gestart met behandeling met MMF welke werd voortgezet tot en met dag 14. De 24-uurs AUC ( $AUC_{0-24}$ ) van MPA en MPAG werd bepaald na een enkele (dag 7) en na meerdere giften MMF (dag 14). Op beide tijdstippen werd geen significant verschil in MPA of MPAG blootstelling gevonden tussen ratten behandeld met ciclosporine of tacrolimus. In een eerder onderzoek met normale ratten (d.w.z. ratten die Mrp2 tot expressie brengen) werden wel significante verschillen gevonden in MPA en MPAG blootstelling tussen ratten behandeld met of ciclosporine of tacrolimus (de blootstelling aan MPA en MPAG was respectievelijk lager en hoger in de met ciclosporine behandelde dieren).<sup>16</sup> Onze resultaten suggereren derhalve dat ciclosporine-gemedieerde inhibitie van de biliare excretie van MPAG door MRP2 het mechanisme is dat verantwoordelijk is voor de interactie tussen ciclosporine en MPA. Desalniettemin kunnen daarnaast ook andere mechanismen een rol spelen. MRP2 komt tot expressie in de proximale niertubuli en ciclosporine-gemedieerde inhibitie van renale MPAG klaring zou de verhoogde MPAG concentraties in met ciclosporine behandelde patiënten kunnen verklaren. Een dergelijk mechanisme lijkt echter geen verklaring te bieden voor de verlaagde blootstelling aan MPA.<sup>17</sup> Daarnaast suggereren recente experimenten in Wistar ratten dat ciclosporine wellicht ook de hepatische glucuronidatie van MPA remt.<sup>18</sup>

Deel 4 van dit proefschrift handelt over de farmacogenetica van calcineurineremmers. Zoals eerder werd opgemerkt is de farmacokinetiek van deze geneesmiddelen sterk variabel tussen individuen. Deze verschillen zijn toegeschreven aan interindividuele variatie in de expressie van de drug transporter P-glycoproteïne en de cytochroom P450 (CYP) isoenzymen CYP3A4 en CYP3A5 welke verantwoordelijk zijn voor het metabolisme van calcineurineremmers.<sup>19</sup> Recentelijk zijn verscheidene, zogenaamde “single-nucleotide polymorfismen” (SNPs) geïdentificeerd in de genen die coderen voor CYP3A4, CYP3A5 en P-glycoproteïne.<sup>20,21</sup> Wij onderzochten de mogelijkheid dat deze SNPs leiden tot een veranderde CYP3A of P-glycoproteïne expressie of activiteit, welke op haar beurt weer resulteert in verschillen in de farmacokinetiek van calcineurineremmers.

Het doel van **Hoofdstuk 4.1** was om te bepalen of genetische polymorfismen in CYP3A4, CYP3A5, en MDR-1 (het gen dat codeert voor P-glycoproteïne) gerelateerd zijn aan interindividuele variabiliteit in ciclosporine en tacrolimus blootstelling. Niertransplantaatontvangers behandeld met ciclosporine (n = 110) of tacrolimus (n = 64) werden gegenotypeerd voor CYP3A4\*1B en \*3, CYP3A5\*3 en \*6, en MDR-1 3435C>T. De voor dosis-gecorrigeerde  $C_0$  werd bepaald en vervolgens gecorreleerd met het genotype. De voor dosis-gecorrigeerde tacrolimus  $C_0$  was significant hoger in patiënten met het CYP3A5\*3/\*3 genotype in vergelijking tot patiënten met het CYP3A5\*1/\*3 en \*1/\*1 genotype. De voor dosis-gecorrigeerde tacrolimus  $C_0$  was ongeveer  $\frac{1}{3}$  hoger in de groep van patiënten die CYP3A5 tot

expressie brachten (patiënten met het *CYP3A5* \*3/\*3 genotype). Draggers van een *CYP3A4\*1B* allel hadden een lagere, voor dosis-gecorrigeerde tacrolimus  $C_0$  dan patiënten met het *CYP3A4* wildtype (\*1/\*1). Er was geen relatie tussen het *MDR-1* 3435C→T polymorfisme en de tacrolimus dosisbehoefte. Geen van de bestudeerde polymorfismen was gerelateerd aan de voor dosis-gecorrigeerde ciclosporine  $C_0$ . Wij concludeerden dat patiënten met het *CYP3A5* \*3/\*3 genotype een lagere dosis tacrolimus nodig hebben om de  $C_0$  streefwaarden te bereiken, terwijl dragers van een *CYP3A4\*1B* allel vergeleken met patiënten homozygoot voor *CYP3A4\*1*, meer tacrolimus nodig hebben om de  $C_0$  streefwaarden te bereiken.

In **Hoofdstuk 4.2** onderzochten wij waarom er door ons wel een correlatie tussen polymorfismen in *CYP3A4* en *CYP3A5* en de tacrolimus dosisbehoefte werd gevonden, terwijl een dergelijke relatie niet kon worden aangetoond voor ciclosporine (de belangrijkste bevinding van **Hoofdstuk 4.1**). Omdat beide CYP isoenzymen een belangrijke rol spelen in het metabolisme van zowel ciclosporine als tacrolimus, was onze hypothese was dat deze ogenschijnlijk tegenstrijdige bevindingen het gevolg waren van een (te) weinig gedetailleerde farmacokinetische analyse (namelijk de voor dosis-gecorrigeerde  $C_0$ ) waardoor een eventueel effect van het *CYP3A* genotype op de farmacokinetiek van ciclosporine mogelijkwijs niet was gedetecteerd. Wij beschreven hiertoe de farmacokinetiek van ciclosporine bij 151 hart- en niertransplantatiepatiënten op een onderhoudsbehandeling met ciclosporine met behulp van “non-linear mixed-effects modeling” (NONMEM) uitgaande van een 2-compartimenten farmacokinetisch model met een eerste-orde absorptie en eliminatie. Alle patiënten werden ge genotypeerd voor de *CYP3A4\*1B* en \*3, *CYP3A5\*3* en \*6, en *MDR-1* 3435C→T polymorfismen. De orale ciclosporine klaring was 9% hoger onder dragers van het *CYP3A4\*1B* allel in vergelijking met patiënten homozygoot voor *CYP3A4\*1*. Dit effect was statistisch significant en bleek onafhankelijk van etniciteit. Toevoeging van het *CYP3A4* genotype aan het NONMEM model leidde slechts tot een beperkte daling van de variabiliteit in orale ciclosporine klaring, wat impliceert dat het *CYP3A4* genotype de sterke interindividuele verschillen in ciclosporine farmacokinetiek slechts voor een klein deel verklaart. Geen van de andere polymorfismen had een significant effect op de bestudeerde farmacokinetische parameters. Wij concludeerden dat dragers van een *CYP3A4\*1B* allel een significant lagere orale ciclosporine klaring hebben dan patiënten homozygoot voor *CYP3A4\*1*. Dit genetische effect op de farmacokinetiek van ciclosporine was echter beperkt en het bepalen van het *CYP3A4* genotype bij transplantatiepatiënten lijkt derhalve geen toegevoegde waarde te hebben bij het bepalen van de dosis ciclosporine.

In **Hoofdstuk 4.3** bediscussiëren wij de huidige status van de farmacogenetica binnen de transplantatiegeneeskunde. Farmacotherapie in deze tak van de geneeskunde wordt gekenmerkt door het frequent optreden van geneesmiddeleninteracties, toxiciteit en de noodzaak tot het nauwgezet controleren van de concentraties van de gebruikte geneesmiddelen om voldoende immunosuppressie te waarborgen. Farmacogenetica heeft het nodige enthousiasme onder transplantatieartsen gegenereerd aangezien het de potentie heeft om “de juiste dosering van het juiste geneesmiddel voor een individuele patiënt” te identificeren. In dit opzicht lijken polymorfismen in het *MDR-1* en *CYP3A5* gen momenteel het meest belovend. De *MDR-1* 3435C→T en 2677G→T polymorfismen lijken weliswaar een beperkte verklaring te bieden voor de interindividuele variabiliteit in calcineurineremmer farmacokinetiek, maar



zij kunnen mogelijk het risico op het ontwikkelen van calcineurineremmer-gerelateerde nefrotoxiciteit voorspellen. Het *CYP3A5\*3* allel, dat leidt tot de afwezigheid van functioneel *CYP3A5* eiwit, is sterk gecorreleerd met de dosisbehoefte van tacrolimus maar niet die van ciclosporine. Patiënten die *CYP3A5* tot expressie brengen (dragers van een *\*1* allel) hebben een hogere dosis tacrolimus nodig om hun streefspiegels te bereiken en hebben, wanneer dit niet wordt onderkend, mogelijk een hoger risico op het krijgen van een acute afstoting.

## KLINISCHE GEVOLGEN

Wat zijn de (klinische) gevolgen van onze bevindingen voor de individuele transplantatie patiënt? Ten eerste tonen onze resultaten aan dat het na meer dan 20 jaar van klinisch gebruik, nog niet duidelijk is wat de optimale strategie voor TDM van ciclosporine is. Ondanks het feit dat recentelijk is aanbevolen om ciclosporine op geleide van de  $C_2$  (of een andere “limited sampling” strategie) te doseren,<sup>7</sup> zijn de onderzoeken waarop deze aanbeveling is gebaseerd sterk bekritiseerd.<sup>10,11,22</sup> Het belangrijkste argument tegen het doseren op geleide van de  $C_2$  is dat er tot op heden geen gecontroleerde klinische onderzoeken zijn verricht die onomstotelijk hebben aangetoond dat een dergelijke strategie tot betere klinische uitkomsten leidt.<sup>10,22</sup> In ons onderzoek kon geen ciclosporine concentratie-effect relatie worden aangetoond. Echter, vanwege de cross-sectionele opzet van dit onderzoek kan een eventueel verband tussen ciclosporine expositie en het optreden van bijwerkingen zijn vertroebeld. Patiënten die ciclosporine niet verdroegen werden mogelijk reeds eerder overgezet op een behandeling met tacrolimus of hun ciclosporine dosis werd gereduceerd. Het doseren op geleide van de  $C_2$  leidde niet tot enige klinisch relevante verbetering maar resulteerde mogelijk zelfs in immunosuppressie. Ondanks het feit dat de waarde van onze bevindingen beperkt wordt door het geringe aantal (geselecteerde) patiënten, suggereren zij dat het doseren van ciclosporine op geleide van de  $C_2$  of  $AUC_{0-4}$  geen voordeel biedt boven het traditionele doseren op geleide van de  $C_0$ , in ieder geval niet bij stabiele patiënten die een onderhoudsbehandeling krijgen. In dit opzicht onderschrijven wij de mening van Campbell en Johnson<sup>10</sup> dat “er momenteel te weinig wetenschappelijk bewijs is om het gebruik van  $C_2$  monitoring te kunnen rechtvaardigen”.

Ten tweede was tot voor kort het enige bewijs voor het bestaan van een farmacokinetische interactie tussen glucocorticoïden en tacrolimus, gebaseerd op *in vitro* onderzoek en dierexperimentele studies. Wij tonen aan dat ook bij de mens, gelijktijdig gebruik van deze geneesmiddelen resulteert in een lagere blootstelling aan tacrolimus. Deze bevinding is in overeenstemming met de recentelijk gepubliceerde bevindingen van twee andere onderzoeksgroepen.<sup>25,26</sup> Daarnaast vormt het werk van Lemahieu *et al.* een bewijs voor het door ons geopperde mechanisme achter deze farmacokinetische interactie, namelijk inductie van het *CYP3A*-gemedieerde tacrolimus metabolisme door glucocorticoïden. De interactie tussen tacrolimus en glucocorticoïden lijkt klinisch relevant aangezien afbouwen van laatstgenoemde (hetgeen tegenwoordig gemeengoed is in de meeste transplantatiecentra) tot een verhoogde blootstelling aan tacrolimus kan leiden, mogelijk resulterend in nefrotoxiciteit, die ten onrechte geïnterpreteerd kan worden als een acute afstoting. *Vice versa*, kan een behandeling van een acute afstoting met een hoge dosis glucocorticoïden

leiden tot een verlaagde expositie aan tacrolimus welke mogelijkwijs aanleiding geeft tot het onterecht vaststellen van een zogenaamde “glucocorticoïd-resistente” afstoting. Het lijkt derhalve geïndiceerd om de tacrolimus concentraties nauwkeurig te controleren gedurende het afbouwen van glucocorticoïden of tijdens een episode van acute afstoting.

Ten derde tonen wij aan dat het gelijktijdig gebruik van ciclosporine en MMF aanleiding geeft tot een verlaagde blootstelling aan MPA. Er lijkt geen belangrijke farmacokinetische interactie tussen MMF en tacrolimus te bestaan. Daarnaast hebben wij het mechanisme dat ten grondslag ligt aan deze interactie opgehelderd, namelijk een door ciclosporine-gemedieerde inhibitie van de transporter MRP2 waardoor de biliare excretie van MPAG wordt geremd. Daarom dient de MMF dosis bij patiënten die gelijktijdig met ciclosporine worden behandeld hoger te zijn dan bij patiënten die tacrolimus gebruiken om een voldoende hoge blootstelling aan MPA te waarborgen. Het staken van de ciclosporine behandeling of een conversie naar een op tacrolimus-gebaseerde immunosuppressieve behandeling kan leiden tot een verhoogde expositie aan MPA en daaraan gekoppelde toxiciteit. Ten slotte kan remming van MRP2 door ciclosporine ook belangrijke klinische consequenties hebben voor het (gelijktijdig) gebruik van andere geneesmiddelen.<sup>28</sup>

Uit onze farmacogenetische studies blijkt dat deze, zich snel ontwikkelende wetenschap behulpzaam kan zijn bij het individualiseren en optimaliseren van de behandeling met calcineurineremmers. Genetische screening van (toekomstige) transplantatontvangers kan helpen bij het identificeren van patiënten met een verhoogd risico op het krijgen van bijwerkingen of onder-immunosuppressie. Deze hypothese dient echter in klinisch onderzoek prospectief te worden getest. Eén mogelijkheid om dit te doen is om patiënten op de wachtlijst voor een transplantatie te genotyperen voor *CYP3A5* polymorfismen en de tacrolimus dosering hierop aan te passen. Op basis van onze data zou de tacrolimus dosering tussen de 30% en 50% hoger dienen te zijn in patiënten die *CYP3A5* tot expressie brengen in vergelijking met patiënten die geen functioneel *CYP3A5* eiwit bezitten. Een dergelijke strategie zou ertoe kunnen leiden dat de tacrolimus streefspiegels sneller worden bereikt en de incidentie van acute afstoting lager wordt. Echter, gezien de lage allelfrequentie van deze polymorfismen in Caucasische en Aziatische populaties, en de lage rejectie incidentie die met de huidige immunosuppressieve behandelingen wordt gerealiseerd, zou er voor een dergelijk onderzoek een groot aantal patiënten nodig zijn om voldoende onderscheidend vermogen te hebben. Hopelijk zijn de verschillende transplantatiecentra bereid de krachten te bundelen om een dergelijk onderzoek uit te voeren. Tot slot is het op dit moment niet duidelijk waarom polymorfismen in *CYP3A* en *MDR-1* wél zijn geassocieerd met de farmacokinetiek van tacrolimus maar niet met die van ciclosporine. De relatieve bijdrage van de verschillende *CYP3A* isoenzymen en *MDR-1* polymorfismen aan de farmacokinetiek van calcineurineremmers, hetgeen tot op heden alleen *in vitro* en met behulp van lever microsomen is bestudeerd, dient derhalve ook te worden onderzocht bij getransplanteerde patiënten in de alledaagse praktijk.<sup>29-32</sup>

## CONCLUSIES

1. De expositie aan ciclosporine, gemeten met de  $AUC_{0-4}$ , is niet gecorreleerd met het optreden van ciclosporine-gerelateerde bijwerkingen bij ontvangers van een solide orgaantransplantaat op een onderhoudsbehandeling met ciclosporine.
2. Het doseren van ciclosporine op geleide van de  $C_2$  bij stabiele levertransplantatiepatiënten leidt niet tot een verbetering van de nierfunctie en is geassocieerd met een risico op immuunactivatie.
3. Gelijktijdig gebruik van glucocorticoiden en tacrolimus verlaagt de blootstelling aan tacrolimus.
4. Gelijktijdig gebruik van ciclosporine en mycofenolaat mofetil resulteert in een verlaagde blootstelling aan mycofenolzuur, terwijl tacrolimus geen klinisch relevant effect heeft op de mycofenolzuur concentraties.
5. De interactie tussen ciclosporine en mycofenolzuur wordt veroorzaakt door ciclosporine-gemedieerde inhibitie van het multidrug resistance-associated protein 2 waardoor de biliaire excretie van mycofenolzuur-glucuronide wordt geremd.
6. Patiënten die *CYP3A5* tot expressie brengen hebben een hogere dosis tacrolimus nodig om de tacrolimus streefspiegels te bereiken in vergelijking met patiënten die *CYP3A5* niet tot expressie brengen. Genetische screening van transplantatiepatiënten op *CYP3A5* expressie kan derhalve behulpzaam zijn bij het vaststellen van de optimale individuele tacrolimus dosis.
7. Genetische polymorfismen in *CYP3A4*, *CYP3A5*, en *MDR-1* hebben geen klinisch relevant effect op de farmacokinetiek van ciclosporine. Het genotyperen van transplantaatontvangers op *CYP3A* en *MDR-1* is dus niet behulpzaam bij het vaststellen van de (start)dosis ciclosporine.

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## ABBREVIATIONS

ABC	ATP-binding cassette
ATP	Adenosine triphosphate
AUC	Area-under the concentration <i>versus</i> time-curve
$C_0$	Predose concentration
$C_{max}$	Peak concentration
CAN	Chronic allograft nephropathy
Cl	Clearance
CN	Calcineurin
CNI	Calcineurin inhibitor
CV	Coefficient of variation
CYP	Cytochrome P450
F	Bioavailability
FKBP	FK-binding protein
IFN	Interferon
IL	Interleukin
MHC	Major histocompatibility complex
MMF	Mycophenolate mofetil
MPA	Mycophenolic acid
MPAG	Mycophenolic acid-glucuronide
MRP2	Multidrug resistance-associated protein 2
NFAT	Nuclear factor of activated T cell
P-gp	Permeability glycoprotein
PLC	Phospholipase C
PTDM	Post-transplant diabetes mellitus
SNP	Single-nucleotide polymorphism
TCR	T cell receptor
TDM	Therapeutic drug monitoring
TGF- $\beta$	Transforming growth factor- $\beta$
$t_{1/2}$	Half-life
$t_{max}$	Time-to-peak concentration

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D.H.

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## **CURRICULUM VITAE AUCTORIS**

Dennis Alexander Hesselink was born on September 19<sup>th</sup>, 1973 in Den Helder, the Netherlands. From 1985 to 1991 he attended the Gymnasium Erasmianum in Rotterdam, the Netherlands. He started his study Medicine at the Erasmus University Rotterdam in 1991. As part of his medical training he worked as a junior research fellow at the department of pediatric nephrology of the Sophia Children's hospital, Rotterdam (Dr. J. Nauta). After graduating as a medical doctor in 1999, he worked as a resident at the department of internal medicine of the Medical Center Rijnmond-Zuid (Dr. A.F. Grootendorst) and the Erasmus MC (Prof. dr. H.A.P. Pols). From 2001 to 2005 he worked as a PhD student at the renal transplant unit of the Erasmus MC where he studied the pharmacokinetics and pharmacogenetics of immunosuppressive drugs (the results of which are described in this dissertation), as well as the immunobiology of dendritic cells (Dr. T. van Gelder and Prof. dr. W. Weimar). He started his training in internal medicine in 2006 at the Havenziekenhuis and Institute for Tropical Diseases, Rotterdam (Dr. P.J. Wismans). In 2007 he continued his training in internal medicine and nephrology at the Erasmus MC. He lives with Judith de Schepper and their two daughters Sophie Quirine and Juliette Frédérique.



